Repurposing Mesalazine as a Potential Treatment Option for Chronic Rhinosinusitis

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DECLARATION

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PRESENTATIONS ARISING FROM THIS THESIS

- Novel treatments for chronic rhinosinusitis, Adelaide Pharmacology Annual Meeting 2020

ABBREVIATIONS

5-ASA	Mesalamine or 5-aminosalicylic acid	CFTR	Cystic fibrosis transmembrane conductase regulator
AERD	Aspirin exacerbated respiratory disease	CFU	Colony forming unit
AFS	Allergic fungal sinusitis	CRSwNP	Chronic rhinosinusitis with nasal polyps
ALI	Air-liquid interface	CT	Computed tomography
ANOVA	One-way analysis of variance	CTLA	Cytotoxic T-lymphocytes associated protein
APC	Antigen presenting cell	CysLT	Cystine leukotriene receptor
BAFF	B-cell activating factor	DAMPS	Damage-associated molecular pattern molecules
BCR	B cell receptor	DCs	Dendritic cells
Bregs	Regulatory B cells	DMSO	Dimethyl sulfoxide
CCL	Chemokine ligand	DNA	Deoxyribonucleic acid
CCR6+	CC motif 6 chemokine receptor protein	EDTA	Ethylenediaminetetraacetic acid
CF	Cystic fibrosis	ELISA	Enzyme-linked immunosorbent assay

EPOS	European position paper on rhinosinusitis and nasal polyps	IL	Interleukin
ESS	Endoscopic sinus surgery	ILCs	Innate lymphoid cells
Exo	Exoprotein	INCS	Intranasal corticosteroids
FDA	Food and drug administration	INF	Interferon
FITC	Fluorescein isothiocyanate-labelled dextrans	IRF4	Interferon regulatory factor 4
FOXP3	Forkhead box P3	iTregs	Induced T regulatory cells
GM-CSF	Granulocyte-macrophage colony-stimulating factor	LDH	Lactate dehydrogenase
GORD	Gastro-oesophageal reflux disease	LK	Lund Kennedy
H&E	Haematoxylin and eosin	LM	Lund-Mackay
HLA	Human leukocyte antigens	LTB4	Leukotriene B4
HNECs	Human nasal epithelial cells	MALDI-TOF	Matrix-assisted laser desorption ionization-time of flight mass spectrometry
HRP	Horseradish peroxidase	mDC	Myeloid dendritic cell
Ig	Immunoglobulin	MHC	Major histocompatibility complex

MIC	Minimum inhibitory concentration	PRR	Pattern recognition receptors
MLST	Multi-locus sequence typing	RANTES	Regulated upon activation, normal T cell expressed and presumably secreted
mRNA	Messenger ribonucleic acid	ROS	Reactive oxygen species
MSU	Monosodium urate	RPMI	Roswell park memorial institute
NaOH	Sodium hydroxide	*S. aureus*	*Staphylococcus aureus*
NF-kB	Nuclear factor-kappa B	SAE	*Staphylococcus aureus* enterotoxins
NK	Natural killer	SLE	Systemic lupus erythematous
ns	Not significant	SNOT-22	22-item Sino-Nasal Outcome Test
OMC	Osteal meatal complex	T cells	T-lymphocytes
OSM	Oncostatin M	T-bet	T-box transcription factor
PAMPS	Pattern-associated molecular patterns	TEER	Trans-Epithelial Electrical Resistance
pDC	Plasmocytoid dendritic cells	TGF	Transforming growth factor
PIC	Poly IC	Th	T helper

TLR	Toll-like receptor	TSLP	Thymic stromal lymphopoietin
TNF-α	Tumour necrosis factor alpha	UC	Ulcerative colitis
TSA	Trypticase soy agar	URTI	Upper respiratory tract infection
TSB	Tryptic soy broth	yo	Years old

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Table 1: *S. aureus* clinical isolate patient summary.

AIMS AND THESIS SUMMARY

The research enclosed in this thesis revolves around investigating the therapeutic viability of repurposing the drug mesalazine into a sinonasal wash for the potential treatment of chronic rhinosinusitis (CRS). This body of work utilises an *in vitro* and *in vivo* experiments to evaluate the safety and efficacy of mesalazine as an emerging treatment option.

This thesis is comprised of 6 chapters, each chapter representing different aspects of developing potential treatments for CRS. This includes exploring the current understanding and treatment options available in CRS, repurposing mesalazine into an appropriate sinonasal wash, testing it's safety and efficacy *in vitro*, developing an appropriate *in vivo* inflammatory model and finally, establishing the effects of mesalazine on sinonasal inflammation *in vivo*.

Chapter 1 functions as an introductory chapter, exploring the fundamental concepts of CRS that lay the foundational work for the coming chapters. This chapter is broken down into six parts. The first and second section provide a concise overview and epidemiology of CRS, respectively. The third section focuses on the aetiology of CRS. The fourth section delves deeper into the immune system and the cells within it that are associated with CRS. The final sections concentrate on the current treatment options for CRS and lastly propose mesalazine as a potential treatment option for CRS.

Chapter 2 is a manuscript examining repurposing mesalazine into a sinus wash, and investigating its safety *in vitro* on human nasoepithelial cells, whilst still retaining its anti-inflammatory function. From this an appropriate dosing range was established which maximised anti-inflammatory effects and minimised cell toxicity.

Chapter 3 is rat sinusitis model. This chapter centered around creating a new inflammatory small animal model that is cheap, easily accessible and non-invasive, and mimics the lymphoplasmacytic histopathology seen in a subset of patients with difficult to treat CRS.

Chapter 4 is a manuscript that ties the two previous chapter together by investigating the effects of mesalazine on sinonasal inflammation in the established rat model. Mesalazine was also compared to current treatments available for CRS *in vivo*. The systemic effects were also investigated.

Finally, in Chapter 6, conclusions are drawn and future directions of research are reflected upon. Mesalazine presents as a potential therapeutic option for the treatment of CRS through its anti-inflammatory effects. Whilst the work embodies in this thesis looks at the effects of mesalazine *in vitro* and *in vivo*, future directions should be aim at investigating its effects in human clinical trials.

CHAPTER 1. INTRODUCTION

1. CHRONIC RHINOSINUSITIS

Chronic rhinosinusitis (CRS) is an inflammatory condition that affects the mucosa membranes of the nasal cavity and paranasal sinuses (Benninger et al. 2003). A clinician can differentiate chronic rhinosinusitis from other nasal conditions by taking a thorough history, performing an endoscopic examination, and reviewing radiological findings. Based on the duration of symptoms, rhinosinusitis may be classified as acute, subacute, recurrent-acute, or chronic rhinosinusitis (Lanza & Kennedy 1997). Historically, the diagnosis was largely reliant on clinical symptoms, however a better understanding of the disease and its broad spectrum suggests that a clinical diagnosis alone may not always be accurate, and current opinion holds that both subjective and objective criteria should be met to diagnose CRS (Benninger et al. 2003; Jackson & Kountakis 2005).

Current European guidelines (Fokkens, WJ et al. 2020) for the definition of chronic rhinosinusitis is:

1. Duration of disease > 12 weeks continuously, with validation by phone or interview.
2. Two or more clinical symptoms present, one of which should be either nasal blockage/obstruction/congestion or nasal discharge (anterior/posterior nasal drip)
    1. ± Facial pain/pressure
    2. ± Reduction or loss of smell (Figure 1)

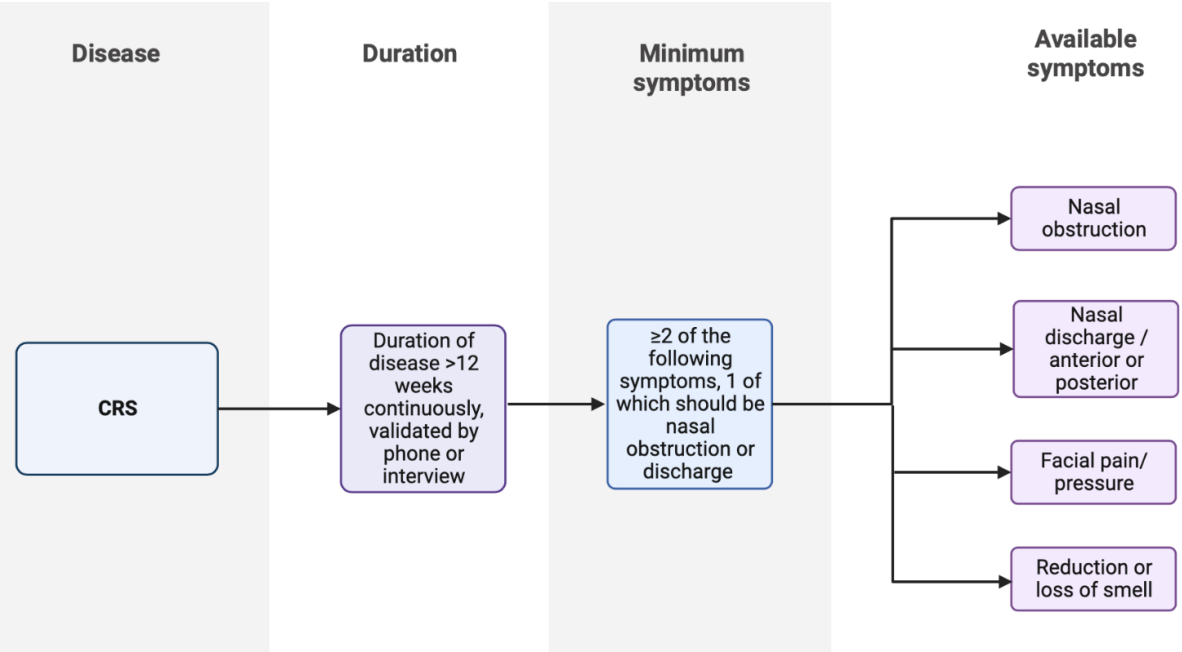

Figure 1. Criteria for CRS diagnosis

The current European guidelines then subclassified CRS into localised and diffuse based on anatomical distribution. In primary CRS, the disease is then divided by endotypes, being type 2 or non-type 2 (type 1 and 3) (Figure 2). Complete resolution before 12 weeks with medical therapy favors a diagnosis of sub-acute or acute rhinosinusitis, and a diagnosis of CRS should not be made (Lanza & Kennedy 1997).

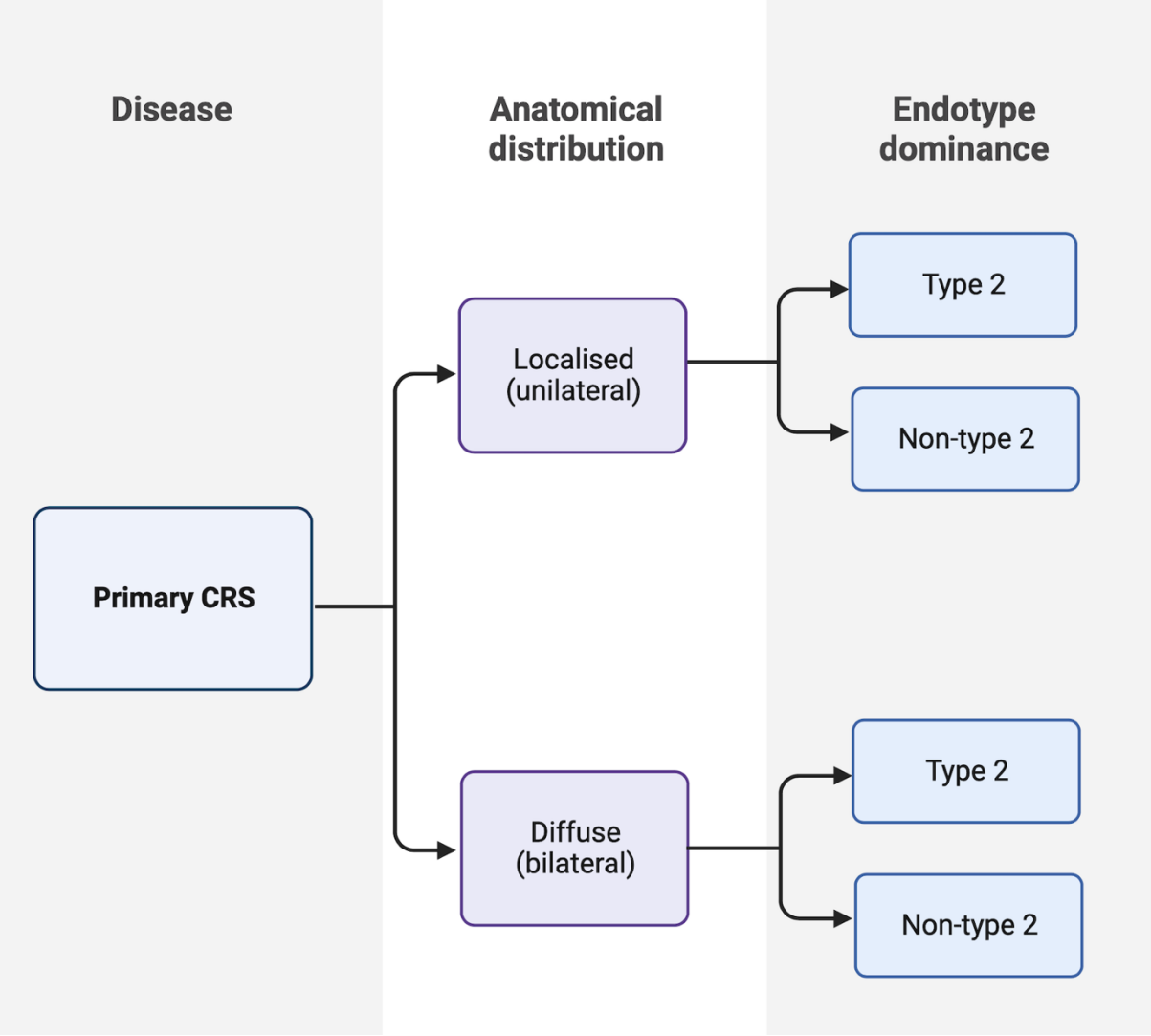

Figure 2. Classification of Primary CRS. Adapted from (Fokkens, WJ et al. 2020) with permission.

Traditionally CRS has been subclassified according to the presence (CRSwNP) or absence of polyps (CRSsNP) (Fokkens, W, Lund & Mullol 2007), with CRSwNP being defined as above, but with bilateral polyps on endoscopic examination, and CRSsNP described as above, but with no visualized polyps. Furthermore, CRS is becoming increasingly more prevalent, and it is an complex disease with various disease types and pathophysiology (Tomassen et al. 2016; Wei, B et al. 2018). As the shift towards classification of CRS into endotypes continues, identification of different endotypes may result in better individualized

therapies against the underlaying pathophysiological processes with more effective treatment options and improved patient morbidity.

2. EPIDEMIOLOGY OF CRS

The prevalence of CRS in Australia is approximately 1.9 million, which represents approximately 9.8% of the population (Habib et al. 2019). It is comparable to Europe, where the overall prevalence of CRS was 10.9% based on the European Position Paper on Rhinosinusitis and Nasal Polyps criteria (Fokkens, WJ et al. 2020). Furthermore, CRS affects an estimated 12.5% of the population in the United States (Hamilos 2011). Health care costs associated with CRS in the US ranged between 6.9 and 9.9 billion dollars, and indirect health care costs were estimated to be 13 billion dollars (Smith, KA, Orlandi & Rudmik 2015).

3. AETIOLOGY OF CRS

Despite its high prevalence, high global health care costs, and increasing research into the disease, the pathogenesis of CRS remains poorly understood. The majority of CRS cases are classified as idiopathic, with a smaller subset being associated with other medical conditions, including aspirin exacerbated respiratory disease, allergic fungal sinusitis, cystic fibrosis, Churg-Straus disease, Wegener's granulomatosis, and Kartagener's syndrome (Goutaki et al. 2017; Hamilos 2016; Kühn et al. 2018). It is becoming more widely recognized that CRS is a multifactorial disease with multiple risks, each contributing to the disease process in a different

manner, and many of these overlapping (Benninger et al. 2003). Many predisposing factors have been described in the literature, which may contribute directly or indirectly to the inflammatory response of the host or to the initiation of an infectious response by bacteria, fungi or viruses, which we will discuss as below.

3.1. Extrinsic factors

3.1.1. Mechanical Barriers

It is important to note that the ciliary function plays an important role in sinonasal homeostasis. A vital function of cilia is to transport mucus from the sinuses, out of the sinus ostia, to the nasal cavity, postnasal space and finally into the oesophagus. A dysfunctional cilium can result in ciliary stasis, a decrease in mucus clearance, and an increase in bacterial activity. The presence of an infectious state can lead to increased inflammation and hypoxia, which in turn impairs cilia function, thus perpetuating the cycle (Fokkens, W, Lund & Mullol 2007). There is an increased incidence of CRS in cystic fibrosis (CF) patients, as well as patients with impaired mucociliary function. Additionally, this increase in CRS prevalence also exists in CF gene carriers who do not manifest the disease (Wang, XinJing et al. 2005; Wang, XinJing et al. 2000). Another structural component of CRS is seen in the nasoepithelial cells' tight junctions, which have been shown *in vitro* to be weaker and to have increased permeability (Antunes, Gudis & Cohen 2009; Den Beste et al. 2013). These findings suggest that the integrity and functioning of the mucosal barrier, plays an important role in sinonasal homeostasis.

3.1.2. Environmental

Multiple environmental factors, such as smoking (Briggs et al. 2004; Chen, Y, Dales & Lin 2003), air pollution (Wolf 2002) and exposure to allergens (Asero & Bottazzi 2001; Gutman et al. 2004) have all been found to be associated with an increase in the incidence of CRS. Both primary and second hand smoking have been connected to increased severity of CRS (Briggs et al. 2004; Goldstein-Daruech et al. 2011; Jaakkola & Jaakkola 2002; Mori et al. 2013; Ramadan & Hinerman 2002; Reh et al. 2009; Tammemagi et al. 2010). Smoking has been shown to inhibit ciliary cells and decrease the mucociliary escalator function (JM 1995; Stanley et al. 1986). Additionally, chemical irritants are exposed to the sinonasal mucosa first, which acts as the first defensive barrier (Reh, Higgins & Smith 2012). Infections and exacerbated CRS may result from injury to the mucosa caused by chemical irritants.

3.1.3. Allergy

It has been shown that allergies, or atopy, are related to CRS, and are associated with poor postoperative outcomes and the need for revision surgery (Dursun et al. 2003). The prevalence of allergies in patients with CRS is estimated to range from 29 to 84% (Alobid, Benítez, et al. 2006; Batra, Tong & Citardi 2013; Emanuel & Shah 2000; Freudenberger, Grizzanti & Rosenstreich 1988; Gutman et al. 2004; Hoover et al. 1997; Leo et al. 2007; Savolainen 1989). It has been suggested that a low grade, chronic mucosal inflammatory state may progress to CRS in patients who have been exposed to additional etiological factors (Pelikan, Zdenek 2009; Pelikan, Z & Pelikan-Filipek 1990).

3.1.4. Microbiome

During the last decade, research had been focusing on the sinonasal mucosa due to the development of 16S rRNA bacterial sequencing methods, which have proven to be highly sensitive and less biased, thus providing a better understanding of the microbiome within the sinonasal environment. It has become increasingly important to focus on the diversity and abundance of commensal bacteria rather than the bacterial burden (Boase, Foreman, et al. 2013). It is the aim of microbiome studies to determine whether or not the components of the microbiome are in balance and how they interact in health versus disease. As a result of a disturbance in the balance of the microbiome, CRS patients suffer from dysbiosis, and their biodiversity is reduced compared to healthy controls (Anderson et al. 2016).

3.1.5. Bacteria

The sinonasal mucosa is colonized by healthy commensal bacteria. Commensal bacteria cultured in healthy individuals include *coagulase negative staphylococcus, coagulase positive staphylococcus aureus* and *Corynebacterium* species (Gordts et al. 2000). Bacterial infection however has been closely associated with CRS, with 83% of cultures reported to be positive for CRS patients (Cleland, Bassiouni & Wormald 2013; Klossek et al. 1998). The most prevalent bacteria in CRS patients' culture is *Staphylococcus aureus* (*S. aureus)*, followed by *Pseudomonas aeruginosa* (Brook 2006). It was found by Cleland *et. al* (2013), that the rate of *Staphylococcus* in culture was 35% and 9% for *Pseudomonas aeruginosa* in a sample of 513 CRS patients. This is similar to the rate reported by Gittleman et al (1991), who reported 35%

for *S. aureus*, and Nadel, Lanza and Kennedy (1998) who reported a rate of 17% for *Pseudomonas aeruginosa*.

It has been shown that *S. aureus* overgrowth is a major contributor to CRS (Wang, XinJing et al. 2000). Furthermore, *Streptococcus pneumoniae*, *Moraxella catarrhalis* and *Haemophilus influenzae* have been associated with biofilm formation in CRS (Hall-Stoodley, Costerton & Stoodley 2004). Biofilms are a matrix of microorganisms in which the bacteria bind to each other as well as the host surfaces. Biofilms are encased in an extracellular polymeric matrix. This gives the bacteria the ability to bind tightly to the mucosal surface and slow their metabolic rate allowing them to evade innate immune responses as well as antibiotics (Hall-Stoodley, Costerton & Stoodley 2004). Most pathogenic bacteria form a biofilm and this allows them as they mature to give off planktonic bacteria, thereby acting as a reservoir and contributing to infection persistence. The formation of biofilms occurs as a bacterial response to cues such as exposure to planktonic cells, low concentrations of antibiotics, decreased nutrition and recognition of surface attachment sites. Of the forementioned bacteria, *S. aureus* has the largest association with biofilm production, as well as with more severe and recalcitrant disease manifestation (Kariya et al. 2014). The rate of biofilm production in CRS is 29-72% and it has been associated with severe disease, mucosal inflammation, increased infections and post operative symptom persistence (Maspero, JF et al. 2020; Tomisa et al. 2019).

3.1.6. Fungal Hypothesis

In the late 1990s Ponikau et al. (1999) proposed that fungus could be the single specific aetiological organism responsible for CRS, the so-called fungal hypothesis. According to this

study, 96% of participants with CRS (202 of 210 CRS patients) had fungus cultured from their nasal secretions (Ponikau et al. 1999). According to histological or culture analyses, most of the patients met the diagnostic criteria for allergic fungal sinusitis (Lampinen et al.).These findings were later confirmed by Braun et al (2003), and they suggested that the presence of fungus resulted in the abnormal eosinophilic mucus that is seen in Allergic Fungal Sinusitis (AFS).

However, the role of fungus as the single most important factor in the development of CRS was not replicated in other studies and the fungus seen in Ponikau's papers were found in similar quantities in the normal control patients. (Braun et al. 2003; Ponikau et al. 1999). Early trials for topical anti-fungals in CRS patients showed promising results (Ponikau et al. 2002; Ponikau et al. 2005). However, these were not seen in properly controlled studies where no beneficial outcomes were seen in objective or subjective disease severity scores, or in the change in pro-inflammatory cytokine profiles from amphotericin B being administered as a topical irrigation in CRS. In addition there were side effects were reported (Ebbens et al. 2006; Liang et al. 2008; Weschta et al. 2004). The role of fungus as a unifying underlying aetiological mechanism has been debunked. However, there is still a role for fungus in subset of CRS patients who develop allergic fungal CRS.

3.1.7. Viral Hypothesis

In the viral hypothesis, a virus is regarded as the triggering event for the development of CRS, but its role in the disease's pathogenesis is unclear. The symptoms of CRS can be exacerbated

when patients have a viral upper respiratory tract infection (URTI). The presence of respiratory viruses in the mucosa has been demonstrated in patients with CRS (Cho et al. 2013; Jang et al. 2006). There is also an association between the presence of virus and the severity of the CRS disease (Goggin, RK et al. 2019). The most common viruses associated with CRS is rhinovirus, followed by coronavirus and influenza (Makela et al. 1998). There is evidence that rhinoviruses can increase bacterial attachment to and colonization of the epithelium (Wang, JH, Kwon & Jang 2009a). Furthermore, viruses have immunomodulatory effects on nasoepithelial fibroblasts (Wang, JH, Kwon & Jang 2009b). Patients with CRS are also induced by rhinovirus to produce pro-inflammatory cytokines in their nasal mucosa (Kim, JH et al. 2015). It has been reported that viruses are associated with increased mucosal eosinophils and lymphocytes in patients with CRS, indicating that infections may be a source of immune dysregulation in these patients (Goggin, RK et al. 2020). The role of viruses in the development of CRS still requires further investigation.

3.2 Intrinsic factors

3.2.1 Anatomical Factors

When Endoscopic Functional Sinus surgery was introduced in the late 1980s by Heinz Stammberger the theory was that narrowing of sinus outflow tracts and other anatomical variations in the sinus bony anatomy could contribute to the development of CRS by impairing mucociliary function and drainage. According to the study by Staikunierne, a third of CRS patients have a single anatomical abnormality, while half of CRS patients have two or more anatomical abnormalities (Staikūnienė et al. 2008). A deviated nasal septum was found to be

the most common anatomical variation in CRS patients on CT scans, followed by unilateral concha bullosas and bent middle turbinates (Aramani, Karadi & Kumar 2014). A study by Aramani et al (2014) found anatomical variations (septal deviation, bilateral concha bullosa, medial deviation of uncinate process, Haller cell, hypertrophic ethmoidal bulla cell and agger nasi cell) were associated with mucosal diseases. This study had a population size of 200, but lacked healthy controls, therefore making the association less impressive (Aramani, Karadi & Kumar 2014). In a further study by Langille et al (2012), 328 consecutive CT's were reviewed and found an association between frontal sinus mucosal thickening and the presence of frontal cells. Conversely, a study by Eweiss & Khalil (2013) found no association between the presence of frontal sinus cells, or agger nasi cells and frontal mucosal thickening.

The controversy as to the contribution of anatomical variants to the development of CRS was added to by a study by Bolger et al (1991), who found no significant difference between the frequency of anatomical variants (particularly the presence or absence of frontal sinus cells) in CRS patients' CTs compared to CTs of patients who had no sinus symptoms. The only anatomical feature that was more prevalent in the CRS cohort was concha bullosa (Bolger, Parsons & Butzin 1991). It was however shown by Zeinrich et al (1988), that osteal meatal complex (OMC) obstruction in CRS patients was not significantly different between patients with and without a concha bullosa. This was further supported by Aktas et al (2003) and Stallman et al (2004) who both found no increase in sinusitis in patients with concha bullosa. Ostia meatal complex (OMC) obstruction was thought to have a significant role in causing CRS, however OMC obstruction is not seen in all CRS patients (Chandra et al. 2010). The conflicting findings for anatomical variants suggest that anatomy likely plays a minor role in the development of CRS.

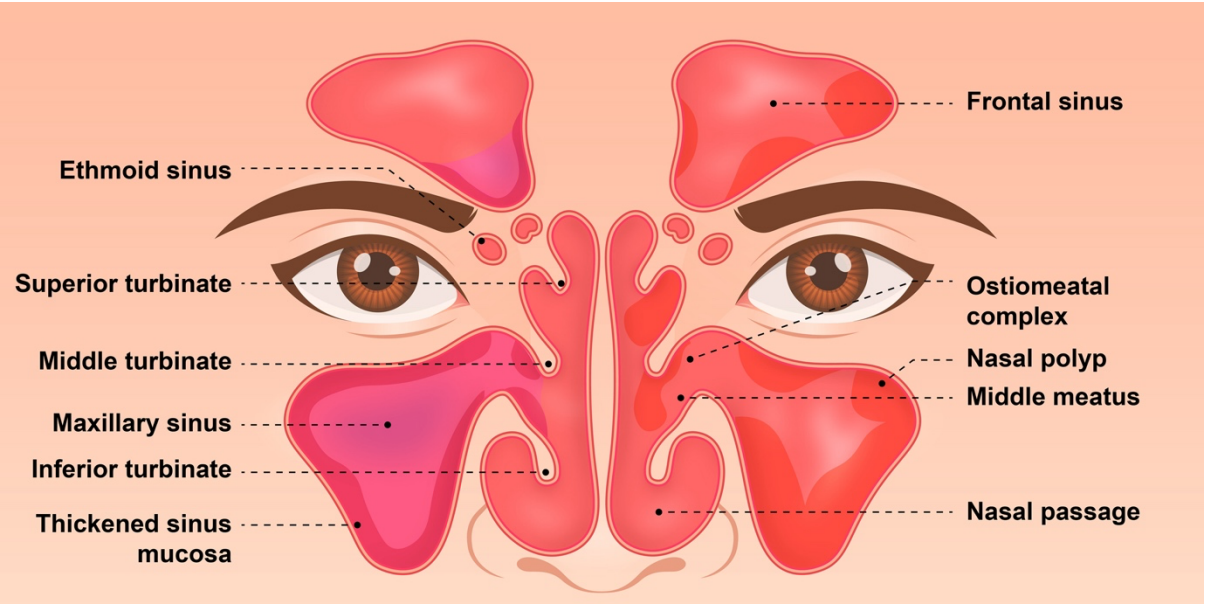

Figure 3. CRS causing anatomical obstruction from mucosal inflammation and polyp formation.

3.2.2. Genetics

Genetic research in CRS has been gaining traction as there are associated genetic predispositions with the disease. Recent technological advancements have enabled DNA studies and improved analysis has shown interesting findings when studying family groups who have a high prevalence of CRS, also having high genetic similarities (Cohen et al. 2006; Greisner III & Settipane 1996; Qu et al. 2007). CRS has been linked to cystic fibrosis, a disease characterized by a dysfunctional Cystic Fibrosis Transmembrane Conductase Regulator (CFTR) gene that inhibits cilia motility by altering mucus consistency by affecting chloride transport to the mucous layer (Wang, XinJing et al. 2005; Wang, XinJing et al. 2000). The study by Wang et al (2000) investigated the most common 16 gene mutations that accounted for 85% of the cystic fibrosis cases. A CF mutation was identified in 7% of CRS patients

compared to 2% of controls out of 146 CRS patients and 123 controls (Wang, XinJing et al. 2000) showing that there was a higher prevalence of CF mutations in CRS patients.

