Clinical Features and Endotypes in Chronic Rhinosinusitis: an Exploratory Study

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A thesis submitted to University of East Anglia for the degree of Doctor of Philosophy

Faculty of Medicine and Healthcare

2021

ABSTRACT

Chronic Rhinosinusitis (CRS) is a complex immune-mediated chronic condition of the upper respiratory system characterised by a variable clinical course and presentation. Clinically it is characterised into CRS with and without polyps (CRSwNP and CRSsNP, respectively). The work described within this thesis aimed to help further advance the understanding of the pathophysiology of CRS, with a focus on biomarkers that optimize patient and treatment selection, and predict therapeutic response. Studies were conducted to identify relevant biomarkers in CRS patients. The first study investigated the clinical features of CRS patients to assess whether there were factors associated with pre- and post-operative compliance. Secondly, biomarkers were identified through a literature search to determine which would be good candidates for this preliminary study. Finally, to determine if identified biomarkers had potential for future clinical application, we explored this set of biomarkers to be used in a clinical trial of CRS patients comparing medical and surgical treatment options. Concurrently, we examined the effects of clarithromycin on the in-vitro expression of selected biomarkers in CRS.

The first study demonstrated that duration of disease, nasal allergy and presence of comorbidities were related to pre- and post-compliance in CRS patients. A total of 36 biomarkers were identified by the literature search. These biomarkers were assessed for their ability to determine endotypes through cluster analysis. From this, CRS was divided into six clusters. Periostin and IL-31 were identified as cut-off points by tree analysis. Our in-vitro results suggested that clarithromycin may be of value in decreasing IL-8 at 4h. These results offer some preliminary data for further research.

In conclusion, these studies add evidence to support the hypothesis that endotypes provide insight into the pathophysiology

of CRS, and enable researchers and clinicians to better characterise and select optimal treatment options in CRS patients.

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ACKNOWLEDGMENTS

Throughout the writing of this thesis, I have received a great deal of support and assistance. First, I express my sincere gratitude to Professor Carl Philpott for allowing me to conduct this research under his auspices. I am especially grateful for his confidence and the freedom he gave me to do this work.

As a thesis supervisor, Professor Carl Philpott supported me in all stages of this work. He is the initiator of this project despite his busy agenda. His expertise, dedication and keen interest to this topic were invaluable in the formulating of the research topic. Without a coherent and illuminating instruction, this thesis would not have reached its present form.

I would like to acknowledge the assistance of Professor Tom Wileman, Dr. Allan Clark and Dr. Jelena Gavrilovic who offered me valuable suggestions and relevant advice for this study. You provided me with the tools that I needed to choose the right direction and successfully complete my thesis. I am deeply grateful to all members of the jury for agreeing to read the manuscript and to participate in the defence of this thesis.

I extend my sincere thanks to all members of the Biomedical Research Centre, Norwich Medical School and Quadram Institute, specially to Dr. Klaus Wellner, and all those who contributed directly or indirectly to this work construction.

I wish to express my gratitude to all the members of the James Paget University Hospital, who helped me to set this study. In particular, I wish to thank the Research Office and ENT surgical team for helping me to resolve administrative issues and for all their help. I also express my gratitude to Jane Woods and Teresa Ferreira for generously sharing their expertise, for their help in all stages, and for their helpful suggestions. I sincerely thank all patients that accept to assist in this work, allowing this study to take place.

Without the support of all members of my family, I would never finish this thesis and I would never find the courage to overcome all these difficulties during this work. My thanks go to my parents for their wise counsel, sympathetic ear and their love during all these years. Finally, there are my friends, especially Fiona, who were of great support in deliberating over our problems and findings, as well as providing happy distraction and encouragement to rest my mind outside of my research.

To Jake, I cannot fully express how grateful I am for your support over the past year. You deserve an award for living with me through this re-submission; you always supported me and never let me give up.

This work was supported by the Sir Jules Thorn Charitable Trust and the NIHR Clinical Research Network.

DISSEMINATION

Manuscripts waiting submission for publication

Waiting submission to the International Forum for Allergy and Rhinology (after thesis submission). Pratas, C., Clark, A., Javer, A., Philpott, C. *Prevalence and associated factors of medication non-adherence in CRS patients following endoscopic sinus surgery.*

Waiting Prospero review (registration number CRD42017056935). Pratas, C., Addsion, A., Clark, A., Philpott, C. *Potential biomarkers in chronic rhinosinusitis: a systematic review*.

Manuscript being prepared for submission to Laryngoscope (research being updated until 2021). Pratas, C., Gokani, S., Mattock, J., Clark, A., Philpott, C. *Potential biomarkers in chronic rhinosinusitis: a systematic review*.

Waiting submission to Rhinology Online (after thesis submission). Pratas, C., Shaida, Z., Gavrilovic, J., Philpott, C. *Time-dependent effect of Clarithromycin on pro-inflammatory cytokines in CRS.*

Manuscript being prepared for submission to JACI. (authors TBC) *Identification of CRS Subgroups Using Cluster Analysis.*

Other publications that the author contributed during the thesis

Hellings PW, Akdis CA, Bachert C, Bousquet J, Pugin B, Adriaensen G, Advani R, Agache I, Anjo C, Anmolsingh R, Annoni E, Bieber T, Bizaki A, Braverman I, Callebaut I, Castillo Vizuete JA, Chalermwatanachai T, Chmielewski R, Cingi C, Cools L, Coppije C, Cornet ME, De Boeck I, De Corso E, De Greve G, Doulaptsi M, Edmiston R, Erskine S, Gevaert E, Gevaert P, Golebski K, Hopkins C, Hox V, Jaeggi C, Joos G, Khwaja S, Kjeldsen A, Klimek L, Koennecke M, Kortekaas Krohn I, Krysko O, Kumar BN, Langdon C, Lange B, Lekakis G, Levie P, Lourijsen E, Lund VJ, Martens

K, Mő Sges R, Mullol J, Nyembue TD, Palkonen S, Philpott C, Pimentel J, Poirrier A, **Pratas AC**, Prokopakis E, Pujols L, Rombaux P, Schmidt-Weber C, Segboer C, Spacova I, Staikuniene J, Steelant B, Steinsvik EA, Teufelberger A, Van Gerven L, Van Gool K, Verbrugge R, Verhaeghe B, Virkkula P, Vlaminck S, Vries-Uss E, Wagenmann M, Zuberbier T, Seys SF, Fokkens WJ. *EUFOREA Rhinology Research Forum 2016: report of the brainstorming sessions on needs and priorities in rhinitis and rhinosinusitis*, Rhinology 2017 Sep; 55(3):202-210.

Conference presentations

Pratas C (2019, Mar). Exploring the possibility of biological signatures for CRS. Oral presentation at the ENT UK Spring Annual Meeting 2019, Chester, UK.

Pratas AC, Ferreira T, Woods J, Clark A, Wileman T, Philpott C (2018, Oct). Exploring the possibility of biological signatures for Chronic Rhinosinusitis. Poster presented at the SERIN 2018, Ghent, Belgium.

Pratas C (2018, Jul). Prevalence and associated factors of medication non-adherence in CRS patients following endoscopic sinus surgery. Oral presentation at the BACO 2018, Manchester, UK.

Pratas C, Addison A, Ferreira T, Woods J, Wileman T, Philpott C (2018, Jul). Defining a core set of biomarkers for patients with CRS. Poster presented at BACO 2018, Manchester, UK.

Pratas C, Addison A, Ferreira T, Woods J, Wileman T, Philpott C (2018, Mar). Exploring the possibility of biological signatures for CRS. Poster presented at the ENT UK Spring Annual Meeting 2018, York 2018.

Pratas C (2018, Mar). Exploring the possibility of biological signatures for CRS. Oral presentation at the BOARS Spring Meeting, York 2018.

Pratas C, Addison A, Philpott C (2017, Nov). Precision Medicine in Chronic Rhinosinusitis in Chronic Rhinosinusitis. Poster presented at the European Rhinology Research Forum 2017, Brussels, Belgium.

Pratas C, Addison A, Philpott C (2017, May). Precision Medicine in Chronic Rhinosinusitis. Poster presented at the 8th annual meeting of the FMH PGR, Norwich, UK.

LIST OF ABBREVIATIONS

A: Adherent

AERD: Aspirin-Exacerbated Respiratory Disease

AFRS: Allergic Fungal Rhinosinusitis

AR: Allergic Rhinitis

ARS: Acute Rhinosinusitis

ASA: Acetylsalicylic Acid

BCA: Bicinchoninic acid

BCA-1: B Cell-Attracting chemokine 1

BSA: Bovine Serum Albumin

CC10: Clara Cell 10kDa protein

CCAD: Central Compartment Allergic Disease

CF: Cystic Fibrosis

CO_2: Carbon dioxide

COPD: Chronic Obstructive Pulmonary Disease

CRS: Chronic Rhinosinusitis

CRSsNP: Chronic Rhinosinusitis without Nasal Polyps

CRSwNP: Chronic Rhinosinusitis with Nasal Polyps

CT: Computed Tomography

Cys-LT: Cysteinyl Leukotriene

DMEM: Dulbecco's Modified Eagle Medium

EAACI: European Academy of Allergy and Clinical Immunology

ECP: Eosinophil Cationic Protein

e-CRS: Eosinophilic CRS

EIA: Enzyme Immunoassay

ELISA: Enzyme-Linked Immunosorbent Assay

eNO: Exhaled Nitric Oxide

ENT: Ear Nose and Throat

EPOS: European Position Paper on Rhinosinusitis and Nasal Polyps

ERS: European Respiratory Society

ESS: Endoscopic Sinus Surgery

ET: Endothelin

FBS: Foetal Bovine Serum

FeNO: Fractional Exhaled Nitric Oxide

FESS: Functional Endoscopic Sinus Surgery

G-CSF: Granulocyte-colony stimulating factor

GP: General Practitioner

H_2O_2: Hydrogen Peroxide

HIV: Human Immunodeficiency Virus

IFN: Interferon

IgE: Immunoglobulin E

IgG: Immunoglobulin G

IL: Interleukin

ILC: Innate Lymphoid Cell

INA: Intentionally Non-Adherent

JPUH: James Paget University Hospital

LM: Lund-Mackay

LPS: Lipopolysaccharides

LT: Leukotriene

LTi: Lymphoid Tissue cell

LTRA: Leukotriene Receptor Antagonist

MIP: Macrophage Inflammatory Protein

MMP: Matrix Metalloproteinase

MPO: Myeloperoxidase

ne-CRS: Non-eosinophilic Chronic Rhinosinusitis with Nasal Polyps

NERD: NSAID-Exacerbated Respiratory Disease

NICE: National Institute for Health and Care Excellence

NINA: Non-Intentionally Non-Adherent

NK: Natural Killer cell

NKT: Natural Killer T cell

nNO: Nasal Nitric Oxide

NP: Nasal Polyps

NS: Not Significative difference

NSAIDs: Non-Steroidal Anti-Inflammatory Drugs

NT: Nasal Tissue

PBS: Phosphate Buffered Solution

PCR: Polymerase Chain Reaction

PNS: ParaNasal Sinuses

PROMs: Patient-Reported Outcome Measures

QOL: Quality of Life

RANTES: Regulated on Activation, Normal T Cell Expressed and Secreted (also known as Chemokine (C-C motif) ligand 5 (CCL5))

RS: Rhinosinusitis

SBV: Set Buffer Volume technique

SCF: Stem Cell Factor

SD: Standard Deviation

SDF: Stromal Cell-derived Factor

SE-IgE: Staphylococcal Enterotoxin-specific IgE

SEM: Standard Error of Mean

sIg: Soluble Immunoglobulin

sIL: Soluble Interleukin

SNOT: Sino-Nasal Outcome Test

SPT: Skin Prick Test

TARC: Thymus and Activation Regulated Chemokine

TIMP: Tissue Inhibitor of MetalloProteinase

TGF: Transforming Growth Factor

T_H: T-helper cell

TNF: Tumor Necrosis Factor

T-PER: Tissue Protein Extraction Reagent

Treg: T regulatory

TSLP: Thymic Stromal LymphoPoietin

VAS: Visual Analogue Scale

WHO: World Health Organisation

WR: Working Reagent

WS: Weighted Sample procedure

I – INTRODUCTION

1.1. Anatomy and pathophysiology of the upper respiratory tract

1.1.1 Anatomy of the nose and the paranasal sinuses

Air from the atmosphere is normally taken in via the nose. The nose is the first part of the upper respiratory tract (Figure 1), and is responsible for warming, humidifying, and, to some extent, filtering inspired air during its passage to the lungs. It also contains the special organ of the sense of smell: by means of the peculiar properties of its nerves, it protects the lungs from the inhalation of deleterious gases and assists the organ of taste in discriminating the properties of food (1).

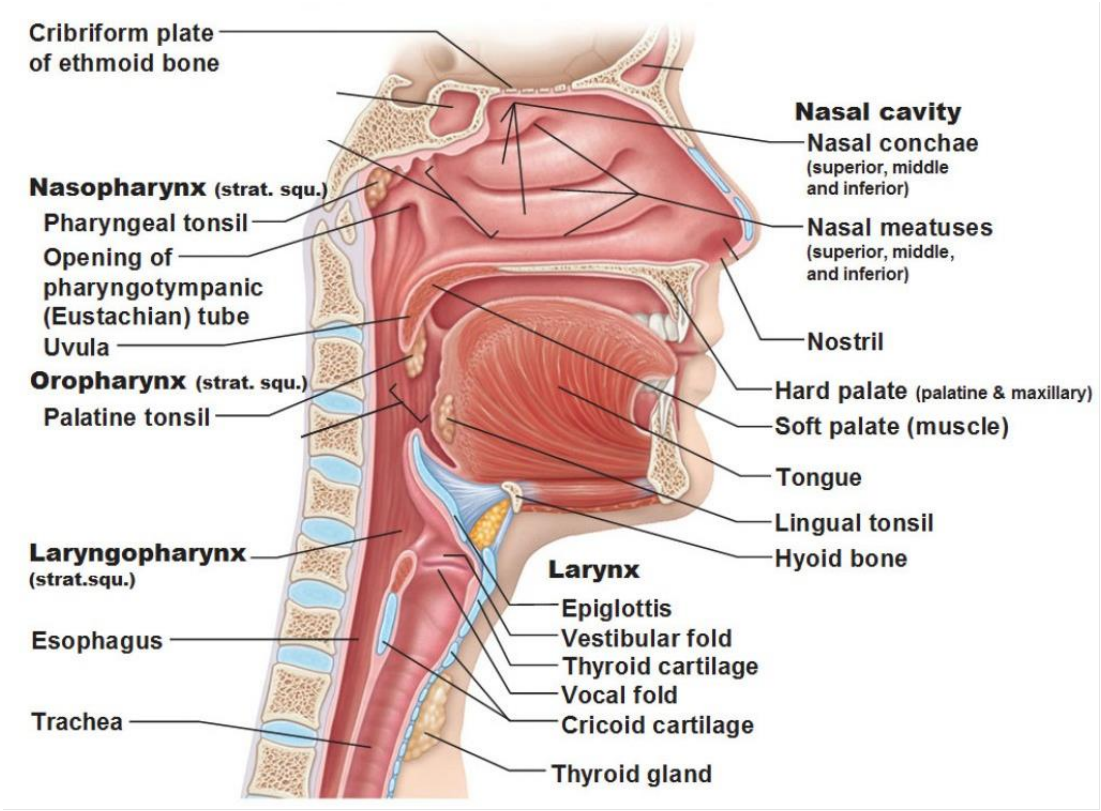


Figure 1: Illustration of the upper respiratory system. Here, we can observe the anatomical structures composing the nose, pharynx, and associated structures. Source: Marieb 2012 (2).

The nose may be subdivided into an external nose, which opens anteriorly to the face through the nostrils, and an internal chamber,

divided by a midline nasal septum into a right and left cavity that opens posteriorly into the nasopharynx. A supporting framework composed of bone and fibro-elastic cartilages houses the nasal cavities. The larger bones in this framework contain air-filled spaces lined with respiratory epithelium, described collectively as the paranasal sinuses (1).

There are 4 pairs of paranasal air sinuses – the ethmoid, sphenoid, maxillary, and frontal sinuses (Figure 2); however, the ethmoid sinuses are a series of separate cells, unlike the other sinuses that are configured as a single chamber with one opening. They all open into the lateral wall of the nasal cavity by small apertures that permit both the equilibration of the air between the various air spaces and the clearance of the mucus from the sinuses into the nose via a mucociliary escalator (1). The next section will explore their function.

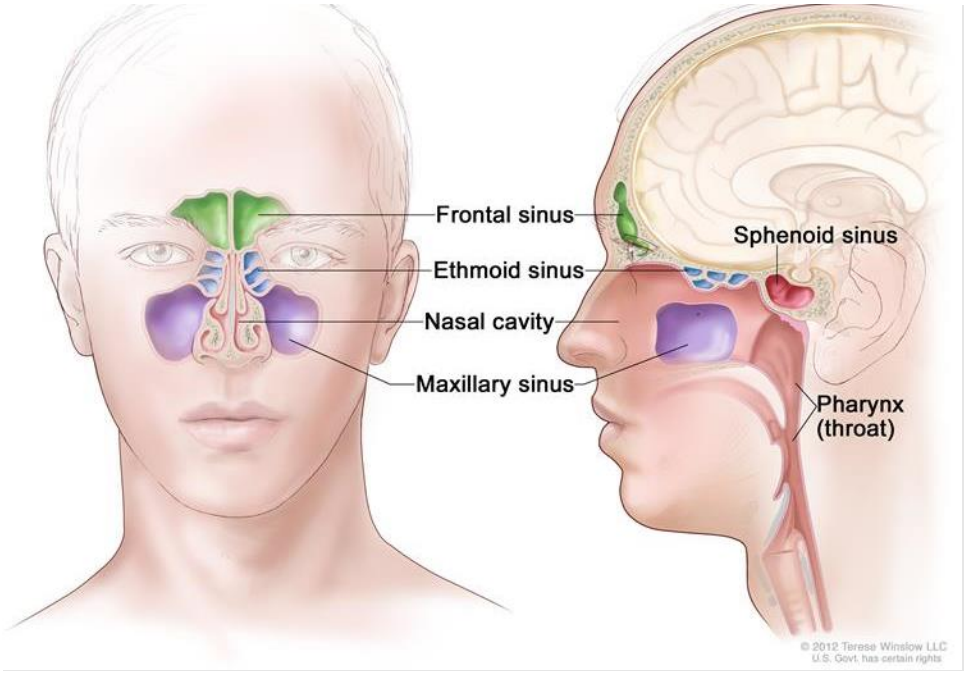


Figure 2: Paranasal sinuses. Illustration of the paranasal sinuses anatomy, frontal view (left) and lateral view of the face (right). Source: https://www.cancer.gov/publications/dictionaries/cancer-terms/def/paranasal-sinus.

1.1.2 Physiology of the nose and the paranasal sinuses

Over recent years, various studies have resulted in a better understanding of nasal physiology. In contrast, however, the role of the human paranasal sinuses remains an enigma today (3).

“Physiological” breathing occurs through the nose. The nasal fossae are considered the front door of the respiratory system, but are also characterised by other peculiar and significant functions, such as: conditioning and moistening of the nasal airflow, filtration of inspired noxious materials, specific and non-specific antibacterial and antiviral activities, reflex action, collection of water from expired airflow and olfactory function (3). No conclusive theory on the role of the paranasal sinuses has been accepted yet. However, some authors have suggested a functional role. The most generally accepted functions are to lighten the skull, impart resonance to the voice, increase the olfactory area, secretion of mucus and also, humidify and warm the inspired air, among others (2,3).

1.1.3 Pathophysiology of the upper respiratory tract

Due to its prominent position, the nose is especially prone to injury and exposure to deleterious substances. This includes fractures and nosebleeds, insertion of objects in the nose, viral infections, allergens and noxious gases from the environment. Therefore, rhinological diseases are very common, with a resulting dysfunction of nasal physiology.

As detailed in section 1.1.2, the nose and the paranasal sinuses are responsible for moistening and filtration of inspired air, as well as, for specific and non-specific antibacterial and antiviral activities, among others. Dysfunction of any of these systems can lead to symptoms such as blockage, rhinorrhoea, facial pressure, headaches, and sinuses infections.

The inflammatory diseases of the nose and the nasal sinuses, which are the main focus of this work, include a wide range of diseases, e.g., rhinitis (both bacterial, viral and allergic), sinusitis,

the common cold and rhinosinusitis. When the mucous membrane of the nose becomes inflamed it is called rhinitis. If this inflammation also spreads to the lining of the sinuses, then it becomes classified as rhinosinusitis (4). Rhinosinusitis is then referred to as a group of disorders characterised by inflammation of the mucosa of the nose and paranasal sinuses (5).

Awareness of the interrelationship between the upper and lower airways has increased; this concept is now known as the unified airway. The respiratory tract is considered to be an integrated system, so whatever processes affect one also affects the other. Hence, changes in the physiology of the nose and paranasal sinuses can and will affect the lower airways and vice versa (6).

1.2. Anatomy of the immune system

The immune system is responsible for protecting the host from constantly evolving microbes, such as external toxic or allergenic substances that enter through mucosal surfaces. The immune system has two fundamental lines of defence: the innate and adaptive immunity. While the innate immune system is responsible for fighting against an intruding pathogen, the adaptive immune system is antigen-dependent and specific, and it has the ability of immune memory. Both of these mechanisms include self-nonself discrimination (7–9).

1.2.1 Anatomy of the upper respiratory immune system

Air exchanges essential to life occur through the upper airways. However, air from the atmosphere is not innocuous, and may contain microbes or particles that can cause respiratory diseases. Therefore, the immune system present in the upper respiratory tract is essential in the prevention and pathogenesis of various respiratory tract diseases. Many of these pathogens infect the upper respiratory tract (nasal passages) prior to dissemination to the lower respiratory tract (airways and lungs) (10–12).

The respiratory immune response consists of multiple tiers of cellular responses that are engaged in a sequential manner to control infections (Figure 3).

The first step of the immune response within the respiratory tract consists of a physical and chemical barrier, including ciliated cells, mucus-secreting goblet cells, club and basal cells. The ciliated epithelial layer that lines the nasal mucosa, is coated with a mucus layer. Here, mucus prevents microorganisms from attaching to the cells by trapping them, before they are swept away by the cilia movements towards the external opening of the nose (13).

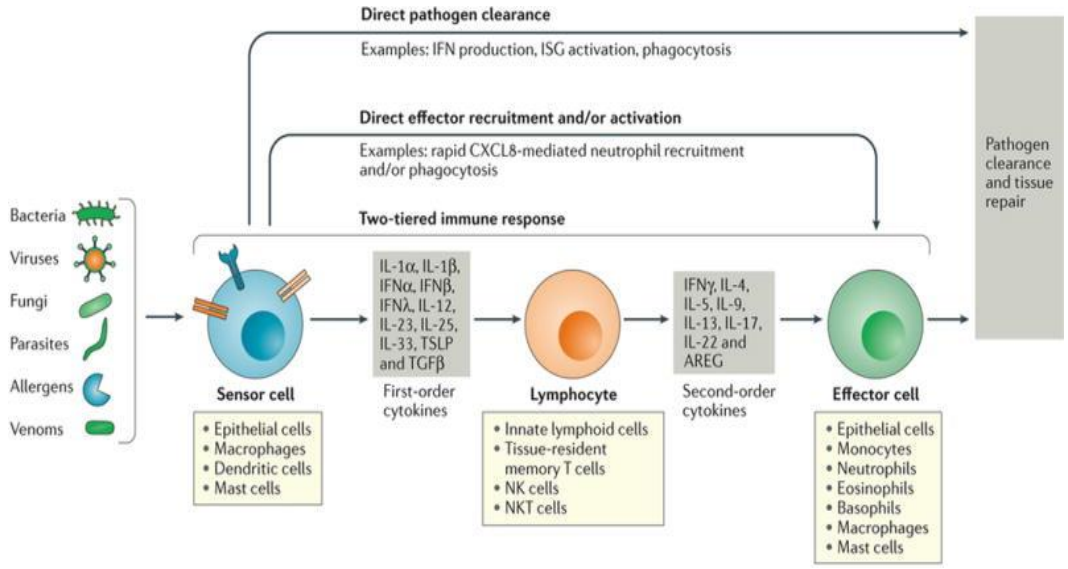


Figure 3: Representation of the step-by-step immune mechanisms following respiratory infection. Source: Iwasaki et al. 2017 (12).

In addition to these mechanisms, when a pathogen manages to breach this barrier, it triggers innate immune responses and the release of first order cytokines that alert local lymphocytes. The second tier of respiratory immune defences is mediated by differentiated lymphocytes including innate lymphoid cells (ILCs), natural killer (NK) cells, innate-like lymphocytes including NKT cells, mucosal-associated invariant T cells, epithelial γδ T cells, and tissue-resident memory T cells. These cells integrate the cytokine signals from local sensor cells and produce effector cytokines that

can enhance and regulate innate and inflammatory responses by recruiting effector cell subsets and eliciting appropriate responses to clear pathogens (12).

According to this, ILCs are classified into three broad groups based on their effector cytokine production and differentiation (Table 1). These cells are emerging as important cells regulating tissue homeostasis, remodelling and inflammation (14,15). Although there are similarities with conventional T-cells, they play different roles in immunity, tissue remodelling and development (16).

Table 1: Innate-like lymphoid cells subsets and characterization (14).

ILC group	ILC populations	Mediators	Functions
1	NK cells	IFN-γ, TNF, perforin, granzymes	Immunity to viruses and intracellular pathogens, tumour surveillance, including cytotoxicity
	ILC1 cells	IFN-γ, TNF	Immunity to extracellular pathogens: viruses, bacteria, parasites
2	ILC2 cells	IL-4, IL-5, IL-9, IL-13; amphiregulin	Immunity to helminths, wound healing
3	LTi cells	LT-α, LT-β, IL-17A, IL-22	Lymphoid tissue development, intestinal homeostasis, immunity to extracellular bacteria
	ILC17 cells	IL-17, IFN-γ	Immunity to extracellular bacteria
	ILC22 cells	IL-22	Immunity to extracellular bacteria, homeostasis of epithelia

These lymphocytes are responsible for producing second order cytokines that will recruit and activate the effector cells, which can, in turn, eliminate or expel foreign stimuli. Moreover, effector mechanisms are also activated in each of the stages to potentially control the infection and prevent activation of subsequent immune responses, thus limiting any inflammatory damage to self (12).

The immune responses produced by an organism are very heterogenous and tailored for the particular stimuli of origin. These responses depend on the differentiation of naïve T helper (T_H0) cells into one (or more) T_H subsets (Figure 4) (14,17). These cells regulate the response to pathogens, and of long-lived memory T cells, which are responsible for stronger and quicker responses when the body encounters the same infectious agent in the future. As detailed in Figure 4, each subset is unique and they share some functions with other cells, including ILCs, and work together to mount an immune response. Investigators find it useful to divide these complex responses into two major types: type 1 and type 2 immune responses. Their respective related cytokines are known as T_H1-type cytokines and T_H2-type cytokines, which will be studied further in this work (14).

Some variability in susceptibility to infection might reflect the association of T_H2 responses with anti-parasite immunity, T_H1 responses with antiviral and intracellular pathogen responses and T_H17 responses with extracellular bacterial infections and the complex counter-regulation among these responses (18) (Figure 5).

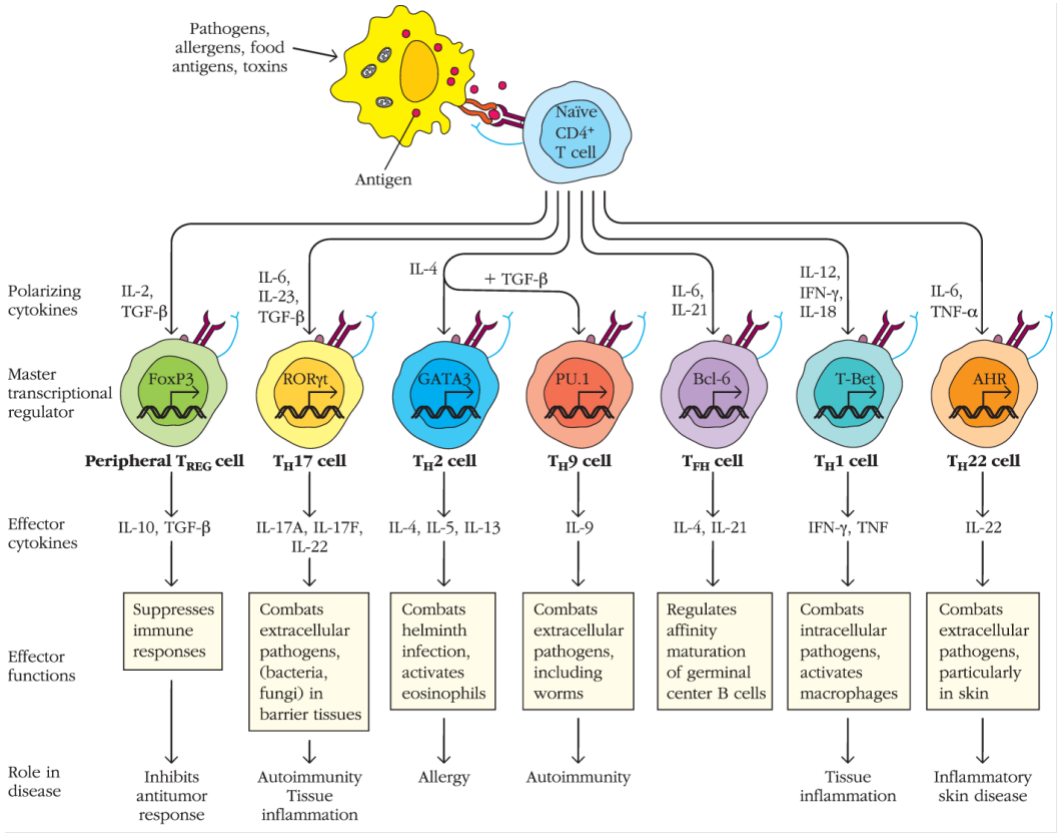


Figure 4: Synthesis of distinct features of T helper subset differentiation and activity on the immune response. Cross-regulation or potential plasticity among subsets is not represented. Source: Punt et al. 2019 (14).

Type 1 responses are triggered by viral and many bacterial infections and polarize CD4+ T cells to the TH1 and TH17 subsets. In conjunction with other immune cells (as ILC1s and ILC3s), they generate TH1-type cytokines, mainly IFN-, and produce proinflammatory responses responsible for clearing these infectious agents and for perpetuating autoimmune responses. Proinflammatory or cytotoxic responses, if excessive, can lead to uncontrolled tissue damage, so there is a need for a counteracting mechanism (Figure 5, Type 1).

On the other hand, type 2 responses are triggered by larger parasites (including worms, protozoa, and allergens), and polarize naïve TH cells to TH2 and TH9 subsets. These subsets, along with other populations of immune cells (e.g., ILC2s), are responsible for producing IL-4, 5, and 13 (Figure 5, Type 2), which are associated

with the promotion of IgE and eosinophilic responses in atopy, and also IL-10, which has more of an anti-inflammatory response. In excess, T_H2 responses will counteract the T_H1 mediated microbicidal action. The optimal scenario would be a well-balanced T_H1/ T_H2 response (14,19).

Type 3 responses are directed against extracellular parasites, as bacteria and fungi. This response is characterised by IL-17 and IL-22, being mediated by ILC3 subset and Th17-associated markers (20)(Figure 5, Type 3).

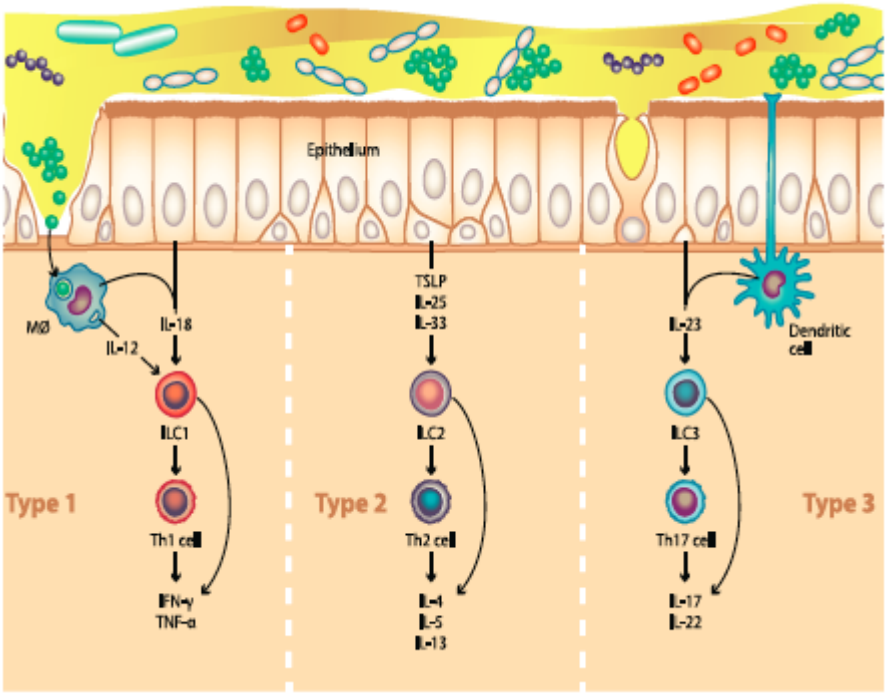


Figure 5: Types of physiologic immune responses across mucosal boundaries. Source: Fokkens et al. 2019 (20).

The importance of such investigations and results will be explored further in this work.

1.3. Rhinosinusitis

1.3.1 Definition

Rhinosinusitis (RS) is an umbrella term that includes continual inflammatory changes in both the nasal mucosa and paranasal

sinuses. Therefore, RS describes many different pathological processes that will be explained in section 1.3.7, that result in mucosal inflammation of the nose and paranasal sinuses. RS can be defined as an acute or chronic disorder depending on the duration of symptoms. Acute Rhinosinusitis (ARS) is clinically defined as symptoms lasting less than 12 weeks with complete resolution; if symptoms persist for more than 12 weeks without complete resolution, it is classified as Chronic Rhinosinusitis (CRS) (5,21,22).

RS must be characterised by at least two sinonasal symptoms as defined in Table 2, one of which should be either nasal blockage/obstruction or nasal discharge and the other should be smell loss or facial pain/pressure (23).

Table 2: Criteria for the diagnosis for RS.

Must have ≥1 of either:	Nasal obstruction/blockage/congestion Nasal discharge (anterior/posterior nasal drip)
and ≥1 of either:	Smell loss or reduction (hyposmia/anosmia) Facial pain or pressure
Symptoms must then be qualified by either CT scan and/or nasal endoscopy	

ARS usually has an infectious origin and it is usually resolved within 4 weeks using antibiotics. After this time, the patient will not show any more symptoms of nasal and PNS inflammation. CRS, on the other hand, is a chronic disease and little is known about its origin or pathophysiology (5,23).

CRS will be the main focus of this work, and therefore from now onwards only CRS will be referred.

1.3.2 CRS

CRS is an inflammatory condition of the nose, the paranasal sinuses and the upper airways that persists for more than 12 weeks without complete resolution of symptoms (23,24). As it is a subtype of RS, it should present the same symptoms as those described for RS, presented in Table 2.

CRS is a multifactorial disease (Figure 6) and the most important predisposing factors are: recurrent upper respiratory tract infections, environmental pollutants, aspirin-sensitivity syndrome, dental infections, sinonasal anatomic variants, nasal allergy, immunodeficiencies, mucociliary abnormalities (such as primary ciliary dyskinesia, Young's syndrome and cystic fibrosis), iatrogenic factors (mechanical ventilation, nasogastric tubes, nasal packing, scar tissue in the ostiomeatal complex as a consequence of sinonasal surgical procedures) and fungi (3).

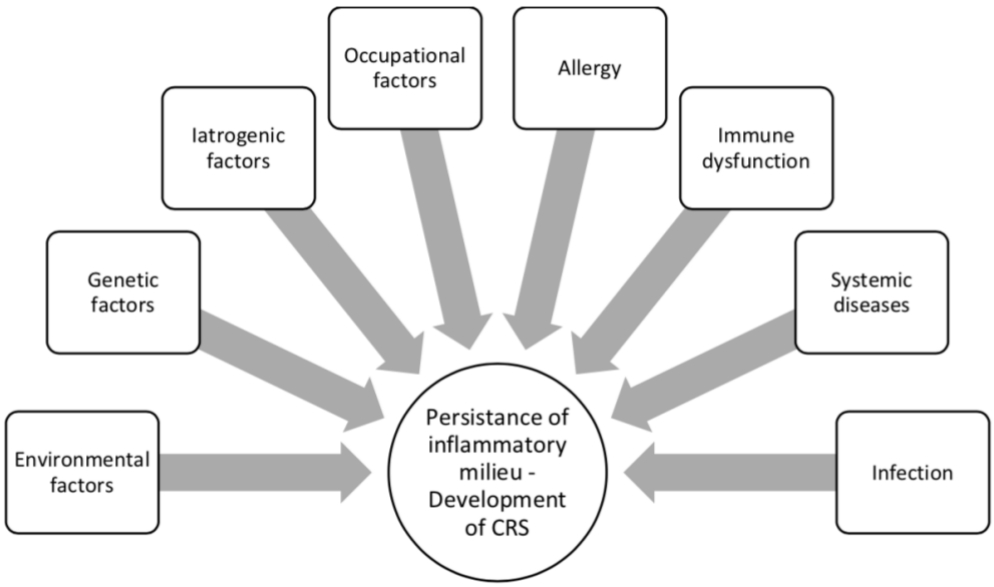


Figure 6: Multifactorial nature of CRS.

Even though little is known about the aetiology of CRS, all the aforementioned conditions may cause the onset of the disease by inducing damage to the sinonasal mucosa (20).

1.3.3 Epidemiology of CRS

CRS is pinpointed as a common health problem with significant morbidity and comorbidity, medical costs, and an impact on general health. Many studies indicate a prevalence of 10-15% in Europe and the United States, placing this condition the second most prevalent among all chronic conditions (23,25–27).

The clinical burden of CRS on an individual can be debilitating (26). However, it is difficult to access the true burden due to the heterogeneity of the disease and the diagnostic imprecision (23). Moreover, CRS potentially produces wide ranging consequences that extend beyond just sinonasal symptoms. These extra-rhinonasal symptoms (poor sleep, productivity and cognitive dysfunction) significantly contribute to the overall health-related burden of disease and decreased quality of life (QOL) (26,28). Aside from its detrimental effects on QOL and productivity, CRS is associated with a lifetime of medical and surgical resource consumption, resulting in significant healthcare expenditures (29).

Some studies have shown that CRS prevalence is associated with increasing age and other respiratory comorbidities (30). Furthermore, in the UK and USA, CRS is slightly more common among females but the presence of nasal polyps and the need to resort to surgery are more common in males; in continental Europe, Korea and Taiwan no changes in prevalence can be seen due to sex or age (23,26,27,31–33).

According to DeConde et al. (2016), CRS presents a significant burden at both the individual and society level due to its high prevalence, vast and burdensome symptom profile, and high indirect costs. It is estimated that the direct cost of CRS is of $23.6 million 2014 USD and indirect costs associated with CRS can go to $13 billion 2014 USD. Regardless of its prevalence, CRS accounts for huge health care expenditures related to office visits, medication

prescriptions, loss of workdays and surgery (26,27,29); but also for over the counter medications and other indirect costs (34).

1.3.4 Clinical subtypes of CRS

Firstly, a disease is classified according to its observable characteristics. These 'visible characteristics' are called phenotypes and can help a physician to divide patients into different groups according to their clinical presentation and subsequent response to treatment. According to Lötvall, and in the context of asthma, a phenotype describes clinical, physiological, morphologic, and biochemical characteristics as well as the response to different treatments (35).

As explained, phenotypes are clinically relevant and help physicians decide on treatment paths. However, they do not elucidate the underlying processes or disease progression, neither improve drug development or diagnosis. Therefore, the term endotype has emerged. Introduced by Anderson, this different form of disease classification helps define distinct subtypes of a condition, describing distinct disease entities with a defining aetiology and/or a pathophysiological mechanism (35).

According to several studies, chronic diseases are composed of several endotypes each one corresponding to a different underlying mechanism. Many of these proposed mechanisms are poorly understood, so research to identify novel therapeutic targets and biomarkers that will help diagnose and prognostic is required. Furthermore, recognising these inflammatory pathways may help predict the response to a determined treatment and thus facilitate treatment decisions (35–37).

As a consequence of its heterogeneity, CRS has numerous heterogeneous clinical presentations (Figure 7). At the beginning of this work, CRS classification was dichotomized based on the presence or absence of NP, with division of further phenotypes based on clinical features (23,24,38).

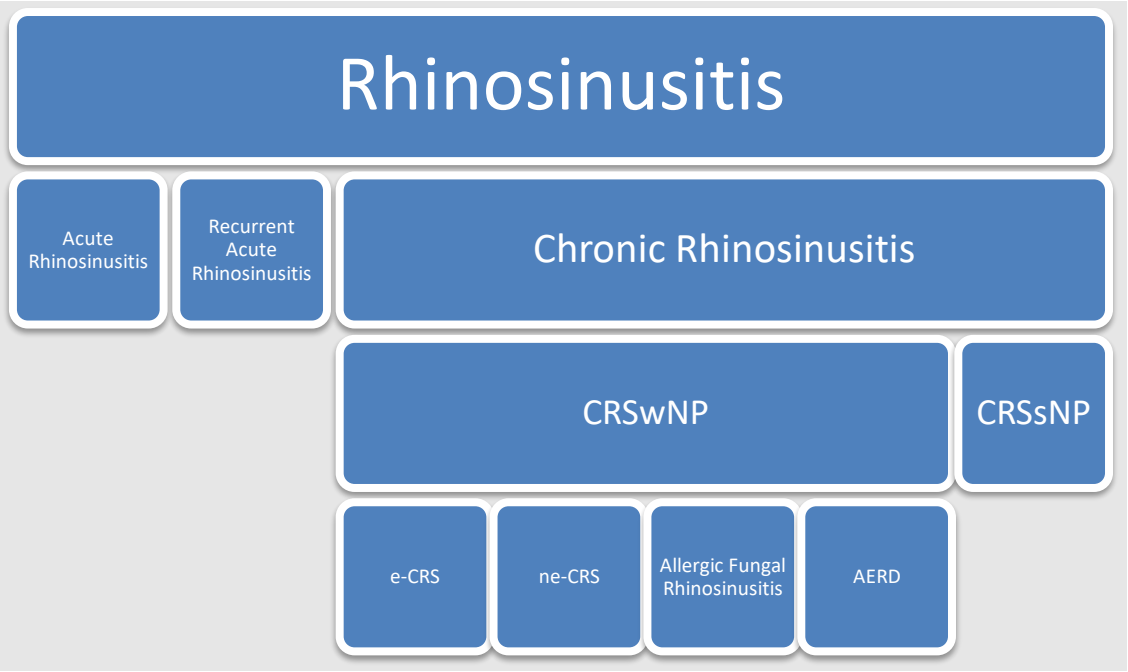


Figure 7: Classification of CRS forms. CRSwNP, CRS with nasal polyps; CRSsNP, CRS without nasal polyps; e-CRS, eosinophilic CRS; ne-CRS, non-eosinophilic CRS; AERD, aspirin-exacerbated respiratory disease.

From the commencement of the present study in 2016, to its completion in 2019, this was the classification of CRS according to its clinical features. However, upon the publication of the 2020 European Position Paper on Rhinosinusitis and Nasal Polyps (EPOS), a new contemporary classification for CRS was proposed. This new classification system approaches CRS as a broad inflammatory airway condition, splitting CRS into primary and secondary forms, and further divided into localised and diffused disease based on anatomic distribution. This new system also considers the inflammatory endotype dominance, which will define treatment options. Primary CRS will be presented here (Figure 8), while secondary CRS will be presented later in section 1.3.8.

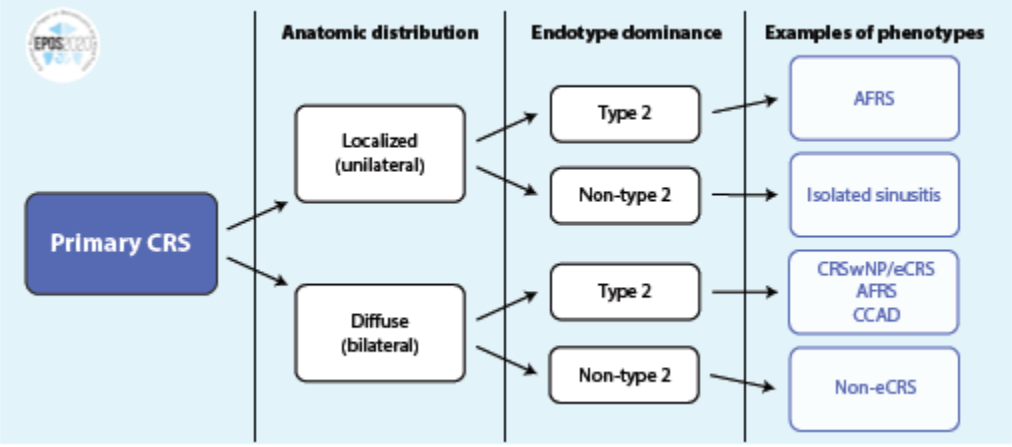


Figure 8: Classification of primary CRS. AFRS, allergic fungal rhinosinusitis; CRSwNP, chronic rhinosinusitis with nasal polyps; eCRS, eosinophilic CRS; CCAD, central compartment allergic disease. Source: Fokkens et al. 2019 (20).

Therefore, endotypes are the next hope for CRS inflammatory pathways, causal-effect relationship, and patients' management. These will be seen further ahead in this work (section 1.4.3).

1.3.5 Diagnosis of CRS

Patients with a history suggestive of CRS are evaluated for evidence of nasal polyps, purulent secretion, or anatomical abnormality, which may be responsible for the symptoms.

The state of health of the paranasal sinuses depends on immunity, mucociliary clearance and the aeration of the sinuses. The middle meatus forms the common drainage pathway for the anterior ethmoidal, frontal and maxillary sinuses, and can be readily examined with a fibre optic endoscope. Endoscopic examination will usually show infected mucus draining from these areas (1).

Standard radiological images are no longer recommended in the diagnosis of RS because of their poor specificity and sensitivity. Computed tomography (CT) scans give a more detailed picture of the sinuses. They are routinely done when endoscopic surgery is anticipated, and sometimes as an additional aid for diagnosis. CT scans define anatomical variations but should not be used in

isolation, since one in three asymptomatic individuals show incidental mucosa changes (1,39).

CT evaluation of patients complaining of CRS is essentially focused on an accurate delineation of those elements – inflammatory mucosal changes and/or predisposing anatomical factors – that may impair mucociliary drainage (3).

Besides the above-mentioned more traditional classification, several efforts have been made to measure the severity of this disease entity based on both clinical manifestations and CT findings. Among the latter, the Lund-Mackay (LM) system is the most popular because of its high inter- and intra-observer agreement rate, as well as its simplicity and reproducibility (40).

The Lund-Mackay classification applies a three-point scale, as shown in Table 3, to each major paranasal cavity (maxillary, frontal and sphenoid sinus, anterior and posterior ethmoid). A score is similarly assigned to the ostiomeatal complex, graded as patent (0) or obstructed (2). The total sum of CT alterations may therefore range from 0 to 24 (3). The Lund-Mackay system relies on a score of 0–2 dependent upon the absence of, partial or complete opacification of each sinus system, deriving a maximum score of 12 per side. Ostiomeatal complex would add a final score of either 0 (no opacification) or 2 (if opacification happens). It was adopted by the Rhinosinusitis Task Force Committee of the American Academy of Otolaryngology Head and Neck Surgery in 1996.

Table 3: Lund-Mackay score.

0	No opacification
1	Partial opacification
2	Total opacification

The impact of CT-based staging systems is still controversial. Several reports failed to demonstrate a direct correlation between patients' complaints at presentation and the imaging findings. In

contrast, some correlation was demonstrated between CT scan severity and endoscopic appearance (41–43). Thus, CT should be considered an invaluable tool for proper surgical planning and anatomic landmark delineation (3).

Therefore, ERS/EAACI guidelines recommend radiographic and/or endoscopic confirmation of disease to prevent misclassification with other diseases that can masquerade as CRS (e.g., primary headache disorder, chronic rhinitis, septal deviation).

1.3.6 Treatment of CRS

Like most chronic diseases, CRS is difficult to control and commonly requires therapy for long periods (44). However, taking medication permanently or for a long-term period might be challenging, especially if the desired outcome is not achieved or if recurrence occurs (45). The European guidelines have been through three iterations with EPOS2020 setting out how patients should be managed; similarly there are other international guidelines such as the International Consensus on Allergy and Rhinosinusitis with similar treatment recommendations (20,46,47). Current therapy for CRS includes intranasal corticosteroids and nasal douching, with select use of oral corticosteroids and with uncertainty about the role of oral antibiotics. In those cases refractory to medical therapy, sinus surgery is considered, but the timing of this intervention remains in doubt (20,48–53). CRS treatment aims to reduce mucosal inflammation, control infection, and restore mucociliary clearance within the sinuses (23,54). Drug options are presented in Table 4.

Table 4: Drug option for CRS patients.

