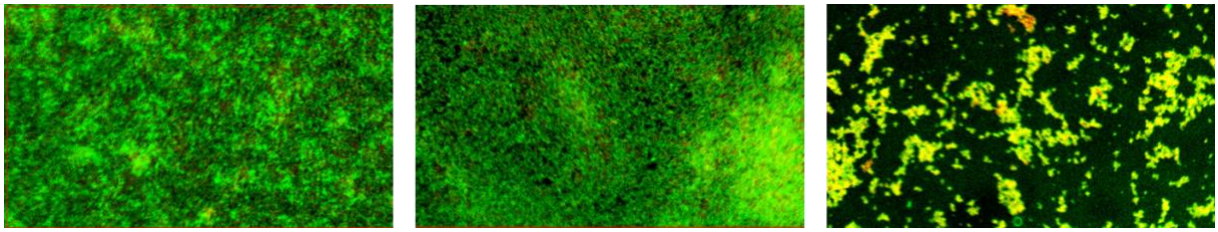
Corticosteroids and the Sinonasal Microbiome



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A thesis submitted for the degree of

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Abstract

Chronic rhinosinusitis (CRS), a disease of multifactorial aetiology characterised by the presence of infection and inflammation. The initial triggering factor/s and the perpetuating factor/s in the disease progression of CRS remains a matter of much debate. As a result of this, the treatment modalities in CRS is directed against infections using antibiotics and against inflammation using oral and topical steroids. Quiet often these therapies are given alone or in combinations for varying duration and frequency to bring clinical improvement in patients. The most unfortunate outcome is the chronicity of this disease despite multiple courses of medical therapies and in many cases, sinus surgeries.

With the emergence of concepts of the microbiome and its implications in health and disease, and many chronic diseases linked to specific microbial alterations, the CRS microbiome have been extensively studied. This has led to the understanding that microbial richness and abundance are very individual specific and there are probably no clear demarcations between a healthy and a diseased sinus. However, a microbial dysbiosis could be associated with an inclination towards unhealthy state. Although antibiotics have been used to effectively to control a number of infections, their irrational use have led to the emergence of multidrug resistant organisms as well as microbial dysbiosis in humans with a potential to develop diseases. CRS is the number one reported condition for which antibiotics are prescribed at the peripheral general practice.

Despite providing excellent clinical outcomes, corticosteroids are often combined with or are replaced by antibiotics in the treatment of CRS. This thesis, attempted to see if corticosteroids in addition to its anti-inflammatory property, had an effect on bacterial growth. We also investigated the usefulness and efficacy of antibiotics over corticosteroid therapy to determine if antibiotics are warranted as first line therapy in the medical management of CRS. Towards this, the aims of the study were:

1. To look at the potential antibacterial action of corticosteroid and the different excipients in a commercial preparation of topical nasal steroid in vitro.

2. To investigate the growth and metabolic response of a common CRS pathogen, *Staphylococcus aureus* in the presence of different concentration of corticosteroid.

3. Finally, to conduct a double-blinded, randomised placebo-controlled trial to better understand the usefulness and efficacy of corticosteroid and antibiotic therapies in CRS along with the probable microbiome changes they bring about and its influence on the clinical outcomes in these patients.

Thesis declaration

I, Lisa Mary Cherian certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Lisa Mary Cherian

Acknowledgements

I will sing to you Lord! I will celebrate your kindness and your justice.

Please help me learn and do the right thing, and I will be honest and fair in my kingdom. Psalms 101

All wisdom comes from the Lord, and so do common sense and understanding. Prov. 2:6

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Publications arising from thesis

Effect of commercial nasal steroid preparation on bacterial growth

Lisa M Cherian, Katharina Richter, Clare Cooksley, Peter-John Wormald, Alkis Psaltis, Sarah Vreugde

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Staphylococcus aureus **metabolises hydrocortisone: effects on anti-inflammatory activity and antimicrobial susceptibility**

Lisa M Cherian, Mahnaz Ramezanpour, Andrew James Hayes, Shari Javadiyan, Hua Hu, Peter-John Wormald, Alkis James Psaltis, Sarah Vreugde

Unpublished and unsubmitted work written in manuscript style

The clinical and microbiome outcomes of medical treatments in Chronic rhinosinusitis: A double-blinded randomised placebo-controlled trial

Lisa M Cherian, Ahmed Bassiouni, Clare M Cooksley, Sarah Vreugde, Peter-John Wormald, and Alkis James Psaltis

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Presentation arising from thesis

1. 57th Australian Society of Medical Research National Scientific Conference, Adelaide, Australia, November 2018: Oral presentation

2. American Rhinology Society, Ohio, USA, October 2018: Oral presentation

3. TQEH Research Expo, Adelaide, Australia, October 2018: Oral presentation

4. Australian Society of Medical Research (ASMR), Annual scientific meeting, Adelaide, Australia, June 2018: Oral presentation

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Abbreviations

CRS – Chronic rhinosinusitis

S. aureus – Staphylococcus aureus

MRSA-Methicillin resistantstaphylococcus aureus

CNS- Coagulase negative staphylococcus

CT- Computerized tomography

ECM- Extracellular matrix

S. epidermidis- Staphylococcus epidermidis

P. acnes- Propionibacterim acnes

C. accolens- Corynebacterium accolens

H. influenza- Hemophilus influenza

S. pneumoniae- Streptococcus pneumoniae

S. pyogenes- Staphylococcus pyogenes

S.haemolyticus-Staphylococcus haemolyticus

S. capitis- Staphylococcus capitis

S. hominis- Staphylococcus hominis

S. warneri- Staphylococcus warneri

S. lugdunensis- Staphylococcus lugdunensis

CNS- Coagulase negative staphylococcus

M. catarrhalis- Moraxella catarrhalis

C. pseudodiphtheriticum- Corynebacterium

BMP- Beclomethasone monopropionate

FP- Fluticasone propionate

MF- Mometasone furoate

BUD- Budesonide

EPOS- European Position Paper on Rhinosinusitis

ARS- Acute rhinosinusitis

qPCR- Quantitative polymerase chain reaction

FISH- Fluorescence in situ hybridization

16sRNA- 16S ribosomal ribonucleic acid

WMS- Whole Metagenome Shotgun

PICRUSt- Phylogenetic Investigation of Communities by Reconstruction of Unobserved States

DS- Dirichlet states

HSDs- Hydroxysteroid dehydrogenases

MIC- Minimum inhibitory concentration

EDTA- Ethylene-diamine tetraacetic acid

SCTD-Sodium citrate tribasic dihydrate

AC- Anhydrous citric acid

Tween 80-Polysorbate 80

pseudodiphtheriticum

K. pneumonia- Klebsiella pneumoniae

C. testosteroni- Comamonas testosteroni

A. aerogene- Aerobacter aerogene

NGS- Next generation sequencing

DNA – Deoxyribonucleic acid

RNA- Ribonucleic acid

OTUs- Operational taxonomic units

RCT- Randomized controlled trial

HPA- Hypothalamo pituitary axis

FDA- Food and Drug Administration

AR – Allergic rhinitis

GORD- Gastroesophageal reflux disease

INCS- Intranasal corticosteroid

BDP- Beclomethasone dipropionate

RCT- Randomised controlled trial

NaCl- Sodium chloride

LDH-Lactate dehydrogenase

CFU- Colony forming units

MAD- Mucosal atomizer device

SNOT 22- Sino-Nasal-Outcome Test 22

HCHS- Hydrocortisone 21-hemisuccinate

HPLC- High performance liquid chromatography

MBIC- Minimal biofilm inhibitory concentration

MTT- Methythiazol tetrazolium

ELIZA- Enzyme-Linked Immunosorbent Assay

QIIME 2- Quantitative Insights In to Microbial Ecology

ADSS- Adelaide disease severity score

LKS- modified Lund Kennedy scoring

PCR- Polymerase chain reaction

LMS- Lund-Mackay score

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Chapter 1- A systematic review of the literature

1.1 Chronic rhinosinusitis – definition

Chronic rhinosinusitis (CRS) is a common complex clinical syndrome of multifactorial aetiology. A large number of guidelines, consensus documents, and position papers have attempted to define CRS[1-5]. Most of these guidelines define CRS as chronic inflammation of the nose and paranasal sinuses lasting for at least 8-12 weeks and having at least two of the following clinical symptoms namely, 1. Nasal obstruction, 2. Anterior or posterior nasal discharge, 3. Facial pain or pressure, and 4. Reduction or loss of sensation of smell. Along with these subjective findings, for a diagnosis of CRS, there should also be objective evidence of nasal polyps, mucopurulent discharge or mucosal oedema on nasal endoscopy or mucosal changes on computerized tomography (CT) of the paranasal sinuses.

CRS can further be classified into two phenotypes based on nasal endoscopy as CRS without nasal polyps (CRSsNP) and CRS with nasal polyps (CRSwNP)[4]. Another classification based on the duration of symptoms distinguishes CRS into acute, subacute, chronic, recurrent acute and acute exacerbations of chronic CRS[6].

1.2 Incidence

Current data from many studies suggests that CRS affects around 5-15 % of the general population[7, 8]. In the western population, the prevalence ranges from 4.5 to 12.5% and is typically less in the developing countries[9]. It affects around as 1.8 million (9.2%) Australians, utilizes a large amount of health expenditure and adversely affects the quality of life of those affected[10]. Some studies have even shown that patients with CRS have a low health state utility values and is comparable to chronic disease processes such as asthma, ischemic heart disease, end-stage renal and liver disease and neurocognitive disorders[11].

1.3 Etiopathogenesis

The etiopathogenesis of CRS with its heterogeneous presentations is largely unknown, even

though a multifactorial mechanism is considered likely. A number of extensively researched hypothesis have been proposed which can be broadly classified into host-derived factors (eicosanoid hypothesis and the immune barrier hypothesis) and environmental factors (fungal hypothesis(12), superantigen hypothesis(13), the biofilm hypothesis(14), the microbiome hypothesis and intracellular bacterial infection(15). The environmental factors are influenced by allergens (16) and toxins(4) and the host factors by genetic predisposition(17) and defective local or systemic immune responses(18).

Inflammation and infection coexist in CRS, questioning the role of either as the initiating factor of the disease. While certain studies describe immune dysfunction, inflamed sinonasal mucosa and sinus outflow obstruction caused by allergic a nonallergic diseases as the triggering elements of inflammation in CRS, other studies demonstrate the role of resident commensal and pathogenic bacteria and their byproducts in the establishment and continuation of inflammation(19, 20). In a small group of patients with diseases like Kartagener's syndrome, cystic fibrosis, Wegener's granulomatosis and sarcoidosis CRS results due to the disease factors affecting the drainage of secretions in the sinuses leading to chronic inflammation(21).

1.3.1 Anatomical variations

The identification of the presence of paranasal sinuses dates back in history to the ancient Egyptians in the 3700 to 1500 BC(22). A better understanding of the anatomy of the nose and paranasal sinuses came about with the basic work describing these structures by Emil Zuckerkandl in the 1870s(23). The paranasal sinuses in humans, generally are classified into maxillary sinuses located below the eye socket, anterior and posterior ethmoid cells, frontal and the sphenoid sinuses. In reality, however, these sinuses are connected to each other or are closely related with regard to their drainage pattern. The area where the sinuses are located is called the lateral wall of the nose and various terminologies are used to define specific cells and spaces. The lateral wall anatomy is exceptionally complicated apart from

having a great number of variations. Middle meatus is a space lateral to the middle turbinate into which the maxillary, frontal and the anterior ethmoidal cells drain. The posterior ethmoidal cells open into the superior meatus and the sphenoid sinus into the sphenoethmoidal recess located medial to the superior turbinate(24).

The majority of the structures of the lateral wall including the sinuses are lined by respiratory ciliated columnar epithelium. Scattered in this layer are goblet cells, eosinophils, mast cells, neutrophils and lymphocytes(25, 26). The mucosal barrier in the nasal cavity comprising IgA and other protective mediators are secreted by the goblet cells (27) and the largest production of nasal secretion in the nasal cavity is mostly from the seromucinous glands present in the submucosa(25). On the septum, the maximum number of goblet cells are seen at the posterior and inferior part. Contrary to this, glands are more abundant in the anterior and superior parts(28). Compared to the other sinuses maxillary sinus has the maximum density of goblet cells.

Where certain studies do not support the contribution of anatomical variants such as concha bullosa, Haller cells, paradoxical middle turbinate, and deviated nasal septum to the development of CRS, certain other studies suggest that anatomical variations may represent one of the multiple factors that influence the expression of the disease. This was more so in patients who developed CRSsNP(29).

1.3.2 Physiology and its dysfunction

Although the exact significance of the presence of paranasal sinuses is debatable, their role in the multiple functions of the nose cannot be ignored. These include vocal resonance, diminution of auditory feedback, air conditioning, pressure damper, reduction of skull weight, flotation of skull in water, mechanical rigidity and heat insulation. However, the major relevance of the presence of paranasal sinus lies in its propensity for causing diseases.

Mucociliary clearance and mechanical defence of the nasal cavity

Mucociliary transport is the physiologic process by which secretions and unwanted particles are eliminated from the nose. This is essentially brought about by the cilia of the respiratory epithelial cells. The cilia consisting of outer nine paired microtubules and an inner paired microtubule propel the mucous blanket backward from the nasal cavity to the pharynx. The energy for this is derived from the conversion of ATP to ADP by the ATPase located at the outer and inner dynein arms of the outer microtubules(30). Particles of size from 3 to 0.5 um are filtered by the nasal mucosa and pushed posteriorly and cleared within 10-20 minutes by the ciliary beat pattern which consists of an effector propulsive stroke and a slow recovery stroke(25). The IgA in the nasal secretions provides the first line of defense against the inhaled bacteria and viruses(25, 27). Apart from the cilia the epithelial cells of the nasal mucosa also play a part in the mechanical defenses by regulating the permeability of mucosa in the event of an inflammatory insult. This is achieved by establishing cell to cell contact in a way that may permit the entry of blood plasma to the lumen but prevent any foreign particles penetrate the airway tissue(27). The surface receptors like CD14 and Toll-like receptors on the epithelial cells can also identify microbial pathogen-associated patterns(27, 31).

Apart from this, the drainage of secretions from the different sinuses also follow specific patterns. In the maxillary sinus, the mucociliary clearance is spiral towards the natural ostium and against gravity. The frontal sinus, ethmoids and sphenoid sinus drainage are downward towards gravity(32). In conditions like cystic fibrosis, Kartagener's syndrome and Youngs disease there is an inherent defect in this mucociliary clearance and so chronic infection of the paranasal sinuses in these patients is a common finding. The defective mucociliary clearance leads to stagnation of secretions which probably facilitates development and persistence of infection in CRS. Outside the genetic conditions, a recent study on a rabbit model of sinusitis has demonstrated a decreased periciliary depth and

mucous transport on the functional micro-anatomy study using micro-optical coherence tomography in the early stages (2 weeks) of CRS development. They also showed that the mucous fermenting microbes (*Lactobacillales, Bacteroidales*) dominated on week 2 and there was a significant shift to potential pathogens (e.g., *Pseudomonas*, *Burkholderia*) by week 14 suggesting that an abnormal mucociliary host defense mechanism could promote the growth of pathogens due to the decreased bacterial clearance capacity(33).

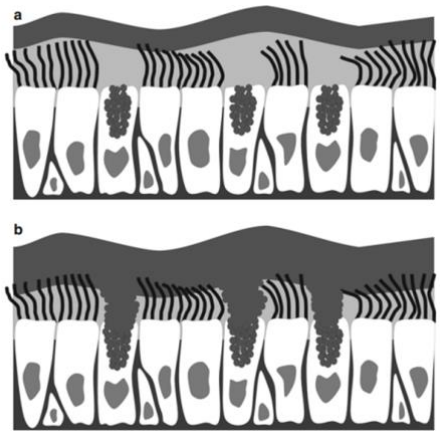

Figure 1: a Normal mucous blanket, b Pathological mucous blanket with mucous hypersecretion(34)

1.3.3 Immunology

The sinonasal epithelial cell layer acts as the first line of defence against foreign invasion. The structural integrity of this barrier is maintained by tight junctions which also control the epithelial permeability. Apart from being a structural barrier, they provide immunological protection through innate toll-like receptors and secretory IgA transport. Tight junctions consist of a number of scaffold adaptors and transmembrane proteins(35). A defect in these structures has been shown to be associated with polyp formation in CRSwNP. Soyka et al. in their study showed a defective barrier function with a decreased expression of TJ proteins and decreased mRNA levels in CRS patients when equated with normal subjects(35).

The composition of the mucous and innate immune response in the nose

Around 80% of the dry weight of the mucous is constituted by glycoproteins. These include sialomucins, fucomucins, and sulphomucins and they can be acidic or neutral. They are responsible for the viscosity and elasticity of the mucus(36). Water and ions enter the mucous through transudation and active ion transport(37). Proteins form another component in the mucus. They are procured from the circulation (complement, α-2rmacroglobulin, C reactive proteins) or are produced by the nasal mucosa and surface cells. Antiproteases like α-antitrypsin, αl-antichymotrypsin, α 2macroglobulin produced by leukocytes are seen to be increased during infection. Lactoferrin found only in the nasal secretions bind to heavy metal ions like iron thereby preventing the growth of facultative and aerobic gram-positive and -negative bacteria and also Candida albicans, which depend on iron for their metabolism (27, 38). Lysozymes are potent bacteriostatic agents against certain bacteria without a capsule and also increases the lysis potential of antibody-activated complements on gram-negative bacteria like E-coli(27, 38). Lactoperoxidases also form toxic molecules to eliminate bacteria(38). The enzymes lysozyme and lactoferrin are however not effective against viral infections(27). Complement system with an array of proteins is involved in the opsonization of pathogens by phagocytes(27). Other notable molecules are secretory leukoprotease inhibitor, uric acid, peroxidase, aminopeptidase, secretory phospholipase A2, and defensins(27). In the event of an insult, the resulting inflammation leads to increased blood flow to the mucosa and also recruitment and activation of inflammatory cells such as neutrophils and macrophages. Immunoglobulins, complement, and proteases are carried to the site to clear the pathogens(39). Neutrophils with toxic granules create an oxidative burst also leading to an increase in the level of nitric oxide in the sinuses which is an important agent in the defense mechanism against pathogens(27).

Adaptive immune response in the nose

Apart from the secretory immunoglobulin Ig A released in response to exposure to antigens,

immunoglobulins are also released against polysaccharide capsule of bacteria like Haemophilus influenza B and Streptococcus pneumoniae[27]. Specific cellular responses occur as a result of presentation and activation of T and B lymphocytes by foreign antigens[38]. A delayed IgE mediated allergic response occurs following initial antigen exposure. Subsequent subjection to the same antigen leads to the release of preformed (like histamine, platelet activating factor) and newly formed (like leukotrienes, prostaglandins) bioactive mediators. The resulting neural and vascular responses lead to allergic symptoms like nasal congestion, postnasal drip, watery rhinorrhoea, nasal and ocular itching, and sneezing[40].

Immunology in CRS

The inflammatory pattern seen in CRSsNP is TH1 predominant and neutrophil biased. Whereas in CRSwNP a Th2 dominant an eosinophil rich inflammation is seen[41]. The eosinophilic pattern seen in CRSwNP, however, is found to vary among the different human races and for example, a non-eosinophilic inflammation is seen in more than half of Chinese patients with CRSwNP[42]. This suggests the involvement of genetic or epigenetic mechanisms in the development of the disease and polyposis. The understanding of innate immune responses and epithelial barrier functions of the host would help us better understand the inflammatory profile seen in the disease and also the mechanisms by which various medical therapies alter these profiles.

Tissue remodelling in CRS

Remodelling is a reorganization or restoration of tissue by extracellular matrix (ECM) production and degradation which occurs following an injury. The end result of this process could be a return to normalcy or a change to pathologic tissue[43]. There are indications of remodelling in CRS and a distinctive histological feature of CRSwNP is albumin accumulation, oedema, eosinophilic infiltration, and pseudocyst formation. Whereas in CRSsNP there is neutrophilic inflammation and marked fibrosis[43, 44]. Presence of collagen

in CRS detected with picrosirius staining of the tissue showed an increase in collagen in CRSsNP compared to CRSwNP[45]. ECM breakdown is modulated by a family of matrix metalloproteinases (MMPs) and their inhibitors, called the tissue inhibitors of metalloproteinases (TIMPs). Both their levels are increased in CRSsNP whereas only MMP-9 is increased in CRSwNP. This might be another reason for the different overt and histopathological differences seen in the CRS phenotypes[46]. In another study comparing early polyp with mature ethmoidal polyp showed that epithelial loss was much pronounced in early polyps. Whereas, in mature polyps junctional proteins E-cadherin, zonula occludens-1 (ZO-1), and occludin showed greater deficits[47]. According to Takabayashi et al.[48], deposition of fibrin mesh in tissue leads to polyp growth. Due to the impaired epithelial expression of tissue plasminogen activator (tPA) polyps have a decreased fibrinolytic activity. Factor XIIIA from M2 macrophages in the tissue is the cause for to fibrin cross-links.

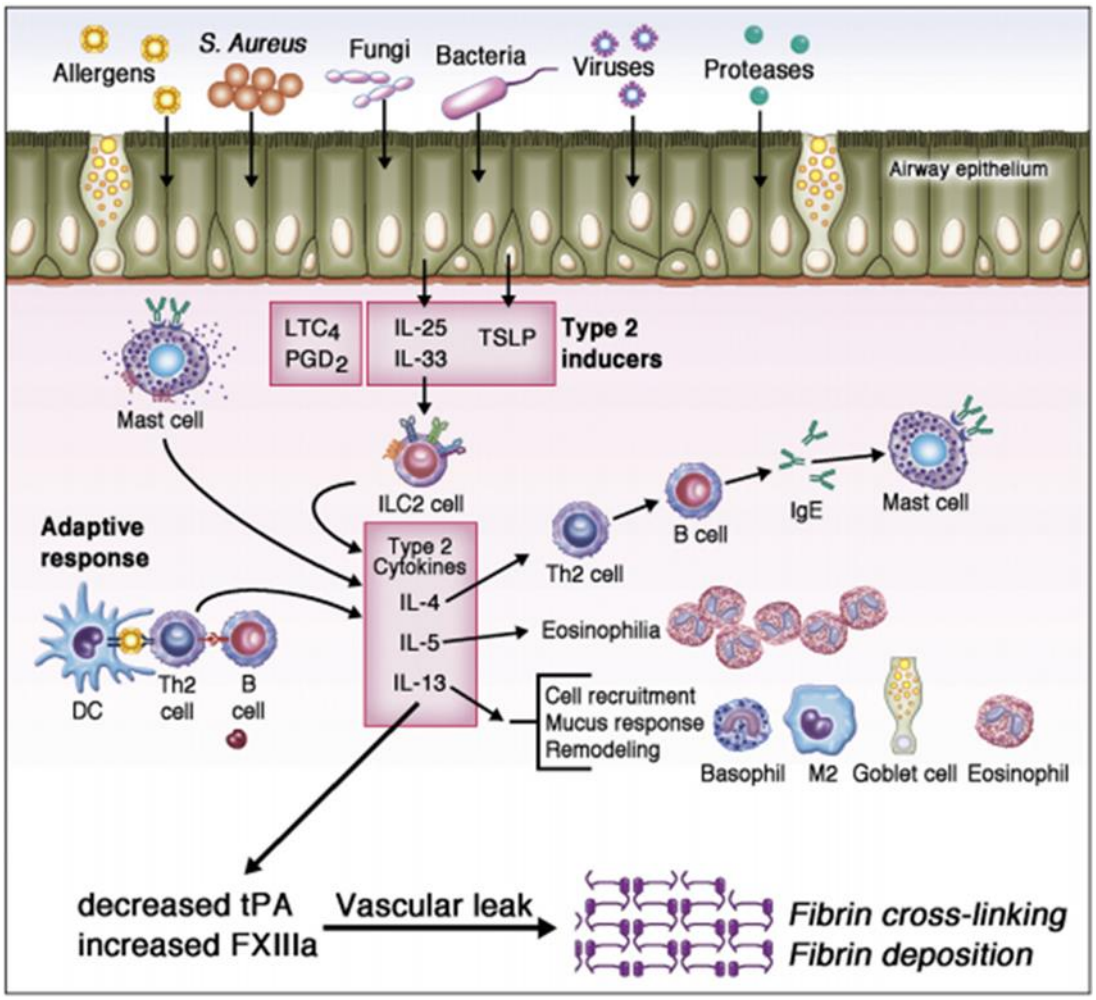

Figure 2: Role of host immune response in chronic rhinosinusitis(49)

Steroids (oral and topical) with their anti-inflammatory properties and antibiotics like Doxycycline with their anti MMP-9 effects potentially intervene with remodelling and affect the pathological sequelae in CRS(50).

1.3.4 Fungus

Fungal elements as an etiological agent were described when positive fungal cultures and eosinophilia was demonstrated in CRS patients. It was hypothesized that fungal elements could become trapped in the nasal mucous secretions and trigger an immune reaction. This would then lead to the recruitment of eosinophils that attacked the fungi and released inflammatory toxic mediators. This whole inflammatory process would then result in the observed polyposis in CRS. The fungal hypothesis, however, failed to hold and currently,

fungi are recognized as physiologic flora of the upper respiratory tract and "innocent bystanders in the majority of cases of CRS. However, fungal colonization does play an important role in patients where the fungus is seen either on microscopy or culture in the thick eosinophilic mucous. This subset of patients is now recognized as "allergic fungal sinusitis"(51). The burden of fungal elements in CRS patients identified using next generation sequencing (NGS) showed that fungal biomass estimated through ITS amplicon concentration correlated with traditional fungal detection techniques and CT double densities thus asserting the current understanding that fungal dysbiosis occurs only in a selected subgroup of CRS patients(52).

1.3.5 Bacteria

Bacteria are prokaryotic organisms which are unicellular. They have a rigid cell wall but they lack nucleus and membrane bound organelles. Usually, a spherical bacterium will have an average diameter of 0.5 to 2 μm and rod or filamentous bacteria 0.25 to 1 μm. The length varies from 1 to 10 μm in the rod-shaped bacterium.

The human body is colonized with a multitude of different microorganisms including bacteria, fungi, and virus. It is considered that the interaction between these microbes is responsible for the balance of various bodily functions. The commensal bacteria are thought to interfere with the pathogenic bacteria through mechanisms like competing for cell surface receptors, thus inhibiting adherence of pathogens, producing antibacterial peptides and other antibacterial metabolites like hydrogen peroxide. An abundance of commensal bacteria may also starve the pathogens for nutrients (exploitative competition)(53). A disruption or shift in the microbial community resulting in dysbiosis could cause an imbalance of the delicate mutual interactions between bacteria and bacteria and host. This is thought to contribute to disease onset, progression, or duration of conditions like antibiotic-associated diarrhoea, bacterial vaginosis, celiac disease, colorectal cancer, cystic fibrosis, oesophageal disease, Crohn's disease and ulcerative colitis (collectively referred to as inflammatory bowel

diseases [IBD]), irritable bowel syndrome, necrotizing enterocolitis, non-bacterial prostatitis, pre-term birth, obesity, pouchitis, and psoriasis where culture-independent sequencing technologies have shown the presence of significant dysbiosis(54).

Despite a large number of studies describing several species of bacteria being abundant in CRS when compared to that of control, there is much debate on the contribution of bacteria in the pathogenesis of CRS. This is due to the number of other studies which fail to identify a specific type of organism with CRS compared to control. It further supports the theory of an ongoing paradigm shift in infectious diseases away from one causative biological agent to a pathogen-dysbiotic microbiome interaction determining the disease outcome(55).

1.3.6 Other factors

*Allergic rhinitis (AR)*has often been associated with the development of CRS. Their symptoms mostly overlap and hence make the distinction between the conditions difficult. AR and CRS are thought to share come common cellular and molecular mechanism in their pathophysiology. This is mostly through eosinophil and mast cell infiltration and IgE expression. Inflammation of the nose in AR would affect the sinuses and show changes on CT as well as endoscopy. However, only AR shows a positive reaction with the nasal challenge of allergens and responds to treatment with allergen immunotherapy. Although current data support a role of on-going allergic inflammation in the development of acute bacterial rhinosinusitis, the data supporting interaction between CRS and AR are insufficient to come to any conclusion(29).

Immune deficiency arising from congenital and acquired causes, chemotherapy in malignancies, hyper immunoglobulin E syndrome, use of biologics like anti-TNF can all cause a sinus infection. These patients almost always have the most refractory and difficult to treat disease. A metanalysis of recurrent uncontrollable rhinosinusitis showed a pooled

IgG, IgA, and IgM deficiency prevalence of 12.7% in patients with recurrent CRS and 22.8% in patients with difficult-to-treat CRS(56).

Dental diseases are known to cause maxillary sinusitis secondary to perinodal infection or dental manipulations.

Gastro-oesophageal reflux disease (GORD) and CRS are often seen to exist together in patients. It is proposed that gastric contents could reflux into the nasal cavity causing mucosal inflammation. Prospective studies have demonstrated the co-existence of GORD and CRS in children and also the improvement in CRS symptoms when patients were treated for GORD(57).

Autoimmune disorders like Churg-Strauss syndrome and selective IgE deficiency have been associated with the development of CRS

Tobacco smoke has been known to adversely affect the sinonasal mucosa in multiple ways. Apart from causing irritation of the eye and nose, nasal congestion, rhinorrhoea, and increased nasal resistance smoke can reduce mucociliary transport, alter innate immune effector proteins, suppress toll-like receptor-mediated pathways, increase squamous metaplasia, and decrease olfactory function(58). Individuals exposed to second-hand smoking during childhood or currently have an increased risk of developing CRS compared to controls. These patients also tend to have worse symptoms and treatment outcomes(59).

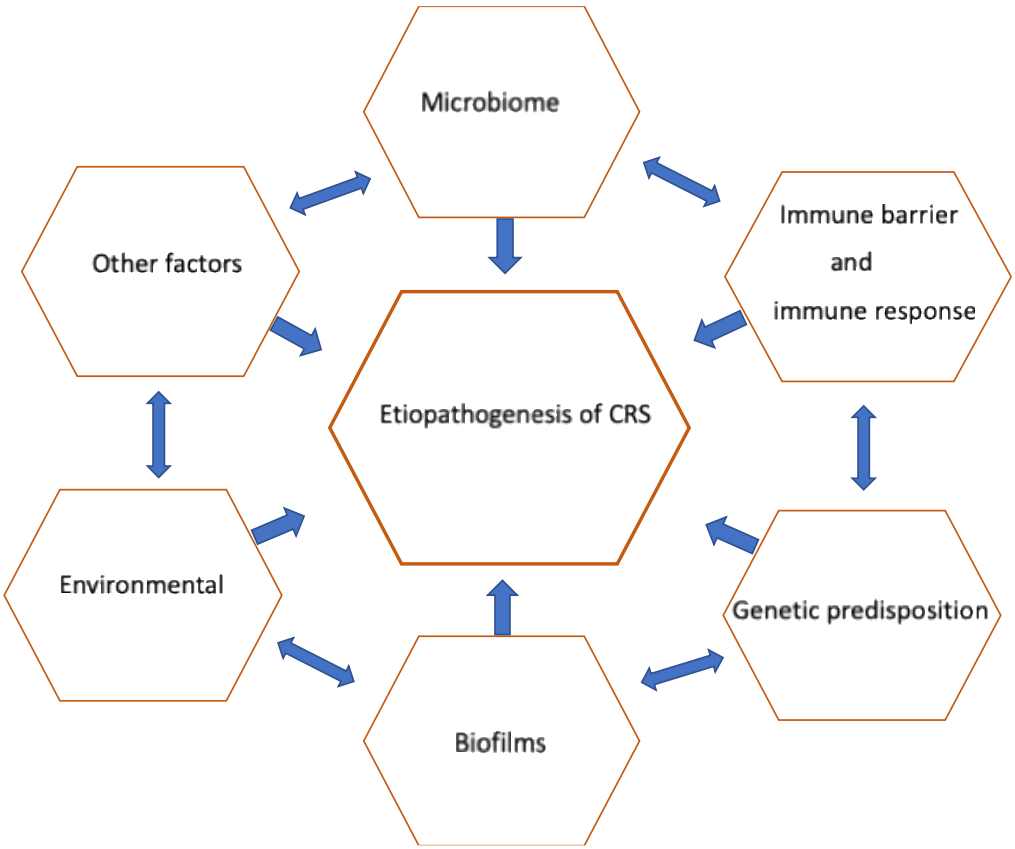

Figure 3: Etiopathogenesis of CRS - the interplay of multiple factors

1.4 Bacteriology in CRS

1.4.1 Bacteria isolation and culture techniques

The prior understanding was that the sinuses were sterile in healthy people. This was, however, proved wrong when bacteria were cultured from the middle meatus and maxillary sinus of healthy individuals(60). These findings led to the theory that bacteria could be present in health and disease of the sinuses.

The isolation of bacteria from the sinuses is using standard laboratories techniques have evolved over the years. Culture-based techniques yield information about the role of the individual organism and their causative mechanisms eliciting disease in the host. There is however a large disparity between various studies trying to isolate the common pathogenic organisms in CRS. The issues could be attributed to the different sampling methods, inability to obtain swabs from specific site (like middle meatus or ethmoid cavity) due to

contamination from the adjacent nasal sites, presence, and absence of polyps, time and method of culture after sample collection, variability in the patient selection including age, recurrent disease, and multiple surgeries. Again, most studies do not quantify the amount of each species of bacteria identified and this would make it difficult to assess the contribution of the organism in the inflammatory milieu. The isolation of anaerobic bacteria requires specific, laborious and more expensive techniques and they are, therefore, often undetected.

Never-the-less, culture based bacteriological isolations remain the standard practice to identify microbes in most clinical settings as it is easy to perform, cost-effective and gives a species level identification and the ability to assess biophysiological responses of the bacteria. Vigorous culture-based techniques in a study were able to identify around 139 distinct bacterial species within most subjects, regardless of disease status. This included *Staphylococcus epidermidis (S. epidermidis), Propionibacterium acnes (P. acnes), Corynebacterium accolens (C. accolens), Corynebacterium tuberculostearicum, Staphylococcus aureus (S. aureus), Propionibacterium avidum, Propionibacterium granulosum, and Finegoldiamagna*. Other common taxa included *Staphylococcus haemolyticus (S. haemolyticus), Staphylococcus capitis (S. capitis), Staphylococcus hominis (S. hominis), Staphylococcus warneri (S. warneri), Staphylococcus lugdunensis (S. lugdunensis), Streptococcus mitis-S. oralis, Streptococcus parasanguinis,* as well as strict anaerobes from the genera *Clostridium, Anaerococcus, Finegoldia, Parvimonas, Peptoniphilus, Veillonella,* and *Fusobacterium*(61).

Figure 4: MALDI-TOF mass spectrometry for identification of microbes from positive cultures(62)

1.4.2 Bacteria in health

The bacterial profile of the paranasal sinuses has been extensively studied and a number of studies have come out with conflicting reports. Axelsson and Brorson in the study showed that *Staphylococci* and *Diphtheroid* species predominated in the nose of healthy controls and *Pneumococci* and *Hemophilus influenza* (*H. influenza*) in sinus aspirates from acute maxillary sinusitis. *Staphylococci* were considered a nasal contaminant rather than a pathogen. However, many of their cultures were growth negative(63). In a study by Jousimies-Somer et al (64)it was seen that in healthy subjects, *H. influenza, Streptococcus pneumoniae (S. pneumoniae), Staphylococcus pyogenes (S pyogenes), and Branhamella catarrhalis* were infrequently isolated. A Pilot study by Allen et al (65)looking at the bacteriology of the nose by analysing nasal lavage fluid samples using traditional bacterial culture methods and microarray techniques showed that there was no change in the bacterial load when the healthy volunteers were infected with rhinovirus. The microarray technique moreover, identified a few bacteria that could not be cultured in the lab. All recruits had *S. epidermidis/coagulase negative staphylococcus* in their nasal sample. Doyle PW and

Woodham JD[66]analysed the nasal biopsy specimens from the ethmoid sinuses using aerobic and anaerobic culture techniques. They saw that the non-pathogenic and the most common clinical isolate was *Coagulase negativestaphylococcus (CNS)*. The most common pathogenic organism isolated was *S. aureus* and this was followed by members of the *Enterobacteriaceae* family. This was quite different from the other studies which showed that *H. influenza* and *S. pneumonia* as the predominant bacteria. Most of these studies, however, looked at the microbiology of the maxillary sinus. Some of these studies also had anaerobes as the predominant organism isolated [67-70].

1.4.3 Bacteria in CRS

A recent study by Zheng-Wei et.al[71] comparing the microbiology of the middle meatus in patients with CRSwNP, CRSsNP and controls found that the most common bacterial species in CRSwNP were *CNS*, *Corynebacterium*, and *S. epidermidis*. In CRSsNP it was *S. epidermidis*, *Corynebacterium*, *CNS*, and *S. aureus* and in the control group, *S. epidermidis*, *CNS and S. aureus* were frequently isolated. Another interesting finding was that they identified a relatively high proportion of *Citrobacter* in the CRSwNP group. This study also tried to categorize the samples into subgroups according to the percentage of eosinophils present in the peripheral blood study. They found that in patients with CRSwNP, the isolation of *S. aureus* was higher in the subgroup which had an increased percentage of eosinophils (>5) whereas *S. epidermidis* was higher in the subgroup with a low percentage of eosinophils. The study concluded that *S. aureus* may promote an eosinophilic inflammatory response, while *S. epidermidis* a non-eosinophilic inflammatory response. A retrospective analysis of the culture results from 513 CRS patients was done by Cleland et al.[72] to determine the impact of surgery and factors like the presence of polyps, asthma, and aspirin sensitivity on the bacteriology in CRS. The study demonstrated that revision patients were more likely to have a positive culture with *S. aureus and P. aeruginosa* being predominant pathogens. The increase in *P. aeruginosa* was also demonstrated in the Zheng-

Wei et.al[71] study suggesting that surgical procedures with the concomitant use of antibiotics promote the colonization of opportunistic organisms. There was also a significantly higher rate of *S. aureus* in CRS patients undergoing revision surgery.

When bacterial isolation was done from sinus tissue biopsy in CRSwNP, *S. epidermidis* and *S. aureus* was found to be a most prevalent organism. This was followed by *S. haemolyticus*, *P. aeruginosa*, *Moraxella catarrhalis (M. catarrhalis)*, *S. pneumoniae*, *S. warneri* and *S. lugdunensis*[73].

The systematic review by Brook I[74] identifies *S. aureus*, *S. epidermidis*, and anaerobic Gram-negative bacteria as the predominant isolates in CRS. The true prevalence of the anaerobe is however questionable as most studies employed inadequate methods for isolation. The isolation rates of anaerobes among twenty studies reviewed were in the range of 8 % to 93%. Despite this, anaerobes as a major pathogen have been supported by a number of studies[75]. It is proposed that the aerobes and facultative organisms in CRS are gradually replaced by anaerobes because of the use of antibiotics which will favour the development of resistant organisms. This leads to persistent oedema and swelling of the sinus cavity resulting in reduced oxygen tension and increased acidity which is an ideal condition for the growth of anaerobes.

CRSwNP is often considered as a separate clinical entity because of the severity of presentation, high recurrence after surgery and recalcitrance. The bacteriology however in these subsets of patients appears to be similar to that seen in CRSsNP with *S. aureus, H. influenzae, and S. pneumoniae* among the common organisms[74].