Human leukocyte antigens (HLA) genes play a role in antigen presentation by B cells, macrophages and dendritic cells. In CRS there are alleles that have been associated with CRS (Jaqueline et al. 2006). The presence of the specific antigen gene HLA-B54 was associated with patients with CRS (Takeuchi et al. 1999), and it was proposed that the HLA-B54 gene may contribute to altered host immunomodulatory responses. Such an impairment could result in increased inflammatory and immune response that can result in CRS. As genetic analysis technology advances so further research in this area will elucidate the roles that genes play in the development of CRS.

3.2.3. Immune hypothesis

Chronic Rhinosinusitis disrupts the normal defensive mechanisms of nasal epithelial host cells on various levels, affecting both mechanical and immune barriers. This impairment of mechanical barriers can occur at the different levels, including the level of mucus production, the mucociliary escalator, and the integrity of tight junctions and adherent junctions on the cells. The immune barriers include the innate and adaptive immune responses. The innate immune response involves cytokine-mediated recruitment of immune cells, complement activation, and the identification and removal of foreign materials. Adaptive immunity encompasses processing antigen recognition, clonal expansion, activation, an effector response for antigen elimination, and the development of memory cells. Disruption of the mechanical and immune barriers can be caused by a range of internal and external factors. However, this

disruption can lead to alterations in the microbiome, increased exposure to pathogens, and an exaggerated immune response, potentially causing further tissue damage (Lam, K, Schleimer & Kern 2015).

Disruption of the mechanical barriers can be observed in CF and CF gene carriers, who have a defective mucociliary escalator and an associated higher incidence of CRS. Weaker tight junctions have been observed in CRS patients *in vitro* (Den Beste et al. 2013). Further, a reduction in genes associated with epithelial barrier maintenance and repair has been observed in CRS patients, suggesting the importance of barrier function and integrity (Richer et al. 2008).

In the innate immune response in healthy sinonasal epithelium, exposure to a pathogen triggers the production and release of antimicrobial molecules. In CRS, many of the underlying mechanisms responsible for this process are compromised (Tengroth, Arebro, et al. 2014; Tengroth, Millrud, et al. 2014). One example of this is the impairment of pattern recognition receptors (PRR), which are associated with the secretion of antimicrobial substances in CRS.

In the adaptative immune response, in Caucasian patients with CRSwNP, an inflammatory profile characterized by T helper Type 2 dominance is observed. This Type 2 inflammation is linked to elevated levels of eosinophils relative to neutrophils, along with increased production of cytokines such as IL4, IL5, IL10, and IL13. Additionally, there is local oedema accompanied by an infiltration of mast cells and B lymphocytes (Berger 2000). On the other hand, the CRSsNP phenotype is more closely associated with T helper Type 1 inflammation (König et al. 2016). T-helper 1 inflammation primarily leads to the production of cytokines like interferon γ, TNF-α, and IL-2 (Seder, Darrah & Roederer 2008). Once inflammation is initiated, it is

sustained through a complex interplay of various cytokines, which are in turn regulated by transcription factors like nuclear factor-kappa B (NF-kB) (Frączek et al. 2013; Henriksson et al. 2001). These intricate interactions are believed to be pivotal in the underlying pathophysiology of CRS.

3.2.4. Inflammatory Endotypes

Traditionally, Chronic Rhinosinusitis has been categorized into two phenotypes: CRSsNP and CRSwNP, constituting roughly 80% and 20% of cases respectively (Benjamin et al. 2019; Fokkens, WJ et al. 2020). As research advances, there has been a growing emphasis on classifying CRS into endotypes based on the specific types of inflammation present, namely Type 2 and non-Type 2 (Type 1 and Type 3) inflammation. This differentiation in CRS endotypes can provide valuable insights into the natural progression of the disease (Bachert et al. 2018). In the Caucasian population, over 80% of individuals with CRSwNP demonstrate a Type 2 inflammation with eosinophils as the predominant cell type (Tomassen et al. 2016; Turner et al. 2018; Van Zele et al. 2006). On the other hand, approximately half of the Asian population who have CRSwP exhibit a predominance of Type 1 inflammation, characterized by a higher presence of neutrophils. This population responds to novel treatments targeting Type 1 inflammation about 50% of the time, suggesting the important for endotype classification for targeted treatments (Cao et al. 2009; Liao et al. 2018; Tomassen et al. 2016; Van Zele et al. 2006; Wang, Xiangdong et al. 2016; Zhang, Y et al. 2017).

3.2.4.1. Non-Type 2 Inflammation

Non-Type 2 inflammation encompasses Type 1 and Type 3 inflammation, and studies on CRS often do not differentiate between these two inflammation profiles. In CRS patients with non-Type 2 inflammation, have elevated levels of TNF-α, IL1-B, interferon γ, IL-6, IL-8, and IL-17, along with increased levels of macrophages, neutrophils, TH1 and TH17 cells (Shi, L-L et al. 2014; Zhang, N et al. 2008).

In Type 1 inflammation, there is a shift in macrophages towards an M1 endotype. This shift leads to the production of IL-1β and reactive oxygen species (ROS) through the activation of NLRP3 inflammasome (Zhong et al. 2021). Neutrophils also play a crucial role in non-Type 2 inflammation by synthesizing and releasing various substances, including LTB4, ROS, IL1-B, IL-8, OSM, myeloperoxidase, elastase, cathepsin G, and proteinase 3 (Zhong et al. 2021). Knowledge about Type 1 inflammation in CRS is lacking at a molecular level, highlighting the need for further research in this area.

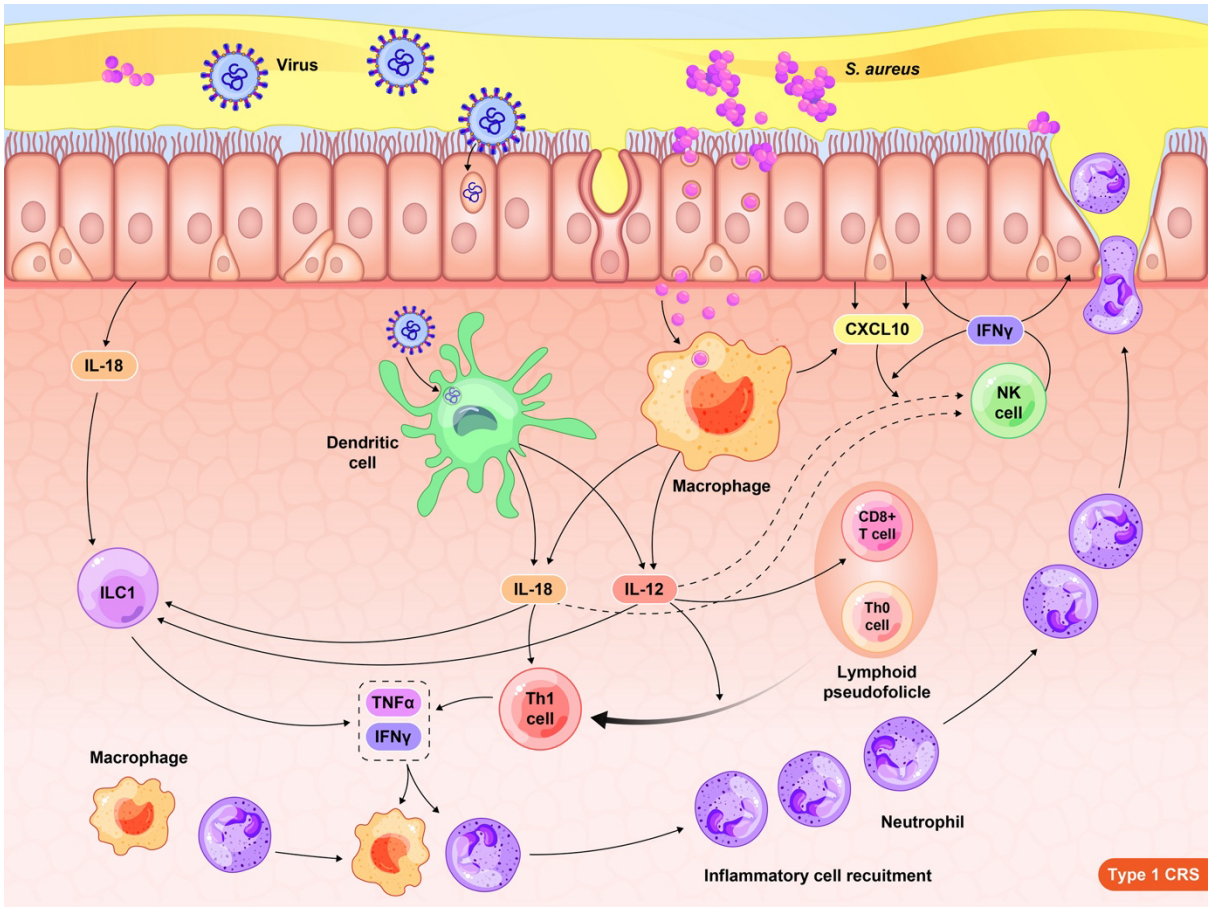

Figure 4. Non-type 2 CRS inflammatory endotype and the cells involved. Adapted from (Fokkens, WJ et al. 2020) with permission.

3.2.4.2. Type-2 Inflammation

Type 2 inflammation in CRS is characterized by elevated expression of IL-4, IL-5, IL-13, and IgE. This type of inflammation is primarily marked by an eosinophilic presence and is strongly associated with the CRSwNP phenotype (Bachert et al. 2020; Fokkens, WJ et al. 2020; Schleimer 2017). Moreover, Type 2 inflammation, particularly when accompanied by eosinophilic dominance, has been linked to severe disease and is considered a risk factor for CRS relapse (Alsharif et al. 2019; Bachert et al. 2020; Fokkens, WJ et al. 2020; Jonstam et al. 2019; Schleimer 2017).

In the Caucasian population, CRS mediated by Type 2 inflammation has been associated with eosinophilic disorders such as allergic rhinitis, asthma, Churg-Straus disease and atopy. The incidence of these conditions in the European population is significantly higher compared to the Asian population (Bachert et al. 2020; Fokkens, WJ et al. 2020; Laidlaw et al. 2021; Liu, Z et al. 2020; Shi, JB et al. 2015).

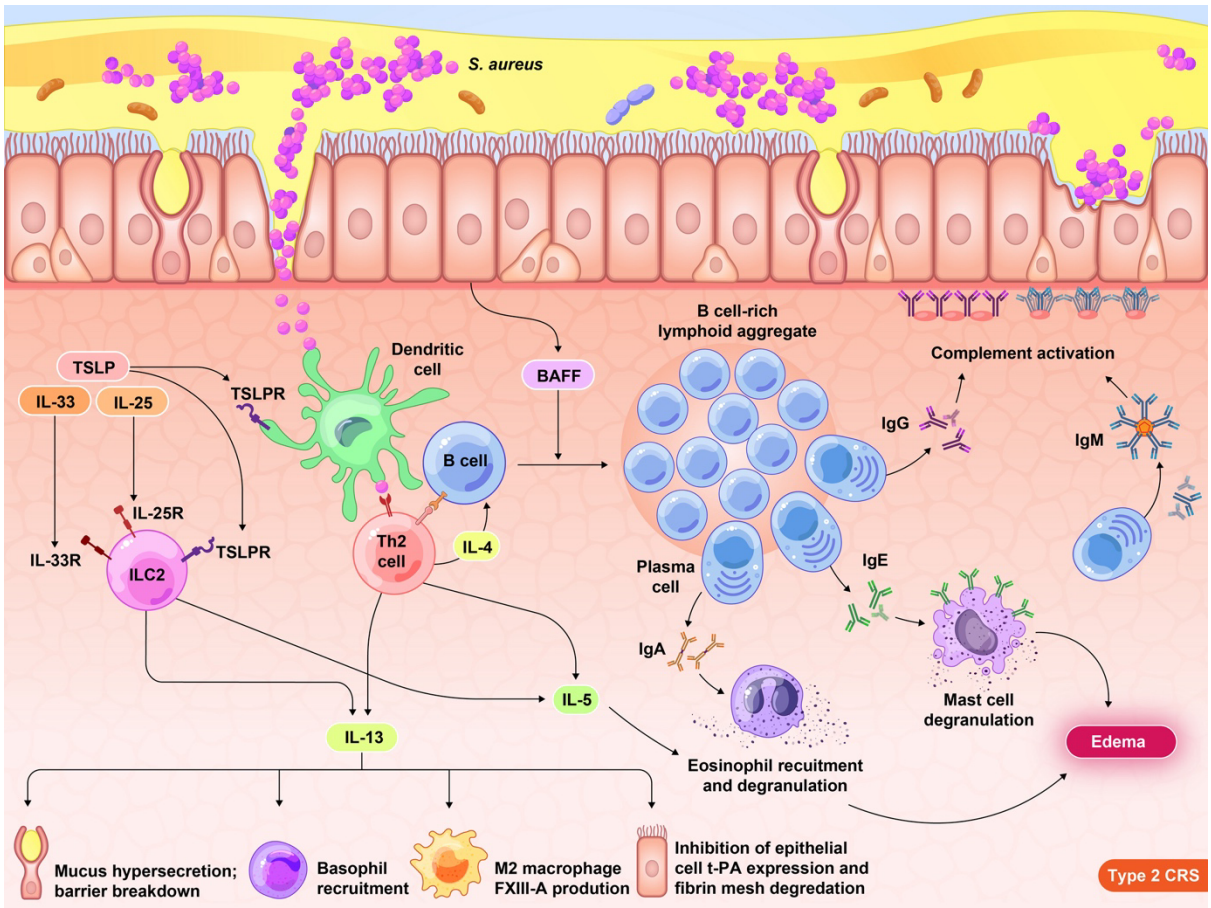

Figure 5. Type 2 CRS inflammatory endotype and the cells involved. Adapted from (Fokkens, WJ et al. 2020) with permission.

4. Immune System and CRS

4.1. Innate Immune System

Innate immunity is the first line of the body's defense against pathogens. The sinonasal epithelium not only provides a physical barrier to pathogens, but also has cells which can sense when pathogens are present and co-ordinate an immune response. The cells lining the mucosa have a diverse range of pathogen sensing receptors, as well as cytokines that can be released as part of an immune response. In healthy individuals, the epithelial cells monitor and sense antigens, once sensed, they release cytokines and chemokines which recruit immune cells to the sinuses. In patients with CRS, there are differences in the innate response, which supports the hypothesis that a dysregulation of the innate immune response is in part contributing to the disease process and inflammation that is central to CRS pathophysiology (Ooi et al. 2010).

4.1.1. Cells of the Innate Immune System

4.1.1.1. Macrophages

Macrophages are effector cells of the immune system. They play a role in maintaining tissue homeostasis, and are located in all tissues (Wynn, Chawla & Pollard 2013). Macrophages come from the mononuclear phagocyte cell line, with progenitor cells in the bone marrow, which produce blood monocytes, and once these enter into the tissue they become tissue macrophages (Hume 2006). Macrophages can be identified by the expression of CD14 and PRR on their surface (Ball & Fanger 1983). Monocytes maturation to macrophages can be identified by the expression of 25f9 cellular marker (Pilling et al. 2009).

Macrophages function by phagocytosing apoptotic cells and debris. Their primary function is immunological, and they are adaptive to their environment. The pathways involved with macrophage function are classified into a classically activated M1 and an alternatively activated M2 pathway (Gordon 2003; Murray, PJ et al. 2014). M1 macrophages are termed this as they are differentiated in response to IFN-γ, which is produced by Th1 in response to intracellular infection. M2 macrophages are similarly termed so, as they response to Th2 cytokines. M2 macrophages are thought to be anti-inflammatory, and respond to parasitic infections (Murray, PJ et al. 2014).

Macrophages were first linked to CRS by Shun et al., (2005) who looked at chemokines that contributed to CRS. Shun et al., (2005) suggested that polyposis in CRS was contributed to by fibroblast stimulated CCL production, which promotes macrophage recruitment. It was discovered by Jung et al. (2019), that tissue from polyps contained a higher amount of NF- Kβ compared to uncinate tissue. This is important as NF-Kβ is an important inflammatory regulator of macrophages. It was also observed that the mRNA of the cytokines responsible for macrophage recruitment, p65, IL-6, IL-8, IL-1β and eotaxin were higher in polyp tissue when compared to uncinate tissue (Jonstam et al. 2019). M2 macrophages have the ability to secrete high levels of CCL18, which is a cytokine that acts as a chemotactic for naïve T cells, Th2 cells and dendritic cells, which may contribute to the underlaying pathogenesis of CRSwNP (Krysko et al. 2011). M2 macrophages further produce factor XIII-A, which induces fibrin deposition and tissue oedema in nasal polyps of Caucasian patients with CRS (Khawar, Abbasi & Sheikh 2016; Peterson et al. 2012; Takabayashi, Kato, Peters, Hulse, Suh, Carter, Norton, Grammer, Cho, et al. 2013; Takabayashi, Kato, Peters, Hulse, Suh, Carter, Norton, Grammer, Tan, et al. 2013). Macrophages in CRSsNP have not undergone extensive study, however they often remain elevated, and likely playing a role in Type 1 inflammation (Kaczmarek et al. 2019;

Khawar, Abbasi & Sheikh 2016; Perić et al. 2015). Macrophages also act to recruit eosinophils and neutrophils to the site of inflammation in CRS (Kaczmarek et al. 2019; Khawar, Abbasi & Sheikh 2016; Perić et al. 2015).

4.1.1.2. Eosinophils

Eosinophils are a variety of white blood cell that have a role in tissue repair and immune defense, especially towards helminths (Abbas, Abul K, Lichtman & Pillai 2019). Eosinophils are derived from bone marrow, where they are released into the vascular system. Once released from the bone marrow, they can be distinguished by a change in their cytoplasmic specific granules (Melo et al. 2008). Stimulation of eosinophils can occur through an IL-5 receptor mediated pathway, which results in the stimulation of Th2 cells, which can in turn lead to the production of TSLP, IL25 and IL-5, causing a positive feedback loop (Clutterbuck, Hirst & Sanderson 1989; Clutterbuck & Sanderson 1988; Mould et al. 1997; Rosenberg, Dyer & Foster 2013). In response to cytokine stimulation, eosinophils release extracellular cytoplasmic granules which have a bactericidal effect (Dvorak et al. 1991). Some of the main proteins that the extracellular granules release are major basic protein 1 and 2, eosinophil cationic protein, eosinophil peroxidase and eosinophil derived neurotoxin (Lehrer et al. 1989). These proteins in turn stimulate other immune cells, which can lead to further bactericidal effects and the production of ROS (Lehrer et al. 1989).

Eosinophils have played a role in chronic inflammatory related diseases, such as asthma and CRS. An eosinophilic inflammation of the airways has been extensively studied in asthma (Lu et al. 2010). In an allergic airway, antigens stimulate cytokine production and Th2 cell recruitment, which result in the chemotaxis of eosinophils (Wardlaw 1999). Chronic inflammation, derived from eosinophils result in hyperresponsiveness, epithelial damage,

mucus secretion, airway remodeling and other chronic inflammatory changes (Wardlaw 1999). Eosinophilic damage was thought to be one of the underlaying pathophysiologic mechanisms in CRS, however it is evident now that non-eosinophilic factors are also present (Harlin et al. 1988; Kaliner et al. 1997; Polzehl et al. 2006).

In CRS, eosinophilia has been prominent, particularly in Caucasian patients (Berger 2000). The incidence of eosinophilia is higher in CRSwNP patients when compared to CRSsNP and controls (Jankowski et al. 2002; Lou et al. 2018; Payne, Borish & Steinke 2011; Szucs et al. 2002). This is also seen in tissue samples where eosinophils are more prominent in CRSwNP samples when compared to CRSsNP (Lou et al. 2018; Payne, Borish & Steinke 2011). Although eosinophils are not as pronounced in CRSsNP compared to CRSwNP, patients with CRSsNP who do have a high eosinophil counts have a higher burden of disease and don't respond to medical or surgical interventions (Soler et al. 2010). This association between eosinophils and polyps, suggest that eosinophils may play a key role in polyp formation. However this relationship is not prominent in polyps from Asian patients and also not in some Caucasian patients, which questions their role in polyposis (Cao et al. 2009; Payne et al. 2011). Regardless, eosinophils act as a biomarker for severe disease with poor prognosis (Tajudeen et al. 2019; Tokunaga et al. 2015).

In CRS, key steps involved in eosinophil recruitment are largely stimulated by epithelial cytokines, complement proteins, proteases, eicosanoids, stem cell factor and Type 2 cytokines (Kowalski et al. 2005; Mulligan et al. 2017; Nagarkar et al. 2013; Smith, SE et al. 2017; Van Zele et al. 2009). Key cytokines that have been shown to be involved with CRSwNP include the regulate on activation, normal T cell expressed and secreted (RANTES) dimer, which is a cytokine that attracts monocytes and T cells and is forty times higher at a protein level in nasal

polyp tissue of CRSwNP patients than the normal nasal tissue of controls (Allen et al. 1998; Crawford et al. 2011). Furthermore, there is also an increase in IL-5, eotaxin and other cytokines and chemokines in CRSwNP, all of which have the ability to stimulate type 2 inflammation and be biomarkers for severe recalcitrant disease with a poorer clinical prognosis. (Luukkainen et al. 2018; Tajudeen et al. 2019; Tokunaga et al. 2015; Yip et al. 2019). ILC2 and Th2 cells are key-players in the signaling pathway that stimulate Type 2 immune responses (Poposki et al. 2017). Eosinophils form extracellular traps at the site of barrier damage and exerting a defense against bacteria; this however may further impair barrier integrity (Gevaert, E et al. 2017). Eosinophils can contribute to oedema formation in CRS, potentially secondary to degranulation, with the mechanism remaining unclear. It is suggested that degranulation may occur through IgA receptors crosslinking, with nasal polyps also containing higher levels of IgA (Feldman et al. 2017; Pleass et al. 2007; Van Zele et al. 2007). Eosinophils are steroid responsive cells, which could also explain why glucocorticoids have an effect in CRS (Schleimer & Bochner 1994). Oral steroids however have significant systemic effects with high morbidity. Targeting features of activated eosinophils have been suggested, with clinical trials using anti-IL-5 having shown clinical benefits and polyp eosinophilia reduction in CRSwNP patients (Bachert et al. 2017). In this study however, only half of the patients demonstrated a reduction in polyp size (Bachert et al. 2017). Contrary to this is the use of dexpramipexole, a small orally available molecule that depletes blood and respiratory tissue eosinophilia, which in CRSwNP patients decreases blood and polyp eosinophil counts, but had no significant impact on symptom scoring or polyp size (Laidlaw et al. 2019). These results suggest that eosinophils have a role in predicting disease severity and prognosis, however they are not necessary for CRS activation and therefore may only contribute partly to the underlaying pathophysiology of the disease.

4.1.1.3. Neutrophils

Neutrophils are the most abundant of granulocytes, and make up 40-70% of the white blood cells in the body (Tigner, Ibrahim & Murray 2020). Neutrophils are important in innate immunity (Mayadas, Cullere & Lowell 2014). They originate from the bone marrow, and their maturation is stimulated by cytokine granulocyte colony stimulating factor (Weisbart et al. 1987). Neutrophils act as the first line of defense for the body (Weisbart et al. 1987). When stimulated, they migrate into the tissue through a process known as diapedesis (Weisbart et al. 1987). Once activated in the connective tissue, they have a lifespan of a few days, and then they undergo apoptosis and are removed by macrophages (Weisbart et al. 1987). Neutrophils express receptors for products from the activation of the complement cascade, as well as antibodies that coat microbes (Abbas, Abul K, Lichtman & Pillai 2019). These receptors and antibodies aid in the ability of neutrophils to phagocytose microbes. Neutrophils are present in nearly all CRS cases, with higher levels observed in CF patients (Derycke et al. 2012). Despite this, the pathophysiology of neutrophils in CRS remains unclear. Neutrophilic infiltratations vary depending on the ethnic group and the absence of polyps. In Caucasians, neutrophilic infiltrate is demonstrated in CRS, with CRSsNP having slightly less infiltrate compared to CRSwNP. CRSsNP's infiltrate appears to be more distinctly neutrophilic in nature, whereas CRSwNP appears more eosinophilic in nature based on the degree of inflammation (Derycke et al. 2012). There is a subgroup of CRSwNP patients who are non-eosinophilic. It is thought that for this patient subtype, neutrophils may play a prominent pathogenic role. This can similarly be seen in other airways diseases such as 'neutrophilic asthma' (Van Drunen et al. 2009). In the Chinese population, there is both an eosinophilic and neutrophilic subgroup, however the rate of eosinophilic polyps are low compared to the Caucasian population (Zhang, N et al. 2006). In the Asian CRSsNP population, non-type 2 inflammation is more prominent,

with neutrophils being the predominant marker associated with disease severity (Kim, DW et al. 2019).

Neutrophils are prominently associated with non-type 2 inflammation; however, they can co-exist with cytokines that predominate in type 2 inflammation. It is postulated that this neutrophilia may represent a physiological response against microbiota in patients with CRS (Derycke et al. 2012; Kim, DW et al. 2019; Kim, DW et al. 2016; Wang, H et al. 2018). This theory may explain why some subgroups of patients have corticosteroid resistance (Pothoven et al. 2017). The contribution of neutrophils to the development of CRS is unclear, however these cells can undergo degranulation, causing tissue disruption and a loss of barrier integrity. Conversely to this, N2 neutrophils release oncostatin M (OSM), which aids in homeostasis and epithelial repair (Morse et al. 2019; Pothoven et al. 2017; Wang, H et al. 2018). Whilst OSM repairs the epithelial lining, excess amounts or decreased follow up signaling has been associated with defective epithelial regeneration, perpetuating CRS (Kim, DW et al. 2016; Pothoven et al. 2017; Wu et al. 2018).

4.1.1.4. Dendritic cells

Dendritic cells (DCs) are immune cells that have a crucial role in the initiation and regulation of immune responses. They play a unique role as antigen presenting cell (APC) in both innate and adaptive immunity. Dendritic cells have PRRs on their surface, which can recognize pathogen-associated molecular patterns (PAMPS) and damage-associated molecular pattern molecules (DAMPS) triggered by antigens (Steinman 2012). Dendritic cells then capture and engulf antigens, degrading them into particles. As a result, major histocompatibility complex (MHC) I and II are upregulated as the dendritic cell matures (Rodríguez-Fernández, Riol-

Blanco & Delgado-Martin 2010). The dendritic cell then presents the antigen particles to naïve T cells, stimulating their activation and proliferation (Rodríguez-Fernández, Riol-Blanco & Delgado-Martin 2010). Dendritic cells are present in high concentrations in lymphoid tissues such as lymph nodes, spleen and thymus, but are also present in lower concentrations in non-lymphoid organs and peripheral blood (Eckert & Schmid 1989; Hackstein et al. 2005). They are characterized by the presence of integrins CD11c, CD24 and CD103 (Schlitzer & Ginhoux 2014). Subclassification of dendritic cells can occur based on function and phenotype, such as plasmocytoid dendritic cells (pDC), and two types of myeloid dendritic cells, mDC1 and mDC2. Antigens can stimulate pDCs, such as influenza and herpes simplex virus through TLR7 and TLR9. pDCs can also be stimulated by microbial DNA and bacteria (McKenna, Beignon & Bhardwaj 2005). Once stimulated, pDCs release a range of cytokines and chemokines including TNF-α, IL-1, IL-6 and INF-α. These cytokines stimulate the activation of Th1 and NK cells, as well as B cells to produce antibodies, thus having a role in both innate and adaptive immune responses (Jego et al. 2003; Megjugorac et al. 2004). A high level of pDCs are found in the nasal mucosa, with higher numbers being found in patients who recently had an upper respiratory tract infection (Hartmann et al. 2006). In CRSwNP patients, there was a higher number of pDC cells in polypoid tissue compared to healthy controls' mucosa (Pezato et al. 2014). Patients with allergies however had lower levels of pDCs in their tissue compared to a healthy control (Hartmann et al. 2006). This was also observed in CRSwNP with allergies also having lower pDC numbers in their tissue compared to CRSwNP alone (Pezato et al. 2014).

Myeloid dendritic cells mDC1 and mDC2 express TLR1, TLR2, TLR4, TLR5 andTLR6 (Kadowaki et al. 2001). Furthermore, mDCs are also responsive to bacterial antigens such as *S. aureus* (Kadowaki et al. 2001). mDC1 express c-type leptin BDCA-1 whilst mDC2 express

BDCA-3, allowing the two to be distinguished from each other (Kadowaki et al. 2001). mDC1 produce cytokines such as IL-10, IL-12 and IL-23, stimulating pDC, cytotoxic T cells and Th1 cells, thus being involved in both the innate and adaptive immune response (Nizzoli et al. 2013; Segura et al. 2012). Whilst mDC2 are associated with a Th2 type immunity, producing mainly interferons and antiviral cytokines (Hayashi et al. 2013; Zhang, S et al. 2013). In CRSwNP, it was shown that patients had a decrease in Th1/Th2 ratio, which correlated with a lower mDC1/pDC ratio (Kirsche et al. 2010). Contrary to this, Pezato et al., (2014) found increased pDCs and mDCs in nasal polyp tissue, however in more severe cases (polyps with asthma), the number of pDCs were reduced. Furthermore, Poposki et al., (2015) found elevated levels of mDCs in CRSwNP and suggest that they may contribute indirectly to inflammation observed in CRSwNP.

4.1.1.5 Mast cells

The development of mast cells begins with the differentiation of hematopoietic stem cells into common myeloid progenitors in the bone marrow. The earliest identifiable mast cell precursors are found in the bone marrow, which express high levels of c-kit stem cell factor CD117 and are CD34 positive (Dahlin & Hallgren 2015; Willheim et al. 1995). They then migrate through the circulatory system via transendothelial migration. Once they migrate to the tissue in which they reside, they are fully matured (Gurish & Austen 2012; Okayama & Kawakami 2006). Mast cells are predominantly found on mucosal surfaces that interact with the environment, such as the sinonasal mucosa and gastrointestinal tract (Gurish & Austen 2012). The surface of mast cells expresses a range of surface receptors such as Fc receptors and TLRs, which are involved in adaptive and innate immunity respectively.