Types of drug	Subtypes
Steroids	Topical nasal steroids
	Systemic steroids
Nasal irrigation	Saline douching
	Steroid nasal irrigation
	Topical antibiotic therapy
	Chemical surfactant (e.g.: baby shampoo)
Antibiotics	
Antifungal therapy	
Others	Decongestants
	Mucolytics
	Antihistamines
	Leukotriene inhibitors
	Analgesics

Endoscopic sinus surgery (ESS) is the main intervention in the treatment of CRS with or without NP refractory to maximum medical treatment (55). It is useful for clearing the sinonasal passages. In this case, a CT scan is mandatory and should be made available, to assess the extent of disease, any previous surgery and any anatomical variants. It is important to make patients aware that ESS may not be a cure for the underlying pathology and that symptoms may recur (56). Therefore, to decrease levels of inflammation and maintain disease control, patients undergo long-term treatment with intranasal steroids and douche prior to and after surgery.

It has been found that many patients do not respond as expected to the “one-size fits-all” treatment approaches, supporting the notion that CRS is a multifactorial disorder with diverse and overlapping pathologies and clinical phenotypes (57). Given the low number of treatment options, and the fact that a significant subset of patients does not respond to these treatments, it is important to determine how to tailor treatment to optimise outcomes, and how to identify

the best suited therapy option for each patient's underlying pathophysiology (58). Therefore, new therapies to control, or ideally heal, these chronic conditions are desperately needed. The most promising direction seems to be more targeted drugs aimed towards specific molecular mediators of inflammation, and research into the cellular and molecular underlying processes of CRS is a prerequisite for such drug development.

Therefore, the identification of CRS endotypes determined by distinct pathophysiological mechanisms, and further characterising the CRS uncontrolled by current treatment regimens, may provide a foundation from which to understand disease causality and ultimately develop efficient management approaches (57).

1.3.7 Pathogenesis/immunity mechanisms of CRS

As previously mentioned, CRS has been divided into two distinct forms: chronic rhinosinusitis with NP (CRSwNP) and chronic rhinosinusitis without NP (CRSsNP). These two distinct disease entities are characterised by the presence or absence of NP, as found on endoscopic examination. This division of CRS patient subsets lacks a complete insight into the underlying pathophysiological mechanisms of these diseases, with many studies investigating whether CRS patients would be better classified based on patterns of symptoms and/or inflammatory mediators (21,59,60).

While the exact mechanisms of CRS pathogenesis are still being elucidated, several trigger factors have been linked to its development (Figure 6). However, due to its multifactorial aetiology and complexity, it has been difficult to define the exact causal relationships of CRS which, in turn, has made identification of suitable treatment very problematic. To address this, researchers have started to focus on the role of specific inflammatory molecules in CRS pathological mechanisms.

A summary of these inflammatory mediators is presented in Table 5, as well as their cell source and biological activity (61,62).

Table 5: Cytokine functions that may be involved in nasal pathogenesis.

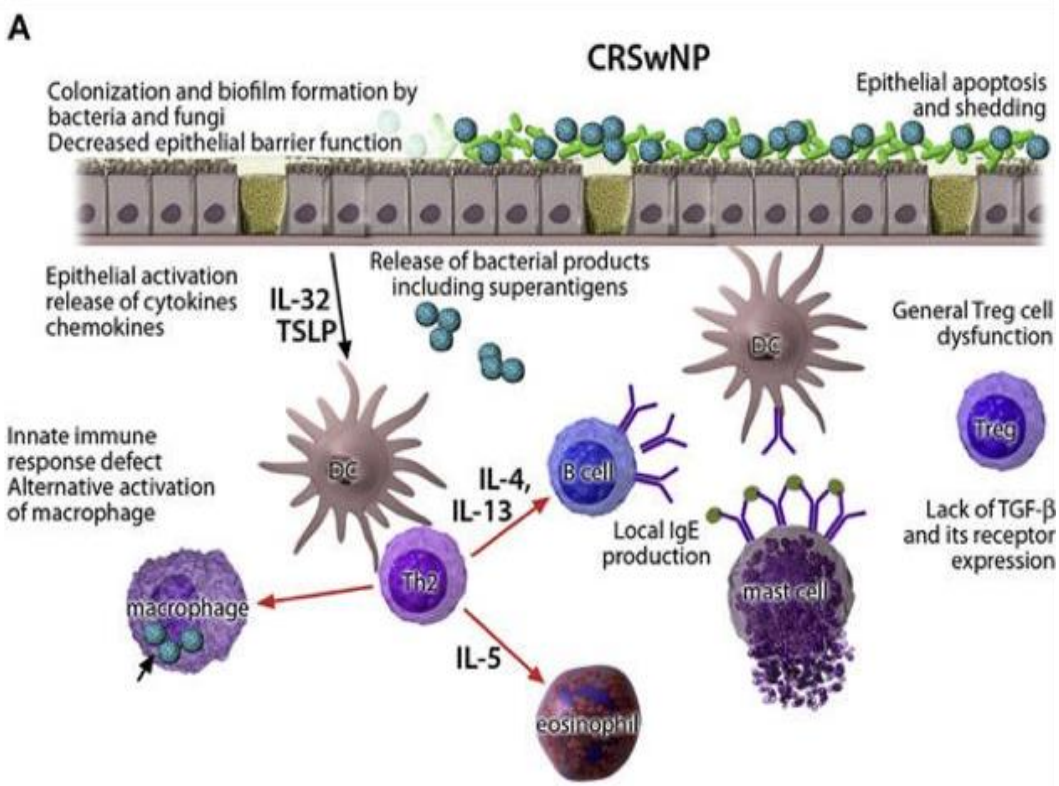
Cytokine	Secreted by	Targets and effects
IL-1	Monocytes, macrophages, endothelial cells, epithelial cells	Vasculature (inflammation); hypothalamus (fever); liver (induction of acute phase protein)
IL-4	T_H2 cells, mast cells	Promotes T_H2 differentiation; isotype switch to IgE
IL-5	T_H2 cells	Eosinophil activation and generation
IL-6	Macrophages, endothelial cells	Liver (induces acute phase proteins); influences adaptive immunity (proliferation and antibody secretion of B cell lineage)
IL-8	Monocytes, macrophages, fibroblasts, keratinocytes, endothelial cells	Mobilises and activates neutrophils; promotes angiogenesis
IL-9	T cells	Mast cell activation
IL-10	T_H2 cells, macrophages	Inhibits production of T_H1 cells and macrophage function
IL-13	T cells	Shares characteristics with IL-4 (isotype switch to IgE); growth factor for B cells
IL-15	T cells and epithelial cells	T-cell growth factor
IL-17A	T_H17 cells	Promotes neutrophil migration and differentiation.
IL-17E or IL-25	T_H2 cells, eosinophils, mast cells	(Pro-inflammatory cytokine) Promotes neutrophil migration and differentiation; promotes T_H2 responses (IL-5 and IL-3 production)
IL-33	Endothelial cells, fibroblasts, smooth muscle cells, keratinocytes	T_H2 development and activation of ILC2s
TNF-α	Macrophages, mast cells	Vasculature (inflammation); liver (induction of acute phase proteins); loss of muscle, body fat (cachexia); induction of death in many cell types; neutrophil activation
TGF-β	T cells, macrophages, other cell types	Inhibits T-cell proliferation and effector functions; inhibits B-cell proliferation; promotes isotype switch to IgA; inhibits macrophages
IFN-γ	T_H1 cells, CD8+ cells, NK cells	Activates macrophages; increases expression MHC class I and class II molecules; increases antigen presentation
G-CSF	Endothelial cells, fibroblasts, macrophages	Neutrophil production
RANTES	T cells, endothelial cells, platelets	Degranulates basophils; activates T cells

Several studies have indicated that these two phenotypes also have distinct immunological mechanisms (29,63,64). While CRSsNP is typically driven by a predominant T_H1 skewed inflammatory response (Figure 9B), a T_H2 response with an enrichment of eosinophils has been found to characterise many cases of CRSwNP (Figure 9A) (21,29,63–70). Recently, some studies have demonstrated considerable differences in the pathological and clinical features of CRS in patients with different racial backgrounds (64,71,72). More specifically, recent studies demonstrated that Asian patients with NPs showed a predominant T_H1/T_H17 response (64,73,74). Even so, there are conflicting reports regarding expression levels of specific mediators in CRSsNP, with some reporting a T_H1-driven inflammatory response and others indicating a T_H2 inflammation, as well as the simultaneous expression of different T_H types within a single phenotype (65,75).

An investigation into inflammatory biomarkers available within nasal tissue and/or mucus can provide insights into the pathophysiology of CRS that may drive the course of patient selection for treatment. Previously, CRS patients were divided into phenotypes according to their distinct clinical findings (NP) and their pathophysiology, so these have also driven treatment paradigms. However, patients from the same group did not respond equally to treatment and, as seen previously in this section, there may well be differing pathophysiology within each phenotype. Furthermore, the discovery of distinguishing inflammatory patterns could enable a more accurate characterisation of individuals with CRS. Such research resulted in the paradigm presented in Figure 5, where three types of immune response are presented. These mechanisms can be involved in CRS pathology by chronically activating one or more of these responses in the sinonasal tissue (20,76,77).

For these reasons, the new EPOS 2020 guidelines present a new model for CRS pathogenesis, which recognises that research interest

is being directed towards understanding the underlying molecular pathways and endotypes associated with CRS (Figure 10), leaving behind the complexity of the host-environment relationship. In the new model, the molecular pathways would be the result of numerous host-environment interactions and would lead ultimately to the phenotype observed in clinic.



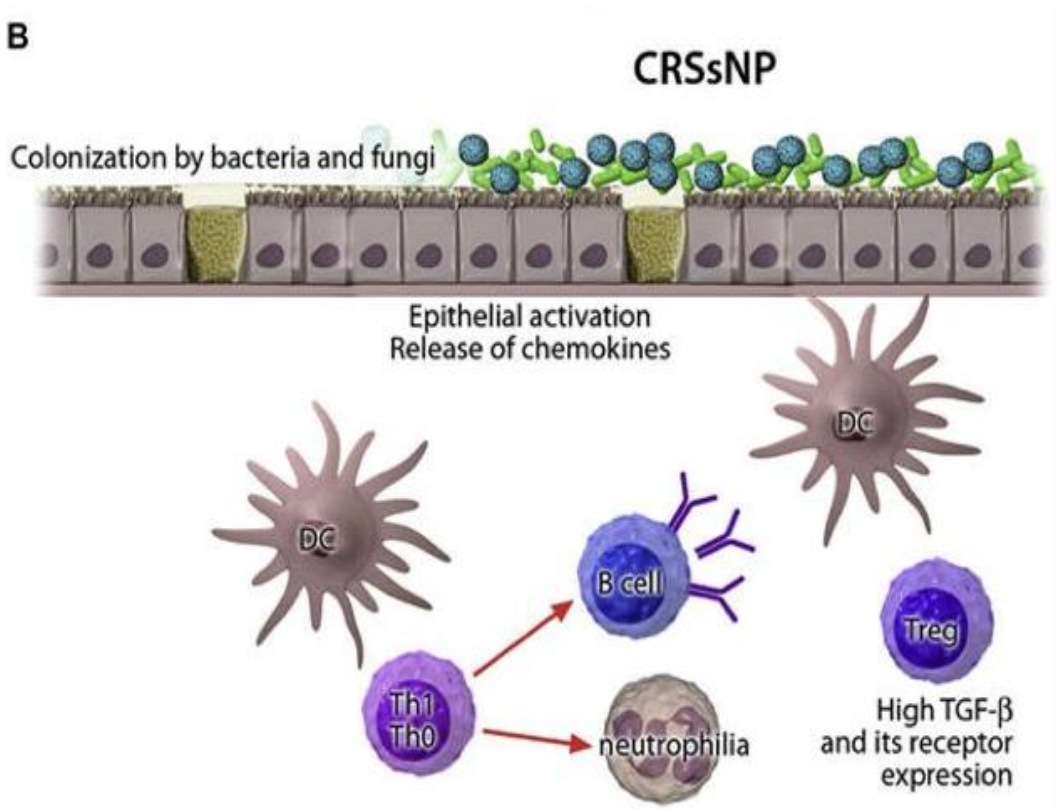


Figure 9: Current understanding of immunological events of CRS phenotypes. A. CRSwNP – T_H2-type microenvironment representing eosinophilia and IgE production. B. CRSsNP, where a T_H1 or T_H0 response prevails. Source Akdis et al. 2013 (21).

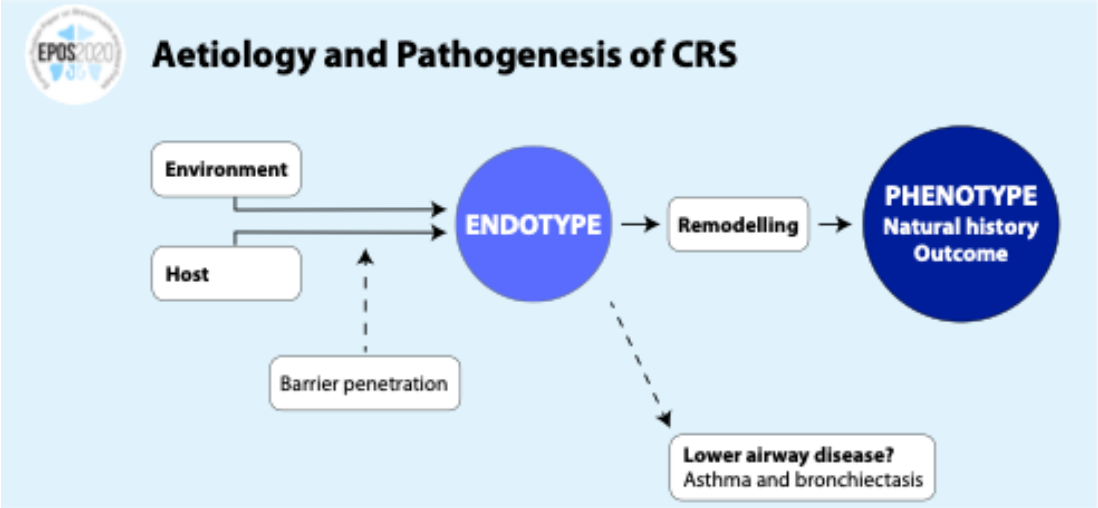


Figure 10: Aetiology and pathogenesis of CRS. Source: Fokkens et al. 2019 (20).

1.3.8 Systemic diseases with Chronic Rhinosinusitis as a common end point

CRS can also arise as an end point for other manifestations that may or may not affect the upper respiratory system. RS in these cases is a result of an underlying systemic disease process or condition that predisposes patients to the development of inflammation in the nasal tract.

Examples of secondary factors include aspirin intolerance (also known as Sampter's triad), immunodeficiency, primary ciliary dyskinesia, and cystic fibrosis. Another common cause is granulomatosis with polyangiitis, which is a systemic vasculitis disease of the small arteries, capillaries, and venules. It can result in nasal disease if granulomas are present in the nasal cavity (78).

The systemic factors associated with the development of CRS are more extensively presented in Table 6 (5).

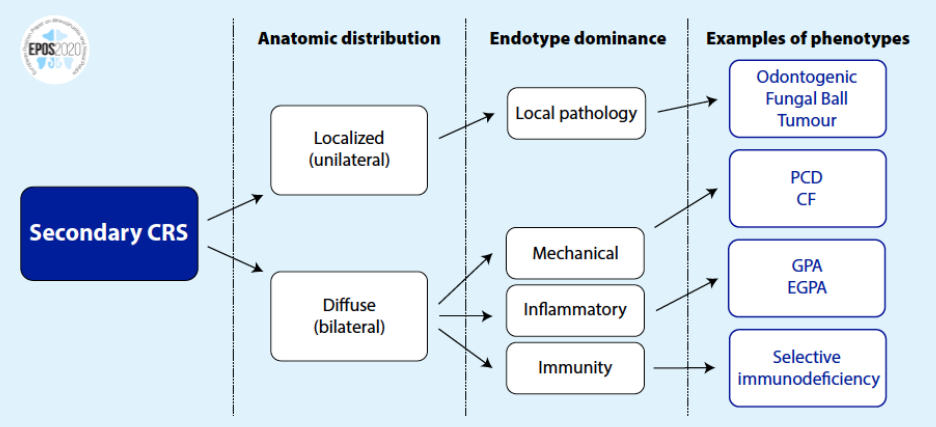
Table 6: Factors predisposing to CRS as a secondary manifestation. Source: Brook 2006 (5).

Secondary factors	
Autoimmune	Sarcoidosis Granulomatosis with polyangiitis
Immune deficiency	HIV Iatrogenic Hypogammaglobulinemia
Allergies	Inhalant/food allergy
Mucociliary disorders	Primary ciliary dyskinesia Cystic Fibrosis
Asthma	ASA intolerance
Metabolic disorders	Diabetes mellitus
Hormonal imbalance	Hypothyroidism Pregnancy

As described previously in section 1.3.4, EPOS 2020 guidelines updated the classification of CRS. This new classification divides

CRS into primary and secondary forms. In here, we investigate the secondary classification in more detail (Figure 11). Secondary CRS would derive from the manifestation/evolution of another systemic disease. As for primary CRS, this form of CRS will be further split into localised and diffused disease based on anatomic distribution, considering inflammatory endotype dominance, which will ultimately define the phenotype of CRS.

Figure 1.2.2. Classification of secondary CRS (Adapted from Grayson et al[154]).



CF, cystic fibrosis; EGPA, eosinophilic granulomatosis with polyangiitis (Churg-Strauss disease); GPA, granulomatosis with polyangiitis (Wegener's disease); PCD, primary ciliary dyskinesia.

Figure 11: Classification of secondary CRS. PCD, primary ciliary dyskinesia; CF, cystic fibrosis; GPA, granulomatosis with polyangiitis (Wegener's disease); GPA, eosinophilic granulomatosis with polyangiitis (Churg-Strauss disease). Source: Fokkens et al. 2019 (20).

1.4. Biomarkers in respiratory diseases

1.4.1 Biomarker definition

Genetic and epigenetic patterns associated with inflammation of the upper respiratory system point to useful biomarkers. The term 'biomarker' has evolved and is now used to define any biologic measurement that could examine or predict underlying physiologic processes or responsiveness to a therapeutic intervention. Biomarkers, broadly defined, are measurable indicators of biological processes, or pharmacological responses to a therapeutic intervention (79,80). A good biomarker should be precise and reliable, and it should be able to distinguish between "normal" and

pathological states. Measurements of biomarkers are traditionally limited to the detection of specific protein ligands in the body fluids and tissues that become altered as a consequence of a biologic or pathologic process. A biomarker may serve different roles such as diagnosis of a disease, severity and/or risk assessment, prediction of drug effects and monitoring of a state or condition (79–82). In other words, biomarkers will help to define populations, predict disease course, improve drug development, monitor the effects of therapy or adverse events, predict clinical outcomes and even identify new biological pathways involved in the disease's pathology, which ultimately will increase knowledge and lead to the identification of new treatments (83).

In addition, an ideal biomarker should demonstrate a clear relationship with a pathophysiological characteristic of a disorder, be reliable and reproducible, inexpensive, sensitive to the desired disease with predictive positive and negative values and measurable changes. Moreover, to be accepted by patients and used widely, its sampling should be simple and easy (83–87).

As a result, it is expected that this approach will enable a better understanding of chronic airway diseases, the identification of relevant disease features and the development of personalised management plans for individuals, which will ultimately improve QOL in respiratory conditions.

1.4.2 Development of biomarkers for respiratory conditions

In clinical practice, the development of biomarkers follows two main approaches. The first one investigates biomarkers for their predictive power, which can help to establish a diagnosis, the stage of the disease and its progression, or even predict responses to treatment. The second approach uses an unbiased 'omics' screening approach that helps to identify adequate biomarkers for both clinical and research applications (84,87).

Biomarker discovery has become an area of growing interest and investigation in recent years, particularly in identifying and attempting to validate new relevant biomarkers in chronic respiratory conditions. Any suitable biomarker to be used in airways disorders should be clinically relevant for the ongoing inflammatory processes or physiological functions of the respiratory system, or have a relationship with the disease progression/regression and the impact of these changes on patients' clinical state and QOL (83).

The first respiratory diseases for which biomarkers have been developed for disease management were asthma and COPD, due to their high burden worldwide. The development of such biomarkers has proved to be extremely useful in the management of asthma, COPD and AR, among others. Therefore, new efforts aim to extend this to other chronic respiratory diseases such as CRS (47,83,84). Moreover, the recognition of airway disease phenotypes and endotypes, assisted by the identification of suitable biomarkers has led to the recognition of respiratory syndromes in asthma and COPD.

Key biomarkers are described in the literature as potential biomarkers for these disorders such as eosinophils, neutrophils, FeNO (fractional exhaled nitric oxide), MPO (myeloperoxidase), ECP (eosinophil cationic protein), matrix metalloproteinase-9/ TIMP (tissue inhibitor of metalloproteinase) ratio, IL-8, H_2O_2 (hydrogen peroxide), Cys-LT (cysteinyl leukotriene), eNO and nNO (exhaled and nasal nitric oxide, respectively). Both eosinophils and neutrophils are validated biomarkers for asthma and COPD in drug development and clinical setting. Their numbers relate to asthma severity and persistence, and eosinophils are also predictors of clinical response to corticosteroids and may predict exacerbations. For this reason, ECP and MPO are also thought to be predictive biomarkers in airway disorders. Although ECP correlates with eosinophils numbers, MPO does not show any relationship with neutrophil levels. Another airway biomarker example is NO which can be measured as FeNO, eNO or nNO. First, FeNO is related to

airway inflammation and likewise it is estimated to predict response to treatment and poor treatment compliance, being used for adjusting treatment in asthma. It is described as high in COPD exacerbations, and possibly when asthma is associated with COPD. Furthermore, eNO could be used for clinical monitoring and early drug development in (allergic) asthma. Finally, nNO has been described as decreased in nasal blockage and NP, and it can be indicated for these particular features. Moreover, Cys-LTs, IL-8 and H_2O_2 were described as being correlated with more severe asthma. Finally, MMP-9/TIMP ratio can be used for monitoring the effects of interventions (81,83,84,87).

In summary, several biomarkers are now available to help individualise treatment for airway diseases, particularly in situations where the disease is severe and irresponsive to therapy. In order to help improve knowledge on immune or allergic pathophysiological mechanisms, and ultimately improve management of airway disorders, biomarkers will need to predict clinical phenotypes and treatment responsiveness in patients.

1.4.3 Biomarkers in CRS

There has been extensive research on biomarkers in CRS. As described in the previous section, the description of an endotype may rely on biomarkers (88,89). Several biomarkers like eosinophils, IgE, and some cytokines, such as IL-5 and IL-13, were described as correlated with the presence of NP. However, there is still no clear relationship between disease events (e.g., severity) and these biomarkers.

CRS occurs in the nose and paranasal sinuses, and therefore, nasal specimens are of extreme importance for the characterisation of its pathophysiological mechanisms. These specimens can be used for measurements of various soluble markers and cell populations by flow cytometry analysis, PCR studies and protein analysis.

Recent studies have revealed several mechanisms that are important in CRS pathogenesis (as seen before). Thus, CRS biomarkers can be used for the following purposes: characterisation of immune mechanisms in CRS; identifying medical therapies; and tracking response to treatment.

This thematic will be discussed in more detail in the next chapters.

1.5. Hypotheses

Nasal tissue contains biomarkers of disease that have yet to be discovered and utilised. A better understanding of inflammatory patterns in CRS may help to refine the classification of sub-groups of patients. This information could be used to develop more specific treatments which target inflammatory pathways that contribute to disease pathogenesis.

This work proposes that by using a liquid bead array approach, with small sample requirements, it will be possible to discover biomarkers of CRS disease and compare them to healthy control populations. Furthermore, these biomarkers may be investigated and further validated in CRS subtypes. By assessing biological and clinical parameters in a CRS cohort, we expect a clearer definition of potential disease endotypes, their relationship to the clinical phenotypes and subsequent management of such groups.

1.6. Aims

CRS is characterised by variable and complex clinical presentation and pathophysiological mechanisms. The identification of immunological subtypes of CRS in individual patients is an important issue, allowing for more focused therapies in the management of CRS. The present thesis was designed to characterise more extensively the inflammatory signatures profiles of CRS patients by assessing biological and clinical parameters. This will allow a clearer definition of potential disease endotypes, their

relationship to the clinical phenotypes and subsequent management of such groups.

The specific aims were to:

1) Investigate the clinical features associated with pre- and post-operative compliance in CRS patients.

2) Search for biomarkers for differential diagnosis (of disease endotypes) leading to subgroup allocation, disease activity, and response to treatment.

3) Define immune profiles in different clinical subtypes of CRS.

4) Identify immune profiles that may predict the likelihood of disease control including compliance with medical treatment after surgical intervention.

5) To test the in vitro effect of clarithromycin on cytokine responses in epithelial cell cultures, that could help to guide CRS subgroups management.

The objectives of this thesis were:

- To provide an overview of the upper respiratory system and its immune response mechanisms, including description of CRS, diagnosis- and treatment-related challenges, and the potential of biomarker-related characterisation of CRS.
- To provide an insight into the problem of medical compliance within a retrospective and prospective cohort of CRS patients.
- To search for possible CRS biomarkers through a literature review.
- To define the rationale underlying the recruitment process and techniques selected to address the research aim.
- To validate a standardised protocol to be used in this work, and to determine sample collection guidance for participants in the MACRO trial.
- To detail the results of immune marker expression according to CRS phenotype-based classification.

- To detail the identified immunological characteristics of CRS patients using the clustering method, and relate those with clinical features, to fully characterise CRS clusters.
- To explore the use of macrolides in cell culture and its action on the immune system.
- To discuss this work in the context of the literature, the strengths and limitations of this thesis, future directions for research and concluding remarks.

II – MEDICAL COMPLIANCE

2.1 Introduction

According to the World Health Organisation (WHO), compliance is defined as the extent to which a person's behaviour corresponds to agreed recommendations from a health care practitioner (90). Medication compliance can be defined as compliance to a prescribed treatment or following the instructions given by a health care practitioner, in accordance with the prescribed interval, dosage, and frequency (91–93). Compliance with medical treatment is a very important issue in medical care since failure to comply with recommended treatments results in a considerable burden to health care systems and health practices, as well as poor medical outcomes (91,94). Therefore, compliance is a key factor in the effectiveness of medical therapies, but it is particularly critical in the treatment of chronic conditions (93,95).

CRS is characterised by a chronic inflammation of the nose and the paranasal sinuses that persists for more than 12 weeks without resolution (23). It is a common health problem affecting approximately 11% of the population (32) with significant morbidity and comorbidity, medical costs and an impact on general health (23,25,26,96). In the UK, it affects about 10% of the population, who then seek treatment from their GPs. They attend an average of four appointments per year and are prescribed a variety of different drugs. Current guidelines recommend ESS as the next step for those who fail medical management alone; however, many patients experience revision surgery after a period of time (97). As a result, CRS is associated with a substantial incremental increase in health care utilisation and expenditures of approximately US$8.6 billion due to increases in office-based costs and prescription expenditures each year. Due to the prevalence and expense of CRS, continued efforts should be directed at determining cost-effective strategies for diagnosis and therapy (98).

Unfortunately, the compliance to treatment regimens in CRS remains persistently low (93). In developed countries, compliance to other chronic illness regimens is low, being around 50% (90,92,99,100). An underlying assumption in most studies is that non-compliance is associated with poorer clinical outcomes (93,101). To improve patient compliance, it is important to evaluate any factors that may lead to non-compliance (99).

The aim of this chapter is to evaluate compliance with and the effectiveness of prescribed treatment in patients with CRS before and after ESS. The secondary study objective is to identify the demographic and clinical factors associated with patient compliance to CRS medication.

2.2 Methods

2.2.1 Population

This study population consisted of adult patients meeting the CRS diagnostic criteria outlined in the European Position Paper on Rhinosinusitis and Nasal Polyps 2012 definition (as was relevant at the time of recruitment) (23) and who had been selected to undergo ESS surgery. Two different databases were used for this analysis; retrospectively recruited patients gave information about post-operative compliance and the prospective cohort allowed the researcher to investigate the pre-operative compliance to medical treatment.

The first population included patients retrospectively recruited who attended the James Paget University Hospital (JPUH) between 2012 and 2016 to undergo ESS and had consented to donation of nasal/sinus tissue during the procedure. All other cases undergoing ESS during this period were excluded. Unfortunately, from the original tissue donors, two had died in the meantime. A sample of 108 out of a total of 172 people agreed to participate and returned the questionnaire. After careful examination of the diagnosis for the included participants, one was withdrawn due to a diagnosis of right

ethmoid fungus ball (mycetoma). Therefore, a total of 107 patients was analysed.

For the second population, patients were recruited for a biomarkers study prospectively between 2016 and 2018. After a careful analysis of their medical records, 6 patients were withdrawn from the analysis. A total of 76 patients was finally included. Both populations' recruitments are shown in Figure 12.

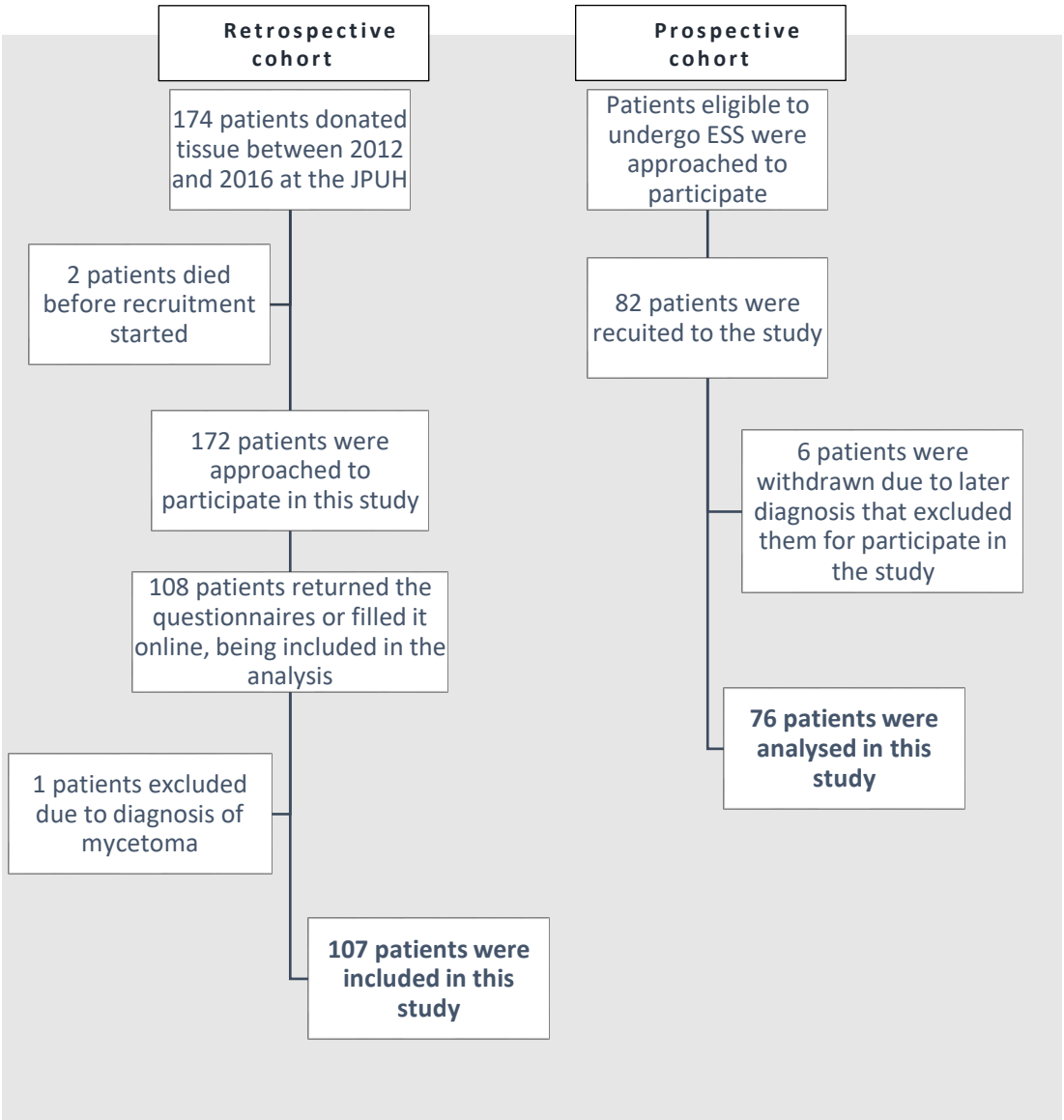


Figure 12: Recruitment of patients for both populations. Flowchart demonstrating the recruitment of patients to both retrospective and prospective populations.

2.2.2 Data extraction

Data were extracted from the hospital electronic medical record system and entered into a standardised collection form. All outpatient clinic visit notes were reviewed. All patients were asked the same 25 questions regarding their demographic characteristics (age, gender, ethnicity), smoking and allergy status, family and CRS history, comorbidities, and the type of treatment they were prescribed with, including their perceptions of treatment and compliance. This questionnaire was study specific and design to collect variables. Any missing data from the questionnaire were filled in, in accordance with the patients' medical notes. If treatment information was not available, treatment was defined as not taken or not useful. In order to fully describe this population, clinical data consisting of pre-operative Lund-Mackay CT scores, SNOT-22 (Sino-Nasal Outcome Test, pre- and post-surgery) were also collected from patients' medical records when available.

The study population was divided into three different groups according to their compliance with the prescribed medication: adherents (A), non-intentionally non-adherents (NINA) and intentionally non-adherents (INA).

The NINA group included participants who did not comply with the prescribed treatment (time or dosage), while participants who admitted to stopping medical therapy deliberately were integrated into the INA group.

2.2.3 Statistical analysis

Statistical analyses were performed using RStudio (version 1.1.383, RStudio, Inc.). For continuous variables, the results were expressed as means and standard deviation, or in box plots. Data distribution was tested for normality using the Shapiro-Wilk test. For dichotomous variables, frequencies and percentages were calculated. In univariate analyses, categorical analyses were compared between adherents and non-adherents using the Chi-

squared test. The distribution of demographic and clinical variables was examined between the different adherent groups. Regression analysis explored the possible relationship between age, time suffering from CRS symptoms, Lund-Mackay pre-operative CT scan, and SNOT-22 (pre- and post-operative) score. A p-value less than 0.05 was considered statistically significant.

2.3 Results

2.3.1 Patients' characteristics

There were 174 patients who underwent ESS between 2012 and 2016 and consented to donate sinonasal tissue to the tissue bank. Only 108 out of 172 participants (62.7%) returned the questionnaire. A total of 107 patients were included in this study (Figure 12). Of these, 61% were male with a mean age of 61 years old, and 79% had another chronic disease.

The second cohort consisted of 76 patients, with a mean age of 53 years old. Forty-five were male and 40 presented some type of comorbidity.

The characteristics of both populations are shown in Table 7.

Table 7: Demographic and clinical characteristics. P-value reflects the association between variables and self-reported compliance.

	Prospective population (n=76)	p-value	Retrospective population (n=107)	p-value	Total (n=183)	p-value
Age (mean±SD)	53.25±14.70	0.44	61.07±9.99	0.26	57.82±12.73	0.37
Male/Female	45/31	0.04*	65/42	0.67	110/73	0.12
Ethnicity		0.65		0.17		0.64
White British	70		100		170	
White Irish	0		2		2	
African	1		0		1	
White and Black African	0		1		1	
Indian	1		0		1	
Caribbean	0		1		1	
Any other Ethnic Group	1		0		1	
Any other Asian background	1		0		1	
Other	2		2		4	
Do not wish to disclose	0		1		1	
Time suffering from CRS, years (mean±SD)	23.25±18.51	3.61e-5*	25.39±13.48	0.39	24.50±15.75	1.20e-12*

Table 7: Demographic and clinical characteristics. P-value reflects the association between variables and self-reported compliance (cont).

	Prospective population (n=76)	p-value	Retrospective population (n=107)	p-value	Total (n=183)	p-value
Family history (%)	21.1	2.65e-15*	29	0.03*	25.7	<2.2e-16*
Smoking (%)	6.6	0.23	3.7	0.12	4.9	0.16
Nasal allergy (%)	39.5	0.002*	60.8	0.68	51.9	1.92e-8*
NSAIDs (Inc. possible, %)	6.6	6.52e-5*	16	0.38	12	4.33e-11*
Comorbidities (%)	52.6	7.61e-7*	78.5	0.09	67.8	2.58e-16*
Under treatment (when answering)	66		107		173	
Lund-Mackay Score	16.34±6.69	0.20	17.96±5.91	0.29	17.38±6.22	0.11
SNOT-22 pre-operative	55.16±20.35	0.81	46.30±24.72	0.57	49.53±23.54	1.00
SNOT-22 post-operative	23.73±16.93	0.12	16.23±14.34	0.65	18.02±15.27	1.20e-7*

* $p<0.05$

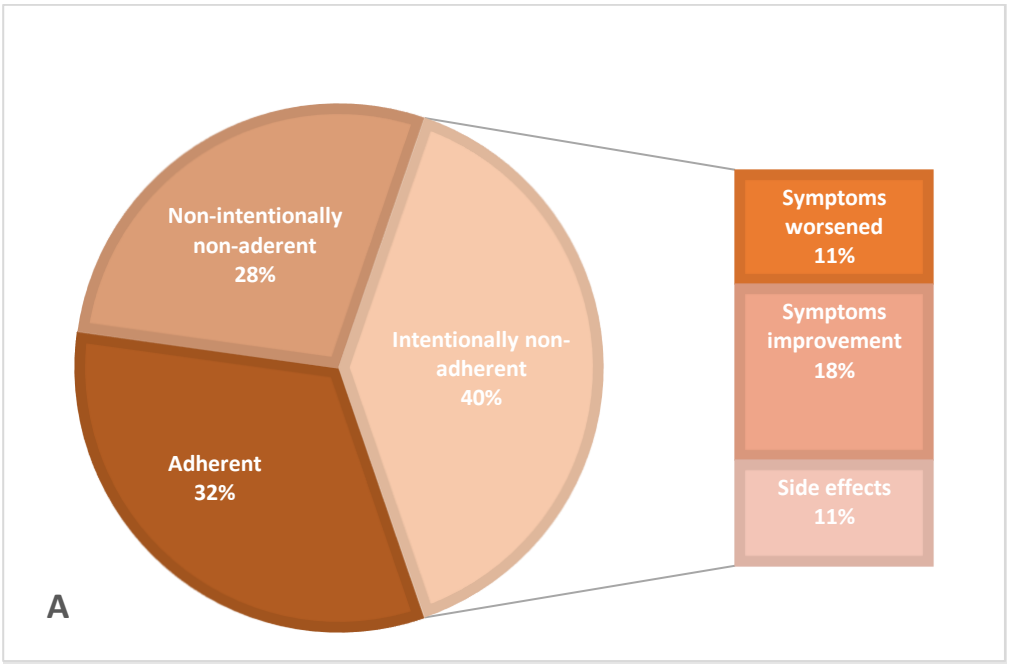
Variables are presented as either count data or means and standards deviations.

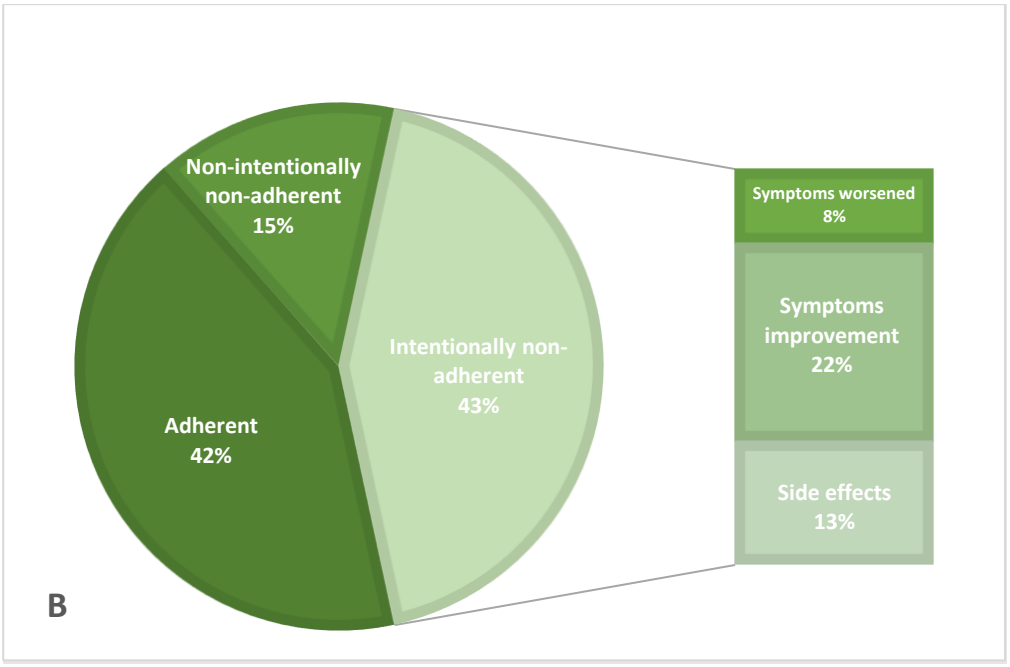
2.3.2 Prevalence of medication compliance

Full compliance to their drug regimen was reported by 68 patients (37%). Non-compliance to their physician's recommendations was described by 115 participants (63%). The reasons for non-compliance included participants who did not comply with the prescribed treatment (time or dosage) (NINA, n=43) and participants who admitted to stopping medical therapy deliberately (INA, n=63). Intentional non-compliance was associated with at least one of the following reasons: improvement of symptoms, symptoms worsened despite medication or side effects experienced during usage.

The most common reason among these participants to stop medication was improvement of symptoms, followed by side effects and deterioration of symptoms; Figure 13 shows their distributions per population, retrospective and prospective recruited patients (Figures 13A and 13B, respectively), and within the total population (Figure 13C).

The results shown in Figure 14 relate to the type of prescribed medication. Steroid nasal spray and sinus rinse showed a mixed response with half of the patient taking it and finding it helpful, and the other half not. Antibiotics were described as the least helpful of all treatments, being also one of the most widely prescribed treatments.


Non-intentionally non-aderent 28%
Intentionally non-adherent 40%
Adherent 32%
Symptoms worsened 11%
Symptoms improvement 18%
Side effects 11%
A


Non-intentionally non-adherent 15%
Intentionally non-adherent 43%
Adherent 42%
Symptoms worsened 8%
Symptoms improvement 22%
Side effects 13%
B

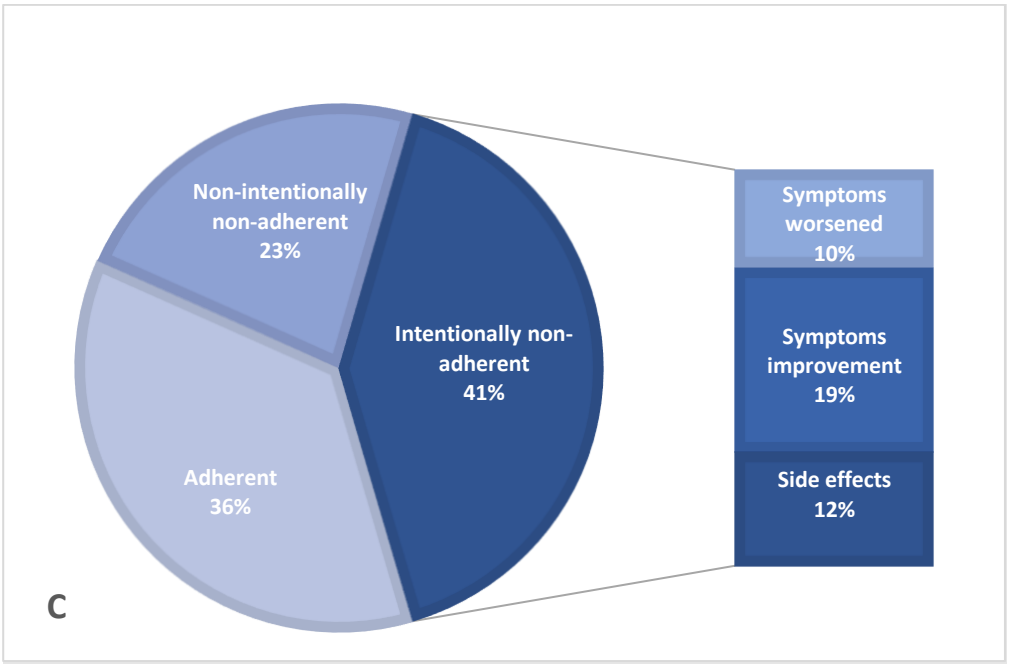
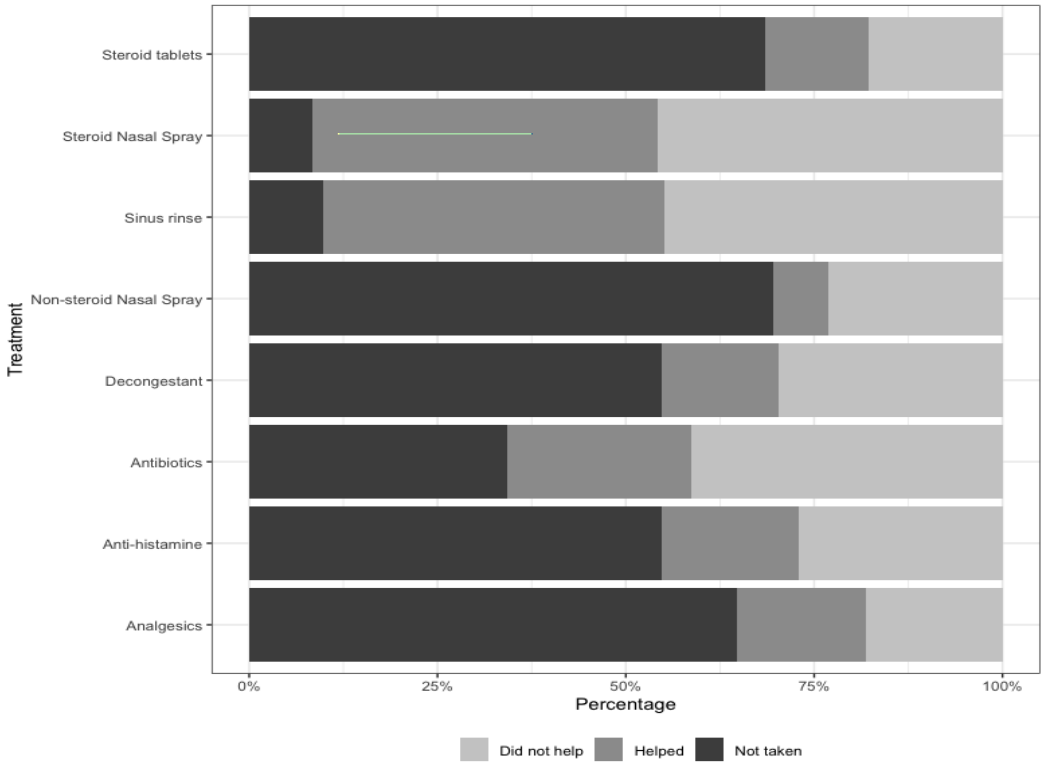
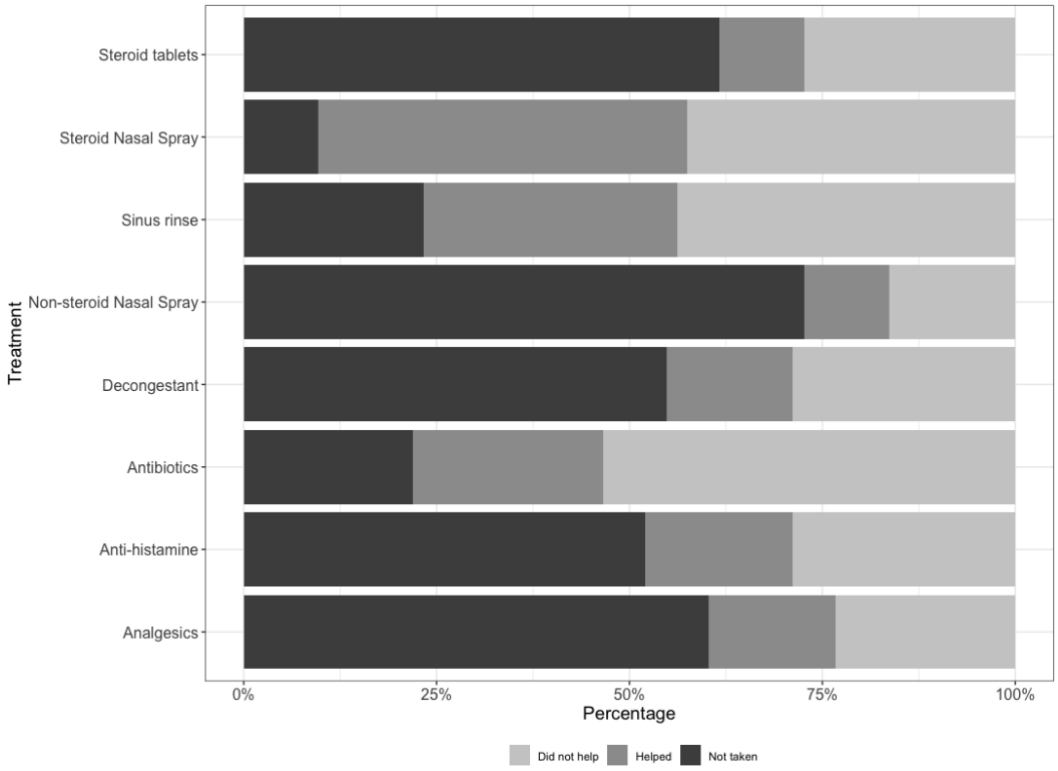


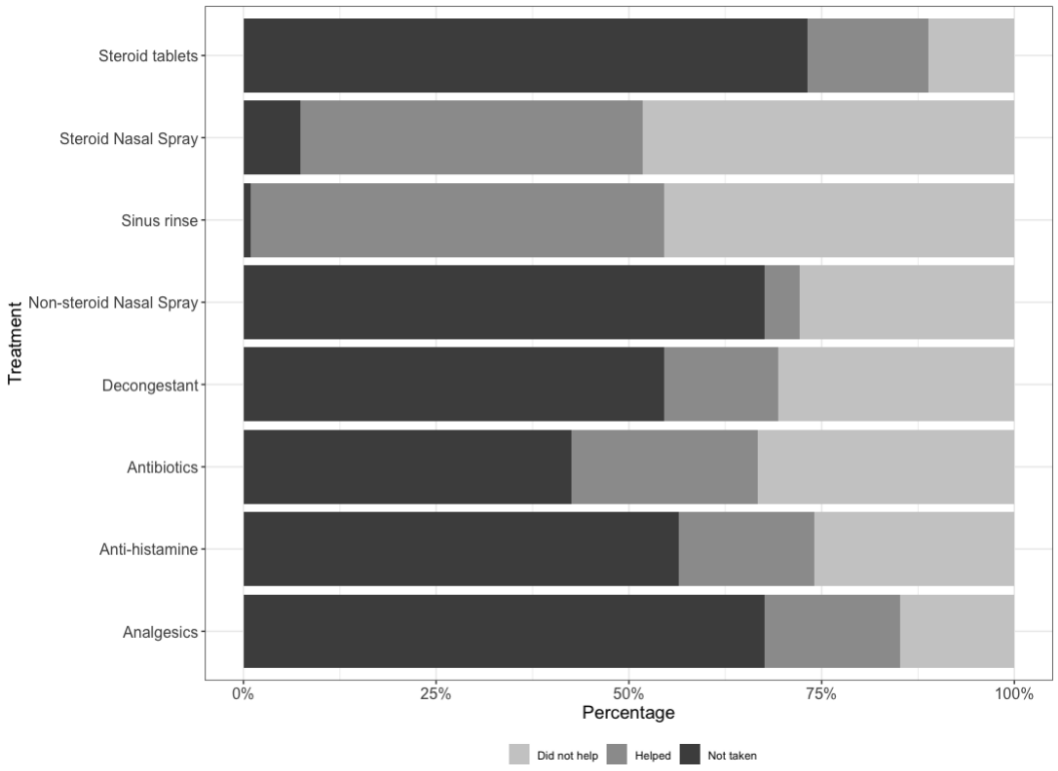
Figure 13: Distribution of compliance with physician's recommendations. The bar shows the reasons to intentionally not comply with recommendations and their correspondent percentages. Each pie chart shows the results correspondent to each population: A, population retrospectively recruited; B, population prospectively recruited population; C, results for the total patients recruited. Missing data was not included.