These results show that in both health and disease there are a group of organisms that can be isolated by traditional techniques from the paranasal sinuses and these differ in the frequency of them of their predominance in the different studies making it difficult to attribute a particular organism in health and in disease.

1.4.4 Biofilms in CRS

Biofilms are bacterial communities embedded in a self-produced polymeric extracellular matrix and attached to biotic or abiotic surfaces or could exist in a floating state (pellicle). In the initial stage of biofilm formation, bacteria switches from planktonic mode to biofilm mode. A rapid phenotypic change and small molecule signalling lead to adhesion and coaggregation of microbes and secretion of a polysaccharide matrix which eventually encapsulates the clusters of bacteria. The process by which a single planktonic bacterium attach to each other is called auto-aggregation. A biofilm is usually polymicrobial, providing the organisms the advantage of passive resistance, synergistic metabolic cooperation, by-product influence, shielding of beta-lactam susceptible organisms by beta-lactamase-producing ones, quorum sensing systems, an enlarged gene pool with more efficient DNA, and many other synergies, giving them a competitive advantage(76). Apart from this the bacteria in the biofilms exist in a low metabolically active state and is often the preferred state of the bacteria in the human body(77).

Biofilms were identified and strongly implicated in the pathogenesis of CRS since 2004(78). Studies have also shown that in the sinonasal cavities biofilms are 10-1000 times less susceptible to antibiotic killing when compared to the planktonic counterpart(18). Although several bacterial species especially occurring as a polymicrobial biofilm formation have been identified in the context of CRS, their exact role in the pathophysiology of CRS is debated due to the identification of similar biofilms in the absence of the diseased state(79). Nevertheless, studies in favour of the biofilm theory in CRS pathogenesis have shown an increased rate of *S. aureus* biofilms and their superantigens in CRS compared to controls. The biofilms in CRS patients tend to co-locate with disruption of the epithelial barrier and have an increased presence of T cells and macrophages(76, 80). Among the CRSwNP patients, *S. aureus* is known to produce the most robust and higher amount of biofilms than *CNS*(73).

While the direct role of biofilms in CRS remains unclear, patients who have biofilm

formation also tend to have a worse prognosis, are more likely to have poorer surgical outcomes and exhibit an increased immune response.

1.4.5 Staphylococcus aureus

S. aureus has been identified as a persistent colonizer of the nasal cavity in 20% of the population and an intermittent carrier in another 30%. It could exist as a benign commensal or could be the major pathogen in many chronic infections including CRS. The pathogenicity is attributed to the various virulence factors expressed by *S. aureus*. These include enterotoxins and toxic shock syndrome toxin which acts as superantigens, cell wall associated proteins, antimicrobial genes, Agr and capsule type and genes involved in biofilm formation, exotoxins that degrade epithelial barrier integrity, affecting complement, antimicrobial peptide production, and adhesion and chemotactic processes(81, 82). In-vitro exposure of human nasal epithelial cells to *S. aureus* or its secretory proteins can induce apoptosis and alter cytokine signaling and nitric oxide production(83). These effects may be responsible for the *S. aureus*-mediated immune modulating effects and the mucosal injury seen due to epithelial damage in CRS. This organism also has the capacity to survive inside the epithelial cells evading the host immune response and antibiotic killing. This is possible by the phenotype switching to metabolically less active small-colony variants (SCVs) which becomes a reservoir of putative pathogens in recurrent recalcitrant CRS resistant to treatments. The intracellular form of *S. aureus* have been identified in up to 75% of CRS patients compared to a 12.5% in controls and have an increased risk of relapse of the disease even after surgery(76). However, there is a recent study which shows no association between *S. aureus* SCVs and CRS with comparable isolation rates of the bacteria in both CRS and controls(84).

Thungberg et al.(81) study found a statistically significant increase in the frequency of *S. aureus* in nares samples from CRS patients than from controls. This they suggested reflects as a risk factor for developing the disease. In this study, however, the prevalence of genes

encoding virulence factors between isolates from patients with CRS and healthy control was not found to be statistically significant.

Despite much controversies in its potential role in the development and progression of CRS, the pathogenicity and virulence of *S. aureus* make it an undesired bacterium in the recalcitrant sinus diseases.

1.4.6 Methicillin resistantstaphylococcus aureus (MRSA)

Methicillin-resistant Staphylococcus aureus (MRSA) was first isolated in England in 1961 and has since then disseminated worldwide with a progressive increase in its incidence. They are also known to form robust biofilms and thus recalcitrant infection. Although its incidence from the anterior nares has been reported to be as high as 53%, it mostly represents colonisation rather than pathogenicity. A retrospective case-control study by Casey et al(85) reported an MRSA incidence of 9.22% in CRS in their study population. The study, however, did not show any association between antibiotic use frequency of previous endoscopic sinus surgery with the incidence of MRSA. To the contrary, MRSA has been associated with recalcitrant disease, revision surgeries and multiple courses of antibiotic use(86).

The treatment of MRSA infection is primarily with a beta lactam compound. Topical therapies have also been proven beneficial in treating MRSA. Topical mupirocin has been shown to eradicate MRSA infection in acute exacerbations of CRS. This can be used as irrigation with Ringer lactate to improve penetration. There is however a concern with the bacteriological profile after mupirocin irrigation as was seen in a study which showed a shift towards an increase in other pathogenic gram negative and positive culturable bacteria(87). Surfactants (baby shampoo) are also effective in removing MRSA colonisation and is shown to have synergistic activity when combined with mupirocin or gentamicin. Another interesting and novel agent which effectively reduces mature biofilms without toxic effect is

Manuka honey which contains methylglyoxal (MGO- identified as the dominant antimicrobial component). Other beneficial topical therapeutic modalities include topical ofloxacin eye drops (0.3%) used intranasally, nasal irrigation containing hypochlorite super-oxidized solution, diluted iodine saline rinse, colloidal silver nanoparticle irrigation, and antimicrobial photodynamic therapy(88).

1.4.7 Staphylococcus epidermidis (Coagulase negative staphylococcus -CNS)

S. epidermidis is considered as a commensal identified in the nasal cavity of healthy controls and having a low virulence. This, however, could be potentially pathogenic when identified as the sole and highly abundant organism. The incidence of their isolation from cultures of CRS can vary from 0% to 60%. This organism is now being more and more recognised as a major pathogen especially if isolated from mucopurulent discharge and represents a single isolate on cultures(89).

1.4.8 Corynebacterium species (sp.)

Corynebacterium sp. is among the most frequently isolated bacteria in both healthy controls as well as CRS patients. While certain studies attribute its presence to the pathogenicity of the disease, certain others associate it with a healthy microbiome(90, 91). Corynebacterium is one of the dominant species in both health and CRS patients. Its reduction in postoperative CRS patients was found to be associated with an increase in the sinus symptom scores(92). C. accolens, a common skin commensal, metabolizes triacylglycerols in nasal secretions to oleic and linoleic acid, which can inhibit the growth of Streptococcus pneumoniae(93).

1.4.9 Bacterial co-occurrence

Different bacterial communities show a plethora of interspecies metabolic exchanges leading to a large number of co-occurrence and co-exclusion relationships. In CRS *S. aureus* is more frequently seen along with *S. epidermidis* and *P. acnes*. While *C. accolens* and *S. aureus* have been seen to mutually facilitated the growth of each other, *Corynebacterium*

pseudodiphtheriticum (*C. pseudodiphtheriticum)* and *S. aureus* were mutually inhibitory. C. accolens is capable of metabolizing triglycerols into free fatty acid and oleic acid which in turn inhibits the growth of Pneumococcus(76).

1.5 Microbiome

As opposed to the previous assumption that the human body harbours from 10 to 100 trillion microbes which greatly outnumber our own human cells, recent studies have shown that the number of bacteria in a 70 kg reference man ($3.8.10^{13}$) is similar to the number of bacterial cells in the body ($3.0.10^{13}$)(94). The combined genetic material of the microorganisms in a particular environment is referred to as the microbiome. This mini ecosystem of communities of symbiotic, commensal and pathogenic organisms can interact with each other and also the host. A microbiome is unique to the host organism and changes in its composition can lead to imbalances in homeostasis. Alteration in the microbiome has been linked to many chronic diseases including inflammatory bowel disease, diabetes mellitus, certain conditions of the skin and some lung diseases. A study(95) from the Danish birth registry which compared 2 million term deliveries either by natural birth or caesarean section showed an increased risk of asthma, systemic connective disorders and inflammatory bowel disease in the cesarean section cohort. A number of other epidemiological studies also demonstrate a link between exposure to microorganisms early in life and a lower incidence of chronic inflammatory disorders later in life(53).

The microbiome of the upper airway and paranasal sinuses is an area of recent research. To date, the studies are relatively small in number, underpowered and differ in terms of sampling technique and site as well as bioinformatic analysis. This has led to a discrepancy in the results obtained with no real consensus achieved on the specific microbiome in health or disease.

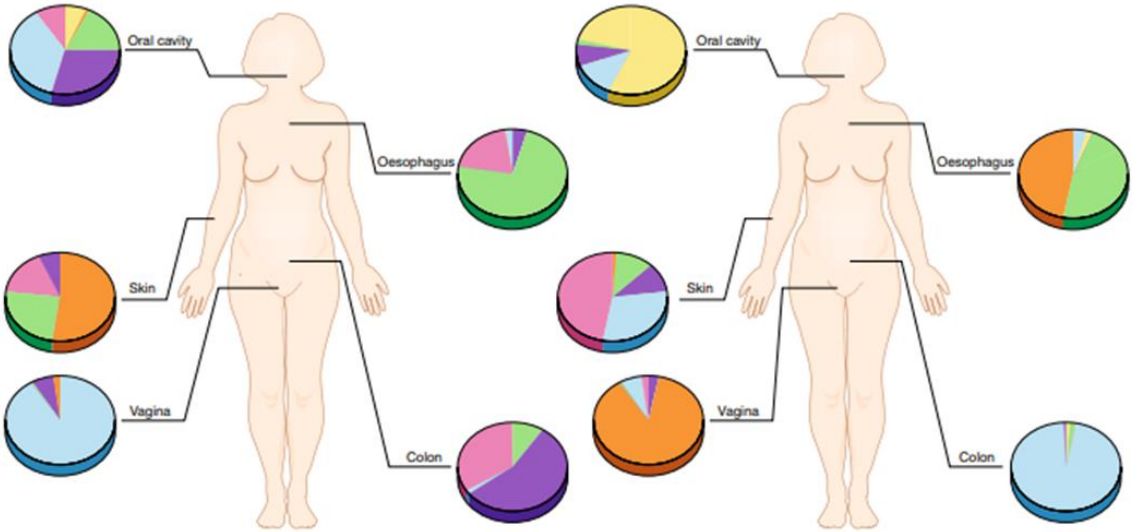

Figure 5: Highly personalised human microbiome.(96)

1.5.1 Microbiome isolation techniques

The microbiome of the nasal cavity has been studied both from swabs and tissue specimens obtained from the sinuses. There are now multiple studies which show that the bacterial DNA load obtained from nasal swabs is comparable to tissue specimens and that the microbiome within the nasal cavity of the same individual remains relatively consistent irrespective of the location from where it was taken. For this reason, the middle meatus is most commonly used as a surrogate for the sinuses and is now the most commonly sampled location in sinus microbiome studies(97).

Recently, there have been significant technological advances in the field of microbiology. Culture-independent techniques have emerged as a much more sensitive and superior modality for understanding the entire micro-ecology of local environments. These techniques allow the detection of previously "un-culturable" bacteria and provide new information on the diversity and relative abundance of resident microbes and potential pathogens. (98). Bacterial detection through culture-independent techniques demonstrate an increased diversity of bacterial species by a magnitude of 10^9 when compared to standard cultures and this is also consistent with other reports in which it is proposed that only 1% to

10% of bacteria encountered in the environment are able to be cultured in the laboratory(99).

These culture-independent techniques rely on matching bacterial DNA isolated from a specimen to libraries of known bacterial sequences. They include quantitative polymerase chain reaction (qPCR), fluorescence in situ hybridization (FISH), mass spectrometry, DNA microarray, and next generation sequencing (NGS) central to the technique.

Next generation sequencing (NGS) emerged in the mid and late 1990s and early 2000s leading to the release of the first commercially available second-generation sequencing system in 2005 thus making it widely available and cost effective(98). NGS relies on sequencing by synthesis design by amplifying the DNA through PCR in the initial step. This is followed by DNA sequencing to determine the DNA sequence. There are several methods of sequencing, each reliant on a different type of chemistry. Pyrosequencing is a method which relies on the production of light when pyrophosphate is released after nucleotide incorporation into a DNA strand. The intensity of light emitted is proportional to the number of specific nucleotides incorporated in a given area and this method can now read DNA sequences of up to 1000 base pairs. Another method is Reversible Terminator Sequencing where DNA is denatured into single strands and washed with the 4 different nucleotides tagged with fluorescent dye and a reversible blocking group. As each base is added at a time this technique is more accurate compared to pyrosequencing. It also has a large output and low reagent cost although the read length is shorter and takes longer run time. Amplicon sequencing is used for an in-depth investigation of the sinonasal microbiome. This involves amplifying and sequencing regions of highly conserved bacterial genes which could then be compared to an existing database to determine the sequence from which the bacterial organisms came. These regions are also called markers (e.g. 16S ribosomal ribonucleic acid subunit -16sRNA, elongation factor). 16s RNA subunit gene is one of the most commonly used regions in view of its cost effectivity and ease of use(100). Although the 16sRNA subunit is conserved amongst all bacteria, there are numerous hypervariable regions within

the subunit which differ among different bacteria. It is these regions that are further sequenced to differentiate the different bacterial genus or species from each other. The sequences obtained through the amplification process are then compared to known genetic sequences in bacteria "banks" or "libraries" to allow identification to occur.

The major downside of 16sRNA is that as some bacteria have multiple copies of the 16S rRNA gene which artificially increase their abundance in a sample. Also, the multiple copies of the 16S rRNA within 1 bacterial genome can be highly divergent and, thus, can be falsely identified as coming from different bacteria, which impacts reported diversity(101). These chimeric sequences can now be identified and removed using tools such as UCHIME and Chimera Slayer. Following this, the sequences identified are designated to operational taxonomic units (OTUs). OTUs refer to clusters of organisms, grouped by DNA sequence similarity of a specific taxonomic marker gene. This grouping allows further community analysis by computational methods to determine abundance, richness, and diversity both within one single population (alpha diversity) and between populations (beta diversity).

Another popular method used is Whole Metagenome Shotgun (WMS) and Metatranscriptomic Sequencing. In this, all the DNA from a given sample (unlike selected portions in 16sRNA sequencing) is sequenced and analysed. This technique is useful in determining the functions of the community(98).

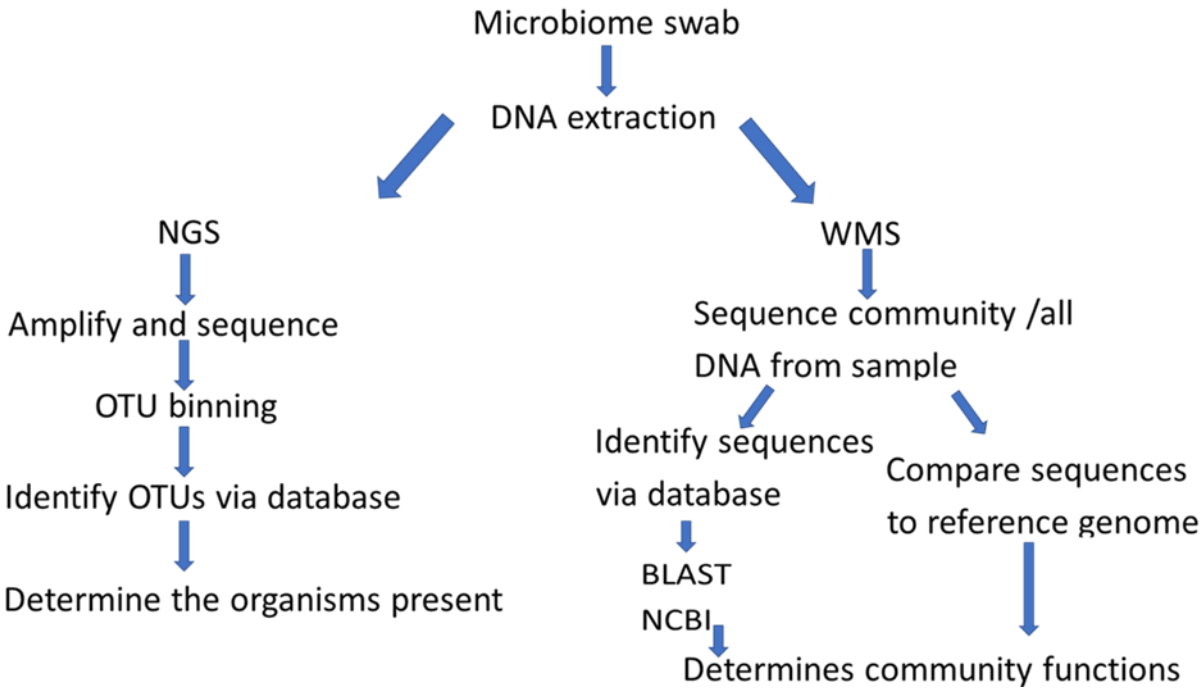

Figure 6: Microbiome analysis using NGS technique and WMS sequencing

1.5.2 Comparison of bacteria by traditional isolation techniques and NGS

Although culture-based approaches can isolate bacterial species accurately, they have only a limited range of defined conditions for bacterial growth. As a result, a number of slow growing, viable but unculturable or difficult to culture organisms often fail to be identified(76). However, analysing species level community differences is not always accurate in next-generation sequencing based studies, which reliably identify taxa only to the genus level.

A cross-sectional study by Fazel et al.(102) on 21 nasal swabs (15 patients with CRS, 5 non CRS, 1 repeat swab from a patient) aiming to compare microbiological culture-based and culture-independent (16S rRNA gene sequencing) methodologies to identify the pathogens in CRS showed that while sequencing generally identified the same pathogens as culture, the opposite was not the case, as culture techniques failed to detect potentially pathogenic members of many genera. The most prevalent organisms in both groups were *CNS* (75%), *S. aureus* (50%), and *P. acnes* (30%). The common isolatesfromgene sequencing was *CNS* (100%), *Corynebacterium sp*. (85.7%), *P. acnes* (76.2%), and *S. aureus* (66.7%). They

concluded that DNA pyrosequencing revealed greater biodiversity than culture, particularly in the anaerobic groups although in most cases culture results represented a subset of the abundant DNA sequence types.

Despite many studies reporting similar dominant bacteria being identified using culture-based and NGS techniques, there are studies which show that dominant bacteria identified on sequencing were identified by culture only approximately half of the time(103). It is known that microbial genetic potential and activity, virulence, and involvement in disease-associated processes can vary significantly at the level of the species and at times even between bacteria of the same genus(104). Hence to fully understand the complexity of bacterial community interactions both overall and at the bacterial species or strain level, studies combining both molecular and culture-based approaches are probably necessary**.**

1.5.3 Microbiome in health

There are nine published studies using NGS which examine the microbiome in sinuses of healthy control patients (75, 102, 105-111). To date, no consensus on the microbiome composition of a normal healthy sinus exists. This is largely because of the differences in study design, including sampling site and method used, different primer selection, sequencing methodology, and bioinformatics analysis. Nevertheless, a systematic review by Andersen et al. (112) showed that members of the phylum *Firmicutes, Actinobacteria, and Bacteroides* phyla were identified in every sample of control patients and patients with CRS. The total bacterial burden and bacterial diversity was similar in both healthy controls and CRS patients. There was also no single phylum that was seen to be particularly enriched in controls. Wagner Mackenzie et al.(113) reviewed all published sinonasal genomic studies and reanalysed the raw data from the available studies in an attempt to standardize the bioinformatic analysis. Although their study did not address the differences in sampling method/site or population differences of the enrolled patient cohorts, it did allow analysis of a much larger patient series facilitating some meaningful comparison.

Their meta-analysis demonstrated an increase in the relative abundance and diversity of some members of the phylum *Actinobacteria* and the genera *Propionibacterium* in healthy sinuses when compared to the inflamed sinuses in CRS patients. Another interesting but previously unreported finding was their observation that the absence of *Burkholderia* and *Propionibacterium* phylotypes appeared to be correlated with an increased network fragmentation of a healthy bacterial community. They postulated that these phylotypes may, therefore, act as possible gatekeepers in the maintenance of a stable sinonasal bacterial population. The potential protective effects of unclassified *Corynebacterium* species borne out from this study is also supported by other studies in the literature. *Corynebacterium*, when applied to the nasal cavity of persistent nasal carriers of S. aureus, resulted in a sizable reduction in nasal load of *S. aureus*. Yet another similar study found an eradication of facultative pathogen with no recolonization on follow up(55). An analysis of microbiome in control patients undergoing trans-sphenoidal pituitary surgery when compared to CRS patients undergoing surgery showed a significant prevalence of *Acinetobacter johnsonii* (82% versus 26%,), *Corynebacterium confusum* (73% versus 26%)(114).

1.5.4 Microbiome in CRS

Jason et al. (115)study proposed that microbial colonization of the gut produces a host antibody response which in turn could influence the microbiome composition and function to benefit the host health through a process they referred to as antibody-mediated immune selection (AMIS). Studies showed that naturally occurring polymorphisms in MHC genes resulted in differences in microbiota composition between different lines of MHC-congenic mice, and this influenced colonization resistance against an enteric pathogen. This emphasize that defects or naturally occurring variability in AMIS among individuals lead to the establishment of unique microbial communities that can influence host susceptibility to inflammatory and infectious disease in the gut. Further studies of germ-free mice also reinforce the hypothesis that bacteria are needed for the development and maintenance of

normal tissue and immune function(116). In one of the studies, the formation of isolated lymphoid follicles which are induction sites for intestinal immune reactions failed to develop in the absence of specific microbial stimulus. The ability of the microbiota to promote substantial changes in gut morphology, including villus architecture, crypt depth, stem cell proliferation, blood vessel density, mucus layer properties and maturation of mucosa-associated lymphoid tissues have also been well described. Invariant NK T cells which are promoters of inflammation is found more abundant in the colon of germ-free mice compared to the NK- like cells suggesting that the gut microbiota promotes homeostasis by decreasing the number of these pro-inflammatory cells. It has been thus proposed that in health a symbiosis between pathogenic bacteria and non-pathogenic bacteria is needed to maintain homeostasis and normal tissue functioning. In chronic diseases it has been shown that a dysbiosis exists (117), leading researchers to postulate that a similar alteration in the sinus microecology may also be responsible for the inflammation seen in CRS.

A number of studies have examined the microbiome of CRS, and similar to the studies in health, no consensus regarding the composition of the microbiome in diseased patients exists.

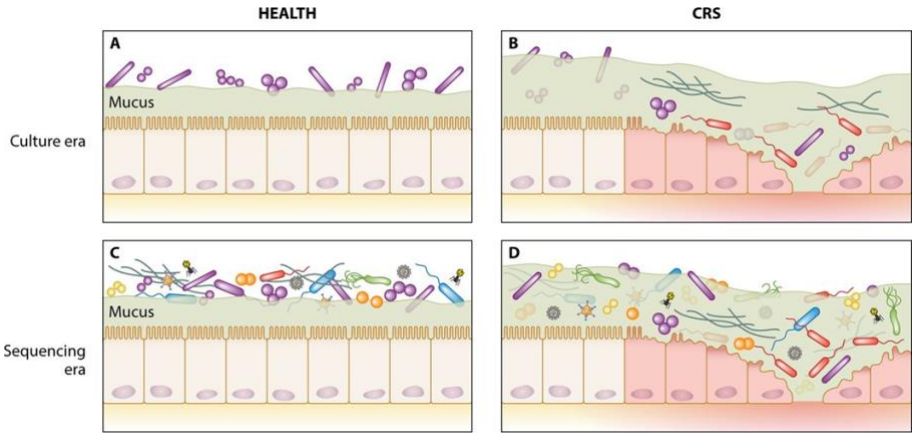

Figure 7: Mucosa associated microbes in health and CRS from culture era and sequencing era.(118)

A common finding of most of the studies however is that although there is no difference in the degree of bacterial burden between the healthy sinus and a diseased sinus, there is a consistent observation of a relative reduction in the markers of bacteria biodiversity, richness and evenness(75, 107, 110, 113, 119). This finding supports the dysbiosis hypothesis as having a possible role in CRS as well. A review of this topic, based on the findings of studies from six independent centers around the world, (120) found a consistent relative reduction in the abundance of commensal bacteria and augmentation of pathogenic strains in the microbiome of CRS. Within the subgroups of CRS, there appeared to be no significant difference in the most commonly isolated bacteria on 16s sequencing between polyp and non-polyp patients, a finding which was different to previously culture dependent studies, that observed bacterial differences between the two phenotypic groups.

Apart from this, various comorbid conditions and patient demographics and habits could also influence the sinus microbiome and the disease profiles. Its observed that CRS subjects are more often asthmatics compared to controls and also CRSwNP is more likely to develop in asthmatics compared to non-asthmatics. Cystic fibrosis patients with CRS with or without polyps are found to have a reduced bacterial community diversity and increased bacterial load compared to healthy controls(90). A history of smoking has been consistently associated with a decrease in bacterial diversity and poor treatment outcomes. However, variables like patient age, allergies, diabetes, ethnicity, gender, polyposis, antibiotic usage in the 6 months prior to surgery, saline washing, intranasal steroid use, and previous surgery was not found to be associated with changes in the bacterial community(91). To better understand the microbiome influence on CRS pathogenesis it might be important to take into account the coexistent comorbidities apart from classifying CRS on the standard phenotypic basis of presence or absence of polyps.

While single gene-based approaches identify the taxonomic diversity of the bacterial community, they do not provide information of the functional capacity of the community.

This can be investigated using computational approaches such as PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States(121). These approaches that sequence all DNA (metagenomics) or all transcribed RNA (metatranscriptomics) or that identify proteins (metaproteomics) or metabolites (metabolomics) will provide greater insights into the true diversity and structure, the full genetic potential and in situ functional activity, of the microbiota. In an attempt to explain the CRS patient heterogenicity on the basis of the presence of distinct sinus microbiota, Cope et al.(122)examined the sinus mucosal microbiome and parallel host immune responses of a cohort of CRS and healthy subjects and related these findings to clinical outcomes of nasal polyposis development. For this, they used an unbiased probabilistic model, Dirichlet-multinomial mixtures (DMM) which identifies clusters of samples based on bacterial communities. Three distinct sample clusters termed Dirichlet states (DSI-III) were identified. All the healthy controls and a few CRS patients clustered into DSI which exhibited relative enrichment of *Streptococcus*. This group was depleted of polyketide and folate biosynthesis and enriched for a pathway responsible for ansamycin biosynthesis, amicrobial secondary metabolite with a broad range antimicrobial activity. DSII (only CRS patients) were the least functionally diverse, the most immunologically active, had the greatest proportion of cystic fibrosis and asthma patients, and was predominated with *Pseudomonadaceae* with relative enrichment for *Fusobacterium, Aggregatibacter, Achromobacter, and Prevotella*. This group was specifically depleted of 67 KEGG pathways for lipid, carbohydrate, terpenoid, and xenobiotic metabolism. DSIII containing CRS patients were further classified into a or b depending on the species dominating the cluster, *Corynebacteriaceae* (DSIII a) and *Staphylococcaceae* (DSIII b). DSIII(a) was characterized by both peroxisome proliferator-activated receptor-γ and retinoic acid-inducible gene-1signaling pathways both of which have been shown to be increased in eosinophilic polyp tissue in CRS patients. DSIII(b) along with DSII was significantly

enriched in bacterial virulence pathways, including two-component response systems, and for fatty acid and tryptophan metabolism pathways associated with inflammation.

It was suggested that patients with *Corynebacteriaceae*-dominated (DS III a) state were uniquely associated with increased IL-5 and IFN-γ gene expression and increased the tendency for development of nasal polyposis and induced TH2-skewed immune responses. On the other hand, IL-1β gene expression was increased in DSI, II, and III(b), which may indicate a role for inflammasome activation in CRS patients with TH1-skewed disease. The study is however limited by the significant number of CRS patients who were on antibiotics prior to their nasal swabs.

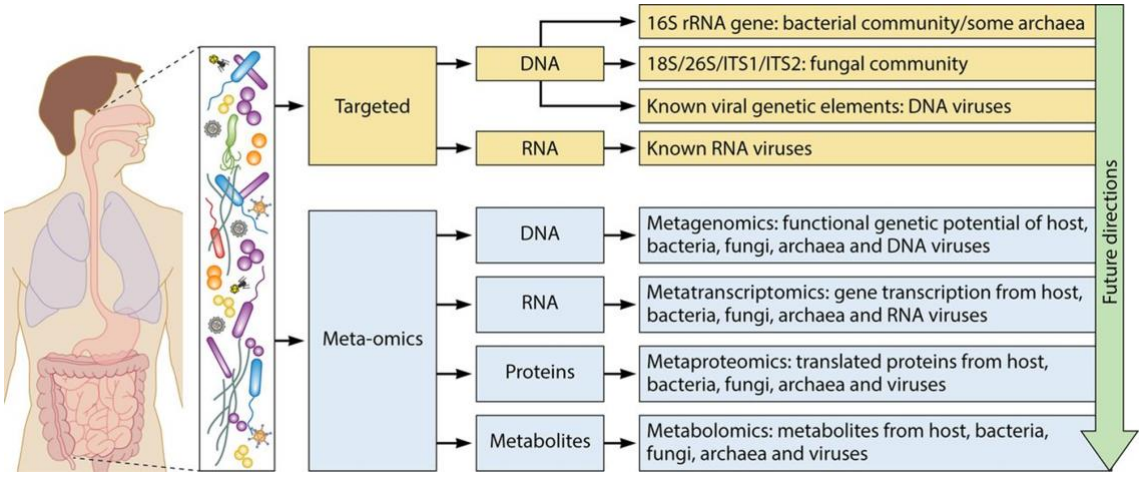

Figure 8: Future directions in microbiological techniques.(118)

In-spite-of all these advances in technology, the exact role of microbiota in the pathogenesis of CRS remains unclear. This is largely because of the substantial technical variation in the methodological approaches used in different studies. The differences in sampling sites and techniques, target gene regions, sequencing platforms, bioinformatics pipelines, and taxonomic assignment databases can all make a meaningful comparison difficult. The present-day consensus from the literature is that specific genera or species of bacteria or bacterial communities may not be directly associated with the development of CRS, rather it

is more common to observe an overall shift away from an apparent "healthy microbiota" which leads to disease. These findings suggest that it is important to identify the possible dysbiosis occurring in sinuses leading to CRS and also to see if the changes are reversible and exhibit long term stability in spite of multiple surgical or medical therapies. Dysbiosis and instability in the community as a whole are likely reflected in the dominance of particular microbes (single pathogen hypothesis) or clusters of microbes (co-occurrence hypothesis), intramucosal invasion and persistence (intramucosal hypothesis), or changes in the membership and activity of the biofilms present(76).

1.5.5 Significance of the different bacteria in health and disease

Identifying and categorizing bacterial communities to different disease entities alone might not be sufficient to understand the significance of the presence or absence of these communities in disease pathogenesis. This would require various metagenomics, metatranscriptomics, and metabolomics/metaproteomic methods to identify the composition and function of host-associated microbiota. Thus, identifying specific microbiome derived molecules that promote the selection of pathogenic microbial communities and drive specific inflammatory responses to help in better understanding of host-microbe interactions.

In all these, the role of commensal bacteria in the sinuses cannot be underestimated. They act as a competitor to pathogenic strains in regard to space, nutrient cell surface receptor and also actively secrete antimicrobial agents like hydrogen peroxide, lactic acid,and bacitracin. It has been shown that bacitracin secreted by *P. acnes*, a commensal isolated from healthy nose possess antibacterial and antifungal properties and also modulate immune responses to pathogenic bacteria. Apart from these properties, commensal bacteria also have important structural and metabolic functions. Studies have established their involvement of regulation of epithelial health and the reinforcement of tight junctions. They are also involved in the sequestration of metals, synthesis of vitamins and fermentation of undigested

polysaccharides and mucus. Even among the patients with CRS, an increased preoperative bacterial diversity has been associated with an improved surgical outcome.

Although it is unclear if dysbiosis drives the initial aetiology or subsequent exacerbation of the disease, the loss of epithelial integrity and inflammations seen in CRS subsequently promotes secondary bacterial, fungal and viral invasion which results in persistent inflammation. The microbial milieu may, therefore, be important in exacerbations and the progression of the CRS, even though not primarily responsible for the initial aetiology. The resident microbiome, host immune responses, therapeutic interventions and the interactions between these factors all eventually contribute to the chronicity of this condition.

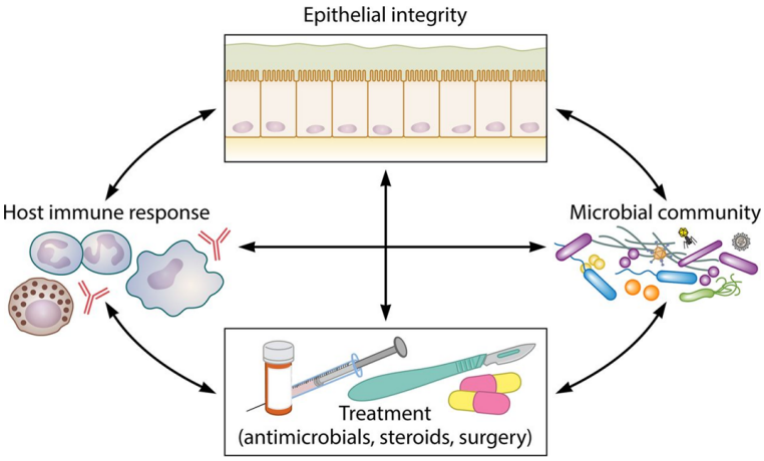

Figure 9: Multifaceted interactions in the development and progression of CRS.(118)

1.6 Medical therapy in CRS

A recent systematic review by Rudmik(123) et al attempted to summarize the highest-quality evidence on medical therapies for adult chronic sinusitis and to provide an evidence-based approach to assist in optimizing patient care. They examined a total twenty-nine studies which met their strict inclusion criteria, including 12 meta-analyses (examining >60 RCTs), 13 systematic reviews, and 4 individual RCTs that were not included in any of the meta-analyses. The results of this well performed systematic review will be detailed in the following section. Topical nasal steroids, oral corticosteroids, oral antibiotics, and saline

nasal irrigations form the main stay of medical therapy in CRS (described below).

Other treatment modalities which are not widely used includes anti-IgE therapy. This involves delivery of recombinant DNA-derived IgG monoclonal antibody which will bind to free IgE preventing its binding to mast cell and basophil receptors. This is given in patients with high serum IgE level(124). Yet another mode of treatment involves the Anti–Interleukin 5 Therapy. Humanized IgG monoclonal antibody is delivered which binds free IL-5 and impairs eosinophilic-mediated inflammation. A systematic review by Soler et al (125)looked at the effect of topical antibiotics like Fosfomycin, N-chlorotaurine, bacitracin, tobramycin, mupirocin, and neomycin for CRS without polyps and evaluated the symptom, quality of life, and endoscopy. The study, however, did not demonstrate any difference in clinical outcomes compared with placebo. It has been shown that there is fungal colonization in the nasal cavity of 96% of CRS patients and healthy controls. Against this background, it was thought that abnormal immunological response to fungi may cause chronic sinusitis. However, studies failed to demonstrate any benefits with topical antifungal (amphotericin) compared to placebo(126). Leukotriene pathway antagonist such as Montelukast had no additional benefit to topical steroids and antihistamines and allergy immunotherapy results in significant symptom relief in CRS(127, 128).

1.6.1 Saline irrigations

The use of saline nasal irrigation as a treatment for nasal ailments can be traced back many centuries. The benefits of saline irrigation are believed to be due to the removal of environmental allergens, clearing excess mucus, and improving mucociliary clearance. A report from pooled data of three RCTs shows that saline nasal irrigations can significantly improve symptom scores and disease-specific quality of life as compared with no treatment(129). Moreover, a review of sixteen RCTs concluded that saline is very beneficial even as a single treatment modality in the treatment of post-surgical CRS(130). Intranasal

saline irrigations also appeared to have a beneficial adjunctive effect on symptom relief for patients(131).

The concentration of saline recommendation varies from hypotonic, isotonic to hypertonic. The efficacy of all the three concentrations remains comparable although it is argued that hypertonic saline may result in nasal irritation. In terms of delivery of the saline, most distribution studies agree that large volume irrigation is superior to sprays or low volume washes in terms of sinus penetration and symptom score improvements.(130).

Saline washes are generally well tolerated by most patients, although reported side effects include nasal irritation/burning, sore throat, otalgia or aural fullness, epistaxis, postnasal drainage, and headache.

1.6.2 Oral antibiotics

Sinusitis was found to be the most common condition for which an antibiotic was prescribed in surveys conducted in 2003 and 2013 in the United States. Despite being widely used as the first line medical therapeutic agent in many clinical practices there is little evidence to support its efficacy in treating CRS. This is because the organisms commonly isolated in sinus disease are also present as harmless commensals in the oropharynx, nasopharynx, and skin. Antibiotic use and benefits are also debatable between CRSsNP and CRSwNP with A-11 grade evidence for use in CRSsNP and B-111 grade evidence for use in CRSwNP from multiple randomized controlled studies(123). Furthermore, a current study suggests that recent antibiotic exposure may be implicated in the development of allergic disease and chronic inflammation in the sinonasal cavities. It is hence argued that CRS is a disease of inflammation alone and not of infection. The propagators of this theory emphasis on the antibiotic use only in cases of acute sinusitis or an acute exacerbation of CRS.

In this contest, amoxicillin-clavulanic acid and second- or third-generation cephalosporins are considered the first-line antibiotic choice. Quinolones are helpful second-line agents for

refractory cases, followed by trimethoprim-sulfamethoxazole, doxycycline, telithromycin, and macrolides[132]. The macrolide group (erythromycin, clarithromycin, and roxithromycin), have received special interest among the CRS research due to its anti-inflammatory effects along with having anti-infective properties. Macrolides inhibit the production of a number of inflammatory cytokines, decrease *Pseudomonas* biofilm formation, decrease secretion of mucous in the airways, blocks transcription factor nuclear factor κB (NF- κB) activation, and facilitates the apoptosis of neutrophils[133, 134].These properties of macrolides may improve clinical outcomes in CRS. Moreover, the presence of biofilms in CRS would also encourage the use of antibiotics early in the disease aiming disease eradication.

In vivo and in vitro studies with macrolides, amoxicillin-clavulanic acid and levofloxacin have all shown potent antibiofilm activity of these compounds against *S. aureus* and *Coagulase negative staphylococcus* in nasal polyposis patients[135]. A study by Zeng et al[136] showed that Clarithromycin at 250mg daily given for a period of 12 weeks produces significant changes in the subjective and objective clinical outcomes in patients, which is comparable to that when Mometasone furoate nasal spray given at 200mcg puff once daily for the same time duration. Moreover, the study also showed significant and similar clinical improvement in both groups at 4weeks of treatment. However, the maximum benefit was seen at the end of 12 weeks in the Clarithromycin group, whereas it was seen earlier in the steroid group.