Mast cells have cell surface receptors including IgE receptors. These allow them to recognize and respond to specific antigens. When the IgE receptor binds to a B cell derived IgE, it triggers a signaling cascade leading to degranulation and release of potent mediators present in the cell's cytoplasmic granules (Siraganian 2003). These mediators include cytokines, histamine, amines, growth factors and proteoglycans. This cascade of reactions mediated by mast cells recognition of antigens, identify them as a key effector cell and important in allergy IgE-mediated hypersensitivity (Williams & Galli 2000). An important IgE-mediated airway disease is asthma. Asthma has associated increased mast cell activation. Epithelial brushings of asthmatic patients have shown increased gene expression for mast cell released proteases (Dougherty et al. 2010). When the bronchoalveolar lavage fluid of asthmatic patients were compared to non-asthmatics, they showed a greater level of tryptase and prostaglandin D2 which are released by mast cells (Fajt et al. 2013). Lung tissue of asthmatic patients also showed increased levels of eosinophils with ultrastructural features of degranulation (Djukanovic et al. 1992).

Mast cells have a longer life span in tissue compared to other immune cells; their ability to replenish their granules that they release allow them to be stimulated repeatedly (Fawcett 1955). Tissue based mast cells can be recruited to inflammation sites. They also have the function of maintaining epithelial barriers, by controlling permeability, cell turnover and progenitor recruitment (Groschwitz et al. 2013). In the gastrointestinal tract, mast cells can be immunosuppressive through cytokines such as TNF-α, which has effects mediated through Treg cells (Ullrich, Nghiem & Khaskina 2007). In CRS patients, mast cell degranulation is elevated in patients with CRSwNP when compared to ARS (allergic rhinitis) inferior turbinate samples, as well as healthy inferior turbinate samples (Drake-Lee & Price 1997; Loesel 2001). This is further supported by the finding that tissue fragments of CRSwNP patients compared

to controls showed upregulated mast cell mediators when stimulated with anti-IgE (Patou et al. 2009). Whilst mast cell degranulation has been associated with CRSwNP, the absolute number of mast cells when compared to healthy patients remains unaffected by CRSwNP (Loesel 2001). Further research is needed in order to characterize the masts cells' involvement within CRSwNP and whether they have an inflammatory or immunosuppressive effect.

4.1.1.6. Innate lymphoid cells

Innate lymphoid cells (ILCs) are a subset of lymphocytes that are antigen receptor negative. They include natural killer (NK) cells, ILC1, ILC2 and ILC3 (Spits et al. 2013; Vivier et al. 2018). ILCs are involved in the production of INFγ, IL-5, IL-13, IL-17A and IL-22 when stimulated (Lim et al. 2017). These cytokines are rapidly produced upon stimulation, making the ILCs a key component of the initial defense of the airway epithelial barrier. ILC2 is among the most studied of the ILC subgroups, with their development being controlled by GATA-3 transcription factor, and once developed, they produce IL-5 and IL-13 (Krabbendam et al. 2018). ILCs can also exhibit plasticity in response to local factors; ILCs can adapt their characteristics to that of other ILCs subtypes (Krabbendam et al. 2018). In response to different chemokines and cytokines, ILC2s can shift their functional characteristics to that of ILC1s to produce INFγ or that of ILC3 to produce IL-17. This plasticity process is complex, with multiple signaling triggers and pathways unknown, and therefore can become easily dysregulated (Krabbendam et al. 2018). The origin of ILCs has been described as having both pan potential ILCs, as well as subset restricted potential ILCs (Lim et al. 2017). The process in which these precursor ILCs give rise to differentiated ILCs remains unclear. Environmental factors have been shown to epigenetically alter ILC's differentiating pathways and therefore alter plasticity (Lim et al. 2017). Despite this plasticity, each ILC group seems to have distinct

cytokine profiles and physiological roles within Type 1, 2 and 3 inflammation (Ebbo et al. 2017).

ILC1 cells stimulate a Type 1 response by producing IFNγ in response to viruses and intracellular bacteria, and activate Th1 cells. Whilst ILC 2 cells foster a Type 2 response through cytokines IL-4, IL-5 and IL-13, in response to parasites and allergies. ILC3 cells are stimulated in response to extracellular organisms and produce a Type 3 response through Th17 cells by producing IL-17 and IL-22 (Kortekaas Krohn et al. 2018; Shikhagaie et al. 2017). In CRS, ILC2 are the most studied due to their association with CRSwNP through their role in Type 2 inflammation. ILC2 have a role in the production of Type 2 cytokines which stimulate inflammation. In CRS, the identified cytokines which stimulate ILC2 cells are IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) (Dogan, Sahin & Yenisey 2019; Golebski et al. 2016; Lam, M et al. 2015; Nagarkar et al. 2013; Nagata et al. 2019; Ouyang et al. 2013; Wang, WW et al. 2018). Whilst Type 1 and Type 3 inflammation, as well as cytokines are present in subsets of CRS patients, the role and contribution of ILC1 and ILC3 is unclear. Further research within ILCs is needed to give a more comprehensive knowledge of their role in CRS.

4.2 Adaptive Immune System

Adaptive immunity is a type of immune response that is specific to a particular pathogen, and it is activated when the body encounters a foreign substance such as a virus or bacteria. This type of immune response involves the recognition of a foreign antigen by immune cells, which then trigger a series of complex reactions, leading to the destruction or neutralization of the invading pathogen. The adaptive immune response has two major components, the humoral response which involves the production of antibodies by B cells, and the cell-mediated immune

response, which is T cell mediated. One of the key features of adaptive immunity is its ability to remember and recognize specific pathogens, which provides long-lasting immunity against future infections by the same pathogen.

4.1.1 Cells of the Adaptive Immune System

4.2.1.1 B cells

B cells are an essential component of the adaptive immune system, responsible for producing antibodies against a wide variety of pathogens. The primary function of B cells is to recognize and bind to antigens on the surface of pathogens or other foreign substances. B cells are formed in the bone marrow from hematopoietic stem cells. The process of B cell development is a complex and regulated process that involves several stages that ensure the production of functional B cells that can recognize and respond to foreign antigens while avoiding self-reactivity (Abbas, Abul K, Lichtman & Pillai 2014). These include pro-B cells, pre-B cells, immature B cells, and mature B cells (Goodnow et al. 2010). Pro-B cells are the earliest stage of B cell development. These cells express the genes that are necessary for B cell development and differentiation. Pro-B cells then differentiate to pre-B cells, which begin to express the B cell receptor (BCR) on their surface (Abbas, Abul K. et al. 2021). The B cell receptor is a protein that is unique to each B cell and helps the B cell recognize specific antigens (Goodnow et al. 2010). The next stage of B cell development is the immature B cell stage. In this stage, the B cell then undergoes a process of negative selection, which eliminates B cells that would react with self-antigens (Janeway et al. 2002). This is important because if B cells were to react to self-antigens, they could cause autoimmune diseases (Janeway et al. 2002). Only B cells that pass the negative selection process continue to mature (Murphy, K & Weaver 2016). Finally,

mature B cells leave the bone marrow and enter the bloodstream, where they circulate throughout the body in search of antigens (Paul 2012).

Upon antigen binding, B cells differentiate into antibody secreting plasmablasts, plasma cells or memory B cells (Ettinger et al. 2005; Tarlinton 2006). The process of B cell activation is complex and involves multiple signaling pathways and checkpoints. The initial activation of B cells occurs in secondary lymphoid organs, such as lymph nodes and spleen, where B cells encounter antigens presented by APCs such as dendritic cells. B cells can also be activated by soluble antigens, such as viral particles or bacterial toxins, which can bind directly to the BCR on the surface of the B cell. The activation of B cells requires the cooperation of several signaling pathways, including the BCR signaling pathway, the CD40-CD40L pathways, and the Toll-like receptor (TLR) pathway. These pathways activate various transcription factors such as NF-Kβ and IRF4, which regulate the expression of genes involved in B cell proliferation, differentiation, and antibody production. B cells can differentiate into different subsets of plasma cells depending on the nature of the antigen and the cytokine environment. For example, B cells can differentiate into short-lived plasma cells that produce low-affinity antibodies in response to acute infections, or into long lived plasma cells that produce high-affinity antibodies and provide long-term immunity against recurrent infections.

In addition to their role in antibody production, B cells have been shown to play a crucial role in regulating the immune system. Regulatory B cells (Bregs) are a subset of B cells that can suppress the activity of other immune cells, including T cells and dendritic cells. Bregs can secrete anti-inflammatory cytokines, such as interleukin-10 (IL-10) and transforming growth factor-beta (TGF-β), which can inhibit the production of pro-inflammatory cytokines and promote immune tolerance. Recent studies have shown that Bregs play a critical role in the

prevention of autoimmune diseases and the maintenance of immune homeostasis. B cells and immunoglobulin deficiencies have been associated with CRS, particularly CRSsNP (Tan et al. 2018). It is suggested that immunoglobulin deficiency can result in recurrent acute sinusitis, which can lead to CRSsNP (Keswani et al. 2017). Immunoglobulin deficiencies however are not present in the majority of CRSsNP patients (Keswani et al. 2017). Conversely, in CRSwNP patients, there is B cell activation and excess antibody production (Hulse et al. 2013; Ickrath et al. 2018; Tsybikov et al. 2015; Wang, M et al. 2019). The polyps from CRSwNP patients have higher levels of B cells and plasma cells, as well as immunoglobulins IgA, IgG, IgM and IgE (Feldman et al. 2017; Gevaert, P et al. 2005; Ickrath et al. 2018; Lau et al. 2017; Tsybikov et al. 2015; Wang, M et al. 2019; Xiao et al. 2016). B-cell activating factor (BAFF) is a cytokine that stimulates B cell antibody production, and is elevated in polyp tissue in CRSwNP patients (Kato et al. 2008). BAFF is also associated with IgE class switching to IgA, and autoantibody production. IgA autoantibody levels are elevated in nasal polyp when compared to controls in CRSwNP patients (Tan et al. 2017). Further research within B cells is needed to give a more comprehensive knowledge of their role in CRS.

4.2.1.2. T cells

T cells have an important role in immunity, and having multiple functions against intracellular and extracellular infections (Abbas, Abul K, Lichtman & Pillai 2019). T cells have a unique T cell receptor that they express on their surface (Germain 2002; von Boehmer, Teh & Kisielow 1989). This is a result of a positive selection process that occurs within the thymus (Germain 2002; von Boehmer, Teh & Kisielow 1989). T cells start out as naïve T cells and undergo a differentiation process into various subtypes. They are activated by APCs through the recognition of specific antigens presented on the APC surface by the TCR. Once they encounter

the antigen, they then display them on their surface via the MHC protein (Townsend & Bodmer 1989). This activation leads to T cell proliferation known as clonal expansion, and subsequent migration to sites where the antigen is present. Activated T cells differentiate into various effector and memory T cell subsets with distinct functions and cytokine profiles. These include CD 4+, CD8+, cytotoxic T cells and helper T cells (Daniels et al. 2006). Once the effector T cells eliminate the antigen, most will undergo apoptosis, however memory T cells will survive and lay dormant in tissue, such as the sinus mucosa, until activated again in response to the same antigen (Sallusto et al. 1999; Sathaliyawala et al. 2013).

4.2.1.3. Th1 and Th2 cells

Th1 and Th2 cells differentiate from CD4+ effector cells (Mosmann, TR et al. 1986). One of their major roles is in signaling other immune cells such as CD8+ killer cells (Abbas, Abul K, Lichtman & Pillai 2019; Sallusto 2016). This is achieved with the interaction between TCR on CD4+ cells and the MHC II on APC (Tao et al. 1997). Then multiple transcription factors are activated, which results in their differentiation into Th1 cells, such as T-box transcription factor (T-bet) (Afkarian et al. 2002; Oestreich, Huang & Weinmann 2011). T-bet promotes the differentiation of Th1 cells, and also has an inhibitory effect on Th17 cells which have opposing roles (Lazarevic et al. 2011). Th1 cells produce IFN-γ, TNF-α and TNF-β, which is involved in the elimination of pathogens (Commins, Borish & Steinke 2010; Sallusto 2016). Th1 cells then recruit phagocytes, including macrophages to the site of inflammation and clear the pathogens (Commins, Borish & Steinke 2010; Melzer et al. 2008; Murray, HW et al. 1985). These cytokines also lead to upregulated antigen presentation, as well as B cell class switching to produce IgG subclasses and neutrophil activation (Commins, Borish & Steinke 2010; Sallusto 2016). Th1 cells additionally produce IL-1, which in combination with TNF-α is

involved in the development of Treg and CD8+ T cells, which are both part of the adaptive immune response (Hernandez-Pando & Rook 1994; Hwang, Hong & Glimcher 2005; Malek 2003; Mitchell, Ravkov & Williams 2010).

Th2 cells are stimulated in response to parasitic infections, and promote an eosinophilic response through IgE and mast cells, resulting in the elimination of the parasites (Abbas, Abul K, Lichtman & Pillai 2019; O'Shea & Paul 2010). CD4+ cells differentiate into Th2 cells through an IL-4 and IL-2 dependent pathway. The transcription factors involved in this process include STAT6, which is activated by IL-4. STAT6 then upregulates GATA3 (Kaplan et al. 1996; Usui et al. 2003). GATA3 acts as a regulator which enhances Th2 cytokine production, whilst down regulates STAT4 and inhibits Th1 differentiation (Usui et al. 2003). Th2 cells are predominantly involved in an eosinophilic response, which is representative of inflammation and allergic response. Common cytokines involved in this are IL-4, IL-5, IL-9, IL10, IL-13, and IL-15 (Henry, Inclan-Rico & Siracusa 2017). Type 2 cytokines stimulate the alternate activation of macrophages, while acting to shut down the classical pathway of inflammation. This shuts down a prolonged inflammatory response, reducing the amount of damage caused. The M2 macrophages produce growth factors which promote fibroblasts, resulting in fibrosis and collagen synthesis, and promote tissue repair. In CRS, this protective function goes awry, resulting in an prolonged and excessive response (Tan, Min & Hulse 2017). Elevated numbers of Th subsets have been associated with CRS (Derycke et al. 2014). In particular, Th2 cells with eosinophils elevation is prominent in Caucasian CRSwNP, whilst Th1 with neutrophilia as more prominent in the Asian population and CF patients (Derycke et al. 2014; Tan et al. 2017; Wang, Xiangdong et al. 2016). It is suggested however that Type 2 inflammation is emerging in Asia (Derycke et al. 2014). This observation occurs more frequently in Asian populations that have adopted a Western lifestyle, however the underlaying pathogenesis of

this is poorly understood. Further studies into this population may yield environmental factors which contribute towards CRS.

Th1 and Th2 cells involvement in CRS were initially described by Hamilos et al., (1995), who showed an increase in cytokines production in both allergic and non-allergic CRS patients. It was shown that tissue eosinophilia was prominent in both allergic and non-allergic subgroups, with higher levels of granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-3, IL-4 and IL-5 in tissues of allergic subgroups of patients, and higher tissue densities of GM-CSF, IL-3 and INFγ (Hamilos et al. 1995). The allergic/Th2 subgroup was associated with atopy (Hamilos et al. 1995). A follow up study by Gevaert et al., (Gevaert, P et al. 2009) showed an IL-5 upregulation was independent of atopy status. A proposed basis for this difference is ethnic origins. In a Caucasian population, INFγ is associated with CRSsNP patients with a Th1 endotype, whereas IL-5 is elevated in patients with CRSwNP and a Th2 endotype (Van Zele et al. 2006). Further studies have shown that for CRSwNP, the Asian population has a predominance of Th1/17 endotypes, whereas the Caucasian population has a more predominant Th2 endotype (Zhang, N et al. 2008). Both ethnic populations however have a Th1 predominance in the CRSsNP population (Fokkens, WJ et al. 1990; Fokkens, WJ et al. 2012). Multiple Th subsets can be activated in a single CRS patient, as stimulation of the involved cytokines may suppress or augment different subsets. This may be a normal immune-response, or it could affect CRS development, clinical presentation, or treatment course. Further research is needed to understand their contribution to CRS (Stevens et al. 2019; Wang, M et al. 2019).

4.2.1.4. Th17 cells

Th1 and Th2 cells were the first CD4+ effector cells to be discovered; more recently however, Th17 cells have been discovered and shown to be different from Th1 and Th2 cells due to their production of IL-17 (Harrington et al. 2005). Th17 cells play a part in the adaptive immune system via the induction of inflammation and can therefore serve a protective or a pathogenic role. Inflammation is an important part of the adaptive immune response, however, sustained inflammation can result in tissue damage and a disruption of homeostasis in the mucosal barrier. CC motif 6 chemokine receptor protein (CCR6+) is a chemokine receptor which distinguishes Th17 cells (Brucklacher-Waldert et al. 2009). Th17 cells cause inflammation through the upregulation of pro-inflammatory chemokines and cytokines (Fossiez et al. 1996). The predominant cytokines produced by Th17 are IL-17A, IL-17-F, IL-21 and IL-22. IL-17A and IL-17F causes inflammation by promoting pro inflammatory chemokines and cytokines (Fossiez et al. 1996). IL-7A's role is thought to be a protective one, whereas IL-7F is thought to be more destructive. IL-17A has been shown to upregulate the release of chemokines that signal the recruitment of fungicidal peptides and neutrophils (Laan et al. 1999; Puel et al. 2012). An *in vivo* inflammatory bowel disease model showed IL-17A modulates T cell mucosal inflammation through the inhibition of Th1 cell differentiation, as well as reinforcing tight junctions by inducing mucin and claudin expression (Gevaert, P et al. 2009; Kinugasa et al. 2000).

The cytokine IL-21 acts as a pro-inflammatory cytokine and results in the stimulation of INFγ, IL-6 and TNF-α (De Nitto et al. 2010; Neurath 2014). In mice, IL-21 was found to be pro-inflammatory by stimulating follicular T helper cells, thus being pathogenic in an ulcerative colitis model (Yu et al. 2015). Conversely, IL-21 knockout mice are largely protected against colitis (Yu et al. 2015). In active SLE patients, there is a 4 fold higher IL-21 mRNA expression

in CD4+ T cells, which could contribute to further generation of plasma cells (Nakou et al. 2013). In Crohn's disease patients, IL-21 is pro-inflammatory through an INFγ -independent mechanism, resulting in ongoing Th1 and Th17 mucosal immune response (Monteleone et al. 2005). IL-22 in contrast, has a protective function; it does this by preventing tissue damage and promoting tissue repair through the induction of proliferative pathways, antimicrobial peptides and anti-apoptotic pathways (Kim, K et al. 2014; Li et al. 2014; Wang, Xin et al. 2014).

Th17 cell responses are associated with a number of auto-immune inflammatory diseases including SLE (Ye, L et al. 2015; Zhao, Y et al. 2010). In CRS, IL-17 is increased in the Chinese population; this however has not been found in the broader population (Cao et al. 2009; Van Bruaene et al. 2008; Wei, P et al. 2014). This suggests that IL-17 and therefore potentially Th17 may have a possible role in CRS in the Chinese population (Cao et al. 2009; Wei, P et al. 2014). Th17 cells have been found to be increased in CRSwNP patients, suggesting they may have an association with polyp development (Miljkovic et al. 2016). Further investigation needs to be conducted in order to characterize the association between Th17 cells and their immunological actions within CRS.

4.2.1.5. Treg

Treg cells are a T cell subset which have a role in modulating the immune response (Lefrançois et al. 2016; Macri et al. 2014; Pant & Macardle 2014; Sakaguchi et al. 2013; Tan et al. 2011; Tsybikov et al. 2015). Treg cells are characterized by the expression of FOXP3 and the surface markers CD25 and CTLA-4 (Sakaguchi et al. 2008; Wing et al. 2008). Differentiation of Treg cells can occur through differences in development. Tregs that develop in peripheral lymphoid

organs that underwent antigen priming are known as inducible Tregs, whilst Tregs released from the thymus are known as tTregs. Treg cells are CD4+.

Treg cells with high expression of CD4, CD25 and CD127 surface markers have a suppressive function on T cell effector responses (Hartigan-O'Connor et al. 2007). They exert this effect through the production of TGF-β and IL-10 cytokines, as well as direct cell contact. TGF-β and IL10 downregulate immune responses and increase self-tolerance (Duchmann & Zeitz 2006; Fowler & Powrie 1999). Treg cells have a function in autoimmune disease such as ulcerative colitis. They have also been observed to have a role in CRS, however there is inconsistency in the literature as to their exact role (Duchmann & Zeitz 2006; Hawrylowicz & O'garra 2005). One study, which defines Treg cells as CD4+CD25+FoxP3+, observed an increase in the number of Treg cells in the mucosa of CRS patients (Sharma et al. 2012). Whilst two other studies, which defined Treg cells as only FoxP3+, observed a decrease FoxP3+ mRNA in CRS patients (Cao et al. 2009; Van Bruaene et al. 2008). Further research is required to understand the role Treg cells have in CRS.

5.Treatment for CRS

The treatment of CRS involves a combination of appropriate medical therapy and surgical management. Since CRS is primarily an inflammatory disorder, topically administered intranasal corticosteroids (INCS) are regarded as the most-effective treatment along with saline nasal irrigation (Fokkens, WJ et al. 2020). In addition to the standard methods of application, such as nasal sprays or drops, the off-label use of glucocorticoid irrigation has shown to have a comparable safety and side effect profile. A recent review even suggests that it may be more

effective than sprays, particularly in a post-operative setting due to increased drug delivery to the sinuses (Grayson & Harvey 2019). In addition to local therapies, antibiotics are often prescribed in cases of CRS, especially during acute exacerbations. However, it is crucial that antibiotics are prescribed after an appropriate swab for culture has been taken to enable culture-based prescribing (Nagi & Desrosiers 2005). In recent years, with an increase in our understanding of the pathophysiology of CRS and high level of evidence studies assessing commonly prescribed medical treatments, there has been a shift in terminology from "maximal medical therapy" to "appropriate medical therapy" before considering surgical intervention (Fokkens, WJ et al. 2020).

5.1. Corticosteroids

Given that CRS is associated with persistent inflammation, corticosteroids play a crucial role in the medical treatment pathway before considering surgical options. Corticosteroids can be administered either topically, orally, or in combination. Oral corticosteroids have demonstrated effectiveness in treating CRSwNP, with several placebo-controlled studies showing significant improvement in symptoms and endoscopic findings (Alobid, Benitez, et al. 2006; Benítez et al. 2006; Vaidyanathan et al. 2011; Vaidyanathan et al. 2010; Van Zele et al. 2010). However, there is a lack of substantial evidence for their efficacy in cases of CRSsNP (Howard & Lal 2013; Poetker et al. 2013). Oral corticosteroids are typically prescribed for short courses, whist on oral corticosteroids patients report significant benefit, however the systemic side effect profile limits prolonged usage (Fokkens, WJ et al. 2012; Van Zele et al. 2010).

5.2. Topical corticosteroids

Topical corticosteroids have demonstrated significant benefits in the treatment of CRS (Jorissen & Bachert 2009). They are often used in combination with or following oral systemic corticosteroids to effectively manage inflammation (Alobid, Benitez, et al. 2006; Benítez et al. 2006; Vaidyanathan et al. 2011). Numerous studies have shown that topical corticosteroids are effective in improving symptoms (Giger et al. 2003; Lund et al. 2004; Parikh et al. 2001) and endoscopic examination findings (Jorissen & Bachert 2009; Woodworth et al. 2004). A meta-analysis from the 2012 EPOS review revealed that topical corticosteroids led to a decrease in the recurrence rate of CRS with polyps, improved nasal airflow, and reduced polyp size (Fokkens, WJ et al. 2012). Studies have shown lower surgical revision rates with corticosteroid sprays, with further improvement in endoscopic scoring observed when corticosteroids were delivered using nasal irrigation (Harvey, RJ et al. 2018). Studies comparing the delivery method between sprays, aerosols, tubihaler, drops and irrigation, favoured nasal irrigation and drops over sprays (Demirel et al. 2008; Harvey, RJ et al. 2018; JOHANSEN et al. 1993; Tos et al. 1998). In post endoscopic sinus surgery (ESS) patients, high volume nasal irrigation achieves significantly better penetration compared to other delivery methods (Harvey, RJ et al. 2008). Nasal corticosteroid irrigation, particularly budesonide, is the most popular choice of topical corticosteroid delivery in post operative patients, showing long term symptom and endoscopic improvements, particularly in patients with tissue eosinophilia (Chen, X et al. 2016; Harvey, RJ et al. 2018; Kang et al. 2017; Snidvongs et al. 2012; Tait et al. 2018).Furthermore, in the EPOS 2020 review, it was suggested that while corticosteroid treatment results in significant improvement in symptom and nasal polyp scores whilst patients are using the medication, the effects likely only last for up to 3 months after treatment cessation (Fokkens, WJ et al. 2020).

The anti-inflammatory effects of topical corticosteroids are thought to be mediated through various mechanisms:

1. Reduction of eosinophils and other inflammatory cells by inhibiting leukocyte adhesion and chemotaxis (Hamilos et al. 1999; Tingsgaard et al. 1999).
2. Enhancement of apoptosis, leading to a decrease in eosinophil viability (Meagher et al. 1996; Mullol et al. 1997; Saunders et al. 1999).
3. Reduction in eosinophil products (Jahnsen et al. 1999; Woodworth et al. 2004)
4. Decrease in pro- inflammatory cytokines, particularly Th2 cytokines (Hamilos et al. 1999; Rudack, Bachert & Stoll 1999; Woodworth et al. 2004)

Additionally, a study by Goggin et al (2014) found that steroids inhibited the growth of *S. aureus* biofilms, potentially due to their inflammatory modulation on the nasal mucosa.

Despite the significant effects of corticosteroids, there remains some uncertainty regarding their long-term effects on CRS. Particularly when ceased.

5.3. Antibiotics

Bacteria are acknowledged to play a possible role in the pathogenesis of CRS. CRS accounts for the fifth most common reason for antibiotic prescriptions (McCaig & Hughes 1995). Current recommendations suggest treating acute infections on top of CRS, as indicated by the presence of mucopus. In cases where mucopurulence is present on examination, a swab should be taken for culture, and the antibiotic regimen should be tailored to the bacterial susceptibility (Fokkens, WJ et al. 2020). However, due to the limited quantity of evidence, it remains

uncertain whether short-term antibiotics have a substantial impact compared to a placebo on long term disease control (Fokkens, WJ et al. 2020; Fokkens, WJ et al. 2012; Soler et al. 2013).

Currently, there is limited data regarding the long-term use of antibiotics in CRS (Soler et al. 2013). Based on current evidence, long-term antibiotic therapy, specifically prolonged macrolide treatment, in addition to standard treatment, may be considered in cases of refractory CRS. This approach has shown effectiveness in improving symptoms, as well as endoscopic and rhinometric parameters, when used for over 12 weeks (Harvey, RJ, Wallwork & Lund 2009; Wallwork et al. 2006). Ragab et al. (2004) also found that long-term erythromycin, when combined with intranasal corticosteroids and alkaline douches, led to improvements in clinical and endoscopic parameters. A meta-analysis by Shen et al. (2018) showed that Asians, who typically have a non-type 2 CRS endotype, that were treated with longterm LDLT macrolides have a better treatment effect compared to non-Asians. A similar effect was also observed by Lin et al. (2020) where CRSwNP patients without tissue eosinophilia showed greater benefit from low dose clarithromycin combined with oral corticosteroids, compared to oral steroids alone over a 12 week period post operatively. Further research into long term antibiotic regimes, especially macrolides, and the associated effects on non-type 2 and type 2 endotypes is required.

Topical antibiotics are generally not recommended as a part of routine treatment for CRS (Soler et al. 2013). They continue to be used however to treat infective exacerbations not responsive to oral antibiotics. If prescribed, topical antimicrobial therapies should be culture directed, with swabs taken with endoscopic guidance preferred compared to swabs taken without endoscopic guidance (Desrosiers & Kilty 2008).

5.4. Anti-Leukotrienes

Anti-leukotrienes, including zafirlukast and montelukast, are often prescribed in cases of aspirin exacerbated respiratory disease (AERD). These medications work by antagonizing the CysLT1 receptor. While anti-leukotrienes are effective in treating asthma, there is limited data supporting their use in chronic rhinosinusitis (CRS), and they may not show a significant difference in efficacy for patients with or without aspirin sensitivity (Smith, TL & Sautter 2014). A review conducted by Smith & Sautter (2014) suggest that montelukast, when used as an adjuvant therapy alongside corticosteroids, may provide some benefit for CRSwNP patients.

5.5. Biologicals (Monoclonal Antibodies)

Monoclonal antibodies represent one of the most recent advancements in CRS treatment. They work by targeting specific cytokines or pathways involved in inflammation and disease progression. However, their current use in CRS is limited due to high costs and accessibility challenges. Dupilumab currently has the highest level of success in CRSwNP, significantly reducing polyp size, Lund-Mackay scores, and severity of symptoms as observed in LIBERTY NP SINUS-24 and LIBERTY NP SINUS-52 phase 3 trials (Bachert et al. 2019). Mepolizumab and reslizumab have shown promise in treating severe polyposis patients by reducing polyp size and eosinophilic cationic protein levels, with the most significant responses observed in patients with elevated IL-5 levels (Gevaert, P et al. 2006; Gevaert, P et al. 2011).

For patients with CRS combined with AERD, elevated IgE levels are commonly associated. Omalizumab, a monoclonal antibody, functions by neutralizing IgE. Research by Gevaert et al. (2013) demonstrated that omalizumab led to a reduction in polyp size and a decrease in the severity of upper respiratory symptoms in individuals with CRSwNP and asthma. However, when used in combination with maximal medical therapy, omalizumab did not provide additional benefits compared to maximal medical therapy alone (Pinto et al. 2010). Currently, biologics are recommended for a limited subset of patients who have bilateral polyps, a history of prior endoscopic sinus surgery, and meet specific criteria, including evidence of Type 2 inflammation, the need for systemic corticosteroids or contraindication to systemic steroids, significantly impaired quality of life, significant loss of smell, and a comorbid diagnosis of asthma (Fokkens, WJ et al. 2020). Recently, in Australia, mepolizumab has been approved for usage in patients with CRSwNP, with severe symptom and endoscopic scores, who have had prior endoscopic sinus surgery. In this subset of patients, mepolizumab has resulted in reduced polyp size, decreased symptom scoring and a reduction in need for further surgery (Fokkens, WJ et al. 2023; Han et al. 2021).

Monoclonal antibodies usage has rapidly increased in the recent years and will continue to increase as regulatory bodies approve it for funding in the treatment of CRS.