A.



B.



C.

Figure 14: Patient's perception of CRS medication. Representation of the percentage of use and patients' perception of usefulness of the different prescribed treatments for CRS: A- total number of patients included in this analysis; B- prospective recruited population reflecting

pre-operative compliance; C- retrospective recruited population reflecting post-operative compliance.

2.3.3 Association between compliance and clinical history or predictors of compliance

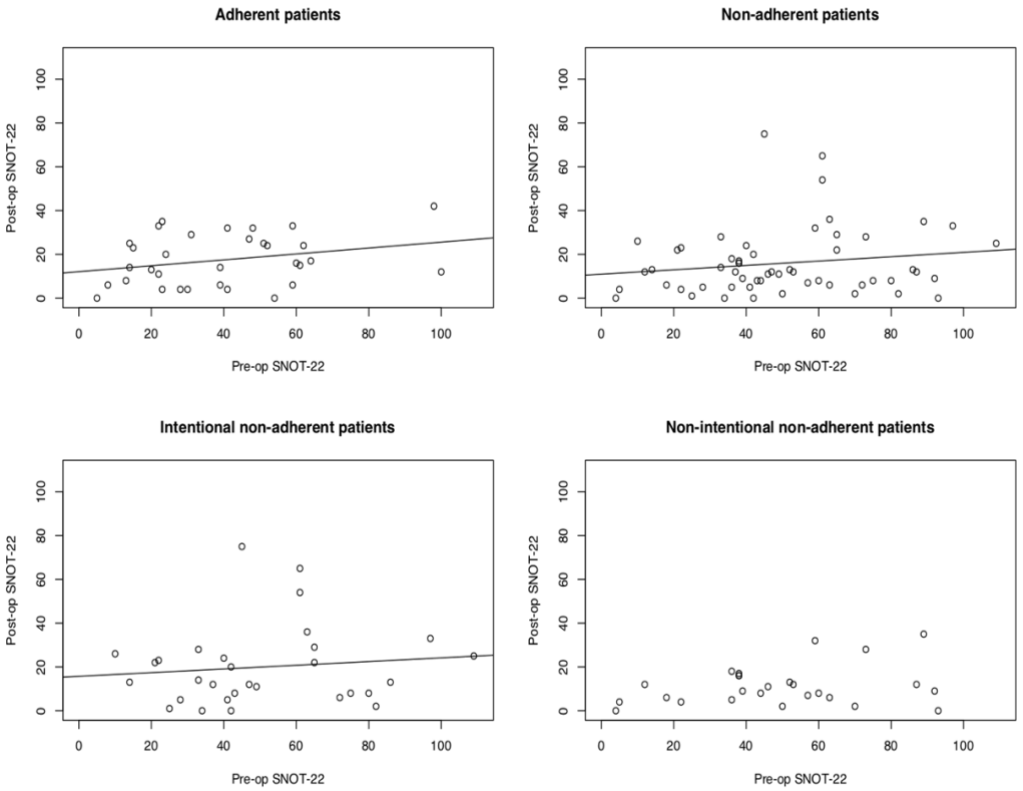
The associations between the demographic and clinical characteristics of the responders and non-compliance are shown in Table 6. When analysing the entire population of this study, several factors are shown to have an association with overall medical compliance. These included time suffering from the disease, the presence of nasal allergy, NSAIDs or other comorbidities, and post-operative SNOT-22. Analysis across the different groups demonstrated that the duration of suffering from CRS symptoms was approximately the same in all three groups. Furthermore, age, pre-operative Lund-Mackay scores and pre- and post-operative SNOT-22 scores had a non-significant distribution between groups (Table 8). According to these results, INA patients presented a lower Lund-Mackay scores, higher QOL scores both pre- and post-surgery, and they had suffered from CRS for longer. In contrast, patients in group A had a higher age, shorter disease duration, lower pre-operative SNOT-22, a mean LM score of 17 and a post-operative SNOT-22 of 18 points.

Table 8: Distribution of continuous variables across compliance groups in the whole population. The results correspond to mean±SD.

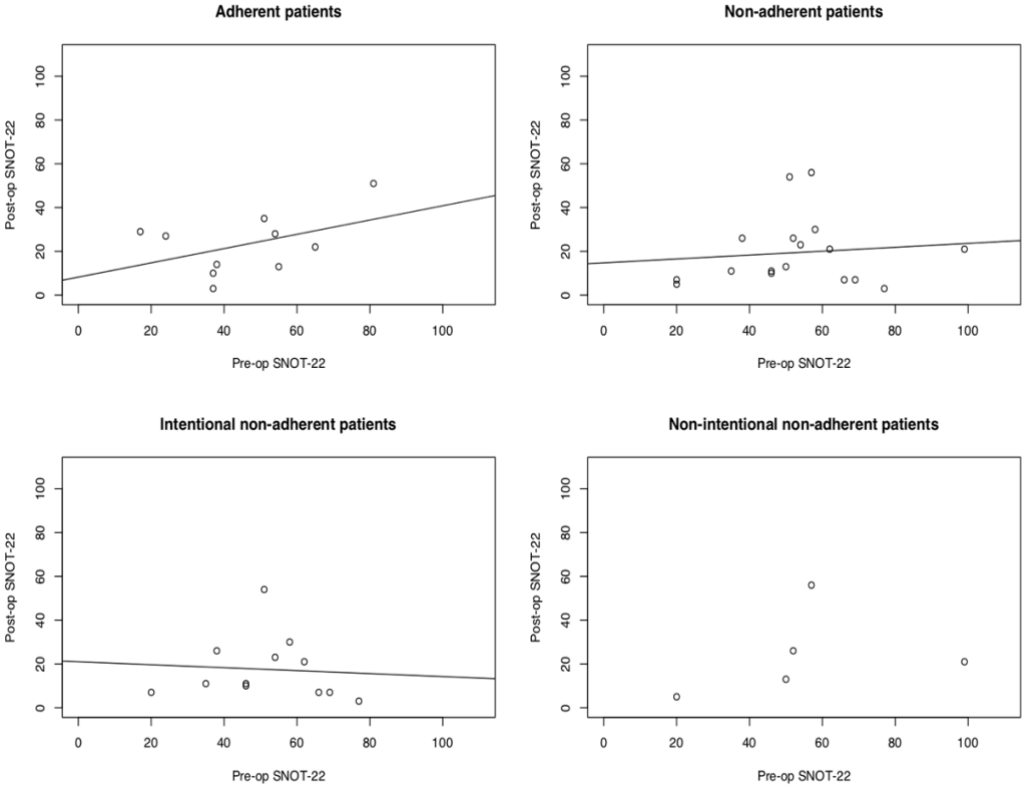
	A	NINA	INA	p-value
Age	58.05±14.59	56.91±11.31	57.62±11.66	0.899
LM	17.42±6.40	18.97±4.72	16.18±6.89	0.113
SNOT-22 pre-op	44.60±23.39	51.69±25.57	51.68±22.42	0.242
SNOT-22 post-op	18.41±12.24	12.71±11.72	20.20±18.10	0.07
Time suffering from CRS (months)	24.54±16.46	25.72±14.93	26.98±14.04	0.658

A – adherent; INA – intentionally non-adherents; NINA – non-intentionally non-adherents; SNOT-22 preop – pre-operative SNOT-22 score; SNOT-22 postop – post-operative SNOT-22 score.

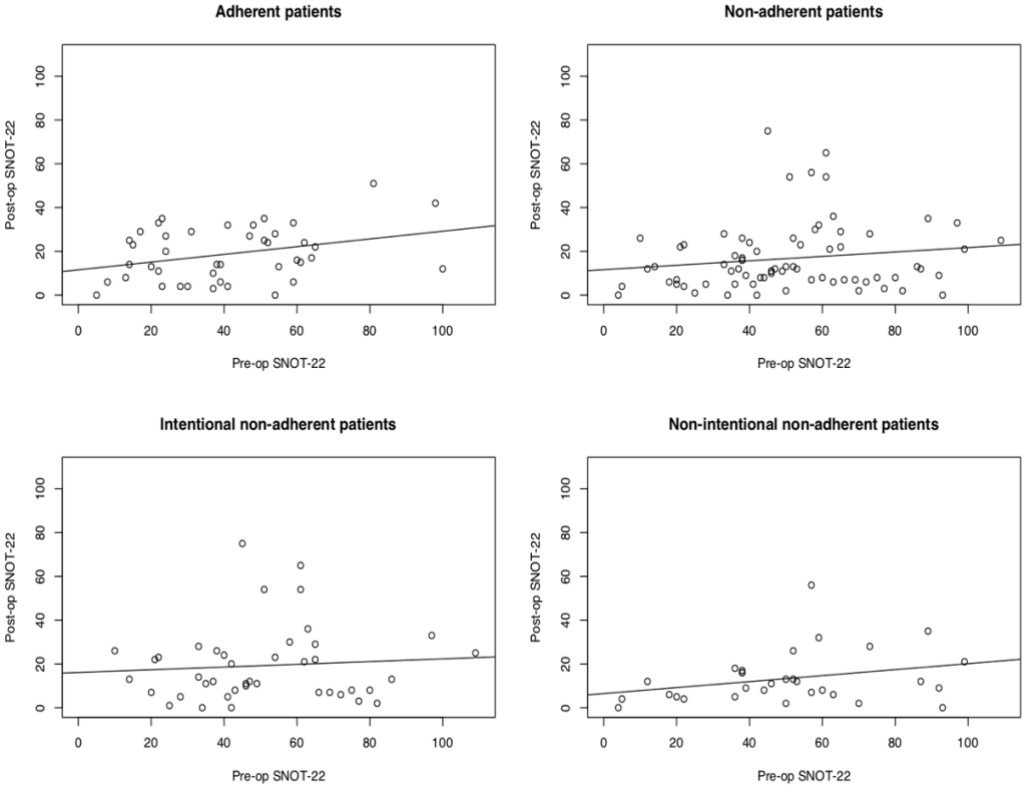
Overall, the SNOT-22 score improved in all groups and no correlation was found between the SNOT-22 scores before and after ESS in any of the groups, suggesting that pre-operative SNOT-22 did not predict the post-operative SNOT-22 score (Figure 15). Further exploration of the SNOT-22 scores across the three groups after ESS revealed the following findings. Patients from group A were all discharged by the 7th visit, while the NINA and INA groups continued to be seen in the outpatient clinic for longer periods (at least until the 11th visit). The plots of the post-operative SNOT-22 scores by group over time are presented in Figure 16. Overall, NINA patients showed a lower mean SNOT-22 that slowly increased one year after surgery, while group A showed a stable post-ESS SNOT-22 mean over time. Lastly, INA patients experienced the worst deterioration of symptom scores after ESS. Although the mean score for INA patients was lower than that for A patients at 3 months post-op, this deteriorated to be the highest mean SNOT-22 score of the 3 groups at 12 months.


Adherent patients
Non-adherent patients
Intentional non-adherent patients
Non-intentional non-adherent patients
Post-op SNOT-22
Pre-op SNOT-22
0
20
40
60
80
100

A.


Adherent patients
Non-adherent patients
Intentional non-adherent patients
Non-intentional non-adherent patients
Post-op SNOT-22
Pre-op SNOT-22
0
20
40
60
80
100

B.



C.

Figure 15: Associations between pre-operative and post-operative SNOT-22. Correlations between pre- and post-operative SNOT-22 among adherent and non-adherent patients (INA and NINA groups) to prescribed medication after ESS. A-total; B-prospective; C-retrospective population.

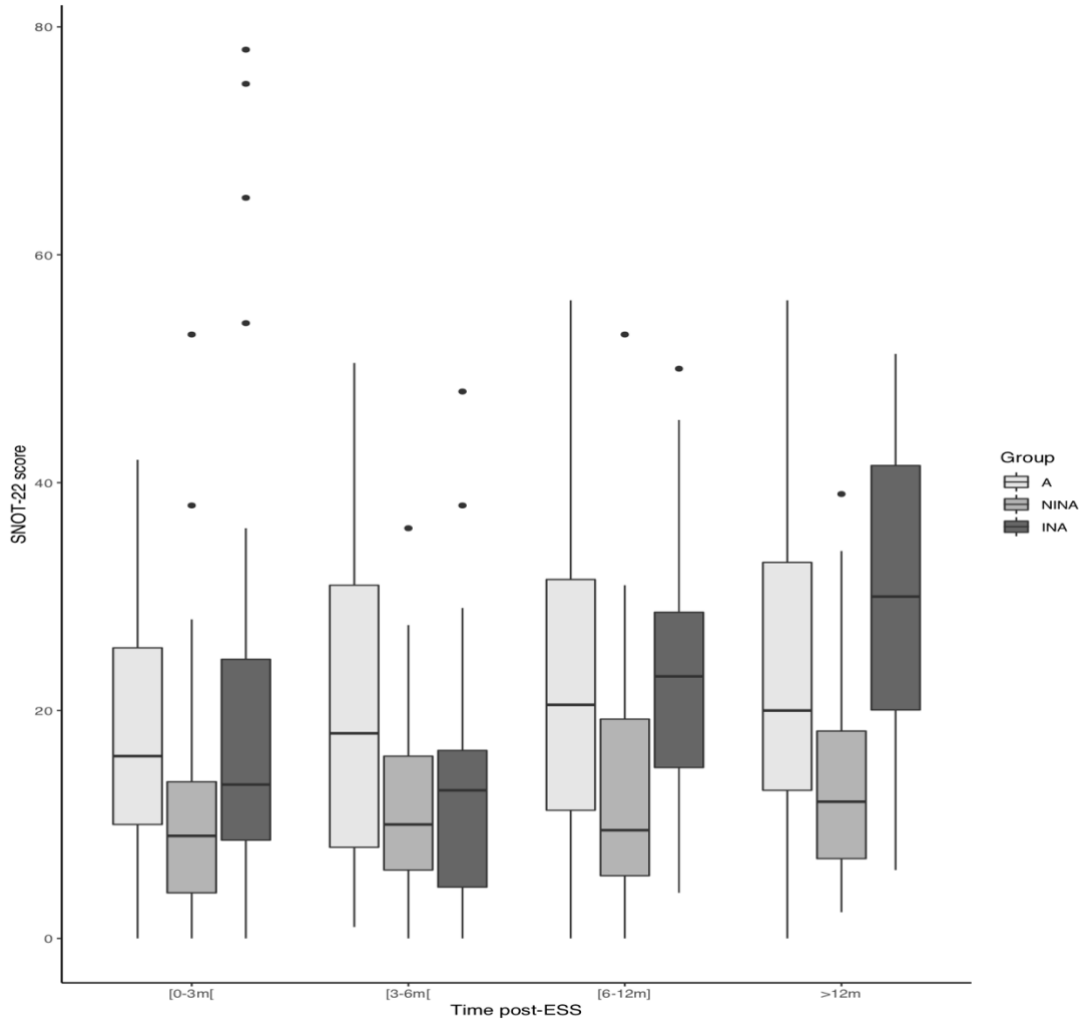


Figure 16: Evolution of SNOT-22 scores. Change in SNOT-22, across the different group of patients, following ESS. Only SNOT-22 scores from retrospective recruited patients are presented. A- Adherent patients, NINA – Non-intentionally non-adherent patients, INA – Intentionally non-adherent patients, m - months.

2.4 Discussion

Compliance to medical treatment post-operatively is a key component of CRS patient management following ESS. The percentage of low compliance to treatment was high in the study participants (65%). Therefore, post-surgical compliance in patients with CRS needs to be improved; however, the key to doing this is to understand and address any factors that drive this poor compliance. These findings are in keeping with reports from other studies on asthma, COPD and other chronic respiratory illnesses (102–105).

However, there is little evidence for compliance in post-operative CRS patients (28,106–111).

Furthermore, in this study, many factors were found to have a statistically significant association with compliance to pre-operative compliance. On the other hand, only family history was found to be associated with post-operative compliance. It seems that older people are more adherent to prescribed medical therapy, perhaps due to other age-related comorbidities or disease complications (112), or simply due to maturity. Therefore, during patient counselling, an emphasis should be placed on the risks of poor compliance to medication in all age groups. Since no other demographic or clinical factor could be associated with poor compliance, further studies need to be conducted to examine a wider range of factors and identify those that may influence compliance to post-ESS medical therapy, such as socioeconomic status and education levels.

In this study, better QOL outcomes after ESS were achieved with adherent or non-intentionally non-adherent behaviour. This may be attributable to high levels of satisfaction with medical therapy and subsequently a strong motivation towards treatment. However, a poorer outcome such as deteriorating symptoms or the occurrence of side effects could result in patients experiencing a lower satisfaction level, which may lead them to intentionally stop treatment.

The main reason uncovered by this study for intentional non-compliance was symptom improvement. This therefore emphasises the need to counsel CRS patients about the chronicity of their disease and to make them realise that even if symptoms improve after surgery, non-compliance with treatment may lead to recurrence of symptoms later with an associated reduction in health related QOL. These findings also highlight the potential additional health costs and resources needed within this population when compliance is poor, thus redirecting interventions to combat this issue should be considered.

The self-reported questionnaire was simple and economical to use. This was a study specific questionnaire designed to collect variables, and not validated presently. However, it included validated quality of life/PROMs, e.g., SNOT-22. A limitation of this method is that it may have elicited only socially acceptable responses (observed in 5% of the responders) and ultimately, it may have overestimated compliance levels. Another limitation of our study is that the levels of education or self-medication were not assessed, and this has been shown to have an influence on compliance to prescribed medication (112). Moreover, insufficient time has elapsed for us to be able to also assess the pre- and post-operative compliance in the same cohort.

Key points:

- Several factors were found to predict pre-operative compliance in this study cohort;
- Only family history was found to be related with post-operative compliance.

III – LITERATURE REVIEW

3.1 Introduction

Previous chapter explored all available tools validated and of great value in monitoring CRS patients; however, they lack predictive value as they are mostly retrospective and do not explain how a patient will evolve or which treatment will be more effective in each particular case. Thus, there is a lack of indicators for patients' subgroup allocation.

Several reports have suggested that some parameters such as eosinophils and neutrophils counts and certain cytokines, such as IL-5 or IL-13, may define patients' phenotype and predict disease activity and outcome. Therefore, these are considered as potential biomarkers for CRS diagnosis and treatment effectiveness. However, not all patients with significant levels of these molecules will respond equally to treatment, which suggests that other mechanisms may be involved supporting the need for novel biomarkers (113).

According to the Biomarkers Definitions Working Group, a biomarker is defined as a characteristic that can be objectively measured and evaluated as an indicator of normal or pathogenic biological processes, or pharmacologic responses to a therapeutic intervention (79). Several articles have reiterated that the discovery, validation and implementation of reliable biomarkers may lead to a personalised approach and treatment and would be of great help when assessing patients with CRS and defining treatment courses (21,114).

For this reason, there has been a boom in publications about CRS. However, these publications explore small numbers of patients or a single feature and there is no consensus about this evidence and its relationship with CRS. Therefore, this chapter performed an exhaustive summary of the literature on this topic under the following research question: "Which parameters could be possible biomarkers for CRS differential diagnosis, activity, patient subgroup

allocation and response to treatment?". This review will allow the researcher to identify possible biomarkers to be used further on.

3.2 Methods

3.2.1 Extensive literature search

A literature search was conducted to explore the latest evidence for CRS endotypes and relevant biomarkers that could be used in this study. This search aimed to assess which parameters could be possible biomarkers for CRS differential diagnosis, activity, patient subgroup allocation and response to treatment. We identified those with sufficient evidence to support inclusion in this study and derived from the pathobiological mechanisms explained earlier in this report. As explained before, the two phenotypes of CRS have distinct immunological mechanisms: the vast majority of CRSsNP patients show a T_H1-skewed inflammatory response, whereas in the CRSwNP group it is a T_H2-type inflammation. Also, these mechanisms can vary between ethnicities or countries. In this study, biomarkers were selected taking these mechanisms into account.

3.2.2 Systematic review

3.2.2.1 Data Sources, Search Strategy, and Selection Criteria

This review was conducted and reported according to the PROSPERO guidelines. We systematically searched three electronic databases (PubMed, Web of Knowledge, and Google Scholar) for studies published between 2006 and 2018. The research question that this review was looking to answer was: "Which parameters could be possible biomarkers for CRS differential diagnosis, activity, patient subgroup allocation and response to treatment?". Therefore, the search included the following terms: 'duration', 'prediction', 'biomarker', 'diagnosis', 'treatment', 'activity', 'total IgE', 'IL-5', and 'eosinophilic count' in combination with 'chronic rhinosinusitis' in individual searches. The three molecular terms ("total IgE", " IL-5" and " eosinophilic count") were chosen due to

the fact that several studies have confirmed their action in CRS pathophysiology (21,23,25,115).

Prior to any search, a search on Cochrane database was performed to ensure the relevance of the present study. The study title, design, the participants' primary condition and the procedure employed were used to select the eligible studies.

Two authors independently selected the studies according to a standardised method, and any disagreement was resolved by discussion with a third author. The study inclusion criteria were as follows: (1) Participants: adult patients with CRS primarily; (2) Outcomes: the study reported the measurement of biomarkers through ELISA or Luminex; (3) Study design: the study had to be a clinical study or trial measuring some biomarkers in human specimens. Studies designed as in-vitro or in-vivo designs were excluded; (4) Only studies in English and published between January 2006 and December 2018 were included.

3.2.3 Data analysis

A narrative synthesis of the findings from the included studies was performed. In addition, if the necessary data were available, subgroup analysis was done for people with the presence or absence of NP separately, or as sub-groups defined by the biomarkers.

3.3 Results

3.3.1 Extensive literature review

The list of possible biomarkers, identified through the previously stated question, consisted of 16 mandatory biomarkers and 20 exploratory ones, as described in Tables 8 and 9, respectively. Mandatory biomarkers had their action well defined in the pathophysiological mechanisms of CRS, as their study is compulsory. Exploratory biomarkers were sometimes contradictory in their action on CRS, or they were supposed to exert a role in CRS due to their role in the immune system. A summary of their

correspondent actions in the immune system, as well as in CRS, are described for each one.

Table 9: Mandatory biological markers and their major functions in CRS.

Biomarker	Action
Eosinophil count	Eosinophils play a major role in the pathogenesis of CRS. (1-5)
Neutrophil count	Neutrophils are considered to play a major role in the pathogenesis of CRSsNP in Caucasian patients. (3)
IL-4	Induction of T_H2 differentiation and IgE class switching. (6-8)
IL-5	Eosinophil-related cytokine. Anti-IL-5 has been proven to ameliorate CRS inflammation. (4,7-9)
IL-13	Responsible for the recruitment, activation and survival of eosinophils and mast cells. (5,9-12)
IL-17A	Induction of pro-inflammatory cytokines and MMP, recruitment and activation of neutrophils. (3,6,8,11-13)
IL-17E or IL-25	Induction of T_H2 responses and inhibition of T_H1 and T_H17 cell responses. (8,9,12,15,16)
IL-32	Induction of pro-inflammatory cytokines; elevated in CRSwNP. (16,17)
IL-33	Potent inducer of T_H2 response. (8,9,12,15,16,18)
Eosinophilic cationic protein (ECP)	A marker of eosinophil activation known to be increased in nasal polyps. (3,13,16,18-20)
Myeloperoxidase (MPO)	Neutrophil-related markers. (13,19,20)
Immunoglobulin E (IgE)	Increased in CRSwNP. (10,12,13,15,19,20)
Staphylococcal enterotoxin-specific IgE (SE-IgE)	Seen to increase along with IL-5, ECP and IgE the risk of comorbid asthma. Also, it has been demonstrated to be correlated with several aspects of disease severity, and the degree and type of inflammation. Shown to be associated with NP intense eosinophilic inflammation and asthma comorbidity. (13,16,19)

Table 9: Mandatory biological markers and their major functions in CRS (cont.).

Biomarker	Action
TGF-β1	Regulation of the differentiation of several Th cell subsets and induction of Treg cells. (12-14,19-21)
IFN-γ	Promotion of T_H1 response and induction of cell apoptosis. (3,7,8,10,11,19-22)
Thymic Stromal Lymphopoietin (TSLP)	Recent evidence shows that it helps to shape the local activation of T_H2. Increased mRNA levels in nasal polyps. (5,12,23-25)

Table 10: Exploratory biological markers and their major functions in CRS.

Biomarker	Action
IL-1β	Described as increased in neutrophilic CRS. (10,13,20)
IL-6	Pro-inflammatory cytokine, responsible for immunity response caused by an infectious agent. (13,18,21)
IL-8	Described as increased in neutrophilic CRS. (13,19,20)
IL-9	Acts on mucus production. (5)
IL-10	Described as augmented when patients suffer from allergic or AERD syndromes. (3,8,10,14,18,21)
IL-15	Prevents neutrophils and eosinophils apoptosis. (14)
IL-31	Correlated with eosinophil levels in serum in allergic asthma and rhinitis, also acts on mucus production. (9)
MMP-7/MMP-9	Remodelling factor of CRSwNP/sNP. Increased levels in both phenotypes. (12)

Table 10: Exploratory biological markers and their major functions in CRS (cont).

Biomarker	Action
RANTES	Described as a possible biomarker for CRSsNP. (3,12,18)
Eotaxin	Important factors in eosinophilic nasal polyps since it recruits and activates eosinophils. (12,18,20)
G-CSF	Increased in nasal polyps. (12)
Neopterin	Found in CRSsNP. (6)
Endothelin (ET) 1	Found in CRSwNP. (6)
MCP-4	Interferes in the recruitment of eosinophils. (12,18)
TNF-α	Pro-inflammatory cytokine. (10,13,20)
Siglec8 or SAF-2	Induces apoptosis of eosinophils. (26)
Periostin	Known in airway eosinophilia and it is described as a predictor of the response of patients to inhaled corticosteroids treatment. (27,28)
Albumin	Involved in mediating nasal polyps' growth. (13)

(1- Akdis et al. 2013; 2- Gitomer et al. 2016; 3- König et al. 2016; 4- Zadeh et al. 2002; 5- Bal et al. 2016; 6- Tsybikov et al. 2016; 7- Bernstein 2004; 8- Xu et al. 2016; 9- Shah, Ishinaga, and Takeuchi 2016; 10- Orlandi et al. 2016; 11- Jiao et al. 2016; 12- Kato 2015; 13- Tomassen et al. 2016; 14- J. H. Kim et al. 2016; 15- Lam et al. 2016; 16- Scheckenbach and Wagenmann 2016; 17- Khawar, Abbasi, and Sheikh 2016; 18- Stevens et al. 2015; 19- X. Wang et al. 2016; 20- Van Zele et al. 2006; 21- Wu, Wang, and Zhang 2016; 22- Sánchez-Segura, Brieva, and Rodríguez 1998; 23- Ito et al. 2005; 24- Kimura et al. 2011; 25- He et al. 2008; 26- Kiwamoto et al. 2012; 27- Parulekar, Atik, and Hanania 2014;28- Pavord et al. 2017)

3.3.2 Systematic review

We identified 2446 potentially relevant study records, of which 163 were relevant to our question (Figure 17). One hundred and forty-three studies were excluded for the following reasons: no full paper published or available; no comparison between different CRS groups; no measure was performed through ELISA or Luminex techniques; or no numeric results were presented.

A total of 20 articles were included in this review all of them referring to CRS as the main topic and analysing biological biomarkers by ELISA or Luminex. The characteristics of the included studies are presented in Table 11. The 20 included studies referred to the analysis of blood (7 studies) and nasal products, including nasal mucosa (14 studies), secretions (1 study) or nasal lavage products (2 studies).

PRISMA Flow Diagram

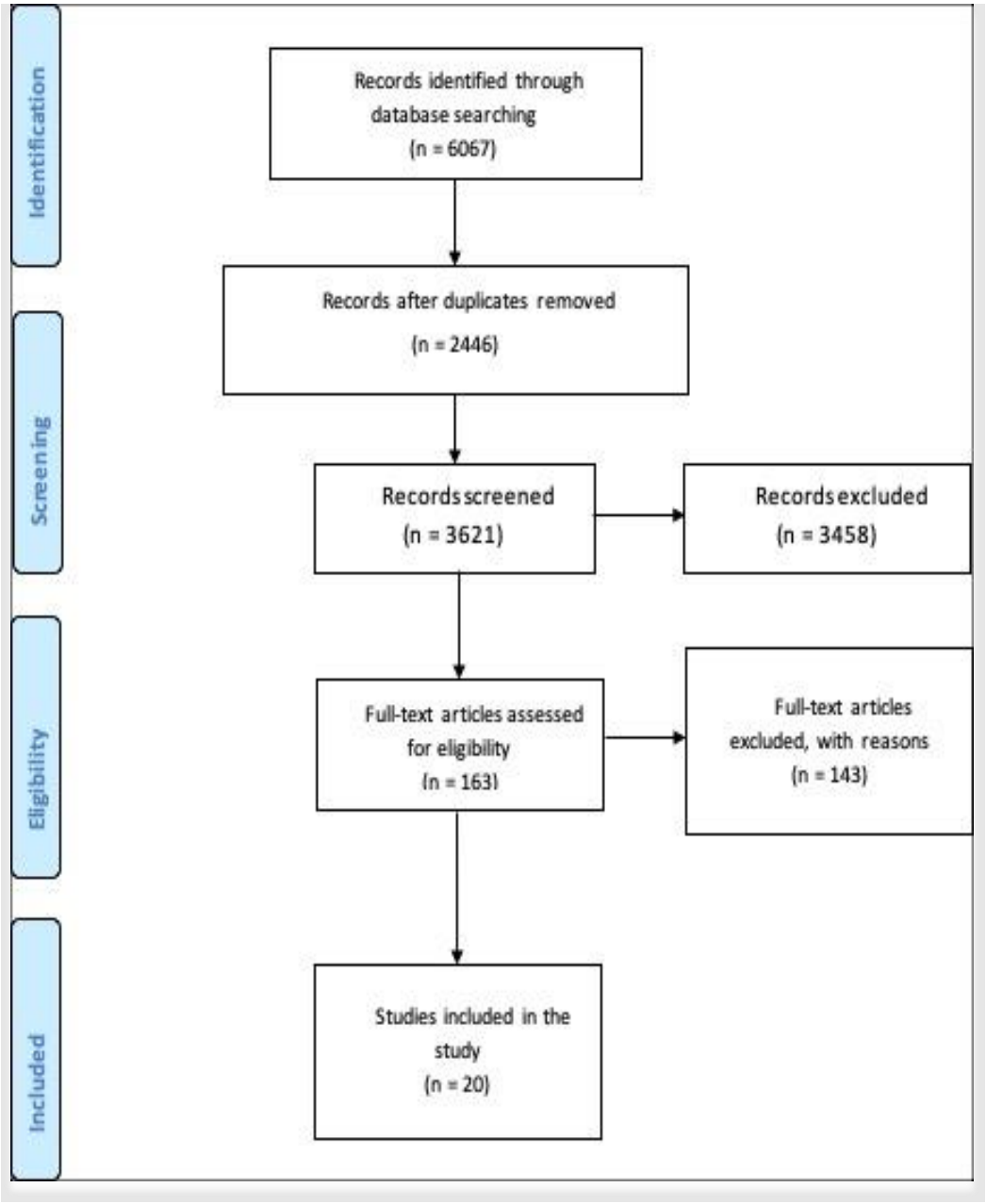


Figure 17: PRISMA flow diagram. (Redrawn from Moher et al. 2009)

New diagnosis and treatment developments in airway diseases are centred on very specific molecules linked to immunologic response pathways. When applied generally without specific disease phenotyping/genotyping knowledge, these may be ineffective. However, when a specific pathway is demonstrated to be active, they

can be quite efficacious. In this review, we analysed 52 biomarkers, and described their expression pattern. Table 12 gives a summary of the evidence on the found biomarkers.

Metalloproteinases and their tissue inhibitors

MMPs play an important role in tissue remodelling associated with various physiological and pathological processes. These proteinases are inhibited by a specific tissue inhibitor of metalloproteinases (TIMPs), which comprise a family of four members. Therefore, it is thought that their function is linked together in CRS. Indeed, our search found a study by Li et al. (134) reporting 3 MMPs (MMP-2, 7 and 9), and four TIMPs. MMP-2 was found to not differ between controls and CRS patients; however, MMP-7 and 9 were increased in CRS patients. In its turn, TIMPs were also increased in CRS patients, more precisely in CRSsNP. Only the concentration of TIMP-3 was below the limits of detection, and possibly may not be expressed in this entity.

Immunoglobulins

CRS is additionally characterised by increases in the local production of several immunoglobulin isotypes, especially IgA and IgE. The levels of sIgA, IgG, and IgE were higher in the CRS groups than in the healthy controls (68,135–138). Furthermore generally, the CRSwNP group were characterised by higher mean levels of sIgA and total IgE than the CRSsNP group (75,135,136,139).

Interferons

Due to their boosting function within the immune system, more precisely the T_H1 pathway, interferons were thought to play a role in CRS. Our study found two described types of interferon: IFN-γ and IFN-α2. Generally, a decreased expression was found among patients with CRSwNP (67,73,140), and a slight increase (non-significant) was found among CRSsNP patients (64,68,141). More,

IFN-γ was found to be decreased in patients with concomitant asthma (140), suggesting that it can provide a protective role for asthma.

Interleukins

Among all studies, 17 interleukins and two receptors (IL-17RB and sIL-6R) were described. IL-1ß, -5, -6, -8, -21 were increased in CRS patients compared with healthy controls (68,73,75,134,135,139–141), especially in patients with comorbid asthma. IL-17A and E had some mixed findings, being increased in nasal secretions of CRS patients (73) and also presenting no differences in serum (142). Therefore, IL-17 receptor B and soluble IL-6 receptor were augmented in CRS patients (143,144). IL-4, -10, -13 and -33 presented contradictory findings, being both increased and decreased in CRS patients in different studies (67,68,73,135,140–142,145). In its turn, IL-12 was described as decreased in CRS patients (73,140), while IL-9 showed no differences among the groups of patients (140). IL-2 was described as boosted in nasal samples of CRS patients, but not in serum samples (135). IL-23, however, was undetectable in the sinus sample of CRS patients (144).

Since IL-6 and sIL-6R were increased in the studied samples (67,135,139,141,144), the authors investigated whether sgp130, which forms a ternary complex with IL-6 and its receptor, was also increased. The results were not surprising as this protein was also increased in CRS patients (144).

Eotaxin

The expression of eotaxin-1, -2 and -3 was investigated across four studies. In all of them, eotaxin was increased in CRS groups when compared with the controls (67,73,140). These results were observed in all nasal specimens; however, their expression was significantly increased in only one study (67).

G-CSF and GM-CSF

Granulocyte-colony stimulating factor and granulocyte-macrophage colony-stimulating factor are both implicated in the proliferation and maturation of neutrophils. Both cytokines were increased in CRS groups when compared with the controls (67,73), demonstrating how these molecules activate different cells and enhance their survival.

Chemokines

MCP-1/4, MIP-1α/ß, TARC and RANTES are all chemokines with different functions within the immune system. MCP-4, MIP-1α and MIP-1ß were increased in CRS patients compared with controls (67,73,140). TARC and RANTES had contradictory findings across different studies, being increased and decreased in CRS groups relatively to the control counterparts (67,73,140). MCP-1 was reduced among the mucosa of CRS patients (67), but increased in nasal secretions (73).

Factors

SDF-1α/ß, SCF, TGF-ß and TNF-α were all described in nasal mucosa. TNF-α was found to be increased among CRS groups (140,141). TGF-ß was generally increased among CRS groups (64,134,139), especially in patients that do not present NP. On the other hand, SCF was described as decreased among CRS patients in both nasal mucosa and lavage (67). Lastly, SDF-1α and ß were decreased in nasal mucosa but increased in nasal lavage, being more expressed in patients with NP (67).

Others

T_H2 regulator thymic stromal lymphopoietin (TSLP) was found to be increased slightly among CRS groups, especially in CRSwNP patients (140). On the other hand, MPO was found to be significantly increased among CRS patients, especially in those who did not

present polyps (64,139). Periostin was significantly increased in CRS patients' serum (146,147). In addition, mean Periostin levels were significantly higher in CRS patients with NP compared to those without polyps (147).

Epithelial-derived neutrophil-activating peptide 78 (ENA-78) is a growth factor implicated in neutrophils activation. It was found to be increased in patients with CRS compared to the controls, being more enlarged in patients who presented NP than in those without (140).

Eosinophil cationic protein (ECP) is a protein released during degranulation of eosinophils and it is related to inflammation. Therefore, it is expected to be increased in patients with high levels of eosinophils, e.g., CRSwNP. Its expression was described across all types of tissues in six studies, and in all of them its levels were higher in CRS when compared with the controls (64,67,68,73,137,139).

B cell-attracting chemokine 1 (BCA-1) was found to be increased generally in nasal mucosa and lavage in CRS patients. However, this study stated that this biomarker was not helpful in defining signature pattern for the study groups (67).

Clara cell 10-kDa protein (CC10) is a multifunction protein with anti-inflammatory and immunomodulatory effects. It was described as significantly decreased in the nasal mucosa of CRS patients when compared with the controls. However, when looking at its expression in plasma, no difference in its expression levels was noted. In addition, CC10 protein levels were inversely correlated with endoscopy scores, overall and total VAS symptom scores, 12 months after the surgery in both CRS groups (141).

Table 11: Characteristics of studies included in the analysis.

Study (Author and Year)	Biomarker	Tissue	Result
van Zele et al. 2007 (136)	IgG (IgG1, IgG2, IgG3, IgG4), total IgE and SE-IgE	Serum and nasal mucosa	↑in CRS groups compared with controls in nasal mucosa, whereas in serum no difference was observed.
Liu et al. 2009 (141)	TNF-a, IL-1B, IL-4, IFN-g and IL-10	Nasal mucosa	↑in CRSwNP and CRSsNP compared with controls.
Li et al. 2010 (134)	MMP-2, MMP-7, MMP-9, TIMP-1, TIMP-2, TIMP-3, TIMP-4 and TGF-B1	Nasal mucosa	TGF-b1, MMP-2, TIMP-1, TIMP-4 increased in CRSsNP, and MMP-7 and 9 increased in CRSwNP. TIMP-2 remains equal across all groups, whereas TIMP-3 was not detected.
Peters et al. 2010 (144)	IL-6, IL-17A and F, and IL-23	Nasal mucosa and nasal lavage	IL-6 ↑in CRSwNP and CRSsNP compared with controls. The other biomarkers were undetectable.
Sejima et al. 2012 (139)	TGF-B, IL-5, IgE, SAE-IgE, ECP, MPO, IL-1B, IL-6, and IL-8	Nasal mucosa	IL-5, IgE, SAE-IgE, ECP, ECP/MPO ratio, IL-8 increased in CRSwNP, whereas TGF-B, MPO, IL-6 and IL-1B increased in CRSsNP.
Derycke et al. 2014 (68)	IL-5, IL-4, IL-17, IL-1B, IFN-g, IgE and ECP	Nasal mucosa	↑in CRSwNP and CRSsNP compared with controls in all biomarkers except IL-17 (equal expression) and IFN-g, where CRSwNP presented a lower expression than controls.
Li et al. 2014 (148)	IL-5, IL-6 and IL-8	Nasal mucosa	IL-5 and IL-6 increased in CRSsNP, while IL-8 was increased in all CRS groups compared with controls.
Xiao et al. 2014 (149)	IL-21	Nasal mucosa	↑in CRSwNP and CRSsNP compared with controls.
Chao et al. 2015 (138)	IL-21, IgE	Serum	↑in CRSwNP and CRSsNP compared with controls.
Cui et al. 2015 (137)	Total IgE, specific IgE and ECP	Serum	↑in CRSwNP and CRSsNP compared with controls.
Shin et al. 2015 (143)	IL-25 and IL-17RB	Nasal mucosa	IL-25 and IL-17RB↑in CRSwNP and CRSsNP compared with controls.

Table 11: Characteristics of studies included in the analysis (cont.).

Study (Author and Year)	Biomarker	Tissue	Result
Stevens et al. 2015 (67)	ECP, IL-4, IL-5, IL-6, IL-10, IL-13, IL-33, eotaxin-1, eotaxin-2, eotaxin-3, RANTES, MCP-4, TARC, SCF, GM-CSF, MCP-1, IFN-a2, IFN-g, BCA-1, SDF-1a and SDF-1b	Nasal mucosa	ECP, MCP-4, IL-13 and GM-CSF increased in CRSwNP. Eotaxin-1,2 and 3, TARC, IL-6, BCA-1, IL-5 and IL-10 increased in CRS groups compared with controls. IFN-a2, SCF, SDF-1a and b, IL-4 and RANTES increased in controls. However, MCP-1 and IFN-g did not show a clear pattern between CRS and controls.
Tsybikov et al. 2015 (135)	IgA, IgE, sIgA, IgG, IL-1B, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10 and IL17A	Serum and nasal lavage	↑in CRS groups compared with controls.
Kim et al. 2017 (145)	IL-33	Nasal mucosa	↑in CRS groups compared with controls.
Konig et al. 2016 (73)	IL-4, IL-5, IL-10, IL-12, IL-13, IL-17, IL-8, GM-CSF, G-CSF, IFN-g, MCP-1, MIP-1a, MIP-1b, eotaxin and RANTES	Nasal secretion	IL-10 and IL-13 were higher in controls than CRS patients; IL-8, MCP-1, eotaxin elevated in CRS groups; MIP-1a, MIP-1b, RANTES, G-CSF, ECP and IL-17 were higher in CRSwNP, whereas IFN-g, GM-CSF and IL-12 were lower; IL-4 and 5 were lower in CRSsNP, where GM-CSF has a higher expression.
Qin et al. 2016 (146)	Periostin	Serum	↑in CRSwNP and CRSsNP compared with controls.
Wang et al. 2016 (64)	IL-5, IFN-g, IL-17A, IL-8, TGF-B1, IgE, ECP and MPO	Nasal mucosa	Mostly, IL-5, ECP, MPO, IgE and IL-8 were elevated in CRS groups, whereas IL-17A was the same across all groups. TGF-B1 gave mixed patterns across the different sites.
Chen et al. 2017 (140)	IL-4, IL-5, IL-9, IL-25, IL-33, IFN-g, eotaxin-1, eotaxin-2, eotaxin-3, IL-8, IL-10, TSLP, MCP-4, TNF-a, ENA-78, RANTES and TARC	Nasal mucosa	↑in CRSwNP and CRSsNP compared with controls, with the exception of IL-9, IL-10 and TARC. More, TNF-a, IL-13, eotaxin-2, eotaxin-3, RANTES were overexpressed in CRSsNP.

Table 11: Characteristics of studies included in the analysis (cont.).

Study (Author and Year)	Biomarker	Tissue	Result
Ozturan et al. 2017 (142)	IL-25 and IL-33	Nasal mucosa and serum	Serum: IL-25 increased in controls. Nasal tissue: IL-25 increased in CRSsNP, and IL-33 increased in control.
Maxfield et al. 2018 (147)	Periostin	Serum	↑in CRS group compared with control.

Table 12: Summary of biomarkers for the diagnosis of CRS.

Biomarker	Full name	Expression pattern	Sample type	Reference
BCA-1	B cell-attracting chemokine 1	↑	Nasal mucosa, nasal lavage	(1)
CC10	Clara cell 10-kDa protein	↓	Nasal mucosa	(2)
		=	Serum	(2)
ECP	Eosinophil cationic protein	↑	Nasal mucosa, nasal lavage, nasal secretion, serum	(1,3-5), (1), (6), (7)
ENA-78	Epithelial-derived neutrophil-activating peptide-78	↑	Nasal mucosa	(8)
Eotaxin		↑	Nasal mucosa, nasal lavage, nasal secretion	(1,8), (1), (7)
G-CSF	Granulocyte-colony stimulating factor	↑	Nasal secretion	(7)
GM-CSF	Granulocyte-macrophage colony-stimulating factor	↑	Nasal mucosa, nasal secretion	(1), (7)
		=	Nasal lavage	(1)
IFN-α2	Interferon alpha-2	↓	Nasal mucosa	(1)
		=	Nasal lavage	(1)
IFN-γ	Interferon gamma	=	Nasal mucosa, nasal lavage	(1)
		↓	Nasal mucosa, nasal secretion	(8), (7)

	↑	Nasal mucosa	(2,4,5)

Table 12: Summary of biomarkers for the diagnosis of CRS (cont.).

Biomarker	Full name	Expression pattern	Sample type	Reference
IgA	Immunoglobulin A	=	Nasal mucosa	(9)
IgE (total and specific)	Immunoglobulin E	↑	Nasal mucosa, serum	(3-5,9,10), (6,9,11)
IgG	Immunoglobulin G	↑	Nasal mucosa	(9,10)
IL-1ß	Interleukin 1 beta	↑	Nasal mucosa, serum	(2-4,9), (9)
IL-2	Interleukin 2	↑	Nasal mucosa, serum	(9)
IL-4	Interleukin 4	↑	Nasal mucosa, nasal lavage	(1,2,4,8,9), (1)
		=	Nasal secretion, serum	(7), (9)
IL-5	Interleukin 5	↑	Nasal mucosa, nasal secretion, nasal lavage, serum	(1,3-5,8,9,12), (7), (1,2), (9)
IL-6	Interleukin 6	↑	Nasal mucosa, nasal lavage, serum	(1-3,9,12), (1,2), (9)
IL-8	Interleukin 8	↑	Nasal mucosa, nasal secretion, serum	(3,5,8,9,12), (7), (9)
IL-9	Interleukin 9	=	Nasal mucosa	(8)
IL-10	Interleukin 10	↑	Nasal mucosa, nasal lavage, nasal secretion, serum	(1,2,9), (1), (7), (9)
		↓	Nasal mucosa	(8)
IL-12	Interleukin 12	↓	Nasal mucosa, nasal secretion	(8), (7)
IL-13	Interleukin 13	↑	Nasal mucosa, nasal lavage	(1)
		↓	Nasal secretion	(7)
IL-17A	Interleukin 17A	↑	Nasal mucosa, nasal secretion, serum	(9), (7), (9)
		=	Nasal mucosa	(4,5)
		n.d.	Nasal mucosa	(2)

Table 12: Summary of biomarkers for the diagnosis of CRS (cont.).

Biomarker	Full name	Expression pattern	Sample type	Reference
IL-17E/IL-25	Interleukin 17E or 25	=	Nasal mucosa, serum	(13)
		↑	Nasal mucosa	(2,8,14)
IL-17F	Interleukin 17F	n.d.	Nasal mucosa	(2)
IL-17RB	Interleukin 17 receptor B	↑	Nasal mucosa	(14)
IL-21	Interleukin 21	↑	Nasal mucosa, nasal lavage, serum	(15), (11), (11)
IL-23	Interleukin 23	n.d.	Nasal mucosa	(16)
IL-33	Interleukin 33	↑	Nasal mucosa	(8,13,17)
		↓	Nasal mucosa, serum	(1,13), (13)
SDF-1α/ß	Stromal cell-derived factor-1 alpha/beta	↓	Nasal mucosa, nasal lavage	(1)
MCP-1	Monocyte chemoattractant protein-1	↓	Nasal mucosa, nasal secretion	(1), (7)
MCP-4	Monocyte chemotactic protein-4	↑	Nasal mucosa	(1,8)
MIP-1a/b	Macrophage inflammatory protein 1 alpha/beta	↑	Nasal secretion	(7)
MMP-2/7/9	Matrix metalloproteinase 2/7/9;	↑	Nasal mucosa	(18)
MPO	Myeloperoxidase	↑	Nasal mucosa	(3,5)
Periostin		↑	Serum	(19,20)
RANTES	Regulated on Activation, Normal T Cell Expressed and Secreted	↑	Nasal mucosa, nasal secretion	(8), (7)
		↓	Nasal mucosa	(1)
SCF	Stem cell factor	↓	Nasal mucosa, nasal lavage	(1)
sgp130	Soluble glycoprotein 130	↑	Nasal mucosa, nasal lavage	(16)
sIgA	Secretory immunoglobulin A	↑ (CRSwNP)	Nasal mucosa	(9)

Table 12: Summary of biomarkers for the diagnosis of CRS (cont.).