Doxycycline a bacteriostatic agent under the Tetracycline group of antibiotics is increasingly being used in CRS due to its broad spectrum of action, safety and good tolerability in patients, excellent penetration into tissue and sinus secretions and most importantly the additional anti-inflammatory action of the compound[137]. The other actions include suppression of cytokine production, inhibition of metalloproteinases (MMPs), suppression of hydrolases, and reduction of pathological tissue damage from reactive

oxygen species[138]. In contrast, studies comparing two antibiotics for 3 weeks or less without a placebo group have shown no difference in outcome between the groups[139].

Antibiotic therapy could potentially destabilise the microecosystem and could facilitate the growth of antibiotic-resistant bacterial strains. Exacerbation of infection following antibiotic therapy has been documented outside CRS in cystic fibrosis. Antibiotic therapy has also demonstrated a predominance of resistant strains in the sinonasal microbiota[140]. This shift in the bacterial diversity was highly individualised from patient to patient and highly variable, again questioning the complex interactions between the microbiota and host leading to disease progression.

1.6.3 Topical antibiotics

Topical antibiotics have been increasingly used in CRS as the biofilm theory of pathogenesis emerged. The theory behind their use in this manner is that higher doses can be delivered directly to the nasal cavity, circumventing first pass clearance and systemic side effects. Although they are still used in acute exacerbations of CRS there is a growing concern for the development of multidrug-resistant bacteria[141]. Despite this, topical antibiotics have a role in treating MRSA positive CRS exacerbations with studies showing 90% eradication at five days and 60% at long term follow up[142]. Its activity against *S. aureus* biofilms has been shown to be superior to that of the oral ciprofloxacin and vancomycin.

Adverse effects of topical antibiotics include postnasal drainage, nasal irritation, dryness, burning, itching, otalgia, and sore throat. Although RCTs do not support the routine use of topical antibiotics in CRS, it does have a role in the culture directed treatment of acute exacerbations[130].

1.6.4 Antifungals

Although early low-level evidence studies initially suggested a role of antifungal medications in the management of CRS, more recent metanalysis have conclusively demonstrated that such agents confer no significant benefits in the management of CRS(143)

Adverse drug effects of topical antifungals include local irritation (nasal burning, dryness, bleeding, itching), muscle aches, facial pain, nasal congestion, rhinorrhoea, and respiratory symptoms (asthma attack, bronchitis, cough)(144).

1.7 Corticosteroids

Corticosteroids are synthetic or naturally occurring compounds structurally related to hydrocortisone and bind to a single group of endogenous corticosteroid receptor mediating an anti-inflammatory action. The basic chemical structure of the corticosteroids contains 3 rings of 6 carbons each and 1 ring of 5 carbons. They also contain a ketone oxygen at position 3, the unsaturated bond between carbons 4 and 5, the hydroxyl at position 11, and the ketone oxygen at carbon 20. Many of the intra nasal corticosteroids (INCS) have halogen groups attached to position 6 and 9. Variations of the corticosteroid at carbon ring D at positions 16, 17, and 21increase topical activity while minimizing systemic adverse effects. In case of mometasone furoate (MF) which is 21-chloro 17(2´ furoate), the chloride at position 21 makes MF resistant to degradation by esterases, and the addition of chloride at position 9 increases its affinity for the corticosteroid receptor(145).

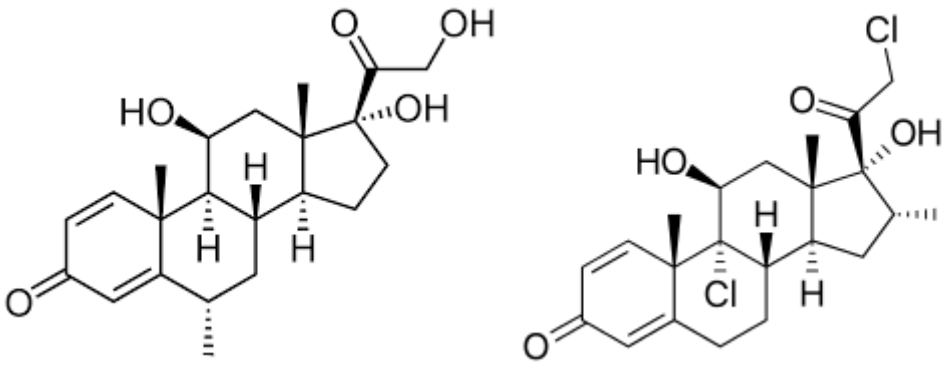

Figure 10a: Methylprednisolone, 3b: Mometasone fumarate

The effects of steroids are brought about by genomic and non-genomic actions(146). The classic genomic or delayed mechanism of action of steroids is mediated through intracellular receptors. The lipophilic steroid molecule is thought to enter the cell by simple diffusion. Once the steroids bind to the ligand binding domain of the steroid receptors the affinity of the receptor for the DNA increases and the glucocorticoid receptors complex is translocated into the nucleus. They then act as transcription factors regulating gene expression by recognizing palindromic hormone response elements (HRE) at the DNA and binding to them. This ligand-receptor complex dependent transcription is initiated in conjunction with the basal transcription complex, different coactivators, repressors, and transcription regulators and is sensitive to inhibitors of transcription and translation(147). Apart from this, gene expression may be modulated by a non-transcriptional action by the interaction of nuclear receptors with sequence-specific transcription factors. This is seen with glucocorticoids, which can affect the activity of NFκB (a modulator of cytokine-induced inflammation). Glucocorticoids genomically can increase the level of inhibitor IκB, which traps NFκB in the cytoplasm(148). In addition, glucocorticoid receptor interacts with p65, a transcriptionally active subunit of NFκB, by protein-protein interaction(149). Glucocorticoids can thus elicit direct actions at the transcriptional level and effects mediated by direct protein-protein interactions, which should be termed non-transcriptional activities of classic steroid receptors.

In contrast to this, the non – genomic effect of steroids has a rapid onset of action (within seconds to minutes) and is insensitive to inhibitors of transcription and protein synthesis. These actions are, however, not uniform and a number of potential mechanisms have been proposed and hypothesized. The Mannheim classification scheme (150)has classified this to better describe and understand the non-genomic actions. This includes 1. Direct steroid action (A) which is nonspecific -A1, 2. Direct steroid action which is specific (AII) and is

mediated through a classic steroid receptor - AIIa, 3. Direct steroid action which is specific (AII) and is mediated through a non-classic steroid receptor – AIIb, 4. Indirect steroid action (B) which is nonspecific – BI, 5. Indirect steroid action which is specific (BII) and is mediated through a classic steroid receptor – BIIa, and 6. indirect steroid action which is specific (BII) and is mediated through a non-classic steroid receptor – BIIb. The receptors responsible for these actions, when present are thought to be located on the cell membrane and are considered structurally similar to the classic intracellular receptors. Direct steroid actions in the absence of a receptor are thought to occur at high concentrations and are considered to occur due to change in membrane fluidity(151).

Most of the adverse effects of oral corticosteroids result from the suppression of the hypothalamic–pituitary–adrenal (HPA) axis function. This leads to the development of iatrogenic Cushing's syndrome (cardiovascular disease, thromboembolic events, weight gain, glucose intolerance, osteoporosis/avascular necrosis of bone, obesity, skin changes, neuropsychological changes, immune deficiency, cataracts, serous chorioretinopathy, and increased intraocular pressure)(130). The modifications created on the carbon ring D at positions 16, 17 and 21 are responsible for increasing the topical activity while reducing systemic side effects of corticosteroids.

In CRS, these actions of steroid can be summed up to its multiple mechanisms by which it decreases the mucosal inflammation, decrease vascular permeability, and reduce glycoprotein release. Corticosteroids thus can reverse histone acetylation of activated inflammatory genes. This leads to a decreased production of cytokines, chemokines, adhesion molecules, inflammatory enzymes, and receptors. Corticosteroids promote transcription of anti-inflammatory proteins and reduce lymphocyte activation, differentiation, and cytokine release by inhibiting macrophage presentation of antigens to lymphocytes(130). Oral and topical steroids can also significantly decrease the expression levels of IL-1, Il-6,and TNF-alpha in nasal polyps(152).

Corticosteroids are known to reduce host immune responses and could potentially affect the body's ability to fight infection. Recent studies, however, provide significant evidence for their use in a wide range of infections, especially in the early stages. Diseases like acute bacterial meningitis, pneumocystis pneumonia, croup, tuberculous meningitis, tuberculous pericarditis and tuberculous pleurisy all have level 1 evidence and grade A recommendation for the use of steroids in the treatment protocols(153). The indications for corticosteroids in rhinosinusitis recommended by the European Position Paper on Rhinosinusitis (EPOS) guidelines include acute rhinosinusitis (ARS), prophylactic treatment of recurrent ARS, CRSsNP, CRSwNP, postoperative treatment of CRS with or without nasal polyps(4).

1.7.1 Oral steroid

The most common oral steroid used in CRS is methyl prednisolone. The utility of oral corticosteroid has been widely acknowledged in cases of CRSwNP while current literature is increasingly showing the benefits of short course oral steroid in CRSsNP cases also. Steroid therapy in CRSwNP has also been described as medical polypectomy due to its remarkable ability to reduce the size of polyps in a sizable number of patients. These patients also report a significant improvement in their sinonasal symptom score as the nasal polyps obstructing the nasal cavity and the sinus drainage reduces. The symptom relief however, tend to return back to the pre-treatment stage after a few weeks of steroid therapy as the polyps have been shown to recur once the anti-inflammatory benefits of oral steroids taper off. The recommended dosage of oral prednisolone over 3 weeks is 25mg every day in the first week followed by 12.5 mg every day for the next week and 12.5mg on alternate days in the third week(154). Oral steroids are however considered superior to antibiotics in the treatment of CRSwNP symptoms. An RCT by Van et.al (155) compared the effect of 200mgofdoxycycline once, followed by 100 mg daily for 20 days with methylprednisolone and placebo for nasal polyposis. They showed that there was an improved polyp score at 12 weeks for the doxycycline group compared to placebo. Methylprednisolone had a larger

improvement in polyp score at 2 weeks compared with doxycycline and placebo. However, after discontinuing the treatment for 12 weeks there was no difference in the symptom score in both the steroid or the doxycycline group.

1.7.2 Topical nasal steroids

Topical nasal steroids are currently the most popular treatment modality for CRS globally. This is due to the advantage of providing high concentrations of localized therapy preventing systemic toxicity, ease of administration, cost efficacy and particularly the clinical benefits observed.

INCS can be delivered through Freon-propelled aerosols, metered dose pumps, aqueous pump sprays, pressurized aerosols, or even as nasal drops and nasal rinses in saline or applied directly into the sinuses in a carrier medium (e.g. chitosan gel). It is estimated that after topical nasal application around 80% of the steroid is available for absorption from the mucosal surface. About 20% of the drug has been shown to remain in the frontal cavity and turbinates for up to 1.5 hours after application. The efficacy of INCS is measured in terms of the therapeutic ratio. An optimal INCS will have the highest ratio of topical to systemic activity as this increases the potential for therapeutic effects relative to undesired systemic effects(156). A portion of INCS applied topically is swallowed and subsequently absorbed at the gastrointestinal tract and also could be absorbed in the circulation at the nasal mucosa. The amount of drug reaching target tissues and exerting therapeutic effect relative to the amount reaching the systemic circulation is a measure of safety of topical drugs. Fluticasone propionate (FP) and Mometasone furoate (MF) are highly lipophilic compounds and are poorly absorbed into the systemic circulation whereas, Budesonide (BUD) which is relatively less lipophilic is better absorbed(157). The systemic availability is however further reduced due to the first pass metabolism of the steroid by the liver.

Once administered the compounds cortisone and prednisone are metabolised into their active form cortisol and prednisolone respectively by the reduction of the ketone group at

position 11. When compared to the corticoid receptor affinity of 1 for dexamethasone the relative binding affinity of beclomethasone dipropionate (BDP) is 0.53, beclomethasone monopropionate (BMP) is 25, BUD is 258, FP is 813 and MF is 1235(145). Half-life (t1/2) is a function of the volume of distribution and the clearance rate of a drug. When multiple doses of a drug are administered the plasma concentration of the drug steadily increases till it reaches a steady state concentration. The highest reported half-life is for FP (7.8) followed by MF (5.8) and then BUD (2.3).Reviews of intranasal administration of BDP, BUD and FP and MF indicate no detectable effects on measures of HPA axis function at recommended doses, which is in agreement with rapid hepatic metabolism of these INCS.(145, 158).

Intranasal corticosteroids currently in use for CRS have a high ratio of topical to systemic activity(159). They are delivered into the nose as aerosols or metered dose pump sprays or aqueous spray pumps or pure powder formulations. Of these, the latter two have a more favourable intranasal drug distribution(160). Lipworth and Seck(161) estimated that around 80%of the drug is available for absorption from the nasal mucosa following intranasal administration. This is because of the nasociliary clearance into the throat. Yet another study using positron emission tomography showed that 10% to 20% of the drug remained in the anterior nasal cavity and inferior turbinate for up to 1.5 hours(162).

As maintenance therapy, topical steroids showed the most benefit in CRS patients with and without polyps. There is also early evidence which shows that high volume nasal irrigations (e.g. BUD rinses) are more effective than the low volume applications (i.e. meter-dosed spray, atomized, or nebulized solutions). A meta-analysis of 6 RCT (all double-blinded) has shown that CRSwNP treated with either mometasone or budesonide showed a significant decrease in polyp size which was directly correlated with a decrease in symptom scores(123). Most of these studies report an improvement in the nasal symptoms of congestion and rhinorrhoea while the improvement in the symptom of altered smell sensation is controversial. There is now increasing evidence supporting the use of nasal steroids for

CRSsNP[163]. Unlike the previous belief that steroids might exacerbate the infection in CRSsNP, studies suggest that the benefits of topical steroids treatment for CRSsNP outweighed the minor adverse effects of epistaxis and headache. The improvement of symptom score seen in these patients was significantly higher than in patients treated with placebo. A recent Cochrane review by Chong et al[164] analysing the use of nasal steroids in CRSwNP and CRSsNP concluded that there was not much information about the quality of life (very low-quality evidence), however, the disease severity for all symptoms improved (low-quality evidence). There was a moderately sized benefit for nasal blockage (congestion) and a small benefit for rhinorrhoea (moderate-quality evidence). Although many studies mentioned the risk of local irritation, the risk could not be quantified (low-quality evidence). The risk of epistaxis was increased (high-quality evidence), but the clinical relevance was unclear.

INCS have been identified as the mainstay of treatment in post-surgical cases of CRS due to their potential to reduce the formation of adhesions and also recurrent mucosal oedema. This has also been demonstrated in molecular biological studies where INCS treatment reduce the expression of cytokines and other growth factors[165, 166]. Fluticasone propionate, a higher generation INCS compound has been shown to reduce the production of type I, III and V collagens and the expression of profibrotic cytokines IL-11 and IL-17 in an RCT where nasal polyp biopsies were analysed before and after treatment[167]. Whereas in another double-blindedplacebo-controlled trial, the same INCS compound was shown to decrease the expression levels of IL-4, IL-13, TNF-alpha and IL-1beta, but with no effects on the expression of endothelial vascular cell adhesion molecule (VCAM)-1 and P-selectin[168].

The reported incidence of side effects of topical nasal steroids in literature is quite a few. The most common symptom is mild epistaxis and this is usually due to incorrect positioning of the sinus rinse bottle towards the nasal septum instead of towards the eye and also the intrinsic vasoconstrictor activity of steroid. The bioavailability of fluticasone and

mometasone is determined to be at 1%, whereas that for budesonide and triamcinolone is closer to 40% to 50%. There is however no evidence currently to show that topical nasal steroids cause HPA suppression at the typical doses given for CRS[130, 169].

1.8 Types of intranasal steroids

1.8.1 Hydrocortisone

Silcox L E used intranasal hydrocortisone alcohol in patients with allergic rhinitis, allergic rhinitis with polyps and acute rhinitis. Formulas with different concentrations of hydrocortisone were used (50mg/100ml,2mg/100ml, 22mg/100mland 20mgmg/100ml with and without vasoconstrictors). The study showed that all the concentrations of hydrocortisone had satisfactory effects on allergic rhinitis with or without polyps. Of the preparations, 20mg/100ml with vasoconstrictors was the most beneficial in the anti-inflammatory action and providing subjective relief[170].

The preparations of hydrocortisone, dexamethasone, and methylprednisolone, are currently only used off-label to treat refractory cases of CRS.

1.8.2 Beclomethasone

Beclomethasone dipropionate (BDP) was the first clinically active aerosolized corticosteroid discovered in 1972. The use of BDP nasal spray especially in children is now not recommended since it was shown to affect the growth rate in children when used for one year.

1.8.3 Fluticasone

Fluticasone propionate (FP) has been approved as a commercial INCS in 1990 for use in adults and children above 4 years of age. The indications include seasonal and chronic allergic and non-allergic rhinitis, perennial rhinitis and CRSsNP and CRSwNP. It is one of the more potent glucocorticoids available for intranasal use. The formulations are available

as metered dose nasal sprays and nasal drops. The daily adult dose of fluticasone is 200 to 400 μg/day in both nostrils.

FP has been used in the treatment of CRSsNP and CRSwNP. Most of the studies focus on CRSwNP with many of them showing improvement on the polyp scores and disease severity[171, 172]. FP, when used as nasal drops, has been shown to decrease the disease recurrence in CRSwNP patients following surgical clearance of the disease. The requirement for surgery in patients with severe polyposis was also found to be significantly reduced following the use of intranasal FP drops[173]. A recent study has shown that fluticasone delivered through an exhalation delivery system significantly improves subjective and objective measures of disease and quality of life scores in CRSsNP patients[174]. A comparison between FP and BDP showed similar effectiveness in terms of disease severity and epistaxis[172].

1.8.4 Mometasone

Mometasone furoate (MF) is among the more recently introduced topical INCS with potency equivalent to FP and almost undetectable systemic availability[145] and was first introduced as a nasal spray in 1998.Its efficacy in the treatment and prophylaxis of seasonal and perennial rhinitis, asthma, ARS and adenoid hypertrophy has been well documented in the literature. In patients with CRSwNP, a daily administration of MF decreases nasal congestion and the size of polyps. It causes minimal or no adverse effects while providing an improvement in the quality of life score and the sensation of smell[171, 175].

A Cochrane review comparing different corticosteroids and their efficacy found no significant difference in the clinical outcomes of nasal obstruction, postnasal drip, anterior rhinorrhoea when FP and MF were used for CRS. The study also found no significant difference between the two drugs for most of the parameters assessed. The analysis also showed that the effectiveness of high and low doses of FP (800μg and 400μg) and MP

(400µg and 200µg in adults, 200µg and 100µg in children) were comparable. However, all the studies reported an improvement in the polyp score with high dose(172).

MF has been shown to be safe for use in pediatric population and in pregnancy. Compared to BDP, MF showed no suppression of bone growth in children after one-year treatment(145). The long-term administration of MF attenuates the inflammatory process decreasing the extent of inflammatory cell infiltration (especially eosinophils) without affecting the epithelial thickness or causing atrophy.

1.8.5 Budesonide

Budesonide was initially developed for use as a respiratory suspension for bronchial asthma. This preparation is now being increasingly used in CRS in an atomizer or drop formulation or in large-volume irrigations to treat uncomplicated as well as recalcitrant CRS. There is now increasing evidence in the literature supporting its use in pre and post-surgical cases of CRS with benefits being superior to traditional nasal sprays in post-operative cases. This is largely due to the ease of administration as a large volume rinse through saline apart from the clinical benefits it offers in the absence any systemic side effects. It is also well tolerated by the patients while giving a high localized concentration of steroid (176).

Budesonide is a glucocorticoid structurally related to 16αhydroxy prenisolone. It is non-halogenated and contains carbon rings. The chemical name of this molecule is 16α, 17α - 22R, S-propylmethylenedioxy-pregna-1, 4-diene-11ß, 21-diol-3, 20-dione. It is a white powder with free solubility in chloroform and sparing solubility in ethanol. It is insoluble in water and heptane. The melting point is 226^0Celcius. It is available as powder inhalers, nebulizing suspensions as respules, metered dose inhalers, metered nasal sprays, ointments, and creams. The two main metabolites of budesonide include 6 beta-hydroxy budesonide and 16 alpha-hydroxy prednisolone.

Budesonide is used in CRS as a pressured dosed nasal spray (e.g. Rhinocort) or as respules.

The respules can be directly instilled into the nasal cavity or used as rinses by dissolving them in saline water or can be applied directly into sinus cavity dissolved in sinus dressings like chitosan gel or gel foams. There is also early evidence which shows that high volume nasal irrigations (e.g. Budesonide rinses) are more effective than the low volume applications (i.e. meter-dosed spray, atomized, or nebulized solutions)(123).

The safety profile of budesonide with regards to HPA axis suppression is currently being investigated. A small prospective study using intranasal budesonide did not show any significant change in intraocular pressure over 22 months although the study reported subclinical HPA axis suppression on prolonged budesonide use(177). A recent prospective cohort study reported low stimulated cortisol levels in 23% of the patients after at least 6 months of treatment with topical budesonide despite being asymptomatic(130). There was also no bone suppression in children after 1-year treatment with budesonide (145). The US Food and Drug Administration (FDA) has classified intranasal steroids as category C with the exception of budesonide, which is category B in early pregnancy.

Table 1: ***Topical nasal applications***

Topical steroid preparations used in nasal application reproduced(130)

Name	Dose (per spray)	Frequency	Notes
Beclomethasone dipropionate	40 or 80 μg	1 spray per nostril bid	
Budesonide spray	32 μg	1–4 sprays per nostril once daily	For CRSwNP – 128 μg (2 sprays per nostril) once daily
Budesonide respules	0.5–1 mg	1. Direct drops (1 mL in each nostril bid) 2. High-volume irrigation bid 3. Mucosal atomizer device, 1 mL (0.25 mg) in each nostril	For irrigation, dissolve 1 mg in 240 mL of normal saline and irrigate 60 mL per nostril bid

		bid	
Ciclesonide	50 μg	2 sprays per nostril once daily	
Ciprofloxacin/ dexamethasone otic drops Prednisolone acetate ophthalmic drops	3 mg/mL ciprofloxacin 1 mg/mL dexamethasone 1 mg/mL prednisolone	2 drops bid per nostril twice daily	Can be tapered to once daily and then every other day
Flunisolide	25 μg	2 sprays per nostril tid	
Fluticasone propionate	50 μg	2 sprays per nostril bid	
Fluticasone furoate	27.5 μg	2 sprays per nostril once daily	May decrease to 1 spray per nostril once daily after symptoms controlled
Mometasone furoate	50 μg	1 spray per nostril bid	
Triamcinolone acetonide	55 μg	1–2 sprays per nostril once daily	

bid- two times a day, tid- three times a day

1.9 Pulmicort in CRS

Pulmicort (Budesonide) respules are commercially marketed in the concentration of 0.5mg/2ml and 1mg/2ml. The other ingredients in the formulation include disodium edetate, sodium chloride, polysorbate 80 (E 433), citric acid - anhydrous (E 330), sodium citrate (E 331) and water for injections. These are also known as inactive ingredients or excipients. Excipients are compounds used in pharmaceutical preparations to solubilize or suspend a drug, for preservation and for the stability of the drug composition for optimal action of the drug. The excipient as such is not considered to be involved in the treatment process as it is present only in very minute quantity.

Pulmicort is widely used off label in the management of CRS and has shown many promising results in comparison to many of the new generation high potency INCS. When treatment with fluticasone nasal spray (group A) or budesonide directly instilled into the nostril using a mucosal atomizing device (group B) or budesonide instilled into the nose in a vertex-to-floor position of the head (group C) was given in post-surgical patients with CRSwNP, the patients in group B showed greater reduction in sinonasal outcome test (SNOTT-22) and Lund -Kennedy scores at six months postoperatively which was closely followed by group C[178]. A recent double-blinded RCT on adult patients to evaluate the incremental effect of adding budesonide to large-volume, low-pressure saline sinus irrigation found that the budesonide group experienced a clinically meaningful reduction in their SNOT-22 scores as well as improvement in the objective endoscopic scoring compared with the control group[179].

The major limitation in the use of budesonide respules in the treatment of CRS (apart from being not FDA approved for use in the nasal cavity) is the cost expense on patients when the treatment has to be given over a prolonged period of time. Intranasal budesonide has been shown to not suppress the hypothalamic-pituitary-adrenal (HPA) axis or affect intraocular pressure when used in sprays, rinses, or respules[178, 180].

1.10 Influence of therapy on sinonasal bacterial profile

Therapy with medications including topical steroids, saline irrigations, oral steroids, and oral antibiotics form the first line of management in CRS. Surgery is indicated as a second line option in patients who do not respond to medications. A number of studies have evaluated the effect of the different medical therapies and surgery on the microbial pattern in CRS.

1.10.1 Invitro studies

The growth of *P. aeruginosa, S. aureus, H. influenzae, S. pneumoniae, M. cattharalis, B. cepaciae,* and *C. albicans* on petri dishes were inhibited in the presence of budesonide 200μg doses and not with fluticasone and beclomethasone. The study attributed this killing effect on the excipients present in budesonide(181).

1.10.2 Animal models

In an attempt to determine whether intranasal steroids help in the elimination of bacteria and thereby modulate the mucosal changes in a rabbit model of acute sinusitis, Cheng You et al(182). showed that intranasal corticosteroids may lessen infiltration by inflammatory cells. The study suggested that steroids could not be a substitute as a single therapy for antibiotics and the combined application of steroid and antibiotic did not decrease the effect of antibiotic but provided better efficacy. Transmission electron microscopic examination of the nasal mucosa in this study showed significant ultrastructure changes in the intranasal corticosteroids group and control group compared to the antibiotic group and hence they concluded that a prompt therapy including an antibiotic is essential to restore the mucosal changes occurring in acute sinusitis. The antibiotic used in the study was ampicillin as intramuscular injection and the steroid budesonide as a nasal spray. *S. pneumoniae* which were used to inflict the acute sinusitis was not detected in the bacterial cultures of all the four groups of rabbit maxillary sinus secretions in the study. The cultures instead, showed different kinds of Gram-negative bacteria which suggested that an original infection could be replaced by opportunistic pathogens.

1.10.3 Clinical trials

1.10.3.1 Pre-surgical

A few studies have evaluated the effect of medical therapy on the sinonasal microbiome in patients who have not had prior sinus surgery. A recent study by Jain et al.(137) compared the

effect of treatment with oral Doxycycline and oral prednisolone for 7 days in patients with uncomplicated CRS. Bacterial communities of *Corynebacterium and Staphylococcus* were found in the pre-treatment swabs of all their patients. The average relative abundance of *Propionibacterium* was significantly increased in the doxycycline group and *Corynebacterium* significantly reduced in the prednisolone group following treatment. They found no significant differences in clinical scores, endoscopic scores, changes in bacterial profiles, and changes in bacterial burden within or between treatment arms.

The administration of nasal steroid spray for allergic rhinitis did not change the *S. aureus* carrier state in these patients(183). The steroid used in this study was triamcinolone acetonide aqueous intranasal spray. When the effect of intranasal fluticasone on the bacterial culture rate and drug sensitivity was assessed in patients with CRSwNP, no significant difference was observed in the bacterial isolation and drug sensitivity of oxacillin and cephazolin before and one month after nasal steroid use. The most common bacteria isolated at both time points were *S. aureus, CNS, and S. pneumonia*. The study suggested that nasal steroids did not facilitate colonization and possible super-infection and antibiotic sensitivity in CRS patients(184).

1.10.3.2 Surgical

Quite a number of studies have evaluated the effect of endoscopic sinus surgery on the microbiota of CRS. Cleland et al.(114) examined the nasal swabs from 23 patients with CRS undergoing surgery and 11 non-CRS patients undergoing transsphenoidal pituitary surgery. Further postoperative swabs were collected in the CRS group. Bacterial identification was done using16S ribosomal DNA bacterial tag-encoded FLX amplicon pyrosequencing. The study showed a higher mean relative abundance of *Acinetobacter* (22 vs 4%) while a lower relative abundance of *S. aureus* in non-CRS controls. In the CRS patients, *S. aureus* was also the most abundant species, followed by *S. epidermidis* (10%), *P. acnes* (8%), *Anaerococcus hydrogenalis* (5%), and *P. aeruginosa* (4%) at the time of surgery. However,

when an analysis of the temporal change of mean relative abundance was done on these patients, there was a significant increase in *A. johnsonii*, from 4% during surgery to 37% at the late postoperative time point and *Acinetobacter schindleri*, which increased from 1% during surgery to 5% at the late postoperative time point. Jain et al.(92)in their longitudinal cohort of 23 patients also reported shifts in bacterial community composition post-surgery. *Staphylococcus* (pre: 95.7%, post: 100%), *Streptococcus* (pre: 95.7%, post: 100%), *Corynebacterium* (pre: 100%, post: 95.7%), and *Propionibacterium* (pre: 95.7%, post: 87%) were the most prevalent genera detected in the 23 patients both before and after surgery. They observed an increase in the number of operational taxonomic units (OTU) in spite of all the patients receiving oral antibiotics for at least two weeks following surgery. This was primarily due to the large increase in *Staphylococcus* (an increase of average relative abundance of 24.7%) while most other genera had reductions in average relative abundance after surgery which was seen consistently with and *Streptococcus* and *Corynebacterium*. A reduction in *Corynebacterium* was seen to be associated with an increase in symptom scores and qPCR replicates postoperatively. A similar result was observed by another study where bacterial burden but not the complexity was highest just after two weeks of antibiotics following surgery suggesting that antibiotics did not eliminate the commensals but in fact increased the bacterial load. A Principal coordinate analysis (PCoA) plot illustrated that the ethmoid microbiota shifted after surgery (day-1) and antibiotic administration (2 weeks) but returned toward the original baseline in many of the patients at 6weeks. The study also compared the nasal swabs from different sites and found that bacterial communities colonizing the ethmoid at 6 weeks postoperatively were most similar to anterior nasal cavity and pre-treatment bacterial profiles, suggesting that these sites may be a likely source for bacterial repopulation, or alternatively, that all sites may naturally return to their baseline states(185).

1.10.3.3 Post-surgical

Liu et al[140]. recruited a small longitudinal cohort study with six patients with patent maxillary antrostomy and active sinus inflammation, and who had not received medical therapy in the form of antibiotics or corticosteroids in the previous eight weeks. Evaluation of the pre and post microbiome after maximal medical treatment showed a consistent decreased total number of unique bacterial taxa among the participants. Patients had a highly specific microbiome pattern both before and after treatment and there was no tendency to incline to a specific microbiota in response to specific therapy. The authors underscore the potential futility of the search for a universal antimicrobial strategy. An interesting finding, however, was that post-treatment, participants frequently became colonized by taxa that are less susceptible to the prescribed antibiotics. Yet another larger study by the same group examined the effect of topical treatments with nasal saline irrigation, topical steroid sprays, or corticosteroid rinses on the microbiota of CRS patients with nasal polyps and controls without active symptoms and signs of sinusitis but with patent antrostomy. The microbiome analysis of the patients showed no association with the use of nasal saline irrigation, with or without added budesonide to a significantly distinct sinonasal microbiota composition. Nevertheless, the use of topical steroid sprays was associated with a distinct microbiota in control subjects[186]. In an analysis by Fazel et al.[102] to examine the factors influencing bacterial biodiversity in CRS patients, they found that asthma and antibiotic use were both associated with reduced microbial diversity and increased *S. aureus* abundance, as measured by specific qPCR. They suggested that asthma and antibiotic use promoted a higher abundance of *S. aureus* in the middle meatus, leading to a decrease in biodiversity.

Many of the studies show a change in the microbial pattern after medical or surgical intervention. It is also seen that while some patients recover from this bacterial community change to return to an initial pretherapy state, others continue to shift toward a new and

different microbial community. These effects possibly depend upon the treatment used and patient immune responses.

1.10.4 Effect of steroids on microbiome or bacteriology of CRS

There are a few studies in the literature which evaluated the effect of steroid therapy on the sinonasal bacteriology. Most of them have focused on the biofilm forms of bacteria. Biofilm formation has been found to be significantly reduced in the presence of corticosteroids mometasone and fluticasone. The reduction was more pronounced when treated with fluticasone. In the same study, isotonic saline was found to be superior to fluticasone in reducing biofilm formation. Among the bacterial strains *S. aureus, P. aeruginosa* and *S. pneumoniae* appeared to be more sensitive to the antibiofilm action of corticosteroids and isotonic saline(73).A study by Goggin et al.(187) investigated the antibacterial potential of corticosteroids like fluticasone, mometasone, and budesonide on *S. aureus* biofilms grown on pegs. Biofilm biomass quantification done using confocal scanning laser microscopy showed a biofilm biomass reduction with the higher doses (clinically significant) of the corticosteroids tested. Both these studies however determined the bacterial biofilm biomass which includes both live and dead bacteria. Hence the different metabolic responses and activity of bacteria to corticosteroids could not be established in the studies. A similar antibacterial action of nasal corticosteroids and saline irrigation was also seen on the planktonic form of bacteria by Liu et al.(186)in subjects with CRSwNP. The study by Nadal et al(89) indicated that *P. aeruginosa* was also more common in those who received systemic steroids.

Presence of biofilms has been associated with significant epithelial destruction and metaplasia which is independent of INCS use in CRSwNP patients. INCS, however, influence the type of inflammatory cell infiltrate seen in the sub-epithelial layers of polyps with a predominant lymphocyte and plasmocytic infiltration seen with INCS use and a predominant polymorphonuclear cells infiltrate seen in the absence of INCS use(166). A

persisting microbial biofilm causes subepithelial inflammatory reaction and consecutive extracellular matrix production in CRSwNP(165). This effect is not affected by INCS use where no significant associations were found with biofilm positivity, biofilm thickness or microscopic architecture of the respiratory mucosa(166).

There has been some concern regarding the long-term use of INCS especially in post-operative cases where the ability of steroids to cause immunosuppression and decrease cytokine production was proposed to facilitate colonization and superinfection by pathogenic bacteria. Contradicting this hypothesis, studies have shown that the use of topical INCS resulted in a decrease in isolation of bacteria from ethmoid sinuses undergoing functional endoscopic sinus surgery(184, 188). The study showed that although the rate of recovery of bacteria, as well as isolation of S. aureus, was higher in revision surgery cases compared to primary surgery, the use of INCS preoperatively decreased the bacterial isolation, especially in the revision cases. The presence of purulent nasal secretions was also less in the group of patients who were on INCS. It was suggested that the anti-inflammatory property of INCS prevented mucosal oedema and sinus obstruction thus decreasing the chance of recurrent bacterial infections after the initial surgical clearance of the disease.

The exact mechanism by which topical steroids and bacteria interact remains unclear. The steroid molecule, as well as the different excipients used in commercial topical steroid, could potentially influence bacterial growth in multiple ways. From a microbiome perspective, if topical steroids are considered to promote bacterial growth due to their immunosuppressive property it could potentially promote both protective and pathogenic bacteria. The final microbial milieu would then be in favour of the bacterial communities which thrive better on steroids and other compounds in topical nasal preparations. This also leads to the potential for development of bacterium that could have steroid resistance and hence could explain the inadequate response to steroids in many patients.

1.10.5 Corticosteroids on microbiome outside the sinonasal cavity

Pragman et al., in their study of the lung microbiome in patients with Chronic Obstructive Pulmonary Disease, demonstrated the separation of microbial clusters based on the use of inhaled corticosteroids (189). Other studies have also shown distinct microbiome profiles in patients with corticosteroid-sensitive vs corticosteroid-resistant asthma, that they attribute to the presence of specific bacteria that degrade corticosteroids and modulate the responsiveness to corticosteroids of inflammatory cells (190)Bacterial colonization with *S. aureus* in lesions of atopic dermatitis has been shown to be eradicated using topical therapy with topical corticosteroid (betamethasone valerate)(191).

An animal study to delineate the functional characteristics of glucocorticoid-mediated changes on gut microbiota and their subsequent repercussions on host mucin regulation and colonic inflammation found a phylogenetic shift in the gut microbiota following treatment with dexamethasone both acutely (10days) and chronically (4 weeks) in healthy SPF mice. This was found notably in the *Actinobacteria* genus, where *Bifidobacterium and Lactobacillus* were significantly elevated after dexamethasone treatment when compared to controls while the colonic mucin degrader, *Mucispirillum*, was noticeably absent aftertreatment. Thus, suggesting a possible relationship between glucocorticoids, altered gut microbiota, and mucin regulation in the intestine. The study also showed a significant decrease in mucin gene expression after dexamethasone treatment. This the authors suggested was protective in the settings of inflammation to maintain the intestinal homeostasis. Further findings in this study were that gut microbiota is necessary and sufficient to regulate mucin gene expression while dexamethasone treatment in the absence of microbes could not produce such effects. Further, they concluded that dexamethasone mediated shifts in gut microbiome confer a protective effect on the development of colonic inflammation when these microbes are conventionalized into the IL10-knock out mouse model(192).

1.10.5.1 Influence of steroids on the antimicrobial activity of antibiotics:

A study by Plotkin et.al. investigated the action of androgens, testosterone, and dihydrotestosterone, and of glucocorticoids, hydrocortisone and dexamethasone, on growth kinetics and antibiotic susceptibility of *Escherichia faecalis*, *Escherichia coli, P. aeruginosa, and S. aureus*. They found an increased sensitivity of *S. aureus* to Erythromycin and *P. aeruginosa* to tobramycin in the presence of steroid hormones. The antibiotic susceptibility changes were not related to the growth kinetics of the organisms(193). However, other studies show that the rate of bacterial growth is inversely proportional to the minimum inhibitory concentration (MIC) of antibiotics, especially those which act on cell functions(194). Ernest Jawetz(195) evaluated the quantitative effects of cortisone on the results of antimicrobial therapy in experimental lethal infections in mice with *Klebsiella pneumoniae (K. pneumonia) and S. pyogenes*. The study showed that cortisone significantly reduced the therapeutic efficacy of penicillin G potassium, streptomycin sulphate, and chlortetracycline hydrochloride. However, in their invitro studies, cortisone did not have any direct effect on the bacterial growth rate and also failed to show any influence on the bactericidal or bacteriostatic action of antimicrobial agents. They hence concluded that the depressive effect of cortisone on antibiotics is mediated through a host dependent mechanism.

1.11 Bacterial and steroid metabolic interactions

Steroids are important signalling molecules essential in many biological processes. In eukaryotic cells, adrenal steroids like mineralocorticoids and glucocorticoids help in electrolyte and glucose homeostasis. Sex hormones like androgens, oestrogens and progesterone help in reproduction. Certain steroid molecules like cholesterol play an inevitable role in the stabilization of the cell membrane in eukaryotes. Steroids are however not known to occur in primitive eukaryotes and prokaryotes like bacteria. These vertebrate steroid compounds (such as sex steroids, cholesterol) and bacterium are ubiquitous in the

environment and has been proposed to serve as a natural source of carbon for the microbes. The literature on the effects of steroids and related compounds on bacterial growth or metabolism is very limited. The complex interactions between bacteria and steroids have been observed in soil and aquatic bacterium and an impressive data on the steroid degradation pathway in terms of steroid intermediate and metabolite identification, as well as the characterization of the diverse enzymes involved, has been reported in the literature. The best-described steroid degrading bacteria is *Comamonas testosteroni* (*C. testosteroni*) which was done by Horinouchi et al.[196]. Steroid transformation and metabolism are often performed by specific hydroxysteroid dehydrogenases (HSDs) that belong to the short-chain dehydrogenase/reductase (SDR) superfamily. Studies on 3α-hydroxysteroid dehydrogenase (3α-HSD) lead to the identification of a novel SDR gene (SDRx) which was found to code for 7α-hydroxysteroid dehydrogenase (7α-HSD) that is involved in steroid degradation in *C. testosteroni*. The 7α-HSD is also known to catalyse the dehydrogenation of the hydroxyl group at position 7 of the steroid skeleton of bile acids found in mammalian liver[197, 198].