5.6. Antifungals

The use of antifungals in the treatment of chronic rhinosinusitis (CRS) gained attention following the proposal of the fungal theory by Ponikau et al., (1999). Subsequently, Ponikau

(2002) suggested that intranasal amphotericin B was effective in treating CRS in a majority of patients (38 out of 51). However, since then, antifungals have undergone extensive research, with multiple randomized controlled trials discouraging their use for CRS treatment (Fokkens, WJ et al. 2020; Sacks IV et al. 2012; Soler et al. 2013). Antifungals are associated with significant side effects and offer minimal benefit in the context of CRS. An exception to this may be AFS. In AFS patients treated with oral itraconazole, multiple retrospective studies have reported improvements in symptoms (Chan, Genoway & Javer 2008; Rains III & Mineck 2003; Seiberling & Wormald 2009). Additionally, Khalil et al. (2011) observed a reduced recurrence of AFS in patients using intranasal fluconazole compared to non-antifungal controls in post-operative patients. However, it is important to note that antifungals come with a high side effect profile, and further research is necessary to determine their appropriate indication in CRS and its subset of AFS patients.

5.7. Saline irrigation

Saline irrigation is a beneficial practice for CRS patients as it helps prevent the accumulation of secretions, enhances mucociliary clearance, reduces obstruction, and improve airway patency (Hauptman & Ryan 2007). The symptomatic improvements in CRS patients are supported by systematic reviews and consensus guidelines (Fokkens, WJ et al. 2020; Harvey, R et al. 2007). There doesn't appears to be a significant difference in treatment effectiveness based on the concentration of saline used; however, hypertonic saline may be associated with nasal burning and irritation (Hauptman & Ryan 2007).

5.8 Surgery

Surgery mainly aims to remove nasal obstruction, improving ventilation and access for postoperative topical therapies. Surgery also facilitates representative tissue sampling within the sinuses to allow for histological analysis and personalized treatment in the post-operative period. However, even after surgical intervention, recurrence rates are high with some studies reporting up to 78.9% in CRSwNP (Calus et al. 2019).

5.9. Treatment Summary

To date, only topical corticosteroids and biologics offer long term benefit in the treatment of CRS. As there is no definitive cure for CRS, pharmacotherapies focus on disease management through inflammation reduction and treatment of infective exacerbations. The most significant challenge in preventing recurrence after endoscopic sinus surgery lies in controlling the inflammation that drives the disease. Therefore, research into medical management strategies is imperative to mitigate disease recurrance.

6.Mesalazine

Mesalazine, also known as mesalamine or 5-aminosalicylic acid (5-ASA), is a medication used to treat inflammatory bowel disease, including ulcerative colitis and Crohn's disease (Böhm & Kruis 2014). Mesalazine was originally created after sulfasalazine was found to be effective in

treating ulcerative colitis (UC) (Svartz 1942). Sulfasalazine has a poor side effect profile, with up to 30% of patients being unable to tolerate it and oligospermia often being a limiting factor in the treatment of male patients. (Nielsen 1982). It was determined by Khan et al (1977) that the 5-aminosalicylate moiety is the active moiety in UC. The 5-ASA was then converted into mesalazine, which lacked the sulphapyridine carrier molecule and the associated side effects. In comparison to sulfasalazine or olsalazine, mesalazine is a relatively safe drug (Loftus Jr, Kane & Bjorkman 2004). Mesalazine is associated with adverse effects in less than 7% of patients with Crohn's disease and UC, which is comparable to placebo (Di Paolo et al. 2001). Side effects include vomiting, nausea, headaches, diarrhea, abdominal pain, and a rash. Rarer adverse effects associated with this drug, include pancreatitis, hepatotoxicity, pneumonitis, and intestinal nephritis (Foster et al. 2003; Ransford & Langman 2002; Stein & Hanauer 2000; Van Staa et al. 2004). Mesalazine can be safely desensitised in patients who have reacted hypersensitively to it (Tolia 1992).

Mesalazine can be administered orally or rectally, with oral formulations demonstrating similar levels of effectiveness. Both oral and topical forms of mesalazine (such as enema, foam, and suppositories) have proven to be effective either as standalone treatments or when combined with oral or rectal administration for managing active ulcerative colitis, as well as for sustaining remission (Marteau et al. 2005). The primary action of mesalazine is believed to be localized at the site of inflammation, particularly within the colon (Iacucci, de Silva & Ghosh 2010). This is partly attributed to the presence of N-acetyltransferases in the epithelial cells of the small bowel and colon, which efficiently metabolize mesalazine into the product acetyl 5-aminosalicylate. It is believed that this metabolite lacks significant anti-inflammatory activity (Allgayer et al. 1989; Ireland, Priddle & Jewell 1990).

Mesalazine is useful in controlling active inflammation and maintaining remission in inflammatory bowel disease (Ye, B & van Langenberg 2015). Recent evidence suggests that the anti-inflammatory properties of 5-ASA may be, in part, attributed to its ability to inhibit the production of key mediators like cyclooxygenase, lipoxygenase, TNF-α, IL-1β, and IL-6, which are notably associated with type 1 inflammation, which does form a subset of patients with CRS (Egan et al. 1999; Kaiser, Yan & Polk 1999; Mahida et al. 1991; Rachmilewitz et al. 1992; Stenson & Lobos 1982). Additionally, it is proposed to operate through mechanisms such as activating peroxisome proliferator-activated receptor-γ (Rousseaux et al. 2005), and acting as an antioxidant and free radical scavenger (Ahnfelt-Rønne et al. 1990; Kruis et al. 2003). Nevertheless, the precise pharmacological mechanisms remain not yet fully elucidated (Böhm & Kruis 2014).

Mesalazine suppositories and enemas are widely recommended as the standard therapy for both induction and maintaining remission in cases of inflammatory bowel disease (Bergman & Parkes 2006). This recommendation is strongly supported by the three largest randomized controlled trials focused on mesalazine in ulcerative colitis. These trials demonstrated that doses equal to or greater than 2g per day were notably more effective in inducing remission in cases of moderately active UC compared to a placebo (Hanauer et al. 1993; Schroeder, Tremaine & Ilstrup 1987; Sninsky et al. 1991). Furthermore, various doses of topical rectal 5-ASA (ranging from 1 to 4.8g/day) have proven effective in treating active distal ulcerative colitis. They also demonstrate clear advantages over placebo in terms of inducing clinical, endoscopic, and histopathological remission (Zhao, X et al. 2017). Additionally, a meta-analysis suggested that the combination of mesalazine and cortisone (5-ASA 1.5 to 2.0g/d +

BDP 3mg/d) had the highest likelihood of being the most effective treatment for inducing clinical and endoscopic remission (Zhao, X et al. 2017).

As mesalazine operates as an aminosalicylate with anti-inflammatory properties, its direct contact with mucosal surfaces prompts the question of whether this pharmacological agent could also provide benefits in the treatment of the mucosal inflammation seen in patient with CRS, particularly those with Type 1 inflammation who are typically steroid resistant.

Given its established safety profile, even in relatively high doses (up to 1000mg) (Dignass et al. 2018), the risk associated with local application on the nasal mucosa and paranasal sinuses is likely to be very low. Mesalazine holds an FDA class B classification for drug safety during pregnancy, and only minimal levels of 5-ASA are detected in breast milk (Biedermann et al. 2012). A study by Bell (1997) examined the safety of mesalazine in 16 pregnant individuals reported no relapses and 19 successful full-term pregnancies without complications. Currently, there is a dearth of literature regarding the safety of topically applied mesalazine on the nasal mucosa, as well as its topical use in patients with chronic rhinosinusitis.

This study endeavors to assess the safety and efficacy of mesalazine as a potential treatment option for CRS, with focus on the type 1 inflammatory endotype.

CHAPTER 2: An *in vitro* study evaluating the safety of mesalazine on human nasoepithelial cells

Statement of Authorship

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Principal Author

Name of Principal Author (Candidate)	William Murphy		
Contribution to the Paper	Primary investigator of the study. This included patient consent, sample collection, carrying out experiment, analyzing results and writing the manuscript.		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	22/1/24

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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An *in vitro* study evaluating the safety of mesalazine on human nasoepithelial cells

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CONFLICT OF INTEREST

The authors declare no conflicts of interest that are relevant to this paper.

AJP is a consultant for Medtronic and Neurent and receives a speaker's honorarium for Sequiris, Storz and Sanofi, GSK and a shareholder for Chitogel.

PJW is a consultant for Neilmed, Stryker, Neurent, receives royalties from Integra and is a shareholder for Chitogel.

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Not Applicable

Abstract

Background:

Chronic rhinosinusitis (CRS) is a disease characterised by inflammation of the nasal and paranasal cavities. It is a widespread condition with considerable morbidity for patients. Current treatment for chronic rhinosinusitis consists of appropriate medical therapy followed by surgery in medically resistant patients. Although oral steroids are effective they are associated with significant morbidity and disease recurrence is common when discontinued. The development of additional steroid sparing therapies is therefore needed. Mesalazine is a commonly used therapeutic in inflammatory bowel disease, which shares a similar disease profile with chronic rhinosinusitis. This exploratory *in vitro* study aims to investigate whether Mesalazine could be repurposed to a nasal wash, which is safe on human nasoepithelial cells, and retains its anti-inflammatory effects.

Methods:

CRS patients' human nasal epithelial cells (HNECs) were collected. HNECs were grown at an air-liquid interface (ALIs) and in a monolayer and challenged with mesalazine or a non medicated control. Transepithelial electrical resistance, paracellular permeability and toxicity were measured to assess epithelial integrity and safety. The anti-inflammatory effects of mesalazine on release of IL-6 and TNF-α were analysed using THP-1 cells.

Results:

Mesalazine did not impact the barrier function of HNEC-ALIs and was not toxic when applied to HNECs or THP-1 cells at concentrations up to 20 mM. Mesalazine at concentrations of 0.5 and 1 mM significantly inhibited TNF- α release by THP-1 cells.

Conclusion:

Mesalazine effectively decreases TNF-α secretion from THP-1 cells, indicating its possibility of anti-inflammatory properties. The safety profile of mesalazine at doses up to 20 mM suggests that it is safe when apply topically on HNECs.

1. Introduction

Chronic rhinosinusitis (CRS) is defined as chronic paranasal sinus mucosal inflammation (Benninger et al. 2003) lasting over 12 weeks with characteristic clinical manifestations. It affects around 10% of western populations, making it an extremely prevalent disease (Habib et al. 2019; Hamilos 2011; Hastan et al. 2011). CRS causes nasal obstruction, discharge, facial pain, headache, and altered or impaired sense of smell and taste (Fokkens, WJ et al. 2020). Patients require long-term monitoring, intensive care, and suffer a substantially diminished quality of life (Rudmik & Smith 2011). Antibiotics, corticosteroids, and saline nasal irrigations are commonly used to release the symptom of CRS. These treatments can be ineffective in some cases. whilst, the emergency of antibiotic resistance can hinder the efficacy of antibiotics in clinical setting (Hsu, Lanza & Kennedy 1998; Young et al. 2012). In such instances, surgical intervention is required (Fokkens, WJ et al. 2020). Surgical procedures may alleviate symptoms and facilitate disease control, although disease recurrence cannot be prevented (Matsuwaki et al. 2008).

Chronic sinonasal inflammation involves the secretion of numerous inflammatory markers and can be associated with the development of nasal polyps (Daines & Orlandi 2010; Phan et al. 2015). Eosinophilic inflammation commonly characterizes the inflammatory response associated with CRS and has been well studied. Recently, the role of macrophages in the

inflammatory process has gained increasing interest. Following an infection, macrophages in peripheral tissue (such as the sinonasal mucosa) function as immune system sentries and produce IL-1β, TNF-a, and IL-6 (Ocaña & Reglero 2012). The secretion of these cytokines is elevated in the context of CRS (Lennard et al. 2000; Xu, R et al. 2007). CRS is also associated with an increase in CD16+ monocytes, which then further differentiate into macrophages (Polasky et al. 2021). This has been supported by the presence macrophage infiltration in CRS (Banks et al. 2014; Shaghayegh et al. 2022). The monocyte-derived macrophages in sinonasal tissue can initiate Th-2 responses associated with CRS (Banks et al. 2014; Ramanathan Jr et al. 2008).

Mesalazine, also known as mesalamine or 5-aminosalicylic acid (5-ASA), is used to treat inflammatory bowel illnesses such as Crohn's disease and ulcerative colitis (Böhm & Kruis 2014). Oral and topical (enema, foam, and suppositories) mesalazine has been shown to be effective for treating active ulcerative colitis and maintaining remission (Marteau et al. 2005). Mesalazine's anti-inflammatory therapeutic efficacy is widely acknowledged in the treating of inflammatory bowel disease. Recent research suggests that the anti-inflammatory effects of 5-ASA are mediated at least in part by a reduction the production of macrophage-derived cytokines such as tumour necrosis factor (TNF)-α, interleukin (IL)-1β, and interleukin (IL)-6 (Kaiser, Yan & Polk 1999; Mahida et al. 1991; Rachmilewitz et al. 1992). Mesalazine, in general, is well tolerated and considered safe for long-term use (Bell & Habal 1997; Dignass et al. 2018). However, its pharmacological processes and mode of action are insufficiently understood (Böhm & Kruis 2014).

Inflammatory bowel disease and certain CRS endotypes share many similar histological features, including neutrophil and plasma cell infiltrates, papillary hyperplasia, basement membrane thickening, oedema, ulcer formation, polypoid formation and some end stage

patients fibrosis and scar formation (Kumar, Abbas & Aster 2013). Although it is known that mesalazine has anti-inflammatory activities and a favourable safety profile in the digestive system, its potential anti-inflammatory effect and safety on the sinonasal mucosa have not yet been studied.

THP-1 cells are a monocytic cell line used to investigate the functions, activities, signalling pathways, nutrition, and drug transport of monocytes and macrophages (Chanput, Mes & Wichers 2014). Therefore, in this study, the safety of various dosages of mesalazine were investigated on *in vitro* cultured human nasal epithelial cells (HNECs), as well as the anti-inflammatory effects on THP-1 cells, which are used to study monocyte/macrophage-related processes.

2. Materials and Methods

2.1 *Mesalazine Preparation*

Mesalazine solution was prepared by dissolving powdered Mesalazine (>99.9% purity) (Sigma Aldrich, Missouri, USA) in sterilised Mili Q water and adjusted to pH to 7 using 1mM sodium hydroxide (NaOH) solution. Mesalazine solution was then diluted into either PneumaCult™-Ex Plus Basal Medium (STEMCELL Technologies, Tullamarine, VIC, Australia), PneumaCult™-Ex Plus 50 x Supplement (STEMCELL Technologies, Tullamarine, VIC, Australia), and penicillin-streptomycin (Thermo Scientific, Walthman, MA, USA) (designated as Ex Plus complete media)] or PneumaCult™-ALI Basal Medium (STEMCELL Technologies, Tullamarine, VIC, Australia); PneumaCult™-ALI 10 x Supplement; penicillin-streptomycin/amphotericin B (Thermo Scientific, Walthman, MA, USA); And PneumaCult™-ALI Maintenance Supplement (STEMCELL, Vancouver, Canada) (ALI complete media)] to

achieve the final concentrations of 0.5 mM, 1 mM, 10 mM, 20 mM and 50 mM. The mesalazine solution was covered with foil paper in all experiments unless specified.

2.2 Human Ethics approval and participant recruitment

Ethics approval for harvesting of nasal brushings was granted by The Central Adelaide Local Health Network Human Research Ethics Committee (reference HREC/15/TQEH/132). Patients undergoing endoscopic sinus surgery (ESS) for chronic rhinosinusitis were recruited to the study. Diagnostic criteria used for CRS were in accordance to American Academy of Otolaryngology and Head and Neck Surgery and the European Position Statement (Fokkens, WJ et al. 2020). All patients provided written informed consent before the study initiation. Samples were de-identified and coded prior to use. The exclusion criteria included: (1) age below 18; (2) pregnancy; (3) active smokers for more than 3 months prior to the recruitment; (4) corticosteroid or antibiotic usage 4-weeks prior to the recruitment; and (5) systemic diseases, including cancer, hepatic and renal failure and other conditions causing immunosuppression or for which immunosuppressant medication was part of their treatment

2.3 Harvesting and Culturing Human Nasal Epithelial Cells (HNECs) in Vitro

Primary HNECs from patients with chronic rhinosinusitis were harvested from nasal polyp mucosa by gentle brushing (Ramezanpour et al. 2016). Extracted cells were suspended in Ex Plus complete media (STEMCELL Technologies, Tullamarine, VIC, Australia). Macrophages were removed by treating the cells with anti-CD68 (Dako, Glostrup, Denmark) coated petri dishes for 20 min at 37℃. Then HNECs were seeded in collagen-I coated T75 cell culture flasks (Corning Incorporated, NY, USA) and grown in Ex plus complete media (STEMCELL

Technologies, Tullamarine, VIC, Australia). The seeded HNECs were incubated at 37 °C with a 95% humidity incubator supplied with 5% CO_2 and inspected daily under light microscopy.

2.4 Air-Liquid Interface (ALI) Culture

Once cells achieved 80-100% confluence, cells were detached by treating with 0.05% trypsin (Thermo Scientific, Waltham, MA, USA) and resuspended in Ex plus complete media after centrifugation. Cell suspensions were then seeded onto collagen IV-coated apical chambers of Transwells (BD Biosciences, San Jose, California, USA). 500 µL Ex plus complete media was added in the basolateral chamber. Cells were cultured at 37 °C with 5% CO_2 and given two to three days to settle. Followed by, the apical chamber medium being removed completely, and the basolateral chamber medium being changed to ALI complete media. The basolateral chamber medium was changed every two to three days. The cells were cultured for 17 to 21 days to allow for differentiation and tight junction formation.

2.5 Measurement of Trans-Epithelial Electrical Resistance (TEER)

The TEER was measured using an EVOM2 epithelial volt-ohm meter (World Precision Instruments, Florida, USA), using ohms per square centimetre (Ω/cm^2). In brief, 100 µL and 500 µL of fresh ALI complete media were applied to the apical and basal chambers respectively. The baseline TEER was measured. Only wells displaying baseline resistance readings greater than 700 Ω/cm^2 were used for the experiments. The ALI complete media from the apical chamber was then removed, followed by adding 100 µL ALI complete media medium containing the final concentration of mesalazine at different concentrations (0.5, 1, 5, 10, 20 and 50 mM). The negative control (ALI complete media) and positive control (10% Triton X-100, Sigma Aldrich, Missouri, USA) were tested alongside. The TEER was measured immediately after the treatment was applied and recorded as time point 0. TEER was then

measured every 15 minutes for the first hour and then in 30-minute intervals for the remaining 6.5 hours. Whilst taking TEER measurements, a heating platform of 37 °C was used. TEER values were normalised against the values obtained at time point 0.

2.6 Measurement of Paracellular Permeability using Fluorescently labelled Dextrans

The paracellular permeability was tested using 4-kDa fluorescein isothiocyanate-labelled (FITC) dextrans (Sigma Aldrich, St Louis, USA). After treating cells with various concentrations of mesalazine for 7 hrs, the media of the basolateral chamber was replaced with fresh medium and the apical chambers medium was removed and cells were washed with PBS. Then, the apical chambers were filled with 3 mg/ml of FITC-Dextran and incubated for two hours at 37 °C. The samples from the basolateral compartment were transferred to a clear bottom black 96-well plate (Corning-Costar Corp., Cambridge, United Kingdom). The fluorescence of the samples was then measured with a FLUOstar Optima 96-well fluorescence microplate reader (BMG Labtech, Ortenberg, Germany) at excitation and emission wavelengths of 485 nm and 520 nm. The experiment was repeated three times with cells from different donors.

2.7 Measuring Cytotoxicity with LDH assay

Following the final TEER measurements, the medium was collected from the basal chambers of each sample and cytotoxicity was determined using the lactate dehydrogenase (LDH) release kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, 50 µL of medium from each condition was transferred to a new plate, 50 µL of LDH reagent was added, and the plate was incubated for 30 minutes at room temperature in the dark. At 490 nm,

absorbance was measured using a microplate reader (BMG Labtech, Ortenberg, Germany). Cells treated with ALI complete media and Triton X-100 served as negative and positive controls, respectively. The relative viability was determined by comparing the LDH levels of negative and positive controls. The experiment was conducted three times.

2.8 Measuring Cytotoxicity with MTT

HNECs and THP1 cells were seeded at $1.2x10^6$ cells/well into 24 well tissue culture plates (Corning Incoperated, Kennebunk, USA) containing 500 µL Ex Plus complete media (Stemcell Technologies) and incubated at 37°C with 5% CO2 until 80% confluence was achieved. Media was then removed and cells were washed with PBS. Cells were then treated with 500 µL Ex Plus complete media (Stemcell Technologies) with the final mesalazine concentration at 0.5, 1 ,5 ,10 ,20 and 50 mM. Cells treated with media and 10% triton were used as negative control and positive control respectively. Sterilised MiliQ water diluted media was used as volume control and 0.25 mg/mL budesonide (0.5 mg/mL, Astrazeneca, Macquarie Park, NSW, Australia) as positive treatment control. 50µg/mL Monosodium urate (MSU, 25mg/mL, Sigma Aldrich, Missouri, USA) was used to stimulate production of TNF-α. Plates were wrapped in foil and incubated at 37°C and 5% CO2 for 24 hours. Cells were then washed with 650 µL of PBS twice. Then 200 µL of media and 50 µL MTT (5mg/ml PBS) was added to each well and then incubated for 4 hours (Mosmann, T 1983). After that, the media and MTT were removed from the wells. Each well was then filled with 400 µL of DMSO and 100 µL of glycine buffer (0.1 M glycine, 0.1 M Nacl adjusted to pH 10.5 with 1 M NaOH). After that, the plates were wrapped in aluminium foil and rocked on a Rocking Platform Mixer (Ratek Instruments, Mitcham, Australia) for 30 minutes. 100 µL was then taken and added in triplicate to new 96-well plates. A FLUOstar optima plate reader (BMG Labtech, Ortenberg, Germany) was used to read the absorbance of the plates at 570 nm.

2.9 Enzyme-linked Immunosorbent Assay (ELISA) IL-6

Samples from the basolateral chambers were collected after 7 hours of mesalazine treatment to determine the Interleukin-6 (IL-6) protein levels using an ELISA assay (BD Biosciences, New Jersey, USA). Briefly, 96-well plates were coated with capture antibody in a 1:250 dilution to a final concentration of 2 µg/ml with coating buffer (0.1 M NaHCO3) overnight at 4°C. The primary antibody was then removed, the plates were washed 2 times and 200 µL of blocking buffer (1x PBS + 2% Bovine serum albumin) was added to each well. 100 µL of samples were then added to wells and incubated at room temperature for 2 hours. After washing, 100 µL of Biotin rat anti-human IL-6 antibody (BD Biosciences, New Jersey, USA) diluted 1:1000 to 0.5 µg/ml in blocking buffer was added into each well. Samples were then incubated at room temperature for 30 minutes. HRP-conjugated streptavidin (Thermo Scientific, Walthman, MA, USA) was diluted 1:10,000 to 125 ng/ml in blocking buffer added to each well and incubated at room temperature for 30 minutes. 100 µL of TMB was then added and the sample was incubated at room temperature for 10 minutes. Stop solution was then added and absorbance was read at 450 nm with a FLUOstar OPTIMA plate reader (BMG Labtech, Ortenberg, Germany). An equal volume of sterilised MiliQ water was diluted into Ex Plus complete media (Stemcell Technologies) and served as volume control and a treatment control of budesonide (0.25mg/mL, Astrazeneca, Nacquarie Park, NSW, Australia) was also used.

2. 10 Enzyme-linked Immunosorbent Assay TNF-α – Cells inflammation model

HNEC and THP-1 cells were seeded into 24 well tissue culture plates as described above. Cells were stimulated with Poly IC (PIC) (10µg/mL in cell culture media), MSU, a volume control and a treatment control was used as described above. TNF-α was then measured using a TNF-α ELISA assay (Invitrogen, Frederick, USA) at 3- and 6-hours post-exposure. Briefly, an ELISA plate was coated with capture antibody (Purified Mouse anti-human TNF-α, BD

Biosciences) and incubated overnight at 4°C. Then plates were blocked with a 100 µL blocking buffer. 100 µL Samples were then added and incubated for 2 hours at room temperature. The plates were then removed and washed 3x times. 100 µL of Biotin mouse anti-human TNF-α (BD Biosciences) was added to each well and incubated for 30 minutes at room temperature. HRP-conjugated streptavidin (Thermo Scientific, Walthman, MA, USA) was added to each well and incubated at room temperature for 30 minutes. 100 µL of TMB was then added after washing and incubated at room temperature for 10 minutes. Stop solution was then added and absorbance was read at 450 nm with a FLUOstar OPTIMA plate reader (BMG Labtech, Ortenberg, Germany).

2.11: Statistical Analysis

Statistical analysis of TEER, FITC dextran, LDH, MTT, IL-6 and TNF-α was assessed with an ANOVA followed by post hoc analysis, using Graph Pad Prism version 9.00 (GraphPad Software, La Jolla, CA, USA). Differences between groups were determined using a one-way analysis of variance (ANOVA). Significance was determined at a p-value < 0.05. Experiments were performed three times. The data is presented using mean ± standard error of the mean.

3. Results

3.1. Mesalazine does not appear to affect the mucosal barrier function of primary human nasal epithelial cell cultures

Mesalazine did not alter the TEER of the HNEC-ALI cultures at all tested concentrations (0.5 mM, 1 mM, 10 mM, 20 mM and 50 mM) at each tested time points (up to 7 hours), compared to the negative control (Figure 1). Furthermore , mesalazine applied for 7 hours, did not appear to have a significant effect on the paracellular permeability of the HNEC-ALI cultures at any of the concentrations tested (Figure 2).

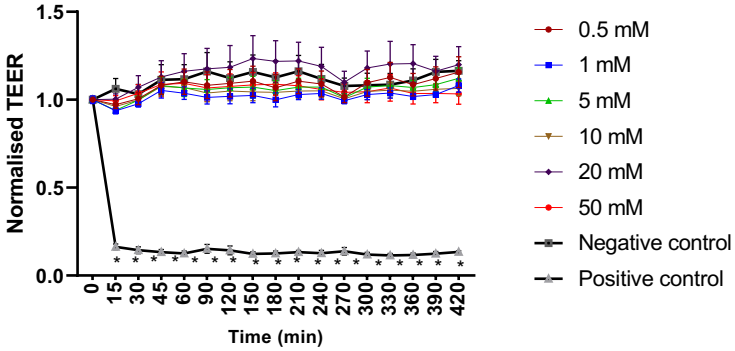


Figure 1. Mesalazine has no significant effect on HNEC-ALI transepithelial electrical resistance. HNEC-ALI TEER measurement after exposure to different concentrations of mesalazine (0.5mM, 1mM, 5mM, 10mM, 20mM and 50mM). ALI complete media and 10% Triton served as a negative and positive control. Statistical significance was determined by comparing each treatment condition to the negative control using one-way ANOVA. The values are shown in mean ± standard deviation (SD) for n = 3. TEER was normalised against the value at time 0. *: $p < 0.05$.

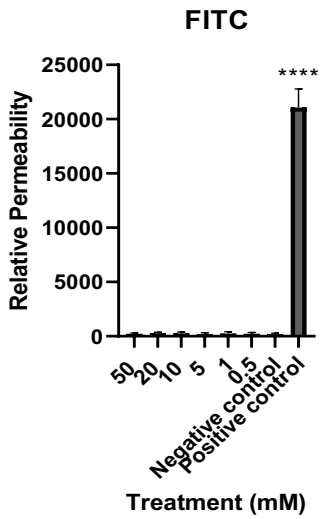


Figure 2. Mesalazine has no significant effect on HNEC-ALI paracellular permeability. The paracellular permeability was measured by the FITC-Dextran Assay at 7 hours, following exposure to different concentrations of mesalazine. ALI complete media served as the negative control and Triton 10% treatment as the positive control. The significance was determined by comparing different treatment groups with the negative control. Experiments were performed in triplicates. ****: $p < 0.0001$.

3.2 Mesalazine does not appear to be cytotoxic to HNECs in concentrations < 50mM

To further evaluate the safety of mesalazine, cell cytotoxicity was measured after 7 hours of mesalazine exposure to HNEC-ALI cultures. None of the tested concentrations of mesalazine induced LDH release compared to the negative control (Figure 3 A). Furthermore, mesalazine at concentrations of up to 20 mM applied for 24 hours to HNECs (Figure 3B) or THP-1 cell cultures (Figure 3C) did not affect cell viability as measured by NAD(P)H-dependent cellular oxidoreductase enzymes activity (MTT assay) compared to any of the tested controls. However, 50 mM mesalazine reduced the cell viability below 80% for both cell types in the

MTT assay ($p<0.0001$) (Figures 3 B and 3 C) this was not seen at any of the lower concentrations.

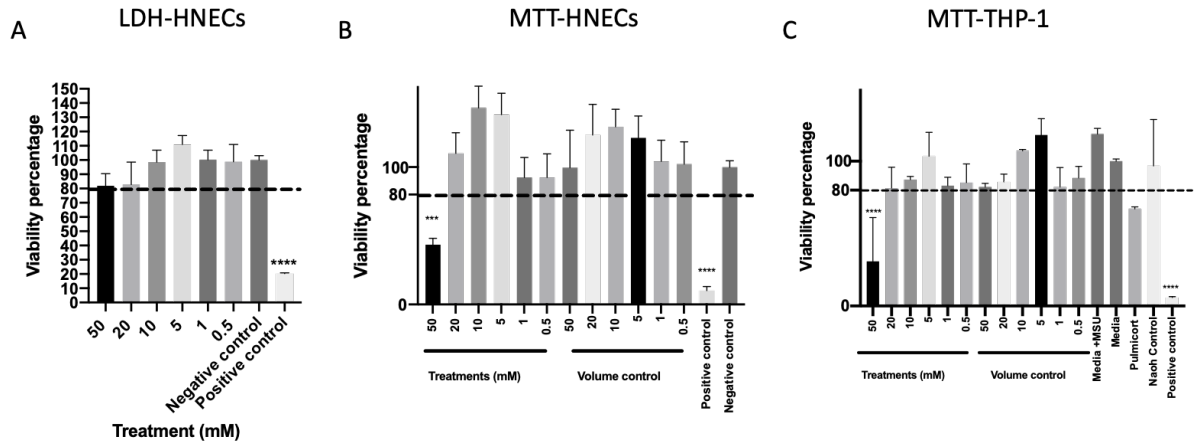


Figure 3: Mesalazine appears to be non-toxic to HNECs and THP-1 Cells in concentrations < 50mM. (A) The Lactate Dehydrogenase (LDH) of HNEC-ALI cultures after 7 hours of treatment with various concentrations of mesalazine. (B) HNECs monolayers and (C) THP-1 cell viability was measured after treatment with various concentrations of mesalazine for 24 hours. Experiments were performed with three replicates. The significance was determined by comparing it with the negative control. ***: $p<0.001$, ****: $p < 0.0001$. Negative control= ALI complete media; Positive control=10% Triton X-100; Volume control= saline 0.9% in media.