Biomarker	Full name	Expression pattern	Sample type	Reference
sIL-6R	Soluble interleukin 6 receptor	↑	Nasal mucosa	(16)
		=	Nasal lavage	(16)
TARC	Thymus and activation regulated chemokine	↑	Nasal mucosa	(1)
		↓		(8)
TGF-B	Transforming growth factor beta	↑	Nasal mucosa	(5,18), (3)
		=		(5)
TIMP-1/2/4	Tissue inhibitor of metalloproteinase 1, 2, 4	↑	Nasal mucosa	(18)
TNF-α	Tumor necrosis factor alpha	↑	Nasal mucosa	(2,8)
TSLP	Thymic stromal lymphopoietin	↑	Nasal mucosa	(8)

(1- Stevens et al. 2015; 2- Liu et al. 2009; 3- Sejima et al. 2012; 4- Derycke et al. 2014; 5- X. Wang et al. 2016; 6- Cui et al. 2015; 7- König et al. 2016; 8- Chen et al. 2017; 9- Tsybikov et al. 2015; 10- Van Zele et al. 2007; 11- Chao et al. 2015; 12- Y. Li et al. 2014; 13- Ozturan et al. 2017; 14- Shin et al. 2015; 15- Xiao et al. 2015; 16- Peters et al. 2010; 17- D.-K. Kim et al. 2017; 18- X. Li et al. 2010; 19- Qin et al. 2016; 20- Maxfield et al. 2018)
n.d., non detectable; ↑, increased; = equal; ↓, decreased.

3.4 Discussion

In this work, we tried to find relevant literature that would allow for more descriptive biomarkers. Since the main goal of this project is to explore the endotypes of CRS and the possibility of biological signatures in specific CRS patient groups, biomarkers were selected taking this into account.

There is a large number of promising biomarkers for CRS, including various nasal and serum proteins, which might fulfil the role of the perfect biomarker for CRS characterisation and early diagnosis. Even the combination of several biomarkers appears promising with regards to the understanding of the immunological mechanisms underlying the different phenotypes of CRS. Nevertheless, further studies are needed to promote validation of novel biomarkers and to further evaluate potential ones.

Key points:

- No ideal diagnostic biomarker for early detection of CRS exists, however IL-5 and IgE have been pointed out as being indicative of the presence of NP;
- Our systematic review identified 52 biomarkers that are supported by different levels of evidence for distinguishing patients with CRS from healthy controls, or identifying different CRS groups;
- 16 mandatory and 20 exploratory biomarkers were found and selected to be analysed subsequently in this project.

IV – SUBJECTS AND METHODS

4.1 Subjects and ethical considerations

In order to fulfil the objectives of this work, a purposive sample of adult patients with or without CRS was recruited from the James Paget University Hospital (JPUH). The exact number of patients was difficult to predict; however, a sample size of 44 participants with CRSwNP, 44 with CRSsNP and 44 controls would give 80% power to detect a Cohen's effect size of 0.6 in the IL-5 expression between any two groups at the 5% level of significance using a two-sample t-test. Cohen's effect size is the mean difference divided by the standard deviation, we used this as we lacked any previous data on i) what the likely standard deviation would be; and ii) what difference was important to detect. A Cohen's effect size of 0.6 would traditionally be considered a 'medium' effect and have an overlap between any two groups of 69%. We aimed to collect 50 patients per phenotype and 50 controls to allow for a 10% drop-out rate. Maximum variation sampling guided recruitment and this ensured a range of ages, genders, ethnic backgrounds, locations, previous treatments and symptom profiles.

The inclusion and exclusion criteria were the following:

- Inclusion criteria:
  - Adult patients with a diagnosis of CRS according to European guidelines who have >12-week history of nasal congestion and/or nasal discharge along with hyposmia and/or facial pressure/pain and confirmation of disease from endoscopy and/or CT scan;
  - CRS patients who were selected to undergo ESS surgery after failing maximal therapy;
  - Control patients consisting of patients aged 18 and above without a diagnosis of CRS or rhinitis and who had undergone a septoplasty surgery for anatomical reasons.
- Exclusion criteria:

- Within 6 months post-operative;
- Rare/complex sinus conditions;
- CRS secondary to systemic disease such as cystic fibrosis and granulomatous disease suspected malignancy;
- Severe asthma (high doses or inhaled steroids, i.e.>1.5mg/day);
- Pregnant/lactating women;
- Immunodeficiency states, including HIV and selective and multiple antibody deficiency states;
- Inability to give consent or to understand and comply with study instructions.

All of the potential patients were sent a letter of invitation, briefly explaining the project and their role in the project. The letter included a participant information sheet (Appendix A) or a control information sheet (Appendix B), a consent form (Appendix C) and a questionnaire (Appendix D). As a second step, patients were approached by the PhD candidate, a research nurse or the ENT surgeon before the procedure and asked whether or not they were willing to participate. Participation was voluntary and the participant could withdraw from the research project at any time. All of the patients were invited to complete an online questionnaire and they were provided with a unique secure PIN to access it. Patients who were interested in participating had their samples collected in clinic or in theatre.

The study was approved by the Research Ethics Committee of East Midlands – Leicester Central and Health Research Authority in October 2016 (Appendix E). All subjects gave written informed consent.

The study included two sets of patients. The first set was derived from a previous tissue bank collection constituted exclusively by CRS patients who had undergone ESS between 2012 and 2016. In that set, all of the patients (if still alive) were contacted. If they

were interested, a recruitment pack, consisting of a patient information sheet (Appendix A), a consent form (Appendix B) and a questionnaire (Appendix C) was sent. If the patient desired to be included, the questionnaire and consent form had to be returned to the investigator. This allowed the researcher to correlate their clinical characteristics with any laboratory findings.

The second set of patients was recruited prospectively and consisted of both CRS and control patients. In this set, the patients were approached verbally in clinic by the ENT doctor or the research nurse. Patients were then given a recruitment pack similar to the other patients. Patients who were interested in participating had their samples collected in clinic or in theatre. CRSwNP and CRSsNP were diagnosed according to the current European position paper (23).

In total, 226 adult subjects filled out the questionnaire. However, seven patients were excluded from the study due to further diagnosis that placed them into the excluded criteria. As a result, 157 CRSwNP, 21 CRSsNP and 41 healthy controls (Table 13) were included in this study.

Table 13: Clinical characteristics of all recruited patients.

	CRSwNP	**CRSsNP**	**Control**
N	157	21	41
Age (mean ± SD)	58.47±11.25	47.81±19.70	39.02±15.7
Male/Female (n)	98/59	10/11	19/22
Duration (mean ± SD)	19.68±13.36	9.12±12.88	-
Comorbidities (%)	72	38.1	56.09
asthma (%)	70	19	12.2
Allergies (%)	56.7	14.3	17.07
History of smoking (%)	47.1	33.3	26.8

4.2 Human samples

4.2.1 Serum collection

Blood was collected before the administration of the anaesthetic agent, in order to prevent any reaction between the assay reagents and the anaesthetic. The collected blood was allowed to clot for at least 30 minutes before separation. The tubes containing the blood were then centrifuged for 10 minutes at 4000 g. Sera were separated from the blood, aliquoted and stored at -80°C until analysis.

4.2.2 Nasal tissue collection

Nasal biological samples were collected during ESS for the patients or during septoplasty for the controls. Nasal samples were kept on ice or at 4°C until the end of the surgery. Samples were later stored at -80°C until analysis or sent to the Biomedical Research Centre (University of East Anglia, Norwich, United Kingdom) and extracted right after reception.

In the case of patients with CRS, tissue samples were taken from the ethmoidal mucosa, NP, mucus, uncinate process, agger nasi cells and mucus. On the other hand, inferior turbinate samples or nasal mucosa were taken from the patients without sinus disease (controls) undergoing septoplasty or septorhinoplasty.

4.3 Molecular biology methods

4.3.1 Bicinchoninic acid (BCA) protein assay

Following the standard protocol for the PierceTM BCA Protein Assay Kit (#23225, Thermofisher), the working reagent (WR) was prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent. A series of diluted albumin (BSA) standards was prepared by dissolving BSA powder in the same solution used for the samples' homogenisation. Each measurement was performed in duplicate in a microplate.

The samples were incubated at 37˚C for 30 min in accordance with the standard procedures. All the absorbances were corrected by the corresponding blank replicate. Absorbance at 562 nm was measured by spectrophotometer (SpectraMax M2e Microplate Reader, Analytical Instruments LLC, Minneapolis, United States).

A standard curve was obtained by plotting the average blank-corrected 562 nm measurements by each BSA standard concentration. Unknown samples were interpolated on the curve. If a sample measurement was outside the curve, it would be more, or less, diluted to fall within the standard curve, since the curve was only assured to be linear within the range.

4.3.2 Enzyme-linked immunosorbent assay (ELISA)

Levels of MPO, Periostin, MCP-4, IgE, Siglec-8, ET-1 and ECP were quantified by commercial sandwich ELISA kits (Thermofisher Scientific, Massachusetts, United States). Detection limits were, respectively, 26, 80, 3, 500, 58, 0.75 and 25 pg/ml. Absorbancies were read with a spectrophotometer (SpectraMax M2e Microplate Reader, Analytical Instruments LLC, Minneapolis, United State) at wavelength 450 nm.

4.3.3 Luminex

In order to study the concentration of biomarkers in both blood and nasal samples, Luminex technology was used. This technology has been described and validated in similar studies (65,67,150).

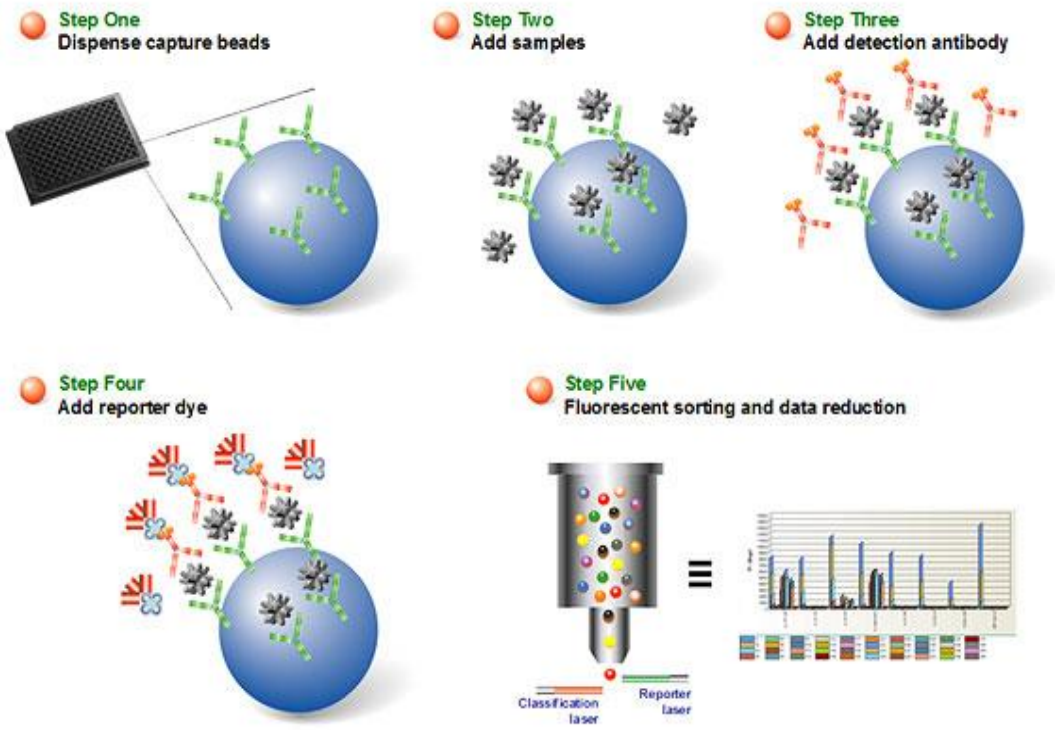
Principle

Luminex™ is a recent technology which, based on the principle of flow cytometry, combines the use of fluorescent microspheres and double reading after excitation by two lasers. This multiplex system consists of a set of microspheres and a flow cytometer with two lasers. The power of this system lies in the fact that it is possible to simultaneously analyse up to 100 types of molecules per well (each

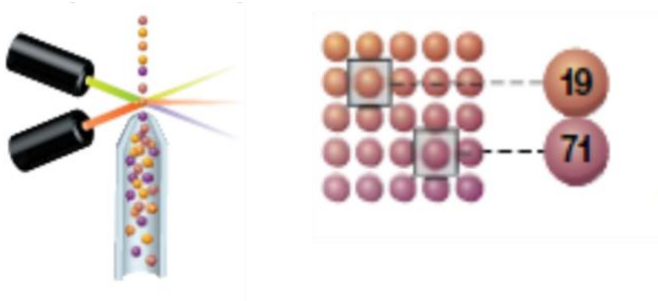
type of bead is recognizable by a different colour code according to its fluorescence), decreasing the amount needed for analysis.

According to the envisaged application, the microspheres are covered either with an antigen, or an oligonucleotide, or a substrate if one wants respectively to reveal an antigen-antibody bond, a molecular hybridization or an enzyme-substrate activity, after addition of a developer labelled with phycoerythrin which gives another fluorescence.

The assay principle can be seen in Figure 18.



A.



B.

Figure 18: Luminex assay presentation. A. Luminex assay operational principle. Source: Bio-Plex® Multiplex Immunoassays, BioRad Laboratories, Hercules, California. || B. Bio-Plex® System: 100 different beads show a red and infrared colour spectrum allowing the determination of 100 different analytes within one sample. The first laser excites the

colour code corresponding to one of the 100 molecules proposed and the second laser allows the quantification of the cytokine to be analysed. Source: Bio-Rad documents.

The system

- Beads

Magnetic beads, with a diameter of 5.6µm and made of polystyrene, are used. They are dyed with different ratios of two spectral distinct fluorophores. By varying the ratio of the two fluorochromes, we obtain a range of 100 categories of beads characterized by a colour code. This colour code is a result of the intensity of the fluorescence emitted by the two fluorochromes in their respective emission spectrum.

- The cytometer

It features a red diode laser (633nm) and a green laser (532nm). While the red laser excites the fluorochromes inside the beads, the green laser excites phycoerythrin. Conventionally, the sample which contains the magnetic beads is aspirated in a sheath liquid and the beads pass one by one in front of the lasers. The red classification laser identifies its spectral address, whereas the green reporter laser quantifies the capture analyte.

Selected assays

The levels of these biological markers will be assessed using multiplex panels (ProcartaPlex, Life Technologies, UK). A human cytokine 17-plex panel (ProcartaPlex Mix&Match Human 17-plex, Invitrogen, Massachusetts, United States) allowed the analysis of IL-1B, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-13, IL-15, IL-17A, IL-31, IL-33, G-CSF, IFN-γ, MMP-7, TNF-α and TSLP. Moreover, MMP-9 and RANTES were measured using a human cytokine 2-plex panel (Human Custom ProcartaPlex 2-plex, Invitrogen, Massachusetts, United States). Singleplex panels of Eotaxin (Human Eotaxin Simplex, Invitrogen, Massachusetts, United States), IL-25

and IL-32 (Bio-Plex Cytokine Assay, Bio-Rad Laboratories, Hercules, California) were used individually due to the impossibility of conjugating them within other panels. This arises from the fact that these beads share a code colour with another molecule.

Validation of this assay

These cytokine assays use fluorescently addressed polystyrene beads with conjugated capture antibodies directed to the aforesaid cytokines. After washing, a fluorescently marked detection antibody builds an immunoassay with the cytokine.

The accuracy and reproducibility of cytokine measurements, made using commercial multiplexing kits, is highly dependent on the availability of high-standard curves (151). These standard points are provided in each Luminex kit and were prepared according to manufacturer's instructions. Standard curves were plate specific to allow us to estimate the protein concentrations of our samples by comparing them to standards with known protein levels. Furthermore, it is important to use a control sample of known concentrations, in order to normalize results between plates for quality control purposes.

Data was collected and analysed using the Luminex 200 system and Luminex XPONENT 3.1 Patch (Luminex Corporation, Austin, USA). Figure 19A shows the sample acquisition software. The superior part of the window gives a real-time reading of sample concentrations, with each line representing the data for each well. At the same time, the system provides the researcher with a histogram, displaying the Doublet Discriminator and events number. A peak must be observed in this histogram for each well, as this validates the reading of the magnetic beads. For instance, if the number of events displayed is low, and no peak is observed, we can deduce that the system displays some type of error, or our beads were aggregated. In such cases, results cannot be validated or extrapolated. The acquisition Density Dot Plot displays a constant

accumulation of events (Figure 19B). In here, each cytokine is identified by a region (grey areas) in the plot, and all collected events for each cytokine are indicated by contrasting colours. For example, Figure 19B shows the identification of 19 cytokines in the same well. Any error associated with sample processing will be displayed on this plot. For instance, if photo-bleaching occurs (as Streptavidin-PE is light sensitive), this can muddle the signal leading to collected events displayed as a diagonal line above the grey areas. Luminex standard curves are obtained from 7 data points plus a blank. In our study, we added another standard point, in order to increase the sensitivity of assays, but it proved irrelevant, as the after 6^{th} dilution, this point showed high variance between replicates and could not be used for the curve construction. This exceeds the number of data points required for accurate logistic regression analysis. The system determines the ideal standard curve range for each assay, ensuring optimal sensitivity and reproducibility of results. A five-parameter regression formula is proposed by the software, in order to calculate the values of unknown samples in relation to the standard (Figure 19C). The researcher should ensure that all results are optimal, and that no errors in sample acquisition occurred. The percent recovery of standards ranged from 70% to 130%, which was used as a detection limit for each protein. The detection threshold was 0.5 pg/ml. Standard curves were validated by the author of this thesis before any results were calculated.

Unknown samples were interpolated on the curve from the steepest part of it, as this is the most reliable. If a sample measurement was outside this area of the curve, it would be re-analysed using a more, or less, diluted sample until it falls within the linear part of the curve.

All 96-well plates included samples from all disease subtypes and controls to minimise inter-assay variation. All obtained tissue protein concentrations were corrected for total protein

concentration, and the results are reported as picograms (pg) of mediator per milligram (mg) of total protein.

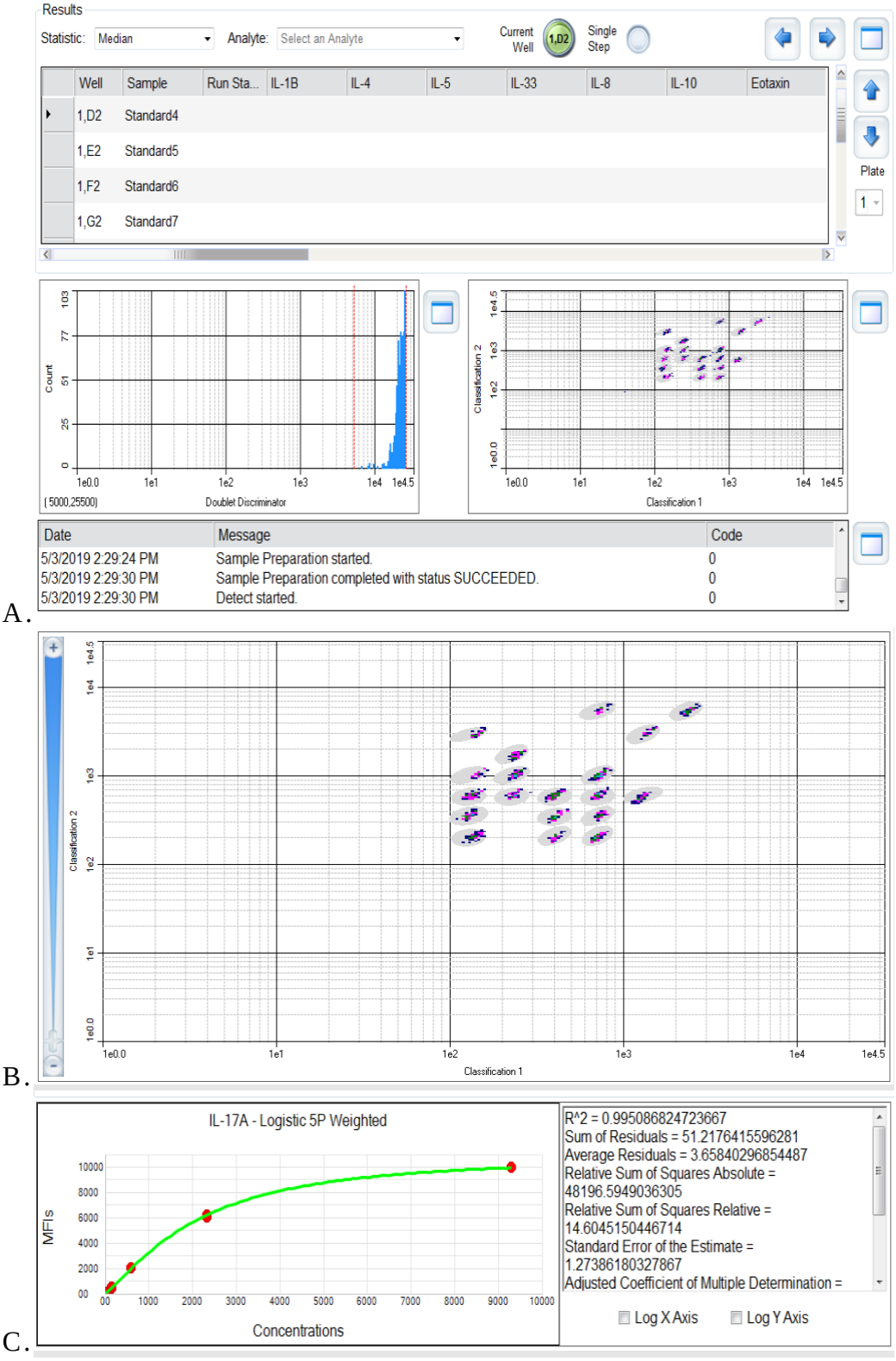


Figure 19: Analysis of samples using Luminex. Outputs of Luminex XPONENT 3.1 analysis during data acquisition. A – Display image during sample reading showcasing the fluorescence histogram and beads region

with beads acquisition; B – Emphasis on the beads acquisition in each of the mediator's region, showing the signal obtained in each one; C – Subsequent obtained graphical disposition using a logistic 5-PL weighted.

V - ASSAY DEVELOPMENT

5.1 Introduction

Chronic RS is associated with a chronic inflammation of the sinonasal tissue. It has a wide prevalence worldwide and it is acknowledged that each phenotype is associated with a different immune mechanism (67). However, several reports differ on the expression pattern of specific mediators in CRS (25,44,64–66,68,123).

In previous chapters, we identified and gave preliminary proof-of-concept studies of the potential biomarkers and discussed the best assay to measure them in clinical practice. While omics is a discovery technology, immunoassays are routinely used for protein biomarker evaluation since they are easy and simple in clinical practice and can be translated into a potential diagnostic assay. The multiplexing of protein assays brings the advantage for simultaneous detection and analysis of several mediators; however, it is limited to standardize assay conditions, less sensitivity over single assays and the quality control of each analyte in the multiplex panel (152). Therefore, this chapter was designed to refine the protocol to be used thereafter in all analysis, giving us guidelines for the future on the standardized protocol for the assessment of potential biomarkers in CRS. For this reason, we evaluate whether the intrinsic conditions of the sample, such as their nature or localisation within the nose, could influence the extraction of our proteins of interest in nasal samples. Also, extrinsic characteristics as extraction techniques or conservation methods were analysed.

5.2 Sample extrinsic characteristics

5.2.1 Introduction

The preparation of samples for protein analysis requires homogenisation, in which the disruption of the tissue will release the cell contents. Selecting the proper technique is, therefore, essential.

In this study, mechanical homogenisation was the chosen one due to its inexpensiveness, ease of use and availability in the laboratory, and most important, its ability to handle whole chunks of tissue, allowing for the removal of further preparatory steps. The bead-based disruption separation technique efficiently homogenises samples. The procedure consisted of a small stainless-steel bead with a homogeniser causing vigorous mixing and turbulence, shearing the sample into small pieces. Next, the sample was inserted into a vial with a buffer solution. The resulting homogenate was semi-solid and after centrifugation/decantation, it was in a liquid form and was treated as serum or cell supernatant.

The first step in this study was understanding which buffer would maximise the recovery and stability of the desired proteins. Therefore, two methods were tested. One consisted of placing the total sample in a known volume of buffer solution. This procedure has been used in our group for in-vivo/in-vitro experiments using murine models, and it has proved to be a valid method. The second one consisted of weighing the sample and adding a proportional amount of buffer to the sample (recommended by the manufacturer's protocol). This comparison allowed us to define the first step of sample treatment after recovery.

Another main concern of this work was the conservation of the sample, both nasal and blood. Therefore, one of the goals was to determine if the biomarkers were conserved when frozen and thawed. This would help to determine the approach needed for handling sample collection for the MACRO trial.

Once we acknowledged the best extraction technique for the sinonasal human samples, we investigated whether the mediator levels would be different if extracted right after collection or extracted after storage at the Biorepository Human Tissue Bank. The latter approach would allow tissues to be stored until the necessary number of samples was reached for the multiplex analysis. Moreover, it would also allow for storage on site (at multiple sites during the MACRO trial) until transport was booked, allowing efficient transportation to the Norwich Biorepository.

5.2.2 Methods

5.2.2.1 Sampling and patient's selection

All patients were recruited as defined in Chapter 4, section 1, and human samples were obtained as previously described.

5.2.2.1 Extraction technique

Samples obtained for the purpose of the step were divided into two segments, one to be use in the set buffer volume technique and the other to be used in the weighted-sample procedure.

Set buffer volume technique (SBV)

Protein was extracted using a small stainless-steel bead per tube, which was pre-incubated for 15 min on ice. After addition of the sample, the tube remained on ice for another 15 min. Afterwards, 1 ml of buffer consisting of T-PER™ Tissue Protein Extraction Reagent (Thermofisher Scientific, Massachusetts, United States) and cOmplete™ Mini EDTA-free Protease Inhibitor Cocktail (Roche, Basel, Switzerland), in a proportion of 1 tablet for 10 ml of T-PER buffer, was added. Later, it was placed in the homogeniser and turned on for 5 min at 50Hz. After homogenisation, the sample was then centrifuged for 10 min at 4000rpm at room temperature and then transferred to a clean propylene tube.

Weighted-sample procedure (WS)

The sample was weighed, and buffer was added in a proportion of 0.1 ml per 1 g of sample. Protein was extracted using a small stainless-steel bead per tube, which was pre-incubated for 15 min on ice. After addition of the sample, the tube remained on ice for another 15 min. After this step, the correspondent amount of buffer was added to the sample and again, it was homogenised for 5 min at room temperature at a rotation speed of 50Hz. After homogenisation, the sample was then centrifuged for 10 min at 4000rpm at room temperature and then transferred to a clean propylene tube.

5.2.2 Temperature-related conservation

When recruiting patients, some of them were randomly assigned to this section of the study. Selection was based on the availability of the sample, the time of the surgery and readiness for extraction. Samples were extracted as per the SBV procedure.

5.2.3 Cytokine and chemokine determinations

The inflammatory mediators were examined as described in Chapter 4, section 3.

5.2.4 Statistics

All data are reported as pg of mediator per mg of protein. A paired t-test with a Bonferroni adjustment was used to compare the expression of protein between both techniques in the same patient. A p-value less than 0.05 was considered significant.

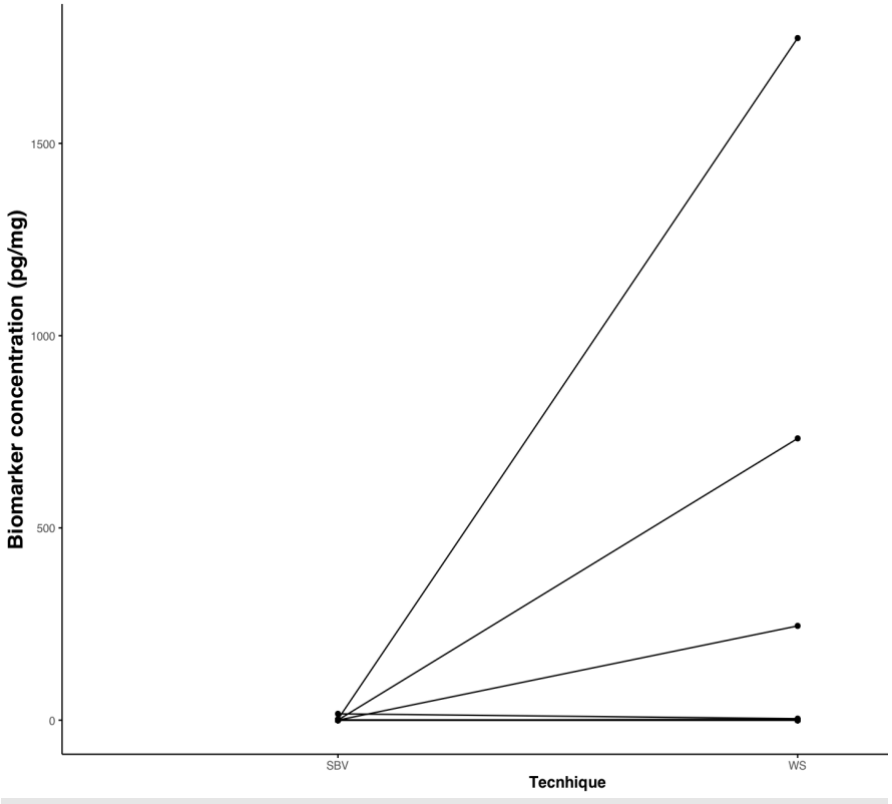
5.2.3 Results

A total of four CRS patients, with and without polyps, were included for assess the validity of the extraction technique. In the paired comparison of protein levels between SBV and WS from the same subjects, there was no significant difference ($p=0.16$). Even, if there was a trend for levels of protein expression in WS to be higher,

this did not reach statistical significance. These higher values are explained by a small amount of overall protein in our samples. Moreover, the volume of WS samples was smaller than the volume obtained in the SBV procedure, which was always around 1ml.

Analysis included IL-5, IL-8, IL-15, IL-17A, IL-33, MMP-9, RANTES and Periostin measurements, allowing for a total of 25 different samples to be analysed (Figure 20). Figure 20B shows this comparison on a logarithmic scale evidencing the scatter of the results and the non-defined pattern among techniques.

A.



B.

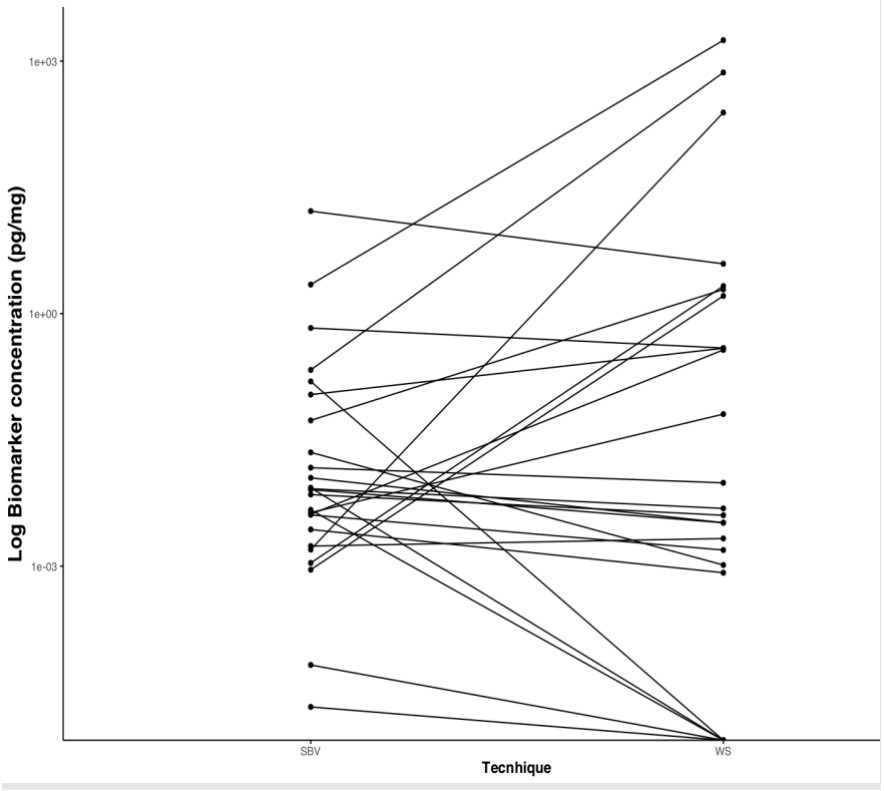


Figure 20: Comparison between the two techniques of extraction: SBV and WS. Results were expressed as pg of protein per ml of solution. Analysis included IL-5, IL-8, IL-15, IL-17A, IL-33, MMP-9, RANTES and Periostin. No significant difference was observed between both techniques (p=0.16). A: Normal comparison; B: Log representation of graphic 1A. SBV: Set buffer volume; WS: weighted sample procedure.

After assessing that no different existed between the extraction techniques recommended for this assay, we turn our attention to the conservation of the samples. The extraction method – SBV, was considered the most practical as tubes can be previously prepared and be ready for the extraction step, and therefore, was selected for the posterior extraction of all protein mediators in this work.

A total of three patients and four samples were available for analysis related to their conservation. The results are presented in Table 14. There was a trend for levels to differ between the fresh and frozen samples, but this did not reach statistical difference except for IL-31 (p=0.016, Table 14). Due to some mediators' availability, not all potential proteins were tested in this study.

Table 14: Comparison of mediators' levels between frozen and fresh samples.

Mediator	Obs (n)	Pairwise t-test (p)	Conclusion
IL-1ß	4	0.62	Non-significant
IL-4	4	0.20	Non-significant
IL-5	4	0.37	Non-significant
IL-6	4	0.71	Non-significant
IL-8	4	0.22	Non-significant
IL-9	4	0.58	Non-significant
IL-10	4	0.30	Non-significant
IL-13	4	0.91	Non-significant
IL-15	4	0.52	Non-significant
IL-17A	4	0.55	Non-significant
IL-31	4	0.02	Significant
IL-33	4	0.35	Non-significant
MMP-7	4	0.35	Non-significant
MMP-9	4	0.29	Non-significant
TNF-α	4	0.45	Non-significant
IFN-γ	4	0.44	Non-significant
G-CSF	4	0.28	Non-significant
Periostin	4	0.35	Non-significant
TSLP	4	0.59	Non-significant

p-values for pairwise t-test comparison between fresh and frozen samples for a same mediator.

Figure 21 shows the expression levels of IL-31 between both conservation techniques for the same sample. Lines represent the variation in expression for the same sample. Therefore, we can see a trend in IL-31 expression – it is higher in frozen samples than in fresh ones. This may be due to the action of proteases which might be inactive when freezing the sample.

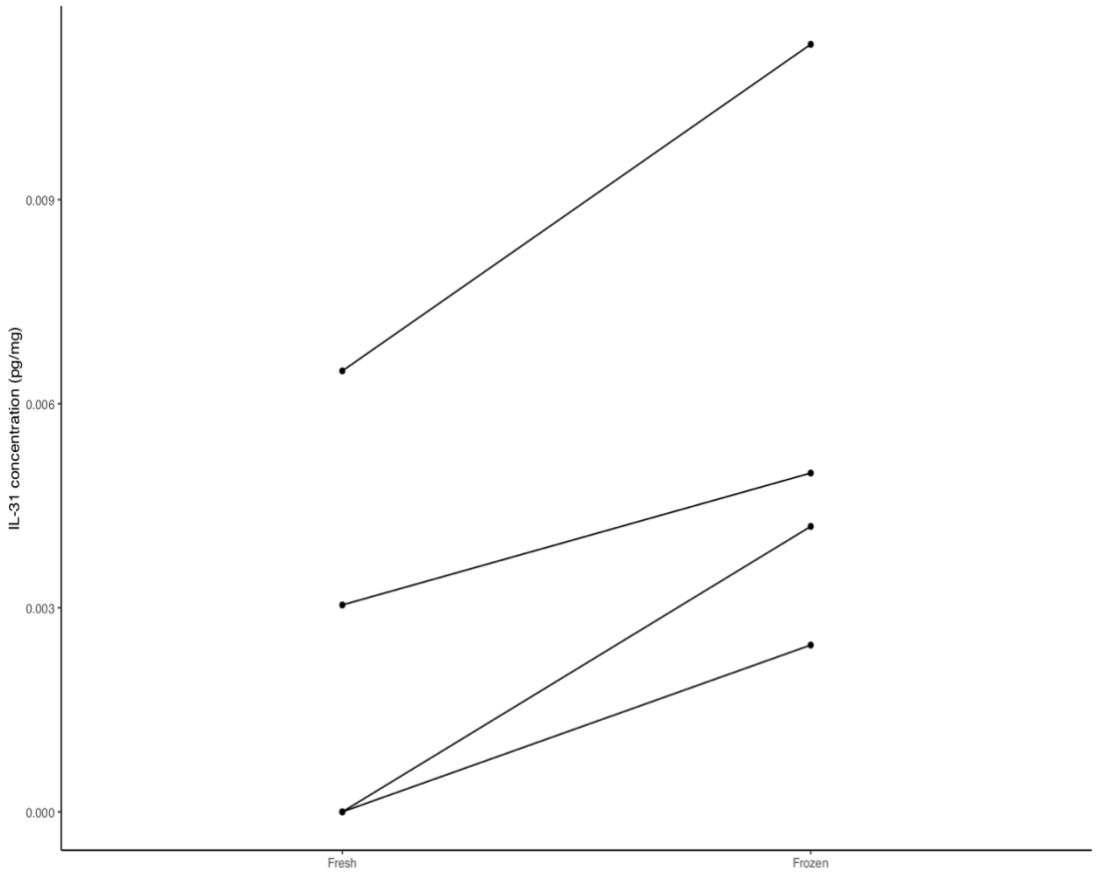


Figure 21: Comparison between fresh and frozen tissue. The expression of IL-31 is compared between fresh and frozen samples (p=0.016).

These results showed us that frozen samples can be used for the assessment of biomarkers in CRS samples. This brings a facility for clinical practice as samples can be collected routinely in clinic and processed at the end of the clinic or when the multiplex assay is complete, decreasing the costs associated with such assay.

5.3 Intrinsic characteristics

5.4.1 Introduction

After having set the protocol for sample analysis, we focused on the optimal sample characteristics that would allow us to routinely analyse CRS patient's samples. All patients included in this study were scheduled for surgery before recruitment. Patients ranged in symptoms severity, extension of the disease and side presentation among other clinical presentations. The collection of samples

occurred during ESS and consisted in nasal (tissue and mucus) and blood samples. All nasal samples collected were removed for treatment purposes. Therefore, tissue samples were taken from either side of the nose independently of the severity and extension of the disease on that side of the nose. Thus, assessment of the consistency of measurements between the 2 sides of the nose is of extreme importance, as it is imperative that one side represents the other in bilateral disease.

Nasal lavage fluid can serve as an alternative and more convenient way to study CRS pathogenesis, allowing for big cohort studies as specimens can be readily obtained without requiring surgical excision. However, it is not known whether mediator levels in lavage fluid accurately represent the levels in the tissue itself. To this end, we measured the same inflammatory mediators in the nasal lavage fluid of patients with CRS (67). Moreover, if patients did not have mucosa alterations that needed surgical excision, mucus would be an ethical alternative. Therefore, the aim was to guide collection procedures by assessing whether samples coming from different sides and tissues of a patient's nose differed in the mediators' expression or pattern.

Simultaneously during ESS and prior to the anaesthetic agent administration, a serum sample was collected. The objective of this was to assess if CRS was a local or systemic inflammation, and if serum alone could give the same information as nasal biopsy. If so, this would also facilitate the collection of samples for each patient and decrease the time for routine analysis.

5.4.2 Methods

Subjects and biological samples

Recruited patients were assigned to this section of the study if they had common samples for the parameters studied – left vs right side, nasal mucosa vs mucus/mucin, and serum vs nasal tissue. All patients were recruited as defined on Chapter 4, section 1, and

human samples were obtained as previously described. Nasal samples were extracted as defined in the SBV procedure, while serum samples were just centrifuged, aliquoted and frozen 30 min after collection.

Measurement of cytokine and chemokine

The inflammatory mediators were examined as described in Chapter 4, section 3. Tissue protein concentrations were normalised to the concentration of total protein concentration (pg/mg of total protein).

Statistics

Differences in concentration of mediators between different sides and tissues samples, as well as between serum and nasal tissue, were evaluated using paired t-test with a Bonferroni adjustment to adjust the significance level for each comparison. A p-value less than 0.05 was considered significant.

5.4.3 Results

To compare the difference in inflammatory mediators related to CRS presentation, we compared levels of expression between left and right tissues within the same patients (Table 15).

Although no significant variation between both sides of the nose was observed and concentrations were similar across both sides, one sample showed a difference on the mediators' levels between the right and left side. This sample corresponded to a patient with CRSsNP and asthma, for whom levels were increased on the right side for MCP-4, Periostin, G-CSF, MPO, IL-1B, IL-15, IL-33, MMP-9 and IFN-γ.

More, there was no significant difference in any of the mediators between nasal tissue (NT) and mucus from the same subjects.

Table 15: Paired comparison of mediators between different nasal side and tissue.

Mediator	Left vs right side			Nasal mucosa vs mucus/mucin		
	n	Pairwise t-test (p)	Conclusion	n	Pairwise t-test (p)	Conclusion
IL-1ß	11	0.20	Non-significant	20	0.25	Non-significant
IL-4	12	0.34	Non-significant	20	0.35	Non-significant
IL-5	14	0.34	Non-significant	21	0.31	Non-significant
IL-6	11	0.99	Non-significant	21	0.67	Non-significant
IL-8	13	0.63	Non-significant	21	0.06	Non-significant
IL-9	11	0.69	Non-significant	21	0.22	Non-significant
IL-10	11	0.97	Non-significant	21	0.5	Non-significant
IL-13	11	0.30	Non-significant	21	0.77	Non-significant
IL-15	13	0.33	Non-significant	21	0.15	Non-significant
IL-17A	13	0.09	Non-significant	21	0.57	Non-significant
IL-25	7	0.33	Non-significant	9	0.22	Non-significant
IL-31	11	0.37	Non-significant	21	0.31	Non-significant
IL-32	8	0.14	Non-significant	7	0.43	Non-significant
IL-33	13	0.73	Non-significant	21	0.17	Non-significant
MMP-7	10	0.63	Non-significant	21	0.37	Non-significant
MMP-9	14	0.37	Non-significant	19	0.52	Non-significant
TNF-α	11	0.19	Non-significant	21	0.61	Non-significant

Table 15: Paired comparison of mediators between different nasal side and tissue (cont.).

Mediator	Left vs right side			Nasal mucosa vs mucus/mucin		
	n	Pairwise t-test (p)	Conclusion	n	Pairwise t-test (p)	Conclusion
IFN-γ	11	0.38	Non-significant	20	0.23	Non-significant
G-CSF	11	0.29	Non-significant	21	0.27	Non-significant
IgE	4	0.46	Non-significant	9	0.53	Non-significant
RANTES	11	0.22	Non-significant	19	0.40	Non-significant
MPO	12	0.78	Non-significant	11	0.42	Non-significant
MCP-4	12	0.18	Non-significant	12	0.16	Non-significant
Periostin	13	0.22	Non-significant	20	0.05	Non-significant
TSLP	11	0.19	Non-significant	21	0.76	Non-significant

p-values for the paired comparison between left and right side of the nose, as well as different nasal samples for a same mediator.

Next, we wanted to ascertain if CRS was a local or a systemic inflammatory process, and if serum alone could give the same information as nasal biopsy. For this, a total of 82 pairs of samples were available for the same mediators as analysed before.

There was a significant decrease in the mediators' expression in serum when compared with nasal tissue expression (p=0.0009; nasal tissue: 1.84 pg/mg protein, serum: 0.11 pg/mg protein). The expression levels of the different mediators in both tissues for the same sample shows a trend where mediators are expressed higher in the nasal tissue than in serum (Figure 22). Therefore, CRS is a predominantly localised inflammation of the nose that cannot be inferred through systemic tests.

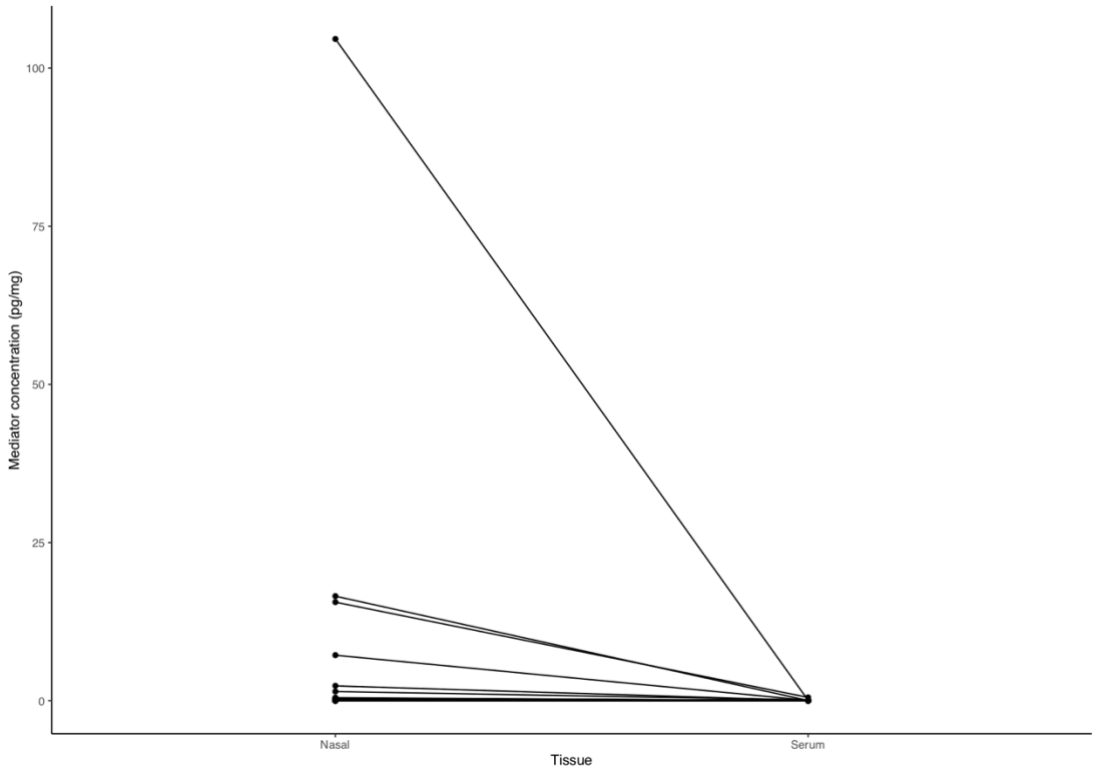


Figure 22: Comparison between the two tissues: nasal and serum. Results were expressed as pg of protein per mg of total protein. Lines represent the variation in expression within the same subject. A significant difference was observed between both tissues (p=0.0009).

5.7 Discussion

The main objective of this work is to elucidate the mechanisms driving CRS pathology. Several studies have examined this, with some focusing on the levels of expression and others on the role of certain cytokines and chemokines in this process. However, as stated previously, there has been an ongoing debate with controversial results across studies. As a result, this chapter aimed to provide the right tools for sample processing and analysis, guiding the following chapters' analysis.

The first goal was to define the best extraction technique that would help to achieve the main goal of this work. We found that there were no differences between the two studied techniques, and from a practicality point of view, the SBV procedure would suit better. This allows a faster and standardized collection and extraction technique for the various samples and inflammatory mediators.

Next, we investigated whether there were any differences between fresh and frozen samples. Of the mediators that were analysed, only IL-31 showed a significant difference between both sets, being higher in frozen samples. Thus, we conclude that samples can be stored frozen after collection until extraction and posterior analysis. These results will permit a better management of patients' recruitment and sample collection and decrease any associated cost with shipment of samples.

We next investigated whether the inflammatory signature could be influenced by the side of the nose, since not all patients experience the same severity level on both sides of the nose. In total, no mediator was significantly elevated or decreased on either side of the nose, as determined by the pairwise t -test. Lastly, the nature of the sample was thought to exert an effect on the levels of biomarkers extracted and measured. Instead, our results showed that even if a trend does exist, no significant differences between both tissues were observed. Therefore, both tissues are satisfactory for analysis,

but, when possible, nasal mucosa will be preferred over mucus, since intra-variability in mucosa samples was less variable. Moreover, nasal mucosa is also easy to extract due to its nature.

In conclusion, we validated our in-house assay proving that nasal tissue, conserved by freezing and extracted by our in-house procedure, is satisfactory for our analysis showing no difference with other procedures or tissues. Guidelines for the MACRO trial include sample collection and extraction instructions that are easy and simple to undertake as a result of this work.