A study aimed at identification of classified functional HSDs and the bioinformatic annotation of these proteins in all complete sequenced bacterial genomes as well as a corresponding phylogenetic analysis found that the dominating phyla that express HSDs were that of *Actinobacteria, Proteobacteria, and Firmicutes* and also some evolutionary microorganisms like *Cyanobacteria and Euryachaeota*. The authors suggested that steroid metabolism is an evolutionarily conserved mechanism that might serve different functions such as nutrient supply and signalling[199]. A similar bioinformatics study which looked at the distribution of aerobic steroid catabolism pathways among over 8,000 microorganisms whose genomes are available in the NCBI RefSeq database identified 265 putative steroid degraders within *Actinobacteria and Proteobacteria*, which mainly originated from the soil, eukaryotic host, and aquatic environments. In many of the actinobacterial genera, particularly in members of the *Corynebacterium,* a pathway for cholesterol degradation was

conserved and for the members of genus *Rhodococcus*, a cholate degradation pathway was present. A pathway for testosterone and, sometimes, cholate degradation had a patchy distribution among *Proteobacteria*. Growth experiments done to confirm this bioinformatics prediction found nine bacterial strains (*Pseudomonas resinovorans, Cupriavidus necator, Sphingomonas wittichii, Shewanella pealeana, Thermomonosporacurvata, Actinoplanes missouriensis, Salinisporaarenicola Amycolicicoccus subflavus, Amycolatopsis sp*.) capable of steroid metabolism. A single ancestral 9,10-seco-steroid degradation pathway was found to be involved in the bacterial steroid metabolisms(200).

Durham et al.(201) examined the effect of different steroids on the growth of *Aerobacter aerogene*(*A. aerogene*) and found that stilbestrol, oestradiol, and progesterone in a concentration-dependent manner enhanced its growth when starch but not glucose, maltose, or raffinose was present as a source of carbon and energy. Cholesterol, estrone, and testosterone did not significantly affect the rate of growth in these experiments. They also suggested that progesterone and oestradiol exerted this effect by influencing the metabolic process of the bacteria while stilbestrol exerts its activity on the enzyme system to enhance bacterial growth. It was also seen that *A. aerogene* was unable to oxidize the steroid compounds or stilbestrol as a sole source of energy.

Raboaun et. al (202)compared the degradation of topical hydrocortisone 17 butyrate in the presence of six different bacteria found on psoriatic skin. The degradation products were analysed using high-performance liquid chromatography (HPLC). They observed that the cocci (*S. aureus, S. epidermidis, Streptococcus agalactiae*) did not change the degradation of hydrocortisone when compared to the 16·6 +/- 7·1% degradation seen in the absence of microbes. Three bacilli, however, increased degradation, *Escherichia coli* 59·1+/-19·4%, *K. oxytoca* 62·1+/-6·7% and *P. aeruginosa* 56·0+/-17·9%.

Steroid compounds are constantly discharged into the environment and act as a substrate for

many organisms. The metabolism of steroid by environmental (soil and aquatic) as well as human pathogenic and commensal bacteria potentially have implications in many disease pathogenesis and progression apart from having important pharmaceutical and health applications.

2 Chapter 2: Effect of commercial nasal steroid preparation on bacterial growth

2.1 Statement of authorship

Statement of Authorship

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Name of Principal Author (Candidate)	Lisa Mary Cherian		
Contribution to the Paper	Conception and design of the project, acquisition of data, analysis and interpretation of data, drafting the article		
Overall percentage (%)	85%		
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	29.06.19

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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Effect of commercial nasal steroid preparation on bacterial growth

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Background: **Topical budesonide (Pulmicort; AstraZeneca AB, Sodertalje, Sweden) is commonly used in the management of chronic rhinosinusitis (CRS). Although its use is due to its perceived anti-inflammatory effect, studies have suggested that it may also have antibacterial properties. To make the hydrophobic steroid molecule suitable for topical administration, pharmaceutical excipients are used in commercial steroid formulations. Herein we investigated the antibacterial action of commercial budesonide and its excipients.**

Methods: **Planktonic and biofilm forms of *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA) were treated with Pulmicort or its excipients at clinically relevant concentrations. Bacterial growth was determined by optical density, resazurin assays, colony-forming unit counts, and Giemsa staining. Minimum inhibitory concentration (MIC) studies assessed excipients' potentiation of antibiotics. Experiments were conducted in triplicate and results analyzed using one-way analysis of variance.**

Results: **There was significant reduction in planktonic and biofilm growth of *S aureus* and MRSA on exposure to budesonide ($p < 0.0001$) and its excipients ($p < 0.0001$). Excipient ethylene diamine-tetraactic acid (EDTA) demonstrated an antibacterial property even at the low concentrations used in topical preparations ($p < 0.0001$). With amoxicillin, excipients exhibited a potential additive/synergistic effect on MIC, whereas erythromycin and aminoglycosides showed an antagonistic action.**

Conclusion: **The commercial product Pulmicort has a direct antibacterial effect on the planktonic and biofilm forms of *S aureus* and MRSA. This effect is at least in part mediated through the excipient EDTA in the product. Excipients also influenced the antimicrobial activity of antibiotics depending on the bacterial strain and antibiotic tested.**

Key Words:
Staphyloccus aureus; MRSA; biofilms; Pulmicort; excipients; EDTA; amoxicillin; aminoglycosides

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Corticosteroids together with antibiotics form the mainstay of medical therapy for chronic rhinosinusitis (CRS), although the specific regimen may vary. Among these, topical corticosteroids are popular due to their absence of systemic side effects.[1] Most therapeutic effects of steroids result from their anti-inflammatory action mediated through glucocorticoid receptors, which suppress inflammatory gene transcription. They also inhibit nuclear factor-κB and activating proteins causing a downregulation of cytokines and inflammatory molecules.[2,3] This action is responsible for decreased mucosal inflammation, improved symptom scores, and reduced recurrence after surgery. It is, however, unclear how sinonasal bacteria respond to topical steroids.

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Effect of commercial nasal steroid preparation on bacterial growth

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2.2 Abstract

Background: Topical Pulmicort (Budesonide) is commonly used in the management of chronic rhinosinusitis (CRS). Although this is due to their perceived anti-inflammatory effect, previous studies suggest that they may also have anti-bacterial properties. To make the hydrophobic steroid molecule suitable for topical administration, pharmaceutical excipients are used in commercial steroid formulations. Here we investigated the antibacterial action of commercial budesonide and its excipients.

***Methods*:** Planktonic and biofilm forms of *S. aureus* and methicillin-resistant *S. aureus* (MRSA) were treated with Pulmicort or its excipients at clinically relevant concentrations. Bacterial growth was determined by optical density, Resazurin assays, colony forming unit counts and Giemsa staining. Minimum inhibitory concentration (MIC) studies assessed excipients' potentiation of antibiotics. Experiments were conducted in triplicates and results analysed using one-way ANOVA.

***Results*:** There was significant reduction in planktonic and biofilm growth of *S. aureus* and MRSA on exposure to budesonide ($p < 0.0001$) and its excipients ($p < 0.0001$). The excipient EDTA demonstrated antibacterial property even at the low concentrations used in topical preparations ($p<0.0001$). With Amoxicillin, excipients exhibited a potential additive/synergistic effect on MIC while Erythromycin and aminoglycosides an antagonistic action.

***Conclusion*:** The commercial product Pulmicort has a direct antibacterial effect on the planktonic and biofilm forms of *S. aureus* and MRSA. This effect is at least in part mediated through the excipient EDTA in the product. Excipients also influenced the antimicrobial activity of antibiotics depending on the bacterial strain and antibiotic tested.

2.3 Introduction

Corticosteroids together with antibiotics form the mainstay of medical therapy for chronic rhinosinusitis (CRS) although the specific regimen may vary. Among these, topical corticosteroids are popular due to their absence of systemic side effects(123).Most therapeutic effects of steroids result from their anti-inflammatory action mediated through glucocorticoid receptors which suppress inflammatory gene transcription. They also inhibit nuclear factor-κB and activating proteins causing a down regulation of cytokines and inflammatory molecules(146, 203). This action is responsible for decreased mucosal inflammation, improved symptom scores and reduced recurrence after surgery. It is, however, unclear how sinonasal bacteria respond to topical steroids.

In an evaluation of the effect of fluticasone, mometasone and budesonide on *S. aureus* biofilms, Goggin et al(187)reported a significant biofilm biomass reduction in the presence of steroids. Since biomass measures both dead and live bacteria together, the metabolic activity and exact response of bacteria to steroids remained unexplored. Similar antimicrobial effects have been identified in other chronic inflammatory conditions, like atopic dermatitis, where topical steroids reduced *S. aureus* colonization.(204)Another study has suggested an indirect antibacterial action in which hydrocortisone increased *S. aureus* sensitivity to erythromycin, without changing the bacterial growth kinetics.(193)

Commercial preparations of topical nasal steroids also contain non-therapeutic compounds known as excipients. In Pulmicort, these include disodium edetate (EDTA), sodium citrate tribasic dihydrate, citric acid anhydrous, polysorbate (tween) 80 and sodium chloride. The

exact concentration of these excipients in Pulmicort and most commercial preparations are unknown. There is, however, a standard guideline for the maximum permissible amount of each excipient that can be used in a pharmaceutical preparation.(205)Excipients are not actively involved in disease treatment but are utilised for preservation, stabilisationand suspension of the active agent. Nevertheless, studies have demonstrated the ability of excipients to reduce bacterial load. EDTA has been shown to eradicate bacterial biofilms from abiotic surfaces.(206, 207)Studies have also shown that combining EDTA with antimicrobials like ethanol complements its killing activity.(208)Tween 80, has been shown to increase the planktonic growth and biofilm biomass of *S. aureus* and slow the growth rate of *P. fluorescens*.(209)Saturated citric acid solution decreased bacterial load on dental plaques(210)and sodium citrate has been found to inhibit growth of many oral cavity bacteria.(211)

Despite being widely used in CRS, the action of excipients on bacteria at the concentration present in commercial nasal steroids and the interaction between excipients and traditional antibiotics currently remains unexplored. We, therefore, hypothesized that steroids and excipients could indirectly modulate inflammation through a direct antibacterial action. We aimed to look at the effect of a commercial nasal steroid and the various excipients both as a preparation and individually on *S. aureus* and MRSA planktonic and biofilm forms. We also investigated the effect of combining the excipients with antibiotics on MIC values against *S. aureus* and MRSA.

2.4 Methods

Bacterial strains:

Staphylococcus aureus ATCC 25923 and methicillin-resistant *Staphylococcus aureus* MRSA JE2 (USA300JE2) were purchased from American Type Culture Collection (Manassas, VA, U.S.).

Drugs and chemicals:

Budesonide 1mg/2ml (Pulmicort respules) was purchased from AstraZeneca AB, Sodertalje, Sweden. The maximum concentration excipient solution (Ex-max) was prepared by combining the maximum permissible food and drugs administration (FDA) approved dosage for each ingredient for topical nasal application in sterile Milli-Q water in the following concentrations: Citric acid anhydrous 0.027% ($HOC(COOH)(CH_2COOH)_2$, ≥99.5%), ethylenediaminetetraacetic acid, 0.02% ($(HO_2CCH_2)_2NCH_2CH_2N(CH_2CO_2H)_2$, 99%), sodium citrate tribasic dihydrate, 0.1% ($HOC(COONa)(CH_2COONa)_2 \cdot 2H_2O$, 99%), Polysorbate (Tween) 80, 0.04% ($C_{64}H_{124}O_{26}$, average M_w~ 79 000), sodium chloride, 1.7% (NaCl, ≥99%). Antibiotics Amikacin, Amoxycillin, Ciprofloxacin, Doxycycline, Erythromycin, Gentamicin, and Vancomycin (all purchased from Sigma-Aldrich, Steinheim, Germany). Water used in the experiments was prepared in a three-stage Milli-Q Plus 185 purification system (Merck Millipore, Darmstadt, Germany).

Determination of presence of budesonide in centrifuged Pulmicort with liquid chromatography- mass spectrometry (LC-MS).

Commercial Pulmicort respule at 1mg/2ml is a cloudy suspension of budesonide in excipients and cannot be used for optical density (OD) measurements. To allow accurate and reliable OD measurements, Pulmicort was centrifuged 10 times for 10 minutes at 4000rpm at room temperature resulting in a transparent supernatant. Pulmicort was diluted 50 fold in acetonitrile and both samples were analysed for the presence of budesonide using a Beckman (Fullerton, CA, USA) liquid chromatograph, consisting of a 126 pump and a 168-diode array detector. A Quattro II, triple-quadrupole mass spectrometer (Micro mass) was used for mass spectrometric analysis. Pulmicort supernatants were used for all treatments measuring the OD for planktonic bacterial growth. For all the other experiments, commercial Pulmicort was used.

The LC-MS analysis showed identical budesonide peaks after removal of insoluble particles in the Pulmicort respules, indicating the presence of budesonide even in the clear supernatant. (Figure 16).

Cytotoxicity assay

The local institutional Human Research Ethics Committee approved the study and primary human nasal epithelial cells (HNEC) were harvested from the inferior turbinate of consented non-CRS control patients. Cell cytotoxicity was determined by measuring the amount of lactate dehydrogenase (LDH) in the medium at 24 hours using the Cytotox Homogeneous Membrane Integrity Assay (Promega, Australia). Briefly, HNECs were grown in Bronchial Epithelial Cell Growth Medium(BEGM, Lonza, Walkersville, MD, USA) in 96-well flat-bottom plates and incubated for 24 hours (37°C, 5% CO2) as described previously,(212)followed by treatment with different concentrations of commercial Pulmicort (5,100 and 200ug/ml) and excipients (starting at a maximum FDA permitted). Cells were incubated for 24 and 48 hours before supernatant collected. 50ul of this supernatant was added to 50ul of LDH reagent, incubated for 30 minutes, and absorbance recorded at 490nm on a FLUOstar Optima plate reader (BMG, Labtech, Ortenberg, Germany). Negative controls included untreated cells and positive controls, cells exposed to Triton X-100 (Sigma Aldrich, Steinheim, Germany).

Pulmicort and excipient effect on planktonic growth of *S. aureus*/MRSA:

Single colonies of bacteria from a 24-hour growth on nutrient broth agar plate at 37^0C, 5% CO2was transferred into 10 ml nutrient broth (NB) and was incubated on a shaking platform (180 rpm) at 37^0C for 14 to 16 hours. A dilution factor was calculated from this overnight broth culture to obtain a starting OD 600 of 0.05. Prior to incubation, this volume was added to tubes containing treatments and NB (making a final total volume of 10ml) in the following proportions.

For the excipient treatment, a solution with maximum FDA approved concentration of the excipients (Ex_{max}) used in Pulmicort was prepared. From this, 2ml of the maximum, half maximum ($Ex_{1/2max}$) and one fourth maximum ($Ex_{1/4max}$) concentration was added to 8ml of NB. For the Pulmicort experiments, treatment A contained two respules of Pulmicort (each 1mg/2ml) in 6ml of NB, treatment B had one respule of Pulmicort in 8ml NB. The controls for A and B contained an equal volume of phosphate buffered saline (PBS; Sigma Aldrich, Steinheim, Germany) in the place of Pulmicort. In the OD measurement experiments, this Pulmicort was the clear centrifuged supernatant (sample 2) and for colony forming unit (CFU) determination and Giemsa staining, it was commercial Pulmicort as is.

i. *Growth curve determination by OD measurements*: OD readings at 600nm were taken every hour for 7 hours and then at 24 hours from each tube to plot a bacterial growth curve. The growth curve plotting was done as 9 independent experiments.

ii. *CFU determination*: Briefly, at the end of 24 hrs, a 20µl volume taken from the tubes with the above-mentioned treatments and serially diluted in PBS and spotted on nutrient agar plates in triplicates. Colony morphology was also determined. The CFU plating was done as three separate experiments.

iii. *Giemsa staining*: Another 20µl from each tube was spread on a glass slide and air dried. This was fixed in methanol for 7 minutes and then stained with 1:20 dilution of Giemsa stain (Sigma-Aldrich, Steinheim, Germany) in sterile Milli Q water for one hour. The slides were rinsed with distilled water, dried and examined under light microscope (Eclipse 90i, Nikon, Japan) at 60 x magnification under oil immersion.

Commercial Pulmicort and excipient effect on *S. aureus*/MRSA biofilm formation:

Alamar blue or Resazurin assay

Single bacterial colonies suspended in 0.9% saline were adjusted to 1.0 ± 0.1 McFarland units (approximately $3 \times 10_8$ CFU/mL) and diluted 1:15 in NB. The resazurin assay (Life Technologies, Scoresby, Australia), was done by inoculating a black 96-well microtiter plate (Costar, Corning Incorporated, Corning, U.S.) with this bacterial suspension. Commercial Pulmicort was added to the wells starting at a concentration of 250µg/ml in the first well and then serially diluted. The excipients were added at a starting concentration of $Ex_{1/2max}$ and then serially diluted. Each excipient at different concentrations was also used individually to treat the biofilms. The final volume in each well was 150µL and this incubated at 37°C for 48 h on a rotating platform (3D Gyratory Mixer, Ratek Instruments, Boronia, Australia) at 70 rpm. Biofilms were then rinsed twice with phosphate buffered saline (PBS) and the viability (metabolic activity) assay was done. For this, 200µL of a freshly prepared 10% resazurin dilution in NB was added to each well, protected from light, and incubated for 7 h at 37 °C on a rotating platform. The fluorescence was measured hourly on a FLUO star OPTIMA plate reader at $\lambda_{excitation}$ = 530 nm/$\lambda_{emission}$ = 590 nm.

Maximum fluorescence was typically reached after 4 h for both S. *aureus* and MRSA. Antimicrobial activity of the treatments used was quantified according to equation 1:

$$\%BK = \frac{F_C - F_T}{F_C} \times 100\ \%$$

Antimicrobial activity of commercial Pulmicort and excipient solution was expressed as the percentage of biofilm killing (% BK), where FC was the fluorescence of the untreated control biofilms (100% bacterial growth) and FT was the fluorescence observed in the treated biofilms. Both FC and FT were corrected for background fluorescence (sterile medium). Viability studies were performed as three independent experiments with six wells per treatment.

Effect of excipients on *S. aureus* and MRSA minimal inhibitory concentration (MIC) of antibiotics:

The MIC values of antibiotics (Amikacin, Gentamicin, Amoxicillin, Ciprofloxacin, Doxycycline, Erythromycin and Vancomycin) and the excipient mixture was assessed against the planktonic *S .aureus* and MRSA using standard methods(213)The MIC value which is the lowest concentration of the drug preventing bacterial growth was determined both visually and also quantitatively by OD measurements. Antimicrobial activity was quantified as:

$$\% \text{ Killing} = \frac{(ODc - ODt)}{ODc} \times 100$$

Where, ODc was the optical density of the untreated control wells (maximum bacterial growth) and ODt was the optical density in the treated wells. Both ODc and ODt were corrected for background absorbance (sterile medium). Viability studies were performed as six independent experiments.

The effect of consecutive treatment with excipients and antibiotics were done in a similar way at their sub-inhibitory concentrations.

Statistical analysis:

All the experiments were conducted at least in triplicate and were presented as mean ± standard deviation (SD) unless stated otherwise. The results were analysed using one-way analysis of variance (ANOVA) following Sidak test (GraphPad Prism version 7.00, GraphPad Software, La Jolla, U.S.). Statistical significance was assessed at the 95% confidence level.

2.5 Results

Effect of Pulmicort and excipients on planktonic growth of *S. aureus* (ATCC 25923) and MRSA (JE2)

OD measurements after 24 hours co-culture with Pulmicort (100 and 200 μg/ml) indicated a significantly reduced *S. aureus* and MRSA growth compared to appropriate volume controls in a dose dependent manner (Figure 11A, $P<0.001$). The maximum concentration excipient solution (Ex_{max}) also showed significant reduction of bacterial growth of *S. aureus* and MRSA (Figure 11B, $P<0.0001$) whilst $Ex_{1/2max}$ reduced growth of the MRSA strain only ($P<0.01$) (Figure 11B). These results were confirmed on CFU counts which demonstrated a similar pattern of anti-Staphylococcal effect from both Pulmicort and its excipients (Figure 11C and 11D). Giemsa staining of the Pulmicort and excipient treated tubes further confirmed findings with a visible reduction in the number of the bacteria when compared to untreated volume control (Figure 11E-G).

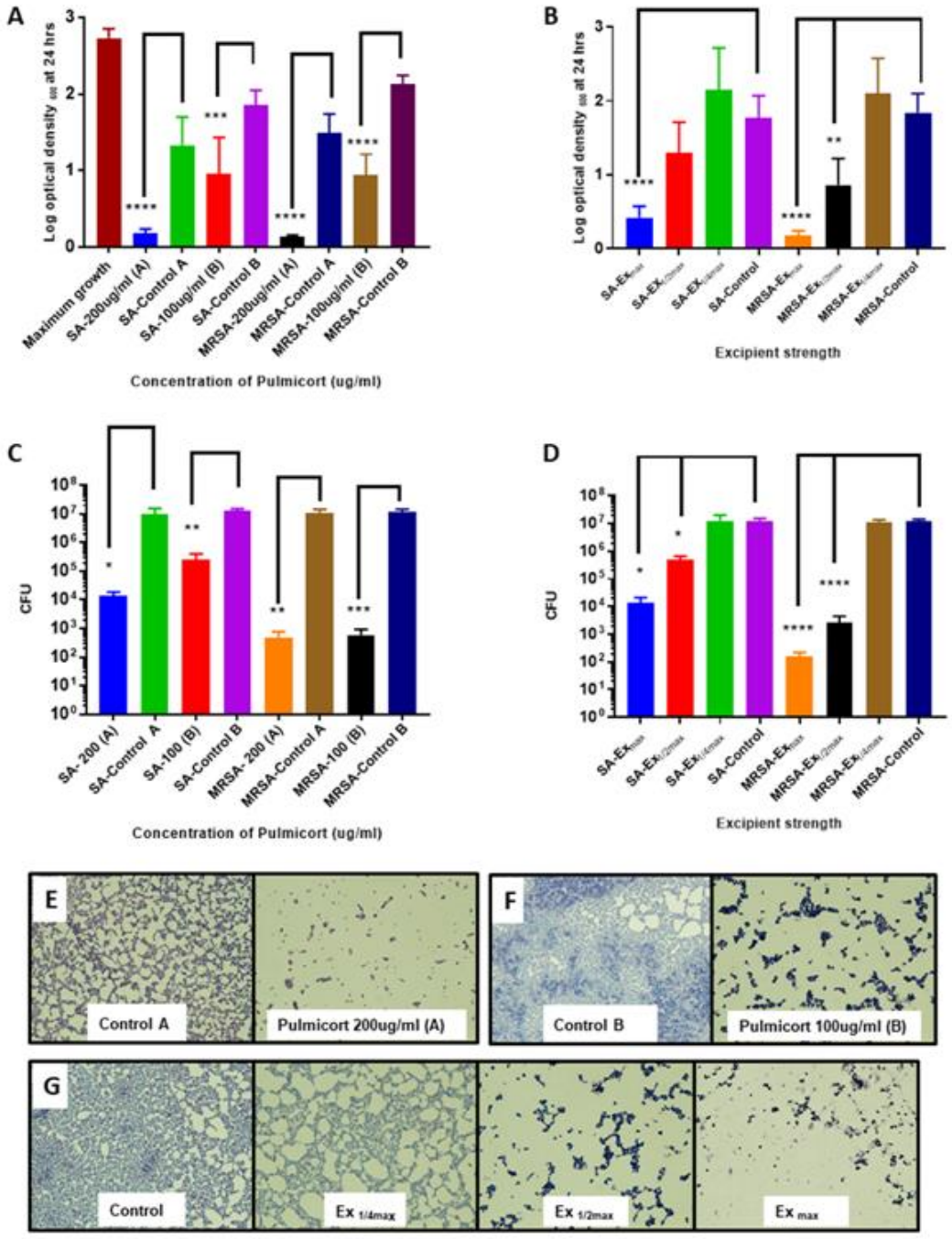

Figure 11: Effect of Pulmicort and excipients on planktonic growth of S. aureus (ATCC 25923) and MRSA (JE2)

(A-D) Log optical density (OD) at 600nm for planktonic growth of *S. aureus* (SA) and MRSA (A-B) or Colony forming unit counts (CFU) (C-D) in the absence (maximum growth) or **(A, C)** presence of Pulmicort at 200ug/ml and 100ug/ml [4 ml or 2 ml Pulmicort in 6 or 8 ml nutrient broth (NB)] compared to volume controls [4 ml or 2 ml phosphate buffered saline (PBS) in 6 or 8 ml NB]; or **(B, D)** 2 ml excipients at different concentrations

(Ex$_{max}$-maximum concentration, Ex$_{1/2max}$-half concentration, Ex$_{1/4max}$-one fourth concentration) in 8 ml NB compared to 2 ml PBS in 8 ml NB (control). Data represent the mean ± SD of three (C, D), six (B) or nine (A) biological replicates. **E, F & G:** Light microscopic image at 60x magnification of Giemsa stained SA after treatment with 200ug/ml (E), 100ug/ml Pulmicort (F) or excipients (G) compared to control. One-way ANOVA, *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; SD, standard deviation.

Effect of Pulmicort and excipients on biofilm formation of *S. aureus* (ATCC 25923) and MRSA (JE2)

We then assessed the effect of commercial Pulmicort or excipients on the formation of *S. aureus* biofilms. Formulations were co-incubated with *S. aureus* or MRSA for 48 hours in plastic plates on a rotating platform followed by *in vitro* resazurin viability assays. Pulmicort incubation showed *S. aureus* biofilm reduction of 91% with 250 µg/ml and 81% with 125 µg/ml compared to maximum biofilm growth (no-treatment control) ($p<0.0001$, Figure 12A). With the MRSA strain the killing was 97%, 99% and 7% when incubated with 250 µg/ml and 125 µg/ml and 62.5µg/ml Pulmicort respectively, compared to the no-treatment control ($p<0.0001$, Figure 12A).

Excipients showed a killing of 92% with Ex$_{1/2\ max}$, 91% with Ex$_{1/4\ max}$ and 57% with Ex$_{1/8max}$ for *S. aureus* compared to the no-treatment control ($p<0.0001$, Figure 12B). For the MRSA strains this was found to be 91%, 88% and 98% respectively ($p<0.0001$, Figure 12B).

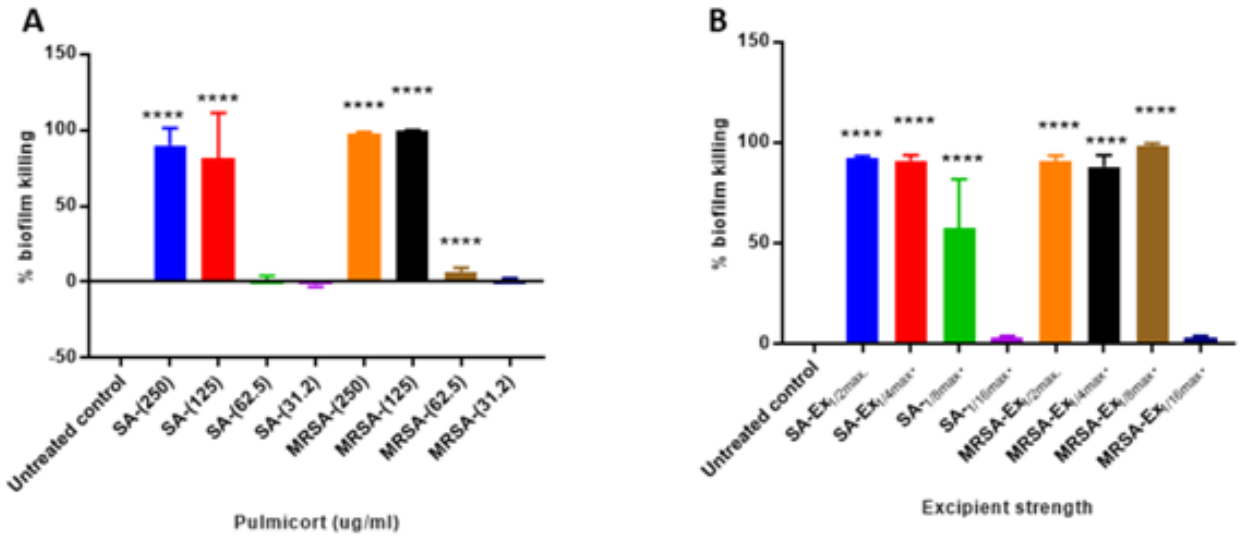

Figure 12: Effect of Pulmicort and excipients on biofilm formation of S. aureus (ATCC 25923) and MRSA (JE2)

Biofilm killing (%) of *S. aureus*(SA) and MRSA on exposure to different concentrations of (A) commercial Pulmicort (250, 125, 62.5, 31.2 µg/ml) or excipients ($Ex_{1/2max}$-half concentration, $Ex_{1/4max}$-one fourth concentration, $Ex_{1/8max}$-one eighth concentration, $Ex_{1/16max}$- one sixteenth concentration) compared to untreated control. Data represents the mean ± SD of three biological replicates. One-way ANOVA, ****, $p < 0.0001$; SD, standard deviation.

Effect of individual excipients on biofilm formation of *S. aureus* (ATCC 25923) and MRSA (JE2)

We then repeated the experiments using different concentrations of individual excipients, to identify the agent responsible for the bacterial killing. Results showed EDTA significantly reduced biofilms of *S. aureus* at concentrations that are approved by the FDA for use in nasal formulations (86% at 0.02%, P<0.0001), and also at much lower concentrations (95% at 0.005%and 65% at 0.0025%, P<0.0001, Figure 13A). MRSA biofilms also responded similarly to EDTA (98% killing at 0.02%, 99% at 0.005% and 61% at 0.0025%, Figure

13B). Furthermore, the compound SCDT at an FDA approved concentration significantly reduced MRSA biofilms (76% at 0.1%, P<0.01, Figure 13B).

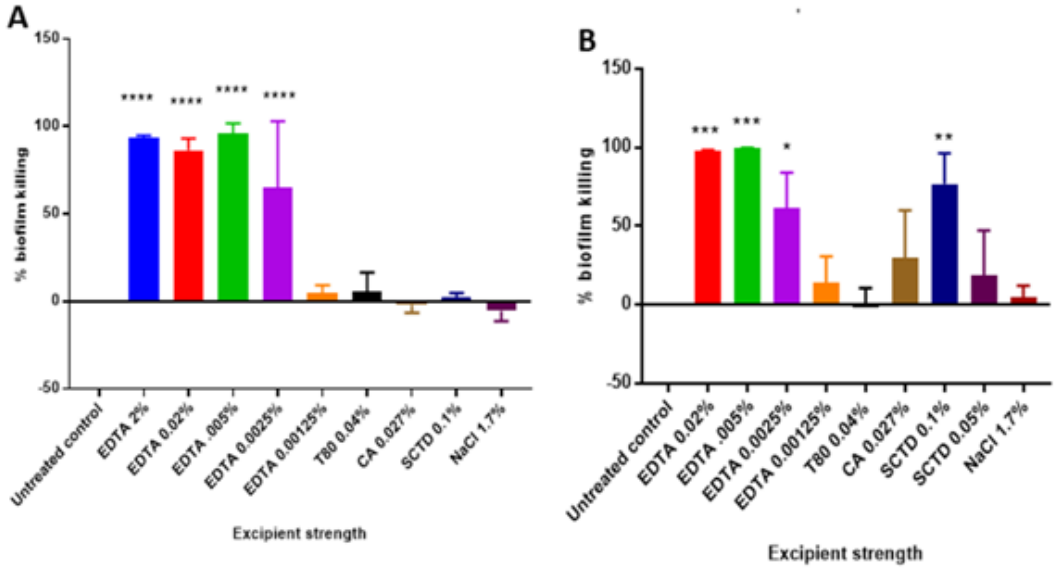

Figure 13: Effect of individual excipients on biofilm formation of S. aureus (ATCC 25923) and MRSA (JE2)

Biofilm killing (%) of *S. aureus* (A) or MRSA (B) on exposure to different concentrations of individual excipients compared to untreated control. Data represents the mean ± SD of three biological replicates. One-way ANOVA, *p < 0.05; **p < 0.01; ***p < 0.001, ****p < 0.0001; SD, standard deviation, CA-Citric acid anhydrous, EDTA-ethylenediaminetetraacetic acid, T80- Polysorbate (Tween) 80, SCTD-sodium citrate tribasic dihydrate, NaCl-sodium chloride.

MIC of excipients

The MIC of the excipients was found to be $Ex_{1/2max}$ for both *S. aureus* and MRSA with > 85% killing. For *S. aureus*, a concentration of $Ex_{1/4max}$ showed 26% killing and for MRSA $Ex_{1/8max}$ showed 44% killing (Figure 14). These concentrations of the excipients that gave less than 50% bacterial killing were combined with antibiotics at concentration lower than its MIC value to assess the effect of antibiotic-excipient combination on bacterial killing.

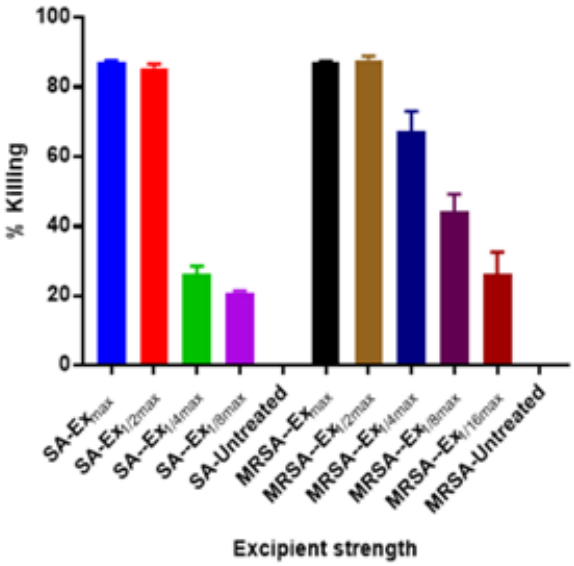

Figure 14: MIC of excipients

MIC of excipients expressed as % killing of *S. aureus* (SA) and MRSA. Ex_{max}- maximum excipient concentration, $Ex_{1/2max}$ – half concentration, $Ex_{1/4max}$ - one fourth concentration, $Ex_{1/8max}$ - one eighth concentration and $Ex_{1/16max}$ – one in 16 concentration. Data represents the mean ± SD of three biological replicates.

Effect of excipients on antibiotic sensitivity of *S. aureus* ATCC 25923 and MRSA JE2

The combination of Amoxicillin with excipients had synergistic effects on the percentage killing of planktonic *S. aureus* (from 43% at ½ MIC amoxicillin and 26% with $Ex_{1/4max}$ to 82% killing when ½ MIC amoxicillin and $Ex_{1/4max}$ were combined, $P<0.05$, Figure 15A).

With the planktonic MRSA, a similar significant increase in killing was seen with the combination of Amoxicillin and excipients (from 55% at ½ MIC amoxicillin and 44% with $Ex_{1/8max}$ to 97% killing when ½ MIC amoxicillin and $Ex_{1/8max}$ were combined, $P<0.01$, Figure 15B). The antibiotic-excipient combination with Amikacin, Gentamicin and Erythromycin on MRSA at ½ MIC however, did not increase bacterial killing. Rather, the combinations demonstrated a potential antagonistic action (from 75% at ½ MIC of

Amikacin to -0.9% killing when ½ MIC Amikacin and $Ex_{1/8max}$ were combined, P<0.0001; from 56% at ½ MIC Gentamicin to -4% killing when ½ MIC Gentamicin and $Ex_{1/8max}$ were combined, P<0.0001; and from 57% at ½ MIC Erythromycin to 8% killing when ½ MIC Erythromycin and $Ex_{1/8max}$ were combined, P<0.001. In all the cases, killing with $Ex_{1/8max}$ alone was 44%, Figure. 15B). This antagonism on MRSA was also found to be significant at higher antibiotic concentrations (1 MIC) for Gentamicin and Erythromycin (from 93% at MIC Gentamicin to 50% killing when MIC Gentamicin and $Ex_{1/8max}$ were combined, P<0.001; and from 90% at MIC Erythromycin to 31% killing when MIC Erythromycin and $Ex_{1/8max}$ were combined, P<0.0001. In all the cases, killing with $Ex_{1/8max}$ alone was 44%, Figure. 15C).

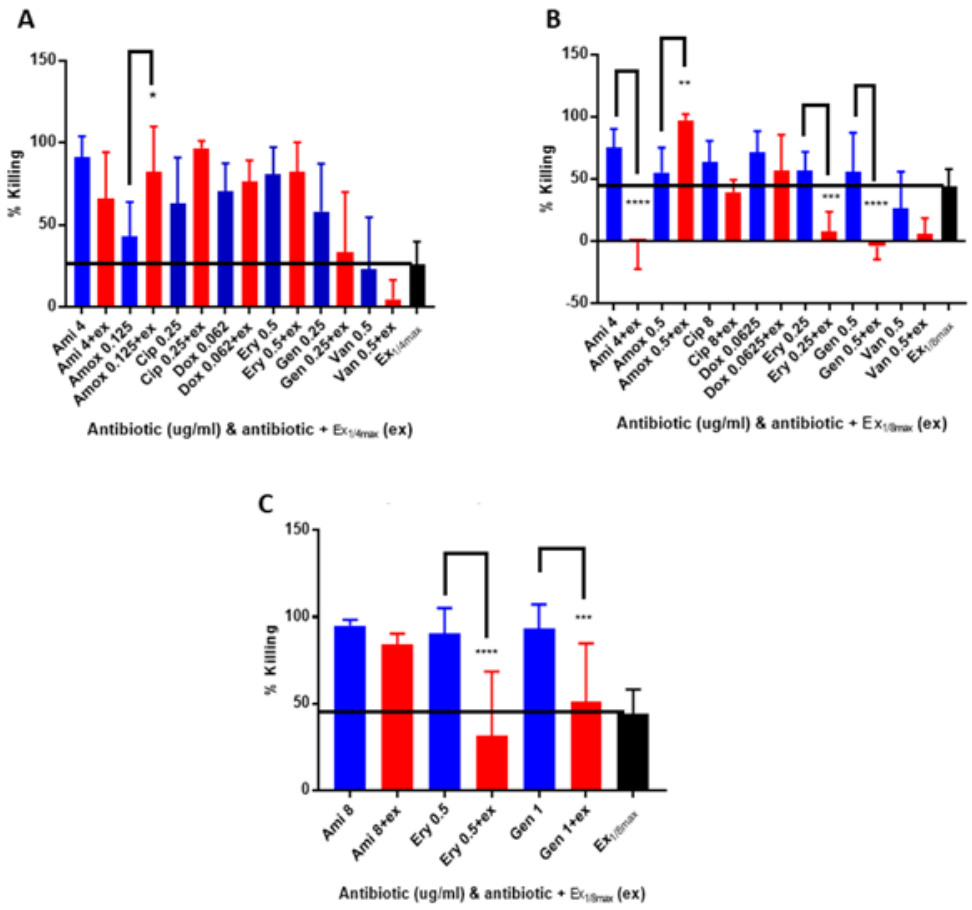

Figure 15: Effect of excipients on antibiotic sensitivity of S. aureus ATCC 25923 and MRSA JE2

MIC expressed as % killing of *S. aureus* (SA) (A) or MRSA (B-C). The blue bars are antibiotics alone and red bars are antibiotic-excipient combinations. $Ex_{1/4max}$ and $Ex_{1/8max}$ - is $1/4^{th}$ and $1/8^{th}$ maximum concentration of excipients. Ami 4- Amikacin 4ug/ml, Amox 0.125 – Amoxicillin 0.125ug/ml, Cip 0.25-Ciprofloxacin 0.25ug/ml, Dox 0.0625- Doxycycline 0.0625ug/ml, Ery 0.5- Erythromycin 0.5 ug/ml, Gen 0.25- Gentamycin 0.25ug/ml, Van 0.5- Vancomycin 0.5ug/ml. Ami 8- Amikacin 8ug/ml, Ery 0.5- Erythromycin 0.5 ug/ml, Gen 1- Gentamicin 1ug/ml. Data represent the mean ± SD of six biological replicates. * $p < 0.05$, **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; SD, standard deviation.