3.3 Mesalazine reduces the release of TNF-α by THP-1 cells

Stimulation with MSU for 7 hours significantly induced TNF-α release by THP-1 cells. Compared with this positive control, addition of 0.5mM and 1mM mesalazine as well as the standard of care therapy (budesonide) significantly reduced TNF-α production by MSU-stimulated THP-1 cells to baseline level. In contrast, higher concentrations mesalazine (from

5mM up to 50 mM) did not reduce TNF-α release compared to the positive control (Figure 4). TNF-α release was not significantly induced in HNEC-ALI cultures (Supplementary figure 1).

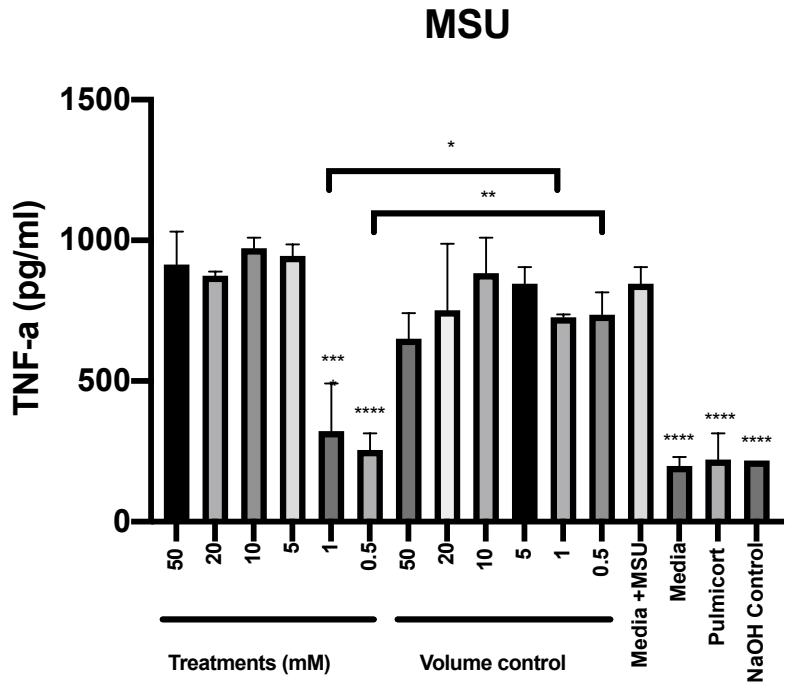


Figure 4: Mesalazine has anti-inflammatory effects on the production of TNF- α by THP-1 cells. The TNF- α production after 7 hours of treatment with various concentrations of mesalazine of THP-1 cells. The significance was determined by comparing it with the control. **: P<0.02*, ***: P<0.01*, ****: $P < 0.0001$. Experiments were performed three times. Negative controls = RPMI media; Volume control= Saline 0.9% in media and positive treatment control= budesonide; Control = NaOH.

3.4 Mesalazine has no effects on the release of IL-6 by HNEC-ALI cultures

To assess the potential for anti-inflammatory effects of mesalazine, the release of IL-6 and TNF-α by HNEC-ALI and THP-1 cultures was measured after stimulation with poly (I:C) or MSU for 7 hours in the presence or absence of various concentrations of mesalazine or budesonide as standard of care control. Compared with the positive control, mesalazine did not show any alteration in IL-6 production by HNECs. In contrast, there was a significant reduction in IL-6 release in cells treated with budesonide, compared to the positive control ($p<0.05$), reducing IL-6 levels to baseline (Figure 5).

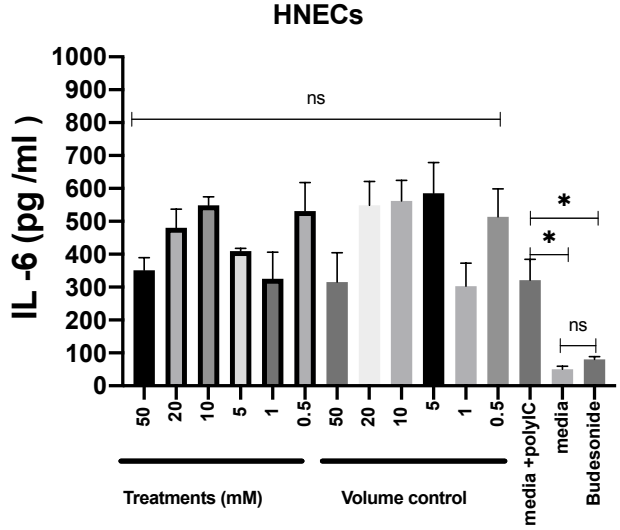


Figure 5: Mesalazine has no effects on the release of IL-6 by HNEC-ALI cultures. (A) The IL-6 production after stimulation with poly (I:C) and 7 hours treatment with various concentrations of mesalazine or volume control. Budesonide was used as standard of care control. Experiments were performed with three replicates. Negative control= Ex plus complete media; Positive control= media plus poly (I:C); Volume control= Saline 0.9% in media and Positive treatment control = Budesonide

4. Discussion

In this study, we evaluated the in vitro safety and potential for anti-inflammatory effects of mesalazine in CRS patient-derived HNEC-ALI cultures and THP-1 monocytes/macrophages respectively. Our findings revealed that mesalazine did not affect the barrier function of HNEC-ALIs and exhibited no cytotoxicity on both HNECs and THP-1 cells at concentrations up to 20 mM. Furthermore, mesalazine at 0.5 and 1 mM reduced TNF-α release by THP-1 cells, indicating its potential for anti-inflammatory effects involving those cell types.

The current treatment for chronic rhinosinusitis routinely comprises intranasal corticosteroids and antibiotics in cases of infectious exacerbations. Short courses of oral corticosteroids are also given in some cases. If these therapies fail, patients are offered surgical intervention (Fokkens, WJ et al. 2020). Therapy choices for chronic rhinosinusitis are therefore limited, and current disease management is often unsuccessful (Matsuwaki et al. 2008).

CRS has recently been attempted to be subclassified into inflammatory endotypes (Fokkens, WJ et al. 2020). The notion is that a variety of inflammatory pathways contribute to a defective interaction at the sinonasal mucosa between the host and the environment (Fokkens, WJ et al. 2020). Three inflammatory endotypes are implicated in CRS, which is characterized by Th1, Th2 and Th17 cells.

Type 2 inflammation is dominated by eosinophilic-driven inflammation and is linked to chronic inflammatory illnesses including asthma (Maspero, J et al. 2022). Whilst type 1 inflammation is driven by IFN-γ and TNF-α and is associated with a neutrophilic/macrophage-driven inflammation (Mayadas, Cullere & Lowell 2014). TLR2 is exhibited at a greater level

in CRS patients, particularly in caucasian populations. Stimulation of TLR2 activates the MYD88-dependent signaling cascade, and the NF-kappa B pathway resulting in a large production of cytokines and chemokines such as TNF-α and IL-6 (Sun et al. 2012). It has been demonstrated that TNF-α is a pro- inflammatory cytokine involved in chronic rhinosinusitis (Lennard et al. 2000; Xu, R et al. 2007). Macrophages play a role in TNF-α production, and are also elevated locally and peripherally in chronic rhinosinusitis (Lennard et al. 2000; Xu, R et al. 2007).

Mesalazine has been employed as a treatment for inflammatory bowel disease for some time. Mesalazine's mechanism of action is currently unknown, however, it is believed to entail the suppression of the NF-kappa B pathway and reduction of TNF-α production (Kaiser, Yan & Polk 1999; Mahida et al. 1991; Rachmilewitz et al. 1992). The obvious question that arises then is whether mesalazine could potentially have an application in certain types of CRS by inhibiting inflammation driven through this pathway.

As mesalazine has never been administered topically in CRS patients, we evaluated the safety and efficacy of mesalazine's anti-inflammatory properties in an *in vitro* setting for the first time. Mesalazine did not significantly alter the TEER during a 7-hour period at concentrations up to 50 mM and did not alter the paracellular permeability, indicating that it has no influence on the integrity of the mucosal barrier. Up to 50mM, there was no significant effect on LDH levels, however, 50 mM mesalazine did reduce cell viability of both HNECs and THP-1 cells in the MTT assay. This indicates mitochondrial malfunction and potential toxicity at this higher dose of mesalazine. However, the fact that 20 mM and lower concentrations did not reduce cell viability suggests that this toxicity is dose-dependent. Furthermore and interestingly, given that the strongest anti-inflammatory effect was seen at lower concentrations of 1 mM and 0.5 mM,

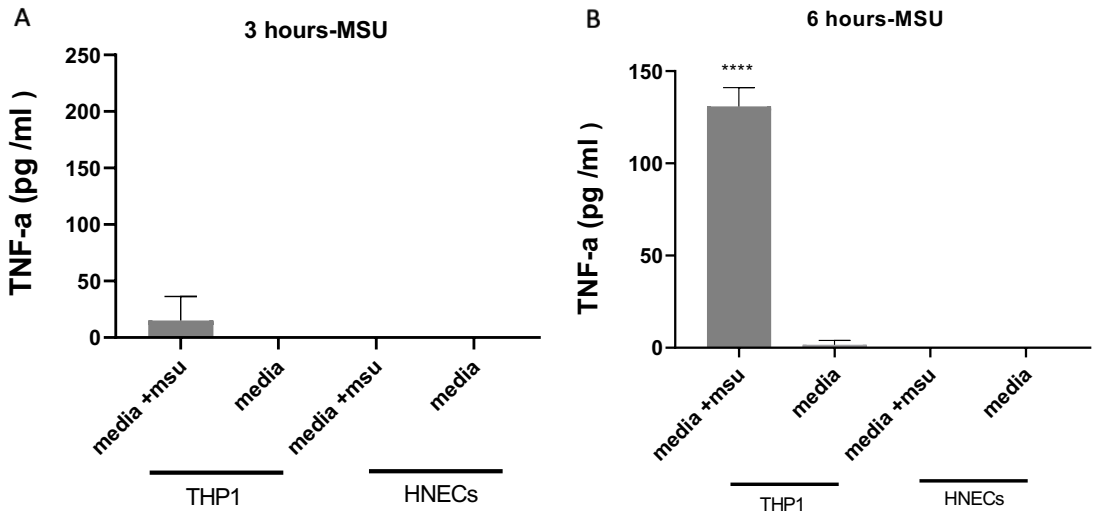
this implies a good *in vitro* safety profile of mesalazine at concentrations that hold promise for their anti-inflammatory effects in the context of CRS. A limitation of this study is that the anti-inflammatory effects were studied on *in vitro* THP-1, as macrophages harvested from the nasoepithelium do not have the ability to replicate and therefore cannot be cultured with the HNECs. Further *in vivo* studies will need to be conducted to validate these promising *in vitro* safety and effectiveness data.

IL-6 is a cytokine produced through the NF-Kappa B pathway (Liu, T et al. 2017). Intriguingly IL-6 showed no significant change when mesalazine was applied in all concentrations up to 50 mM on HNECs in our study. However, mesalazine at doses of 0.5 mM and 1 mM dramatically decreased TNF-α production by THP-1 monocytes/macrophages whereas TNF-α production was inconsistent by HNECs. This supports the notion that mesalazine possesses anti-inflammatory effects that include TNF-α in monocytes/macrophages. In our studies, TNF-α could not be consistently induced in HNECs cultures. This has also been observed in other studies where the secretion of TNF-α by HNECs has been shown to be variable and inconsistent (Ramezanpour et al. 2018). Patient's sinus tissue, in particular CRS tissue is known to be enriched in macrophages and those cells are thought to play an important role in the pathophysiology of this disease (Banks et al. 2014; Shaghayegh et al. 2022). Therefore, this study supports the potential for mesalazine to be used as a topical therapy to reduce inflammation in CRS patients. Given the potent effects of low concentrations of mesalazine seen on reducing the pro-inflammatory TNF-α levels produced by THP-1 cells, further in vivo studies are warranted to validate these findings towards clinical translation.

5. Conclusion

The result of this study indicates that mesalazine effectively reduces TNF-α, suggesting that it possesses anti-inflammatory characteristics. In addition, the excellent safety profile of mesalazine at dosages up to 20 mM suggests that it is not detrimental to HNECs and the mucosal barrier function at these concentrations. Overall, this could suggest that mesalazine is safe on nasoepithelial cells, and as part of a topical treatment for CRS patients could be beneficial in lowering inflammation, however further *in vivo* studies will have to be undertaken to confirm this anti-inflammatory effect in a sinonasal environment.

6. Supplementary Figures



Supplementary figure 1: MSU can stimulate TNF- α in THP-1 cells, but not HNEC. (A) The production of TNF- α after stimulation with MSU for 3 hours for THP-1 cells and HNEC. (B) The production of TNF- α after stimulation with MSU for 6 hours for THP-1 cells and HNEC. Experiments were performed with three replicates. Negative control= Ex plus complete media.

CHAPTER 3: A novel model of *Staphylococcus aureus*-Induced lymphoplasmacytic rhinosinusitis in the rat

Statement of Authorship

Title of Paper	A novel model of *Staphylococcus aureus*-induced lymphoplasmacytic rhinosinusitis in the rat
Publication Status	☒ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
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Principal Author

Name of Principal Author (Candidate)	William Murphy
Contribution to the Paper	Primary investigator of the study. This included experimental design, ethics, carrying out experiment, analyzing results and writing the manuscript.
Overall percentage (%)	80%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date: 22/1/24

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Sha Liu
Contribution to the Paper	Experimental design, tissue harvesting, results analysis and manuscript review
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Signature		Date	29/1/24

A novel model of *Staphylococcus aureus*-induced lymphoplasmacytic rhinosinusitis in the rat

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Keywords: *Staphylococcus aureus*, chronic rhinosinusitis, lymphoplasmacytic inflammatory response, rodent model

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CONFLICT OF INTEREST

The authors declare no conflicts of interest that are relevant to this paper.

AJP is a consultant for Medtronic and Neurent and receives a speaker's honorarium for Glasko Smith Klein, Sequiris, Storz and Sanofi and a shareholder for Chitogel.

PJW is a consultant for Neilmed, Stryker, Neurent, receives royalties from Fusetec and is a shareholder for Chitogel.

FUNDING

Not Applicable

Abstract

Background:

Chronic rhinosinusitis (CRS) is characterised by sinonasal mucosal inflammation. *Staphylococcus aureus (S. aureus)* is associated with severe CRS phenotypes. Different animal models have been proposed to study the association of CRS and *S. aureus*. However, current animal models are expensive due to the use of large animals, have high barriers to ethics approval or require invasive surgical intervention, necessitating a need for a model that can overcomes these limitations. This study aims at establishing a reliable and efficient rat lymphoplasmacytic inflammatory model for rhinosinusitis.

Methods:

Sprague Dawley rats received daily intranasal application of 20μL of saline; *S. aureus* CI_{182} exoprotein (250 μg/mL), or exoprotein CI_{182} in combination with *S. aureus* clinical isolate (CI_{908} or CI_{913}) 10^8 CFU/mL. The rats' sinuses were harvested at 1- and 2- weeks post-intervention. Colony forming units (CFU) and histopathologic examination of inflammation were evaluated.

Results:

S. aureus clinical isolates CI_{908} and CI_{913} in combination with exoprotein (CI_{182}) had higher CFU and caused persistently higher inflammation at both 1- and 2- weeks post-intervention compared to the exoprotein and saline group. The inflammatory cell type observed was lymphoplasmacytic.

Conclusion:

This study provided evidence that the combination of *S. aureus* exoprotein with *S. aureus* induces inflammation that persists for a minimum of two weeks post-intervention. This model is the first known animal model to create the lymphoplasmacytic inflammation subtype of inflammation seen in CRS patients. This offers a cost-effective, accessible, non-invasive, and easy to replicate model to study the causes and treatment of such inflammation.

1. Introduction

Chronic rhinosinusitis (CRS) is characterized by the inflammation of the mucosa lining of the paranasal sinus (Benninger et al. 2003). Approximately 10 % of the Western populations are affected by this disease (Habib et al. 2019). Various studies have linked *S. aureus* to the CRS pathophysiology, in particular the more severe disease phenotypes (Van Staa et al. 2004; Vickery, Ramakrishnan & Suh 2019). *S. aureus* is the most frequently cultured bacteria in patients with CRS exacerbations (Okifo, Ray & Gudis 2022) and influences inflammation by disrupting the epithelial barrier function, impairing mucociliary clearance and inducing innate and adaptive immune responses, which may result in polyp formation (Vickery, Ramakrishnan & Suh 2019). Clinically, rhinosinusitis can be classified according to the duration of symptoms as acute, subacute or chronic (Al-Sayed, Agu & Massoud 2017; Fokkens, WJ et al. 2020). Acute rhinosinusitis is typically virally mediated and lasts for 2-3 days. When symptoms persist beyond 5-7 days, secondary bacterial superinfection is thought likely and in these patients, neutrophilic inflammation predominates (Chow et al. 2012). CRS on the other hand is characterized mainly by the Th1, Th2 and Th17 inflammatory responses. Recently, focus on the understanding of CRS has shifted to endotyping, with further investigation into the underlying inflammatory types and the associated disease outcomes intensifying. On a cellular

level, five phenotypes of nasal polyps have been reported, including eosinophilic, neutrophilic, lymphocytic, plasma cell and a rarer lymphoplasmacytic predominant phenotype (Lou et al. 2016). Attention has primarily surrounded the eosinophilic driven inflammation, as it's the most prominent type observed in the Caucasian population. Little focus has been paid to the rarer mixed lymphoplasmacytic subtype. Eosinophilic driven inflammation is characterized by its responsiveness to steroids, whereas non-eosinophilic subtypes have low responsiveness to corticosteroid (Kirtsreesakul & Atchariyasathian 2006; Wen et al. 2012). CRS with lymphoplasmacytic infiltrate is associated with early polyp recurrence post surgery that is often steroid resistant. These patients often require long term macrolide therapy for disease control (Lou et al. 2016). Interestingly, patients treated with benralizumab for eosinophilic predominant CRS, resulted in a reduction of eosinophilic inflammation and a shift towards lymphoplasmacytic inflammation, potentially suggesting an underlying lymphoplasmacytic inflammation being present beneath the eosinophilic driven inflammation, warranting further exploration of lymphoplasmacytic inflammation in CRS (Ho et al. 2022). Current literature on CRS endotypes and biomarkers suggest that medical treatment should be tailored to the patients, including corticosteroids, antibiotics and biologics (Xu, Z et al. 2022). Further research into the lymphoplasmacytic subtype is clearly needed to better understand the relationship between this inflammatory infiltrate and it's association with CRS to guide disease management.

Experimental research uses animal models mainly in the context of understanding the pathophysiology of diseases, and in preclinical studies to test safety and effectiveness of novel therapies (Barré-Sinoussi & Montagutelli 2015). The development of animal models for sinusitis dates back to the rabbit model developed by Hilding et al. (1933). Subsequent models have also been established in mice and sheep (Hoggard et al. 2017). To date, there is

no established animal sinusitis model focusing on lymphoplasmacytic inflammation. Currently, there is no perfect animal model that faithfully replicates the pathophysiology of acute or chronic rhinosinusitis, with each currently available model having their own deficiencies.

Ethical guidelines categorize animals in research into two groups: small and large animals. Small animals only necessitate approval from a local animal ethics committee, while larger animals, including sheep, require approval from both the central and local animal ethics committees. Many existing sinusitis models entail invasive procedures and surgical interventions, leading to potential harm and discomfort for the animals involved (Lux et al. 2019; Mapara, Thomas & Bhat 2012).

Although mice are commonly used in research because of their low cost, ease of maintenance and genetic modifications, their sinonasal anatomy differs substantially from human anatomy. They lack a true sinus, and their small size can limit tissue sampling and present mechanistic limitations (Lindsay et al. 2006; Lux et al. 2019). Furthermore, they lack essential genes such as Cystic Fibrosis Transmembrane Regulator (CFTR) gene, important in mucociliary clearance, limiting their use for studying the pathophysiology of CRS and other various phenotypes (Lavelle et al. 2016; Lindsay et al. 2006).

Rabbit models present certain advantages, largely based on their size and well-developed sinuses, allowing relatively easy sinus access to create inflammation and sampling tissue. Nevertheless, there are significant costs and ethical considerations that need to be considered. Costs include dedicated housing facilities, as well as the need for well trained and skilled large animal handlers. Complications from instrumentation of the sinuses such as epiphora and pneumonia are not uncommon, and raise ethical concerns on the impact of such models on a rabbit's quality of life (Lux et al. 2019; Marks 1997).

A *S. aureus* biofilm sheep frontal sinusitis model has been established and successfully used in various preclinical safety and efficacy studies (Boase, Jervis-Bardy, et al. 2013; Boase et al. 2011; Ha et al. 2007). Sheep possess sinus anatomy and physiology closely resembling that of humans. Nevertheless, they are subjected to stringent ethical regulations and come with very high housing costs.

Considering the constraints outlined above regarding currently available models, this project seeks to assess the suitability of a rat model for investigating lymphoplasmacytic rhinosinusitis. By creating a rat model with rich lymphoplasmacytic infiltrate, this allows for further characterization of this rarer subtype, and provides a viable *in vivo* model for further investigation into potential underlying disease mechanisms and treatment targets associated with this inflammation type. Although still considered small animals, rats offer several advantages over mice. They are larger in size, translating into easier access to their sinuses, and unlike mice, they also possess (rudimentary) paranasal sinus cavities, and an increased number of submucosal glands which makes them more physiologically akin to humans than mice (Smolich et al. 1978).

2. Materials and Methods

2.1 Animals

Animal procedures were conducted in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes and were approved by the Animal Ethics Committee of the University of Adelaide, Australia (Approval ID M-2022-059). Rats were housed under

standard conditions. Lights were on for 12 hours daily, and cage temperature was 22 ± 1 °C, with continual access to water, standard regular chow and libitum.

2.2 Staphylococcus aureus clinical isolates cultured from patients

Ethics approval for obtaining bacterial swabs was granted by The Central Adelaide Local Health Network Human Research Ethics Committee (reference HREC/15/TWEH/132). Patients were recruited if they were undergoing endoscopic sinus surgery for chronic rhinosinusitis. The diagnostic criteria was based on the European Position Statement on CRS (Fokkens, WJ et al. 2020). Written informed consent was provided by all patients before the study commencement. Clinical history and demographics of patients along with the severity of CRS were recorded. Disease severity was based on completion of the Lund-Kennedy (LK), Lund-Mackay (LM) and the 22-item Sino-Nasal Outcome Test (SNOT-22) (Hopkins et al. 2009; Lund & Kennedy 1997; Lund & Mackay 1993; Naidoo et al. 2013; Psaltis et al. 2014).

The bacteria were harvested using a Transwab (Medical Wire & Equipment, Corsham, Wiltshire, UK) after brushing gently against the middle meatus. The bacteria were then cultured on 1.5% Trypticase soy agar (TSA) (Oxoid, Thebarton, SA, Australia) overnight at 37°C. Individual colonies were re-streaked on 1.5% TSA plates and incubated again overnight at 37°C. Individual colonies were identified using MALDI-TOF. Isolates of *S. aureus* were stored in 50% glycerol stock (Sigma Aldrich, Missouri, USA) at -80 °C for future use.

2.3 Staphylococcus aureus biofilm exoprotein preparation

S. aureus CI_{182} was streaked onto a 1.5% TSA plate and incubated overnight at 37°C. A single colony was then resuspended in 0.9% saline to obtain 1 McFarland (McF) Units, followed by dilution in Tryptic soy broth (TSB) (Oxoid, Thebarton, SA, Australia) at a ratio of 1 in 15. The cultures were then incubated at 37°C for 48 hours in 6-well plates, at 70 rpm to form biofilms. The biofilm supernatants were harvested and filtered using a 0.22 µm syringe filter (PALL Acrodisc, New York, USA) to eliminate any planktonic bacteria and bacterial debris, and obtain exoproteins. The exoproteins were concentrated using a 3k MWCO Pierce Protein Concentrator PES (Thermo Scientific, United Kingdom) at 3000 rpm and 4 °C to concentrations of 200□g/mL.

2.4 Bradford Protein assay

The Bradford protein assay (Biorad, Hercules, CA, USA) was performed according to the manufacturer's instructions to determine the exoprotein concentration. The Bradford protein assay was performed in triplicates for each sample, and the average protein concentration was reported.

2.5 Selection and preparation of S. aureus clinical isolates for inoculation

Two days prior to inoculation, *S. aureus* clinical isolates CI_{908} and CI_{913} were streaked onto 1.5% TSA plates. A single colony was resuspended in 0.9% saline to achieve a concentration of 0.5 McF Units, then cultured overnight in TSB at a 1:100 dilution. The pellets from CI_{908} and CI_{913} were then harvested and resuspended in exoprotein harvested from CI_{182} (as mentioned above) to achieve 2.5 x 10^9 Colony Forming Units (CFU)/mL.

2.6 Rhinosinusitis Rat Model

Sprague Dawley rats (n=21, all male, 6 weeks of age) were divided to receive once per day, into each nostril, applications of 20 µL of saline for 30 days (group 1, n=3), 20 µL of 250 µg/mL *S. aureus* CI_{182} exoprotein for 30 days (group 2, n=6) or 20 µL of 250 µg/mL of *S. aureus* CI_{182} exoprotein for 13 days (groups 3 and 4), followed by 10^8 CFU/mL CI_{908} (group 3, n=6) or 10^8 CFU/mL CI_{913} into 20 µL of 250 µg/mL of *S. aureus* CI_{182} exoprotein from day 14 to day 30 (group 4, n=6) (Figure 1). Rats were monitored daily, and the intervention was stopped on day 30. The rats were humanely euthanized at two-time points, on days 37 and day 44 (7 and 14 days after stopping the intervention), respectively. The nasal cavities were rinsed with 200 µL 0.9% saline, and collected for establishing colony-forming unit counts (CFUs). The nasal tissues were then harvested and placed in 10% EDTA for decalcification for four weeks. After decalcification, the nasal tissues were fixed in 10% neutral buffer formalin for histopathological examination.

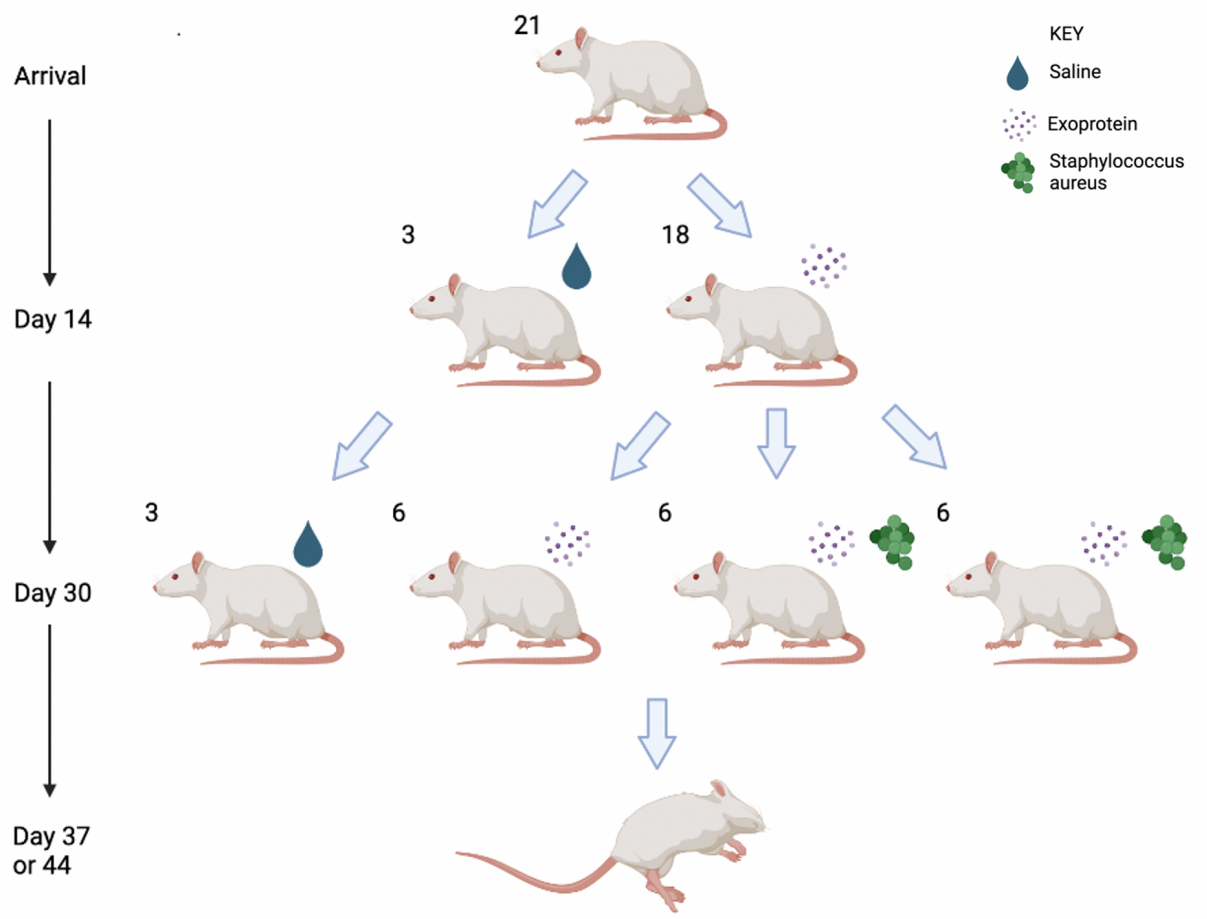


Figure 1. Experiment flowchart.

2.7 Colony forming unit counts (CFU)

The nasal rinses were kept on ice, serially diluted with 0.9% saline and spotted in triplicate on sheep blood agar (Beckton Dickenson, Franklin Lakes, NJ, USA), then incubated at 37°C overnight. CFUs were counted and calculated.