Key Points:

- Samples will be extracted using the SBV procedure in order to obtain approximately 1ml of extracted sample, which will allow for all of our mediator analysis;
- Samples can be stored after collection at -80°C, until later extraction and analysis, without significant difference in the expression levels of the desired mediators;
- Levels from either side of the nose did not have statistical differences, allowing the inclusion of samples from either side of the nose, even if it is not the most severe side;
- Nasal tissue, when available, will be preferred to mucus due to its nature and lower intra-sample variability.

VI – PHENOTYPES BASED ON BIOMARKER ANALYSIS

6.1 Introduction

Chronic rhinosinusitis has been described as a common chronic inflammatory disease of the nasal and paranasal mucosa (23). As its definition indicates, this condition is a chronic inflammatory disease of the upper airways, being similar to asthma or chronic obstructive pulmonary disease, which are chronic inflammatory diseases of the lower airways. As such, its impact on the quality of life is significant as well as the costs associated with it (30,153).

The clinical presentation of CRS has been explored previously in this work, indicating that CRS is primarily a medical problem. Due to its heterogeneity, researchers and clinicians have been trying to classify CRS into phenotypes that can be easily managed and treated. Therefore, several studies have been trying to describe and categorize CRS phenotypes, as this is a critical step in determining optimal treatment, either medical or surgical.

These phenotypes emerged from its visual clinical characteristics, the presence of nasal polyps, as CRSwNP or CRSsNP. Several studies have attempted to characterise the role of various cytokines in the pathogenesis of CRS, describing the expression levels of numerous molecules in both blood and nasal specimens of patients with CRS. The present study investigated the levels of several proteins, in nasal mucosa, and the cell count of eosinophils and neutrophils in blood.

6.2 Methods

6.2.1 Subjects

Adult patients were recruited and included as described in Chapter 4.

6.2.2 Tissue collection

Nasal tissue samples were collected and processed according to the validated method for this study.

6.2.3 Measurement of inflammatory mediators

For tissue analysis, we assessed 27 biomarkers (Table 16) based on our previous literature review. These choices were based on specific inflammatory patterns known on CRSwNP and CRSsNP patients. Concentrations of Periostin, MCP-4, ECP, MPO and IgE were assessed using commercially available ELISA kits from Invitrogen (Thermofisher Scientific, Massachusetts, United States). Concentration of the remaining biomarkers were assayed with the Luminex 200 system (Luminex Corporation, Austin, USA). Concentrations of tissue homogenates were expressed as pg versus mg of total protein (detected by BCA assay). Expression level less than the detection limit were given a value equal to 0 for continuous analysis.

Table 16: Biomarkers analysed.

Category	Biomarker
Anti-inflammatory cytokine	IL-10
Pro-inflammatory cytokines	IL-1ß, IL-6, TNF-α and IL-32
Th1-associated cytokine	IFN-γ
Th2-associated cytokines	IL-4, IL-5, IL-13, IL-17E/25, IL-31, IL-33, TSLP, IgE and Periostin
Th9-associated cytokine	IL-9
Th17-associated cytokine	IL-17A
Neutrophil-associated	IL-8 and MPO
Eosinophil-associated cytokines	Eotaxin, ECP, MCP-4 and RANTES
Lymphoid growth factor	IL-15
Hematopoietic progenitor growth factor	Granulocyte colony-stimulating factor (G-CSF)
Metalloproteinases	MMP-7 and MMP-9

IL, interleukin; IFN-γ, interferon-γ; TSLP, thymic stromal lymphopoietin; G-CSF, granulocyte colony-stimulating factor; TNF-α, tumor necrosis factor α; MMP, metalloproteinase; MCP-4, monocyte chemotactic protein-4; MPO, myeloperoxidase; IgE, immunoglobulin E; ECP, eosinophil cationic protein.

6.2.4 Collection of clinical data

A detailed history was taken from all the participants, as explained previously (Chapter 4). Blood count results were also consulted for each patient, and eosinophil and neutrophil blood count values were extracted and analysed.

6.2.5 Statistical analysis

Statistical analysis was carried out using RStudio version 1.2.13 (RStudio, Inc.). The normality was assessed by Shapiro-Wilks test. Since not all biomarkers followed a normal distribution, non-parametric tests were chosen. The Wilcoxon test was used to compare the levels of interleukins between patients with CRS (CRSwNP and CRSsNP) and controls. Kruskal-Wallis was employed to compare cytokine levels between the different groups of patients (CRSsNP, CRSwNP and control).

The Spearman correlation coefficient was used to assess the level of linear dependence between biomarkers. Data were expressed as mean ± SD. A p-value less than 0.05 was considered as statistically significant.

6.3 Results

One hundred and thirteen patients met the inclusion criteria and had an adequate amount of tissue collected to carry out all intended analysis. Therefore, they were enrolled for cytokine and chemokine processing at this stage. Of these, 78 patients were diagnosed with CRSwNP, 16 with CRSsNP, and 19 were controls. Clinical and demographic characteristics of included patients are presented in Table 17.

Table 17: Demographics and clinical characteristics of cases and control subjects (n=113).

	CRSwNP	CRSsNP	Control
n	78	16	19
Age (mean, years; range, years)	62.8 (33-82)	44.9 (25-80)	39.4 (21-76)
Sex (% male)	57.7	62.5	47.4
Proportion ever smoker (%)	42.3	31.3	57.9
Proportion current smoker (%)	2.6	6.3	21.1
Proportion with allergy (%)	52.6	12.5	10.5
Proportion with asthma (%)	52.6	25.0	10.5
Proportion with aspirin sensitivity (%)	23.1	0.0	10.5

Patients with CRSwNP presented at a significantly higher age. None of the other baseline characteristics differ significantly between groups (results not shown).

The mean concentration for each analyte was compared between groups (Table 18). For the 29 inflammatory markers analysed, paired Wilcoxon tests were performed to calculate the p-values. Of the tested analytes, 14 had statistically significant differences between the three groups (Table 18). These included the following: IL-1ß, IL-4, IL-8, IL-13, IL-15, IL-32, IL-33, IFN-γ, G-CSF, TNF-α, MMP-7, RANTES, Periostin and IgE. Among the 14 biomarkers, 5 biomarkers are cytokines associated with T_H2 immune response. The remaining analytes that were differentially present included pro-inflammatory cytokines, T_H1-associated cytokine, neutrophil- and eosinophil-related chemokine, lymphoid growth factor, hematopoietic progenitor growth factor and a metalloproteinase.

More, we also investigated the differences in concentration levels between the different groups. These are summarised on Table 18.

Table 18: Molecular profiles in sinonasal mucosa of CRS and control groups. Results are presented as mean ± SD.

Mediators	n	CRSwNP	CRSsNP	Control	Overall p-value	Control vs wNP	Control vs sNP	wNP vs. sNP
IL-1ß	108	$7x10^{-4} \pm 1.7x10^{-3}$	$1.7x10^{-2} \pm 6.1x10^{-2}$	$1.4x10^{-3} \pm 2.9x10^{-3}$	**0.02**	NS	NS	**<0.01**
IL-4	106	$1.1x10^{-4} \pm 3.2x10^{-4}$	$9x10^{-5} \pm 2.2x10^{-4}$	$8.5x10^{-4} \pm 1.3x10^{-3}$	**0.01**	**<0.01**	**<0.05**	NS
IL-5	113	0.159 ± 1.394	0.003 ± 0.006	0.002 ± 0.003	0.19	NS	NS	NS
IL-6	108	0.008 ± 0.04	0.016 ± 0.04	0.007 ± 0.02	0.28	NS	NS	NS
IL-8	113	0.004 ± 0.013	0.195 ± 0.786	0.002 ± 0.004	**0.02**	**<0.01**	**<0.05**	NS
IL-9	108	$8.2x10^{-4} \pm 1.8x10^{-3}$	41.0 ± 164.0	$9.2x10^{-4} \pm 1.8x10^{-3}$	0.90	NS	NS	NS
IL-10	108	$5.9x10^{-5} \pm 2.5x10^{-4}$	$9.4x10^{-4} \pm 3.8x10^{-3}$	$1.5x10^{-4} \pm 2.8x10^{-4}$	0.05	**<0.01**	NS	NS
IL-13	107	$7.2x10^{-5} \pm 1.7x10^{-4}$	$1.9x10^{-5} \pm 4.6x10^{-5}$	$1.0x10^{-4} \pm 1.5x10^{-4}$	**<0.01**	**<0.01**	**<0.01**	NS
IL-15	113	$1.5x10^{-3} \pm 0.005$	$2.3x10^{-4} \pm 0.07$	$5.5x10^{-3} \pm 0.01$	**<0.01**	**<0.01**	NS	**<0.05**
IL-17A	112	$3.8x10^{-4} \pm 1.0x10^{-3}$	$2.3x10^{-3} \pm 6.3x10^{-3}$	$6.9x10^{-4} \pm 1.3x10^{-3}$	0.06	**<0.05**	NS	NS
IL-17E/25	70	$1.4x10^{-5} \pm 1.9x10^{-5}$	$7.5x10^{-5} \pm 1.4x10^{-4}$	$6.4x10^{-5} \pm 1.4x10^{-4}$	0.66	NS	NS	NS
IL-31	108	0.002 ± 0.005	0.047 ± 0.16	0.004 ± 0.006	0.14	**<0.05**	NS	NS
IL-32	90	0.004 ± 0.019	0.017 ± 0.027	0.028 ± 0.078	**0.01**	**<0.05**	NS	**<0.05**
IL-33	112	0.07 ± 0.15	0.88 ± 1.32	0.5 ± 1.29	**<0.01**	**<0.01**	NS	**<0.05**
IFN-γ	108	$4x10^{-4} \pm 0.001$	$1.7x10^{-2} \pm 0.043$	$6x10^{-3} \pm 0.016$	**<0.01**	**<0.01**	NS	**<0.01**
TSLP	108	$9x10^{-4} \pm 0.002$	$2.8x10^{-3} \pm 0.006$	$2.5x10^{-3} \pm 0.004$	0.19	NS	NS	NS

Table 18: Molecular profiles in sinonasal mucosa of CRS and control groups. Results are presented as mean ± SD (cont.).

Mediators	n	CRSwNP	CRSsNP	Control	Overall p-value	Control vs wNP	Control vs sNP	wNP vs. sNP
G-CSF	108	0.004 ± 0.02	0.370 ± 1.32	0.009 ± 0.03	**<0.01**	NS	**<0.05**	**<0.01**
TNF-α	108	$7.1x10^{-4}$ ± $2.8x10^{-3}$	$5.1x10^{-4}$ ± $1.0x10^{-3}$	$4.8x10^{-4}$ ± $8.7x10^{-4}$	**<0.01**	**<0.05**	NS	**<0.01**
MMP-7	108	0.08 ± 0.1	0.59 ± 1.6	0.05 ± 0.1	**<0.01**	**<0.01**	**<0.01**	NS
MMP-9	108	0.031 ± 0.089	0.444 ± 1.167	0.037 ± 0.087	0.48	NS	NS	NS
RANTES	107	0.016 ± 0.04	0.103 ± 0.15	0.115 ± 0.21	**<0.01**	**<0.01**	NS	**<0.01**
Periostin	113	2.60 ± 5.47	10.31 ± 15.13	13.7 ± 23.40	**<0.01**	**<0.01**	NS	NS
MCP-4	77	0.008 ± 0.01	0.010 ± 0.01	0.020 ± 0.04	0.75	NS	NS	NS
MPO	104	15.48 ± 53.18	76.19 ± 223.80	24.60 ± 47.09	0.61	NS	NS	NS
IgE	90	0.96 ± 2.56	2.28 ± 8.79	1.23 ± 5.09	**<0.01**	**<0.01**	NS	**<0.01**
ECP	74	17.19 ± 39.77	92.77 ± 293.43	68.58 ± 154.54	0.85	NS	NS	NS
Eotaxin	61	0.004 ± 0.016	0.002 ± 0.003	0.003 ± 0.005	0.86	NS	NS	NS
Eosinophils	98	0.352 ± 0.35	0.220 ± 0.19	0.192 ± 0.11	0.39	NS	NS	NS
Neutrophils	98	5.736 ± 2.66	4.388 ± 1.44	4.051 ± 1.14	0.05	**<0.05**	NS	NS

IL, interleukin; IFN-γ, interferon-γ; TSLP, thymic stromal lymphopoietin; G-CSF, granulocyte colony-stimulating factor; TNF-α, tumor necrosis factor α; MMP, metalloproteinase; MCP-4, monocyte chemotactic protein-4; MPO, myeloperoxidase; IgE, immunoglobulin E; ECP, eosinophil cationic protein. An overall p-value, indicating the difference between CRS patients and controls, was obtained using the Wilcoxon test. Overall p-value <0.05 indicates that at least one CRS group was different from the controls. Control vs wNP: p-value between Control group and CRSwNP; Control vs sNP: p-value between Control group and CRSsNP; wNP vs. sNP: p-value between CRSwNP and CRSsNP group.

Patients presenting CRS with nasal polyps had significantly decreased levels of IL-15, IL-32, IL-33, IFN-γ, RANTES and IgE, in comparison with controls and other cases without nasal polyps, whereas TNF-α was statistically significantly higher in the CRSwNP group than in the other two groups ($p < 0.001$). More, we could also observe that this group had significantly lower concentrations than control subjects of Il-4, IL-8, IL-10, IL-13, IL-17A, TSLP, and Periostin, and had a significant higher concentration of MMP-7 and neutrophils than controls.

On the other hand, patients with CRSsNP presented significantly higher levels of G-CSF than control or CRSwNP subjects. Moreover, CRSsNP subjects also presented higher concentration levels than controls of the following biomarkers: IL-13, IL-8 and MMP-7, and lower levels of IL-4. IL-1 ß presented different levels only between CRS groups, being markedly increased in patients with CRSsNP.

Significantly higher levels of T_H2-associated cytokine (IL-13) was found in controls than in both CRS groups (Table 18).

Correlation of cytokines with clinical features

According to our results from Chapter 3, we analysed the potential relationship of our mediators with one another and with some key clinical features. Results from the Spearman's correlation analysis are presented in Table 19.

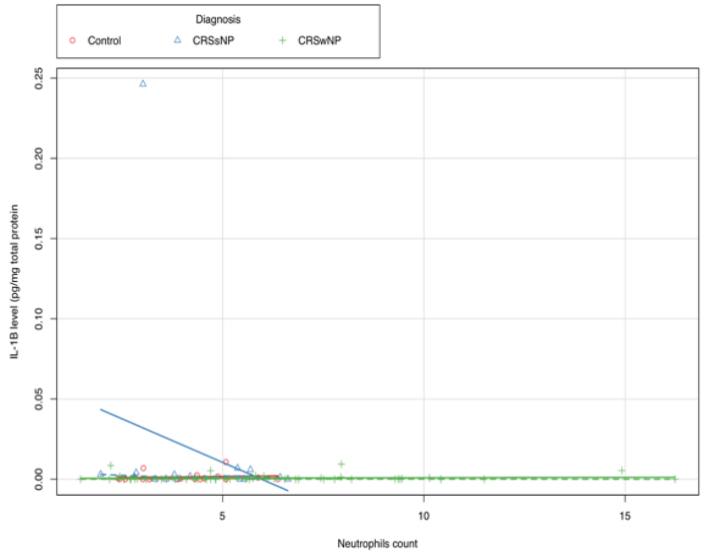
The strongest positive correlations appeared to be between IL-1ß/IL-8 and MMP-7/MMP-9. Negative correlations were noted between IL-1ß/neutrophil count, IL-15 with both cells (neutrophil and eosinophil count), IL-31 with allergy and asthma, Periostin/eosinophil, MPO/neutrophil count and MCP-4/eosinophil count, with only this one being significative. All other correlations were weakly positive and not significant.

Table 19: Correlation between relevant biomarkers.

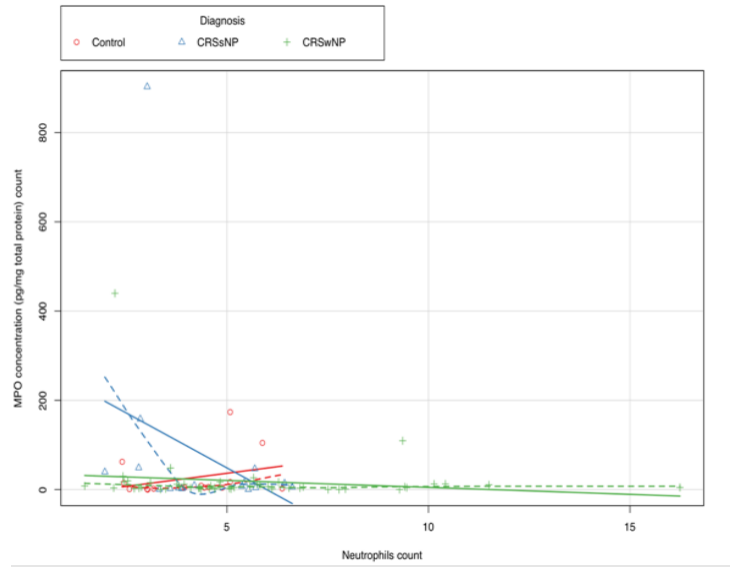
	p-value	Correlation coefficient
IL-1ß with neutrophil count	NS	r=-0.14
IL-5 with eosinophil count	NS	r=0.3
IL-8 with neutrophil count	NS	r=0.16
IL-8 with IL-1ß	**$2.2x10^{-6}$**	r=1
IL-13 with eosinophil count	NS	r=0.12
IL-15 with eosinophil count	NS	r=-0.14
IL-15 with neutrophil count	NS	r=-0.072
IL-31 with allergy	NS	r=-0.16
IL-31 with asthma	NS	r=-0.06
MMP-7 with MMP-9	**$1.4x10^{-5}$**	r=0.41
Periostin with eosinophil count	NS	r=-0.079
MCP-4 with eosinophil count	**0.0069**	r=-0.32
MPO with neutrophil count	NS	r=-0.1
ECP with eosinophil count	NS	r=0.14
Eotaxin with eosinophil count	NS	r=0.19

A p-value ≤ 0.05 was considered significant; NS, not significant; r, spearman correlation

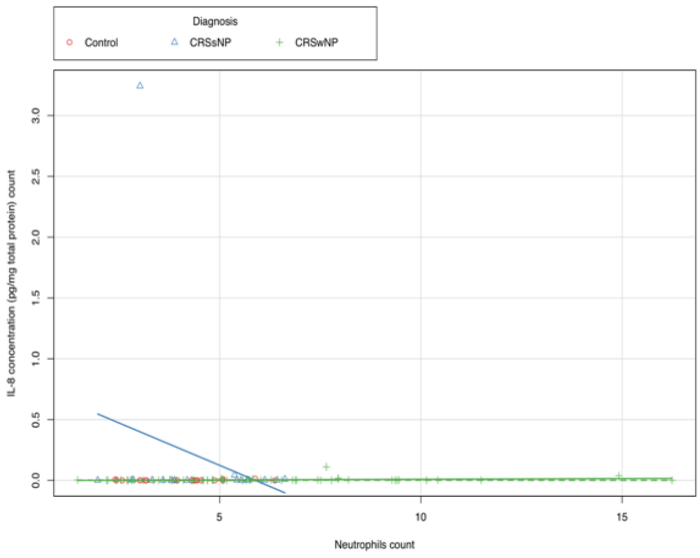
To understand who was driving these “tendencies”, we analysed these correlations within the specific groups (or phenotypes). Results can be seen in Figure 23.


Diagnosis
Control
CRSsNP
CRSwNP
IL-1B level (pg/mg total protein
0.00
0.05
0.10
0.15
0.20
0.25
5
10
15
Neutrophils count

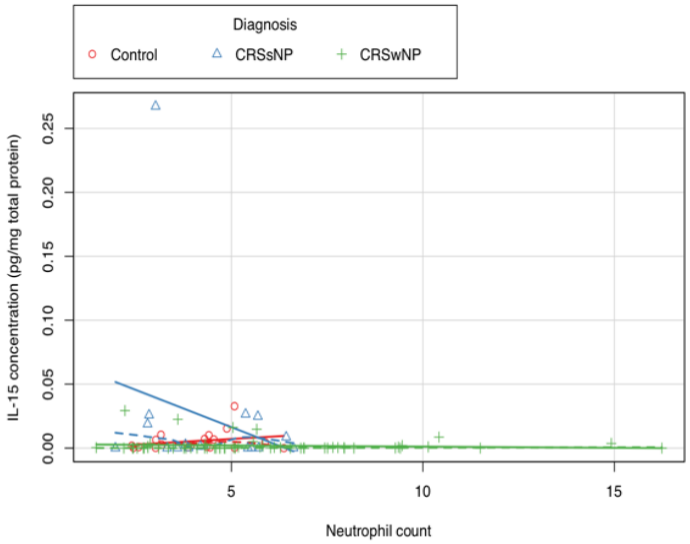
A.


Diagnosis
Control
CRSsNP
CRSwNP
MPO concentration (pg/mg total protein) count
0
200
400
600
800
5
10
15
Neutrophils count

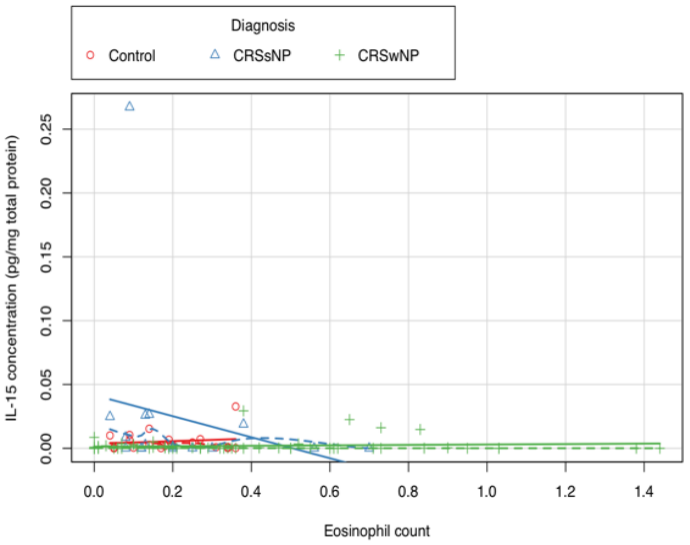
B.


Diagnosis
Control
CRSsNP
CRSwNP
IL-8 concentration (pg/mg total protein) count
0.0
0.5
1.0
1.5
2.0
2.5
3.0
5
10
15
Neutrophils count

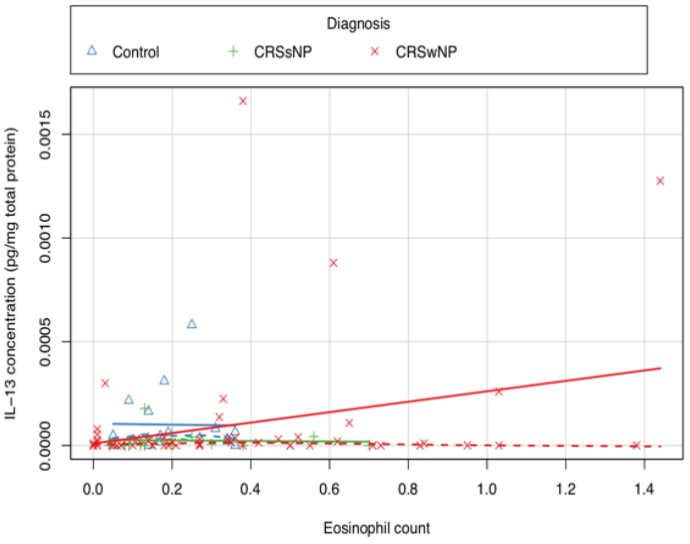
C.


Diagnosis
Control
CRSsNP
CRSwNP
IL-15 concentration (pg/mg total protein)
0.00
0.05
0.10
0.15
0.20
0.25
5
10
15
Neutrophil count

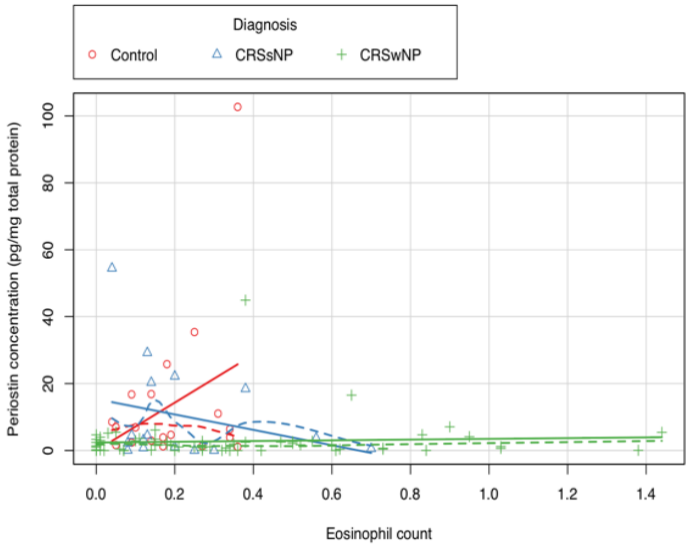
D.


Diagnosis
Control
CRSsNP
CRSwNP
IL-15 concentration (pg/mg total protein)
0.00
0.05
0.10
0.15
0.20
0.25
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Eosinophil count

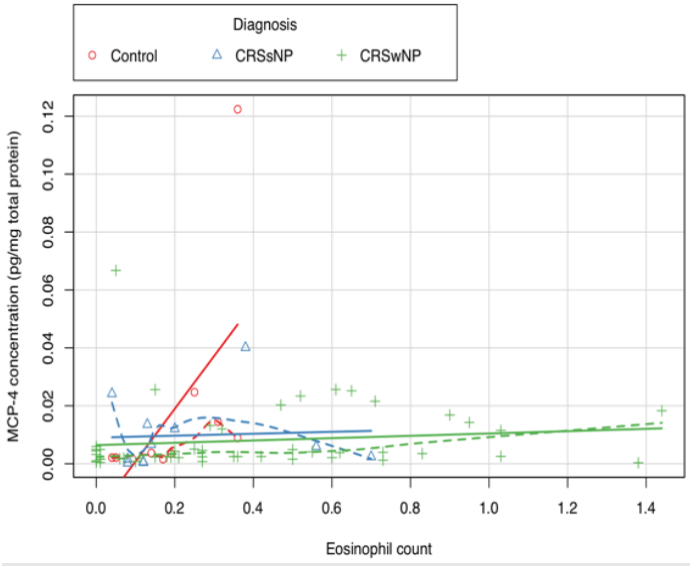
E.


Diagnosis
Control
CRSsNP
CRSwNP
IL-13 concentration (pg/mg total protein)
0.0000
0.0005
0.0010
0.0015
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Eosinophil count

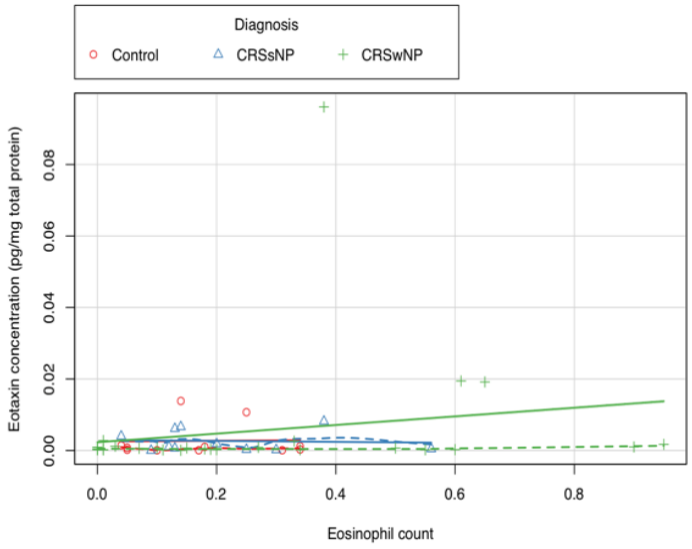
F.


Diagnosis
Control
CRSsNP
CRSwNP
Periostin concentration (pg/mg total protein)
0
20
40
60
80
100
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Eosinophil count

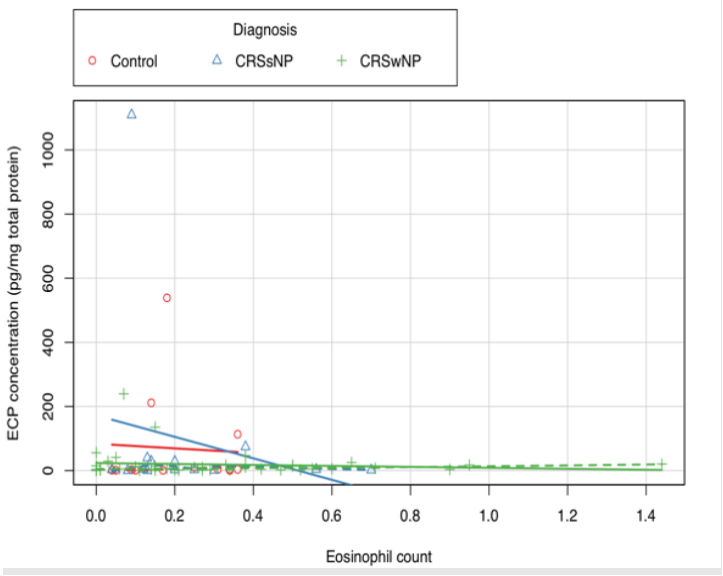
G.


Diagnosis
Control
CRSsNP
CRSwNP
MCP-4 concentration (pg/mg total protein)
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Eosinophil count

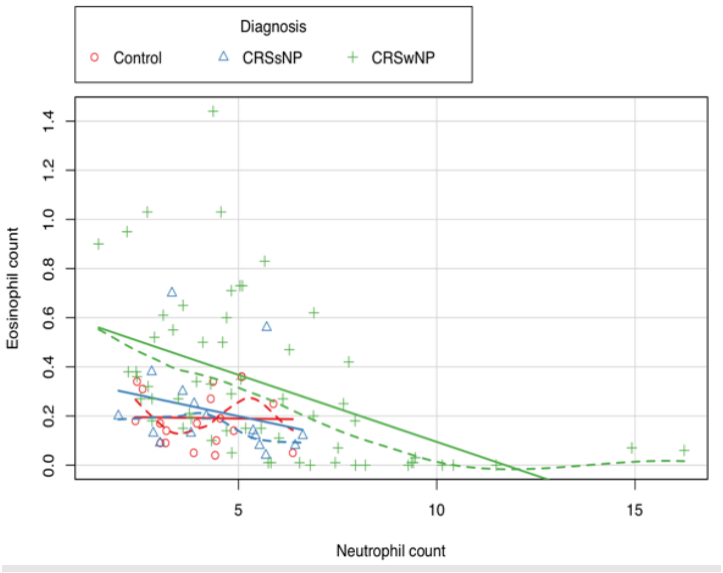
H.


Diagnosis
Control
CRSsNP
CRSwNP
Eotaxin concentration (pg/mg total protein)
0.00
0.02
0.04
0.06
0.08
0.0
0.2
0.4
0.6
0.8
Eosinophil count

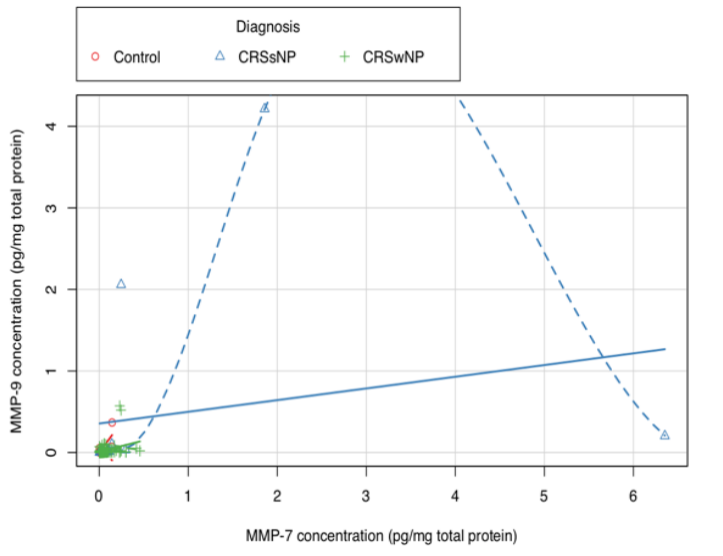
I.


Diagnosis
Control
CRSsNP
CRSwNP
ECP concentration (pg/mg total protein)
0
200
400
600
800
1000
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Eosinophil count

J.


Diagnosis
Control
CRSsNP
CRSwNP
Eosinophil count
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
5
10
15
Neutrophil count

K.


Diagnosis
Control
CRSsNP
CRSwNP
MMP-9 concentration (pg/mg total protein)
0
1
2
3
4
0
1
2
3
4
5
6
MMP-7 concentration (pg/mg total protein)

L.

Figure 23: Correlation analysis: between IL-1B/neutrophil count (A), IL-8/neutrophil count (B), MPO/neutrophil count (C), and IL-15/neutrophil count (D), IL-15/eosinophil count (E), IL-13/eosinophil count (F), Periostin/eosinophil count (G), MCP-4/eosinophil count (H), Eotaxin/eosinophil count (I), ECP/eosinophil count (J), eosinophil/neutrophil counts (K), and MMP-9/MMP-7 (L). Graphical representation in each individual group.

In general, CRSsNP was responsible for driving the correlation between different biomarkers and neutrophil counts (IL-1ß, IL-8, IL-15, MPO). Moreover, it was also the one group showing the correlation found on Table 19 between the two metalloproteinases and between ECP and neutrophil counts. CRSwNP was the phenotype forming a distinctive pattern in Eotaxin/eosinophil count (Figure 23I) and between eosinophil/neutrophil count (Figure 23K).

In addition, looking into each specific group for Periostin/eosinophil count, only the control group presented its known correlation (147). CRSwNP displayed a small positive correlation coefficient, showing that eosinophils might increase with Periostin in these patients (Figure 23G); while MCP-4/eosinophil count suggested that eosinophils increase with MCP-4 expression in all CRS groups (Figure 23H).

To continue with our investigation, we turn now to the correlation between know mediators and its associated clinical characteristics that have been described into chapter 3. We correlated the presence of allergic and AERD syndromes with IL-10 levels (Figure 24), which showed that IL-10 tended to be decreased in the presence of these comorbidities.

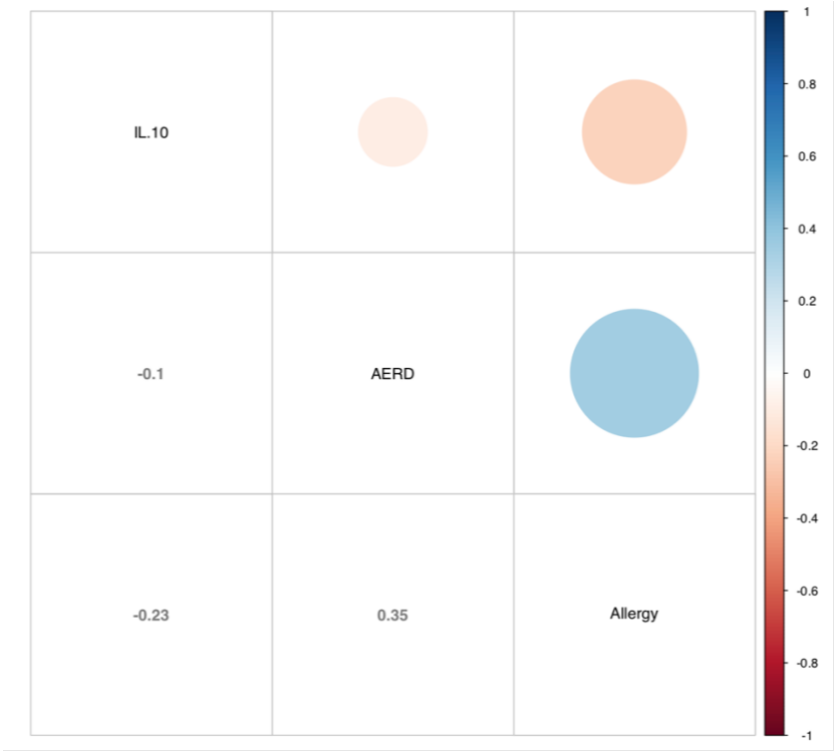


Figure 24: Correlation matrix of IL-10, AERD and allergy syndrome. The area of the circles in the upper part of the figure shows the absolute value of the corresponding correlation coefficients, and the lower part shows the exact correlation coefficients between each pair.

As both TNF-α and IL-6 are pro-inflammatory cytokines, and in chapter 3, IL-17A was described as an inducer of both cytokines, we investigated the relationship between the three. The results are presented in Figure 25. The Spearman correlation coefficients of IL-17A with both pro-inflammatory cytokines suggest that IL-17A increases their presence when it is increased itself. Moreover, the Spearman coefficient for TNF-α/IL-6 suggested that TNF-α tended to increase along with IL-6.

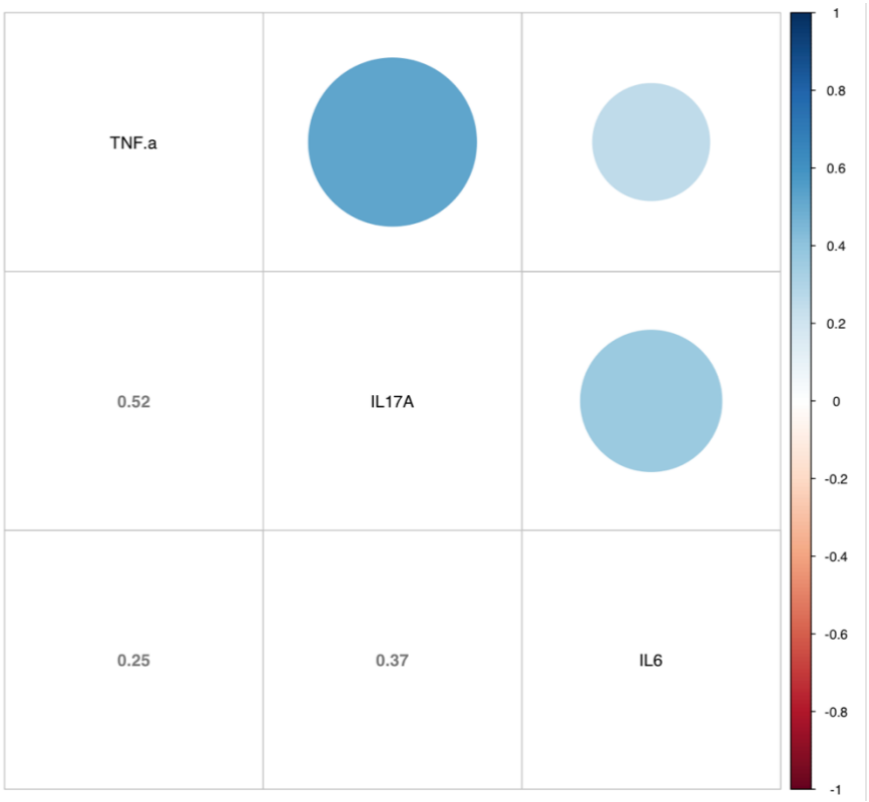


Figure 25: Correlation matrix of TNF-α, IL-17A and IL-6. Circle area in the upper part of the figure show the absolute value of corresponding correlation coefficients, and the lower part shows the exact correlation coefficients between each pair.

6.4 Discussion

In the present study, we compared the profiles of different immunologic markers according to the clinical phenotypes (CRSsNP and CRSwNP). These biomarkers were identified previously through a systematic review (Chapter 3), which resulted in 35 biomarkers to be analysed. However, six biomarkers could not be tested in these analyses: Staphylococcal enterotoxin-specific IgE (SE-IgE), transforming growth factor beta 1 (TFG-ß1), neopterin, endothelin (ET) 1, Siglec-8 or SAF-2, and albumin. While SE-IgE, Neopterin and albumin were not tested due to a lack of resources (e.g.: lack of funding or commercial detection kit not available), the other three were not tested due to the limited sample availability.

It has been reported that CRSsNP is driven by a predominant T_H1-skewed response, whereas CRSwNP were classified into 2 subtypes (e-CRSwNP: T_H2-dominant response with eosinophil infiltration;

and ne-CRSwNP: mixed T cell subsets with neutrophilic infiltration) (21,63,65–70,75). Both CRSwNP and sNP exhibit considerable heterogeneity in the nature of the inflammatory response between individuals due to genetic, epigenetic, and environmental factors (Chapter 1). Therefore, in this study, we investigated the patterns of sinus mucosal inflammation in adult patients with CRS to verify the accuracy of these findings. Although we recruited patients only at one site, we believe these patients to be representative of the current CRS UK population as this population which is increasingly mixed and varied in severity and clinical features.

A total of 113 patients were tested in this study across all analysed biomarkers. However, some samples produced a result of "not a number", which could not be analysed statistically and are therefore not included in these analyses. In our study, we employed the same techniques validated in other studies - Luminex technology (as explained previously) and ELISA technique, which allowed us to analyse up to 27 human proteins related to inflammation in CRS and healthy subjects in one single sample.

In this study, we started by investigating the T_H1 and T_H2-associated cytokines. The first group, composed by IFN-γ, acts to promote the T_H1 immune response and to induce cell apoptosis (63,66,70,73,75,120–122,126). Across groups, its expression levels were overall significantly elevated in CRSsNP and decreased in patients presenting nasal polyps, presenting significant difference between CRS groups agreeing with initial studies by Van Zele et al. in Belgium (66,154) and disagreeing with other groups, which do not describe an elevation of IFN-γ in CRSsNP (58,67,155,156).

The latest group, which included IL-4, IL-5, IL-13, IL-17E/25, IL-31, IL-33, TSLP, IgE and Periostin, presented different patterns across groups. Firstly, IL-4, one of our mandatory biomarkers and found to have a role in the induction of T_H2 differentiation and IgE class switching (chapter 3), was discovered to be significantly decreased in our CRS population (both groups) when compared with

the controls. This finding disagrees with most literature studies (65), and with what is known on CRS treatment, as Dupilumab is known to block IL-4 signal, which in our cohort was not increased. IL-5 level was almost 100-fold superior in CRSwNP when compared to the other groups and significantly higher than controls. This agrees with several other studies (65,77,123,157), as it is associated with eosinophils (115,117,120,121) and therefore, it was expected to be increased in patients presenting NP. Due to its role in the pathophysiology of CRS, we correlated its levels with eosinophils, but the low correlation observed suggests that IL-5 variation is unexplained by the eosinophil count in this cohort, with no particular group being responsible for this (figure not shown). The next T_H2-associated cytokine was IL-13, described previously as responsible for the recruitment, activation and survival of eosinophils and mast cells, and it is expected to be increased in inflammatory and allergic reactions. Several studies have described its increase in CRS disease, more so on patients presenting with CRSwNP (155,158,159); however, our results showed that patients with CRS presented lower levels of IL-13 than the controls, with patients with NP showing a higher level than patients without NP. With this, we also investigated the relationship between eosinophil and IL-13 levels and observed that IL-13 tended to increase along with the eosinophils count only on CRSwNP patients, which agrees with the relationship described in the literature between the two (159). IL-17E or IL-25 was described as an inducer of T_H2 responses while inhibiting the T_H1 and T_H17 cell responses (chapter 3), and eliciting an overproduction of IL-4, IL-5, and IL-13 (160). Moreover, it has been described as playing a crucial role in the pathogenesis of CRSwNP in Asian patients (143,161). However, due to the limited amount of analysis, these correlations were not investigated, and our results showed the opposite, but our population was not in its majority Asian, which can explain such differences.

IL-31 was previously described as associated with eosinophil levels in serum in allergic asthma and rhinitis (115), as well as exerting a function on mucus production. Therefore, in this study we studied its possible action on CRS. We observed that it was increased in patients without NP and decreased in patients with NP. Even if this mediator has not been described in CRS yet, it has been reported to act in immune mediated and allergic diseases such as asthma, allergic rhinitis, urticaria, dermatitis, among others (115,162,163). Here, we correlated IL-31 levels with the presence of asthma and allergy to verify this action within CRS. However, our results suggested that IL-31 tended to be decreased in the presence of allergy or AERD.

Another potent inducer of T_H2 responses described on Chapter 3 was IL-33. Its expression is elevated in nasal tissues of patients with CRSwNP, however in our study this was not verified. CRSsNP presented the higher level of this marker and CRSwNP patients presented a significant decrease in IL-33 levels when compared with the remain groups. Its increase in patients without NP can be due to its previous association with T_H1/ T_H17 cytokines (IFN-γ and IL-1β), which were higher on CRSsNP patients; however, the remaining inflammatory and neutrophil recruitment markers were not studied here.

Thymic stromal lymphopoietin helps to shape the local activation of T_H2 responses. Moreover, it has been shown previously that its mRNA levels are increased in NP (127–129). Since we did not measure mRNA, but it is a precursor of protein synthesis, we still expected its protein levels to be increased in patients suffering from NP. However, our investigations showed that TLSP levels were also significantly higher in patients without NP than in patients with them. Furthermore, TLSP expression was significantly decreased in patients with NP when compared to its physiological expression. Further studies would be needed for this biomarker in order to understand if the protein levels follow a similar result at mRNA

level or if, there is a regulatory mechanism that decreases protein levels even if its mRNA levels are increased during nasal polyposis, as described before.

In Chapter 3, we reported the increase of IgE in CRSwNP patients from Europe and Asia (63,65,74,75,123,136). However, our findings showed that IgE was elevated in CRS patients without NP and decreased in CRSwNP.

Periostin was the last T_H2-associated mediator to be studied in here. Periostin is known in airway eosinophilia and as a biomarker for nasal polyps in CRS (147). Nevertheless, in here, we observed its levels to be increased within CRSsNP patients and significantly decreased in the CRSwNP group, when compared with the control group. More, we were interested in investigating the correlation between this biomarker and the level of eosinophils. The Spearman correlation coefficient suggested that eosinophils decrease when Periostin increases, which contradicts our literature review. However, when looking at the different groups' behaviour, we can observe that only data points from the CRSsNP group showed this relationship, while the control group and CRSwNP presented its known association.

After investigating T_H1 and T_H2-mediators expression, we moved to describe the behaviour of T_H9- and T_H17-markers in our population. T_H9-associated cytokine, IL-9, was described by Bal et al. as having an action on mucus production. More, it has been described as overexpressed in CRS, specifically in CRSwNP (164,165); however, in our results, CRSsNP presented the higher levels of IL-9, contradicting the literature findings that IL-9 would be involved in allergy and asthma (165), as this group presented the least proportion of it. T_H17 only studied mediator in this study, IL-17A, induces the activation/formation of pro-inflammatory cytokines, such as IL-6 and TNF-α, and MMPs, and to recruit and activate neutrophils to the site of inflammation (Chapter 3). Therefore, we expected it to be increased in CRSsNP and positively

correlated with neutrophil and neutrophil-related markers and pro-inflammatory cytokines. We observed that its levels were increased, even if not statistically significant, in patients with CRSsNP and decreased in patients with CRSwNP, when compared with physiological levels. When, we investigated the relationship between the three cytokines: IL-17A, IL-6 and TNF-α, the correlation coefficients suggest that increasing IL-17A levels lead to an increase when it is increased itself of the other two cytokines, as observed by Chen et al (166).

Next, we turn our attention to the mediators that have a pro- and anti-inflammatory functions. Our pro-inflammatory mediators, which included IL-1ß, IL-6, IL-32 and TNF-α, have not been all studied within CRS. However, due to its function when the immune system is triggered by an infectious agent, and the lack of explanation for CRS immunopathology, we investigated IL-6 and TNF-α equally across both CRS patient and controls. All mediators apart from IL-32 were increased in CRS cases compared to controls. IL-32, which induces the activation/formation of pro-inflammatory cytokines, such as IL-6 and TNF-α, was expected to be increased in patients with CRSwNP (44,125). However, the expression of IL-32 was lower within CRS groups, being the lowest within the CRSwNP group with no explanation. Regarding IL-6 and TNF-α, these were both increased in both CRS phenotypes compared with controls, demonstrating that CRS might have some infectious underlying cause, or activate this pathway during its course. Moreover, the correlation between these two inflammatory markers proved that they move together in CRS inflammatory environment. Lastly, IL-1ß was increased in both CRS groups, which correlates with the Belgian studies (65,66). Since it is known that IL-1ß increases in CRS patients with increased levels of neutrophils (Chapter 3), we correlated the two measurements. The obtained correlation suggests that IL-1ß tended to decrease when the neutrophil count increased, which is not in agreement with the current literature in the field.

Therefore, we looked into this trend within the specific groups and only the CRSsNP group presented IL-1ß decrease associated with neutrophil count increase with no known justification. Therefore, we suggest further studies to be performed to confirm this trend and possibly correlate it with another biomarker or clinical feature not studied here.

Only IL-10 has anti-inflammatory characteristics and has been described previously (Chapter 3) as being elevated when patients suffer from allergic or aspirin-exacerbated respiratory disease (AERD) syndromes. In our analysis, the levels of IL-10 were increased in our population of patients without NP and decreased in our population of patient with NP. However, our population of CRSsNP patients showed the lowest percentage of allergy, showing that IL-10 was not increased in the presence of allergy in our cases. To verify this in our study, we correlated the presence of allergic and AERD syndromes with IL-10 levels, concluding that IL-10 was decreased in the presence of allergy or AERD, which would explain the results in our cohort.