Cytotoxicity assay

A 24 and 48-hour exposure of HNEC from non-CRS patients (n=3, 2 males and 1 female, aged 35-65 years old) to Pulmicort and excipients showed no significant cell toxicity (Supplementary Figure 17A &17B).

2.6 Discussion

The presence of biofilms in CRS and their influence on disease severity and treatment outcomes is well established.(214-216)Previous research from our department has shown that topical steroids may directly reduce biofilm production in an in vitro environment outside of the inflammatory milieu.(187)This study builds on this research, demonstrating that Pulmicort's non-therapeutic excipients have independent antibacterial actions on both planktonic and biofilm forms of *S. aureus* and MRSA. Moreover, our study shows that those excipients influence the antimicrobial activity of commonly used antibiotics.

The excipients used in commercial preparations help to suspend the active drug and act as a preservative and stabilizing agent. Because they are used in minimal concentrations, they are largely considered as non-therapeutic. Previous studies do suggest however that when

used at higher concentrations these agents may have an effect on bacterial growth. EDTA for example can potentiate the cell walls of bacteria and destabilize biofilms by sequestering calcium, magnesium, zinc, and iron.(217)Unlike previous studies which suggest this only occurs at higher concentrations(218, 219)our study demonstrated EDTA to reduce *S. aureus* and MRSA biofilm formation at FDA approved and much lower concentrations. A similar antibiofilm effect on an MRSA strain was also seen for sodium citrate dibasic trihydrate (SCDT), another commonly used excipient. Like EDTA, SCDT acts as a metal chelator inhibiting biofilm formation. (211)Although the other excipients in Pulmicort such as NaCl, citric acid and tween 80 have all been shown to influence bacterial growth independently in other research,(209, 220, 221)none of them had any effect on bacterial growth in our study. This is most likely secondary to the low concentrations used. For example, although NaCl has been shown to cause physical damage to the cells at high concentrations, (222)*S. aureus* has been reported to be osmotolerant at low and moderate concentrations of NaCl due to its ability to accumulate osmoprotectants such as proline and glycinebetaine in the cells.

Budesonide, when used in mucosal atomiser device (MAD) or incorporated into gel (e.g. Pulmicort respules mixed with saline or chitosan gel) for application into the sinuses deliver concentrations from 0.25mg to 1mg and when used as a nasal spray (e.g. Rhinocort) delivers a maximum of 256µg/day into the nasal cavity. These concentrations of the steroid with its excipients are comparable to that used in our study. However, when budesonide is used as a large volume nasal rinse, the concentration of the drug and excipient is much lower and did not demonstrate the antibacterial action which was seen at the higher concentrations in this study. A study by Thamboo et.al.(180)comparing the effect of budesonide nasal rinses (high volume, low concentration) with budesonide delivered through MAD (low volume, high concentration) in post-operative CRSwNP patients found that the patients using MAD had significant improvement in the SNOTT-22 scores compared to baseline when used for a short term (up to 60 days) without causing any adrenal suppression. This clinical

improvement could be due to the additional antibacterial action of the commercial topical nasal steroid and the excipients used in them which was demonstrated in our study. Each topical nasal preparation of budesonide contains different excipients, but most of them have compounds like tween 80 and EDTA. The FDA concentrations of the EDTA found in the different products can vary from 0.5% to 0.0001%, which is also in the range of the concentrations analysed in our study.(205)

Antibiotics are often given in combination with topical steroids in CRS. When drugs or compounds are combined, they could produce desirable or undesirable interactions. Additive effect in a combination is the sum of individual effects. Synergism occurs when the combination effect is higher than the additive effect and antagonism refers to an opposite action where the combination effect is lesser than additive effect.(223)Here, the steroid or the excipient could potentially interact additively, synergistically or antagonistically with antibiotics influencing the MIC values. To demonstrate these interactions, we used a sub-inhibitory concentrations of both excipient and antibiotic as per previous studies.(224)In our study, we found that although the excipient solution demonstrated an inhibitory effect on the growth of both *S. aureus* and MRSA, the combination of antibiotic-excipient showed an antagonistic trend on MIC for all the antibiotics tested except amoxicillin.

For the Amikacin/Gentamicin-excipient combination in our study, the antagonistic action seen could be due to the divalent and monovalent ions in the excipient solution competing with the antibiotics at the receptors for uptake into bacteria as have been shown in previous studies.(225, 226)Compounds like sodium chloride and citric acid also influence cell viability at the transport level affecting aminoglycoside action.(226)EDTA, however, synergises the activity of Gentamicin against *S. aureus* biofilms by sequestering the divalent and monovalent cations.(227)This effect was not seen in our study and is attributed to the low

EDTA concentrations compared to that of the total divalent and monovalent ions in the excipient solution.

Our results also demonstrated that the addition of excipients to Erythromycin reduced its antibacterial effect on MRSA whilst leaving its effect on *S. aureus* unchanged. This finding was consistent with previous MIC studies that combined Erythromycin with plant-based products,(228)compounds targeting bacterial fatty acid biosynthesis(229)and other protein synthesis inhibitors.(230)

Antagonistic effects while combining bactericidal and bacteriostatic compounds have been reported by previous studies.(231)Bacteriostatic compounds could inhibit cell activity rendering them less susceptible to the bactericidal compounds. It is possible that in our study, the excipient solution has a bacteriostatic property inhibiting the action of most of the antibiotics tested.

Amoxicillin inhibits cell wall penicillin binding proteins (PBP) 1 and 3 which is required for the formation of the cross linkage between peptidoglycan polymer chains in bacterial cell wall. MRSA utilises PBP 2a to perform essential cell wall crosslinking functions. PBP2a in turn requires teichoic acid to locate and orient the enzyme onto bacterial cell.(232)It has been shown that the enzyme lipoteichoic acid synthase, responsible for the production of lipoteichoic acids in *S. aureus* is inhibited by EDTA.(233)This mechanism could explain the synergistic effect of Amoxicillin-excipient combination on *S. aureus* and MRSA seen in our study.

No evidence of cytotoxicity was observed in this study for the excipient or Pulmicort concentrations tested. Many of the excipient used in commercial preparations have however been shown to be cilio-toxic to varying degrees. This depends on the compound, its concentration and also the combination with other agents. Previous studies have shown that budesonide based nasal sprays with EDTA at a concentration of 0.1% are notcilio-toxic and

had no hazardous effect on ciliary beat frequency(234)We can also, therefore, assume that it would be unlikely that at the lower concentrations used in this study, the excipients would have an adverse effect on the cilia.

A number of limitations within this study raise questions for further investigation. The direct effect of budesonide on bacteria could not be ascertained due to its insolubility in physiological media. The exact mechanism of antibiotic-excipient effect on MIC could not be explained as the excipients used in the study, and commercially, are usually combinations of different compounds. The possibility of multiple combination effects and the species of bacteria targeted should be considered before concluding whether the antibiotic-excipient combination is potentially synergistic or antagonistic on MIC.

2.7 Conclusion

In conclusion, topical application of low volume, high concentration of Pulmicort had an antibacterial effect on *S. aureus* and MRSA. This action was mediated through the excipient EDTA for *S. aureus* and EDTA and SCDT for MRSA used in its commercial topical preparation. This implicates that antibiotic-resistant strains like MRSA are more susceptible to excipient killing, possibly through multiple mechanisms.

The combination of excipients with antibiotics like Amikacin, Gentamicin and Erythromycin appeared to have an antagonistic trend on MRSA killing. Nevertheless, the action of Amoxicillin, the most commonly used antibiotic in CRS against gram positive organisms was found to be potentiated in the presence of excipients for both *S. aureus* and MRSA.

2.8 Acknowledgments

We kindly acknowledge the services of Flinders Analytical laboratories, for the assistance with liquid chromatography- mass spectrometry (LC-MS).

Figure 16:

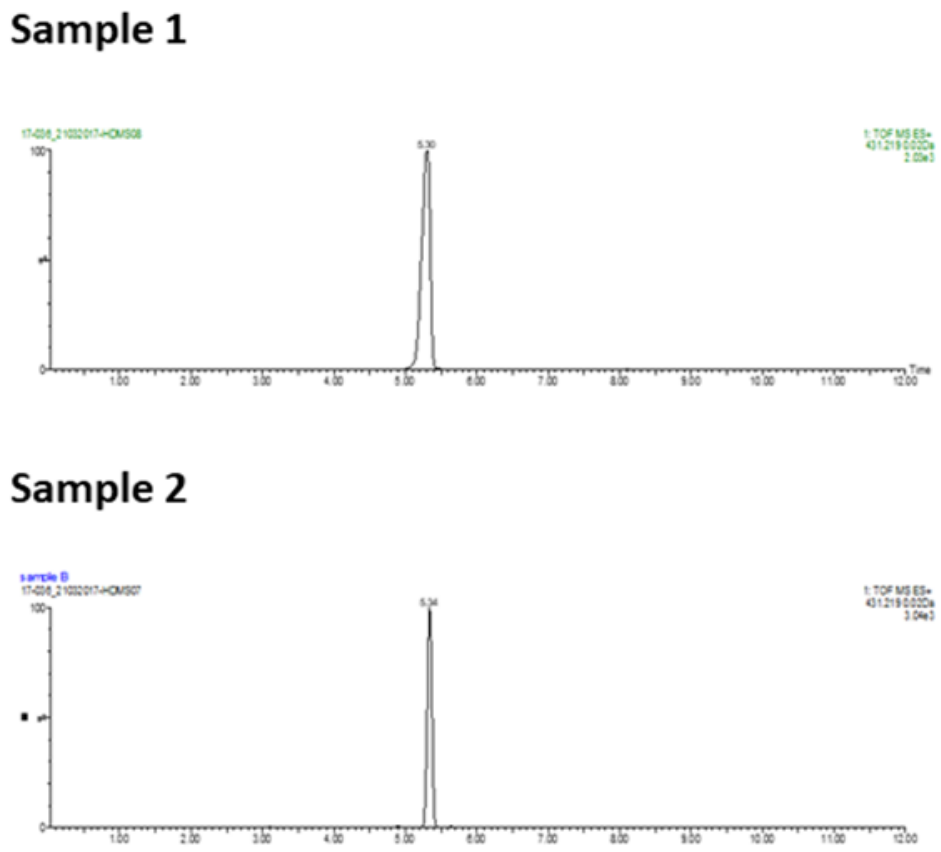

Figure 16: Determination of presence of budesonide in centrifuged Pulmicort with liquid chromatography- mass spectrometry (LC-MS).

Mass chromatograms of the molecular ion of budesonide from sample 1 or uncentrifuged pulmicort and sample 2 or centrifuged pulmicort . Peaks at 5.30 minutes (sample 1) and 5.34 minutes (sample 2).

Figure 17:

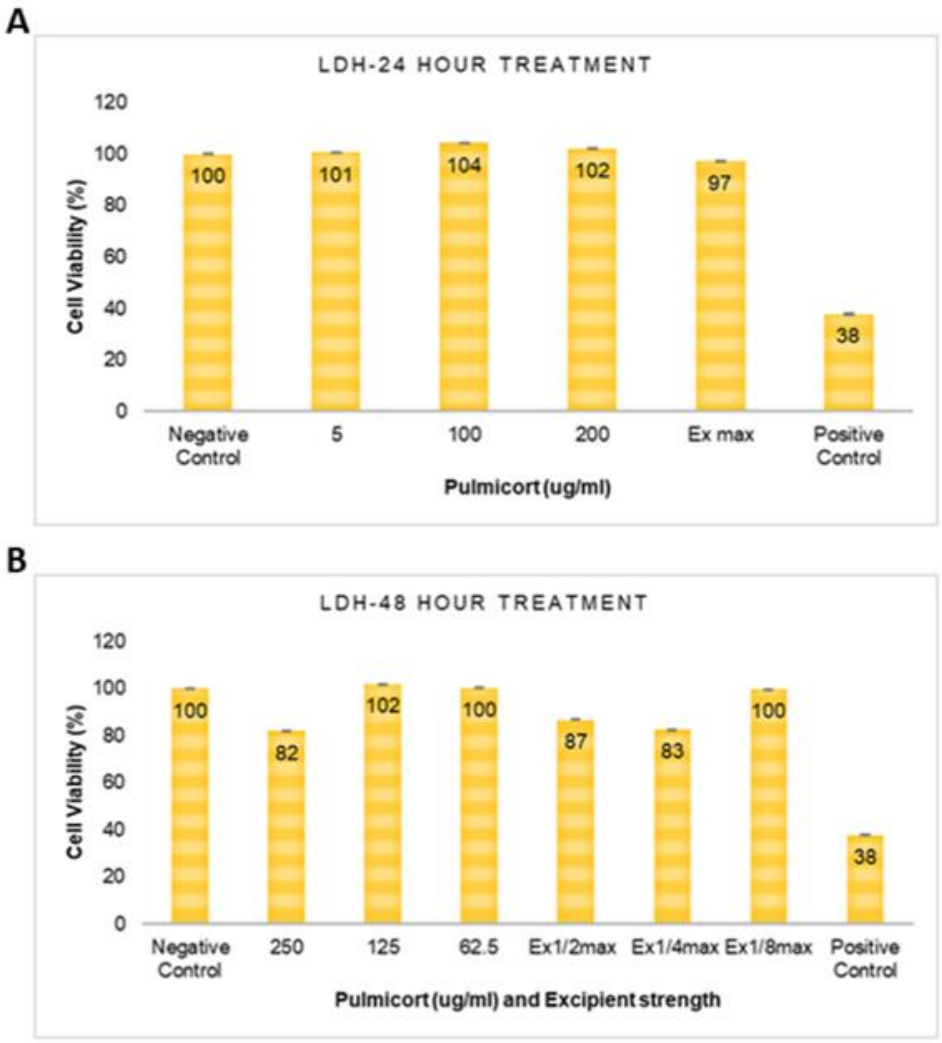

Figure 17: Cytotoxicity assay for Pulmicort and excipients

2A: Cell viability using LDH assay after 24-hour treatment with commercial Pulmicort (5,100 and 200 ug/ml) and Maximum concentration excipient solution (Ex_{max}). Negative control is untreated cells and positive control is HNECs + 10% Triton X-100. Data represents the mean ± SD of three biological replicates.; LDH, lactate dehydrogenase; SD, standard deviation.**2B**: Cell viability using LDH assay after 48-hour treatment with Pulmicort (250,125 and 62.5 ug/ml) and concentration excipient solution ($Ex_{1/2max}$-half concentration, $Ex_{1/4max}$-one forth concentration, $Ex_{1/8max}$-one eighth concentration). Negative control is untreated cells and positive control is cells +10% Triton X-100. Data represent the mean ± SD of three biological replicates.; LDH, lactate dehydrogenase; SD, standard deviation

3 Chapter 3: *Staphylococcus aureus* metabolises hydrocortisone: effects on anti-inflammatory activity and antimicrobial susceptibility

3.1 Statement of authorship

Statement of Authorship

Title of Paper	Staphylococcus aureus metabolises hydrocortisone: effects on anti-inflammatory activity and antimicrobial susceptibility
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Lisa M Cherian, Mahnaz Ramezanpour, Andrew James Hayes, Shari Javadiyan, Hua Hu, Peter-John Wormald, Alkis James Psaltis, Sarah Vreugde. Unpublished data

Principal Author

Name of Principal Author (Candidate)	Lisa Mary Cherian		
Contribution to the Paper	Conception and design of the project, acquisition of data, analysis and interpretation of data, drafting the article		
Overall percentage (%)	85%		
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	02.07.2019

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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3.2 Abstract:

Background- Glucocorticoids are used in a wide range of inflammatory conditions including infections. Although the anti-inflammatory action of steroid is fairly well understood, the effect of steroids on the metabolic function of human pathogens is unclear. Here, we investigated the metabolic responses of planktonic and biofilm forms of *S. aureus* at different concentrations of hydrocortisone 21-hemisuccinate (HCHS)

Methods- The effect of HCHS at different concentrations on planktonic and biofilm growth of *S. aureus* ATCC 25923 was evaluated using optical density, colony forming units (CFU), Giemsa staining, Resazurin assays and confocal laser scanning microscopy. Minimum biofilm inhibitory concentration was determined to assess the influence of HCHS on antibiotic killing of *S. aureus* biofilms. High performance liquid chromatography (HPLC) was used to determine the bacterial degradation of HCHS. The effect of HCHS in the presence and absence of bacteria on poly (I:C) induced IL-6 production by Primary Human Nasal Epithelial Cells was evaluated using Enzyme Linked Immunosorbent Assays.

Results- There was a dose-dependent increase in CFU counts in HCHS treated planktonic *S. aureus* compared to untreated control. Bacterial biofilms were found to be less susceptible to antibiotics at low steroid concentrations and more susceptible at high concentrations. The degradation of HCHS to active cortisol was found to be accelerated by *S. aureus* and this was associated with a significant reduction in poly (I:C) induced IL-6 secretion.

Conclusion- HCHS influenced *S. aureus* multiplication, aggregation, biofilm formation, antibiotic susceptibility and metabolic activity in a concentration dependent manner. *S. aureus* was capable of metabolising HCHS to its active compound cortisol thereby potentiating its anti-inflammatory action.

3.3 Introduction:

Glucocorticoids are a class of steroid hormones secreted by the adrenal cortex and are involved in the carbohydrate metabolism and regulation of various cellular functions like growth, homeostasis, metabolism, cognition and inflammation.(235)Due to their wide range of actions spanning from anti-inflammation to immune suppression, glucocorticoids are universally prescribed in a number of clinical conditions. These actions make glucocorticoids the mainstay of treatment for conditions like asthma, allergy, septic shock, rheumatoid arthritis, inflammatory bowel disease, and multiple sclerosis.(236)The main clinical benefit of steroids is thought to be due to its anti-inflammatory action. They decrease vascular permeability and reduce the migration of lymphocytes to the site of infection, inhibit the transcription of inflammatory genes and promote the encoding of anti-inflammatory proteins.(235, 236)Apart from these, corticosteroids have been shown to be beneficial and at times life-saving when used in certain infections. In infections, steroids have been shown to reduce the painful erythema in cellulitis, joint swelling in bacterial arthritis, reduce the inflamed lymph node in tuberculous lymphadenopathy and accelerate the resolution of effusion in tubercular pleurisy, pericarditis and chronic middle ear effusion.(237)Contrary to this, literature also shows that there is a dose dependent increase in serious and opportunistic infections especially with long term steroid use.(238) Although the beneficial effects of steroids could be due to their perceived anti-inflammatory effect, their influence on the bacteria present in these infections is unclear.

Steroid compounds are ubiquitous in the environment as they are excreted by humans and animals. They thus provide an easily available substrate for microorganisms. It has been shown that certain soil and aquatic bacteria have highly specialised mechanisms by which they can utilise steroids as a carbon source for growth and multiplication.(200)This could be true with many pathogenic and non-pathogenic bacteria in humans as well, where administration of steroids has a positive or negative outcome on disease progression. When

applied as a topical cream for atopic dermatitis, hydrocortisone reduced the bacterial colonisation of *Staphylococcus aureus* in eczema and atopic dermatitis.[239]Steroids have also been shown to increase the sensitivity of bacteria to erythromycin.[193]Some bacteria are also capable of metabolizing sex steroid hormones through the activity of distinct enzymes, thereby playing a role in the regulation of sex hormone levels.[240]Given the important role of sex steroid hormones in regulating the activity of immune cells, altering the balance between active and inactive steroids by steroid-degrading bacteria might therefore have both physiological and pathological implications.[240]The effect of sex steroid hormones on infectious diseases varies depending on the infective species where men are more susceptible to certain pathogens such as *Mycobacterium tuberculosis,* whilst women are more susceptible to infections with for example *Pseudomonas aeruginosa* or *Salmonella typhimurium*.[240, 241]

The effect of glucocorticoids on the growth dynamics of *S. aureus*, a common human pathogen, is however not known. The objective of this study was to determine metabolic responses of planktonic *S. aureus* and their biofilm counterparts to hydrocortisone 21-hemisuccinate (HCHS, a water-soluble steroid). We also determined the effect of steroid at different concentrations on the antibiotic susceptibility of biofilms.

3.4 Methods:

Bacterial strains and tissue:

Staphylococcus aureus ATCC 25923 (*S. aureus*) was purchased from the American Type Culture Collection (Manassas, VA, U.S.). Human nasal epithelial cells (HNEC) were obtained from patients in accordance with guidelines approved by the Human Ethics Committee of the Queen Elizabeth Hospital and the University of Adelaide. All patients gave written informed consent (reference HREC/15/TQEH/132) and all samples obtained were anonymised and coded before use.

Drugs and reagents:

Hydrocortisone 21- hemisuccinate sodium salt (HCHS), and sodium succinate were purchased from Sigma-Aldrich, Steinheim, Germany. Giemsa stain (Sigma-Aldrich, Steinheim, Germany), Resazurin dye/ Alamar blue (Life Technologies, Scoresby, Australia) and Methyl thiazolyl diphenyl-tetrazolium bromide (MTT; Sigma Aldrich, Missouri, USA) were obtained from their respective suppliers. Antibiotics used in minimal inhibitory concentration (MIC) and minimal biofilm inhibitory concentration (MBIC)assays were Amikacin, Amoxycillin, Ciprofloxacin, Doxycycline, Erythromycin, Gentamycin, and Vancomycin (all purchased from Sigma-Aldrich Steinheim, Germany).

Effect of Hydrocortisone 21- hemisuccinate (HCHS) on planktonic growth of *S. aureus*:

Bacteria were grown on a 2% nutrient broth agar plate (NBA) for 24 hours at 37°C. A single colony from this plate was transferred into 10 ml nutrient broth (NB) and incubated at 37°C for 14 to 16 hours with shaking at 180 rpm. The overnight culture was subsequently diluted into NB to obtain a starting OD 600 of 0.05. Prior to incubation, this volume was put in 50ml falcon tubes containing the treatments and NB (making a final total volume of 10ml). HCHS was added to NB at concentrations of 5, 2.5and 0.625mg /ml.

Growth determination and Colony forming unit (CFU) determination:

To identify the bacterial growth pattern in the presence of HCHS, OD 600nm of the tubes was measured at 0-hours and then hourly for 7 hours before a final measure at 24 hours for each condition. A colony forming unit (CFU) count was also performed at 24 hours from both the supernatant and the sediments. Briefly, at the end of 24 hours, the tubes were left to stand for one hour at room temperature. A 20µl volume taken from the supernatant without disturbing the pellet at the bottom was serially diluted in phosphate buffered saline (PBS) and then spotted on NBA plates in triplicates. The supernatant from the tubes was then

carefully aspirated and the pellet resuspended in 500µl of PBS to be used for CFU counting as mentioned above. In addition, colony morphology was also determined. All experiments were carried out 3 times independently.

Giemsa staining:

Giemsa staining was performed on the sedimented pellet at the bottom of each falcon tube (from the above experiment). In brief the pellet was resuspended in 500µl of PBS before 20µl was taken from each tube, spread on a glass slide and air dried. This was followed by fixing in methanol for 7 minutes and then staining with a 1:20 dilution of Giemsa stain in sterile Milli Q water for one hour. The slides were rinsed with distilled water, dried and examined under a light microscope (Eclipse 90i, Nikon, Japan) at 60 x magnification under oil immersion.

Effect of HCHS on *S. aureus* biofilm formation:

1. *Alamar blue or Resazurin assay*

Resazurin assay was performed as per the protocol described previously.[224, 242] In brief, single colonies of bacteria suspended in 0.9% saline were adjusted to 1.0 ± 0.1 McFarland units (approximately $3 \times 10_8$ CFU/ml) and were diluted 1:15 in NB. The resazurin assay was done by inoculating a black 96-well microtiter plate (Costar, Corning Incorporated, Corning, U.S.) with the diluted bacterial suspension. HCHS was added to the wells at a starting concentration of 22.8mg/ml and was serially diluted till 0.71mg/ml. Sodium succinate in concentrations comparable to that present in HCHS was measured and used as control treatment for each concentration of HCHS tested. The final volume in each well was 150µl and this incubated at 37°C for 48 h on a rotating platform (3D Gyratory Mixer, Ratek Instruments, Boronia, Australia) at 70 rpm. Following the incubation with treatments, biofilms were rinsed twice with PBS and the viability (metabolic activity) assay performed. For this, 200µL of a freshly prepared 10% resazurin dilution in NB was added to each well

and was incubated, protected from light, at 37 °C on a rotating platform. The fluorescence was measured every 30 minutes on FLUO star OPTIMA plate reader at $\lambda_{excitation}$ = 530 nm/$\lambda_{emission}$ = 590 nm till maximum fluorescence of the untreated well was attained.

Bacterial metabolic activity (viability) following the treatment was quantified after one-hour incubation with resazurin dye according to the equation:

$$\%MA = 100 - \left(\frac{F_C - F_{TX}}{F_C} 100\ \%\right)$$

The viability of bacteria on coincubation with HCHS was expressed as the percentage of metabolic activity (% MA), where FC was the fluorescence of the untreated control biofilms and FT was the fluorescence observed in the HCHS treated biofilms. Both FC and FT were corrected for background fluorescence (sterile medium).

The reads were taken and plotted one hour after exposure to Alamar blue dye. The increased metabolic activity of the biofilms expressed as % viability (metabolic activity) was plotted above or below 100% activity of the untreated well. These studies were performed as three independent experiments with three wells per treatment

1. *Methythiazol tetrazolium (MTT) assay*

The methythiazol tetrazolium (MTT) assay was done to complement the findings of the metabolic activity of steroid-treated bacteria from the Alamar blue assay. In this, biofilms were prepared in the same manner as described for Alamar blue assay, but in a 96 well clear bottom cell culture microtiter plates (Cellstar, Greiner Bio-One GmbH, Frickenhausen, Germany). After washing the biofilms twice with PBS, the wells were filled with 150µl of PBS. To this, a 50µl of 0.3% MTT dye prepared in PBS was added and the plate incubated for 2 hours at 37°C. The dye was then suctioned out and 150µl of dimethyl sulfoxide (DMSO; Sigma Aldrich, Missouri, US) and 25µl of glycerine buffer (0.1 M, pH 10.2) was

added into the wells. This was then incubated for 15 minutes at room temperature with gentle agitation. The absorbance of the solution was measured at a wavelength of 540nm using a Microplate Reader Model.

2. *Quantification of viable bacteria:*

To quantify the biofilms in the HCHS treated wells, a CFU count was done. Briefly, biofilms were grown with different concentrations of HCHS on a clear 96 well flat bottom plate in the rotating chamber for 48 hours. After a washing step with PBS, the wells were filled with 200µl of PBS and sonicated for 15 minutes. A 20µl aliquot was then taken from each well and serially diluted and plated on NBA plates and incubated for 24 hours followed by CFU counting and colony morphology determination.

3. *Giemsa staining of biofilms*

Giemsa staining was done to visually demonstrate the morphology and density of the HCHS and sodium succinate treated biofilms. Briefly, a 20µl aliquot was taken from the wells of the above experiment (CFU count) after the sonication and spread on a glass slide, air-dried, methanol fixed and then stained with Giemsa to be examined under a light microscope as described previously.

5. Confocal scanning laser microscopy for live/dead staining of S. aureus biofilms

S. aureus ATCC 25923 were grown on an 8-chamber Falcon Culture Slides (In Vitro Technologies, Noble Park, Australia) in NB in the presence and absence of HCHS for 48hours at 37°C and with shaking. Following a wash with 0.9% saline, the wells were treated with 5% glutaraldehyde fixative (Sigma Aldrich, Steinheim, Germany) for 45 minutes at 21_0C. After one more wash to remove the excess fixative, the slides were immersed in MilliQ water (Millipore, Billerica, MA, USA) containing 1.5 µl/ml each of both LIVE/DEAD BacLight stains SYTO9 and propidium iodide (Invitrogen Molecular Probes, Mulgrave, Victoria, Australia). The slide was incubated in the dark at 21°C for 15

minutes and then washed with 0.9% saline to remove excess stain, mounting oil (Invitrogen Molecular Probes, Mulgrave, Victoria, Australia) applied and then covered with coverslips. Samples were visualized by using an LSM700 confocal scanning laser microscope (Zeiss Microscopy, Jena, Germany). The excitation/emission wavelengths of the BacLight staining were 485/530 nm and 485/630 nm, respectively.

Effect of HCHS on *S. aureus* planktonic minimal inhibitory concentration (MIC) to antibiotics:

The minimal inhibitory concentration (MIC)of antibiotics was assessed by measuring the optical density (OD) at 595nm for different concentrations of the tested antibiotics as described in previous published protocols.(213)MIC was also measured in the presence of different concentrations of HCHS.

Effect of HCHS on *S. aureus* biofilm minimal biofilm inhibitory concentration (MBIC) to antibiotics:

S. aureus inoculate was prepared in 0.9% saline at a density that was adjusted to a 0.5 McFarland turbidity standard (10_8 colony-forming units [CFU]/ml) and diluted 1:100 in NB. From this 100µl was put into wells of a 96-well black microplate followed by incubation on a rotating platform for 48 hours at 37°C for biofilm formation. Following incubation, the supernatant was aspirated and wells were washed twice with PBS to remove planktonic bacteria. Treatments consisting of 60µl of Miller Hinton broth (MHB) and 60 µl of various antibiotics at different concentrations prepared in MHB were subsequently added to the wells. The wells were then incubated for 20 hours and washed once to remove the excess treatment. To these wells, Alamar blue staining was applied at 10% in MHB and fluorescence measured as describe above.

To determine the effect of HCHS on the biofilm MBIC, the above experiment was done with HCHS (22.8mg/ml and 1.4mg/ml) added to the broth at the time of incubation for

biofilm formation and the rest of the experiment was the same as described above. The MBIC was then determined by doing an Alamar blue assay and the result was represented as a percentage biofilm killing and a cut off value of 90% was taken, as was described previously.(243, 244)

Harvesting and Culturing Primary Human Nasal Epithelial Cells

The local institutional Human Research Ethics Committee approved the study (HREC/15/TQEH/132) and primary human nasal epithelial cells (HNEC) were harvested from the inferior turbinate of consented non-CRS control patients and all samples were anonymised and coded before use according to a previously described protocol.(245)Briefly, the nasal brushings were suspended in PneumaCult-EX Plus basal medium (05008, Stem cell Technologies, Cambridge, UK). The nasal brushes were flicked against each other with force to extract the cells, which were then depleted of monocytes using anti-CD68 (2021-04, Dako, Glostrup, Denmark) coated culture dishes. This was followed by the expansion of HNECs in routine cell culture conditions of 37 °C humidified air with 5% CO_2 in collagen coated flasks (156367, Thermo Scientific, Walthman, MA, USA).

Lactate dehydrogenase (LDH) assay

Cell cytotoxicity was determined by measuring the amount of lactate dehydrogenase (LDH) in the medium after 24 hours treatments using the Cytotox Homogeneous Membrane Integrity Assay (Promega, Australia). Briefly, HNECs were grown in in 96-well flat-bottom plates for 24 hours (37°C, 5% CO2). Then HNECs were treated with different concentrations of HCHS (22.8mg/ml and 1.4mg/ml) in the presence or absence of bacteria (*S. aureus* ATCC 25923). A 50 µl sample of the supernatant was added to 50 µl of LDH reagent and incubated for 30 minutes at 37°C. The absorbance was recorded at 490nm on a FLUOstar Optima plate reader (BMG, Labtech, Ortenberg, Germany).

Determination of bacterial degradation of HCHS using High-performanceliquid chromatography (HPLC)

The amount of residual HCHS and cortisol produced in the presence or absence of *S. aureus* ATCC 25923 was determined using High-performanceliquid chromatography (HPLC). Briefly, HCHS was incubated in the presence or absence of *S. aureus* ATCC 25932 at 37°C and at room temperature for 48 hours. This was then centrifuged briefly at 3000 rpm for 3 minutes to separate the bacterial pellet. The supernatants obtained were analysed on a HPLC system (HPLC, Agilent 1200 series, USA) with diode array UV detector on a Luna C18(2) column (150x3mm, 5u, Phenomenex, Torrance, CA, USA) based on a modified method described by Zhang et al.(246)Gradient conditions were 10%B (0-5min), 30%B (5-15min), 40%B (15-20min) and 100%B (20-35min) with mobile phases A and B consisting of 15% and 70% acetonitrile respectively in 0.1% formic acid/water. Mobile phase flow rate was 1ml/min. Peak data was collected at 245nm with cortisol eluting at 11.9min and HCHS at 18.8min.

Enzyme-Linked Immunosorbent Assay (ELISA)

The supernatant was collected from HNECs that were treated for 24 hours with different concentrations of HCHS (22.8mg/ml and 1.4mg/ml) in the presence or absence of *S. aureus* ATCC 25923. The Interleukin-6 (IL-6) protein levels were estimated with an ELISA kit using rat anti-human IL-6 antibodies (BD Biosciences, New Jersey, USA), according to the manufacturer's instructions. All measurements were performed in duplicate using a FLUOstar OPTIMA plate reader (BMG Labtech, Ortenberg, Germany).

Statistical analysis

All experiments were conducted at least in triplicate and are presented as mean ± standard deviation (SD) unless stated otherwise. The results were analysed using one-way analysis of variance (ANOVA) with Sidak test. MIC analysis was performed using two-way ANOVA

(GraphPad Prism version 8.00; GraphPad Software, La Jolla, CA). Planktonic growth assays were done using Kruskal-Wallis analysis with Dunn's correction. Statistical significance was assessed at the 95% confidence level.

3.5 Results

Planktonic bacterial growth with HCHS

To determine the effect of steroids on planktonic growth of bacteria, *S. aureus* ATCC 25923 was grown in NB containing differing concentrations of Hydrocortisone21 hemisuccinate (HCHS)or control with NB in the absence of HCHS for 24 hours. HCHS was found to be completely soluble in NB and did not precipitate when incubated at similar conditions. There was a non-significant increase in total CFU (sediments CFU + supernatant CFU) for the highest concentration of HCHS treated *S. aureus* compared to control (*Figure 18Ai*). After incubation of *S. aureus* with HCHS for 24 hours however, an increasing sedimentation of the bacteria was observed with supernatants becoming clearer. A CFU count was done to further evaluate the bacterial numbers in the sediments and supernatants. This confirmed these observations with increased CFU counts of bacteria in the sediments (significant at 5mg/ml, p=0.01) and decreased CFU counts in the supernatants (significant at 5mg/ml, p=0.02), compared to the control, as the concentration of steroids increased (*Figure 18Bi &ii*). In line with this, the CFU counts of HCHS treated *S. aureus* samples were higher in sediments compared to supernatants at the highest concentration(p=0.001) whereas in control samples, CFU counts were non-significantly higher in supernatants than in sediments (*Figure 18Aii*). Giemsa staining of the sediments showed aggregation of bacteria in the HCHS treated samples compared to control (*Figure 18C*).

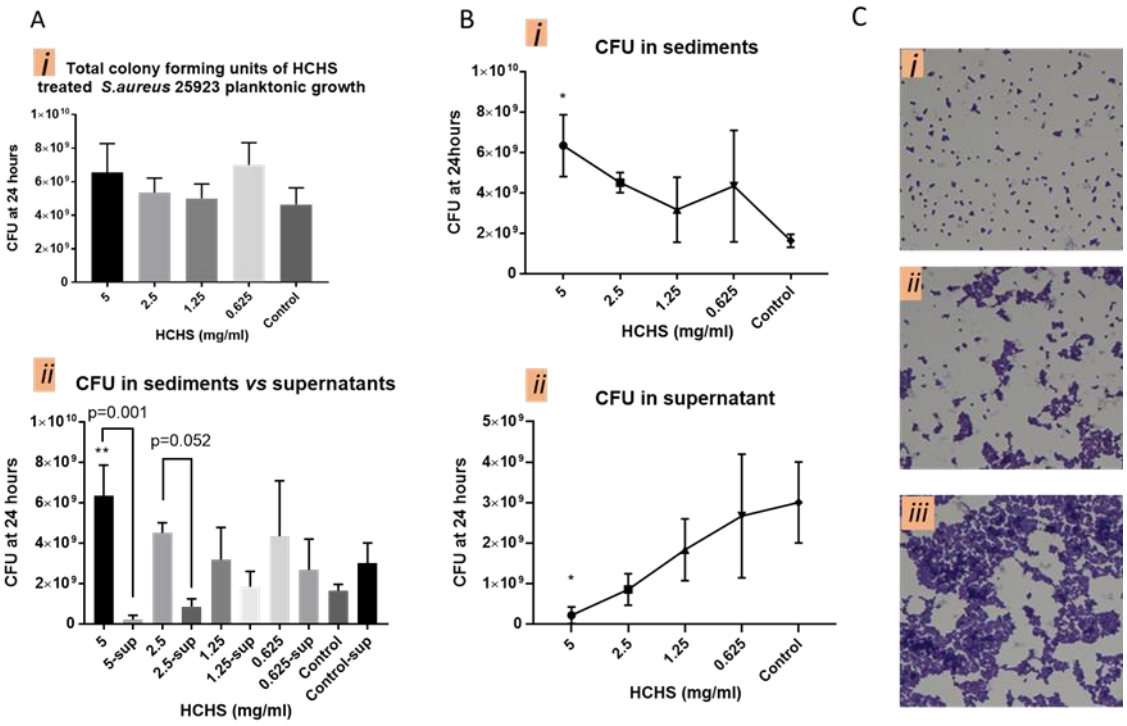


Figure 18: Effect of Hydrocortisone 21 hemisuccinate (HCHS) on the planktonic growth of S. aureus ATCC 25923.