2.8: Histopathology examination with haematoxylin and eosin and Gram staining

The decalcified rat heads were embedded in paraffin, and 6µm sections were cut and stained with haematoxylin and eosin (H&E) or gram staining using standard protocols. Ten areas were selected and graded from 0 to 3 for inflammation for both the anterior and posterior segments in accordance with Houtak et al (2023).

2.9 Genomic DNA extraction and sequencing

For the *S. aureus* isolates from the exoprotein group, as well as $CI_{182,}$ CI_{908} and CI_{913}, whole genome sequencing was performed before and post intervention. The genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, 69504, Hilden, Germany) following the manufacturer's guidelines. Genomic DNA was sequenced using the Oxford Nanopore Technologies (ONT) Gridion Device (Oxford Nanopore Technologies, Oxford, UK). The SQK-RBK 114.96 Rapid Barcoding Kit (Oxford Nanopore Technology) was used with R10.4.1 MinION flowcells (Oxford Nanopore Technology). Base-calling was conducted with Dorado v 0.4.0 in super accuracy mode, using the 'r10.4.1 e8.2 400bps_sup@v4.2.0 configuration (Oxford Nanopore Technology).

2.11 Bioinformatics

Adapters and barcodes were removed from long reads using Porechop (Wick, Volkening & Loman 2017), with long-read-only assemblies created using Flye v2.9.2 with the option "–

nano-hq." (Kolmogorov et al. 2019). Sequence types were assigned using multi-locus sequence typing (MLST) in the MLST program (Seemann 2020).

2.12 Statistical analysis

Statistical analysis of the data was performed using GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA). Statistical significance was determined using an one-way analysis of variance (ANOVA), and a p-value of < 0.05 was considered significant.

3: Results

3.*1 Staphylococcus aureus* clinical isolates selection.

Clinical isolates (CI_{182}, CI_{908} and CI_{913}) were obtained from patients with CRS. They were selected from patients with high CRS disease severity scores (Table 1). All patients (1 male, 2 female) had CRS with nasal polyps (CRSwNP) and asthma, one patient also had gastro-oesophageal reflux disease (GORD) and another aspirin sensitivity.

Table 1: *S. aureus* clinical isolate patient summary. CI = Clinical isolates; yo = Years old; GORD = Gastro-oesophageal reflux disease; CRSwNP = Chronic rhinosinusitis with nasal polyps; ADSS = Adelaide disease severity score; SNOT22 = Sino-nasal outcome test; LM = Lund Mackay; LK = Lund Kennedy

CI 182	**CI 908**	**CI 913**
Male	Female	Female
62yo	73yo	53yo
GORD, Asthma	Asthma	Asthma and aspirin sensitivity
CRSwNP	CRSwNP	CRSwNP
SNOT 22 – 34	SNOT 22 – 71	SNOT 22 – 69
LM : 20	LM : 20	LM 24
LK: 18	LK : 18	LK 20

3.2 Rat nasal cavity colony forming unit (CFU) count.

The number of CFUs of *Staphylococcus aureus* harvested from both nostrils at day 37 and day 44 time point were investigated. Results for CFUs were similar between both time points and hence the datapoints were merged. Rats challenged with exoproteins only as well as with any of the CIs in exoproteins had higher CFUs compared to saline treated group which did not grow any *S. aureus* strains ($p<0.05$). Rats challenged with CI_{908} had significantly higher CFUs than those challenged with CI_{913}, and both were higher than exoprotein only treated rats or control. Long-read sequencing identified *Staphylococcus aureus* in the exoprotein

only treated group as CI_{913}. This suggests the presence and subsequent significant difference in CFUs between the exoprotein group and the saline group, which was likely due to a cross-contamination of the exoproteins only treated group with CI_{913}.

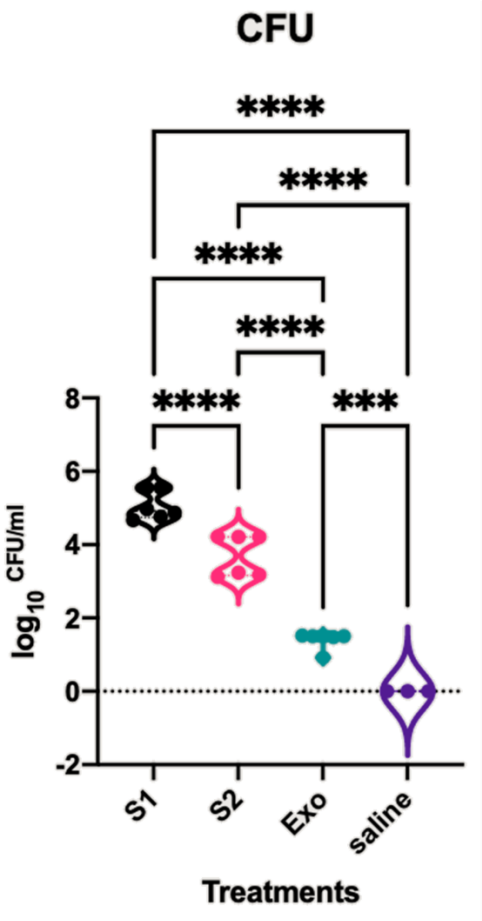


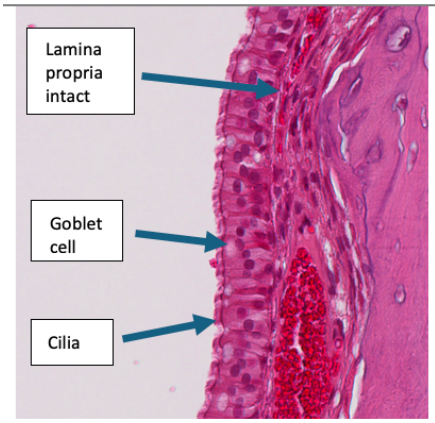
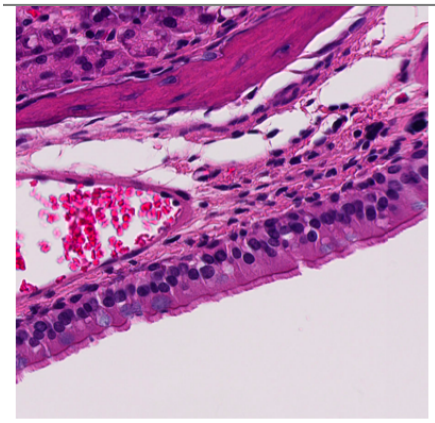
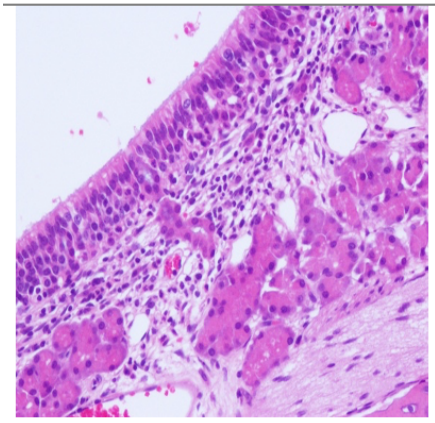
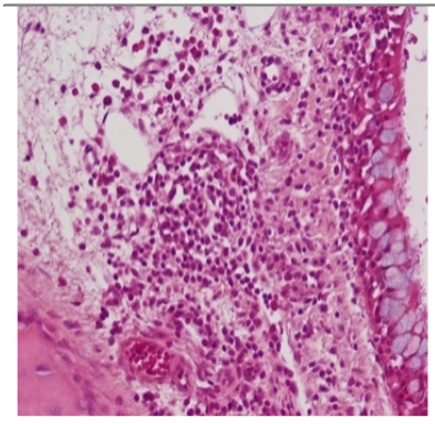
Figure 2. *S. aureus* colony-forming units (CFU). The CFUs of *Staphylococcus aureus* strains CI_{908} (S1), CI_{913} (S2), exoprotein (Exo), and saline control in the nasal cavity were analysed, and significance was determined using ANOVA. The asterisk (*) denotes statistical significance at $p < 0.05$ with *** $p<0.001$ and **** $p<0.0001$.

3.3 S. aureus clinical isolates and exoproteins induced significant inflammation.

The inflammatory infiltrate, primarily consisting of lymphoplasmacytic cells, was prominent and infiltrated the epithelium, leading to disruption of the lamina. Inflammation was present in both the respiratory and olfactory epithelium (Supplementary Figure 3). There was non-specific peribronchial infiltrate observed in exoprotein and both *Staphylococcus* groups. The kidney and spleen had no observable changes in any of the rats (Supplementary Figure 4).

For the assessment of inflammation, ten areas with the highest degree of inflammation were selected from each animal. The severity of lymphoplasmacytic infiltrate in the lamina propria was graded by a pathologist blinded to the treatment groups on high-power (X40) fields ranging from 0 to 3, indicating no inflammation, mild, moderate, or severe inflammation, respectively. Representative images for each grade (0 to 3) were presented in Table 2. The examination revealed significant inflammation in the rats inoculated with *S. aureus* and/or exoprotein, particularly in the *S. aureus* CI groups when compared to the exoprotein and saline control group. Additionally, the exoprotein group exhibited significantly greater inflammation than the saline control group (Figure 3a & Table 3). The inflammation was observed both anteriorly and posteriorly without any significant difference between both locations (Figure 3b).

Table 2: Inflammatory infiltrate scoring. The representative image (X40) was selected to represent each score.

Histology	Grade
	Grade 0 – Unaffected area of pseudostratified ciliated columnar epithelium with a normal number of goblet cells and minimal inflammatory infiltrate. The lining epithelium was intact.
	Grade 1 – Mild lymphoplasmacytic infiltrate in the lamina propria.
	Grade 2 – Moderate lymphoplasmacytic infiltrate in the lamina propria with lymphocytic infiltration of the lining epithelium resulting in some disorganization and disruption of the epithelium and loss of cilia.
	Grade 3 – Severe lymphoplasmacytic infiltration of oedematous lamina propria. The respiratory epithelium was disrupted, and sometimes ulcerated (top right of image), with marked epithelial lymphocytic infiltration and goblet cell hyperplasia.

A B

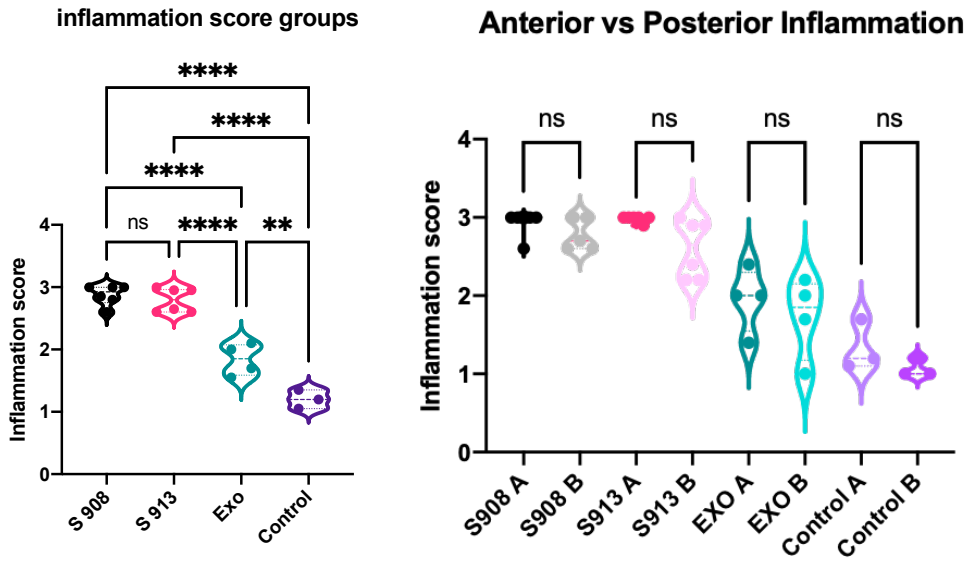
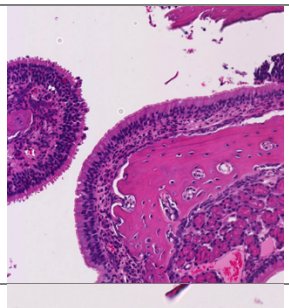
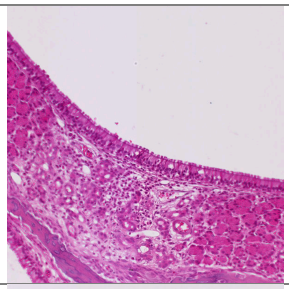
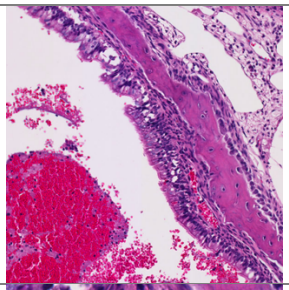
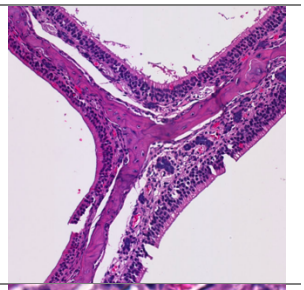
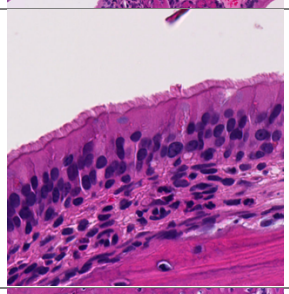
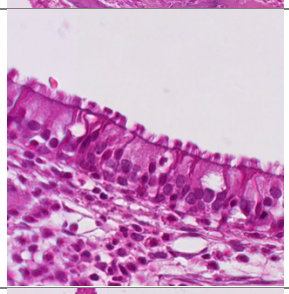
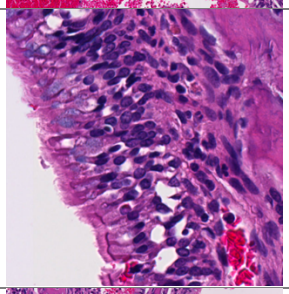
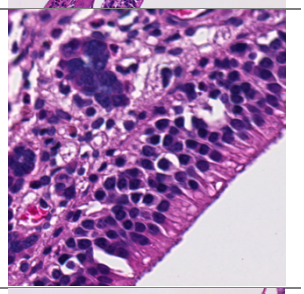
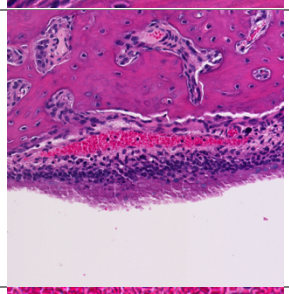
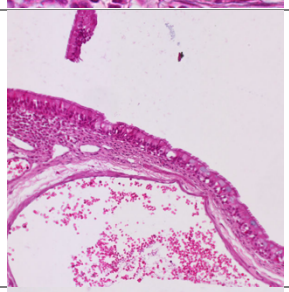
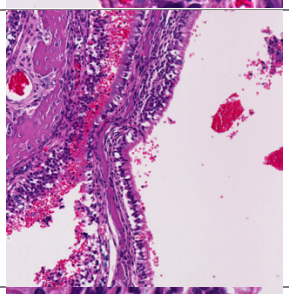
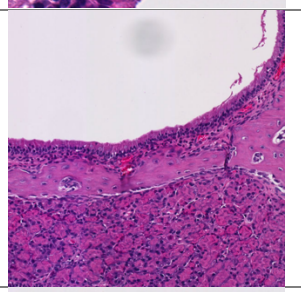
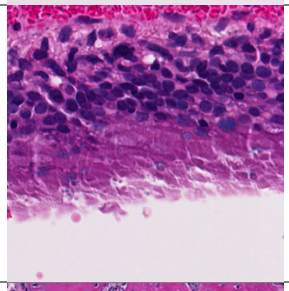
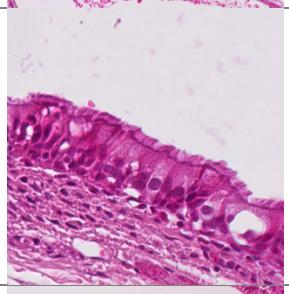
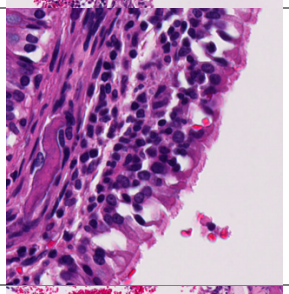
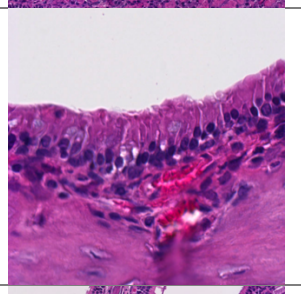
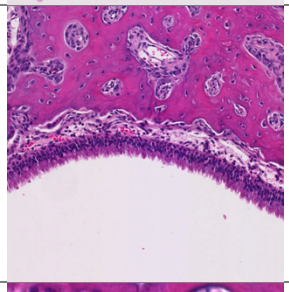
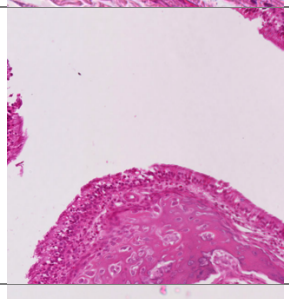
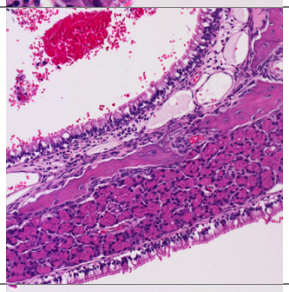
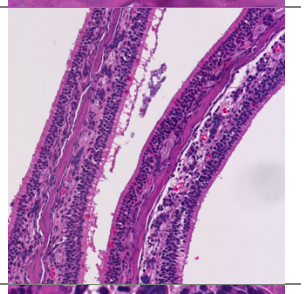
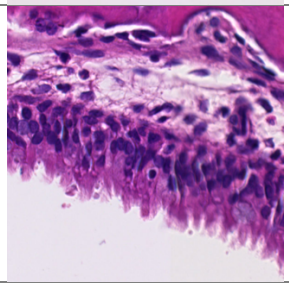
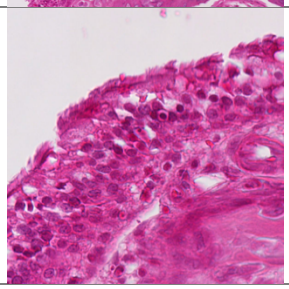
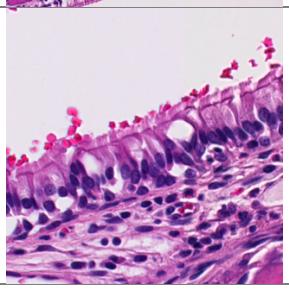
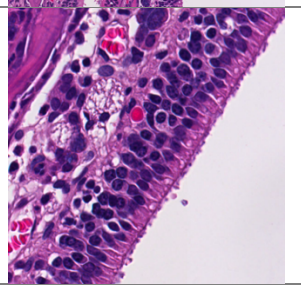


Figure 3: *S. aureus* and exoprotein induced inflammation *in vivo*

A) Inflammation scores of the sinonasal cavities for rats challenged with CI908, CI913, exoprotein or control (saline). B) Anterior (A) and posterior sections (B) were compared with the samples from different segments of the same rat. S 908 n=6; S 913 n=6; Exo n=4 and Control n=3. Significance was determined by comparing the results with the saline control. Asterisks indicate statistical significance (**: $p<0.01$; ****: $p<0.0001$), ns = not significant.

Table 3: representative inflammation for control (saline), exoprotein, CI_{908} and CI_{913} treated rats. There are 3 representative areas of inflammation for each sample, with a low-powered (10X) view and high powered (40X) view of each of those areas.

	Saline	Exoprotein	Staph CI_{908}	Staph CI_{913}
10 X # 1				
40 X # 1				
10 X # 2				
40 X # 2				
10 X # 3				
40 X # 3				

3.4 S. aureus invades the nasal mucosa.

The results of the Gram staining procedure confirmed the presence of *S. aureus*. This bacterium was observed to colonize the epithelial layer of the sinuses, which constitutes the outermost cellular lining of the nasal passages and was identified within epithelial cells. Further investigation revealed that *S. aureus* possessed the capability to also breach the lamina propria, a thin layer of connective tissue located beneath the epithelium and infiltrate the subepithelial region. These findings suggest that the bacterium exhibits the capacity to penetrate "damaged" nasal tissue (Figure 4).

4. Discussion

In this study, our objective was to develop a rat sinonasal inflammation model that mimics inflammation seen in *S. aureus* CRS in humans. This is the first animal model that reflects the inflammatory milieu of the less common and less well described lymphoplasmacytic subtype of CRS. As focus on CRS shifts towards endotyping and targeted treatment options, this model provides value in establishing the first *in vivo* model that is characterised by inflammation, reflective of that seen in lymphoplasmacytic predominant CRS and can be used to help further characterise this rarer inflammatory subtype. By abstaining from surgical procedures and invasive techniques, we not only prioritised the well-being and comfort of the rats but also aimed to create an easy-to-replicate inflammatory model, different to previous animal models which often require surgical interventions. We deliberately chose a rat model given its smaller

size than the rabbit and sheep, making it easier and cheaper to house and to mitigate many of the availability and ethical concerns with using larger animals.

We utilized *S. aureus* exoprotein, both individually and in conjunction with two distinct strains of *S. aureus*. The results demonstrated that both the exoprotein alone and the combination of exoprotein with *S. aureus* strains induced notable and widespread inflammation. The combination of exoprotein with *S. aureus* strains demonstrated markedly elevated levels of inflammation in comparison to both the control group and the exoprotein-alone group. This suggests that although exoprotein can in itself generate an inflammatory response, additional bacterial factors produced by live bacteria further exacerbate the inflammation. We indeed observed significantly higher CFU counts in the rats treated with both CIs of *S. aureus* compared to both the control group and the exoprotein-alone group. This finding was corroborated by Gram staining, which confirmed that the inoculated *S. aureus* bacteria successfully colonized and invaded the sinonasal mucosa membrane. Even though *S. aureus* was also cultured in the exoprotein-only group, likely due to contamination of the exoproteins by live bacteria, CFUs were lower, along with significantly reduced levels of inflammation compared to the groups treated with clinical isolates. This suggests that the level of inflammation is indeed linked to the bacterial load. These findings align with microbiome studies that have shown the severity of CRS to be directly related to increased bacterial load (Hoggard et al. 2017; Psaltis & Wormald 2017; Wagner Mackenzie et al. 2017).

Histopathological analysis revealed significant lymphoplasmacytic infiltrate with goblet cell hyperplasia in both anterior and posterior segments of the nasal tissue in the *S. aureus* infected rats. Although there was no overall difference between the anterior and posterior regions, there was a tendency for the inflammation to be more widely distributed in the former, possibly due

to anatomical differences between these two regions, with anterior segments receiving more consistent exposure compared to deeper segments.

The inflammation observed was consistent with prior studies showing increased inflammation in the subepithelial layer and lamina propria of nasal mucosa after exposure to *S. aureus* (Boase et al. 2011; Jia et al. 2014). Such severe inflammation with goblet cell hyperplasia is also indicative of epithelial remodelling after chronic antigen exposure (Redington 2000), and were observed in CRS, chronic airway diseases, cigarette smoke exposure and cystic fibrosis (Ramadan & Hinerman 2002; Sethi 2000; Silva & Bercik 2012; Spurzem et al. 1991). The lymphoplasmacytic infiltrate was observed most prominently in the *S. aureus* infected groups, consistent with the inflammatory milieu observed in CRS (Al-Sayed, Agu & Massoud 2017). Although lymphoplasmacytic infiltrate is considered rarer compare to the eosinophilic subtype, Mariano et al. (2019) examined 277 CRS patients and found lymphoplasmacytic infiltrate to be the prominent infiltrate in 111 patients, potentially making it a more important inflammatory subtype in CRS than originally believed. Plasma cells (effector B cells) originate from B lymphocytes and secrete antibodies in response to antigens (Khonsary 2017). B cell activation and excess antibody production is associated with CRS, most prominently with CRSwNP (Hulse et al. 2013; Tsybikov et al. 2015; Wang, M et al. 2019) and is consistent with increased levels of plasma cells and B cells in polyp tissue from CRSwNP patients (Feldman et al. 2017; Gevaert, P et al. 2005; Lau et al. 2017; Tsybikov et al. 2015; Wang, M et al. 2019; Xiao et al. 2016). T-lymphocytes (T cells) differentiate into effector T cells, such as CD4+, CD8+, regulatory, cytotoxic or helper T cells. Different effector T cell subtypes are involved in CRSsNP and CRSwNP (Hamilos et al. 1995; Miljkovic et al. 2016; Sharma et al. 2012). A meta-analysis by Shen et al. (Shen et al. 2018) showed different inflammatory infiltrates and treatment responsiveness based on the country of origin, with nasal polyps in the Asian

population characterized by infiltrates more linked to type 1 inflammation, exhibited a greater response to macrolides. In contrast, the western population's polyps are more associated with type 2 inflammation, demonstrated more responsiveness to steroids. Further investigation into the lymphoplasmacytic cell subtype could improve the understanding of the pathogenic relationship between *S. aureus* and CRS, and further exploration could lead to more accurately targeted therapies tailored for this subgroup of patients. Interestingly, in our study, there was no significant difference in the extent of inflammation that was observed between the 2 time points, taken one week apart, even though the rats did not receive further *S. aureus* during this time. Together, our findings indicate the activation of a more chronic immune response in the rats with robust lymphoplasmacytic immune cell infiltration and goblet cell hyperplasia.

The use of exoprotein alone induced significant inflammation, suggesting the importance of the secreted inflammatory proteins in the development of this response. This could be due the effect of exoprotein impairing mechanical barriers, such as tight junctions, which contribute to the development of CRS, or possibly due to antibodies to *S. aureus* exoprotein acting as a superantigen (Antunes, Gudis & Cohen 2009; Chen, J-B et al. 2017; Gevaert, P et al. 2005). *Staphylococcus aureus* enterotoxins (SAE) are a well described component of *S. aureus* exoprotein. They can function as superantigens, resulting in the production of SAE-specific antibodies that can potentially establish sustained inflammation. Our study's findings support this in addition to the direct disruptive effect the exoprotein has on the nasoepithelium. This breach is likely to promote the subepithelial colonization of *S. aureus* in patients with sinusitis and possibly prolongs the duration of inflammation. It should be mentioned however our finding of the presence of *S. aureus* in the exoprotein only treated group suggests cross-contamination, possibly due to the rats also being housed in the same room, despite being in separate cages. The extent to which this contributed to inflammation seen in this group cannot

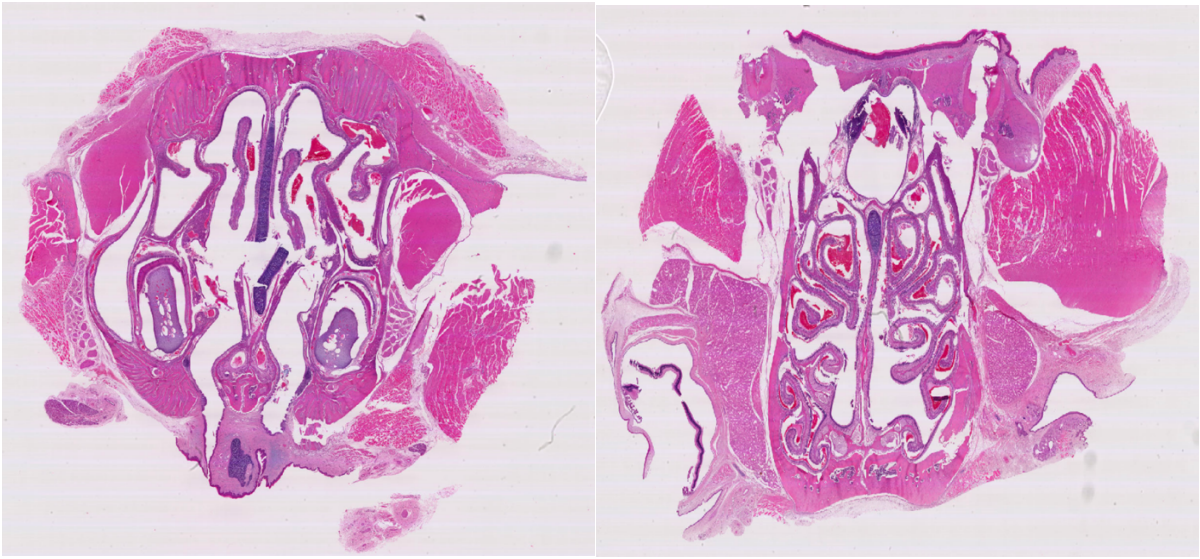
be discounted and does suggest that further experiments mitigating contamination are required to confirm our results.

While the inflammatory environment observed in this study aligns with that observed in the context of CRS in humans, it's important to note a limitation. Although the cell types present in the inflammatory milieu mirror those seen in CRS, the designated time interval of 3 months of symptoms, as outlined by current guidelines for humans, was not met. As a result, future investigations will aim to determine if the inflammation persists three months post-intervention.