Due to their major roles in the pathogenesis of CRS described previously in Chapter 3, we investigated the circulating levels of both neutrophils and eosinophils across the different groups of patients. Both cell types were increased in CRS patients when compared with the control group confirming their roles described in several important studies. Later, we observed the correlation between these two cells suggesting that eosinophils tend to decrease when neutrophils increase. This is observed in both CRS groups defending the separation of CRS in eosinophilic and neutrophilic CRS forms, as the control group showed no correlation between both cell types. We investigated therefore, the neutrophil- and eosinophil-associated biomarkers. Regarding the neutrophil-related markers, they were increased in the CRSsNP group than in the other groups. In Caucasian patients, CRSsNP has been described as neutrophil-mediated inflammation (167,168), which we here confirm

such findings as all neutrophil-associated markers studied were increased in this group, that was composed mostly by white patients. Further, the described functions of IL-8 in CRS are the same as for IL-1ß (Chapter 3). As such, we correlated the levels of IL-8 with neutrophils and its related cytokine, IL-1ß. For the first correlation, it suggested that IL-8 tended to increase along with the neutrophils count, especially in CRSsNP, which was expected according to the literature (155). The correlation between IL-8 and IL-1ß suggests a perfectly monotonically relation, proving that both are associated as described previously on our literature review. Lastly, we also looked at the correlation between MPO and neutrophils. The Spearman correlation coefficient showed this relation to be inversed, meaning that when MPO levels increase, the neutrophil cell count decreases. This was unexpected and we looked at all groups individually. This is pronounced in the CRSsNP group, while physiologically (control group) the direct relationship between MPO and neutrophils was demonstrated. As such, this suggests that our CRSsNP patients present some factor that is influencing this relationship and as such, further studies should be undertaken to understand this at a genomic level (mRNA) and relate it to other features of CRSsNP.

On the other hand, the eosinophil-related markers - ECP, MCP-4 and RANTES -, were all decreased in patients presenting NP. As described previously on Chapter 3, all of these biomarkers, expect for RANTES, are expected to be increased in NP, more precisely in eosinophilic CRSwNP. An explanation for such decrease could be the nature of the CRSwNP, perhaps in this case non-eosinophilic, even if eosinophil count was higher on this group. In order to confirm our findings, further studies where patients are divided according to the eosinophilia level should be considered. As we did not conduct histological examinations, it is not possible to do such stratification in this study and will be discussed as a limitation further on. RANTES, on the other hand, has been described previously as a potential biomarker for CRSsNP (Chapter 3). Here,

taking the control group as descriptive of physiological conditions, RANTES was slightly increased in the CRSsNP group, and decreased in patients with NP. This proves that RANTES can discriminate between both groups, as described previously (67,73,123).

Eotaxin is an important factor in eCRSwNP, being responsible for recruiting and activate eosinophils (Chapter 3). Prior to any analysis, we expected its levels to be increased in the CRSwNP group. This was confirmed in our analysis, as the levels of eotaxin were higher in the CRSwNP patients and decreased in CRSsNP, when compared with the control group.

As said before, all these were markers associated with eosinophils. We, therefore, wanted to assess this relationship between biomarkers and eosinophils count in our groups. MCP-4, Eotaxin and ECP all correlated positively with eosinophils, demonstrating the known relationship between them. When looking at the different groups, we can observe that for each biomarker the group demonstrating this pattern differed. MCP-4 showed a positive correlation for all groups, independent of the condition, proving this association to happen even on physiological conditions. For ECP, only points from the CRSsNP group showed a negative association with eosinophils and the controls displayed a flat line, showing that within control patients, ECP had no correlation with this cell type. This might suggest that the positive relationship between ECP and eosinophils described in the literature might be correct for NP. Lastly, for Eotaxin, we observed that only the CRSwNP group showed this distinct pattern, while CRSsNP displayed a flat line.

According to Kim et al. (124), IL-15 prevents the apoptosis of eosinophils and neutrophils, and in their study, its levels did not differ significantly between CRS and controls. However, our results show that it was significantly higher in CRSsNP and decreased in CRSwNP group, when compared with controls. Therefore, we investigated the correlation between this cytokine and the levels of

eosinophils and neutrophils. These analyses suggested that IL-15 tended to decrease along with both cells count.

Kato et al. (123) described G-CSF to be increased in NP; nevertheless, in our analysis it was significantly increased in patients without NP, contradicting this report. Further studies should be considered to confirm either point.

We included in our analysis; two metalloproteinases described in Chapter 3 as having some function on CRS and increased in both CRS groups (123). Our investigations found MMPs to be increased in CRS, especially in the group without nasal polyps. MMP-9 was slightly decreased in CRSwNP patients compared with the control group; however, this was not significant. As both metalloproteinases are described as having a similar function, and the expression results also had alike patterns, we investigated the relationship between both MMPs. Their correlation coefficient suggests that MMP-7 tended to increase along with MMP-9. When looking at the different groups, we can observe that only data points from the CRSsNP group showed this relationship.

Collectively, these results indicate that patients with CRSsNP show an increased mixed T_H cell ($T_H1/T_H2/T_H17/T_H9$) immune response with increased neutrophilic- and eosinophil-associated inflammation, whereas CRSwNP presented an IL-5 dominated response accompanied by eosinophil and neutrophil infiltration. We can also conclude that our results were varied across groups and did not always agree with the known literature. We are uncertain if this can be explained by our cohort, which was not restricted to specific clinical features, or by the fact that all patients included received preoperative treatment, which could modify their local molecular profiles.

Recently, extensive work has been focusing in characterising the inflammatory profiles of CRS patients instead of classifying them according to clinical characteristics (e.g.: nasal polyps). Great variation both between and within CRS populations has challenged

the dichotomy of CRS diagnosis in CRSwNP and CRSsNP. Endotyping CRS based on the underlying pathophysiology has emphasised even more the heterogeneity of this complex disease, revealing mixed inflammatory profiles coordinated by a network of inflammatory cell types and molecules. Our attempt to verify a dichotomy according to phenotypes is proved to be inefficient and therefore, we will move towards a more molecular approach, defining clusters of patients that share similar molecular profiles. Such better understanding of CRS aetiology will aid the development of more personalised therapeutic strategies targeting key inflammatory markers and reducing the reliance on traditional treatments, which are more and more proving to be inefficient (167,169–171).

Key points:

- Several biomarkers were found to have significant differences in their expression across groups, although some like IL-8, IL-10, MMP-7 and neutrophil count were not discriminatory between CRS groups;
- The following biomarkers were found to be significantly increased in CRSsNP patients in comparison with CRSwNP: IL-1ß, -15, -32, -33, IFN-γ, G-CSF, RANTES, Periostin and IgE;
- TNF-α was the only biomarker significantly increased in CRSwNP patients in comparison with CRSsNP patients;
- All of the above referred biomarkers could possibly be used to separate the two phenotypes;
- IL-4 and IL-13 were decreased in CRS patients when compared with controls ($p<0.05$), contrary to the literature;
- The actual dichotomy of CRS phenotypes is not enough to explain all the variations within the CRS population.

VII – CLUSTER ANALYSIS: DEFINITION OF ENDOTYPES

7.1 Introduction

In the last chapter, we tried to define groups by phenotype. However, there was no clear separation in the biomarkers' expression between different entities of CRS, neither between them and the control patients. Recently, a new line of thought has arisen trying to define complex disease entities using endotypes instead of phenotypes.

The research performed over the last few years attempted to cluster patients by a multitude of possible features. This allowed a further characterisation regarding the pathophysiological mechanisms present in the different groups. Asthma is a good example of this, presenting endotypes such as AERD and allergic bronchopulmonary mycosis, among others. The understanding of these mechanisms together with the identification of relevant biomarkers allows a more individual approach to diagnosis and treatment, improving the outcomes of these patients (37,172).

In this chapter, the paradigm is the same. As CRS is considered to be analogous to asthma in the upper respiratory tract, we attempted to define endotypes by clustering biomarker expression within CRS patients. The aim of this study was to 1) use clustering methods to identify endotypic subgroups from a large cohort of patients with CRS, 2) describe clinical differences in identified clusters, and 3) perform discriminant analysis to define a decisional tree to classify patients into identified clusters.

7.2 Methods

7.2.1 Subjects

Subjects were recruited for this study as stated in Chapter 4.

7.2.2 Baseline clinical characteristics

All demographic and clinical characteristics of clusters were assessed before surgery. Nasal samples were collected as described previously on Chapter 4.

7.2.3 Measurement of molecular markers in tissues

The protein concentrations of detected mediators were measured as validated in Chapter 5.

7.2.4 Statistical analysis

Data were analysed using the RStudio version 1.2.13 software (RStudio, Inc.). For continuous variables, results are expressed as mean and standard deviation. Data distribution was tested for normality using a Shapiro-Wilk test. As the variables were not normally distributed, the following statistical tests were used: for dichotomous variables, a chi-square test was used to determine difference between groups; while, for continuous variables, a Kruskal-Wallis test was used for between-group comparison. If expression level of biomarkers were below the detection limit, it was replaced by the value 0 for analysis purposes. A p-value of <0.05 was considered significant.

For cluster analysis, a hierarchical cluster analysis was used, which attempts to identify relatively homogeneous groups of patients based upon selected characteristics. A number of biomarkers have been examined as potential sources of information regarding cluster identification. In this study, the subjects were sorted into groups using the k-means method, based on the correlation ratio and mixed principal component analysis. The optimal number of clusters was determined using the NbClust package in RStudio and the Elbow method. All available patients will be used in this analysis.

To define the number of biomarkers to be used, several simulations were run increasing the number of mediators to be

analysed without decreasing greatly the number of patients clustered. Twenty-one biomarkers were analysed using cluster analysis and nine clinical variables were measured to investigate the clinical patterns associated with the different clusters.

When performing the analysis using 21, 23 and 24 mediators, the number of clusters obtained was always the same (n=3), being an optimal number of three clusters obtained for our population of CRS subjects (Figure 26).

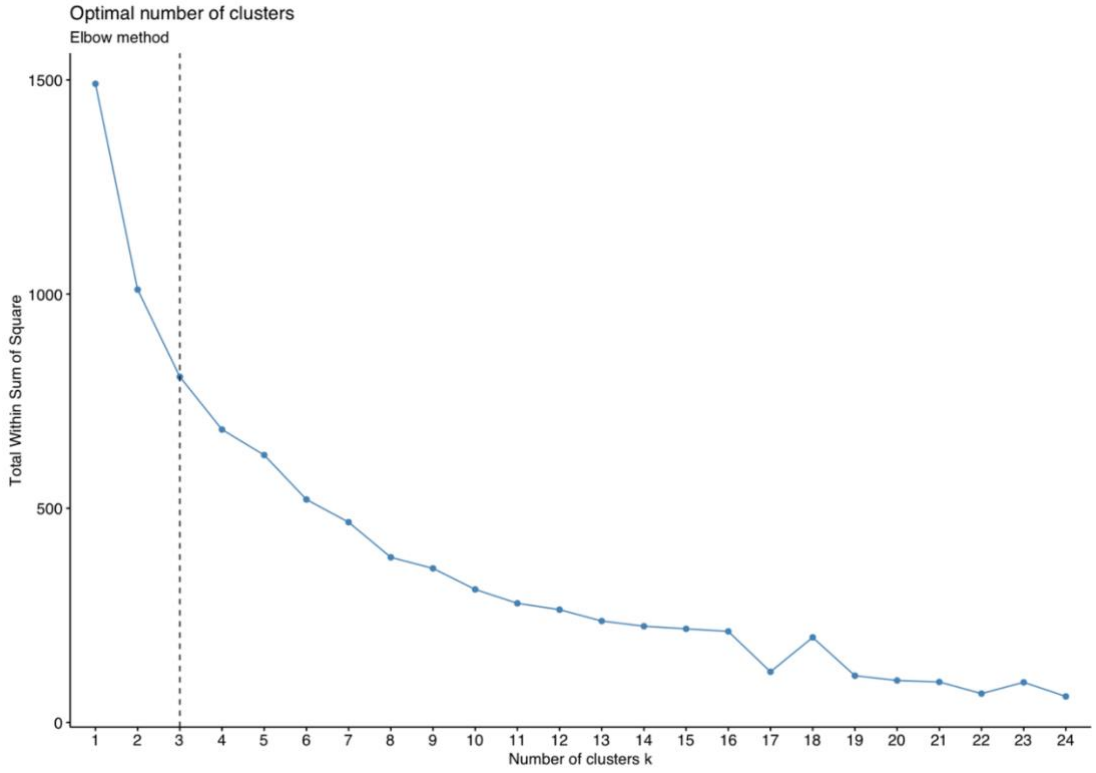


Figure 26: Graphical representation of the Elbow method in our dataset. This methods of interpretation and validation of consistency within cluster analysis helped us finding the appropriate number of clusters. The same graphic was obtained when using data from 21, 23 or 24 biomarkers.

All simulations were run using a different number of patients (73, 57 and 33 patients for 21, 23 and 24 biomarkers, respectively) as some biomarkers' data were not available for all patients. However, the clusters were defined by the same patients as observed in Figure 27 and 29. Figures 28 and 30 show the space distribution of our found clusters.

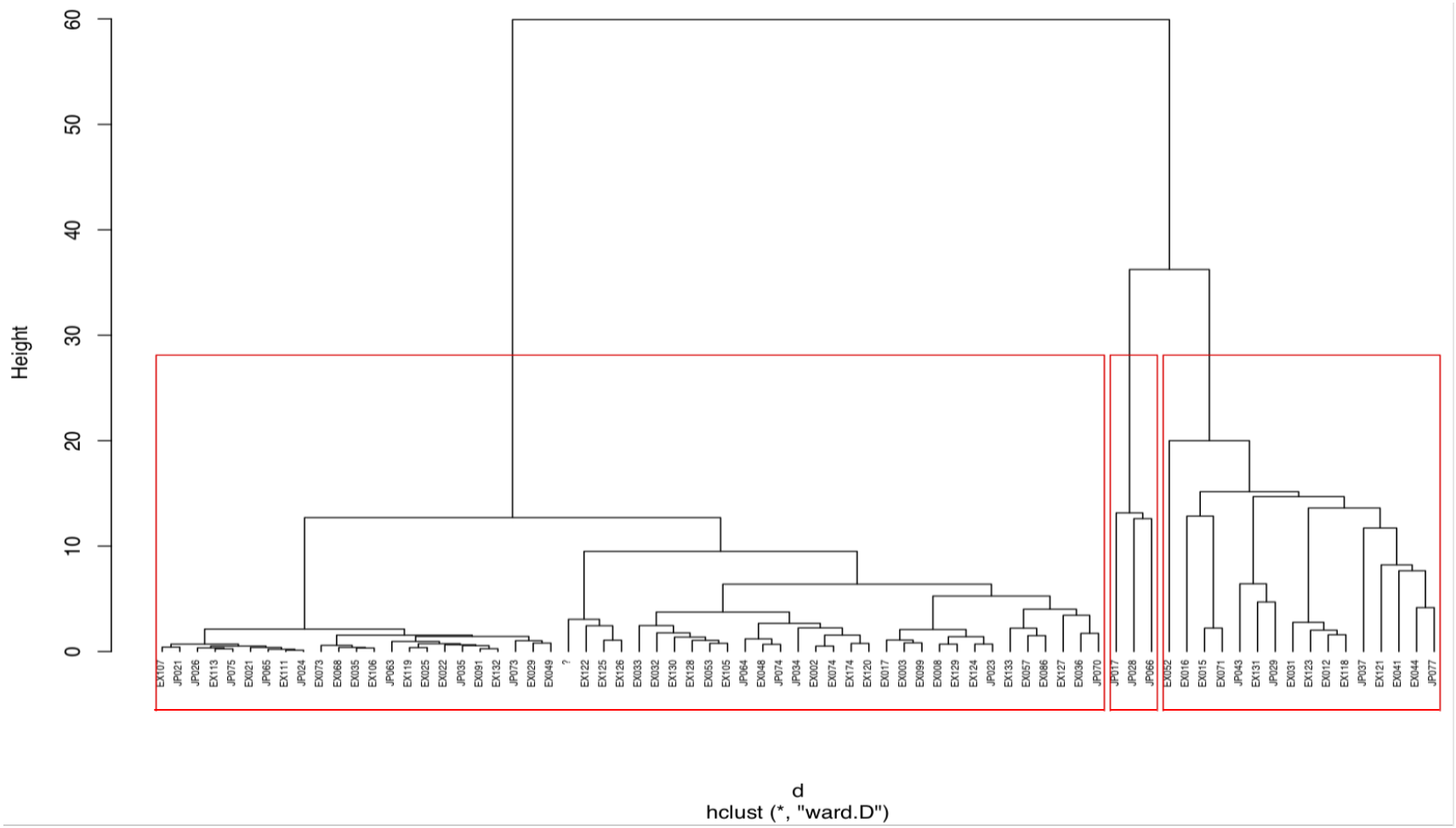


Figure 27: Dendrogram showing the hierarchical relationship of 21 biomarkers between 73 patients. Here, hierarchical binary cluster tree is showed with three clusters observed comprised of 54, 3 and 16 patients, respectively. Each red rectangle defines a cluster.

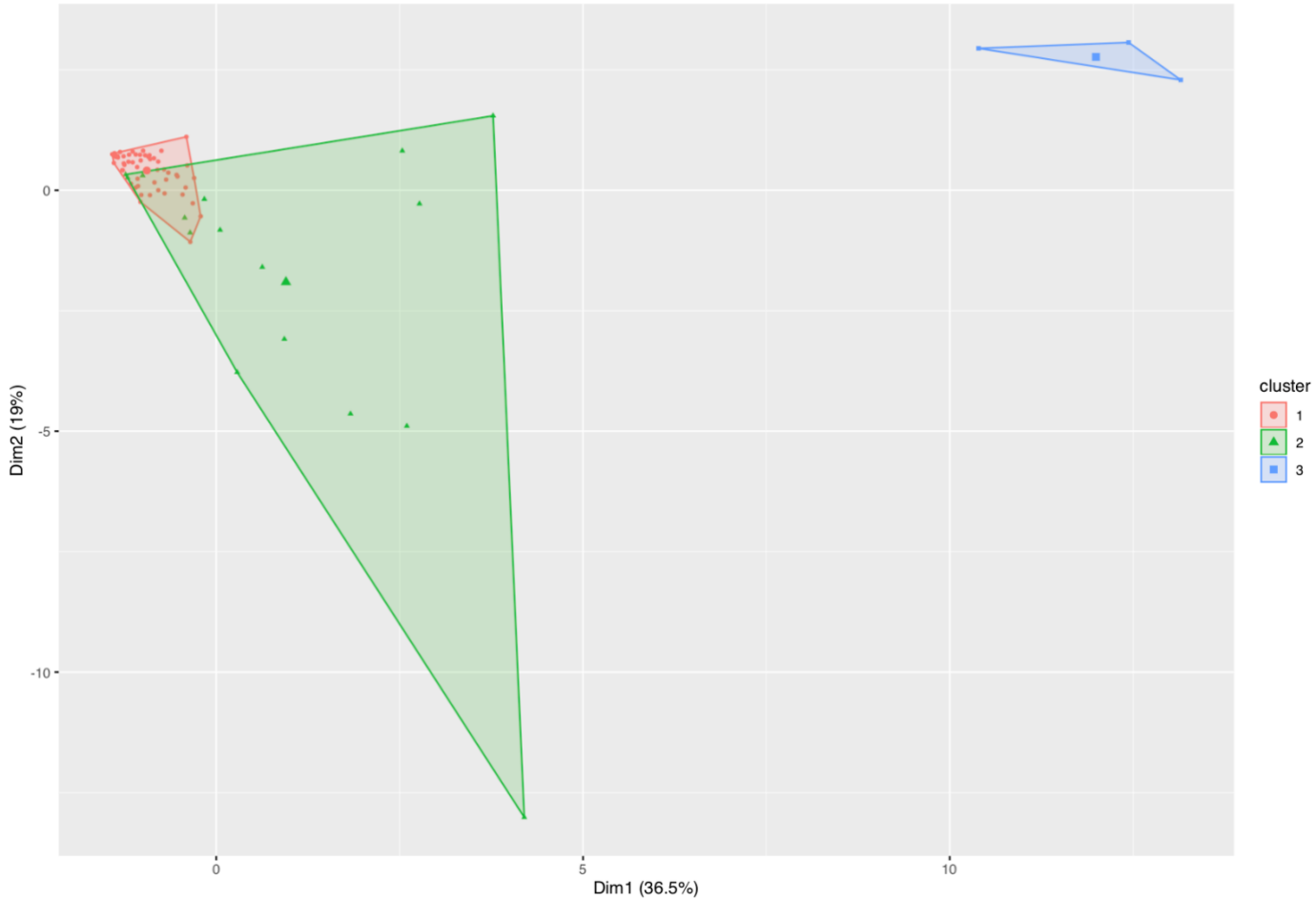


Figure 28: Scatter plot showing distinct groups from the analysis of 21 biomarkers. These correspond to the Figure 27 clusters (cluster 1 – 54 patients, red; cluster 2 – 16 patients, green; cluster 3 – 3 patients, blue).

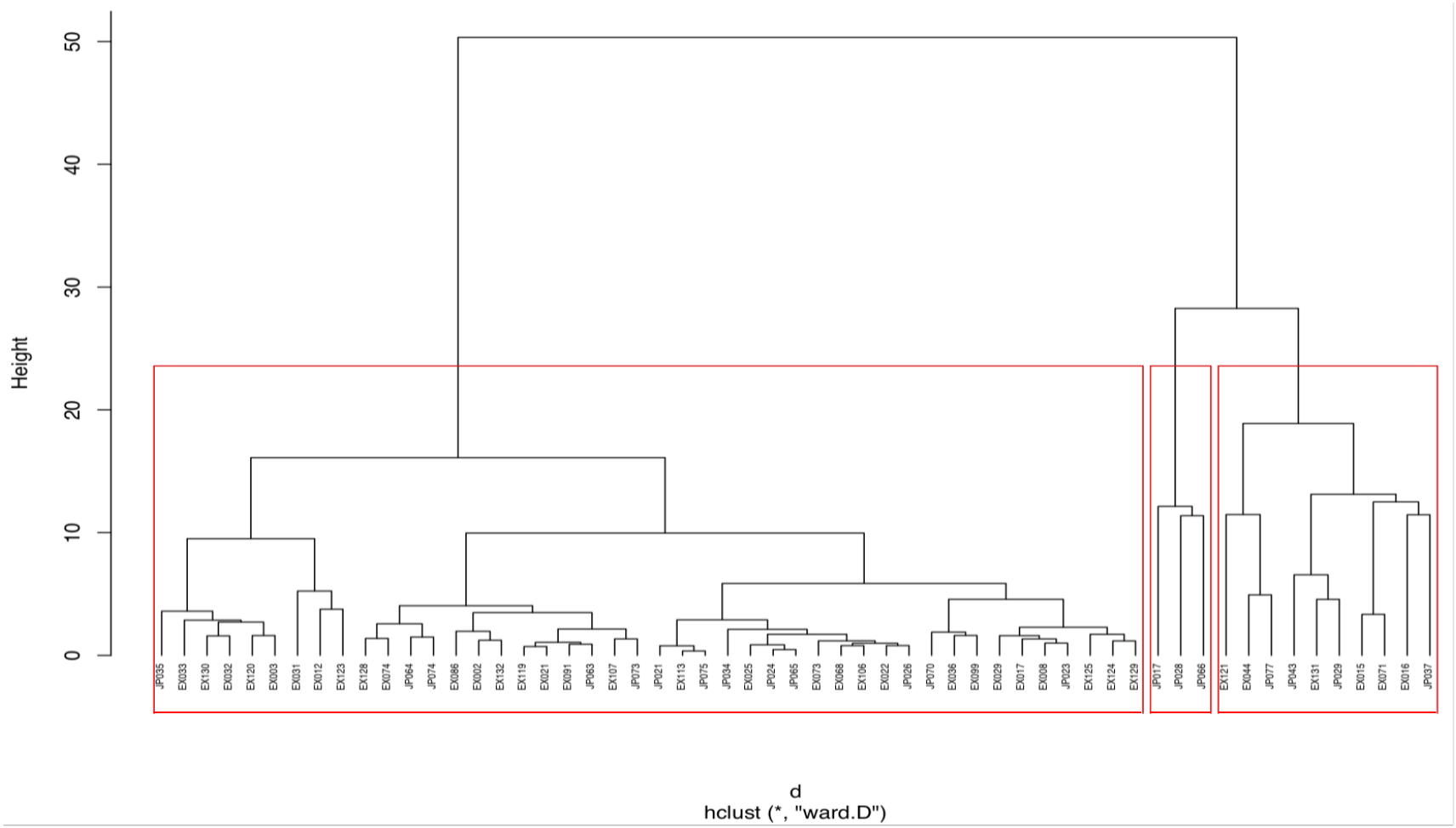


Figure 29: Dendrogram showing the hierarchical relationship of 23 biomarkers between 57 patients. Here, hierarchical binary cluster tree is showed with three clusters observed comprised of 44, 3 and 10 patients, respectively. Each red rectangle defines a cluster.

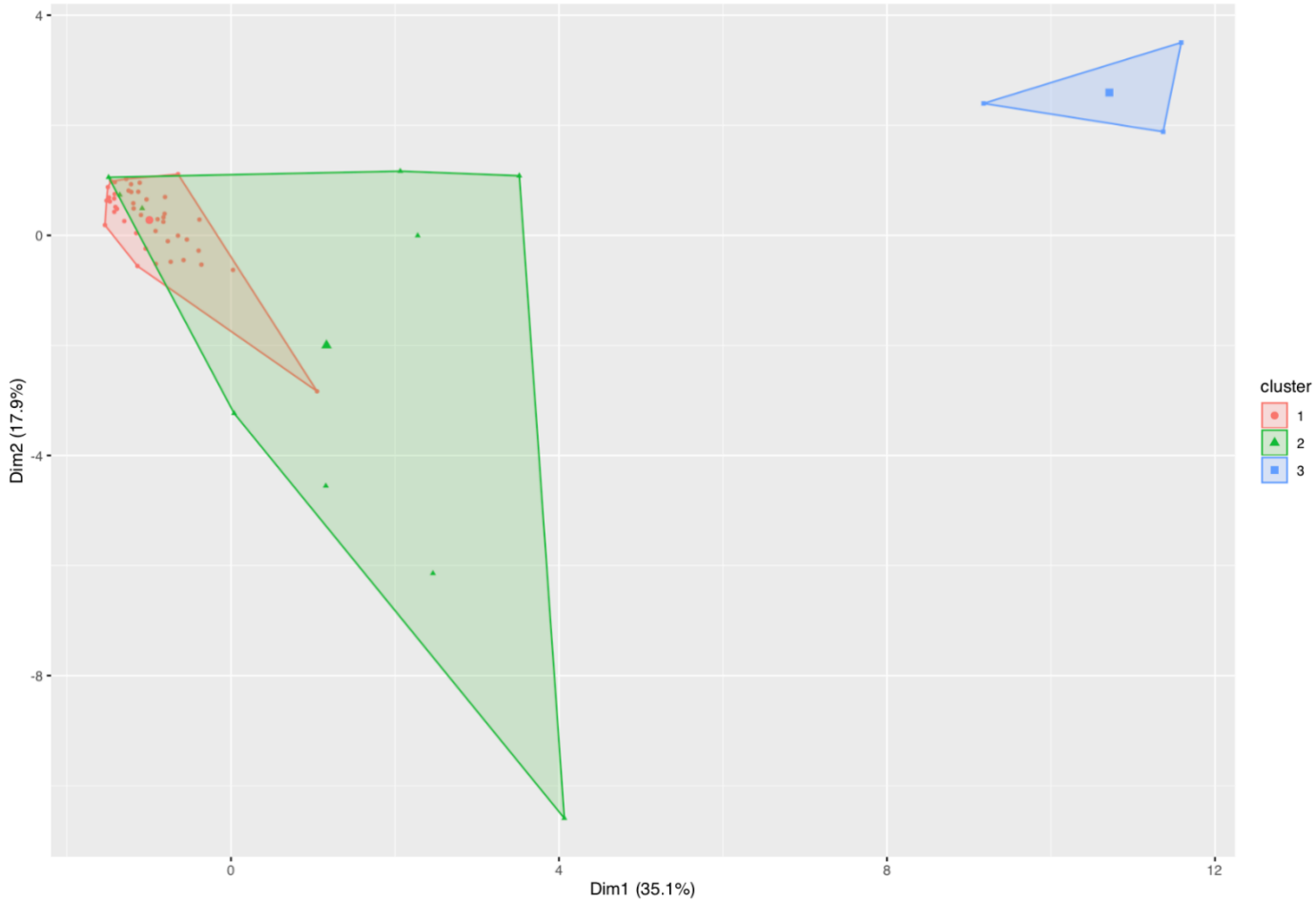


Figure 30: Scatter plot showing distinct groups from the analysis of 23 biomarkers. These correspond to the Figure 29 clusters (cluster 1 – 44 patients, red; cluster 2 – 10 patients, green; cluster 3 – 3 patients, blue).

7.3 Results

A total of 73 CRS patients were enrolled in this analysis, as these had a complete set of readings for the desired biomarkers. This was the chosen simulation since the number and the configuration of the clusters were similar across all performed simulations, and this one included the highest number of patients. Therefore, using the clustering approach outlined above, a dendrogram was generated as shown in Figure 27. A three-cluster reduction was chosen to describe our cohort and its scatterplot is presented in Figure 28. Differences across clusters are presented for demographic factors and medical comorbidities, exposure history and biomarkers' assessment (Table 20 and 21). Interestingly, traditional clinical features such as presence of nasal polyps, atopy, asthma, or aspirin sensitivity did not significantly differ across clusters. None of the demographic or measures of CRS severity (both objective and patient reported) differed either between clusters.

Cluster 1

Seventy four percent of patients are grouped into this cluster. This cluster is characterised by the highest percentage of patients with NP, AERD and smoking history (past or present). Moreover, patients in this cluster reported the highest SNOT-22 score, which meant the highest impact on QOL for CRS patients, being also the oldest cluster (mean age of 60 years). While, this cluster presented the highest neutrophil blood count, it had the lowest tissue levels of IL-1ß, IL-8, IL-15 and MPO, all markers of neutrophilic CRS. Furthermore, the levels of IL-10, which are described as being associated with AERD syndrome were 0 pg/mg. Nonetheless, this cluster presented the highest SNOT-22 scored by patients, showing that this group had the highest impact on QOL.

Cluster 2

Cluster 2 contains 22% of patients, with an even distribution of male and female patients. Most patients in this cluster presented NP as well (87%). This cluster had the highest frequency of co-existence of asthma and atopy. Along with the high frequency of atopy in this cluster, these subjects also manifested the highest eosinophil blood count. Compared with the other clusters, these patients presented the higher tissue levels of IL-10 (anti-inflammatory marker associated with allergic syndromes); IL-4, IL-5 and IL-13 (Th2-associated cytokine); TNF-α (pro-inflammatory marker); and IL-9 (Th9-associated). IL-9 was described as acting on mucus production, however only half of these patients were described as having mucopus or eosinophilic mucin. Objective disease severity measures were the highest in this cluster, with a mean CT or LM score of 17.3, which might explain the higher level of TNF-α in these subjects, whereas it presented the lowest impact in QOL (lower SNOT-22 score; Table 20).

Cluster 3

Cluster 3 is the smallest group (n=3; 4% of patients), along with the lowest scores for both SNOT-22 and LM. The majority of subjects in this cluster did not present any NP. This cluster also contained the youngest age range, and there was no history of smoking, asthma, atopy or AERD syndrome. Its eosinophil and neutrophil blood count were also the lowest of all clusters. The SNOT-22 score was almost higher than in cluster 1; however only one patient had this information available. This cluster presented the highest levels of IL-1ß, IL-8, MPO, Periostin, RANTES, TSLP, IFN-γ, G-CSF, IL-31, IL-33 and IL-17A, with the subsequent higher expression of MMP-7 and 9.

The subjects included in this analysis and subsequently in all clusters were predominantly men.

Table 20: Demographic and clinical characteristics of CRS clusters.

	Cluster 1 (n=54)	Cluster 2 (n=16)	Cluster 3 (n=3)	Overall p-value
Gender, male (%)	33 (61.1)	8 (50.0)	2 (87.0)	0.66
Age	59.92±15.24	57.4±15.21	46.3±21.46	0.50
Nasal polyps, n (%)	50 (92.6)	13 (87.0)	1 (33.3)	0.07
Atopy, n (%)	23 (42.3)	11 (69.0)	0 (0.0)	0.07
Smoking history, n (%)	30 (44.4)	6 (37.5)	0 (0.0)	0.22
Asthma (%)	55.6	69	0	0.08
AERD (%)	26	12.5	0	0.47
SNOT-22	51.5	35.4	51	0.23
LM score	16.05	17.3	12.3±11.5	0.72
Blood Eosinophil count ($x10^9$/L)	0.31	0.45	0.18	0.58
Neutrophil count ($x10^9$/L)	5.71	4.86	3.58	0.25

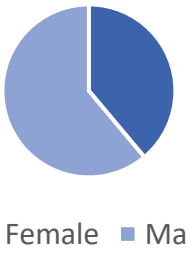
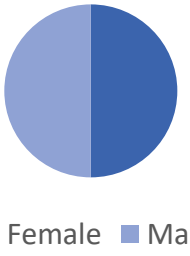
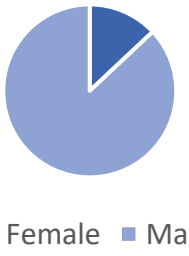
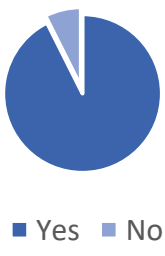
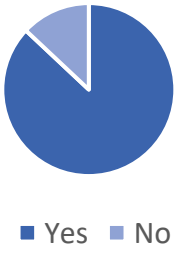
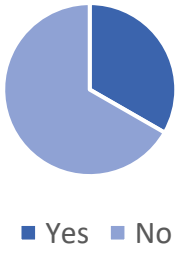
AERD, aspirin-exacerbated respiratory disease; LM, Lund Mackay.
For continuous variables, results are displayed as mean ± SD (standard deviation).
Categorical variables are expressed as frequency and percentage. An overall p-value, indicating the difference between the three clusters, was obtained using a Kruskal-Wallis test for continuous variables and chi square test for categorical variables. The overall p-value <0.05 indicate that at least one cluster was different from the others.

Table 21: Molecular profiles in sinonasal mucosa of CRS clusters. Results are presented as mean±SD. All levels are expressed as x10^{-3}, except for Periostin and MPO.

	Cluster 1 (n=54)	Cluster 2 (n=16)	Cluster 3 (n=3)	Overall p-value
IL-1ß (pg/mg)	0.2±0.4	0.9±1.3	**6.1±1.8**	<0.01
IL-4 (pg/mg)	0.04±0.1	**0.3±0.6**	0±0	0.02
IL-5 (pg/mg)	0.3±0.4	**771.9±298**	1.4±1.2	<0.01
IL-6 (pg/mg)	2.2±47	**17.5±41.9**	**16±15**	<0.01
IL-8 (pg/mg)	1.3±1.7	**2.6±3**	**2.6±0.7**	0.04
IL-9 (pg/mg)	0.4±0.8	**2.7±3.9**	0.7±0.8	0.03
IL-10 (pg/mg)	0±0	**0.2±0.5**	0±0	0.06
IL-13 (pg/mg)	0±0.1	**0.2±0.3**	0 ± 0	<0.01
IL-15 (pg/mg)	3±0.8	3.5±6.1	**27±2**	<0.01
IL-17A (pg/mg)	0.1±0.1	1.3±1.5	**2.5±1.8**	<0.01
IL-31 (pg/mg)	0.8±1.1	5.1±4.1	**6.7±3.2**	<0.01
IL-33 (pg/mg)	63±83	144.9±388.8	**2230±950**	0.01
G-CSF (pg/mg)	0.9±2.1	2.4±3.1	**90±61.5**	<0.01
MMP-7 (pg/mg)	58±57	180±140	**210±50**	<0.01
MMP-9 (pg/mg)	15 ± 26	60±130	**930±810**	<0.01
IFN-γ (pg/mg)	0.1±0.2	0.5±0.7	**26.4±18.8**	<0.01
TSLP (pg/mg)	0.5±0.6	2.2±3.3	**9.1±7.8**	0.09
RANTES (pg/mg)	13.4±22.7	40±70	**360±89**	<0.01
TNF-α (pg/mg)	0.1±0.2	**1.6±3.6**	0.3±0.2	<0.01
Periostin (pg/mg)	1.86 ± 1.88	4.2±5.57	**42.8±10.4**	0.01
MPO (pg/mg)	7.93 ± 15.04	11.96±11.32	**214.86±165.47**	<0.01

IL, interleukin; G-CSF, granulocyte colony-stimulating factor; MMP, metalloproteinase; IFN-γ, interferon-γ; TSLP, thymic stromal lymphopoietin; TNF-α, tumor necrosis factor α; MPO, myeloperoxidase.
An overall p-value, indicating the difference between the three clusters, was obtained using a Kruskal-Wallis test. The overall p-value <0.05 indicate that at least one cluster was different from the others. The numbers in bold indicate the highest value of a particular variable among the three clusters.

Figure 31 shows a summary of the clusters.

	CLUSTER 1	CLUSTER 2	CLUSTER 3
IL-1B	0.0002	0.0009	0.0061
IL-4	0.00004	0.00035	0
IL-5	0.0003	0.7719	0.0014
IL-6	0.0022	0.0175	0.0158
IL-8	0.0013	0.0026	0.0026
IL-9	0.0004	0.0027	0.0007
IL-10	0.00001	0.00024	0
IL-13	0.00004	0.00021	0
IL-15	0.0003	0.0035	0.0266
IL-17A	0.0001	0.0013	0.0025
IL-31	0.0008	0.0051	0.0067
IL-33	0.06	0.14	2.23
IFN-G	0.0001	0.0005	0.0264
MMP-7	0.06	0.18	0.21
MMP-9	0.015	0.056	0.927
MPO	7.93	11.95	214.86
G-CSF	0.0009	0.0024	0.0900
TNF-A	0.0001	0.0016	0.0003
TSLP	0.0005	0.0022	0.0091
RANTES	0.013	0.038	0.362
PERIOSTIN	1.86	4.20	42.86
Gender			
Nasal polyps			

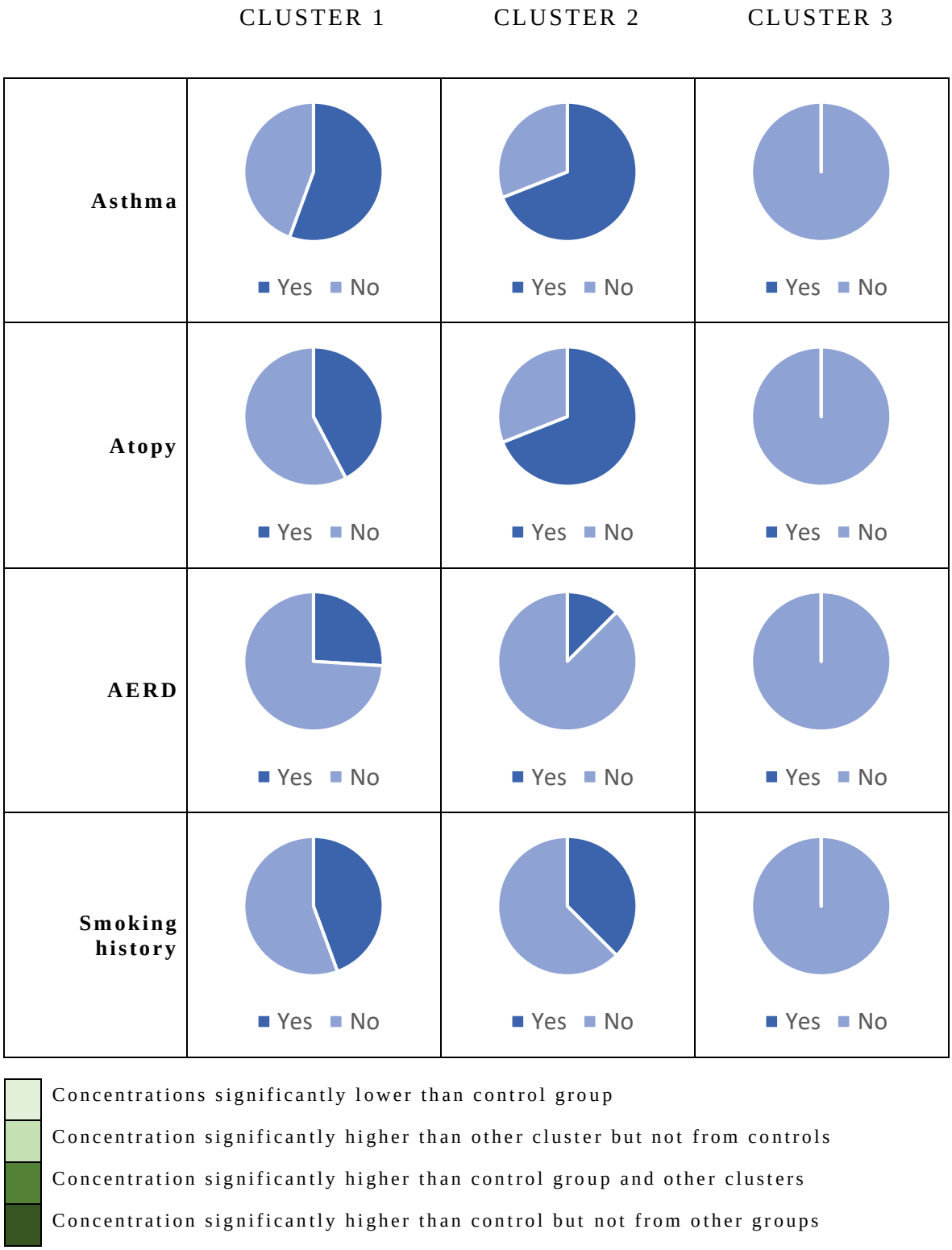


Figure 31: Diagram of identified clusters. Summary of all clusters' clinical features.

Cluster 1 was composed of 54 patients, and visually, one could argue that it could be the compilation of some sub-clusters. To verify this, we ran another simulation with just the patients belonging to Cluster 1. Figure 32 shows the dendrogram with the obtained clusters, while Figure 33 shows the space distribution of our newly identified clusters.

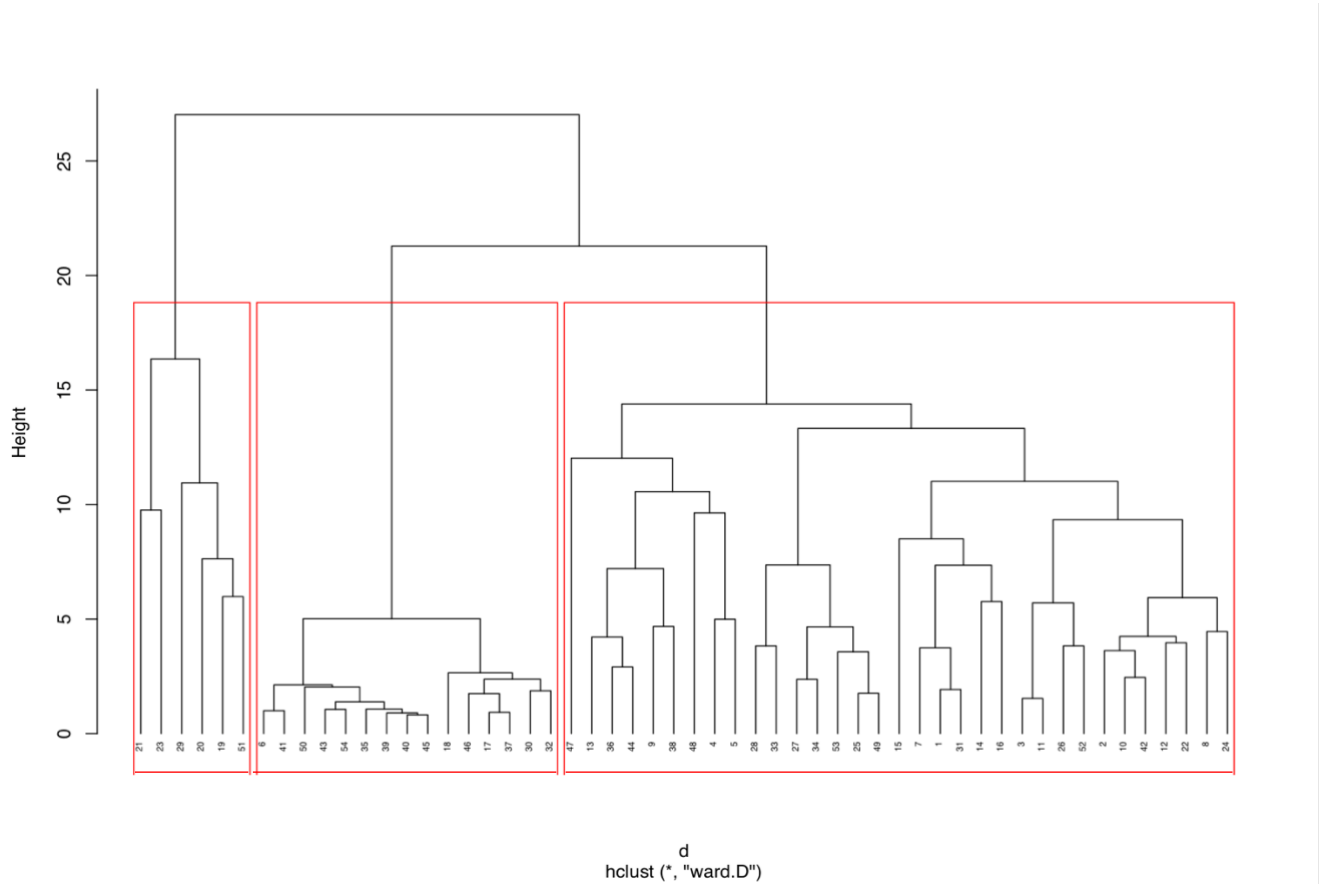


Figure 32: Dendrogram showing the hierarchical relationship of 21 biomarkers between the 54 patients from Cluster 1. Here, hierarchical binary cluster tree is showed with three clusters observed comprised of 6, 15 and 33 patients, respectively. Each red rectangle defines a cluster.

Figure 33: Scatter plot showing distinct groups from the analysis of 21 biomarkers. These correspond to the Figure 32 clusters (cluster 1 – 33 patients, red; cluster 2 – 15 patients, green; cluster 3 – 6 patients, blue).

Differences across sub-clusters for clinical, demographic features and biomarkers' expression are presented in Table 22 and 23.

Cluster 1A

The vast majority of patients in this group had NP. This cluster had the lowest frequency of AERD and smoking history (past or present). Moreover, patients in this cluster reported the lowest LM and SNOT-22 score, which meant the lowest impact on QOL for CRS patients and the less severe clinical presentation. While, this cluster presented the lowest neutrophil and eosinophil blood count, it presented the highest tissue levels for almost all markers of neutrophilic CRS, except MPO. Furthermore, this group had the highest levels of eosinophilic markers, proving that this group has a mixed immune response.

Cluster 1B

Most patients in this cluster were female. Moreover, these patients presented the lowest mean age, and the lowest frequency of NP. This cluster had the highest frequency of co-existence of atopy and AERD. Along with the high frequency of atopy in this cluster, these subjects also manifested the highest neutrophil blood count. Compared with the other clusters, these patients presented the lowest tissue levels for all biomarkers investigated, not showing any specific immune response. This cluster also had the highest LM and SNOT-22 score, while presenting the highest frequency of smoking history.

Cluster 1C

The majority of subjects in this cluster presented NP, and they also had the highest age of all sub-clusters. This cluster also contained the lowest frequency of smoking history. Its eosinophil blood count was the highest of all clusters. This cluster presented the highest levels of IL-6, IL-33, MPO, G-CSF, MMP-7 and TSLP.

This led us to suggest that this cluster is characterised predominantly by a T_H2 immune response.

Table 22: Demographic and clinical characteristics of Cluster 1 sub-clusters.

	Cluster 1A (n=6)	Cluster 1B (n=15)	Cluster 1C (n=33)	Overall p-value
Gender, male (%)	4 (66.7)	7 (46.7)	23(70)	0.23
Age	56±11.47	55±19.30	63±12.4	0.31
Atopy, n (%)	2 (33)	11 (73)	13(39)	0.10
Smoking history, n (%)	3 (50)	9 (60)	15 (45)	0.85
Asthma (%)	2 (33)	7 (47)	20 (61)	0.84
AERD (%)	1 (16.7)	5 (33)	7 (21)	0.76
Nasal polyps, n (%)	5 (83)	12 (80)	29 (88)	0.77
SNOT-22	39±14.6	55±22.1	52±25.4	0.45
LM score	12.6±9.4	17.1±6.3	15.5±6.8	0.75
Blood Eosinophil count ($x10^9$/L)	0.16	0.31	0.34	0.84
Neutrophil count ($x10^9$/L)	5.11	6.25	5.25	0.64

AERD, aspirin-exacerbated respiratory disease; LM, Lund Mackay.
For continuous variables, results are displayed as mean ± SD (standard deviation).
Categorical variables are expressed as frequency and percentage. An overall p-value, indicating the difference between the three clusters, was obtained using a Kruskal-Wallis test for continuous variables and chi square test for categorical variables. The overall p-value <0.05 indicate that at least one cluster was different from the others.

Table 23: Molecular profiles in sinonasal mucosa of Cluster 1 sub-clusters. Results are presented as mean. All levels are expressed as $x10^{-4}$, except for Periostin and MPO.