24-hour planktonic growth of S. aureus ATCC 25923 treated with different concentrations of (HCHS) followed by CFU counts of supernatant + sediments(1A) sediments vs supernatants (1Aii), sediments (1Bi) or supernatants (1Bii). Giemsa staining of control (1Ci), 2.5mg/ml HCHS treated S. aureus (1Cii) or 5mg/ml HCHS treated S. aureus (1Ciii). CFU- colony forming units, HCHS-Hydrocortisone 21 hemisuccinate, Control-maximum growth (untreated), Sup-supernatant, Kruskal- Wallis analysis with Dunn's correction *p < 0.05, **p < 0.01.

Effect of HCHS on the metabolic activity of *S. aureus 25923* biofilms

A resazurin assay (Alamar blue) was performed to assess the effect of HCHS on *S. aureus* biofilm metabolic activity. Compared to sodium succinate control, the different concentrations of HCHS had variable effects on the metabolic activity of biofilms.

We found a significant increase in metabolic activity of *S. aureus* biofilms in a concentration dependent manner. At the highest concentration tested, (22.8mg/ml), there was a 2.4-fold increase in metabolic activity ($p<0.0001$). At the intermediate concentrations, the metabolic activity showed an increase of 1.6 and 2-fold for the 5.7 mg/ml ($p<0.05$) and 11.4 mg/ml ($p<0.0001$) respectively (*Fig.19)*. In the presence of HCHS all the bacterial strains showed aggregation at the higher concentrations tested, which was seen as sedimentation formation at the bottom of the wells and was demonstrated using Giemsa staining (*Fig. 20C*).

We then did MTT assays and CFU counts to confirm the findings of increased metabolic activity and determine the number of viable bacterial cells in the presence of increasing HCHS concentrations. MTT assays mirrored Alamar Blue assays showing a dose-dependent increase in the metabolic activity of biofilms in the presence of HCHS (*Fig20A)*. A significant increase in CFU (to 1.6 $x10_6$) was seen in bacteria treated with 1.4mg/ml HCHS compared to control ($2.2X10_5$, $p=0.0002$) *(Fig 20B)*. However, CFU counts gradually decreased with increasing HCHS concentrations and at the highest concentration, CFUs were similar to the untreated control. Giemsa staining showed increased cell numbers when bacteria were treated with HCHS at a concentration of 1.4mg/ml with increasedbacterial aggregation at the higher concentration of HCHS (22.8mg/ml) *(Fig 20C)*.

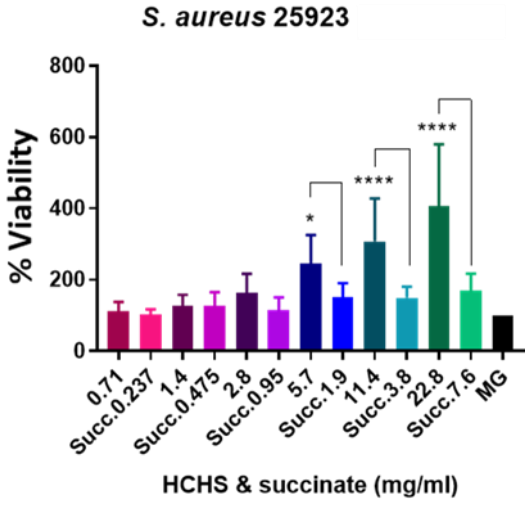


Figure 19:Metabolic activity of S. aureus ATCC 25923 on exposure to increasing concentrations of HCHS compared to succinate as determined by Resazurin assay

All values are relative to untreated controls, normalised to 100%. Data represent the mean ± SD of three biological replicates. One-way ANOVA, *p < 0.05; ****p < 0.0001; SD, standard deviation; Succ, sodium succinate salt; HCHS- hydrocortisone 21 hemisuccinate; MG, maximum growth.

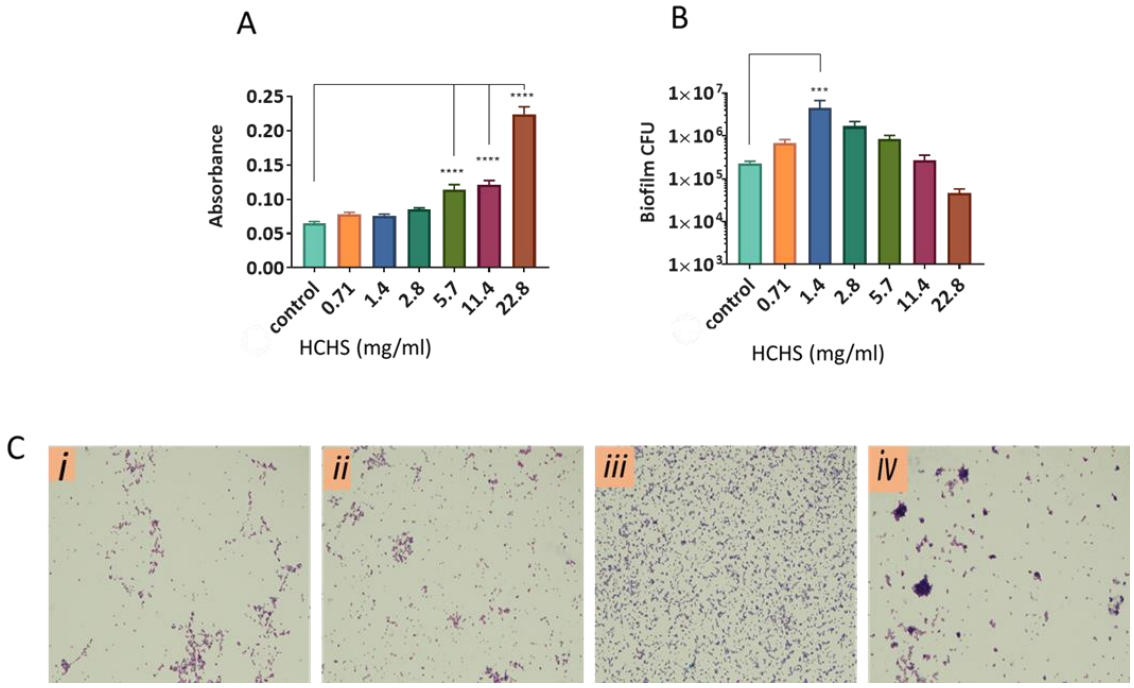


Figure 20:MTT assay (A), CFU count (B) and Giemsa staining (C) of S. aureus ATCC 25923 biofilms

Biofilms treated with different concentrations of HCHS. Control in experiment A and B is sodium succinate. (C) i- Giemsa staining of i-untreated biofilms, ii- biofilms with sodium succinate 7.6mg/ml, iii- biofilms with 1.4mg/ml HCHS, iv- biofilm with 22.8mg/ml HCHS. Data represent the mean ± SD of three biological replicates. One-way ANOVA, **p < 0.01; ***p < 0.001; ****p < 0.0001; SD, standard deviation, CFU- colony forming units, HCHS- hydrocortisone 21 hemisuccinate; control, untreated well.

Confocal scanning laser microscopy

We then did Live/Dead staining and confocal scanning laser microscopy to analyse the effect of HCHS on *S. aureus* ATCC 25923 biofilms. At the higher concentrations (11.4 mg/ml and 22.8 mg/ml of HCHS) tested, there was evidence of clumping and change in morphology of the biofilms. The arithmetic mean intensity showed an increase in both the live and dead staining as the concentration of HCHS increased to 5.7 mg/ml and thereafter showed a decrease as the concentrations increased *(Fig. 21, Table. 2)*

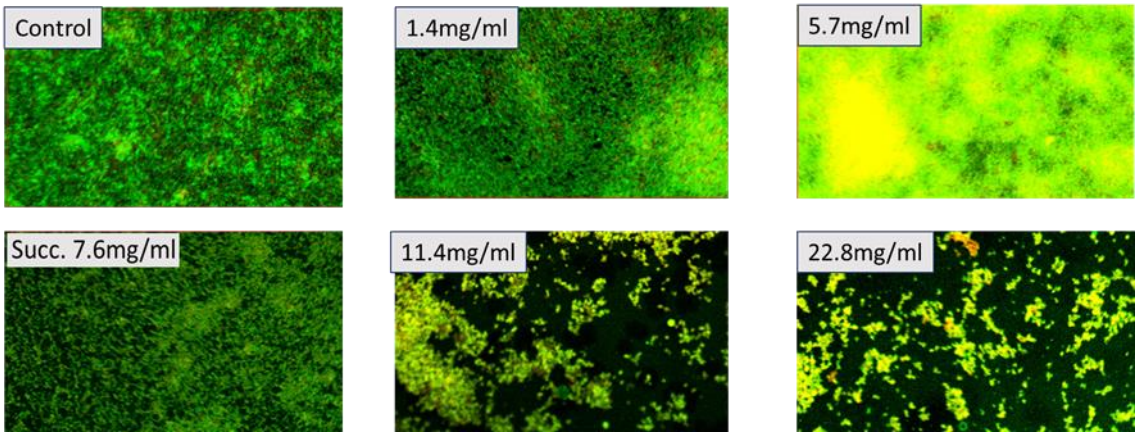


Figure 21:Confocal scanning laser microscopy of S. aureus ATCC 25923 biofilms after LIVE/DEAD BacLight staining

Untreated control and Sodium succinate control compared to biofilms grown at different concentrations of HCHS (1.4mg/ml, 5.7mg/ml, 11.4mg/ml, and 22.8mg/ml as marked on the picture). The green cells represent the live bacteria, red cells represent dead bacteria. HCHS, hydrocortisone 21 hemisuccinate, Succ.7.6mg/ml – Sodium succinate 7.6mg/ml, Control- maximum bacterial growth with no treatment

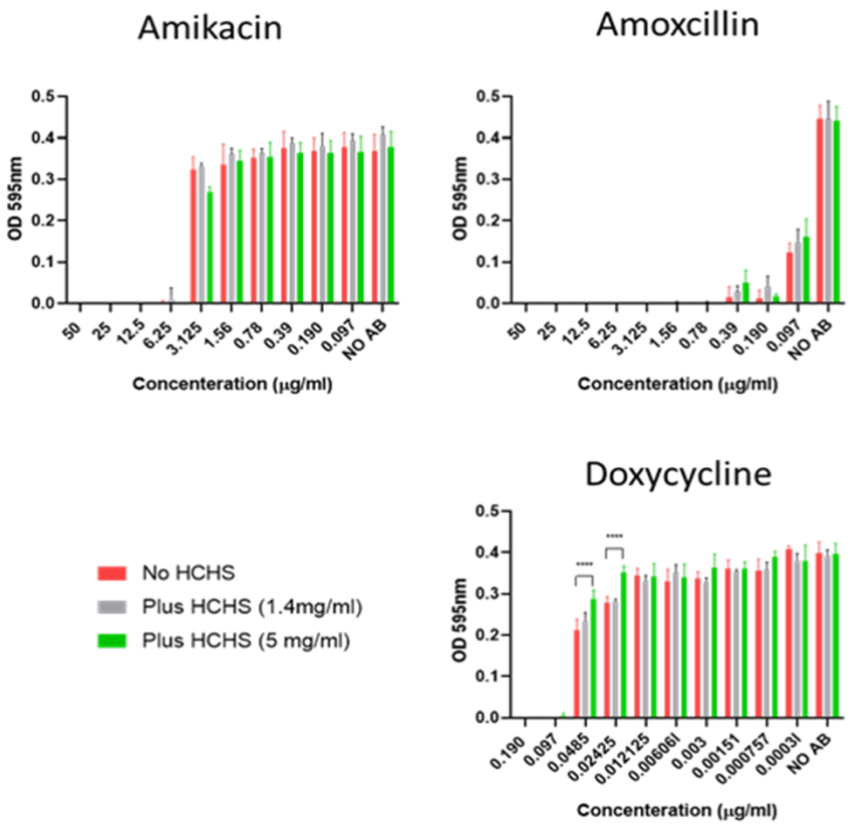
Table 2: Arithmetic mean intensity of live (CH-1) and dead (CH-2) staining. HCHS, hydrocortisone 21 hemisuccinate

HCHS (mg/ml)	Staining intensity CH-1(live)	Staining intensity CH-2(dead)

Control (no HCHS)	94.9	44.08
1.4	121.66	65.86
5.7	242.44	149.7
22.8	86.2	49.75

Effect of HCHS on *S. aureus* ATCC 25923 Minimal Inhibitory Concentration (MIC) of antibiotics:

None of the tested concentrations of HCHS shifted the MIC for any of the seven tested antibiotics. However, compared to control, increased planktonic growth rates of the bacteria were observed with subinhibitory concentrations of ciprofloxacin, doxycycline, erythromycin and gentamicin in the presence of the two concentrations of HCHS tested ($p < 0.0001$) (Figure 22).



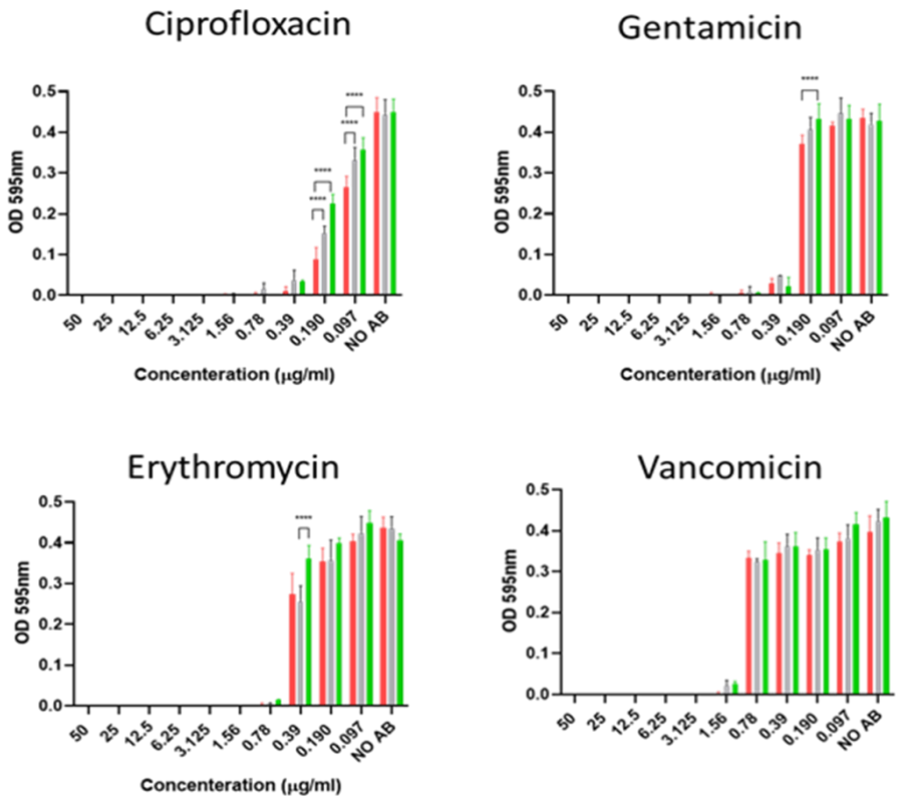


Figure 22:MIC of planktonic S. aureus 25923 compared to its MIC in the presence of different concentrations of HCHS.

Data represent the mean ± SD of three biological replicates. two-way ANOVA, ****p < 0.0001; SD, standard deviation, OD595- optical density, HCHS- hydrocortisone 21 hemisuccinate; NO AB- no antibiotic.

Effect of HCHS on *S. aureus* ATCC 25923 Minimal biofilm inhibitory concentration (MBIC) of antibiotics:

We then wanted to assess whether differences in metabolic activity and phenotype affected the susceptibility of *S. aureus* biofilms to antibiotics. Following treatment with different antibiotics, the MBIC value was represented as the concentration which gave more than 90% biofilm killing. In the absence of HCHS, ATCC 25923 biofilms were susceptible to all the 7 antibiotics tested at different concentrations. In the presence of 1.4mg/ml HCHS however, bacteria within the biofilm were resistant at the highest concentration tested for

5/7 antibiotics. In contrast, when incubated with HCHS at 22.8mg/ml, the MBIC demonstrated a consistent decrease for all the antibiotics tested and none were found to be resistant to any of the antibiotics tested *(Table 3).*

Table 3: MBIC of S. aureus 25923 biofilms compared to MBIC in the presence of different concentrations of HCHS.

Antibiotic	MBIC (µg/ml)	MBIC (µg/ml) with 1.4mg/ml HCHS	MBIC (µg/ml) with 22.8 mg/ml HCHS
Percentage killing	>90%	>90%	>90%
Ami	8	>32	2
Amox	2	16	1
Cip	1	>32	0.5
Dox	0.5	>32	0.25
Ery	2	>32	0.5
Gen	1	>32	0.5
Van	8	8	2

Effect of *S. aureus* ATCC 25923on the degradation of HCHS

High-performanceliquid chromatography (HPLC) analysis was done on the media in the presence of HCHS with and without *S. aureus* ATCC 25923 to determine if *S. aureus*was capable of metabolizing HCHS. HCHS in media degraded into cortisol (active compound) when incubated at 37$_0$C for 48 hours (26% and 7% cortisol for 1.4 mg/ml and 22.8 mg/ml HCHS respectively). In the presence of bacteria, there was a manifest increase in the relative cortisol production (65% and 23% cortisol for 1.4 mg/ml and 22.8 mg/ml HCHS respectively) *(Table 4)*

Table 4: Degradation of HCHS at different concentrations in the presence and absence of bacteria

The concentration of remaining HCHS and the active metabolite, cortisol was averaged for two samples.

Sample Concentration (mg/ml), at 37^0C for 48 hours	HCHS (mg/ml)	Cortisol (HC) (mg/ml)
1.4	0.67	0.24
1.4 + bacteria	0.3	0.57
22.8	15.53	1.27
22.8 + bacteria	10.93	3.4

Enhancement of anti-inflammatory action of HCHS in the presence of *S. aureus* ATCC 25923:

Given the increased production of cortisol in the presence of *S. aureus*, we evaluated whether the anti-inflammatory effect of HCHS was enhanced in the presence of *S. aureus.* The exposure of HNECs to poly (I:C) elicited a strong induction of IL-6 production, as expected.(212)The addition of both low and high concentrations of HCHS (1.4mg/ml and 22.8mg/ml respectively) decreased IL-6 production which was significant at the high concentration (p=0.0001). Compared to HCHS, adding the supernatants of *S. aureus* treated with identical HCHS concentrations, IL-6 production was further reduced which was significant at the low steroid 1.4 mg/ml concentration (p=0.0008) (Figure 23).

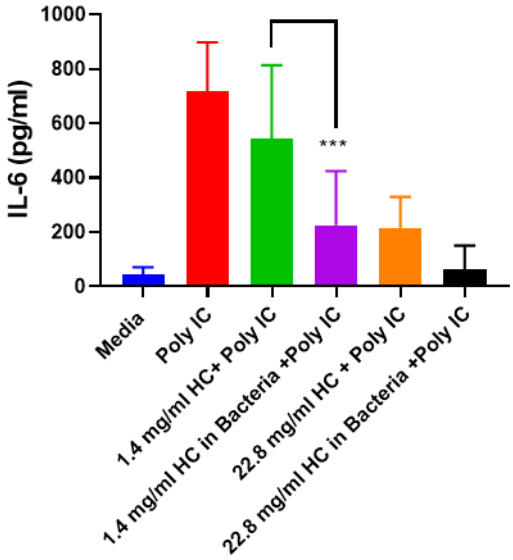


Figure 23: IL-6 secretion in the absence or presence of poly (I:C) LMW and different concentrations of HCHS or supernatants of S. aureus treated with identical HCHS concentrations

IL-6 secretion by Primary Human Nasal Epithelial Cells (HNECs) in the absence (media) or presence of poly (I:C) LMW and different concentrations of HCHS or supernatants of S. aureus treated with identical HCHS concentrations**.** Data represents the mean ± SD of three biological replicates; IL-6, Interleukin 6; HNEC, Human nasal epithelial cells; HC &HCHS, hydrocortisone hemisuccinate; Media, 10µl nutrient broth + 90µl of HNEC media-PneumaCult-EX Plus basal medium; SD, standard deviation

Lactate Dehydrogenase (LDH) assay:

Cell viability was assessed by measuring LDH release from HNEC- cultures. A 24-hour exposure of different concentrations of HCHS or supernatants of *S. aureus* treated with

identical HCHS concentrations showed no significant increase in LDH release in HNECs ($p > 0.05$) (Figure24).

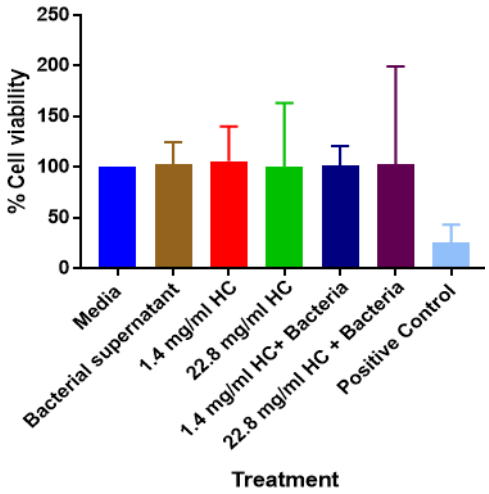


Figure 24: Cell viability using LDH assay

Cell viability using LDH assay after 24-hour treatment with bacterial supernatants in the presence (1.4mg/ml HC +bacteria, 22.8mg/ml HC+ bacteria) and absence (1.4mg/ml HC, 22.8mg/ml HC) of HCHS and bacterial supernatants. Negative controls included untreated cells and positive controls; cells exposed to Triton X-100. Data represents the mean ± SD of three biological replicates.; LDH, lactate dehydrogenase; HC &HCHS, hydrocortisone 21 hemisuccinate; SD, standard deviation

3.6 Discussion:

The use of corticosteroid as an oral or topical agent in chronic and acute infections remains controversial. Steroids have been shown to be beneficial and at times life-saving in certain infections while their use have also been associated with an increase in infection severity leading to life threatening complications.

In this *in vitro* study, we demonstrate that HCHS increases the metabolic activity of *S. aureus* ATCC25923 in a dose-dependent way accompanied with increased aggregation of

planktonic and biofilm bacteria. An increased CFU count was observed when treating *S. aureus* ATCC25923 biofilms with 1.4 mg/ml HCHS, however, a gradual decrease in CFU counts was seen with increasing HCHS concentrations. The changes in metabolic activity were associated with changes in antibiofilm activity of antibiotics with a reduced susceptibility at 1.4 mg/ml and an increased susceptibility at the highest concentration of 22.8 mg/ml HCHS. *S. aureus* ATCC 25923was capable of metabolizing HCHS, thereby increasing the cortisol production and boosting the anti-inflammatory properties of HCHS. An increasing metabolic activity with increasing steroid concentration which was then followed by a decrease in metabolic activity at the highest steroid concentration was also observed in the study done by Raab,(247)where he tested the action of methylprednisolone sodium-hemisuccinate and methylene prednisolone diethylamino acetate on *S. aureus*. This response differed depending on the organism and the glucocorticoid tested. *Pseudomonas aeruginosa* under similar conditions showed an increase in metabolic activity with increasing steroid concentration, without a drop at the highest concentration tested, which they attributed to the esters in the steroid molecule. Steroid compounds like progesterone have been shown to both inhibit and promote the growth of *Neisseria gonorrhoeae* depending on the dosage of treatment. The promotion of bacterial growth is thought to be due to the subversion of the activity of the host serine-threonine kinase Akt by the gonococcal phospholipase D.(248)In another study, HCHS injected intra-allantonically to chick embryo infected with intra-allantonic *Staphylococcus epidermidis* increased the mortality at doses of HCHS 10µg and above. This was, however, attributed to the teratogenic effect as HCHS did not affect the growth or virulence of the bacteria. Interestingly, at the lower HCHS dose of 1µg, the increased mortality was associated with an increase in the tissue concentration of bacterial strains.(249)

We observed that the planktonic *S. aureus* 25923 and its biofilms showed an increase in aggregation as the concentration of HCHS increased. A similar finding was reported where

pathogenic and non-pathogenic *Pseudomonas* species were incubated in a tobacco suspension.(250)Microscopy showed a decrease in the free-floating bacteria with an increase in the bacterial aggregates and this phenomenon required the presence of both bacteria and plant cells. Treatment with streptomycin inhibited this aggregation, suggesting that bacterial protein synthesis is required for this response. Extracellular polysaccharides and cell wall proteins are factors which are necessary for bacterial aggregation which is considered to be an important step in biofilm formation.(251)Bacterial aggregation has also been shown to be influenced by the number of bacterial cells in the media. Although this phenomenon is linked to bacterial adaptation and virulence development, these aggregates also develop in the presence of compounds like nickel where this is considered to be mechanism to reduce the surface area and circumvent nickel toxicity.(252)The size of the aggregates increased and their number decreased with higher concentration of nickel and it was vice versa with lower test concentrations. In this current study we could not establish the viability of all the cells in the aggregates nor the molecular mechanisms involved in the formation of the aggregates in the presence of HCHS. Namely, we observed an increase in the CFU of biofilms till a concentration of 1.4mg/ml HCHS and there after a decrease with the higher concentrations of HCHS even though the metabolic activity was increased at higher concentrations. It could be that the bacterial aggregates were not able to be dispersed adequately with sonication and a proper dilution and hence an accurate CFU was not attained.

The anti-inflammatory action of steroids and its benefits in a vast number of infections have been well described. McGee et.al(237)reviewed 190 trials using steroids along with antibiotics in conditions where infections play a major role and showed that steroid therapy improved survival, provided long term benefit and also relieved symptoms in a large number of infections. The benefit was seen more so in the most severe infections with greater morbidity. In some viral infections however, steroid therapy was either ineffective or at times harmful. Hydrocortisone was the steroid used in 4 % of these trials with the dose

varying from 10mg prednisolone equivalent to more than 1300mg administered as a single dose to more than 400 days in certain infections. In clinical settings, hydrocortisone can be used intravenously for infections like severe community- acquired pneumonia as bolus doses up to 200mg followed by infusions at 10mg/hr for several days.(253)Hydrocortisone is also used as a topical agent in haemorrhoids (5mg/g to 25mg), inflammatory bowel diseases, inflammatory conditions in dermatology, ophthalmology and otology at concentrations ranging from 1% 2%, 10% as drops, creams, ointments or foams.(254-257)These concentrations are comparable to the ones used in our study. Gong et.al,(239)in their study observed a decrease in the skin colonization of *S. aureus* in eczema and atopic dermatitis with both corticosteroid-antibiotic ointment and corticosteroid ointment alone. The results of our study showed that the increased biofilm metabolic activity at highest tested HCHS concentrations (clinically relevant doses) also increased the *S. aureus* biofilm susceptibility to antibiotics thus further supporting its use in specific infections. A similar action with cortisone and hydrocortisone acetate was seen in a *Mycobacteriumlepraemurium* infected rat study, where the steroid treatment decreased the bacterial infection in the spleen of these rats.(258)In contrast, systemic administration of glucocorticoids has been shown to increase the relative risk of developing certain bacterial, fungal and viral infections in a population-based study.(259)Bacterial aggregation and biofilm formation have been associated with an increased tolerance to antibiotics. In the current study, we found this to be true at the low steroid concentration. However, at higher concentration, the biofilms were more susceptible to antibiotics and this we propose could be due to molecular mechanisms linked to the increased metabolic state in biofilms in the presence of HCHS, providing optimal conditions for the antibiotics to act.

We also observed that in the presence of *S. aureus* 25923, there was an increase in the dissociation of the compound HCHS into the active metabolite cortisol, as well as an increased anti-inflammatory action as evidenced by a decrease in the inflammatory marker

IL-6. Analysis of the degradation of hydrocortisone 17-butyrate in the presence and absence of microorganisms by Rabouan-Guyon et al[202]showed an increased steroid degradation with bacilli compared to cocci like *S. aureus* and controls. Bacteria have been shown to utilise steroids as growth substrate and multiply. Enzymes involved in 21-dehydroxylation or 16α-dehydroxylation of steroids such as corticosteroids and sex hormones have been identified in intestinal microbiota.[260]A metabolomic study demonstrated a reduction in steroid metabolites from the gut of streptomycin treated mice, suggesting that the reduction in faecal bacteria may be responsible for this.[261]Apart from this, literature shows that many soil and aquatic bacteria have specialised mechanisms by which they metabolise steroids. A study done to delineate the distribution of aerobic steroid catabolism pathways using both hidden Markov models and reciprocal BLAST identified 265 putative steroid degraders within Actinobacteria and Proteobacteria.[200]The strain *S. aureus* 25923 in our study was capable of degrading/utilising HCHS and producing cortisol, however the complex mechanisms involved in this process was beyond the scope of this study.

3.7 Conclusion:

This study identified for the first time the effect of HCHS on increasing the metabolic activity and aggregation capacity of *S. aureus* ATCC 25923 biofilms. HCHS affected the susceptibility of the biofilms, but not planktonic cells, to antibiotics. This is the first report on the potential of *S. aureus* to metabolise HCHS thereby potentiating the anti-inflammatory action of HCHS. Whilst further studies are required, testing the relevance of our findings using clinical isolates in the *in vivo* setting, our findings suggest that the clinical benefit of using steroids as adjuvants in infections might depend on the concentration of the steroid used and influenced by the host microbiome composition.

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4 Chapter 4: The clinical and microbiome outcomes of medical treatments in chronic rhinosinusitis: A randomised double blinded placebo-controlled trial

4.1 Statement of authorship

Statement of Authorship

Title of Paper	The clinical and microbiome outcomes of medical treatments in Chronic rhinosinusitis: A double-blinded placebo-controlled randomised controlled trial
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	Lisa M Cherian, Ahmed Bassiouni, Clare M Cooksley, Sarah Vreugde, Peter-John Wormald, and Alkis J Psaltis

Principal Author

Name of Principal Author (Candidate)	Lisa M Cherian		
Contribution to the Paper	Ethics and design of the project, acquisition of data, analysis and interpretation of data, drafting the article.		
Overall percentage (%)	90%		
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	07.07.19

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	Ahmed Bassiouni		
Contribution to the Paper	Statistical analysis, interpretation of data and manuscript editing		
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Name of Co-Author	Clare M Cooksley		
Contribution to the Paper	Acquisition of data and manuscript editing		
Signature		Date	07.07.19

Name of Co-Author	Sarah Vreugde		
Contribution to the Paper	Conception of the project, critically revising the article		
Signature		Date	07.07.19

Name of Co-Author	Peter-John Wormald		
Contribution to the Paper	Conception of the project, critically revising the article		
Signature		Date	07.07.19

Name of Co-Author	Alkis J Psaltis		
Contribution to the Paper	Conception and design of the project, critically revising the article		
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4.2 Abstract

Background

The mainstay of treatment in chronic rhinosinusitis (CRS), a complex disorder of infection and inflammation, is topical steroid, oral steroid, antibiotic or a combination of these. The clinical outcomes vary among patients and are possibly dependent on these therapies as well as the initial phenotypic presentation. Among the different parameters implicated in the disease progression is sinonasal microbial dysbiosis. This double blinded, randomised placebo-controlled trial investigates the effect of the three different medical therapies in CRS on the clinical outcomes and explores the associated microbiome shifts.

Methods

Fifty eligible CRS patients were randomised into 3 active treatment arms consisting of oral prednisolone, topical Pulmicort or antibiotic, each arm with appropriate placebo. Patient symptom scoring, endoscopic grading, microbiome swabs and bacterial swabs were performed on enrolment (time_0), at the completion of treatment (time_3) and at week 6 (3 weeks after the completion of treatment). 43 patients completed the trial and were included in the final analysis. Microbial communities were characterized using 16S ribosomal RNA (rRNA) gene-targeted amplicon sequencing. Analysis of bacterial abundance, diversity and stability were done using Quantitative Insights In to Microbial Ecology (QIIME 2) platform.

Results

There was significant improvement of clinical outcomes in all subjective and objective patient assessment scores in both the prednisolone and topical pulmicort arms at treatment completion, but not sustained at 6 weeks [*sinonasal outcome test 22 (SNOT 22) p=0.012, p=0.008; Lund Kennedy score (LKS) p=0.013, p=0.025 respectively]*. Similar improvement was seen also on the sub group analysis with a more pronounced difference in CRS with

nasal polyp patients (*SNOT 22 p=0.06, prednisolone; p=0.013, pulmicort*). Antibiotics, on the contrary, worsened symptoms. An increase in *Corynebacterium* was seen in Pulmicort and antibiotic and decrease with Prednisolone. There was an increase in Faiths phylogenetic diversity at 3-weeks (not at 6 weeks) in the Pulmicort arm. Microbial stability assessed using Rank variability and Difference variability was suggestive of an unstable microbiome in the antibiotic arm.

Conclusion

This study shows comparable clinical improvement with oral prednisolone and pulmicort topical rinses. Topical nasal rinses are, however, recommended as it can be given longer and is devoid of systemic side effects. Although microbiome changes were observed with the different treatments, we could not ascertain its consistency and clinical significance. In the light of our findings, and based on the potential local and systemic effects on microbiome and the emergence of multidrug resistant bacteria that is associated with antibiotic use, we do not recommend it in the routine treatment of CRS patients especially those presenting with polyposis.

Short title: Impacts of medical therapies in sinusitis.

Key words: *Microbiome, Prednisolone, Pulmicort, Doxycycline, Patient outcome assessment*

4.3 Introduction

Chronic rhinosinusitis (CRS) is a disease of multifactorial aetiology where infection and inflammation often co-exist in the sinonasal cavity. The triggers of sinonasal mucosal inflammation in the context of CRS vary and may include epithelial barrier dysfunction, immune dysfunction, sinus outflow obstruction caused by anatomical variations and allergy and colonisation with pathogenic bacteria.[18, 19]The resultant clinical manifestation of CRS is variable however, with polyps evident in some patients, while purulence and oedema predominate in others.

There is a growing interest and mounting evidence in literature to the contribution of the resident microbiome to health and disease state. Studies performed on the microbiome of the upper airway are relatively small in number, underpowered and differ in terms of sampling technique and site as well as bioinformatic analysis. This has led to a discrepancy in the results obtained with no real consensus achieved on the specific microbiome in health or disease. However, a relatively consistent observation is that of relative reduction in the markers of bacterial biodiversity, richness and evenness in CRS patients compared to controls. [74, 107, 110, 113, 262]The present-day consensus of expert opinion is rather than specific genera or species being directly associated with the development of CRS, CRS patients more often exhibit overall shift away from an apparent "healthy microbiota" into a state of dysbiosis. It is unclear as to whether the dysbiotic state changes over time, and in particular how factors including current medical treatments influence this change. [118]Oral antibiotics and corticosteroids, represent the pillars of the medical management of CRS. Rhinosinusitis is the most common disease for which antibiotics are prescribed.[263]The literature on the usefulness of antibiotics in CRS, however, is unclear with some reporting benefits on short term antibiotics and others reporting no added benefits over other modalities of medical management.[264, 265]Oral antibiotics have been shown to influence microbiome composition of the gastrointestinal tract affecting the host immune system and at times with deleterious

effect[266]Antibiotic use has also been associated with depletion of specific bacteria in the gastrointestinal tract and this in turn has been associated with the development of diseases like type-1 diabetes mellitus, bronchial asthma and obesity in animal and human studies[267]Whilst the effects of antibiotic use on the gut microbiome have been studied extensively, only a few small studies have evaluated the microbiome changes in the paranasal sinuses in response to antibiotic therapy. Liu et al[140]demonstrated a decrease in the bacterial diversity following antibiotic therapy in recalcitrant CRS patients while Merkley et al[268]contrastingly observed an increased diversity and decreased abundance following antibiotic therapy. It is important to note that these studies were conducted in post-operative patients or patients undergoing sinus surgery. Given that sinus procedures have been shown to influence microbial ecology, this confounding factor may need to be considered when interpreting the above studies' findings.[92]

Oral and topical corticosteroids exert their clinical benefit in CRS patients through anti-inflammatory action, which is mediated through glucocorticoid receptors present within the host cells[235, 236]In addition to their direct anti-inflammatory action, corticosteroids can also potentially influence inflammation by acting on the bacterial growth either directly[187], or through the excipients used in their commercial preparation[269]Little research has been performed however into the influence of corticosteroids on the sinonasal microbiome and the resultant clinical outcomes.

Literature provides ample evidence for symptom improvement with topical steroid use and short term benefits with oral steroid therapy in CRS patients, particularly those with nasal polyps[270, 271]Antibiotics on the other hand, despite being associated with development of allergic diseases and chronic inflammation in the paranasal sinuses on long term use[90, 272]continue to be routinely used in clinical practise of CRS management.

The primary aim of this study is to evaluate the independent influence of commonly prescribed medications, namely oral steroids, topical steroids and oral antibiotics on the

sinonasal microbiome in un-operated patients with CRS. We secondarily examined the effect of each of these medical treatments on symptom and endoscopic scores. To the best of our knowledge this is the first randomised double-blinded placebo-controlled trial evaluating the microbiome changes in CRS patients receiving maximal medical therapy and who have not undergone any prior sinus surgery.

4.4 Methods

Ethics statement

An informed written consent was obtained from all the study participants enrolled, in accordance with the declaration of Helsinki. This study was approved by the Central Adelaide Local Health Network Ethics Committee (HREC/15/TQEH/177).

Study design

This was a randomised double-blinded placebo-controlled trial, recruiting patients with chronic rhinosinusitis (CRS) who attended the ENT out-patient department between December 2016 and December 2018. Randomisation was performed centrally by The Queen Elizabeth Hospital (TQEH) Pharmacy Clinical Trial department. The pharmacist used a computer-generated table of random permutation of 20 numbers to randomise into permuted blocks of 9 to the 3 different treatment interventions. The permuted block size or the active agent prescribed was not known to the investigator. All clinical trial medication (active and placebo) for the study was prepacked prior to study commencement by TQEH Pharmacy Clinical Trial pharmacists in a double-blind fashion. This ensured allocation concealment of randomisation. Allocated pack numbers corresponding to the randomisation list were then available to dispense to trial participants with the allocation numbers provided to participants in the order of enrolment into the study. All parties remained blinded until final data analysis and statistical testing.

Study participants

Patients who were diagnosed with CRS as per the criteria outlined in the European Position Paper on Rhinosinusitis and Nasal Polyps (EPOS) 2012[4]were randomised into three different medical treatment groups. All patients self-administered their oral and irrigation medication daily for 3 weeks. All the patients were given 3 ‘treatments’ with one being the active medication they were allocated.

The first group received oral prednisolone (Panafcortelone, Aspen Pharmacare, St Leonards, NSW), oral placebo for antibiotic and 2 times daily 240ml Neilmed saline sinus irrigations (Santa Rosa, CA) with placebo representing budesonide respules added to the rinse. The second group received oral placebo (representing oral prednisolone in tapering dose), oral placebo (representing antibiotic) and 2 times daily Budesonide, 0.5mg/2mL (Pulmicort respules, AstraZeneca AB, Sodertalje, Sweden) diluted in 240ml Neilmed saline sinus irrigations. The third group received oral placebo (representing oral prednisolone in tapering dose), oral doxycycline (Doxylin, Alphapharm, Millers Point, NSW) and 2 times daily 240ml Neilmed saline sinus irrigations with placebo representing budesonide respules added to the rinse. Contents of each treatment arm is represented in *Table 5*. The placebo oral medication was prepared by Professional Pharmaceutical Packaging Pty, Ltd (VIC, Australia) and the placebo for Pulmicort respules was 2mL water for injections (Pfizer, Brooklyn, USA). The treatment was randomised and repacked by the pharmacology clinical trials unit at TQEH. Neilmed saline sinus irrigation was used as the base nasal irrigation for all three groups with patients getting the sachets they had to add to the rinses. The inclusion and exclusion criteria for the study is outlined in (*Table 6*). The patients were followed up at 3 weeks (treatment completion) and at 6 weeks (3 weeks after treatment completion). Patient symptom scoring, endoscopic grading, microbiome swabs and bacterial swabs were performed on enrolment (time_0), at week-3 at the completion of treatment (time_3) and at week 6 (3 weeks after the completion of treatment) (time_6). Radiological severity of CRS was scored for all patients using the Lund-Mackay score (LMS)[273]prior to recruitment into the study.