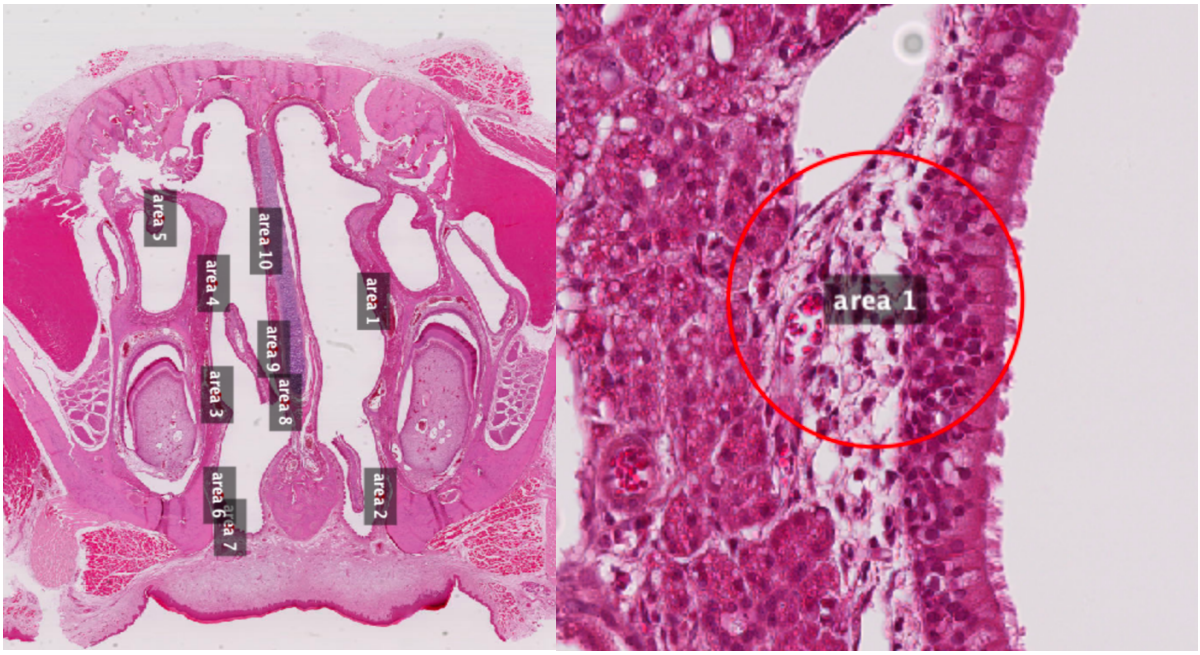
5. Conclusion

Our study successfully establishes the first rat model with a focus on lymphoplasmacytic inflammation in the sinuses. The combination of *S. aureus* exoprotein with live *S. aureus* bacteria induces inflammation that persists for a minimum of two weeks post-intervention. This model suggests the possible creation of a self-sustaining inflammatory response at least in the short term. Through the effective generation of a robust lymphoplasmacytic infiltrate, this model opens the door for additional research into this rarer inflammatory subtype. It presents a valuable tool for investigating mechanisms and interventions related to sinusitis in a preclinical setting.

6. Supplementary figures



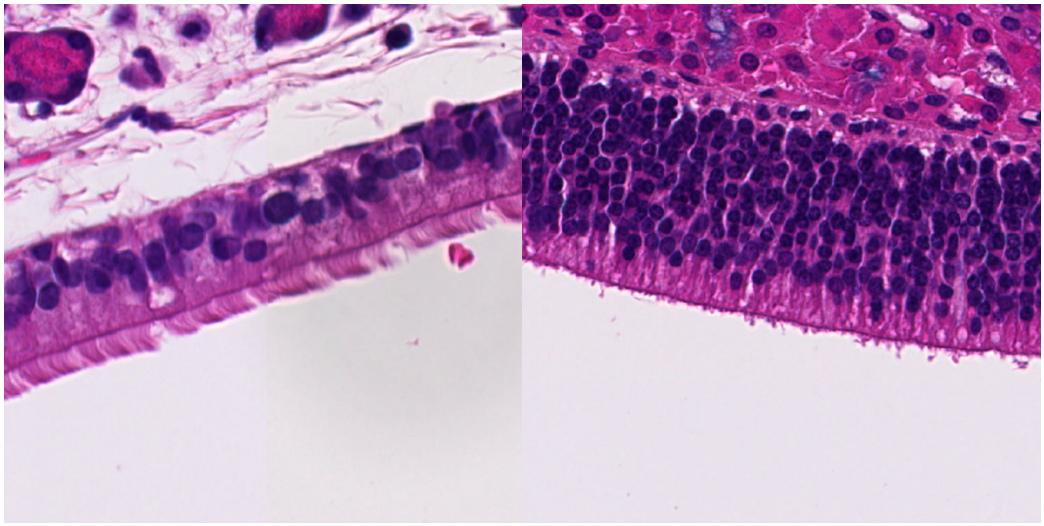
Supplementary figure 1. Anterior (left) and posterior (right) sections of the rat nasal cavity in H&E staining.



Supplementary figure 2. A completely labelled slide of areas of maximal inflammation mapped (left). A labelled area of maximal infiltrate (right).

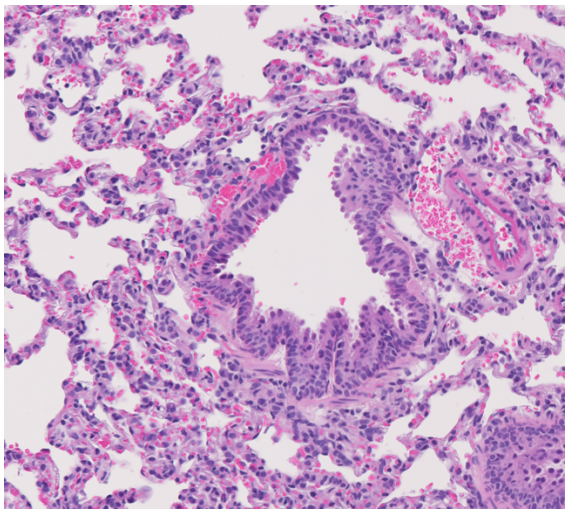
Supplementary table 1. Long read sequences.

Group	**Sequence type**
Exoprotein	Type 976
CI_{908}	Type 1290
CI_{913}	Type 976
CI_{182}	Type 20



Supplementary figure 3. Respiratory epithelium (Left) and olfactory epithelium inflammation (Right)

A



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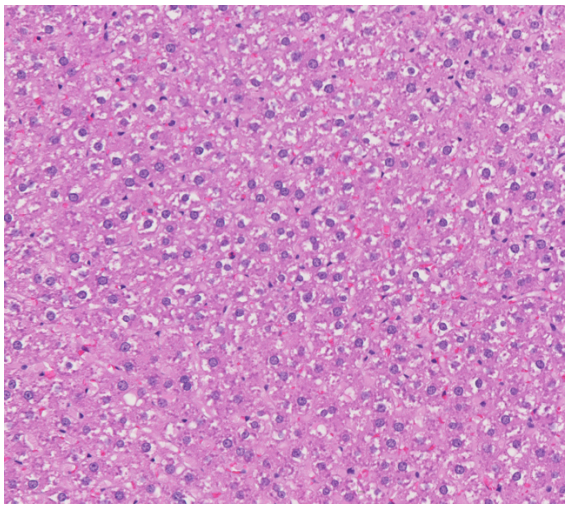
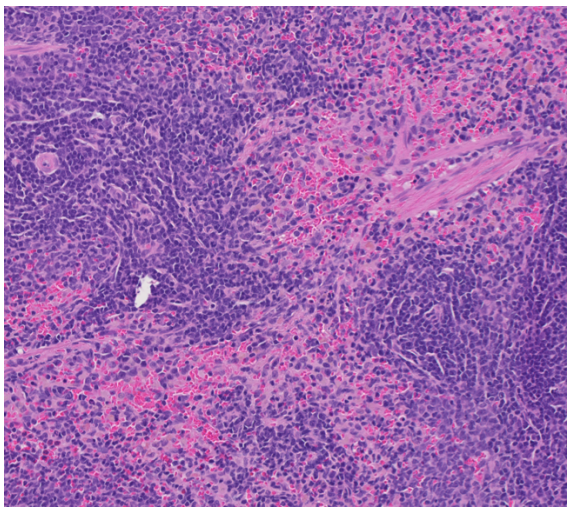


C



Supplementary figure 4. (A) Lung histology with non-specific bronchioalveolar infiltrate. (B) Liver histology. (C) Spleen histology.

CHAPTER 4: Mesalazine is effective at reducing *Staphylococcus aureus*-induced lymphoplasmacytic inflammation in vivo

Statement of Authorship

Title of Paper	Mesalazine is effective at reducing *Staphylococcus aureus*-induced lymphoplasmacytic inflammation *in vivo*
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	

Principal Author

Name of Principal Author (Candidate)	William Murphy		
Contribution to the Paper	Primary investigator of the study. This included experimental design, ethics, carrying out experiment, analyzing results and writing the manuscript.		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	22/1/24

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Sha Liu		
Contribution to the Paper	Experimental design, tissue harvesting, results analysis and manuscript review		
Signature		Date	22/1/24

Name of Co-Author	Sholeh Feizi
Contribution to the Paper	Experiment conduction, tissue harvesting

Signature		Date	22/1/24

Name of Co-Author	Kaviya Kalyanasundaram		
Contribution to the Paper	Experiment conduction, tissue harvesting		
Signature		Date	23/1/24

Name of Co-Author	Karen Hon		
Contribution to the Paper	Experiment conduction, tissue harvesting		
Signature		Date	22/1/24

Name of Co-Author	John Finnie		
Contribution to the Paper	Pathology assessment		
Signature		Date	22/1/24

Name of Co-Author	Kenny Yeo		
Contribution to the Paper	Results analaysis		
Signature		Date	22/1/24

Name of Co-Author	George Bouras		
Contribution to the Paper	Results analaysis		
Signature		Date	23/1/24

Name of Co-Author	Gohar Shaghayegh

Contribution to the Paper	Tissue harvesting, manuscript review		
Signature		Date	23/2/24

Name of Co-Author	Professor Wormald		
Contribution to the Paper	Supervising, methodology, manuscript review		
Signature		Date	26/1/24

Name of Co-Author	Professor Vreugde		
Contribution to the Paper	Supervising, methodology, manuscript review		
Signature		Date	25/1/24

Name of Co-Author	Professor Psaltis		
Contribution to the Paper	Overall supervision, conceptualization, methodology and manuscript review		
Signature		Date	29/1/24

Mesalazine is effective at reducing *Staphylococcus aureus*-induced lymphoplasmacytic inflammation in vivo

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Running title: Mesalazine's effectiveness at reducing sinonasal inflammation

Keywords: Mesalazine, *Staphylococcus aureus*, chronic rhinosinusitis, lymphoplasmacytic inflammatory response, treatment

CONFLICT OF INTEREST

The authors declare no conflicts of interest that are relevant to this paper.

AJP is a consultant for Medtronic and Neurent and receives a speaker's honorarium for Glaxo Smith Klein, Sequiris, Storz and Sanofi and a shareholder for Chitogel.

PJW is a consultant for Neilmed, Stryker, Neurent, receives royalties from Fusetec and is a shareholder for Chitogel.

FUNDING

Not Applicable

Abstract

Background:

Chronic rhinosinusitis (CRS) is characterised by sinonasal mucosal inflammation and has considerable morbidity for patients. The mainstay of treatment is topical corticosteroids and oral antibiotics in the context of infectious exacerbations. Courses of oral corticosteroids are given at times to reduce the inflammatory load, as are low dose long term macrolides for patients with lymphoplasmacytic inflammation . These medications can have severe side-effects and novel, safe and effective alternative medications are needed. Mesalazine may help address this therapeutic need, with recent in vitro studies showing safety on nasoepithelial cells whilst retaining its anti-inflammatory function. The objective of this study is to establish the anti-inflammatory effects of mesalazine in vivo.

Methods:

Sprague Dawley rats (n=48) were divided into 8 groups (n=6 per group). Group 1 received saline daily. Groups 2-8 were inoculated with 20µL of 250µg/mL of *S. aureus* CI_{182} exoprotein for 13 days followed by 20µL of 250µg/mL of *Staphylococcus aureus (S. aureus)* CI_{182} exoprotein containing 10^8CFU/mL CI_{908} from day 14 to day 30. Group 2 received no further treatment. Rats in groups 3-6 were treated with mesalazine, 1mM from day 14 (Group 3), or from day 30 (Group 5) and 10mM from day 14 (Group 4), or from day 30 (Group 6). Groups 7 and 8 were treated with clarithromycin and Pulmicort respectively from day 14. Rats were humanely euthanised at day 44, followed by determining colony forming units (CFUs) and histopathology to evaluate inflammation.

Results:

Mesalazine at 1mM significantly reduced lymphoplasmacytic inflammation when given from day 14 and day 30, and 10mM when given from day 14 compared to positive control *S. aureus* CI_{908} alone. Mesalazine was equally effective as pulmicort and clarithromycin at reducing inflammation. Mesalazine had no significant effect on *S. aureus* CI_{908} CFUs, whilst both clarithromycin and Pulmicort significantly reduced *S. aureus* CI_{908} CFUs compared to control.

Conclusion:

The findings from our study provide compelling evidence that mesalazine at 1mM successfully reduces lymphoplasmacytic inflammation caused by *S. aureus* CI_{908}. The lack of a reduction in S.aureus CFU, suggests that this is purely through an anti-inflammatory action. This indicates that mesalazine may have potential as an anti-inflammatory treatment option for CRS.

1. Introduction

Chronic rhinosinusitis (CRS) is characterized by chronic inflammation of the sinus mucosa with clinical signs and symptoms lasting 12 weeks or more (Benninger et al. 2003). CRS classically causes nasal obstruction and discharge, facial pressure, headaches, and impairment of sense of smell, resulting in a reduction in the quality of life (Rudmik & Smith 2011). It is a highly prevalent disease affecting approximately 10.9% of Western populations and has high associated healthcare costs (Fokkens, WJ et al. 2020).

CRS has been classified into CRS without nasal polyps (CRSsNP) or CRS with nasal polyps (CRSwNP). Recently, there has been a call to further classify CRS based on inflammatory types, including type 2 and non-type 2 inflammation (Fokkens, WJ et al. 2020). The routine medical management of CRS involves intranasal corticosteroids, saline washes and antibiotics when required. Short courses of oral corticosteroids are also sometimes given to patients, in particular those with CRSwNP. Although these current treatment options are widely available, there is an emerging threat of antibiotic resistance. Steroids may be contraindicated in some individuals , and in others can lead to long term local and/or systemic toxicity. (Hsu, Lanza & Kennedy 1998; Young et al. 2012). Where medical management is ineffective at treating CRS, surgical intervention may be required for symptom control (Fokkens, WJ et al. 2020). Despite surgical interventions, disease recurrence can still occur, thus better long-term treatment modalities are needed to combat CRS (Matsuwaki et al. 2008). Treatments have classically focused on managing the symptoms of CRSsNP and CRSwNP; however, as further understanding of inflammatory endotypes arises, a space opens for personalised treatments that depend on the inflammatory milieu present. Current literature on CRS endotypes suggests that

treatment should be tailored to the patient (Xu, Z et al. 2022), and therefore, further research on treatments based on endotypes is warranted.

Mesalazine, also known as mesalamine or 5-aminosalicylic acid (5-ASA), is an anti-inflammatory agent currently used to treat inflammatory bowel diseases, mainly ulcerative colitis (Böhm & Kruis 2014). Oral and rectal formulations of mesalazine are effective in treating active ulcerative colitis and keeping it in remission (Marteau et al. 2005). The mechanism of action of mesalazine is not fully understood, however, it has been suggested to be involved in regulating the production of cytokines such as tumour necrosis factor (TNF)-α, interleukin (IL)-1β, and interleukin (IL)-6 (Böhm & Kruis 2014; Kaiser, Yan & Polk 1999; Mahida et al. 1991; Rachmilewitz et al. 1992). These cytokines are also elevated in CRS and are involved in type 1 inflammation (Lennard et al. 2000; Xu, R et al. 2007). A previous study by our team indicated that mesalazine demonstrated anti-inflammatory effects in concentrations of 0.5 to 1mM *in vitro* and was proven safe when applied to human nasoepithelial cells in concentrations up to 20mM (Murphy et al. 2024). Whilst this was observed *in vitro*, further investigation is needed to establish the safety and efficacy of topical mesalazine to reduce sinonasal mucosal inflammation *in vivo*. Therefore, in this study, the safety and anti-inflammatory activity of varying dosages of mesalazine was investigated in a rat model of sinonasal inflammation.

2. Materials and Methods

2.1 Mesalazine preparation

A solution of mesalazine was prepared by dissolving powdered mesalazine (>99.9% purity, Sigma Aldrich, Missouri, USA) in sterilized MilliQ water. The pH was adjusted to 7 using a 1 mM sodium hydroxide (NaOH) solution. The mesalazine solution was then filter-sterilized using a 0.22 µm syringe filter (PALL Acrodisc, New York, USA). Subsequently, mesalazine was added to *S. aureus* biofilm exoproteins prepared according to our previous study (Murphy et al. 2024) to achieve concentrations of 1mM and 10mM mesalazine in 200□g/mL *S. aureus* exoproteins. Mesalazine solution was covered with foil paper for all experiments unless otherwise stated.

2.2 Budesonide (Pulmicort) and clarithromycin preparation

A solution of budesonide (Pulmicort) was prepared (10mg/L, AstraZeneca, Macquarie Park, NSW, Australia), serving as a topical, steroid-based treatment control. Final concentrations delivered to rats was 0.01mg/ml, comparable to clinical concentrations used in humans (Bernstein et al. 2023; Thamboo et al. 2014). A clarithromycin solution was prepared by combining sterilized water with clarithromycin suspension pellets (Mylan Health, Carole Park, QLD, Australia), and shaken to form a suspension with a concentration of 50mg/ml. This served as an additional treatment control, centred on the antibiotic effect.

2.3 Ethics approval

Animal procedures were conducted under the Australian Code for the Care and Use of Animals for Scientific Purposes and were approved by the Animal Ethics Committee of the University of Adelaide, Australia (Approval ID M-2022-059). Ethics approval for obtaining bacterial swabs from patients was granted by The Central Adelaide Local Health Network Human Research Ethics Committee (HREC/15/TWEH/132).

2.4 Selection and preparation of exoprotein and S. aureus clinical isolate for inoculation

Patients were recruited if they were undergoing endoscopic sinus surgery for chronic rhinosinusitis. The diagnostic criteria for CRS were based on the European Position Statement on CRS (Fokkens, WJ et al. 2020). Written informed consent was provided by all patients before the study commencement. Clinical history and demographics of patients along with the severity of CRS were recorded. The severity of the disease was determined using the Lund-Kennedy (LK), Lund-Mackay (LM), and 22-item Sino-Nasal Outcome Test (SNOT-22) (Hopkins et al. 2009; Lund & Kennedy 1997; Lund & Mackay 1993; Naidoo et al. 2013; Psaltis et al. 2014).

S. aureus clinical isolates CI_{182} and CI_{908} , were chosen to establish the rat sinusitis mode using previously established protocol (Murphy et al. 2024; Shaghayegh et al., 2023). They had were chosen based on their association with a severe phenotype of CRS disease Briefly, the clinical isolates were harvested from CRS patients, grown on blood agar and mannitol salt

agar plates (Oxoid, Basingstoke, UK), and identified using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS, Bruker® MBT, SA Pathology, Adelaide, Australia). Clinical isolates were stored in 50% glycerol stock (Sigma Aldrich, Missouri, USA) at -80 °C for future use.

For exoprotein preparation, *S. aureus* clinical isolate CI_{182} was grown for 48 hours, and the supernatants were harvested, filter-sterilised, and then concentrated to 250□g/mL according to protocols detailed in both Shaghayegh et al. (2023) and Murphy et al. (2024). Briefly, preparation of *S. aureus* clinical isolates for inoculation was achieved by culturing CI_{908} overnight in TSB, and then the bacteria were centrifuged at 1800 x g for 10 minutes, the TSB removed, and the bacteria were resuspended in CI_{182} exoprotein to achieve a 2.5 x 10^8 Colony Forming Units (CFU)/mL in 200□g/mL exoprotein.

2.5 Rhinosinusitis rat model

This experiment was performed using a previously validated and published rhinosinusitis rate model (Murphy et al. 2024). Sprague Dawley rats (n=48, all male, 6-7 weeks of age) were housed under standard conditions. Lights were on and off on a 12-hour cycle, and cage temperature was 22 ± 1 °C, with access to water and standard regular chow ad libitum 24 hours daily.

Rats were divided into 8 groups according to Figure 1.

1. Group 1 (n=6) received 20 µL of saline once daily for 44 days.
2. Groups 2-8 (n=42) were challenged with 20 µL of 250 µg/mL of *S. aureus* CI_{182} exoprotein for 13 days, followed by 10^8 CFU/mL CI_{908} in 20 µL of 250 µg/mL of *S.*

aureus CI_{182} exoprotein from day 14 to day 30. From day 30- day 44, rats did not receive further *S. aureus* or exoproteins. Rats (n=6/group) were randomised to receive:

1. Group 2 (positive control): no treatment.
2. Group 3: 20□L of 1mM mesalazine twice a day (BD) from day 14 to 44.
3. Group 4: 20□L of 10mM mesalazine BD from day 14 to 44.
4. Group 5: 20□L of 1mM mesalazine BD from day 30 to 44
5. Group 6: 20□L of 10mM mesalazine BD from day 30 to 44.
6. Group 7: 20□L of Pulmicort solution (1mg/100ml) BD from day 30 to 44
7. Group 8: Clarithromycin (50mg/kg daily) from day 30 to 44.

Inhaled isoflurane was used for anaesthesia at all times that rats received intranasal installation. Rats were monitored daily, and the intervention was concluded on day 44, at which point the rats were humanely euthanized.

Following this, the nasal cavities were rinsed with 100□L sterile 0.9% saline each side, totalling 200□L per rat, then the rest of the nasal cavity was harvested and placed in 10% neutral buffered formalin (Sigma Aldrich, Missouri, USA) for further testing. Rats' organs, including lung, liver, spleen, heart, and kidney were harvested, and placed in 10% neutral buffered formalin.

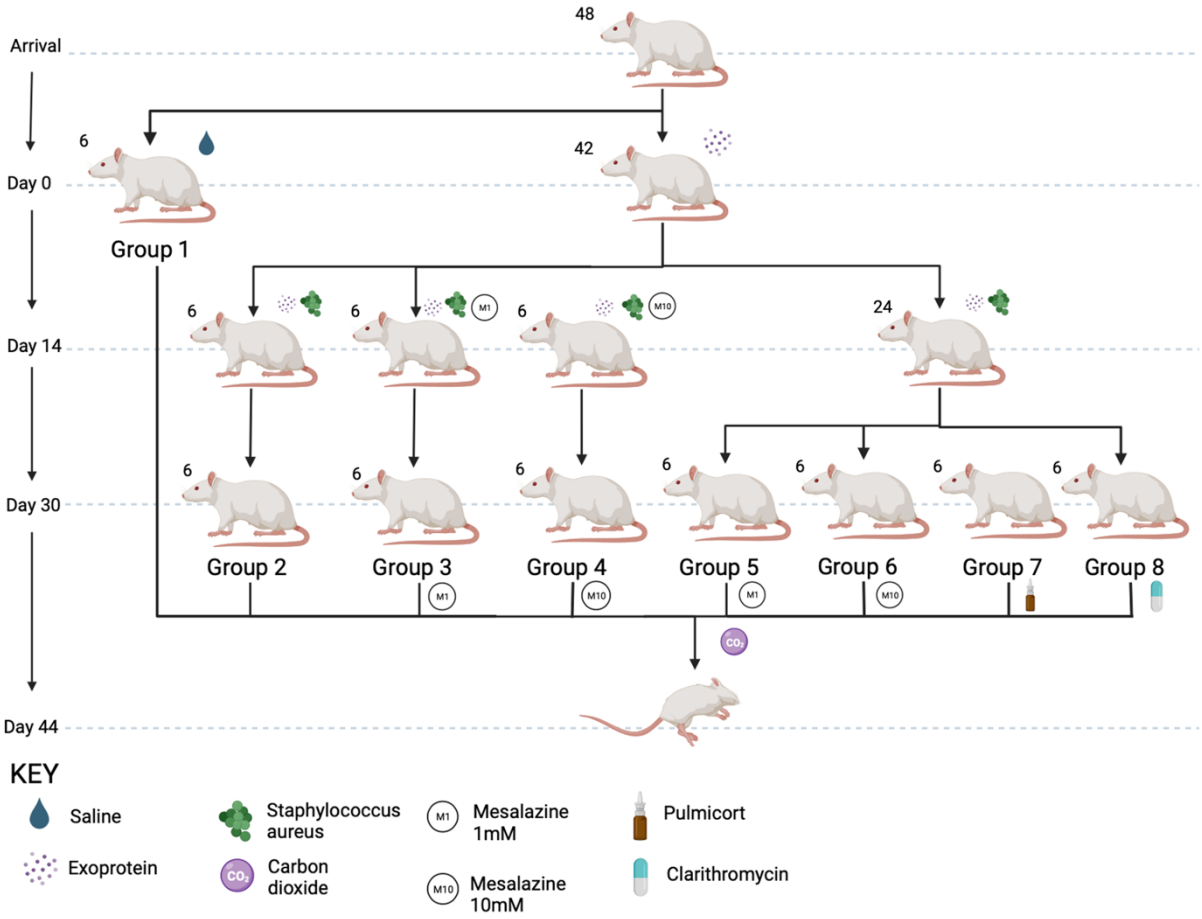


Figure 1. Flow chart of experimental groups. Group 1 received saline from day 0 to 44. Groups 2-8 received CI_{182} *S. aureus* exoprotein from day 0 to 13, and CI_{908} *S. aureus* in CI_{182} exoprotein from day 14 to 30. Group 2 received no additional treatment (positive control). Groups 3 and 4 were treated from day 14 to day 44 with 1mM mesalazine (Group 3), 10mM mesalazine (Group 4). Groups 5-8 were treated from day 30-44 with 1mM mesalazine (Group 5), 10mM mesalazine (Group 6), Pulmicort (Group 7) or Clarithromycin (Group 8). Rats were humanely euthanised on day 44.

2.6 Colony forming unit counts (CFU)

Nasal rinses were kept on ice. Rinses were serially diluted with 0.9% saline and spotted onto TSA in triplicates and incubated overnight at 37□C. CFUs were counted and calculated to obtain CFU/mL.

2.7 Histopathology examination with haematoxylin and eosin, and Gram staining

The rats' nasal cavities were decalcified in 10% EDTA (Sigma Aldrich, Missouri, USA) for four weeks, with a replacement of 10% EDTA every 4 days. Following decalcification, the rats' nasal cavities, as well as harvested organs, were embedded in paraffin, and 6µm sections were prepared. These sections were then stained with hematoxylin and eosin (H&E) following standard protocols. A pathologist graded inflammation in ten selected areas from 0 to 3 for both the anterior and posterior sections where maximal infiltrate was observed (Supplementary Figure 1).

2.8 Minimum inhibitory concentration (MIC)

S. aureus was streaked onto TSA plates. The MIC of clarithromycin against *S. aureus* 908 was assessed using the Clinical and Laboratory Standard Institute microdilution method (MA 2006). Concisely, single colonies of *S. aureus* CI 908 were resuspended in nutrient broth and adjusted to 0.5 McFarland units. After this a dilution of 1:100 bacteria in nutrient broth was prepared. Then 96-well microliter plates were inoculated with 50□L aliquots of diluted bacterial suspension. After, 50□L aliquots of different concentrations of clarithromycin were

added and then incubated for 24hrs at 37□C. MIC was reported as the lowest concentration that no visible turbidity was shown.

2.9 Statistical analysis

Statistical analysis of inflammation scores was assessed with an ANOVA with Dunnett post hoc test using Graph Pad Prism version 9.00 (GraphPad Software, La Jolla, CA, USA). Significance among groups was determined at a p-value < 0.05. CFUs were also analysed using a one-way analysis of variance (ANOVA), and a p-value of < 0.05 was considered significant.

3. Results

3.1 Selection of *S. aureus* clinical isolates

S. aureus clinical isolates were selected from patients with high CRS disease severity scores. Both patients (1 male and 1 female) had CRS with nasal polyps and asthma, and the male patient also had gastro-oesophageal reflux disease (GORD). Details of patient demographics and disease severity scores are listed in Table 1.

Table 1: *S. aureus* clinical isolate patient summary. CI = Clinical isolates; yo = Years old; GORD = Gastro-oesophageal reflux disease; CRSwNP = Chronic rhinosinusitis with nasal polyps; SNOT22 = Sino-nasal outcome test; LM = Lund Mackay; LK = Lund Kennedy

CI 182	**CI 908**
Male	Female
62yo	73yo
GORD, Asthma	Asthma
CRSwNP	CRSwNP
SNOT 22 – 34	SNOT 22 – 71
LM: 20	LM: 20
LK: 18	LK: 18

3.*2 S. aureus induced a lymphoplasmacytic sinonasal inflammation*

Challenging the rats with 250 µg/mL of *S. aureus* CI_{182} exoprotein for 13 days, followed by 20 µL of 250 µg/mL of *S. aureus* CI_{182} exoprotein containing 10^8 CFU/mL CI_{908} from day 14 to day 30 induced a robust sinonasal inflammation at the time of tissue harvest 2 weeks later (day 44). The rat sinonasal inflammatory infiltrate observed in all groups challenged with *S. aureus* primarily consisted of lymphocytes, with few plasma cells (lymphoplasmacytic), consistent with Murphy et al. (2024). When inflammation was robust (grade 3), lymphocytes infiltrated the lining respiratory epithelium (supplementary figure 2), sometimes associated with the disruption of the epithelium and goblet cell hyperplasia (supplementary figure 3).

Systemically, the lung, kidney, heart and spleen had no observable changes in any of the rats (supplementary figure 4).

3.3 Mesalazine, Pulmicort and clarithromycin decrease inflammation equally

The administration of mesalazine at a 1mM and at 10 mM concentration from day 14 and mesalazine at 1 mM concentration from day 30 demonstrated a statistically significant reduction in inflammation scores compared to the positive control ($p<0.05$). Similarly, clarithromycin and Pulmicort administered from day 30, significantly decreased inflammation compared to the positive control ($p<0.05$). Average inflammation scores were similar between rats treated with clarithromycin, Pulmicort, or 1mM mesalazine administered from day 14 and from day 30 onwards, or 10 mM mesalazine administered from day 14 onwards ($p>0.05$). Interestingly, mesalazine at 10mM when administered from day 30 did not result in a significant decrease of inflammation compared to the positive control (Figure 2).