	Cluster 1A (n=6)	Cluster 1B (n=15)	Cluster 1C (n=33)	Overall p-value
IL-1ß (pg/mg)	**8.8**	1.9	1.7	0.03
IL-4 (pg/mg)	**1.9**	0.1	0.3	0.16
IL-5 (pg/mg)	**3.8**	0.8	3.4	0.10
IL-6 (pg/mg)	24.1	5.6	**28.7**	0.01
IL-8 (pg/mg)	**28.2**	5.0	13.1	0.14
IL-9 (pg/mg)	**18.5**	2.2	2.4	<0.01
IL-10 (pg/mg)	**0.5**	0.03	0.1	<0.01
IL-13 (pg/mg)	**1.2**	0.06	0.4	<0.01
IL-15 (pg/mg)	**5.1**	0.7	3.8	0.02
IL-17A (pg/mg)	**0.82**	0.4	0.77	0.50
IL-31 (pg/mg)	**9.3**	4.9	9.2	0.68
IL-33 (pg/mg)	256	248	**871**	0.01
G-CSF (pg/mg)	9.8	2.3	**11.9**	0.39
MMP-7 (pg/mg)	295	307	**754**	0.02
MMP-9 (pg/mg)	**335**	19.5	172	<0.01
IFN-γ(pg/mg)	**3.1**	0.2	0.8	<0.01
TSLP (pg/mg)	2.5	2.4	**7.4**	0.04
RANTES (pg/mg)	**516**	48	104	0.01
TNF-α (pg/mg)	**2.93**	0.17	0.34	0.03
Periostin (pg/mg)	**4.6**	0.7	1.9	<0.01
MPO (pg/mg)	**25.4**	2.27	7.32	<0.01

IL, interleukin; G-CSF, granulocyte colony-stimulating factor; MMP, metalloproteinase; IFN-γ, interferon-γ; TSLP, thymic stromal lymphopoietin; TNF-α, tumor necrosis factor α; MPO, myeloperoxidase.
An overall p-value, indicating the difference between the three clusters, was obtained using a Kruskal-Wallis test. The overall p-value <0.05 indicate that at least one cluster was different from the others. The numbers in bold indicate the highest value of a particular variable among the three clusters, while the italics indicate the lowest value.

Discriminant analysis

Discriminant analysis using the same 21 variables used in the cluster analysis was performed. This analysis would enable the identification of those measures which best separate patients into clusters. Based on this, a decision tree was developed for the three initial identified clusters. This permutation test identified the cut-off values for each node, in which patients were assigned to the appropriate cluster. Using adjustment due to the size of clusters, 72.6% of subjects in this cohort were assigned to the appropriate cluster. The resulting algorithm is presented in Figure 34. Appropriate classification ranged from 43.8% to 100% in Cluster 2 and 3, respectively. The total number of patients on each termination and its distribution against the cluster assignment is shown below each node (5 to 9) as a histogram.

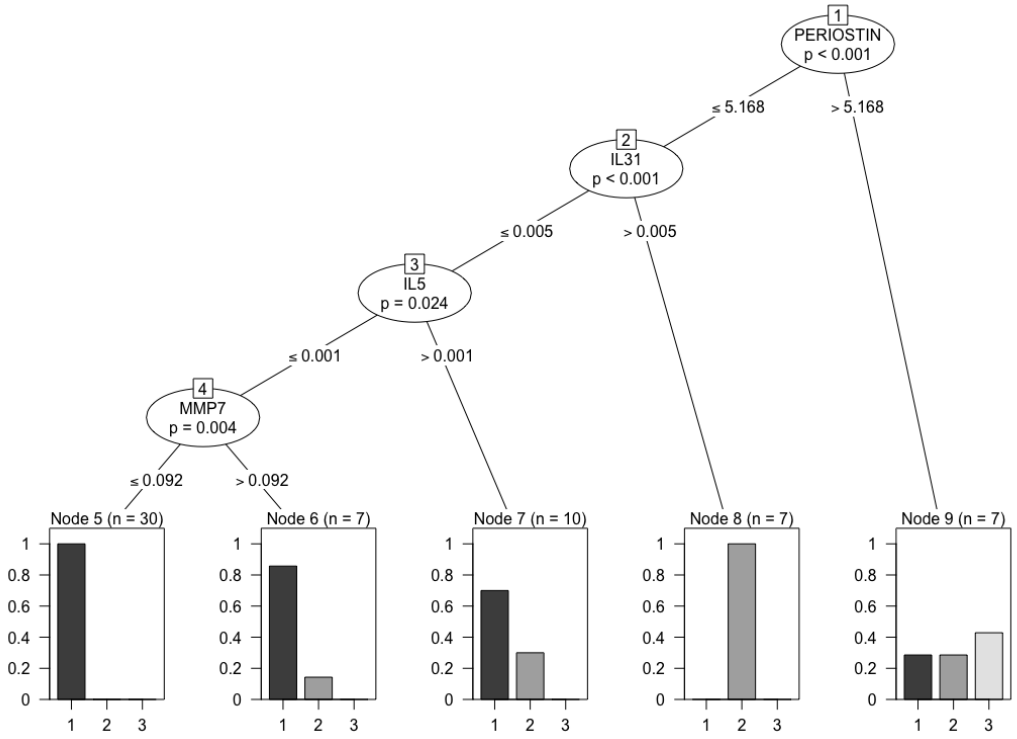


Figure 34: Tree analysis for the 3 initial identified clusters. Using four variables (Periostin, IL-31, IL-5 and MMP-7), subjects can be assigned to the 3 clusters: 1, 2 and 3.

As further hierarchical clustering allowed for a sub-division of cluster 1, this analysis was repeated for the total of 5 identified

clusters. Again, in here, the same 21 variables were analysed. The discriminant analysis showed that the strongest discriminatory variables were Periostin, IL-31, IL-9 and IL-13. A tree analysis was performed and is presented on Figure 35.

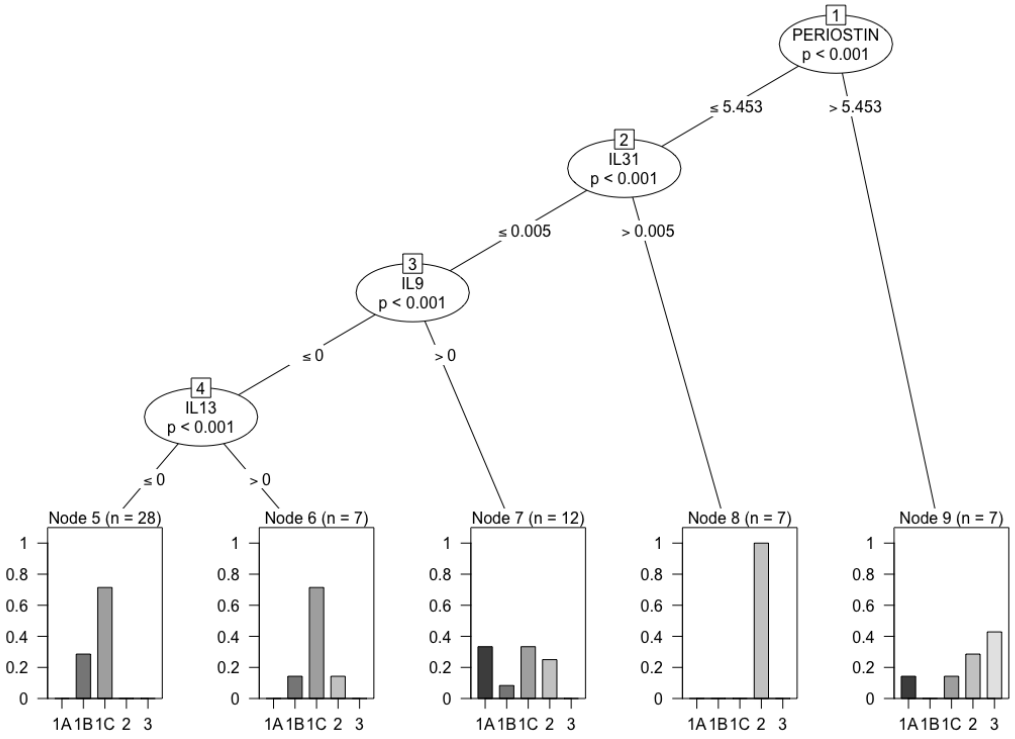


Figure 35: Tree analysis for the 5 identified clusters. Using four variables (Periostin, IL-31, IL-9 and IL-13), subjects can be assigned to the 5 clusters: 1A, 1B, 1C, 2 and 3.

However, in this tree, only 53.4% of subjects in the current cohort were assigned to the appropriate cluster. To note, the first two nodes of both trees contain the same molecules and provide the same results for cluster assignment, suggesting that a simple method for endotyping CRS subclasses can be based on these two biomarkers.

7.4 Discussion

This study was a retrospective analysis of the expression of exploratory and validated biomarkers, with the aim of exploring the hypothesis that biomarkers may identify previously unrecognised CRS endotypes. We used data from 21 pre-identified biomarkers within a cohort of 73 CRS patients. Through cluster analysis, we

attempted to identify endotypes of CRS with distinct profiles of immune features and identify associations between these endotypes and clinical characteristics. As the immune system is known to intervene in the pathophysiology of CRS, these biomarkers were identified as potentially reflecting the active molecular processes in disease affected tissues, e.g., nose and nasal mucosa.

Clustering of all patients with CRS based on cytokine measurements, irrespective of their phenotype, resulted in an optimal outcome of 5 distinctive clusters. As discussed previously in this work, CRS is not a homogeneous inflammatory disease, but instead it presents a wide diversity of inflammatory profiles. A similar study by Tomassen et al. (65), also observed considerable data variability within a CRS cohort, demonstrating that multiple clusters could be distinguished. Three initial clusters were identified with different inflammatory profiles and different clinical phenotypes, reflecting the importance of endotypes in clinical practice.

CRS was classified based on its clinical characteristics, as nasal polyps. A shift in this paradigm has been observed lately, with research focusing on defining endotypes for CRS. The major assumption of the previous classification was that all patients within a specific phenotype have similar disease characteristics and should be managed with the same therapeutic regimen. As seen in chapter 1, CRS patients within the same phenotype do not respond similarly to treatment. Thus, the purpose of this study was to improve the understanding of CRS mechanisms, and to develop a classification algorithm that would reflect the heterogenous pathophysiology of CRS. To accomplish this, a cohort of CRS patients was recruited and analysed for 21 biomarkers. This cohort included a variety of CRS manifestations, to truly reflect and analyse the whole spectrum of CRS manifestations.

To date, identification of CRS subtypes was performed through the stratification of phenotypes, or the study of a small number of

molecules in a restricted CRS group. Several studies have focused on eosinophilic and non-eosinophilic inflammation in CRS and its associated molecules, like IL-5 and IgE (20,115,157,159,168,173), guiding the therapeutic management of such patients through biologics (174–177). The cluster analysis described in this study identified five groups of patients with CRS who differ in clinical and inflammatory variables. Cluster 1 contained the highest number of patients and the highest variability of biomarker expression across subjects. Almost all subjects in Cluster 1 presented polyps, and it reported the highest neutrophil count and SNOT-22 score. According to Succar et al, decreased quality of life in CRS patients is associated with high neutrophilic inflammation (178), which might explain the results in our cohort as well. This cluster had the highest mean age. A previous study associated elderly CRS patients with a proinflammatory neutrophilic endotype (179), which could also be identified in Cluster 1. Differing from this study, pro-inflammatory cytokines were not increased in this cluster, which could have been caused by the sample type preferred in our study (nasal tissue instead of mucus).

Cluster 2 was characterised in our study by a high eosinophilic count and LM score. Previous studies have reported eosinophilic CRS with higher LM scores (180–182). This group also presented the highest levels of pro- and anti-inflammatory markers, T_H9- and T_H2-associated cytokines. Subjects presented the highest atopy levels but, as IgE was not included in this analysis due to incomplete assessment, this could not be confirmed. However, we did measure the IL-10 levels, which is related to allergic and AERD syndromes (63,67,73,121,124,126), and these were increased in this group. This cluster presented a mixed immune response, confirming that CRS is a heterogeneous inflammatory disease that activates numerous pathophysiologic pathways.

Lastly, cluster 3 was the smallest one, including only 3 patients. According to the analysis, this cluster can be composed of outliers

only, as this group presented several biomarkers which were expressed at far higher rates than controls or other clusters. Many of the inflammatory markers studied in this work were increased in this group, though no particular incidence of clinical features were observed in this group. This cluster was the most homogenous for clinical characteristics, as it was composed of only 3 subjects, and none presented atopy, AERD, asthma or smoking history. As a suggestion for further studies, it would be interesting to see if any of these clinical features can impact upon the expression of inflammatory markers, even on a genetic level, as individuals without these clinical features presented the highest expressions of biomarkers, while presenting no respiratory comorbidity.

Next, a classification tree to help allocate patients into clusters was performed. Similar clustering techniques have been performed in CRS, asthma and other inflammatory diseases (59,60,65,183). Traditionally, they are classified into clusters based on clinical characteristics, however, this algorithm is not good for data with missing values for machine learning. Therefore, our analysis could not include clinical variables for analysis, as they contained missing values, and only the 21 laboratory variables were included. Of the 21 studied variables, three were associated with T_H2 (IL-5, IL-31 and Periostin) and another one was a metalloproteinase (MMP-7). From these results, we could see that Periostin was a good selector for our cluster 3, as all subjects went into this same node. Also, cluster 3 had a highly successful appropriate classification rate. Cluster 2 showed the lowest appropriate classification percentage; however, using IL-31 as a cut-off value seems a good classifier for this cluster, as only these patients are distinguished at this division.

There was one large group (cluster 1), compared with the other clusters. Further hierarchical clustering showed us that cluster 1 was comprised of 3 sub-groups, cluster 1A, cluster 1B and cluster 1C.

Cluster 1A was comprised of patients presenting mixed immune responses, as both eosinophilic and neutrophilic markers were highly

expressed in this group. This challenges the dichotomy of eosinophilic/non-eosinophilic CRS, and encourages further studies to characterise this classification. These results support previous research findings by demonstrating that overlap of T_H2-mediators, even if partial, can be observed between e-CRS and ne-CRS (150). However, this classification is traditionally based on histological analysis (173), which was not performed in our study, so no firm conclusion can be drawn here. As this cluster has a small number of subjects, this cluster could present an abnormal expression of biomarkers, so these results require validation with bigger cohorts.

Cluster 1B could be classified as neutrophilic CRS, as it presented the highest value of these cells among the 3 sub-clusters. As seen previously when describing Cluster 1, this is the group driving the possible correlation between neutrophil blood count and severity scored by LM and SNOT-22 score (178).

We observed that Cluster 1C contained the highest eosinophil blood count, but presented none of its associated biomarkers. Again, no histological analysis was performed in this study to relate the blood levels with in-situ levels, which are currently used to characterise e-CRS.

More, our results confirmed that nasal polyposis is a condition that affects older men (184), as our results demonstrated an increase in the frequency of polyps with age in male patients.

The binary classification tree, constructed for the totality of clusters, presents the same final results: same number of classifier variables (n=4) and distribution of clusters per node. However, of the identified variables, three were associated with T_H2 (IL-31, IL-13 and Periostin) and one was associated with T_H9 pathway (IL-9). The beginning of our tree confirms that Periostin and IL-31 allow us to characterise our population. Nonetheless, these results would need to be validated at a higher scale (higher cohort), to address potential outliers as explained previously.

These binary trees can be very useful in characterising inflammatory entities and have been used previously in asthma characterisation. However, here they were only used as an exploratory theory as some of our small clusters could be outliers, and their existence may affect this model significantly since the sample size is small.

Clinical data are not included in the cluster analysis since only a small subset of subjects had these variables completed. A *post-hoc* analysis of these variables within the cluster's subjects provided potential insight into the clinical manifestations of CRS that might be related to the different molecular groups. One of the goals of this work was to correlate the identified groups to CRS evolution. However, follow-up 1-year postoperatively was not possible for all subjects, due to the small duration of this study. Future studies should investigate and characterise these variables.

As stated previously, other groups have reported similar modelling approaches to investigate CRS endotypes (65,150). The overall purpose and methodology can be diverse, but the size and demographics of the cohorts, and the number and type of variables used, differ from other studies. The cluster analysis by Tomassen et al. has similarities to this study, but was performed in a larger cohort (n=173) and used fewer variables to generate the clusters (14 versus 21 in this study) (65). Although some variables were the same as those used in this study (8 variables overlapped), we took an innovative approach by using a larger pool of biomarkers identified through our systematic review.

The results from the present study look promising, using a relatively large cohort with an also relatively high number of biomarkers. In the present study, our initial cohort of subjects can be classified as relatively big, compared with similar studies (75,155); however, such studies are normally very restrictive in their inclusion criteria, which generates more consistent results of a

certain CRS manifestation. Similar studies with larger cohorts (64,65) looked for a small number of biomarkers than our study.

In order to verify this study results and further characterise CRS groups, future studies should aim for larger cohorts, keeping or increasing the number of variables to investigate. More, the focus on classification binary trees should be undertaken, as such models can provide cut-off values that can guide clinicians and researchers on the management of CRS. The MACRO study will permit for a bigger recruitment of patients across several centres, allowing for further investigations of these preliminary results.

Key points:

- Three initial clusters of patients were found that described our population of 73 subjects;
- Three more clusters were identified within one of the initial clusters, leading to a total of 6 clusters in our population;
- Molecular profiles were defined for each cluster, and some biomarkers could be related to typical clinical features, for instance, neutrophil count with high SNOT-22 score, eosinophils with high LM scores, or IL-10 with allergic and AERD syndromes.

VIII – Time-dependent effect of Clarithromycin on pro-inflammatory cytokines in human A549 cell line

8.1 Introduction

Despite its prevalence and impact on health, chronic rhinosinusitis aetiology remains incompletely understood (185). Diagnosis is primarily based on clinical symptoms, with endoscopy or computerised tomography (CT) scans used to assist diagnosis by showing presence of mucosal changes and nasal polyps. This enables a division into phenotypes – CRSwNP and CRSsNP, which frequently guides diagnosis, prognosis, and treatment. However, this classification does not reflect heterogeneity of CRS in clinical presentation, pathology and therapeutic response (185,186), as discussed in chapter 1.

As seen previously in this work, the pathophysiological mechanisms underlying CRS involve cellular infiltration of neutrophils, macrophages and proinflammatory cytokines associated with helper T cell type 1, 2 and 17 (187). More, research has been undertaken to analyse the cytokine signatures associated with T_H1, T_H2 and T_H17 inflammation to help us subtype according to endotype rather than phenotype. A more robust expression of these cytokine markers could serve as an aid for guiding therapeutic decisions in the future.

Recently, it has been suggested that several macrolides, such as erythromycin, azithromycin, and clarithromycin, are effective for the treatment of respiratory diseases including CRS. Macrolides inhibit and kill pathogens and down-regulate pro-inflammatory mechanisms (188,189). While long term low dose macrolide therapy is traditionally used in the management of CRS, the exact role macrolides play in its management is unclear. The National Institute for Health and Care Excellence (NICE) has no clear guidance on dosage, duration of therapy or the patient groups most likely to

benefit, based on an international consensus statement published in 2016 (63). Systematic reviews published in recent years have concluded that macrolides may be effective in improving endoscopic and CT scores in CRS patients compared to baseline, but that effectiveness is likely to depend on appropriate patient selection (190–192). The anti-inflammatory mechanisms of macrolides in CRS management are an area of active research, with much of the evidence based on studies of relatively small sample sizes. A 2019 systematic review of 22 randomised controlled trials identified that, in CRS patients, macrolides may downregulate expression of pro-inflammatory cytokines IL-1β, IL-6, TNF-α and IL-8, amongst others (193), and that a decrease in T_H-2 cytokines were reported more frequently than a decrease in T_H1. In this review, authors recommended further research to explore the exact mechanisms underlying the immunomodulatory effects of macrolides, to optimise usage and identify appropriate patient groups who may benefit.

Evidence examining the relationship between time-dependent macrolide exposure or concentration-dependent macrolide exposure and cytokine responses in CRS is extremely limited. Two studies examining cytokine expression in mice following macrolide therapy suggested that different exposure times to macrolides may result in different changes in cytokine expression (194,195). Duration of macrolide therapy may be a factor in responsiveness to macrolide therapy in CRS, but further studies are required to establish whether variation in expression of cytokines implicated in CRS occurs depending on duration of macrolide exposure.

While macrolides may be effective in some cases of CRS, a lack of understanding and guidance on appropriate antibiotic usage has resulted in unnecessary prescription of antibiotics. This raises concerns as this can have long-term consequences by promoting antimicrobial resistance to antibiotics, with one study demonstrating an increase in proportion of macrolide resistant streptococci following macrolide therapy (188,196). This risk appears to be

greater in long term therapy compared to short term therapy (197). Antimicrobial resistance is considered a significant threat to patients' safety in Europe (198) and promotion of appropriate antibiotic usage is part of the UK's 5-year antimicrobial resistance strategy (199). Other concerns with macrolide usage include the risk of adverse effects, such as Clostridium difficile colitis and effects on cardiac conduction (200).

A better understanding of macrolides involved in CRS, such as their mechanisms of action and time dependent effects, could enable us to predict patient responses to macrolide therapy and enable a more personalised treatment plan (73,201). For instance, it will help us understand whether high doses in the short term are more beneficial than lower doses in the long-term (188,202).

In this study, clarithromycin was selected rather than erythromycin, since two reviews have raised concerns about its cardiac toxicity, especially in patients presenting a long QT interval (203,204). Moreover, clarithromycin was selected rather than other macrolides due to its characteristic of therapeutic serum concentrations and high tissue concentrations (205), and has been described as having an immunomodulatory effect on respiratory diseases and also CRS (206–208), through the inhibition of neutrophilic inflammation and macrophage activation (193).

In summary, clarithromycin was preferred since erythromycin has poor tolerability, previous randomized controlled trial revealed poor efficacy with azithromycin administration and roxithromycin has limited availability in the UK. Clarithromycin, on the other hand, is readily available in the UK with a reasonable side-effect profile (209) and it is currently recommended as an option by EPOS and ENT-UK rhinosinusitis commissioning guidelines for the treatment of CRS patients in a secondary care setting (23,210).

Therefore, the aim of this chapter was to explore and compare the anti-inflammatory effect of clarithromycin in vivo on different

inflammatory mediators, with direct correlation on different phenotypes of CRS.

8.2 Materials and Methods

8.2.1 Ethics statement

All experiments were evaluated and approved by East Midlands - Leicester Central Research Ethics Committee (Appendix E). Due to methodological issues, the A549 cell line rather than patient samples were used to obtain the final results. All patient tissue samples were disposed of according to the principles of the Human Tissue Act 2004.

8.2.2 Cell culture

The cell line used in this work was A549 adenocarcinoma human alveolar basal epithelial cell line, which is used nowadays for both basic research and drug discovery. It was deemed as a good replacement for epithelial cells culture from CRS patients due to its similarity to the nasal mucosal tissue. The line was obtained from another research team (Chris Morrison's group, BMRC, University of East Anglia) and cultured in Dulbecco's Modified Eagle's Medium - low glucose (Sigma Aldrich, Merck, Germany). This media was supplemented with 10% foetal bovine serum (FBS) and 1% of penicillin and streptomycin.

Cells were incubated under conditions of 5% CO2 and 37°C, and medium was changed daily. All cell culture work was performed under sterile conditions. The trypLE (a cell dissociation enzyme similar to trypsin) and phosphate buffered solution (PBS) were purchased from Thermo Fisher.

When the A549 cell line is cultured, a monolayer is formed, which becomes adherent to the base of the culture flask. This type of monolayer growth allows for the calculation and uniform distribution of the cells within a 24-well plate.

8.2.3 Cell counts and viability

All freshly isolated cells, for tissue culture or cryopreservation were counted and assessed for viability, based on trypan blue dye exclusion. A small volume (10µl) of the sample was transferred to a well of a round bottom 96 well plate (Nunc) and diluted 1:1 with 0.4% trypan blue (Sigma-Aldrich). The cells were then counted using a hemocytometer (Neubauer) to enable a viability count under a light microscope (Olympus, Japan) using the x40 objective. Assessment was made according to the appearance of cells under the microscope: cells with a phase bright appearance were viable cells, whereas cells staining densely with trypan blue were dead. The blue colour is caused by the intake of trypan blue when cell membranes are disrupted. The percentage viability was calculated by counting live and dead cells.

8.2.4 Cytokine selection

A literature search was conducted on MEDLINE OVID for systematic reviews published between the years 2000-2018 examining the immunopathology of CRS. Five cytokines were selected for inclusion in this study: IL-1β, IL-4, IL-5, IL-8, GM-CSF. As this is an exploratory study, these five cytokines were chosen among numerous others related to CRS. Selection was based upon the action of cytokines on distinctive CRS phenotypes identified within the literature search. While IL-5 is widely studied and associated with eosinophilic CRSwNP, GM-CSF, IL-1B and IL-8 are known actors on neutrophilic CRS. IL-4 induces the T_H2 differentiation and IgE class switching and was proven in our work to be associated greatly with CRS.

8.2.5 Clarithromycin

The pharmacy department of the James Paget University Hospital (Gorleston, United Kingdom) supported this work by providing the clarithromycin used in the experiments (Clarithromycin 500 mg

powder for concentrate for solution for infusion, Hameln, Germany). Reconstitution of powder was performed according to the manufacturer's instructions. The obtained solution of 50mg/ml clarithromycin was further diluted to 0.064mg/ml, using the same solution of PBS and DMEM used for the establishment of the cell culture. This dilution was chosen as it corresponds to the minimum inhibitory concentration of clarithromycin (211).

8.2.6 Cell culture and clarithromycin treatment

To generate epithelial cell media, A549 cells, at an initial count of 0.05x106 cells, were grown in 24-well culture plates (Nunc) until confluent. Before treatment, supernatants were harvested from each well and stored at -80°C, to form our baseline samples. After incubation with 0.064mg/ml clarithromycin solution for 4, 12, 24, 48 or 72 hours, supernatants were collected and stored at -80°C. The cells were then washed twice with PBS, and media were replaced with fresh culture media solution to avoid direct effects of clarithromycin on cells survival. Supernatants were again gathered after an additional 24h of incubation and stored at -80°C until analysis.

8.2.7 Enzyme-linked immunosorbent assay (ELISA)

Cell-free supernatants were quantified for levels of IL-1ß, IL-4, IL-5, IL-8 and GM-CSF using a human cytokine 5-plex panel (ProcartaPlex Mix&Match Human 5-plex, Invitrogen, Massachusetts, United States), according to the manufacturer's instructions. Detection limit was 0.5 pg/ml. All data was collected and analysed using the Luminex 200 systema and Luminex XPONENT 3.1 Patch (Luminex Corporation, Austin, United States). The median fluorescence intensity of the unknown sample was then converted into a value (pg/ml) based on the known cytokine concentrations of the standard curve using a 5-parameter regression formula.

8.2.8 Statistical analysis

Statistical analyses were performed using RStudio (version 1.4.1106, RStudio, Inc.). For continuous variables, results were expressed as means and standard deviation in box plots. Data distribution was tested for normality using the Shapiro-Wilk test. Comparisons were calculated by using Kruskal-Wallis and Benjamini-Hochberg corrected p-value for multiple comparisons. A p-value less than 0.05 was considered statistically significant.

8.3 Results

8.3.1 Cell cultures

The trypan blue dye exclusion test was performed on the culture supernatants after incubation. There was no significant difference in cell viability between the treatment and control specimens (data not shown).

8.3.1 Effects of clarithromycin on in-vitro cytokine and chemokine expression

Clarithromycin in the dose used in these experiments did not exhibit significant effects on cell viability (results not shown).

We started by analysing the effects of clarithromycin on in-vitro release of cytokines (GM-CSF, IL-1β, IL-4 and IL-5) and chemokine (IL-8) by A459 cells.

It was seen that these cells produced IL-1B, IL-4 and IL-8 even without stimulation (Figures 36-40, incubation time 0). However, the concentrations of IL-5 and GM-CSF were low or below the detection threshold in each group and we were unable to analyse the potential immunomodulatory effect of clarithromycin.

After 4 hours of incubation with clarithromycin, there was a reduction in IL-1β and IL-4 concentrations. Removal of treatment increased levels closer to baseline, but these results were not statistically significant. More, IL-8 concentrations significantly

reduced after 4 hours of incubation with clarithromycin. This did not persist, and the IL-8 concentration increased after removal of clarithromycin. However, this continued to be a significant result compared with the baseline result (Figure 36).

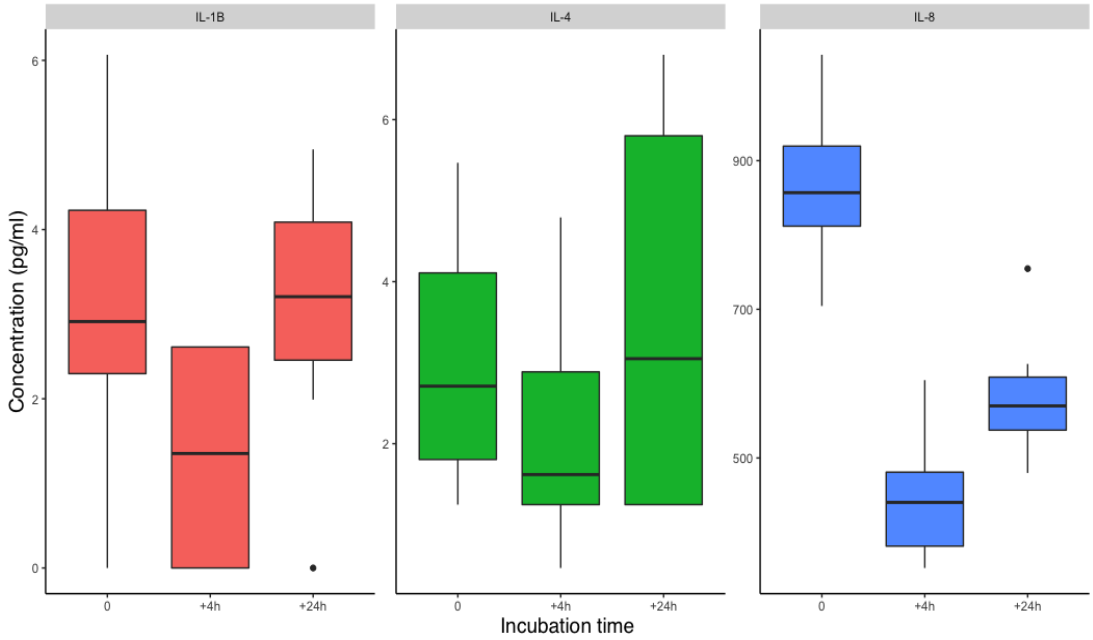


Figure 36: Incubation for 4 h - clarithromycin impact on pro-inflammatory mediators' secretion. Figure shows the impact of clarithromycin, respectively, on IL-1β, IL-4 and IL-8 release by A459 cells. Before (incubation time 0), after co-incubation (incubation time +4h), and 24 h after wash-out (incubation time +24h), cell culture supernatants were analysed for the presence of the indicated cytokines by Luminex®. Values are expressed as mean ± SEM from 8 independent experiments.

To analyse if a longer incubation time with clarithromycin would cause similar patterns, cell cultures were incubated with treatment for 12 h (Figure 37).

Clarithromycin at 12h inhibited IL-1B production, but this was reversed 24h after removal of treatment, returning to values close to baseline levels. On the other side, there was little difference in concentrations of IL-4 following incubation with clarithromycin. Twenty-four hours after removal of treatment, we could observe a slightly significant decrease on IL-4 levels (p=0.046). After incubation for 12 hours, the IL-8 concentrations reduced. After

subsequent removal of clarithromycin, IL-8 concentrations reduced even further. This result was significant (p=0.0154) when compared to the other two samples (Figure 37).

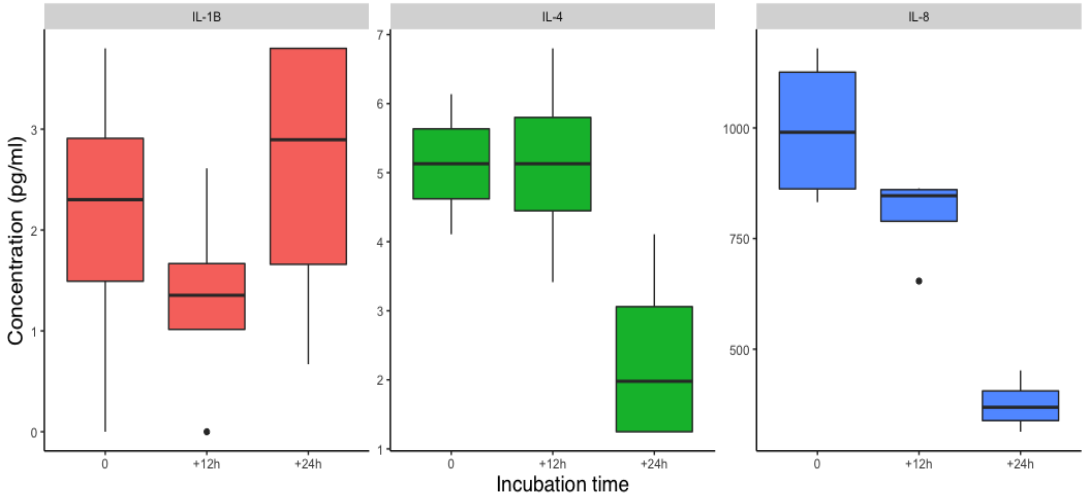


Figure 37: Incubation for 12 h - clarithromycin impact on pro-inflammatory mediators' secretion. Figure shows the impact of clarithromycin, respectively, on IL-1β, IL-4 and IL-8 release by A459 cells. Before (incubation time 0), after co-incubation (incubation time +12h), and 24 h after wash-out (incubation time +24h), cell culture supernatants were analysed for the presence of the indicated cytokines by Luminex®. Values are expressed as mean ± SEM from 4 independent experiments.

After 24 hours of incubation with clarithromycin, exposure led to an increase in IL-1β concentrations, which decreased following removal of clarithromycin. None of these results were significant (Figure 38). After longer exposures of 48 and 72 hours, clarithromycin decreased Il-1β concentration, and the effect persisted following removal. These results were also not statistically significant (Figure 39 and 40).

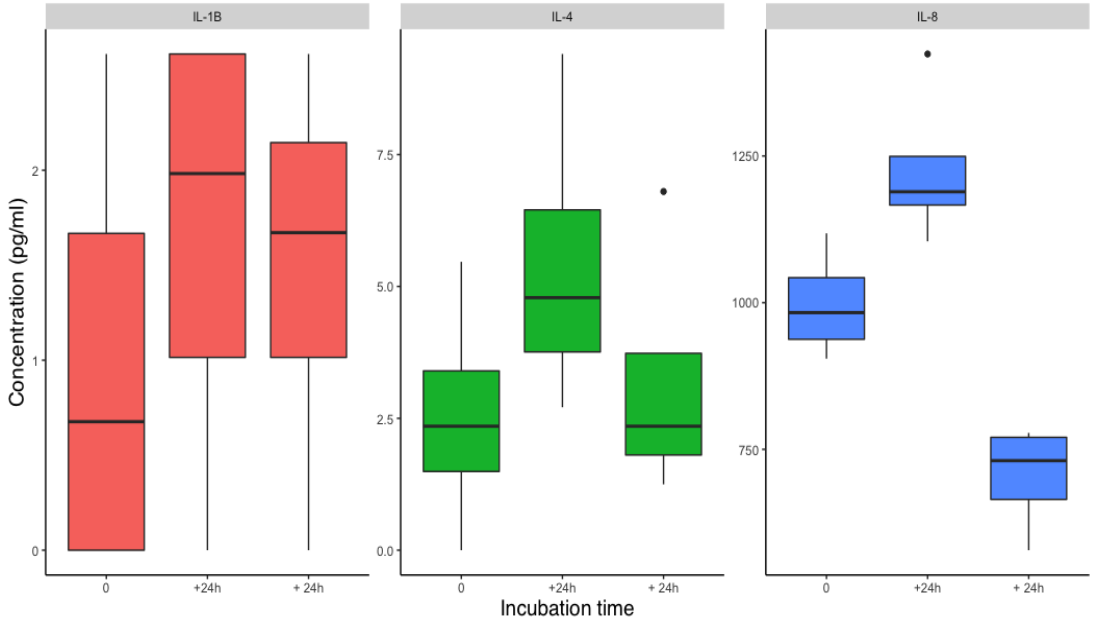


Figure 38: Incubation for 24 h - clarithromycin impact on pro-inflammatory mediators' secretion. Figure shows the impact of clarithromycin, respectively, on IL-1β, IL-4 and IL-8 release by A459 cells. Before (incubation time 0), after co-incubation (incubation time +24h), and 24 h after wash-out (incubation time + 24h), cell culture supernatants were analysed for the presence of the indicated cytokines by Luminex®. Values are expressed as mean ± SEM from 4 independent experiments.

After 24h, 48h and 72h incubation periods, interleukine-4 concentrations increased after treatment and decreased following removal of clarithromycin (Figure 38, 39 and 40). This was statistically significant in the 72 hours incubation group (p=0.03, Figure 40). IL-8 expression levels followed the same trend as IL-4, with its concentrations increasing after treatment and decreasing once clarithromycin was removed (Figure 38, 39 and 40). At 24h and 48h of incubation time, levels after treatment were significantly increased compared with baseline and after washout, whereas at 72h of incubation, all group medians were significantly different from each other.

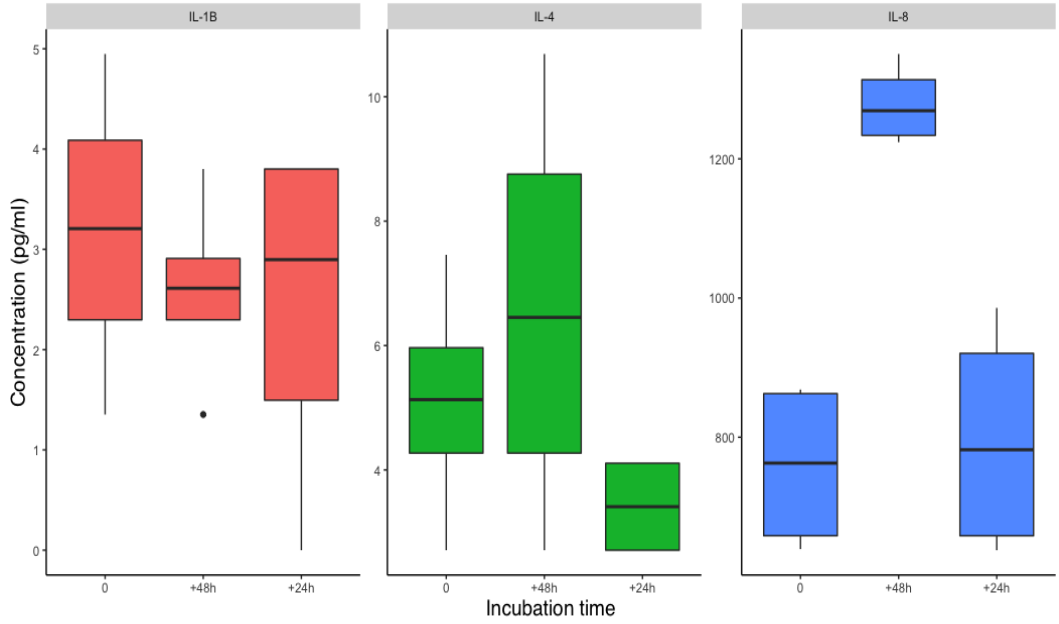


Figure 39: Incubation for 48 h - clarithromycin impact on pro-inflammatory mediators' secretion. Figure shows the impact of clarithromycin, respectively, on IL-1β, IL-4 and IL-8 release by A459 cells. Before (incubation time 0), after co-incubation (incubation time +48h), and 24 h after wash-out (incubation time +24h), cell culture supernatants were analysed for the presence of the indicated cytokines by Luminex®. Values are expressed as mean ± SEM from 4 independent experiments.

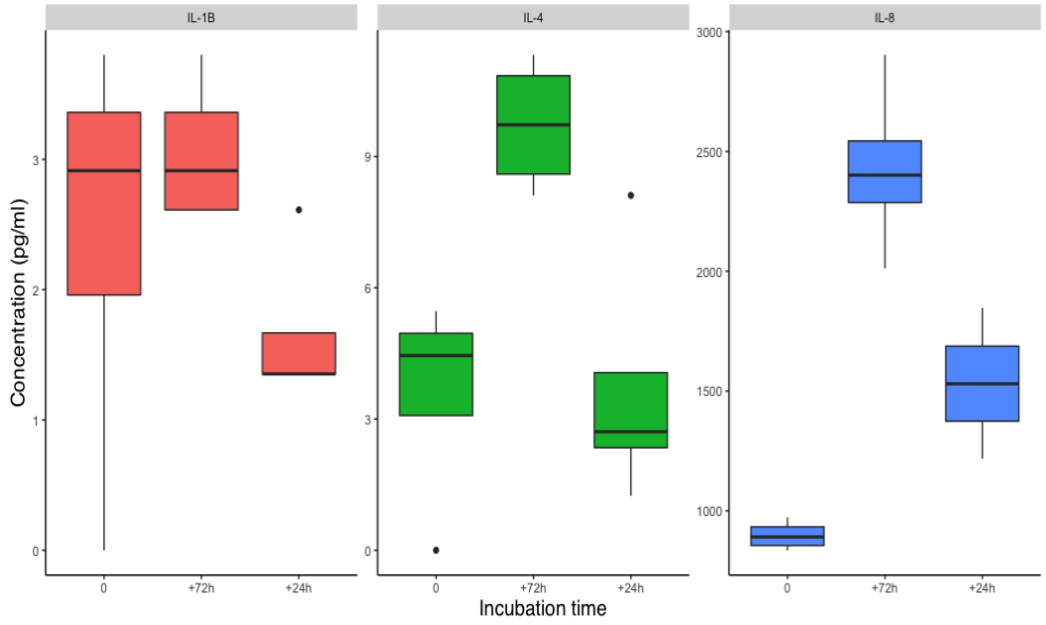


Figure 40: Incubation for 72 h - clarithromycin impact on pro-inflammatory mediators' secretion. Figure shows the impact of clarithromycin, respectively, on IL-1β, IL-4 and IL-8 release by A459 cells. Before (incubation time 0), after co-incubation (incubation time +72h), and 24 h after wash-out (incubation time +24h), cell culture

supernatants were analysed for the presence of the indicated cytokines by Luminex®. Values are expressed as mean ± SEM from 4 independent experiments.

8.4 Discussion

Macrolides are an important therapeutic option in the treatment of many chronic inflammatory diseases due to their immunomodulatory effects, and therefore they may be clinically effective in CRS (193). However, little is known about how macrolides affect specific pathophysiological features of CRS and in particular which CRS patients stand to benefit the most from taking them. In this study, the anti-inflammatory activity of clarithromycin and its immunomodulatory function was investigated. Also, we wanted to assess the duration of its effects. Therefore, this macrolide was administered for 4, 12, 24, 48 and 72 hours, and mediators' levels were measured before and after incubation, and after a washout period of 24h.

The aim of this chapter was to evaluate the action of clarithromycin on the selected cytokine levels – GM-CSF, IL-1β, -4, -5 and -8. Previously, Courcey et al. (212) performed a similar experiment using nasal epithelial cells that were stimulated in order to assess cytokine concentrations. The study design adopted in the present study was identical to the one adopted by Courcey et al. (212), so their protocol was tested. However, we were unable to obtain primary sinonasal epithelial cells in culture and an immortalised respiratory cell line was privileged to carry out this work. Currently, there are no immortalized nasal cell lines from CRS patients or individuals without CRS. As such, we investigated the possibility of using currently available cell lines that could mimic the upper respiratory cells. Previous studies established primary nasal fibroblast cultures, while others used immortalized lower respiratory epithelial cell lines, such as A549 or BEAS-2B, given the similarities between the upper and lower airways (213). The A549 adenocarcinoma human alveolar basal epithelial cell line is

used nowadays for both basic research and drug discovery, and it was widely available at our laboratory. Given its similarity to the nasal mucosal tissue (213), it was suitable to act as a surrogate for an epithelial cell culture from CRS patients.

A literature research was performed to select the mediators of interest in this work. The selected cytokines were IL-1β, -4, -5, -8 and GM-CSF (68,214–219).

Recent studies have clearly demonstrated that, in addition to antibacterial effects, macrolides may also have anti-inflammatory effects. Previous research has shown that reduction of IL-1β may be a potential mechanism of macrolides (206,208,220). Our results also demonstrate reduced expression of IL-1β after incubation with clarithromycin, although these results reverted to levels close to baseline 24-hour after removal of treatment. This decrease after incubation was only significant at 4 hours. This is likely due to small sample sizes, and more samples measured at 4 hours than other timeframes. We elected to focus on the 4-hour period as previous research has suggested that there may be a more observable effect, as peak tissue levels of Clarithromycin occurred at 4 hours after administration (205) and therefore more 4-hour samples were measured. IL-1β is associated with neutrophilic CRS, and clarithromycin may impair production or secretion of these cytokines. This may result in reduced neutrophil accumulation in the sinus mucosa, thereby reducing the inflammation underlying CRS. This may be of particular use in Asian CRSwNP populations, with research suggesting a tendency towards neutrophilic inflammation in Chinese patients (167). Multiple studies in Asian populations have described patients with neutrophilia as difficult to treat, despite treatment with endoscopic sinus surgery and poor response to corticosteroid treatment compared to patients with predominantly eosinophilic inflammation (57,168,221). Macrolide therapy may be an alternative or adjunctive treatment option in these patient groups.

In cell sample groups which had been exposed to clarithromycin for 24, 48 or 72 hours, concentrations of IL-1β did not return to baseline levels, and the reduction of IL-1β persisted. This was most noticeable in the samples which had been incubated with clarithromycin for 72 hours. Although these results are not statistically significant, the trend demonstrated highlights an area for future research. There is no existing research examining the relationship between IL-1β and time dependent clarithromycin exposure. If found to be statistically significant in studies with larger sample sizes, this may give us an indication that longer durations of treatment may result in longer term suppression of pro-inflammatory cytokine IL-1β. This may help us answer the question of whether short term high dose macrolide therapy or long-term lower dose therapy is most appropriate in relevant patient groups, thus enabling the development of appropriate guidance for clinicians.

When investigating IL-4 levels and clarithromycin exposure, the data obtained demonstrated a reduction in IL-4 after 4 hours, with concentration of IL-4 returning to above baseline levels upon removal. At 12 hours, there was little change when clarithromycin was introduced, while removal resulted in a reduction in IL-4 concentration. After incubation with clarithromycin for 24, 48 and 72 hours however, IL-4 concentrations increased and returned to near baseline levels upon removal. The increase from baseline and decrease following removal of clarithromycin was significant ($p<0.1$).

Existing research suggests that macrolides may reduce levels of IL-4 (193). While incubation with clarithromycin for 4 hours demonstrated a reduction in IL-4, this was not statistically significant, and incubation for other times demonstrated an increase in IL-4 following addition of clarithromycin, significant after incubation for 72 hours ($p<0.1$). However further research with larger sample sizes should be conducted to determine whether the

trend observed at 24 and 48 hours is significant. As IL-4 has been associated with epithelial barrier dysfunction in CRS (222), and epithelial barrier dysfunction has been implicated as a pathological mechanism in CRS, IL-4 is an appealing drug target. If future studies find that IL-4 expression is significantly increased following clarithromycin exposure for 24, 48 or 72 hours, defining the optimum duration of treatment or development of other therapies to target IL-4 may be important.

After 4-hour incubation with clarithromycin, IL-8 concentrations were significantly decreased compared to baseline concentrations ($p<0.05$). Following removal of clarithromycin, the levels increased, although not to baseline levels, and this result was not statistically significant.

Similarly, exposure to clarithromycin for 12 hours also reduced IL-8 levels, although not significantly. Concentrations of IL-8 were further reduced following removal of clarithromycin, and this was significant ($p<0.1$). This may suggest that incubation with clarithromycin for 12 hours is sufficient for the initial reduction to persist. After 24, 48 and 72 hours of exposure to clarithromycin, IL-8 levels appeared to increase, and removal reduced IL-8 concentrations close to baseline levels. The reduction following removal was significant at 24 hours, and the increase in IL-8 and reduction following removal was also significant at 48 and 72 hours ($p<0.1$). These results support Shinkai et al.'s research examining macrolide antibiotics in COPD (223). They found that IL-8 levels decreased over 6 hours and then increased at 12-72 hours after exposure to clarithromycin and found similar results with azithromycin at 24 and 48 hours, although in our study incubation for 12 hours was associated with an initial reduction in IL-8. Further research should focus on examining this result for longer time periods to examine whether this trend continues. This may enable us to determine whether macrolides are appropriate for patients with neutrophilic-predominant CRS.

No conclusions can be drawn from the results of the present study relating to the relationship between IL-5 and time dependent clarithromycin exposure. Many of our samples had IL-5 concentrations below the detection threshold of our assay. Future studies examining IL-5 in this cell line may need to consider stimulation of the A549 cells with TNF-α or IFN-γ (224). Previous studies have demonstrated a decrease in IL-5 in response to macrolide therapy (193), though this was not apparent in this study with this cell line. More, even with stimulation, low levels of IL-5 were detected in another study examining IL-5 production by human airways epithelial cells (224). For this reason, further studies with larger sampling, stimulated or not, are needed to detect any statistically significant difference. Other cell lines might also prove to be more adequate in such conditions than the one used in this study.

Many of the samples tested for GM-CSF yielded undetectable results. Previous studies examining GM-CSF in lung cancer and inflammation in airway epithelium have detected little or no GM-CSF in resting A549 cells (225,226). Future studies examining GM-CSF and time dependent clarithromycin exposure using A549 cells as a model for CRS will need to stimulate the cells with IL-1β and use more samples to provide meaningful results (227). While GM-CSF was detected in a small number of samples, these levels were low and differences between groups were not found to be statistically significant.