Table 5:Contents of each treatment arm of the RCT

Oral steroid	Topical steroid	Oral antibiotic
Panafcortelone ® (25mg/day for 1 week then 12.5mg/day for 1 week then 12.5 mg every other day for 1 week) + 240ml isotonic saline with water for injection (2ml respules) as placebo delivered intranasally 2 times a day + oral placebo for antibiotic 2 tablets on day one, followed by one tablet once daily for 3 weeks	Pulmicort ® (0.5mg/2ml respules) washes of the nasal cavities and sinuses delivered intranasally 2 times a day in 240 ml isotonic saline + oral placebo for antibiotic 2 tablets on day one, followed by one tablet once daily + placebo for oral steroid in tapering doses for three weeks	Oral Doxylin® (antibiotic) tablets 2 tablets on day one, followed by one tablet once daily + placebo for steroid in tapering doses + 240ml isotonic saline with water for injection (2ml respules) as placebo delivered intranasally 2 times a day for 3 weeks

Table 6:Inclusion exclusion criteria of the RCT

Inclusion	Exclusion	Withdrawal
1.Have symptoms and signs of CRS and require medical management AND 2.are over 18 years of age AND 3.are able to give written informed consent AND 4.are local patients who will be returning to this centre for follow-up care	1.Requirement for a specific corticosteroid or antibiotic treatment based on symptomatology 2.allergy to steroid or antibiotics 3.pregnant or breastfeeding 4.uncontrolled diabetes 5.on other CYP450 inhibiting drugs 6.liver disease	1.Patients who do not return to this centre for follow up 2. who exhibit an allergic reaction to any test treatments 3. who requests withdrawal from the study; no reason will be required 4.development of pus (confirmed secondary bacterial infection) in patients from groups 1 or 2 (patients will be commenced on an antibiotic and withdrawn from the study). 5.increasing size of nasal polyps causing a worsening of nasal blockage (patients will be commenced on steroids and withdrawn from the study).

Patient scoring

The patient symptoms were scored using the validated Adelaide disease severity score(274)

Sinonasal outcome test-22 (SNOT-22).(275)Endoscopic assessment was performed using the validated modified Lund Kennedy scoring(276)

Sample collection

A guarded flexible nasopharyngeal flocked swab (Copan Italia S.p.A., Brescia, Italy) was used under endoscopic guidance to prevent contamination from the rest of the nasal cavity. The swab was rotated in the middle meatus 7 times for maximum bacterial yield. Following this the swab heads were immediately transferred into a sterile container, transported on ice to the laboratory and stored at -80^{0}C.

DNA extraction

For DNA extraction from collected swabs, we used the Qiagen DNeasy Blood and Tissue kit (Hilden, Germany) with modifications. Briefly, using sterile forceps, the swabs are held by the remaining part of the shaft and with sterile scissors the swab head cut into small pieces, allowing the pieces to drop into a 2 ml microcentrifuge tube. Lysozyme (Sigma-Aldrich, St Louis, Missouri, USA) was added to the lysis buffer at a concentration of 20 mg/ml. From this,180 µl was transferred into to each tube and left at room temp overnight. The following day, a 5 mm stainless steel bead, Qiagen was added to each tube. The Qiagen Tissue Lyser was used to beat the swabs with the steel bead for 20 seconds at 15 Hz. The steel beads were removed from the tubes and replaced with 50mg of 0.1 mm glass beads (Sigma Aldrich) A second round of bead beating was performed in the Tissue Lyser for 5 mins at 30 Hz. To this, 25 µl proteinase K and 200 µl Buffer AL (without ethanol) were added and mixed by vortexing. Samples were incubated at 56^{o}C for 30 mins. Tubes were pulsed briefly in a centrifuge after incubation to collect the beads and the supernatant was transferred to a new tube. The protocol was continued according to the manufacturer's instructions and the DNA eluted in 100 ul elution buffer. Samples were quantified using the NanoDrop spectrophotometer (ThermoFisher scientific, Massachusetts, USA).

PCR amplification of 16S rRNA gene and pyrosequencing

Polymerase chain reaction (PCR) amplification and sequencing was performed by the Australian Genome Research Facility. By amplifying the V3 to V4 (341F–806R) hypervariable region of the 16S rRNA, gene libraries were generated. PCR amplicons were generated using the primers CCTAYGGGRBGCASCAG in the forward sequence and GGACTACNNGGGTATCTAAT in the reverse sequence, using AmpliTaq Gold 360 Master Mix (Life Technologies, Mulgrave, Australia) and following the local protocol. The resulting amplicons were measured by fluorometry (Invitrogen Picogreen; Thermo Fisher Scientific, Waltham, MA) and normalized. The equimolar pool was then quantified by quantitative PCR (KAPA Biosystems, Capetown, South Africa) and set up for sequencing on the Illumina MiSeq (Illumina Inc., San Diego, CA) with 300 base paired end chemistry.

Bioinformatic pipeline

Demultiplexed fastq files were received from the sequencing facility. We used the new QIIME 2 (version 2018.11)(277)for our bioinformatics pipeline. Forward and reverse reads were joined using PEAR(278)through the QIIME 2 plugin q2-pear (https://github.com/bassio/q2-pear). Joined sequences were then quality-filtered using the QIIME 2 plugin q2-quality-filter(279)with minimum quality parameter of 20. Denoising and Amplicon Sequence Variant (ASV) formation were done using deblur(280)through the q2-deblur plugin with setting "trim-size" = 435 and with otherwise default parameters. Taxonomy assignment was done against the Greengenes 16S reference database (the 99% clustered similarity sequences),(281)version 13.8 (August 2013) using the BLAST-based classifier implemented in QIIME 2 (q2-feature-classifier)(282)and which implements a Lowest Common Ancestor (LCA) consensus algorithm. The SATé-enabled phylogenetic placement (SEPP) technique(283)was used for insertion of the ASVs into the high-quality tree generated from the 99% OTUs Greengenes reference database.

A rarefaction depth cut-off was chosen at 400 before downstream analysis. Taxa were compared at the genus level with additional species-level analyses for Staphylococcus

aureus and epidermidis. The taxonomic assignment of the two DNA-negative control samples containing extraction reagents only was explored. The bacterial genus Flavobacterium was common to both samples and was present in relatively low abundance in many samples, so this genus was excluded before downstream statistical analyses. Mean relative abundance as well as prevalence of the genera were calculated for each group. Shannon's diversity and Faith's phylogenetic diversity index[284]were used for alpha diversity. Diversity metrics were generated through sci-kit bio version 0.5.3.

Microbiome stability studies were conducted according to methods described by Martí et al. Rank variability (RV) is a per-sample index, and a surrogate for microbiome stability. It is defined by Martí et al. as "the absolute difference between each taxon rank and the overall rank".[285]Differences variability (DV) is another index defined as "the absolute difference between each taxon rank at a given time and the value it had in the previous time step, averaged over all taxa present". These were calculated using a Python implementation of the equations described in the original paper.

Statistical analysis

We analysed outcomes using a "longitudinal analysis of covariance" (longitudinal ANCOVA) model specification, which controls for baseline value of the outcome measure as a covariate, as specified in the methods of Twisk et al[286]A Repeated measures model without the treatment variable, but with the interaction between treatment and time specified as a covariate, was used to corroborate the longitudinal ANCOVA results and to compare the outcome values at baseline "time_0" to outcomes at follow-up timepoints after randomization and administration of treatment(s), and allow plotting of all three timepoints (including baseline). Estimated marginal means, standard errors (SEs), 95% confidence intervals, and contrasts between study groups were extracted from model results using the R package "emmeans".[287]For all models mentioned, a linear mixed modelling approach was used (through the R packages lme4 and lmerTest)[288, 289]to adjust for the dependency of

repeated observations within each patient enrolled in the trial by specifying the patient variable as a random effect, following the paper of Twisk et al. All statistical analyses were performed using R[290](R Foundation for Statistical Computing, Vienna, Austria) and the Python scientific stack[291]through the Jupyter notebook interface.[292]

4.5 Results

Patient cohort

A total of 178 patients referred from general practitioners with a preliminary diagnosis of chronic rhinosinusitis were screened for recruitment from the outpatient clinic of the Queen Elizabeth Hospital, Adelaide between the period Dec 2016 and Dec 2018. Among these, 69 patients met the eligibility criteria with 50 agreeing to be enrolled in the trial with randomization into the three treatment arms. The patient recruitment and flow chart details are summarized in (*Figure 25*). From this randomized cohort, 5 patients were excluded due to withdrawal or non-compliance with the prescribed treatment. Analysis after unblinding revealed that 1 of these patients was from the active oral steroid arm, 1 from the active nasal steroid irrigation arm and 3 from the oral antibiotic arm. All the patients who completed the trial reported compliance to the 3-week therapy. The demographics and baseline clinical characteristics of the 45 patients who participated in the trial are summarized in *Table7*. Post-trial follow-up was possible for 43 patients with only 2 patients, both from the active oral antibiotic arm lost to follow up following their 3-week trial visit. Of the patients who were followed up for at least 6 months post trial completion 33 desired and proceeded to surgical intervention. There was however no between the treatment group in proceeding to surgery *($p= 0.39$)*. There were also no statistically significant differences between the three groups in terms of baseline disease severity (*LKS, $p= 0.45$, LMS, $p= 0.17$*).

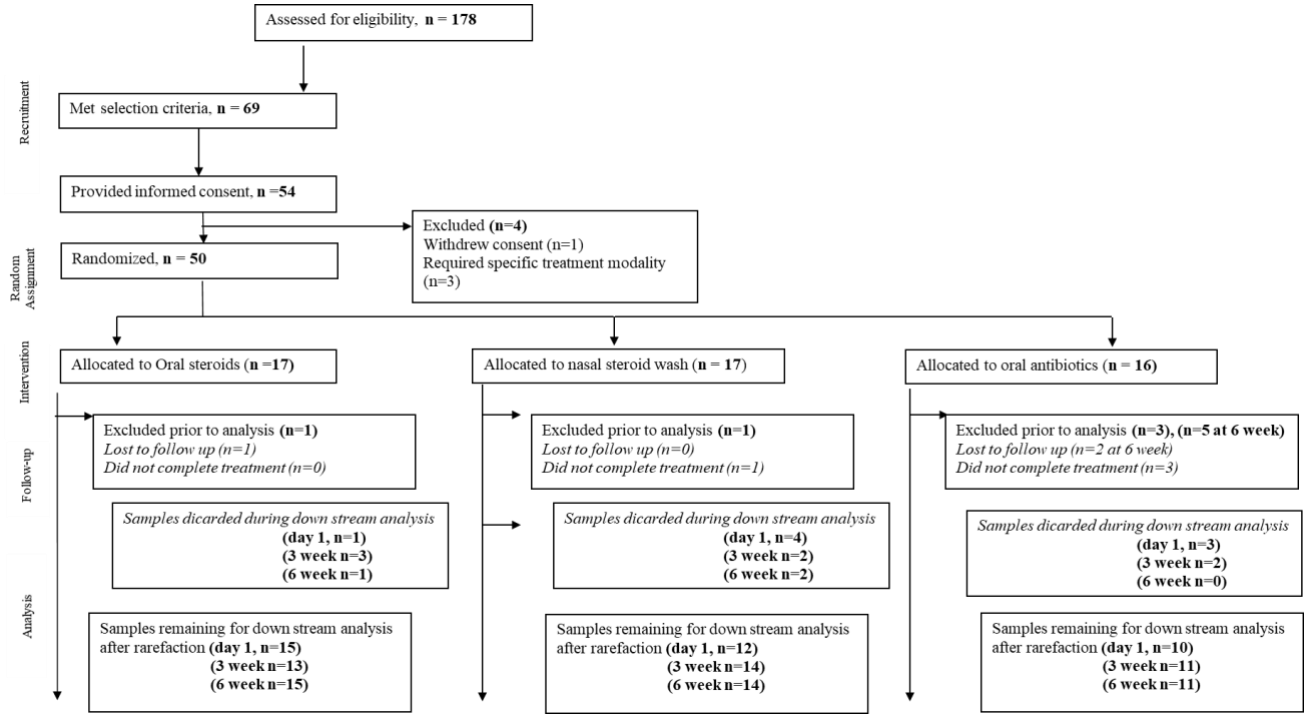

Figure 25: CONSORT flowchart

Table 7:Patient Demographics

	Oral steroid	Topical steroid	Oral antibiotic	P value
Number (n= 45)	n=16	n=16	n=13	
Mean Age yrs. (SD)	44.18	41.25	39.00	
Male/Female	9/7	12/4	8/5	
CRSsNP/CRSwNP	10/6	8/8	10/3	
SNOT-22 mean (SD)	60.37 (21.7)	49.62 (20.2)	63.69 (21.7)	0.178
ADSS mean (SD)	14.75 (2.8)	12.81 (3.3)	13.46 (2.2)	0.169

LKS mean (SD)	5.12 (2.8)	6.37 (4.1)	4.69 (4.2)	0.457
LMS mean (SD)	12.56 (4.0)	13.00 (5.6)	9.84 (4.3)	0.179
Patients requiring surgery after medical therapy (n=43)	11 (68.75%)	12 (75%)	10 (90.9%)	0.399

CRSsNP; Chronic rhinosinusitis without polyps, CRSwNP; Chronic rhinosinusitis with polyps, SNOT 22; Sinonasal outcome test 22, ADSS; Adelaide disease severity score, LKS; Lund Kennedy score, LMS; Lund Mackey score, SD; Standard deviation.

Clinical response of each treatment arm:

Symptom Scores*:* We observed a decrease in the SNOT 22 and ADS scores in all the treatment arms with a significant reduction in the oral and topical steroid group. A repeated measures model was used for estimation of treatment effects of each arm across different timepoints, in comparison to baseline. These comparisons revealed a reduction (from baseline) of the SNOT-22 mean score of 12.9 [CI 3.0, 22.7] (p = 0.012) for oral prednisolone and 14.4 [CI 3.9,24.9] (p = 0.008) for Pulmicort rinse immediately post treatment at 3-weeks. In comparison, the 3-week score for oral doxycycline decreased only 4.6 [CI -7.4, 16.5]. These improvements were not sustained at 3-week post treatment, with a recurrence towards baseline in all groups. (*Figure: 26A & B*). Changes in the Adelaide Disease Severity Score mirrored that of the SNOT-22. (*Figure: 26D*).

Endoscopic Scores: Similarly for the Lund-Kennedy score, we notice some improvement in the scores immediately post treatment at 3 weeks for the oral prednisolone (1.1 [CI 0.2, 1.9], p = 0.013) as well as the Pulmicort rinse arms (1.0 [CI 0.1, 1.9], p = 0.025), while the oral doxycycline arm had a comparatively negligible reduction (0.3 [CI -0.7, 1.3]). Again, this observable improvement in LKS for the first two treatments arms at the first follow-up appointment was not sustained at 3-week post treatment, with a movement toward baseline. (*Figure: 26C*)

Comparison of clinical response to treatment between groups:

Difference between the three treatment arms at 3 weeks were compared using longitudinal ANCOVA. There was a clinically significant reduction in SNOT-22 between treatment with oral prednisolone versus oral antibiotic (-8.7, CI [-23.9, 6.4], p = 0.253) and between treatment with Pulmicort rinse versus oral doxycycline (-11.1, [CI -26.7,4.5], p = 0.159) at the 3-week follow-up appointment.

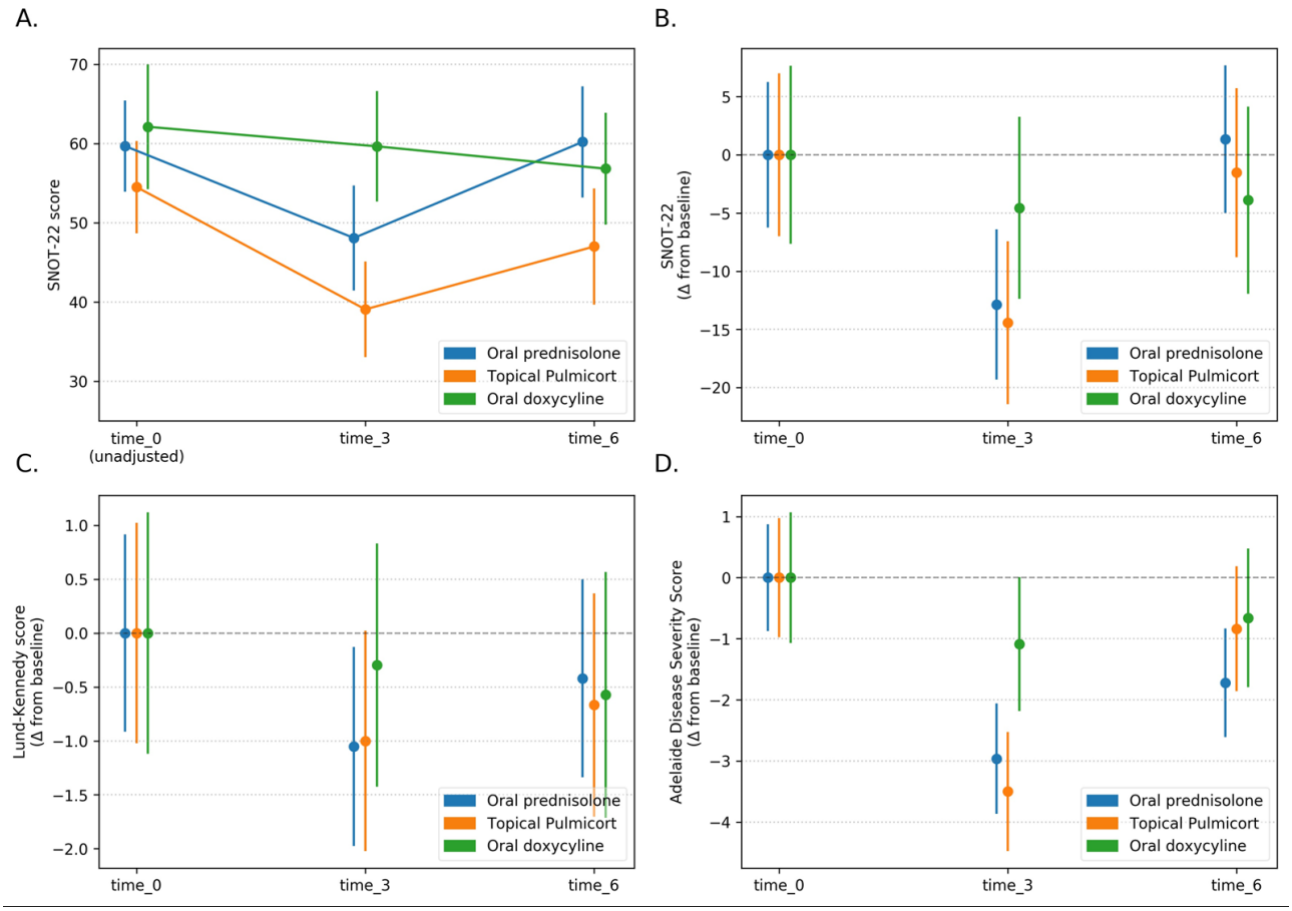

Figure 26: Clinical outcomes of the trial

Clinical outcomes of the trial: the oral and topical steroid groups showed some temporary improvement at 3 weeks, while there were poorer outcomes for the oral antibiotic group. Data represented as means (circles) and Standard Errors (error bars). (A) SNOT-22 scores: unadjusted raw data at baseline (time_0) and values as inferred from longitudinal ANCOVA at follow-up timepoints time_3 and time_6. (B) SNOT-22 scores as inferred from repeated measures model, demonstrated as change from baseline. (C) Lund-Kennedy scores as inferred from repeated measures model, demonstrated as change from baseline. (D) Adelaide disease severity scores (ADSS) as inferred from repeated measures model, demonstrated as change from baseline. SNOT-22 = Sinonasal outcome test-22; ADSS = Adelaide disease severity score; LKS = Lund Kennedy score; time_0 = baseline (day 0);

time_3 = immediate post therapy at 3 weeks; time_6 = 3-week post treatment completion; ANCOVA; Analysis of covariance.

Microbiome outcomes: taxonomy

We observed a differential abundance trends across the follow-up timepoints with different treatments. Corynebacterium and Staphylococcus species were the most common taxa in all 3 patient groups at all assessed time points. In the oral antibiotic treatment arm, a tendency towards an increase in the relative abundance of Corynebacterium and Staphylococcus was observed immediately after treatment. At 3 weeks post oral antibiotic treatment, the tendency for increased relative abundance of Corynebacterium persisted 17.4% [CI -1.6, 36.4], while the relative abundance of Staphylococcus returned to pre-treatment levels.

In patients randomised to the Pulmicort irrigation treatment arm, a tendency towards an increase in the relative abundance of Corynebacterium was also observed immediately post treatment and at 3 weeks post treatment it remained high at 16.7% [CI -0.3, 33.8]. Similar trends to increases in the relative abundance of *Staphylococcus* were also observed in this treatment arm at both measured time points with 9.8% [CI -7.2, 26.8] at 3 weeks post treatment.) *Figure 27*

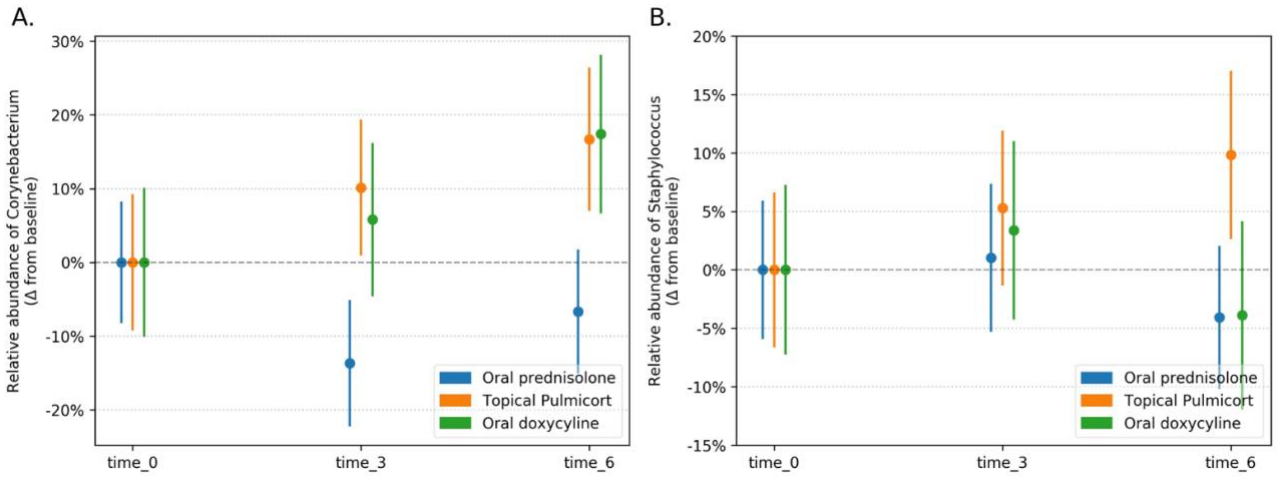

Figure 27: Relative abundance of Corynebacterium and Staphylococcus

Relative abundance of Corynebacterium and Staphylococcus, showing an increasing Corynebacterium with topical Pulmicort and oral doxycycline and decreasing Corynebacterium with oral prednisolone at 3 and 6 weeks. Data represented as means (circles) and Standard Errors (error bars). (A) Relative abundance of Corynebacterium, as inferred from repeated measures model, demonstrated as change from baseline. (B) Relative abundance of Staphylococcus, as inferred from repeated measures model, demonstrated as change from baseline. time_0 = baseline (day 0); time_3 = immediate post therapy at 3 weeks; time_6 = 3-week post treatment completion

Microbiome outcomes: diversity and stability studies

We measured Faith's Phylogenetic Diversity index (Faith's PD) and Shannon's index as measures of phylogenetic and non-phylogenetic alpha diversity, respectively for all three treatment groups. Pulmicort rinses increased Faith's PD immediately after treatment, a 1.1 increase [CI 0.1, 2.1], compared to baseline), but with a rebound towards baseline value at the 6-week timepoint. No significant changes were observed in Shannon's index for Pulmicort rinses and the oral steroid arm, while there was a trend towards a slight decrease in Shannon's index at 6-weeks with oral antibiotic treatment. (*Figure: 28)*

We used Rank variability (RV) and Differences variability (DV) as surrogates for assessing microbiome stability according to Martí et al.(285) When a large number of bacterial taxa change their ranking in terms of abundance compared to the average, the RV and DV becomes higher (and the microbiome becomes, supposedly, more labile i.e. less stable). In our trial, the oral steroid and topical steroid rinse arms demonstrated a (temporary) reduction in RV at the 3 weeks marks. Comparatively speaking, oral antibiotic treatment had higher RV and the highest DV at all follow-up timepoints.

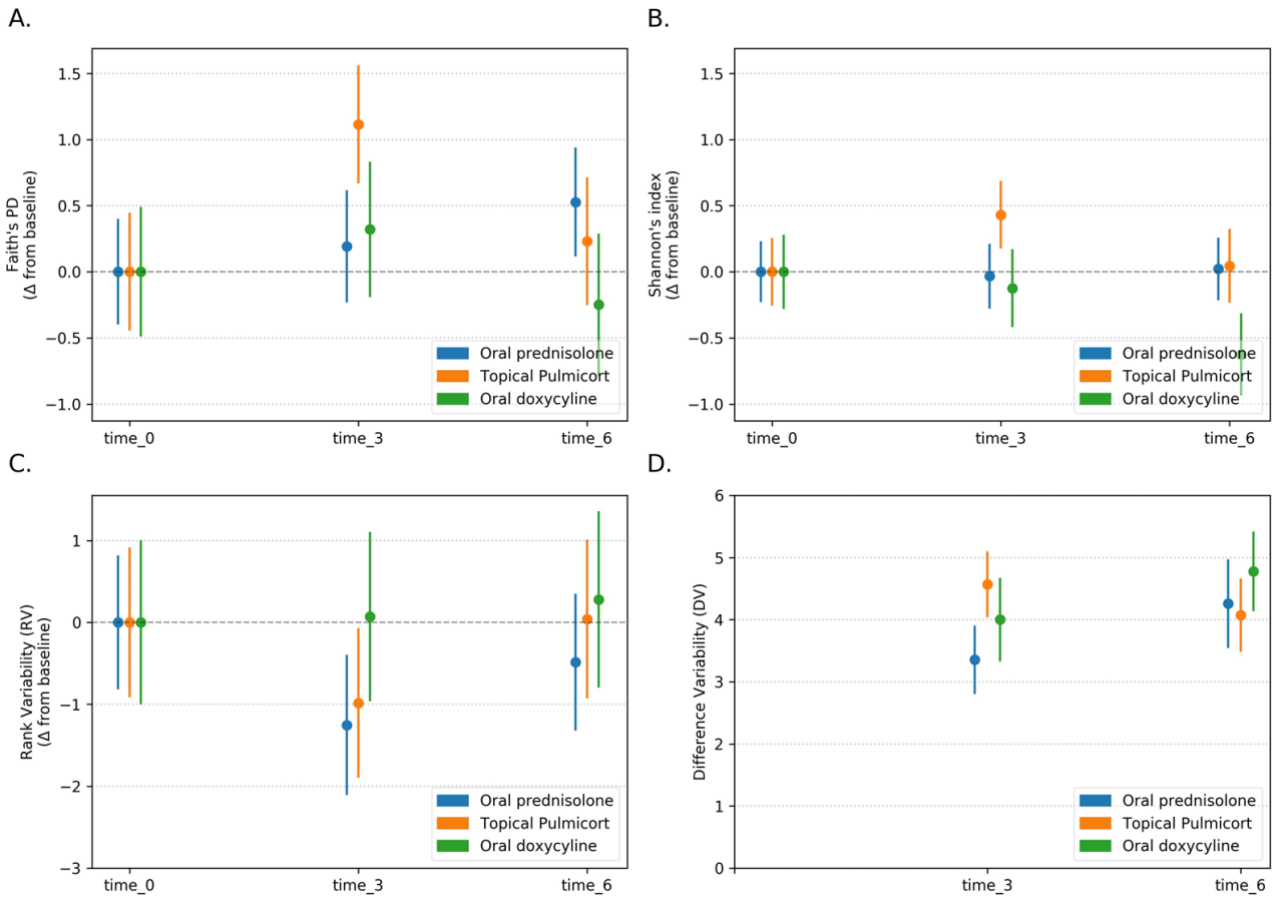

Figure 28: Microbiome alpha-diversity and stability comparisons

Microbiome alpha-diversity and stability comparisons. Data represented as means (circles) and Standard Errors (error bars). (A) Faith's PD, as inferred from repeated measures model, demonstrated as change from baseline. (B) Shannon's index, as inferred from repeated measures model, demonstrated as change from baseline. (C) Rank Variability, as inferred from repeated measures model, demonstrated as change from baseline. (D) Difference Variability (DV) raw scores at time_3 and time_6 follow-up timepoints. Faith's PD = Faith's phylogenetic diversity index; RV = Rank Variability; time_0 = baseline (day 0); time_3 = immediate post therapy at 3 weeks; time_6 = 3-week post treatment completion

Subgroup analyses: polyps versus non-polyps

To investigate effect of nasal polyps on outcomes, we conducted separate repeated measures models for the CRSsNP and CRSwNP subgroups. The improvement in SNOT-22 scores for CRSsNP at 3 weeks compared to baseline were: 11.0 [CI -1.9, 23.9] for the oral steroid group; 7.9 [CI -6.1, 21.8] for the topical steroid group; and 6.6 [CI -6.9, 20.0] for the oral antibiotic group. On the other hand, the improvement at 3 weeks was more pronounced in the CRSwNP subgroup for the oral steroid (15.8 [CI -0.8, 32.4], p = 0.06) and the topical steroid groups (21.3 [CI 5.1, 37.6], p = 0.013), while the antibiotic had an overall average worsening of symptoms at 3 weeks. Again, a rebound phenomenon was observed for both steroid groups 3 weeks after completing the treatment course. *(Figure 29)*

Figure 5 also shows that there appears to be no consistent explanation or association of the symptomatic outcome with the microbiomic parameters at any particular time point in our cohort (demonstrated are Faith's PD, relative abundance of Corynebacterium and Staphylococci).

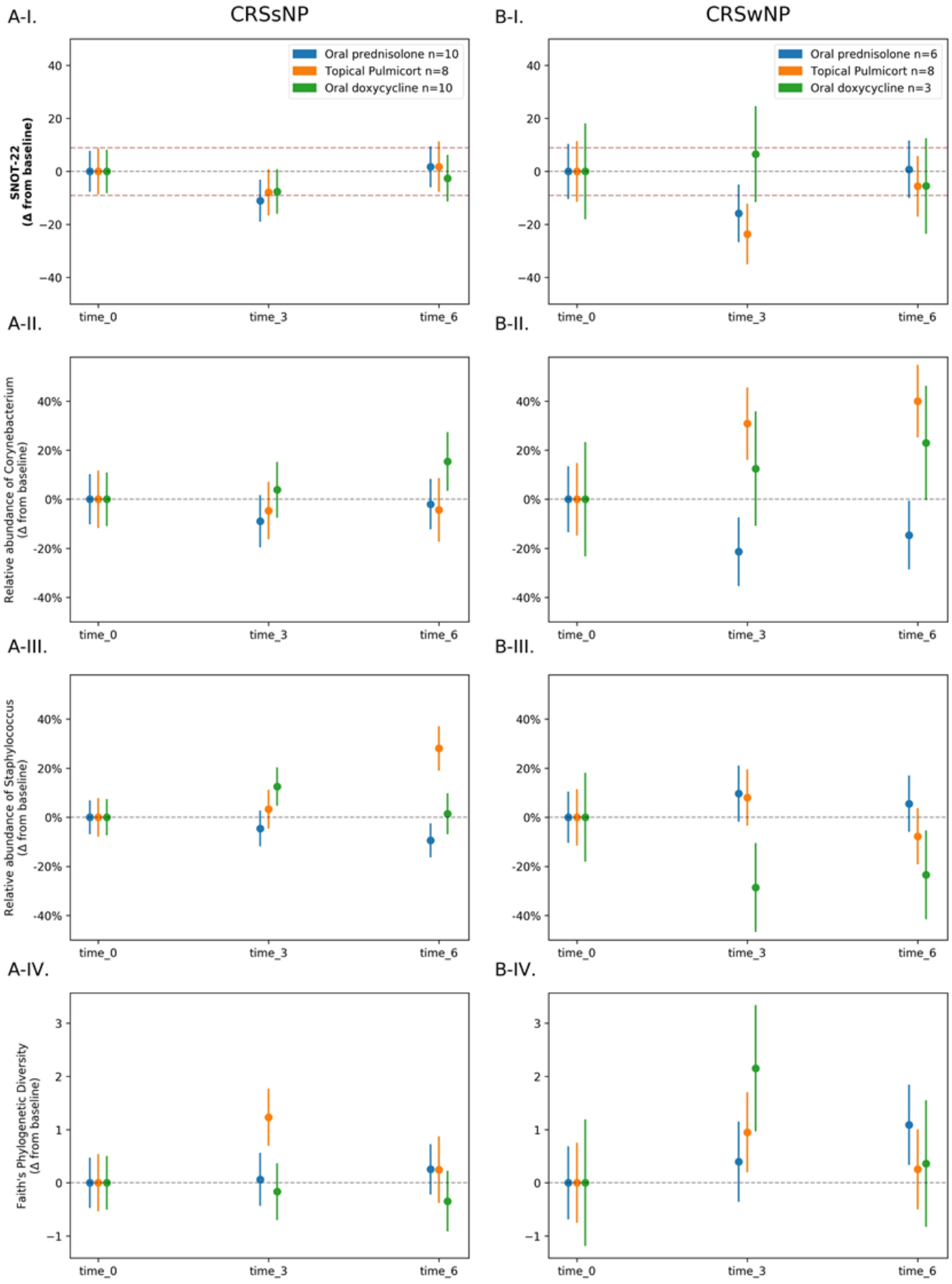

Figure 29:Subgroup analysis of CRS

Subgroup analysis demonstrating SNOTT 22 score improvement on treatment with oral and topical steroids, and a worsening with oral antibiotics in CRSwNP patients at 3 weeks. Data represented as means (circles) and Standard Errors (error bars) and the results inferred from a repeated measures model. The maroon dotted line indicates minimal clinically important difference (MCID)[293]SNOT-22 = Sinonasal outcome test-22; CRSsNP= Chronic rhinosinusitis without polyps; CRSwNP= Chronic rhinosinusitis with polyps; Faith's PD = Faith's phylogenetic diversity index; time_0 = baseline (day 0); time_3 = immediate post therapy at 3 weeks; time_6 = 3-week post treatment completion.

4.6 Discussion

The pursuit of defining a healthy and diseased microbiome in the paranasal sinuses has led to the present day understanding of microbial dysbiosis in disease development and progression in CRS. Our study, further investigates this, to better understand the changes in microbial communities and their effect on clinical outcomes in response to currently recommended medical therapies for the treatment of CRS.

To our knowledge, this is the first double blinded, randomised placebo-controlled trial to compare the clinical outcomes in the three different medical therapies in CRS and explore the microbiome shifts with these treatments. We observed an immediate post-treatment significant improvement in the subjective (SNOT-22 & ADSS) and objective (LKS) clinical scores of CRS patients treated with either oral or topical steroids for 3 weeks. This improvement however, was not sustained and the clinical symptoms returned to base line at 3 weeks post cessation of treatment. No such improvement in any clinical parameters were observed following 3 weeks of oral doxycycline treatment. With regard to the microbiome outcomes in these patients, we observed that the group which received topical steroid showed a tendency towards increasing relative abundance of *Corynebacterium* and *Staphylococcus* at the end of 3 and 6 weeks. However, we could not conclusively associate

this with the improving clinical outcome as, the antibiotic group also demonstrated a similar increase in *Corynebacterium* while the oral steroid group decreased relative abundance of *Corynebacterium* and *Staphylococcus*. A further subgroup analysis differentiating patients with and without nasal polyps, demonstrated an improvement of clinical symptoms with topical or oral steroids and worsening with oral antibiotics in CRSwNP patients. These outcomes also did not appear to have any specific association with the microbiome outcomes.

In the treatment for CRS, steroids seemed to outperform antibiotics in terms of clinical improvement and objective reduction in endoscopic inflammation. The anti-inflammatory action of steroids by modulation of inflammatory gene transcription is well documented in literature(235, 236). This along with the possible indirect and direct antibacterial effects of steroids is probably responsible for the immediate post therapy clinical benefits observed in our study(187, 294). Most antibiotics on the other hand act by eliminating the pathogen and thereby the infection. The antibiotic doxycycline, apart from its broad-spectrum anti-infective property have also been shown to decrease inflammation by modulating gene expression of proinflammatory cytokines.(138)The increased benefit with steroids over doxycycline in our trial could be due to the fact that steroids have a more potent anti-inflammatory property and also that the antibiotic treatment was not culture directed.

A number of RCT's have reported improvement in symptoms with the use of topical steroids in CRSsNP. The systematic review and meta-analysis of nine RCT's by Kalish et al. investigating the effect of topical steroids on CRSsNP, suggests that although there is insufficient evidence to demonstrate an overall clinical benefit, topical steroids should be included in management protocols of CRSsNP as they appear to be safe and provides some symptomatic benefits.(295-298)Contrary to this, Parikh et al(299)in their double blinded, placebo controlled RCT, could not find any difference in clinical symptoms with the use of topical

fluticasone and placebo and so do not advocate their use in routine treatment of CRS. In this study, however, the patient sub-classification into those with and without polyps was not defined which might attribute to their findings. In CRSwNP, a systematic review and metanalysis by Rudmick et al(300)on 19 randomised placebo controlled studies using topical steroids showed that mometasone, fluticasone, and budesonide provided reduction in symptoms in these patients. The duration of treatment in the studies ranged from a minimum of 4 weeks to 270 weeks and outcome measured as improvement in nasal symptoms. Fluticasone propionate nasal drops in a 12-week double blinded RCT on CRSwNP patients showed a decrease in the number of patients going in for surgery when compared to the placebo group. However, the assessment in this study was done immediately post therapy and hence do not account for further recurrence of symptom. (173)The use of oral steroids in CRSsNP is not sufficiently supported in literature (297, 298, 301).In CRSwNP oral steroids have been shown to reduce the inflammatory markers, radiological scores, endoscopic scores and the size of the polyps, thereby improving patient symptoms on an immediate (2 week) follow up(270) as well as lasting to up to 12 weeks with oral steroid taper, followed by maintenance with intranasal corticosteroids(271, 301, 302). In our trial we found that budesonide rinses, although not approved by the U.S food and drug administration (FDA) for use in CRS, had comparable clinical benefits to oral steroids. This was also observed in our subgroup analysis with a significant improvement in patient symptomatology in the CRSwNP group. A number of studies including double blinded placebo-controlled trials have investigated the effect of budesonide in CRS symptoms in post and pre surgical patients. These studies with follow up ranging from 3 months to one year have consistently demonstrated benefits with budesonide rinses.(303-305) Studies evaluating the safety profile of budesonide rinses also demonstrated that there was no evidence of hypothalomo-pituitary-axis suppression (follow up of 38.2 months), changes in intraocular pressure or adrenocorticoid hormones levels (follow up of one year).(305, 306) Despite the having

significantly higher chances of systemic side effect, oral steroids are FDA approved for use on CRSwNP patients whereas topical budesonide rinses remains off label.(4)

Oral doxycycline (non-culture directed) compared with oral steroid in a double blinded trial in CRSwNP have shown a comparable reduction in polyp size, inflammatory markers and most of the nasal symptom scores till a 12 week follow up.(155) Our study failed to demonstrate clinical improvement in terms of symptoms and endoscopy with doxycycline in the CRS cohort as well as in the CRSwNP group. This could be due to the comprehensive patient well-being assessment with SNOT 22 used in our study. Antibiotics continue to be used in the management of CRS with a low level of evidence and many of the available studies define cure rates as eradication of original pathogen with or without recolonization with non-pathogenic flora.(4, 301). Based on our findings and the fact that antibiotics have potential local and systemic side effects on local and remote microbiomes, we could not recommend antibiotics as routine in maximum medical therapy of CRS.