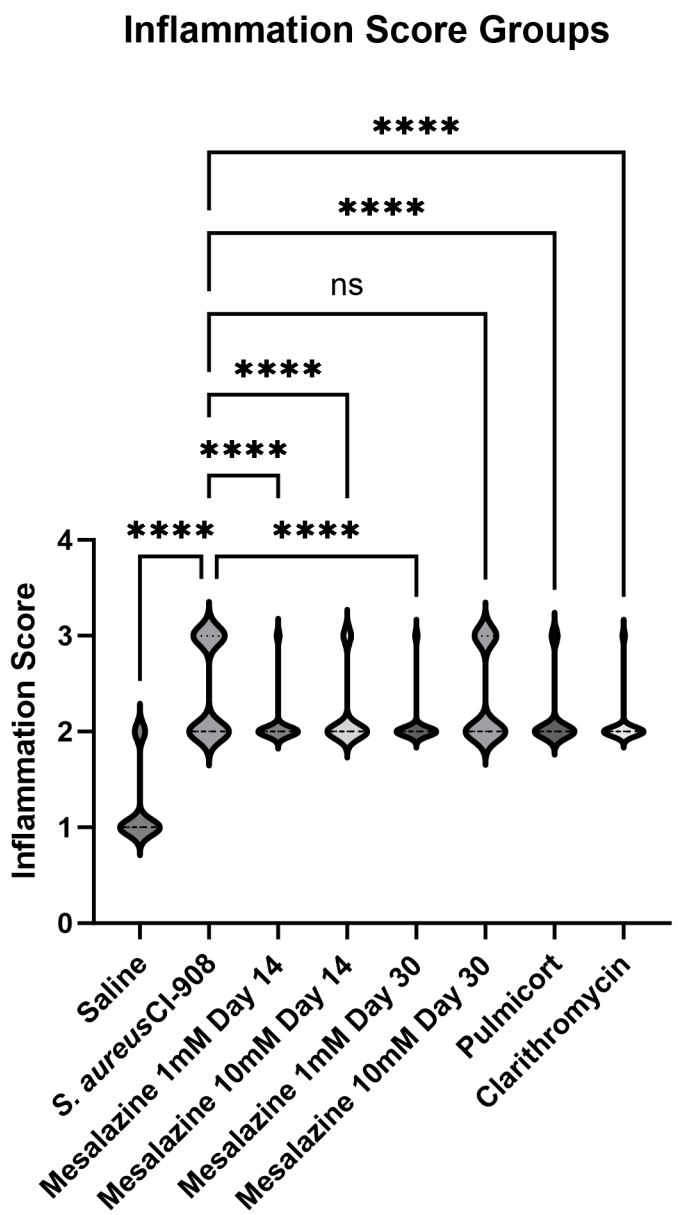


Figure 2: Inflammation scores of the sinonasal cavities. Mesalazine 1mM from day 14 n=6; Mesalazine 10mM from day 14 n=6; Mesalazine 1mM from day 30 n=6; Mesalazine 10mM from day 30 n=6; Positive control (*S. aureus* CI908) n=6; Pulmicort n=6; Clarithromycin n=6; Saline n=6. Significance was determined by comparing the results with the saline control. Asterisks indicate statistical significance (****: $p<0.0001$), ns = not significant.

3.5 Mesalazine does not significantly affect S. aureus colony forming units.

MIC of clarithromycin was determined to be 0.25μg/ml. The number of CFUs of *Staphylococcus aureus* from each group was determined at day 44. The administration of mesalazine at 1mM and 10mM at both the 14-day and 30-day time point did not affect CFU counts compared to *S. aureus* CI908. Clarithromycin and Pulmicort significantly reduced *S. aureus* CI908 compared to control.

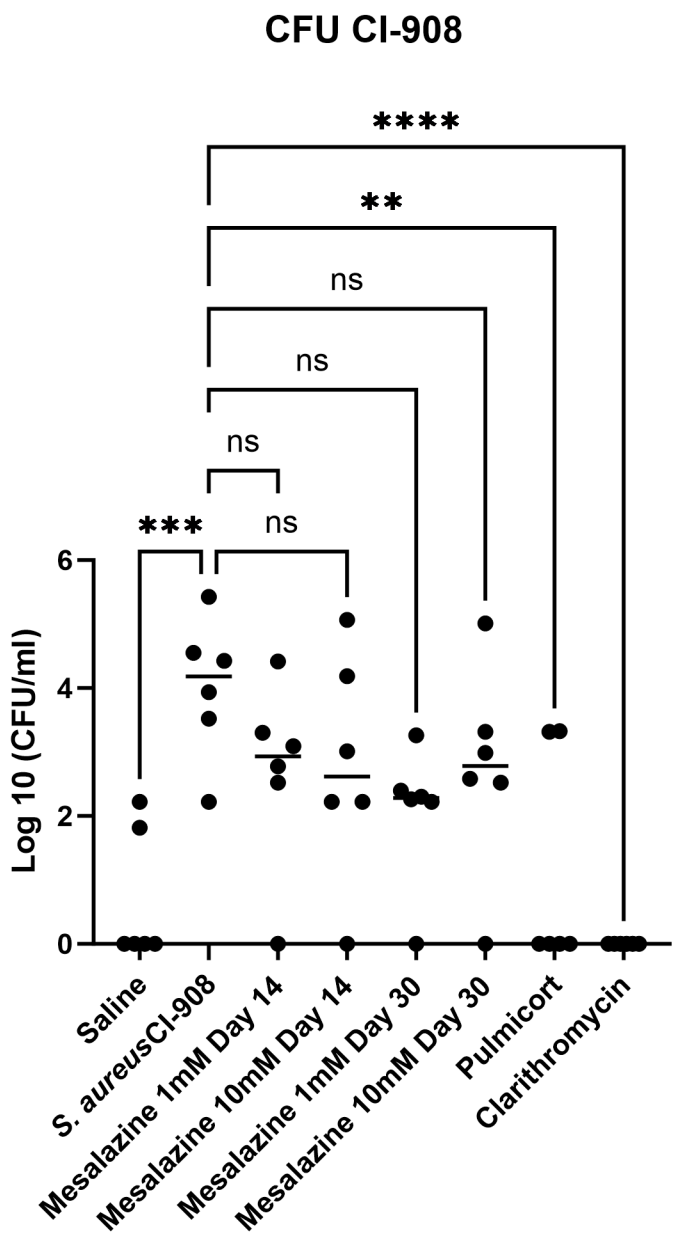


Figure 3. Colony forming units of each group. Saline n=6; Positive control (n=6); Mesalazine 1mM from day 14 n=6; Mesalazine 10mM from day 14 n=6; Mesalazine 1mM from day 30 n=6; Mesalazine 10mM from day 30 n=6; Pulmicort n=6; Clarithromycin n=6. Significance was determined by comparing the results with the *S. aureus* CI_{908} positive control. Asterisks indicate statistical significance (**: $p<0.01$; ***: $p<0.001$; ****: $p<0.0001$), ns = not significant.

4. Discussion

In this study, we evaluated the safety and anti-inflammatory effect of mesalazine in an *in vivo* setting in the context of the lymphoplasmacytic subtype of inflammation that is seen in some patients with CRS. Our findings suggest that mesalazine at 1mM and 10mM concentration, whilst not reducing *S. aureus* infection compared to control, could significantly reduce inflammation when given at day 14, and when given at 1mM from day 30. The anti-inflammatory effect of 1mM mesalazine was similar to that of Pulmicort and to clarithromycin. Together, these results support the potential of mesalazine to reducing lymphoplasmacytic mucosal inflammation.

Current treatment for CRS involves appropriate medical therapy followed by surgical intervention. Medical therapy often involves intranasal and/or oral corticosteroids patients and in some patients a course of antibiotics. If there is minimal response symptomatically, surgery is often then offered (Fokkens, WJ et al. 2020). Post surgical intervention, patients are generally prescribed Pulmicort washes in an attempt to decrease disease recurrence (Snidvongs et al. 2012). Although this has become a useful way in facilitating long term disease control, some patients do not tolerate topical steroids due to local side effects and in those with predominantly lymphoplasmacytic inflammation it may be less or ineffective.

Mesalazine has been successfully used topically for the treatment of inflammatory bowel disease for many years. The mechanism of action is currently unknown, however it is postulated to involve the suppression of NF-kappa B resulting in the reduction of TNF-α production (Kaiser, Yan & Polk 1999; Mahida et al. 1991; Rachmilewitz et al. 1992). In CRS, it is proposed that the stimulation of TLR2 activates the MYD88-dependant signalling

cascade, stimulating the NF-kappa B pathway and resulting in the production of TNF-α and IL-6 (Sun et al. 2012). It has been shown that TNF-α is a pro-inflammatory cytokine involved in CRS and has been involved in type 1 inflammation (Lennard et al. 2000; Xu, R et al. 2007).

A recent in vitro study in our department showed that a mesalazine-based sinus wash can inhibit TNF-α secretion without being toxic to human nasoepithelial cells (Murphy et al. 2024).This in vivo study confirms our previous in vitro study that mesalazine is non-toxic when applied into the nose of rats and could reduce inflammation significantly when compared to positive control.

In this study, challenging rats with intranasal *S. aureus* secreted exoprotein followed by instillation with S. aureus clinical isolates resulted in robust sinonasal lymphoplasmacytic inflammation that lasted at least 2 weeks after instillation of bacteria had ceased, consistent with prior rat sinonasal inflammatory models, as inflammation histopathologically similar to that present in CRS (Liang et al. 2008; Marino et al. 2019). It was observed that mesalazine in the concentration of 1mM was effective at reducing inflammation when started at both day 14 and day 30, which is consistent with the *in vitro* effects observed by Murphy et al. (2024). As a comparison, mesalazine when given in the context of IBD, is given at much higher concentrations of up to 4.8g daily to induce remission of an active flare in humans, which is 1000 fold higher than the daily dosing given to the rats (Karagozian & Burakoff 2007). Interestingly we found that although Mesalazine administered at a higher concentration of 10mM also reduced inflammation when administered from day 14, this was not the case when this concentration was administered from day 30. The lack of effect on inflammation for 10mM mesalazine given from day 30 is consistent with our *in vitro* study (Murphy et al. 2024), where mesalazine at the concentration of 10mM did not reduce TNF-α levels

compared to control. This apparent inverse dose response might be secondary to precipitation of the drug at high concentrations. Mesalazine at 10mM concentration given from day 14 did however reduce inflammation which might be explained by the effects of mesalazine on attenuating biofilm formation Dahl et al., (2017). Giving mesalazine from day 14 as opposed to day 30, will mean mesalazine has longer to attenuate biofilm formation and could potentially explain the reduction of inflammation that is observed at day 14 but not when given from day 30 at concentrations of 10mM.

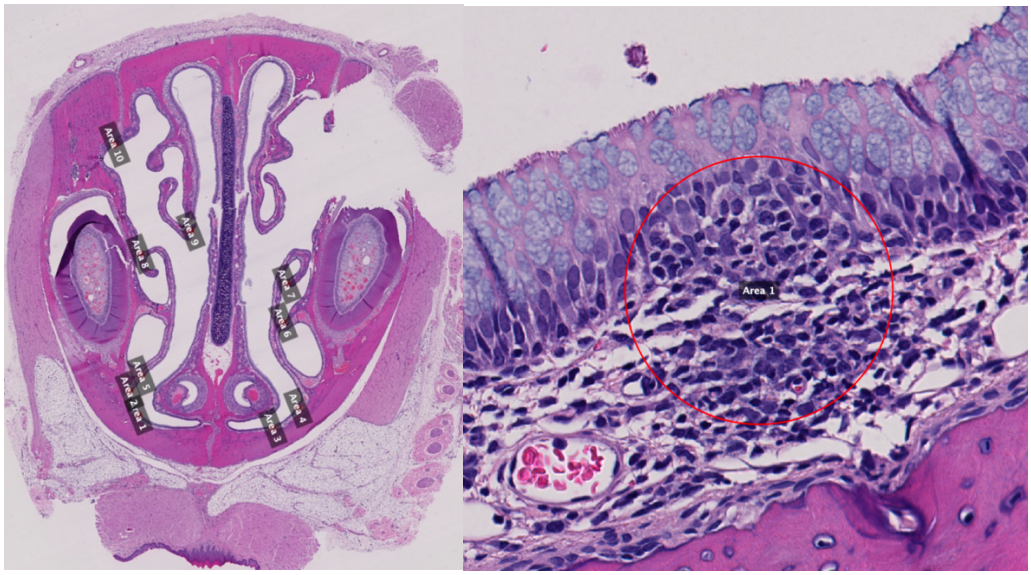
Mesalazine had no significant effect on CI_{908} CFUs when compared to *S. aureus* positive control. This is consistent with current literature on mesalazine which does not have any direct bactericidal properties against S. aureus (Kruszewska, Zareba & Tyski 2002). Although not significant, mesalazine at both 1mM and 10mM compared to the positive control trended to having lower CFUs. This may be due to the low power of this exploratory/pilot study or may truly reflect that Mesalazine's anti-inflammatory action is independent on any effect on the local sinonasal microbiome. Clearly further larger studies and those sampling the sinonasal microbiome are required and are currently being planned. Treatment with clarithromycin resulted in no CI_{908} CFUs in any of the rats, consistent with clarithromycin's known antibacterial effects. In the Pulmicort group, only 2 rats still had C_{908} grown, which could be explained by its known antibacterial effect on S. aureus (Cherian et al. 2019). Interestingly, despite the absence of *S. aureus* colonizing rats in clarithromycin treated rats, there were no differences in inflammation scores between this group and rats treated with mesalazine 1 mM where the reduction in *S. aureus* CFUs was not significant. This indicates that the inflammation in these rats was at least in part due to the stimulation with *S. aureus* exoproteins, as seen in in vitro experiments (Panchatcharam et al. 2020).

The limitations of this study, include the low number of isolates tested and the low power of this pilot study. Furthermore this inflammatory model was based on the inflammatory milieu observed in CRS, as opposed to the human definition of the diagnosis, where symptoms must persist for longer than 3 months in current guidelines. A further limitation was the presence of bacterial contamination of two rats in the saline group. This limitation may be addressed in future by having rats housed in separate rooms, as well as using separate PC2 rooms for installation.

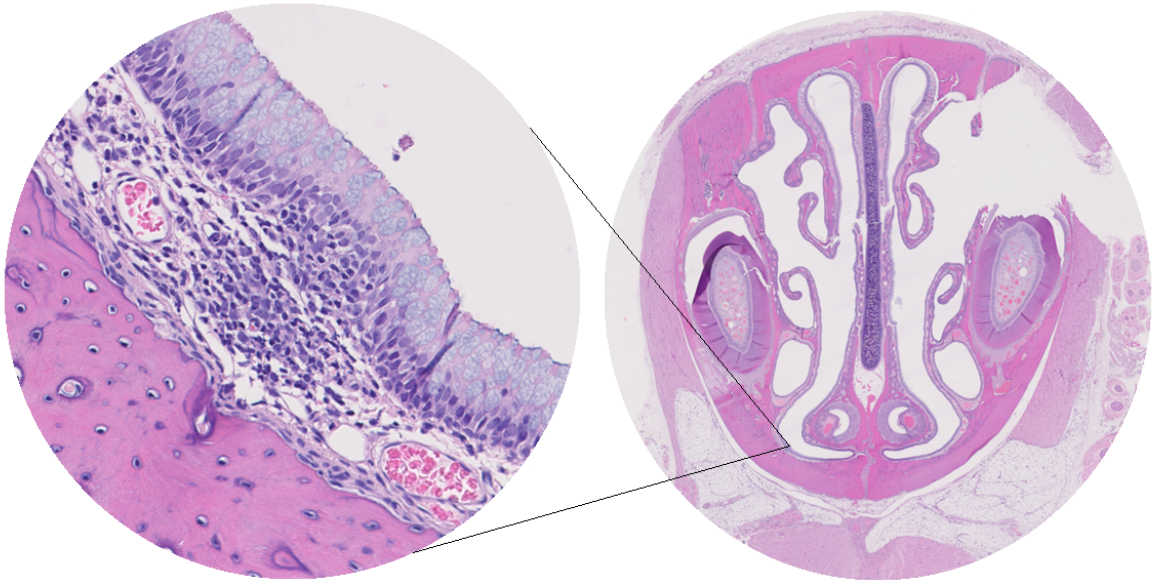
5. Conclusion

The results of this study indicate that mesalazine effectively reduced sinonasal inflammation caused by *S. aureus* and exoproteins in a rat sinusitis model. This suggests that mesalazine has anti-inflammatory properties in an *in vivo* setting at 1mM, and at the higher dose of 10mM, its anti-inflammatory property varies depending on the time of administration. Mesalazine's anti-inflammatory effect is comparable to treatment standards pulmicort and clarithromycin. In addition, mesalazine had no observable effects systemically. Overall, this suggests that mesalazine is safe to use *in vivo,* and may have potential usage in the topical treatment of sinonasal inflammation. Further human trials would need to be undertaken to confirm its anti-inflammatory effects in CRS patients.

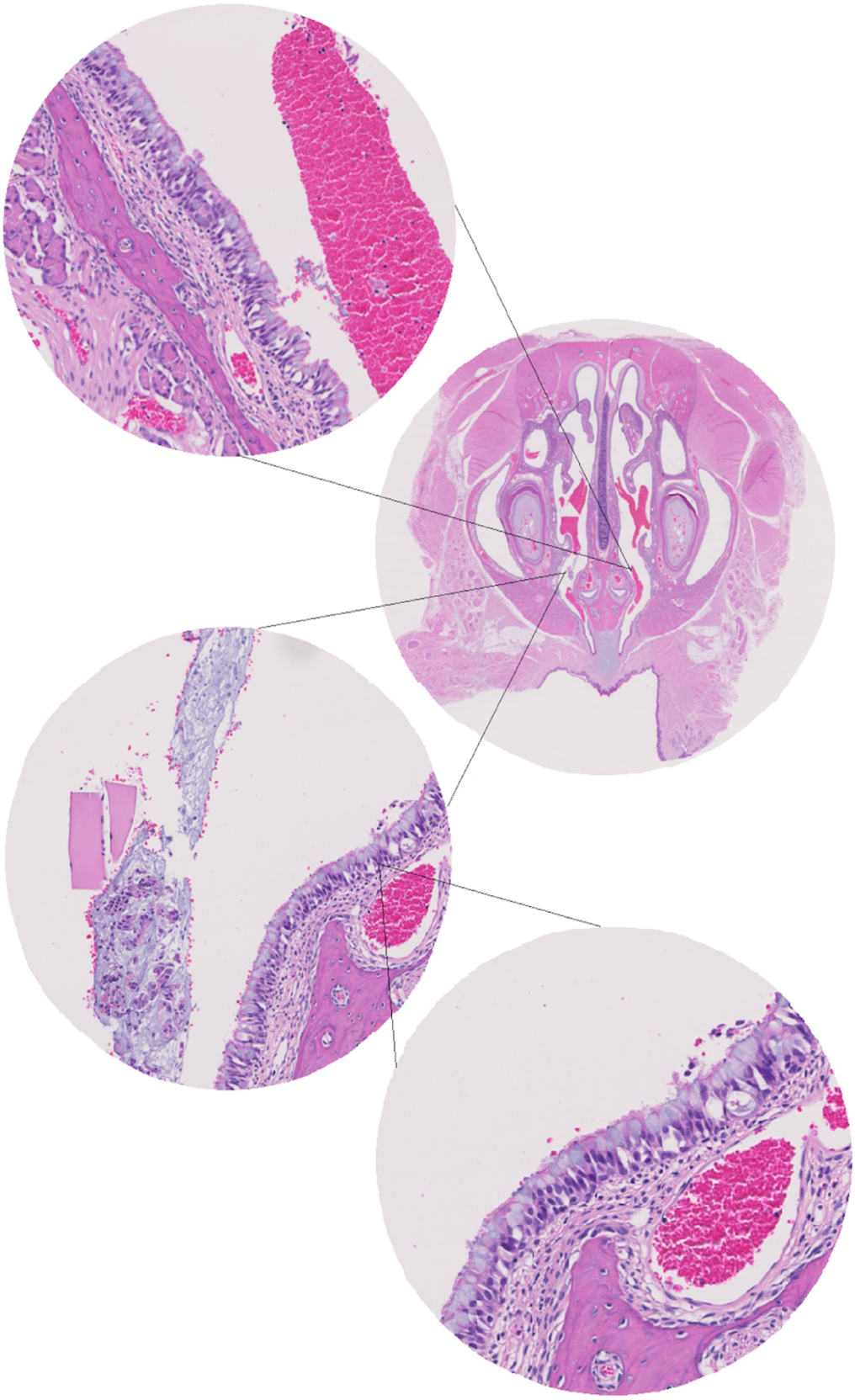
6. Supplementary Figures



Supplementary Figure 1. A labelled slide of areas of maximal inflammation mapping. A labelled area of maximal infiltrate (left). A labelled area of maximal infiltrate (right).

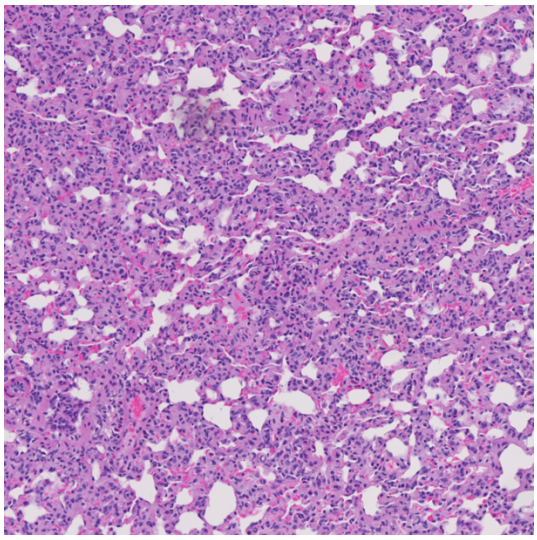


Supplementary Figure 2. Heavy lymphoplasmacytic infiltrate, with goblet cell hyperplasia in *S. aureus* CI_{908}.



Supplementary Figure 3. Goblet cell hyperplasia with an active section in *S. aureus* CI_{908}.

A



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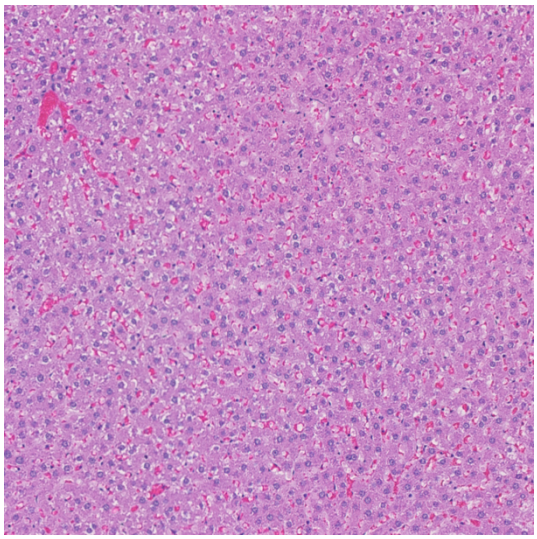

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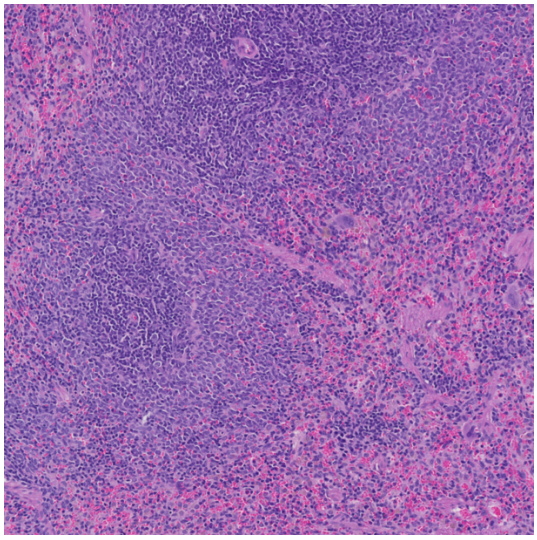

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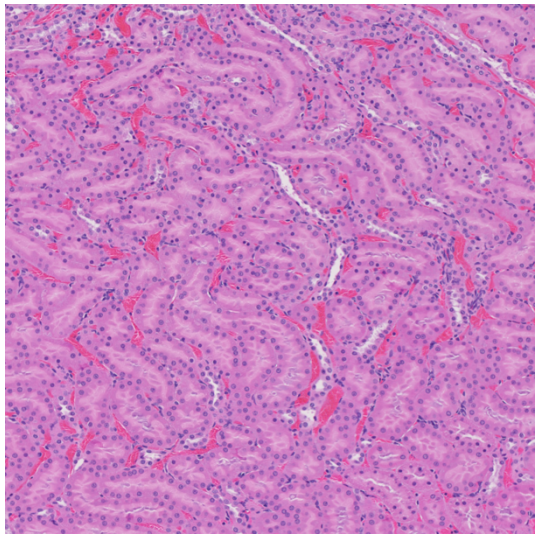

Supplementary Figure 4. Organ histology from 10mM mesalazine groups. (A) Lung histology (B) Liver histology. (C) Spleen histology. (D) Cardiac histology

CHAPTER 5: SYNOPSIS

The research confined in this thesis has progressed the knowledge of potential novel treatment options for CRS. It has also provided a new inflammatory small animal model that is cheap, easily accessible, and non-invasive that mimics the lymphoplasmacytic histopathology seen in a subset of patients with difficult to treat CRS. As discussed in this thesis, CRS is a disease with a diverse range of potential aetiologies and contributing factors. The current treatment modalities involve appropriate medical therapy followed by surgery. However, there is often poor disease control and a recurrence of the disease despite this. Traditionally, CRS has been divided into disease categories depending on the presence or the absence of nasal polyps. Recently however, there has been a larger emphasis placed on the inflammatory endotypes that are present within CRS, being type 2 and non-type 2 (type 1 and 3) inflammation. Classically, treatment has been focused on type 2 inflammation, largely driven by eosinophils, however treatment targeted at type 1 non-eosinophilic inflammation is lacking. Following an extensive literature review, further assessment of the drug mesalazine, a drug currently used in IBD, was warranted. The research within this thesis, therefore, includes firstly the solubilization and formulation of mesalazine into an appropriate sinus wash, and an investigation into its *in vitro* safety on nasoepithelial cells whilst retaining its anti-inflammatory properties. Secondly, the development of an appropriate animal inflammatory model that represented the inflammatory milieu involved in CRS histopathologically. Finally, an assessment of mesalazine's effects on inflammation, as well as its safety in an *in vivo* environment. This was done in an effort to address the question as to whether mesalazine could be used as a potential future treatment option in CRS.

In vitro evaluation of mesalazine

Initially, mesalazine was repurposed to a sinus wash, at an appropriate pH for the nasal cavity. Following this an *in vitro* study was conducted to evaluate the safety of the mesalazine wash on nasoepithelial cells, as well as its anti-inflammatory effects on THP-1 cells, which are used to study type 1 inflammation caused by macrophages *in vitro.* Macrophages arise from progenitor cells in the bone marrow and cannot replicate in their matured state, therefore the THP-1 cell line was used as it is acts like a macrophage, but still retains the ability to replicate. In this *in vitro* study, mesalazine's safety on nasoepithelial cells was assessed using toxicity studies as well as mucosal barrier structure and function tests. Results showed absence of toxicity with mesalazine applied at concentrations up to 20mM. Mesalazine's anti-inflammatory effects were tested by measuring the levels of TNF-α produced by THP-1 cells in the presence and absence of mesalazine at various concentrations. It was shown that mesalazine significantly reduced TNF-α levels at concentrations of 0.5mM and 1mM but not at higher concentrations. This apparent inverse dose response was thought to be secondary to precipitation. This suggested that mesalazine was safe to use on nasoepithelial cells in concentrations up to 20mM, whilst retaining its anti-inflammatory effects at 0.5mM and 1mM.

Establishment of an appropriate animal model

After establishing the safety and efficacy of mesalazine *in vitro*, it then needed to be tested in an environment that represented the complex interactions in living organisms. To do this, an animal model needed to be established that histopathologically mimicked CRS inflammation in an *in vivo* setting that was cheap, easy to access, whilst also prioritising the animal's

wellbeing by being non-invasive. This model was established using Sprague Dawley rats, who underwent a total of 30 days of exoprotein installation, or 14 days of exoprotein installation followed by 16 days of exoprotein and *S. aureus* installation. Two different *S. aureus* strains were used, C_{908} and C_{913}. Selection of these isolates was based on severe patients' disease severity scores. Both C_{908} and C_{913} strains caused sinonasal mucosal inflammation as evaluated on days 37 and 44. The inflammation observed was lymphoplasmacytic in nature and was representative of inflammation found histopathologically in a subset CRS patients. The inflammation was present both anteriorly and posteriorly, however trended towards a higher amount of inflammation anteriorly, which suggested that the bacterial load was greater at this location. CFU counts were also reflective of the inflammation scores observed. This set the foundation where the inflammation was present for 2 weeks after ceasing instillation of bacteria and paved the way for testing mesalazine at a BD dosing within that time period.

Mesalazine's anti-inflammatory effect *in vivo*

Once a sinonasal inflammatory model was established, the safety and efficacy of mesalazine was then investigated. It was established that mesalazine, given in twice daily dosing in concentrations of 1mM from day 14 and 30, as well as 10mM given from day 14, was effective at decreasing sinonasal inflammation by day 44. There was no significant difference on inflammation for mesalazine compared to positive controls treated with budesonide and clarithromycin. No significant effect of mesalazine was observed on CFU counts, consistent with mesalazine not having direct antibacterial effects on *S. aureus*. There were no systemic effects observed on histopathology, and no adverse events for all rats involved in the study.

This suggests that mesalazine *in vivo* had a significant effect on inflammation, and had similar anti-inflammatory effects compared to current treatment standards.

Concluding statement

This thesis establishes the safety and efficacy of mesalazine in both an *in vitro* and an *in vivo* environment. While there are currently multiple treatment options available, poor control and recurrence of CRS often occurs and there is a need to further investigate other treatment options. As we gain a further understanding of CRS, there is a growing focus on the underlying disease inflammatory phenotypes, as opposed to the classical differentiation of CRS with or without nasal polyps. As there is a shift in the understanding of CRS as different phenotypes, treatment options should increasingly be targeted at the underlying inflammation present. Mesalazine has strong anti-inflammatory effects on lymphoplasmacytic inflammation in concentrations of 1mM, and partial anti-inflammatory effects at 10mM, as well as an excellent safety profile up to 10mM in an *in vivo* setting. Mesalazine therefore holds potential as a treatment option for CRS. Ultimately, further research needs to be undertaken to assess if the safety and efficacy of mesalazine in the *in vivo* models translate into clinical practise in humans. Further research could also focus on a multimodal treatment, where mesalazine is used in combination with other treatment modalities. Whilst there are multiple topical treatment options for CRS that are proposed or were in use for both antimicrobial and anti-inflammation purposes, there still remains significant space for further high-quality research re-evaluating these treatment options.

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