One previous study has considered the relationship between time dependent exposure to macrolides and GM-CSF levels, while examining how macrolide antibiotics modulate ERK phosphorylation and cytokine production in patients with chronic obstructive pulmonary disease (COPD) (223). These researchers found that clarithromycin increased GM-CSF at 48 hours. This is an unexpected result, given that other research has proposed that clarithromycin may reduce levels of GM-CSF (228), suggesting time dependent

exposure may have a role. Examining the expression of GM-CSF in response to different time lengths of clarithromycin exposure warrants further research. GM-CSF is proved to be elevated in asthma (193). Given that CRS is thought to share some of the pathophysiological mechanisms of asthma in the upper airways and that GM-CSF is elevated during symptomatic exacerbations in patients with CRS (229), more research based on the recommendations from this study may provide useful information which could be used to guide management of CRS.

Clarithromycin in the doses used in these experiments did not exhibit significant effects on cell viability. These findings outline a specific and dose-dependent impact of clarithromycin on the inflammatory response in CRS.

8.5 Conclusions and future work

This study leads us to conclude that the effect of clarithromycin exposure on the cytokines included in this study varies over time. Several interesting patterns have emerged in this study. While some cytokines presented no real change, or no obvious patterns of change, others showed trends which were not statistically significant. Elsewhere, others exhibited significant changes. Regarding the lasting effect of clarithromycin, we could note two different patterns: a reversal of the clarithromycin effect or a prolongation of the effect. This could indicate that macrolides may have a long-lasting and time-dependent effect on immune mediators beyond the duration of the macrolide therapy being given.

This study showed changes in the cytokine expression profile associated with exposure to clarithromycin in a particular cell line – A459. At the outset, our underlying assumption was that different patients will have different patterns of cytokine expression, and different changes in cytokine expression profile when exposed to clarithromycin. Further studies on samples of upper respiratory nasal mucosa from different individuals may produce a different

pattern of cytokine change, so unnoticeable cytokines in our study should not be excluded from further studies.

The results which have emerged, combined with suggestions from previous research examining macrolides in other airways diseases, warrant further investigations into the time dependent effects of macrolide antibiotics in CRS. This is an area which has been little investigated but may enable development of appropriate guidance for macrolide prescribing in CRS patients. It may be possible that exposure to clarithromycin for certain amounts of time leads to a persistent reduction in cytokine expression. It is also possible that clarithromycin increases cytokine levels. Therefore, duration of therapy may also explain the differences in responsiveness to macrolide therapy observed in the literature.

Future studies should aim to overcome the limitations present in this study. These include the choice of the cell line. Ideally, studies should use isolated cells from healthy and CRS patients, however in the present study, we were unable to create a derived stable cell line from patients' samples in the available time. Nonetheless, the A549 cell line can be an appropriate choice, provided that cytokine levels of resting cells are assessed prior to any testing. Additionally, time controls, that would account for the growing of initial assay cells over time, should also be assessed since cells can evolve over time. Also, stimulation might need to be considered if resting levels of mediators are below detection, as seen on De Courcey et al.(212), which used stimulation in primary human nasal epithelial cell cultures or Jang (206), that studied clarithromycin on rhinovirus-infected A549 cells. Future studies should also build upon our study and examine additional mediators implicated in CRS, to give a broader understanding of the cytokines that have time dependent responses to clarithromycin. This will be useful in developing recommendations for the duration of therapy, but also in selecting appropriate patients for macrolide therapy.

Determining appropriate duration and dose of macrolide therapy is essential to enable clinicians to achieve a persistent anti-inflammatory effect, with minimal treatment duration. As existing research suggests that mechanisms of actions of different macrolides may vary between each other (201), studies examining other time dependent immunomodulatory effects on these cytokines with other macrolide antibiotics should also be conducted. This would explore whether the effects observed are exclusive to clarithromycin, or also present in other macrolides.

Key points:

- A specific and dose-dependent impact of clarithromycin on the inflammatory response in CRS was observed;
- Further studies are needed, with possible modification of cell line or stimulation prior to incubation, to confirm and extrapolate these results;
- Further studies with additional "actors" in CRS pathophysiology should be considered.

IX – GENERAL DISCUSSION

CRS is a common chronic disease impacting QOL. CRS presents a burden at both individual and societal levels due to its high incidence, vast and difficult symptoms, and high indirect costs.

The aim of this work was to extensively characterise CRS patients, both clinically and molecularly, and to correlate both characterisations to fully characterise CRS endotypes and enable evaluation of responses in the MACRO trial according to endotypes. Moreover, we were interested to know if whether clusters could predict a better or worse surgical outcomes on SNOT-22. To address such aims, three studies were developed during this thesis: 1) search for biomarkers or clinical features that could be used for differential diagnosis (of disease endotypes) leading to subgroup allocation, disease activity, and response to treatment; 2) definition of immune profiles in different clinical subtypes of CRS; 3) identification of immune profiles that may predict the likelihood of disease control including compliance with medical treatment after surgical intervention.

The first research goal was addressed by conducting a questionnaire-based study concerning medical compliance and its factors in a cohort of CRS patients pre- and post-surgery (detailed in chapter 2) and a systematic review (reported in chapter 3) into the available literature at the beginning of this work.

The results demonstrated that non-compliance to medication was found to be common in this study as in others and is associated with poor outcomes (230). It is therefore urgent to improve the current non-compliance rates. To improve compliance, the source problem needs to be first identified. Patient's self-reporting of non-compliance is specific and predicts future adverse outcomes. Here, we identified the major reason for non-compliance as symptom improvement. Typically, in clinical practice, the "one size fit all" management approach is currently used; however, with medication,

some patients experienced side effects and symptom worsening, and even the recurrence of symptoms. Thus, we believe that biomarkers would help to predict clinical phenotypes, treatment responsiveness, and even recurrence, which would, in turn, lead to better management of CRS, decreasing the burden on health care systems. To help us search for the ideal CRS biomarkers, we conducted a systematic review of the literature (chapter 3). The results demonstrated that no ideal diagnostic biomarker for early detection of CRS exists, however IL-5 and IgE have been extensively described as being indicative of the presence of NP (76,89,113). The 20 included studies enabled the description of 52 biomarkers for CRS that are supported by different levels of evidence. These biomarkers are described as optimal for distinguishing patients with CRS from healthy controls or for identifying different CRS groups. However, we chose to analyse further in this project only 36 biomarkers. Sixteen mandatory biomarkers were selected as they correspond to established molecules, and their actions are well defined in the pathophysiological mechanisms of CRS. The twenty exploratory biomarkers were selected as they had been described in the literature as having varied and contradictory actions in CRS, or as they were expected to exert a role in CRS due to their overall role in the immune system. Thus, this research goal identified an apparent lack of adequately powered studies investigating the relationship between molecular markers and CRS. Consequently, there remains a need for research to address potential biomarkers for endotyping in CRS and to potentially guide diagnosis and treatment, that can provide the foundation for future exploratory trials.

CRS is a challenging entity with several underlying mechanisms. Current guidelines recommend dividing it into phenotypes according to the presence or absence of NP (23). Attempts to further define its classification have been made by the identification of biomarkers. Thus, better recognition of sub-groups with the specific clinical

characterisation might permit individualised diagnosis and therapy, with the potential for patient-driven care.

Elucidating the mechanisms driving CRS pathogenesis has been the subject of numerous studies, with some focused on the role that various cytokines and chemokines may play in the disease. However, there are conflicting reports regarding the expression levels of specific inflammatory mediators in CRS, which may in part be secondary to different types of specimens and/or methodology used. As a result, the second research goal included the definition of immune profiles in different clinical subtypes of CRS, or the identification of specific CRS molecular identifiers that would allow one to recognize an early-stage CRS patient from the overall nose-sufferers' patients. As for any robust research, defining your methodology and population is crucial. Several high-profile studies for CRS investigation have very narrow cohorts, with very specific requirements and consequently low numbers of samples (67,119,150,165). In here, we opted for a broader inclusion criteria that would be comprehensive of a wider pool of CRS patients. As explained on Chapter 1, CRS is a very complex entity with several manifestations and aetiology, and restrictions on the included population would condition our characterisation. The originality of our work is on the recruitment process as, contrary to other similar studies, no CRS patient was excluded from this study. This was done to reflect the heterogeneity of the disease to try and capture and reflect the varied pathophysiology of CRS. More, we did not restrict our studied variables preferring a broader pool of variables to characterize the heterogeneous mechanisms of CRS than focalise on one specific variable. The immune system is a network of signalling molecules so, it is only expected that one variable can influence another and together they can act on a certain pathway/mechanism. Therefore, focalising on certain molecules only could distort our perception of CRS.

To be able to analyse all samples equally, and to provide guidelines for collection and storage of nasal samples for the MACRO national trial, we needed to validate our methodology. This method needed to be easy, simple and cost-effective, in order to be widely applicable. The selection of our laboratorial methods was based on similar studies, since ELISA and multiplex assays have been widely used in the area to analyse a small sample for a multitude of parameters (65–67,150). UniCAP is also widely described on such studies, however it was not available at our institution. Due to the main goal of the study (characterise CRS subgroups) and, to ease its implementation and acceptance by both clinicians and patients, histological analysis was not selected in this study. We investigated the effect of different conditions on the biomarker's stability, achieving an easy and optimal protocol to be performed by all centres, preventing any inter-sample variability. The biomarker development process is a methodical effort in which the evidence supporting its use increases as the intended purpose of the biomarker moves from research to clinical practice. It starts with biomarker discovery, moving to develop and validate its method of detection before it can be use in clinical practice (231). We identified the relevant biomarkers through our literature review, as referred previously, and needed now to develop and validate its method of detection. As stated previously, we tested the method for collection and storage conditions, but also for intrinsic characteristics of the collected sample. Reference studies in the area differ on the sample use for their analysis – nasal mucosa, polyps or mucus. However, as seen not all patients have the same CRS characteristics, so we were interested in assessing possible differences across different nasal tissues, in order to optimise the protocol. Also, we asked ourselves if severity could impair or condition the expression of the relevant biomarkers. All variables were taken into account and our results showed no significant differences across all tested variables. However, IL-31 demonstrated

a higher expression level when freeze. An explanation for this could be some cleavage activity present in the sample, perhaps by another cytokine, that is inactivated during the freezing process. Further studies into this cytokine need to be considered to confirm this issue. As of now, IL-31 is reported as regulating T_H2 cytokine levels in NP (232), but these cytokines did not present any problem on testing, showing that the problem might be specific to IL-31. To dismiss a possible over-assessment of IL-31, it could be considered to assess its mRNA levels instead of protein levels to understand the exact mechanisms around this cytokine. This addressed, we defined a procedure that was tested across the whole thesis and that proved the easiness and cost-effectiveness of the methodology. Guidelines were sent across to the MACRO investigators for approval and use during the trial by all sites.

After determining the optimum protocol for our study, we focused on our second research goal - definition of immune profiles in different clinical subtypes of CRS. In chapter 6, we compared the profiles of different immunologic markers according to the clinical phenotypes (CRSsNP and CRSwNP). Our goal was to study 35 variables, but six could not be included due to constraints associated with the sample or availability of the detection assay. In total, we studied 29 biomarkers across our cohort of CRS patients and controls. It has been reported that CRSsNP is driven by a predominant T_H1-skewed response, whereas CRSwNP were classified into 2 subtypes (e-CRSwNP: T_H2-dominant response with eosinophil infiltration; and ne-CRSwNP: mixed T cell subsets with neutrophilic infiltration) (21,63,65–70,75). Therefore, in this study, we investigated the patterns of sinus mucosal inflammation in adult patients with CRS to verify the accuracy of these findings. In this study, we investigated the T_H1, T_H2, T_H9, T_H17-associated cytokines, pro- and anti-inflammatory molecules, neutrophil- and eosinophil- associated markers, as well as lymphoid and hematopoietic progenitor growth factors, and metalloproteinases.

Although CRSwNP and CRSsNP show certain differences in biological and clinical manifestations as revealed by previous studies and our current study (21,23,71), the present recommended classification does not accurately reflect the heterogeneous characteristics of CRS. Our results indicated that patients with CRSsNP show an increased mixed Th cell ($T_H1/T_H2/T_H17/T_H9$) immune response with increased neutrophilic- and eosinophil-associated inflammation, whereas CRSwNP presented an IL-5 dominated response accompanied by eosinophil and neutrophil infiltration. Results were varied across groups and did not always agree with the known literature in the field. Several reasons can explain this: 1) the broader inclusion criteria; 2) any preoperative treatment received that could modify biomarkers expression; 3) the heterogeneity nature of CRS.

A review by Ahern and Cervin (167) confirmed this heterogeneity of the disease, highlighting the need for CRS characterisation associated with pathophysiology rather than the presence of nasal polyps. Our results also verify this statement, justifying the mixed responses to treatment and experiences of our cohort. In order to characterise the CRS subgroups and develop a more tailored treatment for CRS patients, we continued our work by revealing the inflammatory profiles present in our cohort, trying to classify them into subgroups that can predict the likelihood of disease control. Moreover, this classification can also provide clinicians with the tool to identify such groups easily and manage them efficiently.

Recently, cluster analysis has been used to further characterise the classification of CRS. Chapter 7 presents inflammatory endotypes of CRS based on a cluster analysis of biomarkers. As the immune system is known to intervene in the pathophysiology of CRS, these biomarkers were identified as potentially reflecting the active molecular processes in disease affected tissues, e.g., nose and nasal mucosa. Several studies have identified clusters of CRS patients based on a limited number of markers and clinical features

(57,59,60,65,233,234). In the present study, we used 21 pre-identified variables to generate six clusters (5 distinct ones) through hierarchical cluster analysis of biomarkers. Interestingly, we observed that our generated clusters presented a typical T_H2 and mixed immune response. Even though our study is not the first to integrate a higher number of biomarkers into one cluster analysis, it is the first to present both a high number of assessed variables and a broad cohort of CRS subjects. This allows to reflect the heterogeneous nature of CRS.

Here, we identified five distinct groups, contesting some previous findings that might not be comparable due to the difference in the ethnicity of the cohorts (59,65,150). Therefore, we could not confirm our clusters with other studies; however, our focus was to define endotypes solely based on biological characteristics and then to compare these with clinical parameters. However, our results allowed us to confirm some literature statements on CRS. First, our cluster 1 established that nasal polyposis is a condition predominantly affecting older men (184). This was demonstrated through an increase in the frequency of polyps with age and prevalence of men presenting polyps. Then, we showed that decreased quality of life in CRS patients is associated with high neutrophilic inflammation, as previously stated (178). Moreover, elderly CRS patients present a pro-inflammatory neutrophilic endotype in cluster 1, validating Morse's results (179).

Cluster 2 instead reported the known association between eosinophilic CRS with higher LM scores (180–182). This cluster also added more evidence to the fact that CRS is a heterogeneous inflammatory disease that activates numerous pathophysiologic pathways, by presenting mixed immune responses. Our clustering approach brings novel insights into CRS endotypes, highlighting the importance of several biomarkers in select groups of patients. In recent years, the development of biological agents targeting the T_H2 pathway and IgE has increased for the treatment of CRSwNP. In light

of our results, we would expect the anti-IL-4 and IL-13 receptor antibody to show promising effects for the treatment of cluster 2, whereas IL-1ß and IL-6 blocking are expected to have an effect on clusters 3 and 1, respectively. Therefore, our study provides additional information for the identification of proper biomarkers to guide current and future biologic treatment for CRS.

Clinical data are not included in the cluster analysis since only a small subset of subjects had these variables completed, but they were used *post hoc* to look for differences across the groups. Our findings suggest that the "one size fits all" management approach used currently in clinical practice is unlikely to be appropriate for all CRS patients, and that treatment would need to be tailored according to each cluster's characteristics. Therefore, this analysis aimed to correlate the identified groups with CRS evolution. Unfortunately, we were unable to characterise the possibility of recurrence or even performed corelations to follow-up 1-year postoperatively, since our time frame did not allow for a longitudinal study.

A simple discriminant analysis was used in this study to place each patient into a cluster, and confirm the previous clusters identified by hierarchical clustering. Use of this clinical algorithm is crucial for the clinical applicability of these findings. Similar clustering techniques have been performed in CRS, asthma and other inflammatory diseases (59,60,65,183). Traditionally, they are classified into clusters based on clinical characteristics, however, this algorithm is not good for data with missing values for machine learning. Therefore, our analysis could not include clinical variables for analysis, as they contained missing values, and only the biomarkers assessment was included. The beginning of our tree confirms that Periostin and IL-31 allow us to characterise our population. However, as explained previously, our analysis yielded some small clusters and therefore, this analysis is designed to serve as a foundation for future, more widespread, multi-institutional studies containing larger cohorts.

The results from the present study look promising. Using a relatively large cohort with an also relatively high number of biomarkers will validate our findings to provide clinical guidance The originality of this study was in the inclusion of wide CRS cohort and broad pool of studied variables. Previous studies favour either large pool of variables or large pool of subjects, differing from our study.

In order to validate the results of this study, and further characterise CRS groups, larger cohorts should be used, in order to provide some degree of replication. More, the focus on classification binary trees should be continued, as such models are crucial for clinical applicability. As stated before, the MACRO study will enable a multi-centre recruitment of patients, allowing for further investigations of these preliminary results.

After describing our cohort of patients and its groups, we wanted to assess the pertinence of macrolides as a therapeutic agent for our CRS cohort. Macrolides are an important therapeutic option in the treatment of many chronic inflammatory diseases due to their immunomodulatory effects, and therefore they may be clinically effective in CRS (193). However, their mechanism of action remains unclear. This study (chapter 8) aimed to provide some guidance into macrolides used within CRS, and analyse their use within our cohort. More specifically, we wanted to evaluate the action of clarithromycin on the selected cytokines – GM-CSF, IL-1B, -4, -5 and -8; and assess the time-effect of clarithromycin on these molecules. These cytokines were selected through a systematic review, acknowledging that neutrophilic CRS has been described as difficult to treat by endoscopic sinus surgery or corticosteroid treatment (55,164,210). Therefore, macrolide therapy may be an alternative or adjunctive treatment option in these patient groups.

We were unable to directly test clarithromycin's action in CRS samples, and instead a cell culture was used to see its action on CRS inflammatory markers. Clarithromycin was administered for 4, 12,

24, 48 and 72 hours, and mediators' levels were measured before and after incubation (at different points in time), in order to assess its action and time-effect.

Our findings were preliminary and will need further validation. In this exploratory study, we observed that results varied according to the mediator and time of incubation. Although IL-1β results were not statistically significant and levels reverted close to baseline 24-hour after removal of treatment, we hope that longer durations of treatment may result in longer term suppression of pro-inflammatory cytokine IL-1β. This may help us answer the question of whether short term high dose macrolide therapy or long-term lower dose therapy is most appropriate in relevant patient groups, thus enabling the development of appropriate guidance for clinicians.

Further research should focus on examining these results with time controls, as cytokine levels fluctuated depending on the exposure time to clarithromycin. This may enable us to determine whether macrolides are appropriate for patients with neutrophilic-predominant CRS. These findings outline a specific and dose-dependent impact of clarithromycin on the inflammatory response in CRS. Further studies using other cell lines, stimulated or not, should be designed to confirm and further characterise these findings.

Lately, Doxycycline, a tetracycline antibiotic, has been described as having immunomodulatory properties in CRS (235). More specifically, it was described as having similar immunomodulatory effects on metalloproteinases, ECP, MPO and proinflammatory cytokines, such as TNF-α, IL-1β and IL-6, in CRSwNP (235). Therefore, studies examining time dependent immunomodulatory effects on these cytokines with Doxycycline should also be conducted. This would allow further exploration of the potential medical management of CRS, and further determine which groups of patients would benefit from macrolides or tetracycline treatment.

According to Tomassen et al., CRS-related inflammation should be considered as a mixture of T_H1, T_H2, T_H17,

eosinophilic/neutrophilic, pro-inflammatory and T_H22 immune responses. Due to the recent advances in biologics targeting CRS mechanisms, we anticipate that an approach based on non-invasive biomarker-defined clusters would help to tailor treatment options by identifying responders to them (174,177).

Future research based on the recommendations from this study may provide useful information to guide clinical practice on the management of CRS.

Applicability of the study

This study was designed to explore the possible existent relations between clinical features, observed in the clinic, and immunity mechanisms implicated in CRS, by dosage of present biomarkers in the biological samples. It moves away from current literature that focusses on either clinical or molecular characteristics and more traditional treatment approaches. By recognising the key chemical mediators occurring in the inflammatory mechanism of CRS and how pattern recognition could be established to help to test biotherapies to be used in this pathology, we aimed to take the next steps towards personalised medicine for CRS, in particular when selecting treatment regimens for CRS patients. The future application of this approach in a clinical setting would be to determine key biomarkers from an initial nasal biopsy to enable endotyping of patients. Through comparison between biomarkers profile and decisional trees identified in this work (chapter 7), clinicians will be able to match patients to the most appropriate cluster for their immunological profile. This could then be used to guide the most appropriate treatments for selected patients enabling more refined and targeted treatment programs to be established and thus more efficient use of resources as well as minimising the possibility of adverse effects from ineffective medications or interventions.

Thesis limitations and strengths

Limitations

It is important to consider the findings of the thesis in light of the limitations and strengths of this study. However, such limitations are a useful consideration for future research.

The medical compliance study (chapter 2) was based on a self-completed questionnaire. The patient's perceptions of help/non-help could not be assessed objectively, being subject to some bias. More, questionnaires for the prospective population were collected during preoperative visit or before surgery, introducing possible inherent biases due to the Researcher of Clinical Staff being present within the room. This might have precipitated more compliant responses from this specific population. To help address this, questions were repetitive and constructed to avoid such "socially acceptable" responses. More, this questionnaire was not validated as it was a study specific questionnaire designed to collect variables. However, it included validated quality of life/PROMs (e.g., SNOT-22) in its construction.

The systematic review carried out in chapter 3 was limited to our methodology and to studies published in English. Although multiple databases were searched, it is possible that relevant articles were missed. More, the study focused on published papers until 2018, and, as CRS is a field in constant evolution, this could lead to a potential reporting bias in the results. The lack of validated biomarkers in CRS led to a broader search, which could account for the heterogeneity in the included studies, and to the impossibility of examining our results through a meta-analysis. However, a narrative description was possible which revealed important gaps in the evidence base.

Similar to previously published cluster analysis studies of CRS patients, the subjects in our study were those presenting in secondary care for surgery after medical therapy had failed. In addition, more CRSwNP patients were enrolled than CRSsNP patients (3:1).

Therefore, the extrapolation of these findings to the general CRS population must be done with care. Besides, given the described immunopathological differences between Asian and Caucasian patients, our findings might be restricted to relevance in the white British population (most of our cohort). Since our analysis wanted to define CRS subgroups based on biomarkers expression and hierarchical clustering, bias was reduced. Although these findings have the potential to aid in the development of individualised diagnosis and treatment strategies for the management of CRS patients, these need to be confirmed in further studies with long-term monitoring and larger cohorts.

Another potential criticism is the fact that all our subjects were recruited in a single centre and therefore our findings would need validation/replication across multiple centres. A new study has been set up nationwide - MACRO, based upon the results of this thesis, and this will bring new insights to the area. Therefore, the results of the present study at this stage are very preliminary and have an observational character only, and further investigations are needed for validation. Also, other studies performing functional studies are needed. These would test pharmacologic targeting of selected biomarkers and whether they can modulate the clinical features of the disease. Furthermore, despite our efforts to be objective, there were several areas of subjectivity, including our selection of variables to cluster or the decision over the number of clusters. We were unable to cover all the molecules identified previously as critical for the pathogenesis of CRS due to limited resources, limited sample amount and limited number of analysed samples. Like other CRS cluster studies, our current study does not address the question of within cluster stability, which is important to conduct longitudinal studies to evaluate the stability of clusters over time (57). In addition, our data should be interpreted cautiously as the evaluation of a large number of biomarkers in a relatively small number of subjects (notably, cluster 3) might lead to the inference

of biased results from the collection or manipulation of the data. Ultimately, application of our results to a larger population must be validated through the use of additional patient cohorts. Our simple categorisation binary tree algorithm could be used potentially in clinical practice to aid in individualized decision making. However, at the present time, findings should be limited to the patient population studied.

Concerning the assays used in this study, we favoured the Luminex technique whenever possible. As explained previously in Chapter 4, this technique has been validated and used by other research studies in the area, and allows the simultaneous study of several parameters in one sample/assay. However, due to the financial restrictions of this study and the parameters of the research grant, only protein assays were used. Once the primary research goal was fully achieved, no funding was available to verify these results through the adoption of another technique, such as qPCR. Crucially, the use of protein assays alone was predetermined by the research project. Even if the research grant allowed for validation through another technique, the current pandemic situation precluded further laboratory work. Future work should aim to validate these findings through genetic assays.

Finally, our macrolide studies presented some limitations of their own. These included small sampling and the use of a cell line instead of CRS samples. Studies using larger sample sizes may produce less variation in their results, and therefore produce more representative results. More, studies should use isolated cells from healthy and CRS patients. However, in the present study, we were unable to create a derived stable cell line from patients' samples in the available time. Nonetheless, a cell line can be an appropriate choice, provided that cytokine levels of resting cells are assessed prior to any testing. Also, we chose to not stimulate our cells prior to treatment, this might potentially have biased our results, especially for IL-5 and G-CSF, that were undetectable prior to treatment. Stimulation might

need to be considered if resting levels of mediators are below detection.

Strengths

The systematic review included multiple databases and was focused upon the research question. A comprehensive research strategy using three biological terms was employed, and it enabled the emergence of further biomarkers from the review of the literature. Due to the heterogeneity of identified studies, we filtered them to include only studies where comparisons between CRS groups and proteomics techniques were performed, allowing for an original literature review. Therefore, this literature review describes the possible parameters for determining CRS subgroups (endotypes), activity and response to treatment associated with the different groups, reflecting its heterogeneity.

Our research into the medical compliance of CRS patients demonstrated that older age was the only determinant of compliance with treatment for CRS before and after ESS, despite considering other clinical and demographic variables and opens the door for biological profiling to assist clinicians in selecting the best treatments that may aid compliance.

This study also allowed for the inclusion of a wider cohort of CRS patients, which could theoretically include every possible manifestation of CRS, in conjunction with a broader pool of studied variables. This complements the literature in this field, as traditionally studies have chosen to either restrict the CRS phenotype studied, or the variables analysed.

As stated before, the outcome of many research studies in CRS are restricted to a very similar phenotypic characteristics, which might represent a same endotype of CRS. In here, our study distanced itself from this approach, allowing for a possible wider identification of subgroups. Here we used different clustering approaches to identify mechanisms that were endotype/subgroup specific, illustrating the

diversity in inflammatory profiles for CRS. Furthermore, our choice of biomarkers covers several classes of biomarkers, and not just one class in particular (e.g., T_H2-associated markers), allowing for future directions in therapeutics mAbs.

Lastly, our clarithromycin study outlined the specific and dose-dependent impact of clarithromycin on the inflammatory response in CRS.

Overall, this thesis benefited from a multi-disciplinary team with a variety of expertise. This allowed for a quick translation of our laboratory results into clinical practice, and for the constant correlation of biological markers and clinical characteristics. This allowed for the translation of laboratory results into recommendations for clinical practice in each study phase.

Furthermore, this work has been extensively presented across the UK and Europe, revealing the impact this original work has had on the research area internationally.

Concluding remarks and future directions

Our study has provided further evidence of endotype profiling for CRS and how this could be utilised in practice. It is unlikely that phenotypes or endotypes can be defined by using a single biomarker expression, but instead a combination of several biomarkers, and their relationships, is needed, and thus our study set up the premise for further cluster analysis to be undertaken to refine these profiles.

Firstly, a systematic review demonstrated there was a large number of promising biomarkers for CRS, which might be adequate biomarkers for CRS categorisation and early diagnosis. The included studies demonstrated that immunological mechanisms for CRS phenotypes may result from a combination of several biomarkers. This revealed the need for future research to validate novel biomarkers and further evaluation potential ones.

Secondly, five endotype clusters of CRS patients, which had distinct biological profiles could be defined in our cohort. Our results represent evidence that CRS can be characterised by several biomarkers that can better characterise individuals with several disease phenotypes. Our definition of endotypes was entirely based on inflammatory biomarkers, and further studies are needed that incorporate clinical characteristics, genetics, and treatment responses to further characterise these endotypes. Given our data, further endotypes might be identified, or current ones might be merged together.

The five identified endotypes within our cohort of CRS patients may provide a more precise description of the underlying mechanisms of CRS than phenotypes only and enable a more personalised treatment regimen. Some interesting results arose from this analysis, and suggestions for further studies include: 1) replicate this study in a much larger cohort in order to provide validation of the clusters; 2) observe if any clinical features can impact upon the expression of inflammatory markers in CRS; 3) the use of classification binary trees should be verified, as such models can provide cut-off values that can guide clinicians and researchers on the management of CRS.

Other study designs should be considered to address the limitation of the present study. In this work, proteomics was preferred and used across all studies. However, these preliminary results will need to be confirmed through other techniques, such as genetic or histological analysis.

Lastly, future studies should aim to overcome the limitations present in the macrolides' study. Ideally, this study should be replicated using larger sampling and with isolated cells from healthy and CRS patients. Also, stimulation might need to be considered if resting levels of mediators are below detection, as seen here for IL-5 and GM-CSF. Future studies should also build upon our study and examine additional mediators implicated in CRS, to give a broader

understanding of the cytokines that have time dependent responses to clarithromycin. This will be useful in developing recommendations for the duration of therapy, but also in selecting appropriate patients for macrolide therapy.

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Appendices

Appendix A - Participant Information Form

James Paget University Hospitals NHS
NHS Foundation Trust
Lowestoft Road
Gorleston
Great Yarmouth
Norfolk
NR31 6LA

Main switchboard: 01493 452452
Direct line: 01493 452832
E mail: brenda.peck@jpaget.nhs.uk
www.jpaget.nhs.uk
https://www.uea.ac.uk/rhinology-group/home

Exploring Endotypes in Chronic Rhinosinusitis

IRAS Number: 202585
Participant Information Form v1.0 10.09.2016

Project Sponsor: University of East Anglia

Chief Investigator: Carl Philpott
Co-Investigators: Ana Pratas
Tom Wileman

Location: University of East Anglia and its associated NHS Hospitals
James Paget University and Norfolk and Norwich University NHS Foundation Trusts

Dear _____

Introduction

You are invited to take part in this research project that is investigating the condition chronic rhinosinusitis (CRS). This is because you have been identified as suffering with CRS.

Please take a few moments to read this document that will help you decide whether you wish to take part.

What is chronic rhinosinusitis?

CRS is a condition where the lining of the sinuses and also the nose becomes swollen and the resulting symptoms have lasted for at least 3 month. At present, when patients with CRS present to Ear, Nose and Throat doctors, they will typically examine the nose to see whether or not there are any polyps (grape like swellings) sitting in the nasal cavity. This leads to 2 main groupings of CRS with nasal polyps (CRSwNPs) and CRS without nasal polyps (CRSsNPs) – these are known as phenotypes.

What are endotypes?

As opposed to phenotypes, which are determined by the physical characteristics of the disease found on examination, endotypes are determined by chemicals produced in the sinus/nasal tissue and mucus. There is increasing evidence that certain groups of patients with CRS have certain patterns of chemicals that they produce and that these patterns will also help to predict which treatments will work best for these specific groups. We believe there are a much larger number of endotypes, as compared to the two basic phenotypes we have described above, but this has not yet been clearly defined and as such

current treatment recommendations are largely based on whether or not a patient has polyps present in their nose. In practice, we know that within these two groups, patients can respond very differently to treatment, so by defining the underlying endotypes, we hope to understand why that is.

What does participation in this research involve?

As you have CRS and have chosen to undergo endoscopic sinus surgery, we are asking permission to keep some of the swollen tissue removed from your sinuses for research. This is tissue that we would normally get rid of at the end of the operation. In addition to this we would want to capture some of the mucus present in your nose and sinuses as well as a blood sample. All of this tissue will then be transferred to the tissue bank for storage until we are ready to test it in our laboratory. In addition to your permission to use these samples for research, we would also want to look through your medical records to see what treatments such as nasal sprays and antibiotics that you have already had, so that we can match this information with the findings in the laboratory. By doing this we hope to be able to learn more about why some patients respond better than others to the same treatments that are commonly used for CRS. If you have already donated tissue on a previous occasion, we are contacting you just to get your permission to look at your health records and conduct the laboratory tests on your existing samples.

We hope that you will be able to help us but we do of course understand if you do not wish to participate and will fully respect this decision.

What are the benefits to you of taking part?

There are no direct benefits to you from taking part in this project. This is scientific research aimed at understanding the biology of the condition so that we will better be able to treat the condition in the future.

What are the disadvantages or risks of taking part?

You will be asleep (under anaesthetic) during the procedure, so you will not experience any discomfort from these samples being removed beyond that which you would expect from the surgery itself. We will be looking at your personal health records relating to your CRS, but will maintain the confidentiality you would expect

How will we tell you what we have found?

This study will form the basis of a PhD studentship for Ana Pratas and the results will be included in her thesis held at UEA. We have an existing research website that we will use to announce results. We will also contact our patient charity Fifth Sense, so that they can make announcements on their website and their newsletters. We will use this to update participants on the progress of our research and to let you know of any of our findings. We hope to publish any findings of significance in scientific journals. We plan to utilise the initial findings to help further refine our investigations during a national trial of CRS treatment known as the MACRO Programme: Defining best management of adult chronic rhinosinusitis.

Further information

What will happen if I don't want to take part?

There will be no impact on any care that you may need.

How will my information be kept confidential?

The information about your tissue will be kept in confidential in hospital and university computers. The information will be coded so as to be only available to the research team.

Who is organising and funding this study?

This study is organised in Norfolk by a team of ENT surgeons and scientists. It is funded from scientific research grants that have been competitively awarded by the Sir Jules Thorn Charitable Trust.

Will I be paid for taking part?
No. We are seeking volunteers to help us with this study. We may be in a position to refund reasonable travel expenses if these are necessarily incurred.

What will happen to the tissue/blood samples that I give?
The tissue sample will be sent to the Norwich Biorepository and stored in a freezer at -80°C. If our initial work in the laboratory suggests that fresh samples provide more useful information, some samples may be chilled only and taken straight to the laboratory. The blood sample will be sent to a specialist laboratory at the Norwich Biorepository where it will be stored. We have no immediate plans to undertake any genetic testing but the sample will be stored for 10 years as to be available for future ethically approved research when the DNA can be extracted.

Who has reviewed the study?
All research that involves NHS patients or staff, information from NHS medical records or uses NHS premises or facilities has to be approved by an NHS Research Ethics Committee before it goes ahead. This study has been reviewed by the xx NHS Ethics Committee. Approval does not guarantee that you will not come to any harm if you take part, however approval means that the Committee is satisfied that your rights will be respected; that any risks have been reduced to a minimum; have been balanced against possible benefits and that you have been given sufficient information on which to make an informed decision.

What if I am harmed by the study?
Nothing in this study is expected to cause you any harm. In the event that something does go wrong and you are harmed during the research study there are no special compensation arrangements. If you are harmed and this is due to someone's negligence then you may have grounds for a legal action for compensation but you may have to pay your legal costs.

The normal National Health Service complaints mechanisms will still be available to you through:-

Tracey Noakes
Complaints Manager
James Paget University Hospitals NHS Foundation Trust
Lowestoft Road
Gorleston
Great Yarmouth
Norfolk
NR31 6LA
Tel: 01493 452019
complaints@jpaget.nhs.uk

Contact for further information
If you have any queries or concerns about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions. In this situation please don't hesitate to contact:

Mr Carl Philpott
Honorary ENT Consultant
James Paget University Hospital
NR31 6LA

Clinical Senior Lecturer
Norwich Medical School
University of East Anglia
Norwich
NR4 7LT

Independent sponsor contact:
Professor Michael Frenneaux
Dean of Norwich Medical School
University of East Anglia
Norwich
NR4 7LT

Appendix B - Control Information Form

James Paget University Hospitals NHS
NHS Foundation Trust
Lowestoft Road
Gorleston
Great Yarmouth
Norfolk
NR31 6LA

Main switchboard: 01493 452452
Direct line: 01493 452832
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www.jpaget.nhs.uk
https://www.uea.ac.uk/rhinology-group/home

Exploring Endotypes in Chronic Rhinosinusitis

IRAS Number: 202585
Participant Information Form for Controls v1.0 24.10.2016

Project Sponsor: University of East Anglia

Chief Investigator: Carl Philpott
Co-Investigators: Ana Pratas
Tom Wileman

Location: University of East Anglia and its associated NHS Hospitals
James Paget University and Norfolk and Norwich University NHS Foundation Trusts

Dear _____

Introduction

You are invited to take part in this research project that is investigating the condition chronic rhinosinusitis (CRS). This is because you have been identified as **NOT** suffering with CRS and DO NOT have nasal allergy but are due to undergo an operation called a septoplasty.

Please take a few moments to read this document that will help you decide whether you wish to take part.

What is chronic rhinosinusitis?

CRS is a condition where the lining of the sinuses and also the nose becomes swollen and the resulting symptoms have lasted for at least 3 month.

What does participation in this research involve?

We would like to capture some of the mucus present in your nose and sinuses as well as a blood sample. We would also like to take a small piece of tissue from one of the structures in your nose (turbinates). All of this tissue will then be transferred to the tissue bank for storage until we are ready to test it in our laboratory. In addition to your permission to use these samples for research, we would also want to look through your medical records to see what treatments such as nasal sprays and antibiotics that you have already had, so that we can match this information with the findings in the laboratory. By doing this we hope to be able to learn more about why patients with CRS respond differently to those without CRS.

We hope that you will be able to help us but we do of course understand if you do not wish to participate and will fully respect this decision.

What are the benefits to you of taking part?
There are no direct benefits to you from taking part in this project. This is scientific research aimed at understanding the biology of CRS so that we will better be able to treat the condition in the future.

What are the disadvantages or risks of taking part?
You will be asleep (under anaesthetic) during the procedure, so you will not experience any discomfort from these samples being removed beyond that which you would expect from the surgery itself. We will be looking at your personal health records relating to your previous treatment, but will maintain the confidentiality you would expect

How will we tell you what we have found?
This study will form the basis of a PhD studentship for Ana Pratas and the results will be included in her thesis held at UEA. We have an existing research website that we will use to announce results. We will also contact our patient charity Fifth Sense, so that they can make announcements on their website and their newsletters. We will use this to update participants on the progress of our research and to let you know of any of our findings. We hope to publish any findings of significance in scientific journals. We plan to utilise the initial findings to help further refine our investigations during a national trial of CRS treatment known as the MACRO Programme: Defining best management of adult chronic rhinosinusitis.

Further information

What will happen if I don't want to take part?
There will be no impact on any care that you may need.

How will my information be kept confidential?
The information about your tissue will be kept in confidential in hospital and university computers. The information will be coded so as to be only available to the research team.

Who is organising and funding this study?
This study is organised in Norfolk by a team of ENT surgeons and scientists. It is funded from scientific research grants that have been competitively awarded by the Sir Jules Thorn Charitable Trust.

Will I be paid for taking part?
No. We are seeking volunteers to help us with this study. We may be in a position to refund reasonable travel expenses if these are necessarily incurred.

What will happen to the tissue/blood samples that I give?
The tissue sample will be sent to the Norwich Biorepository and stored in a freezer at -80°C. If our initial work in the laboratory suggests that fresh samples provide more useful information, some samples may be chilled only and taken straight to the laboratory. The blood sample will be sent to a specialist laboratory at the Norwich Biorepository where it will be stored. We have no immediate plans to undertake any genetic testing but the sample will be stored for 10 years as to be available for future ethically approved research when the DNA can be extracted.

Who has reviewed the study?
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against possible benefits and that you have been given sufficient information on which to make an informed decision.

What if I am harmed by the study?
Nothing in this study is expected to cause you any harm. In the event that something does go wrong and you are harmed during the research study there are no special compensation arrangements. If you are harmed and this is due to someone's negligence then you may have grounds for a legal action for compensation but you may have to pay your legal costs.

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Complaints Manager
James Paget University Hospitals NHS Foundation Trust
Lowestoft Road
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Tel: 01493 452019
complaints@jpaget.nhs.uk

Contact for further information
If you have any queries or concerns about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions. In this situation please don't hesitate to contact:

Mr Carl Philpott
Honorary ENT Consultant
James Paget University Hospital
NR31 6LA

Clinical Senior Lecturer
Norwich Medical School
University of East Anglia
Norwich
NR4 7LT
Independent sponsor contact:
Professor Michael Frenneaux
Dean of Norwich Medical School
University of East Anglia
Norwich
NR4 7LT

Appendix C - Consent form

James Paget University Hospitals NHS
NHS Foundation Trust

IRAS Study Number: 202585

Participant Identification Number for this study:

CONSENT FORM (Controls and CRS patients)

Title of Project: Exploring Endotypes in CRS

Name of Researcher: Carl Philpott and Ana Pratas

Please initial box

1. I confirm that I have read the information sheet dated 10.09.2016 (version 1.0) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that relevant sections of my medical notes and data collected during the study, may be looked at by individuals from the research team, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. I understand that the information collected about me will be used to support other research in the future, and may be shared anonymously with other researchers. ☐

5. I agree to my General Practitioner being informed of my participation in the study. ☐

6. I agree to take part in the above study. ☐

Name of Participant	Date	Signature
Name of Person taking consent	Date	Signature

When completed: 1 for participant; 1 for researcher site file; 1 (original) to be kept in medical notes.
Exploring endotypes consent form v1.0 10.09.2016

Appendix D - Questionnaire

James Paget University Hospitals NHS Foundation Trust

Exploring Endotypes in CRS

1. Welcome to the 'Exploring Endotypes in CRS' Study

Welcome to the 'Exploring Endotypes in CRS' Study. Please read the following information carefully before completing the questionnaire. You will have been provided with a Participant Identification Number (PIN) by the study team when you agreed to take part in the study. You will need this PIN number in order to complete the questionnaire. You have been invited to take part because you are either a patient with Chronic Rhinosinusitis or a control subject. If you have volunteered to take part in the study because you are undergoing septoplasty (control subject), not all questions will be relevant to you but please answer the questions wherever possible. If you have previously donated tissue when you had sinus surgery at the James Paget University Hospital, please answer the questions in relation to how you were at the time of coming in for surgery. If you experience any difficulties with this questionnaire or you have any queries related to the study or do not know your PIN number, please contact Jane.Woods@jpaget.nhs.uk.

2. Participant Details

1. Please tell us if you have been invited to complete this questionnaire as a patient with Chronic Rhinosinusitis or as a Control Subject:

- ☐ Patient with Chronic Rhinosinusitis
- ☐ Control Subject

2. Please enter your Participant Information Number (PIN number) - this will have been provided to you by the study team:

3. Please enter your date of birth:

DD/MM/YYYY

4. Please select your gender:

- [ ] Male
- [ ] Female

5. Please select your ethnicity:

- **White**

- [ ] British
- [ ] Irish
- [ ] Other
- **Asian or Asian British**
- [ ] Indian
- [ ] Pakistani
- [ ] Bangladeshi
- [ ] Any other Asian background
- **Mixed**
- [ ] White and Black Caribbean
- [ ] White and black African
- [ ] White and Asian
- [ ] Any other mixed background
- **Black or Black British**
- [ ] Caribbean
- [ ] African
- [ ] Any other black background
- **Other Ethnic Group**
- [ ] Chinese
- [ ] Any other Ethnic Group
- [ ] I do not wish to disclose my ethnic origin

3. Allergies and Smoking History

6. Do you have any allergies?

- [ ] House Dust Mite
- [ ] Timothy Grass

- [ ] Dog
- [ ] Cat
- [ ] Tree Pollen
- [ ] Weed Pollen
- [ ] Aspergillus
- [ ] Alternaria
- [ ] Mixed Moulds
- [ ] Other (please specify):

7. What is your smoking status?

- [ ] Current Smoker
- [ ] Ex-Smoker
- [ ] Non-Smoker

8. If you are a current smoker, how much do you smoke in an average week?

9. If you are an ex-smoker, how long ago did you stop smoking?

Years
Months

4. Medical and Family History

10. Do you have any other medical conditions (for which you receive medical help or take medication)?

11. Is there any family history of medical problems which affect the nose/lungs/sinuses?

5. History of Chronic Rhinosinusitis

12. For how long have you been experiencing symptoms of Chronic Rhinosinusitis (Blocked nose, runny nose, poor sense of smell, facial pressure)?

Years
Months

13. Have you ever received medication for Chronic Rhinosinusitis?

- Yes
- No
- I don't know/I'm not sure

14. Which of the following medications have you received for Chronic Rhinosinusitis?

	Currently taking:	Have taken in the past:	Helped my symptoms:	Did not help my symptoms:
Steroid Nasal Spray				
Non-Steroid Nasal Spray				
Antibiotics				
Steroid Tablets				
Sinus Rinse				
Nasal Decongestant				
Antihistamine				
Analgesics (Pain relief such as Paracetamol or similar)				

15. If you have taken any of the following medications in the past for Chronic Rhinosinusitis, how many courses have you taken? (Please leave blank if zero).

	Number of Courses/Spells
Steroid Nasal Spray	
Non-Steroid Nasal Spray	

	Number of Courses/Spells
Antibiotics	☐
Steroid Tablets	☐
Sinus Rinse	☐
Nasal Decongestants	☐
Antihistamines	☐
Analgesics (such as paracetamol or similar)	☐

16. Have you experienced any side effects from current or previous treatments?

17. Have you experienced periods of time where your symptoms of Chronic Rhinosinusitis were well controlled?

- ☐ Yes
- ☐ No
- ☐ I don't know/I'm not sure

18. If you answered yes above, why do you think it was that your symptoms of Chronic Rhinosinusitis were well controlled during this period?

19. Have you ever forgotten to take your medication?

- ☐ Yes
- ☐ No

20. Do you always take your medication at the time indicated?

- ☐ Yes

- [ ] No

21. Do you ever stop taking your medication because your symptoms are worse?

- [ ] Yes
- [ ] No

22. Do you stop taking your medication because your symptoms are better?

- [ ] Yes
- [ ] No

23. Do you ever stop taking medication because of side effects?

- [ ] Yes
- [ ] No

24. In the last week how many times have you not taken your medication?

- [ ]

Appendix E – Ethical Approval

NHS
Health Research Authority

Mr Carl Philpott
Norwich Medical School, Chancellor's Drive
University of East Anglia, Norwich
Norfolk
NR4 7TJ

Email: hra.approval@nhs.net

05 December 2016

Dear Mr Philpott

Letter of HRA Approval

Study title:	**Exploring Endotypes in Chronic Rhinosinusitis (ExpRess)**
IRAS project ID:	**202585**
REC reference:	**16/EM/0468**
Sponsor	**University of East Anglia**

I am pleased to confirm that HRA Approval has been given for the above referenced study, on the basis described in the application form, protocol, supporting documentation and any clarifications noted in this letter.

Participation of NHS Organisations in England

The sponsor should now provide a copy of this letter to all participating NHS organisations in England.

Appendix B provides important information for sponsors and participating NHS organisations in England for arranging and confirming capacity and capability. **Please read *Appendix B* carefully**, in particular the following sections:

- *Participating NHS organisations in England* – this clarifies the types of participating organisations in the study and whether or not all organisations will be undertaking the same activities
- *Confirmation of capacity and capability* - this confirms whether or not each type of participating NHS organisation in England is expected to give formal confirmation of capacity and capability. Where formal confirmation is not expected, the section also provides details on the time limit given to participating organisations to opt out of the study, or request additional time, before their participation is assumed.
- *Allocation of responsibilities and rights are agreed and documented (4.1 of HRA assessment criteria)* - this provides detail on the form of agreement to be used in the study to confirm capacity and capability, where applicable.

Further information on funding, HR processes, and compliance with HRA criteria and standards is also provided.

It is critical that you involve both the research management function (e.g. R&D office) supporting each organisation and the local research team (where there is one) in setting up your study. Contact details

and further information about working with the research management function for each organisation can be accessed from www.hra.nhs.uk/hra-approval.

Appendices

The HRA Approval letter contains the following appendices:

- A – List of documents reviewed during HRA assessment
- B – Summary of HRA assessment

After HRA Approval

The document *"After Ethical Review – guidance for sponsors and investigators"*, issued with your REC favourable opinion, gives detailed guidance on reporting expectations for studies, including:

- Registration of research
- Notifying amendments
- Notifying the end of the study

The HRA website also provides guidance on these topics, and is updated in the light of changes in reporting expectations or procedures.

In addition to the guidance in the above, please note the following:

- HRA Approval applies for the duration of your REC favourable opinion, unless otherwise notified in writing by the HRA.
- Substantial amendments should be submitted directly to the Research Ethics Committee, as detailed in the *After Ethical Review* document. Non-substantial amendments should be submitted for review by the HRA using the form provided on the HRA website, and emailed to hra.amendments@nhs.net.
- The HRA will categorise amendments (substantial and non-substantial) and issue confirmation of continued HRA Approval. Further details can be found on the HRA website.

Scope

HRA Approval provides an approval for research involving patients or staff in NHS organisations in England.

If your study involves NHS organisations in other countries in the UK, please contact the relevant national coordinating functions for support and advice. Further information can be found at http://www.hra.nhs.uk/resources/applying-for-reviews/nhs-hsc-rd-review/.

If there are participating non-NHS organisations, local agreement should be obtained in accordance with the procedures of the local participating non-NHS organisation.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please email the HRA at hra.approval@nhs.net. Additionally, one of our staff would be happy to call and discuss your experience of HRA Approval.

IRAS project ID	202585

HRA Training
We are pleased to welcome researchers and research management staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

Your IRAS project ID is **202585**. Please quote this on all correspondence.

Yours sincerely

Miss Lauren Allen
Assessor

Email: hra.approval@nhs.net

Copy to: *Mrs Yvonne Kirkham (Sponsor contact)*
Ms Joanne Lucas, James Paget University Hospital NHS Foundation Trust (Lead NHS R&D contact)

NIHR CRN Portfolio Applications Team