The microbiome changes with the different treatments had differential outcomes in our study. The pre-treatment microbial communities were predominated with *Corynebacterium* and *Staphylococcus* as has been observed in previous studies. The topical steroid showed a tendency towards increasing relative abundance of *Corynebacterium* and *Staphylococcus* at the end of 6 weeks, oral antibiotic showed similar increase in *Corynebacterium* while the oral steroid group demonstrated a trend of decreasing *Corynebacterium* and *Staphylococcus* at 6-weeks. A very recent study by Jain et al (307) observed similar effects of oral steroids and oral doxycycline on the *Corynebacterium* and an opposite effect on *Staphylococcus* average relative abundance in CRS microbiome. Unlike our trial, this study did not show any difference between the patient symptom scores across the treatments. The CRS patients in their cohort however, was not double blinded and received treatment only for 7 days which could account for the difference seen from our study. Despite this, similar to our results, their study also did not show any bacterial taxa that significantly corelated with

SNOT 22 or Lund Kennedy scores. A pilot study by Ramakrishnan et.al(308) aiming to look at the microbiome changes associated the use of intranasal corticosteroids (INCS) or topical antibiotic in non CRS volunteers observed an increase in the relative abundance of *Corynebacterium*, *Staphylococcus & Gonordia* and a decrease in *streptococci* and *Moraxella spp* with INCS. Although a similar shift in microbial diversity was observed in our trial, this did not persist after treatment cessation as observed in their study. Ramakrishnan et al's study however, included only 5 patients with 4 in INCS arm and 1 in topical mupirocin arm and was non blinded or randomized. Despite our findings on the bacterial changes with the different treatment modalities, the exact influence of the individual bacteria and its potential changes in abundance or diversity, and its association with clinical outcomes, could not be explained in our study. Furthermore, the inability of current sequencing technology to allow accurate speciation below the genus level is a significant limitation of microbiome research at present. With improvement in bioinformatic pipelines and analysis techniques, higher resolution and speciation may become possible and yield more useful information in this area.

A relatively new concept in healthy and diseased state is microbial stability or variability over time. This theory, based on Taylors law was applied to explore the temporal stability of the microbiotas under different conditions to understand how this is related to the health status of the subjects by Martí el al. (285)They found statistically increased microbial variability for grade III obese patients, subjects taking antibiotics, subject who had a salmonella infection, subject who had travelled abroad, and the patients with a diagnosis of inflammatory bowel disease. In our study we observed an increasing trend in the Faiths phylogenetic diversity at 3weeks for the topical (not sustained at 6 weeks) and oral steroid groups, and a trend towards a decrease in the Shannon's index in the antibiotic group at 6weeks. Most interestingly, the RV and DV index of microbial stability suggested the

development of a progressively unstable microbiome with oral antibiotic use at 3 and 6 weeks which was not seen in the steroid groups.

The major drawback of our study is the small sample size. The patients were already diagnosed with CRS before they visited our tertiary hospital, and a number of them were already on medical therapies when assessed for eligibility and hence could not be recruited.

Although topical and oral steroid groups showed similar clinical outcomes in our study, their bacteriological profiles with regards to *Corynebacterium and Staphylococcus* were different, suggesting that the immediate clinical benefits are mostly due to anti-inflammatory action of steroids. However, the lower RV index with steroids (oral and topical) demonstrated in our study, suggests that the microbial stability could also contribute to clinical outcomes. With the data we have available and the limitations of our study, we cannot ascertain with certainty whether the microbiome changes we have observed do have any clinical significance. In fact, we could perhaps argue that our results make us believe, with the limits of current technology, that symptomatic improvement was somehow "independent" of these changes that we looked at in the microbiome. e.g. steroids mainly work because of their anti-inflammatory action.

4.7 Conclusion

The report from our study show that steroids (topical and oral) provide symptomatic improvement, in a cohort of CRS patients, as long as they continue to be given. Although there were some associated microbiome changes with the various treatments, we could not ascertain the consistency of these and whether they do have a clinical significance at all. Budesonide nasal rinse reduced the symptoms to a similar extend in patients with and without polyps and could possibly be used for longer durations without the dangers of systemic side effects of oral steroids. Oral antibiotic on the other hand, failed to bring symptomatic benefits, and in patients with CRSwNP worsened the symptom scores. In the

light of our findings and based on the fact that antibiotics have potential local and systemic side effects on local and remote (e.g. gut) microbiomes, we could not recommend antibiotics as a routine in the maximum medical treatment of CRS patients especially those presenting with polyposis.

4.8 Acknowledgement

We acknowledge The Queen Elizabeth Clinical Trials for their help with the drug randomisation and dispensing in this trial. We thank the microbiology department of South Australian Pathology for their help with the isolation of bacteria from nasal swabs.

Thesis summary

The thesis begins with a detailed and systematic review into the literature on chronic rhinosinusitis, its etiopathogenesis, current recommended medical therapies and especially focuses on microbiome in CRS and the factors influencing it *(chapter 1)*. Chronic rhinosinusitis (CRS) is a common complex clinical syndrome of multifactorial aetiology where infection and inflammation coexist. It commonly presents with symptoms of nasal obstruction, discharge, facial pain and altered sensation of smell. CRS can further be classified into two phenotypes based on nasal endoscopy as CRS without nasal polyps (CRSsNP) and CRS with nasal polyps (CRSwNP). Among the various proposed factors in the etiopathogenesis of CRS, the most prominent ones are bacterial infection and host immune responses and these have been extensively researched. Literature reviews have shown an association between microbiome profiles in healthy and diseased states in a number of chronic inflammatory disorders. It is proposed that complex interactions between the resident microbes as well as between the microbe and host immune system are responsible for normal and healthy state of the various bodily functions. Along with the different factors associated with disease development, surgical or medical interventions could also potentially alter this harmony leading to local or remote microbial dysbiosis. Studies have shown this to be true in many conditions like inflammatory bowel disease, bronchial asthma, diabetes and obesity where newer treatment modalities are targeted towards restoring microbial harmony. According to the current understanding, a similar process is believed to underlie the etiopathogenesis and disease progression in CRS.

The medical management in CRS is mostly directed against the inflammation and infection with oral steroids, topical nasal steroids, oral antibiotics or a combination of these. Rhinosinusitis has been found to be the most common condition for which antibiotics are prescribed in general practice. Despite this, a large number of patients, respond inadequately to medical therapies and eventually require multiple surgical interventions and prolonged

medical management. The response to medical therapies varies among patients, depending on the treatment, its duration and initial phenotypic presentation in CRS. It is often observed in literature that, despite having proven bacterial colonization and biofilms in the mucosa, CRS patients respond well to topical steroid therapies. Corticosteroids have well established anti-inflammatory mechanisms by which they bring about the clinical benefits. The complex interactions between corticosteroids and microbiome in the sinuses of patients and the influence of these interactions on the disease outcome is, however, not clearly understood. An insight into this would ultimately, lead to a greater understanding of the way in which medications influence the microbial species that contribute to disease pathogenesis and this could lead to tailored therapeutic approaches and improved outcomes in patients with CRS.

The first part of this project investigated the effect of a commercial nasal steroid (pulmicort) and the individual components used in its preparation on methicillin sensitive and methicillin resistant *Staphylococcus aureus* (*S. aureus and MRSA respectively*) which are common pathogens in CRS, in an *in vitro* study *(chapter 2)*. We observed that pulmicort, which contains the corticosteroid budesonide and excipients, inhibited the biofilm formation of *S. aureus* and MRSA strains tested. Interestingly, the excipient EDTA, which is a component present in commercial topical nasal steroid preparations, also inhibited the biofilm formation at FDA approved concentrations and concentrations much below. The presence of the excipients also influenced the antimicrobial activity of antibiotics differentially and were also found to be non-toxic to human nasal epithelial cells. This study showed that commercial nasal steroid preparations, apart from the anti-inflammatory action of its active ingredient steroid, probably possessed properties which inhibited biofilm formation, which was mediated through the excipients present in them, to bring about the clinical benefits in patients. This understanding is important because it could potentially prevent the unwarranted combination of topical steroids with antibiotic in selected CRS patients.

The second part of the project (Chapter 3) focused on evaluating how corticosteroid on its own, influenced the various metabolic responses and growth dynamics that occur in *S. aureus* This invitro study was done using a water-soluble corticosteroid hydrocortisone hemisuccinate (HCHS). We found a differential growth response in the bacteria depending on the concentration of HCHS. There was an increase in the metabolic activity and aggregation of *S. aureus* planktonic forms as well as biofilms in the presence of HCHS in a dose dependent manner. At the highest HCHS concentrations (clinically relevant), the biofilms were found to be more susceptible to all the antibiotics tested, while the presence of intermediate HCHS concentrations rendered the biofilms tolerant to antibiotics even at the highest antibiotic concentration tested. Yet another finding in this study was the increased dissociation of HCHS into the active metabolite cortisol in the presence of *S. aureus*, which in turn resulted in a greater drop in the levels of the inflammatory marker IL-6 induced in primary human nasal epithelial cells. This suggests that *S. aureus* was probably capable of utilizing steroid as a growth substrate, thereby causing dissociation of HCHC, and eventually potentiating its anti-inflammatory action. The information on the usefulness of corticosteroids in infections in the literature is controversial. While it has been shown to be beneficial and at times life-saving when used in certain infections there are also studies demonstrating increase in serious and opportunistic infections especially with long term steroid use. Our study had demonstrated for the first time that corticosteroids can variably affect the bacterial metabolic and growth responses in human pathogenic bacteria, depending on the steroid concentration and the bacterial strains tested. This expands our understanding on the possible causes for the differential responses of steroid therapy in infections. This project provides new scope of research where steroids could be utilized in specific concentrations with antibiotics to contain a number of chronic inflammatory infections in humans.

For the final part of this project (Chapter 4), we conducted a double-blinded randomized placebo-controlled trial (RCT), aiming to evaluate and compare the clinical and microbiome outcomes of various medical treatments commonly employed in the "maximal medical therapy" of CRS patients. The three treatment arms included oral prednisolone, topical budesonide nasal rinses (pulmicort) and oral doxycycline, along with appropriate placebo, administered for a of duration of 3 weeks. Patients were evaluated at baseline, then followed up at 3 weeks post intervention, / and thereafter 3 weeks after completion of treatment. Patient's symptom scoring, endoscopic grading, microbiome swabs were done at all these visits. We observed an immediate and significant improvement in the subjective (SNOT-22 & ADSS) and objective (LKS) clinical scores of CRS patients treated with either oral or topical steroids for 3 weeks. The changes were not sustained at 6weeks.There was no improvement in the scores at any time point in the doxycycline group.

With regard to the microbiome outcomes in these patients, we observed that the group which received topical steroid showed a tendency towards increasing relative abundance of *Corynebacterium* and *Staphylococcus* at the end of 3 and 6 weeks. However, we could not conclusively associate this with the improving clinical outcome as, the antibiotic group also demonstrated a similar increase in *Corynebacterium* while the oral steroid group decreased relative abundance of *Corynebacterium* and *Staphylococcus*. A further subgroup analysis differentiating patients with and without nasal polyps, demonstrated an improvement of clinical symptoms with topical or oral steroids and worsening with oral antibiotics in CRSwNP patients. These outcomes also did not appear to be have any specific association with the microbiome outcomes. There were also no statistically significant differences in Shannon's and Faith's diversity indices between the three treatment groups, although the doxycycline group had slightly lower diversity indices. Rank Variability (RV) and Difference Variability (DV) which are indices of microbial stability suggested the

development of a progressively unstable microbiome with oral antibiotic use at 3 and 6 weeks, which was not seen in the steroid groups.

This research enhances our current knowledge on the association between the clinical outcomes of CRS patients with the most widely used medical therapies and the changes that occur in the microbiome communities in the sinuses. Steroid therapies could potentially alter the microbial pattern in CRS patients, without causing a reduction in the bacterial diversity and show clinical improvement during the time of administration. Antibiotics however, could decrease the bacterial diversity thus potentially leading to microbial destabilization with unclear clinical benefit. Literature provides ample evidence on the harmful effects of antibiotics on local and remote microbial communities in the human body and also on the deleterious results of multidrug resistant bacteria emerging due to unjustified antibiotic use. Oral steroids, on the other hand, while providing reasonable clinical benefits in CRS, is associated with a number of complications when used for a prolonged period. Topical steroids, however, have been shown to be safe and bring about excellent symptom relief in CRS patients. Based on the above facts, and the findings from this PhD research we encourage the use of topical steroid nasal rinses as first line medical therapy in CRS patients and discourage the routine use of antibiotics in these patients especially those presenting with polyps. The outcomes from the studies conducted in this project informs daily clinical practice, and would help to tailor, modify and potentially develop better topical nasal therapeutic agents in CRS which would efficiently control infection and inflammation while maintaining the microbial harmony.

Appendix

Government of South Australia
SA Health

CENTRAL ADELAIDE LOCAL HEALTH NETWORK
The Queen Elizabeth Hospital

Participant Information Sheet

Title: **The Effect of Topical and Oral Corticosteroids on the Sinonasal Microbiome.**

HREC number: HREC/15/TQEH/177
Ethics Application / Approval Number:

INVITATION TO PARTICIPATE

We invite you to participate in a research project which we believe is of potential importance. You are being invited because you require medical management of chronic rhinosinusitis symptoms. However, before you decide whether or not you want to participate, we need to be sure that you understand **why we are doing it**, and **what it would involve if you agreed.** We are therefore providing you with the following information. Please read it carefully and be sure to ask any questions you have. The Doctor conducting the research will discuss it with you and answer any questions that you may have. You are also free to discuss it with outsiders, (i.e. family, friends and / or your local doctor) You do not have to make an immediate decision. Your participation is purely voluntary. Should you agree to enter the trial, you may change your mind and withdraw at any stage.

PARTICIPATION IS VOLUNTARY

Participation in any research project is voluntary. If you do not wish to take part, you are not obliged to. If you decide to take part and later change your mind, you are free to withdraw from the project at any stage without providing a reason. Your decision to take part, not to take part or to withdraw will not affect your position on the surgical waiting list, your routine treatment, your relationship with those treating you, or your relationship with this institution.

BACKGROUND TO THE STUDY

What is the research about?
Chronic inflammation of the sinuses and nose (“chronic rhinosinusitis” or “chronic sinusitis”) causes headache, pain over the face, blockage of the nose and discharge from the nose. The cause of this condition is not fully understood. Finding out what factors are important in triggering the inflammation, and the body’s response to the inflammation is

important to enable the development of new treatments. Corticosteroids (or steroids) are the main medical treatment for chronic rhinosinusitis.

The aim of our study is to determine the effect of steroids (either as oral tablets, or as a nasal wash) on the bacteria living on the mucosal lining of the nose and sinuses (the sinonasal microbiome). The information gained from this research can then be used to guide new treatment approaches.

Why is the research being done?
Disorders such as inflammation of the nose and sinuses are not easy to live with. These disorders can be seen in different forms in the population and there are many factors contributing to the severity of the disease. One of the causes of severe sinus disease is microbial infections. Unfortunately, for many people with these disorders, current treatments do not work very well.

The Ear, Nose & Throat Department at The Queen Elizabeth Hospital is actively working to try to better understand the nature of the microbes (including bacteria and fungus) living in the sinuses and their effect on sinus health and disease. From our previous research work into microbes, our department has found new treatments to be used in chronic sinusitis, which are being tested and developed.

To study the microbiome in chronic rhinosinusitis, the doctors working in the Ear, Nose & Throat Department need to obtain nasal swab samples from the lining of the nose. These swab samples will be taken while you are in clinic. The samples will be then taken to the laboratory and studied to identify the microbes in it.

If you consent, your samples will be collected and stored in the ENT Department and used in studies approved by the Ethics Committee. The donated samples will only be used for research within the Ear, Nose & Throat Department at The Queen Elizabeth Hospital laboratories, and the department will not transfer its ownership to other institutions. Occasionally, the department may need to send some of your stored samples to specialized labs for certain tests (for example, to characterize the bacteria from your nose). If some of your samples are transferred to other labs for special tests, this will happen only in a way that will not reveal your identity, so your privacy will always be protected. No genetic information about your DNA will be studied.

Who is sponsoring it, and are they paying the researcher or his/her department to do the research?
There is no commercial sponsor. This research is part of the activities of the Ear, Nose & Throat (ENT) Department at The Queen Elizabeth Hospital and the University of Adelaide. The department is supported by research grants from the Government and private Research Foundations.

How and why have I been chosen as a possible participant in the research?
Your doctor is currently seeing you in the outpatient clinic because you are suffering from sinus symptoms. As part of this study, we require samples from individuals who have inflammation of the nose and sinuses.

How many other people have been asked to consider participating?
Every individual attending the outpatient clinic and is diagnosed with chronic sinusitis will be asked to participate. A total of 45 patients will be involved in the trial.

PROCEDURES AND TREATMENT

What treatment will I get if I do take part? Will this be different from the treatment I would get otherwise? If so, how and in what ways?
After signing the consent form, you will be randomly allocated into one of three groups to receive one of the routine treatments for chronic rhinosinusitis:

Doxylin® (also known as doxycycline) – an antibiotic that is effective against many types of microbes, which may be contributing to your current symptoms. It is a commonly-used medication in tablet form that has been shown to be effective against chronic rhinosinusitis symptoms, and is approved by the Therapeutic Goods Administration (TGA).

Pulmicort® (also known as budesonide) – the same type of medication found in steroid spray(s) you may have used previously such as Rhincort® or Nasonex®. Budesonide is a steroid solution that is TGA-approved to treat asthma symptoms in asthmatic patients.

Panafcortelone® (also known as prednisolone) – a member of the same family of medications as Pulmicort®/budesonide (i.e., steroids). Prednisolone is a steroid tablet that is TGA-approved to treat a variety of conditions including asthma.

You will be provided with the Consumer Information leaflets for each of these treatments.

The treatment will be provided to you in a way such that you will not know which treatment you are receiving and which treatment group you belong to. This way of research while “not knowing” is called blinding.

We will see you at the end of the 3-week treatment period, and then again 3 weeks later, which is the standard review time.

Your confidentiality is of utmost importance and will not be compromised. There will be no other changes to your care.

Will I have to come back to the clinic more often or remain in hospital for longer than would normally be the case?
No. You will be asked to attend the clinic at three weeks after your first appointment, then after six weeks, which is the normal standard of care. Nasal swab samples will be collected at each visit.

What will I be asked to do at each visit?
Initially, we will take CT images of your nasal cavities and sinuses and do an examination with a small telescopic camera (endoscope) in order to ascertain the severity of your disease. We will also ask you to complete a questionnaire about your nose or sinus condition, medications that you are taking, past history and allergies, as another measure of this. At each visit (initial, 3- & 6-week reviews), we will use an endoscope to take a swab

of the tissue surface inside your nose. We anticipate that the extra time that this will take during each clinic visit will be 10 minutes.

How long will my participation in the study last?
The study will finish after the 6-week visit, and medical management of your condition will continue as normal.

What procedures will I be asked to submit to including exposure to radiation and what will be the likely effects?
The extra procedures you will have are 3 swabs taken of the tissue surface inside your nose. This is a very gentle procedure and we do not expect any adverse effects.

If I decide not to take part what other treatments are available to me?
The main medical treatment received in each group is considered part of the routine treatment administered for chronic rhinosinusitis.

In the event that patients in group 2 and 3 develop pus (confirmed secondary bacterial infection), you will need a specific oral antibiotic therapy, and will therefore be commenced on an antibiotic and excluded from the study.

If the need arises for steroid treatment in group 1 participants (for example, increasing size of nasal polyps causing a worsening of nasal blockage, or worsening of sinusitis symptoms), then you will be commenced on steroids and you will be withdrawn from the study.

Will the decisions about my treatment be made by my usual doctor or by someone else?
Your usual treating doctor will still make all the treatment decisions with you.

Are there any factors, which would exclude me from participating, like pre-existing illness, the possibility of becoming pregnant or other drugs being taken?
Your surgeon will have screened you for any medications or conditions that would make the participation risky or inappropriate for you.

In general, these are the criteria that would exclude you from participating:

- the use of corticosteroids and/or antibiotics in the period of 6 weeks prior to study initiation
- the need for the use of a specific form of steroid (either oral or topical nasal corticosteroids) for specific indication (e.g. continued severe growth of nasal polyps) during the study period
- the need for the use of oral or topical nasal corticosteroids for other indications (for example, asthma or allergic rhinitis) during the study period
- the need for the use of antibiotics during the study period- either for presence of pus/secondary infection in the nose upon endoscopic examination, or for other indications
- history of previous sinus surgery
- pregnancy/breastfeeding
- age < 18 years
- conditions causing immunosuppression

MEDICINES AND DRUGS

What are the names and amounts of the drugs which I will be given?

Group 1. Oral antibiotics (200mg on day 1 then 100mg daily thereafter) and placebo for steroid, + 240ml isotonic saline delivered intranasally 2 times a day for 3 weeks

Group 2. Oral placebos for antibiotic and steroid, + 0.5mg topical steroid in 240ml isotonic saline delivered intranasally 2 times a day for 3 weeks

Group 3. Oral placebo for antibiotic, and steroid (25mg/day for 1 week then 12.5mg/day for 1 week then 12.5 mg every other day for 1 week) + 240ml isotonic saline delivered intranasally 2 times a day for 3 weeks

Note: The placebo is a capsule similar in appearance to the steroid and antibiotics but does not contain any active ingredients.

PATIENT MANAGEMENT

What would happen if I were to feel severe discomfort or pain?

In the unlikely event that you experience pain or severe discomfort please contact your treating doctor or the researchers to discuss the situation. If you feel more comfortable seeing your GP this is also an option, as is the emergency department of the QEH or any other hospital.

DISCOMFORTS, RISKS AND SIDE EFFECTS

Will there be any discomforts, such as additional needles, biopsies, or pain?

A nasal swab will be taken in clinic and this is a minimally invasive procedure with no side effects and is done safely in clinics or on the wards. All samples will be taken by your doctor under the guidance of the endoscope camera for increased safety. All samples will be obtained after a nasal local pain-killer spray is sprayed into your nose, to minimize any discomfort or pain.

Are there likely to be side effects from the research procedures, and if so what are they?

There are no overt risks perceived to be associated with this project to participants randomized to receive either antibiotic therapy (group 1) or steroid therapy (groups 2 and 3). These treatments are considered part of the routine medical therapy for chronic rhinosinusitis.

Specific side effects

Doxycycline: The most likely side effect of doxycycline (Doxylin®) use is increased sensitivity to UV light – this is why it is important that you avoid sun exposure during the study. Other potential side effects of doxycycline include nausea, vomiting, diarrhoea, stomach burning, tooth discolouration, and reduced growth of tooth enamel. Less common side effects include rashes, inflammation in the mouth, bone deformity, fungal overgrowth. Rare side effects include lifting off of the skin or nails and nail discolouration, oesophageal ulcers (due to partly swallowed tablets – thus you will be asked to refrain from lying down after taking medications), fever, abdominal cramping/pain, dehydration,

inflammation and fat accumulation in the liver, increased pressure in the head, allergic reactions such as anaphylaxis, & joint stiffness.

Budesonide: There are some side effects which may occur, these include: Nose/throat dryness or irritation, cough, sneezing, headache, nosebleeds, and unpleasant taste/smell, ear infections, respiratory infections, viral infections, gastroenteritis, vomiting, diarrhoea, abdominal pain, conjunctivitis. The product information sheet provided by AstraZeneca (the manufacturer of Pulmicort®) indicates an adverse reaction rate of about 3% or more. Tell your doctor immediately if any of the following rare, but serious side effects occur: vision problems, loss of taste or smell, pain/sores in your nose, or signs of too much corticosteroid (such as unusual acne, menstrual period changes, puffy face).

Prednisolone: The prednisolone medication (Panafcortelone®) used in this study is unlikely to cause any problems during short term use. Possible side effects of prednisolone include reduced adrenal function, increased susceptibility to infection, masking of signs of infection, sodium and water retention, swelling, high blood pressure, low blood potassium, high blood sugar, changes in blood lipid levels, osteoporosis, fractures, increased appetite, indigestion, delayed wound healing, skin atrophy, bruising, facial flushing, hair growth, growth retardation in children, muscle weakness and wasting, fat redistribution, weight gain, menstrual irregularity, amenorrhoea, psychiatric effects, posterior subcapsular cataracts. These effects are more common with long-term treatment courses, whereas for this study, you will take prednisolone for 3 weeks only. As for budesonide, tell your doctor immediately if you notice any of the rare but serious side effects noted above.

Who should I contact if I am worried about any effects that I experience?
You can contact the treating doctor, the researchers or your GP.

Would I be withdrawn from the study if my condition became worse or if any extra risks came to light during the course of it?
If there were any concerns you would be withdrawn from the study and your condition monitored closely.

In the event that patients in group 2 and 3 develop pus (confirmed secondary bacterial infection), you will need a specific oral antibiotic therapy, and will therefore be commenced on an antibiotic and excluded from the study.

If the need arises for steroid treatment in group 1 participants (for example, increasing size of nasal polyps causing a worsening of nasal blockage, or worsening of sinusitis symptoms), then you will be commenced on steroids and you will be excluded from the study.

Pregnancy and breastfeeding
There are no known harmful effects of these treatments to pregnant women or the unborn child; however we have chosen to exclude pregnant and breastfeeding women at this stage of the trial. If you are breastfeeding, may be pregnant, or are planning to get pregnant please kindly inform us.

Are there any activities I should refrain from during and in the period following the research and for how long, e.g., blood donations, taking other medication, sexual activity

(with or without attempting to achieve a pregnancy) exposure to sunlight, driving, taking part in other studies?
We encourage participants to practice protected sex during the duration of this study for the reason outlined above. Women of childbearing age should use effective contraception. Because the antibiotic used in this study is sometimes associated with skin sensitivity, you are advised to avoid exposing your skin to the sun while taking these medications. Also, we will ask that you refrain from lying down (e.g., going to bed) for a period of time after taking your medications to avoid problems from incomplete tablet digestion.

WHAT WILL HAPPEN TO THE INFORMATION COLLECTED?

How will my confidentiality be protected – will the information and results be de-identified?
You will be assigned a code which will be attached to all the study forms thus de-identifying your study results. A list of patient's names matching the allocated codes is kept in a locked research area of the ENT department and only accessible to the researchers. Your name will not be identified in any written results from the trial.

Will I be informed about the results of the study?
If you are interested, you are welcome to have access to the results of the study once they have been collected, analysed and published. Please contact Dr Psaltis on the number below (see WHAT IF I HAVE A QUESTION ABOUT THE STUDY?).

How long will my information be stored for?
Your information will be stored for 15 years before being destroyed.

What are my rights?
If you become injured during this study, and your injury is a direct result of the effects of study procedures, The Queen Elizabeth Hospital will provide reasonable medical treatment. Your participation in this study shall not affect any other right to compensation you may have under common law.

How can I obtain more information?
If you have any specific questions about your legal rights you may ask the researchers or the executive officer of the ethics committee – contact details are below.

Is there any payment for participation?
No payments or reimbursements (e.g., for parking costs) will be made to participants.

BENEFITS OF THE RESEARCH

Is there any chance that the proposed research will be of benefit to me personally, or to future patients with the same condition?
It is unlikely that there will be an immediate benefit to you for participating. However, as research provides a better understanding of sinus disease, the eventual development of new treatments in the future may benefit other people in the community suffering from sinus disease.

WHAT IF I HAVE A QUESTION ABOUT THE STUDY?

If you wish to speak to one of the research team about this study you can contact a member of the Research Team (lisamarycherian@gmail.com) or Dr Psaltis at TQEH on (08) 8222 7158, or after hours on (08) 8222 6000.

All research in Australia involving humans is reviewed by an independent group of people called a Human Research Ethics Committee (HREC). The ethical aspects of this research project have been approved by the Central Adelaide Local Health Network HREC.

This project will be carried out according to the National Statement on Ethical Conduct in Human Research (2007) incorporating all updates. This statement has been developed to protect the interests of people who agree to participate in human research studies.

Should you wish to speak to a person not directly involved in the study about it, you may contact The Executive Officer of the HREC, on (08) 8222 6841.

Participants or other concerned individuals who would like to make a complaint about this study should contact the HREC Secretariat

Ph:
Email: hrec@adelaide.edu.au
Post: Human Research Ethics Secretariat
c/- Research Branch
The University of Adelaide
SA 5005

INSTRUCTIONS – SINUS RINSE

Step 1: Wash your hands. Fill the clean bottle with the designated volume (240mL) of previously boiled water.

Step 2: Cut the SINUS RINSE mixture packet at the corner and pour its contents into the bottle. Tighten the cap and tube on the bottle securely. Place one finger over the tip of the cap and shake the bottle gently to dissolve the mixture.

Step 3. Select one plastic Budesonide 0.5mg/placebo plastic vial and squeeze the contents into the prepared SINUS RINSE bottle and shake again gently.

Step 4: Standing in front of a sink, bend forward to your comfort level and tilt your head down. Keeping your mouth open, without holding your breath, place the cap snugly against your nasal passage. SQUEEZE BOTTLE GENTLY until the solution starts draining from the OPPOSITE nasal passage. Some may drain from your mouth. For a proper rinse, keep squeezing the bottle GENTLY until 1/2 (120 mL) of the bottle is used. Do not swallow the solution.

Step 5: Blow your nose very gently, without pinching nose completely to avoid pressure on eardrums. If tolerable, sniff in gently any residual solution remaining in the nasal passage once or twice, because this may clean out the posterior nasopharyngeal area, which is the area at the back of your nasal passage. At times, some solution will reach the back of your throat, so please spit it out. To help drain any residual solution, blow your nose gently while tilting your head forward and to the opposite side of the nasal passage you just rinsed.

Step 6: Now repeat steps 4 and 5 for your other nasal passage.

Step 7: Clean the bottle and cap. Air dry the SINUS RINSE bottle, cap, and tube on a clean paper towel

Government of South Australia
SA Health

CENTRAL ADELAIDE LOCAL HEALTH NETWORK
The Queen Elizabeth Hospital

CONSENT FORM

Title: Effect of Steroids on the Sinonasal Microbiome **Protocol Number**:

I, the undersigned .....

hereby consent to my involvement in the research project explained above.

- I have read the Participant Information Sheet or someone has read it to me in a language that I understand.
- I understand the purposes, procedures and risks of the research described in the project.
- I have had an opportunity to ask questions and I am satisfied with the answers I have received.
- I freely agree to participate in this research project as described and understand that I am free to withdraw at any time during the project without affecting my future care.
- I understand that I will be given a signed copy of this document to keep.
- I give permission for my general practitioner to be informed of my participation in this study

I <u>do/do not</u> have an allergy to steroids (please circle)

I <u>do/do not</u> have an allergy to tetracycline antibiotics (please circle)

PATIENT SIGNATURE ..... DATE ...../...../.....

INVESTIGATOR DECLARATION I have given a verbal explanation of the research project, its procedures and risks and I believe that the participant has understood that explanation.

INVESTIGATOR SIGNATURE ..... DATE ...../...../.....

Screening Visit Assessments and Checklist

At Recruitment:

☐ Review of inclusion and exclusion criteria
☐ Confirm informed consent form signed
☐ Swab for bacterial culture
☐ Endoscopic evaluation and completion of Modified Lund-Kennedy questionnaire
☐ Microbiome swab
☐ Lund-Mackay CT scoring
☐ Book for CT scan if not previously done
☐ Review appointment booked

Pre-Treatment Checklist

☐ Recording demographic information and contact details
☐ Review of medical history
☐ Dispensed treatment agent with instruction sheet

Follow-up Checklist

☐ Phone call review after week 1: Check drug administration, dosing & compliance, symptom relief/persistence, adverse events

☐Phone call review at week 3: Check drug administration, dosing & compliance, symptom relief/persistence, adverse events, reminder for follow up.

☐Phone call review at week 6: Check drug administration, dosing & compliance, symptom relief/persistence, adverse events, reminder for follow up.

Post-Treatment at 3- and 6-weeks Checklist

☐☐ Has the participant returned all treatment agent (used and unused vials)

☐☐ Physical examination

☐☐ Review symptom relief/persistence and adverse event

☐☐ Completion of SNOT22

☐☐ Repeat microbiome and bacteriology nasal swabs to be taken

☐☐ Endoscopic evaluation and completion of Lund-Kennedy questionnaire by Investigator

Staff Initials: _____ Date (DD/MM/YYYY): ____/_____/_____

Inclusion & Exclusion Criteria Review

<table>
<tr><th>Inclusion Criteria</th><th colspan="2">Criteria Met</th><th>Date</th></tr>
<tr><td>Each participant must meet the following criteria to be enrolled:</td><td>Yes</td><td>No</td><td></td></tr>
<tr><td>1.Participant must have had at least two of the following symptoms of chronic rhinosinusitis that has been previously persistent for greater than 3 months.
<table>
<tr><td></td><td>Anterior /PND</td><td>Nasal block</td><td>Facial pressure</td><td>Smell</td></tr>
<tr><td>Day-1</td><td></td><td></td><td></td><td></td></tr>
<tr><td>Week-3</td><td></td><td></td><td></td><td></td></tr>
<tr><td>Week-6</td><td></td><td></td><td></td><td></td></tr>
</table></td><td></td><td></td><td></td></tr>
<tr><td>2. Participant must show evidence of chronic sinusitis by direct endoscopic examination.</td><td></td><td></td><td></td></tr>
<tr><td>3. Participant must show evidence of chronic sinusitis on CT scan</td><td></td><td></td><td></td></tr>
<tr><td>4. Participant must have the ability to take oral drugs and administer nasal lavage twice daily for the duration of the treatment period.</td><td></td><td></td><td></td></tr>
<tr><td>5. Participant must be between the ages of 18 and 70 years.</td><td></td><td></td><td></td></tr>
<tr><td>6. Participant must be able to give written informed consent.</td><td></td><td></td><td></td></tr>
<tr><td>7. Participant must have the ability and willingness to attend several visits to the study center
Participant must be willing to present to study center at Day 21 AND Day 42 of recruitment?</td><td></td><td></td><td></td></tr>
<tr><td>8. Participant must be willing for nasal microbiome swab and bacterial culture swab at the time of recruitmentand at Day 21 AND Day 42 of recruitment</td><td></td><td></td><td></td></tr>
<tr><td>If the answer to any of the inclusion criteria is No, the participant is not eligible for participation.</td><td></td><td></td><td></td></tr>
</table>

ADELAIDE SYMPTOM SEVERITY SCORE Symptoms∗ Nasal obstruction, Rhinorrhoea, Post-nasal drip, Headache or facial pain, Sense of smell, (all scored 1 to5), Quality of life† How do your symptoms affect your quality of life? Scale of 0 to7 ∗1 = no symptoms, 2 = mild, 3 = moderate, 4 = severe, 5 = extreme. † 0 = no effect; 7 = maximal effect

Exclusion Criteria	**Criteria Met**		**Date**
Each participant must NOT meet the following criteria to be enrolled:	Yes	No	
1. Participant who have used oral steroid and/or oral antibiotic and/or nasal steroid spray within the period of 6 weeks prior to study			
2. Female participant who is pregnant or breast-feeding.			
3. Participant who is immunocompromised.			
4. Participant who is an active smoker			
5. Participant who is taking any CYTP450 inhibitors			
6. Participant with liver disease			
7. Participant with a previous history of sinus surgery			
8. Participant has a clinically significant laboratory abnormality.			
9. Participant with known allergy to Doxycycline, Prednisolone or Budesonide			
10. Participant is unlikely to comply with the study protocol or, in the opinion of the investigator, would not be a suitable candidate for participation in the study.			
If the answer to any of the exclusion criteria is Yes, the participant is not eligible for participation.			

Staff Initials: _____ Date (DD/MM/YYYY): ____/_____/_____

I have reviewed all Inclusion/Exclusion criteria and it is in my medical judgment that the participant is eligible for participation in the study.

Assigned to Cohort #: _____

Signature Principal Investigator/Sub-investigator: _____

Date (DD/MM/YYYY): _____

Scoring and collection

<table>
<tr><td>CT -Lund – Mackey score</td><td></td><td></td></tr>
<tr><td>Was a CT scan taken?
And

Scored</td><td><table>
<tr><td>Sinus</td><td>Left</td><td>right</td></tr>
<tr><td>Maxillary</td><td></td><td></td></tr>
<tr><td>Ant/ethmoid</td><td></td><td></td></tr>
<tr><td>Post/ethmoid</td><td></td><td></td></tr>
<tr><td>Frontal</td><td></td><td></td></tr>
<tr><td>Sphenoid</td><td></td><td></td></tr>
<tr><td>OMC</td><td></td><td></td></tr>
<tr><td>Total points</td><td></td><td></td></tr>
</table></td><td>Date Performed:

_____/_____/_____
DD MM YYYY</td></tr>
<tr><td colspan="3">Bacterial Culture Swab</td></tr>
<tr><td>Was a bacterial nasal swab taken?
Day 0

3 weeks

6weeks</td><td>☐ Yes ☐ No

☐ Yes ☐ No

☐ Yes ☐ No</td><td>Date Performed and Time:

DD/MM/YYYY (HH:MM)</td></tr>
<tr><td>Microbiome swab</td><td></td><td></td></tr>
<tr><td>Was a microbiome swab taken?
Day 0

3 weeks

6weeks</td><td>☐ Yes ☐ No

☐ Yes ☐ No

☐ Yes ☐ No</td><td>Date Performed and Time:

DD/MM/YYYY (HH:MM)</td></tr>
</table>

Additional Notes:

Recruitment– Day 1

Demographics

Date of Visit: _____/_____/_____

DD MM YYYY

Date of Birth: _____/_____/_____

DD MM YYYY

Gender: ☐ Male ☐ Female

UR number:

Race:
- ☐ American Indian or Alaskan Native
- ☐ Asian
- ☐ Black or African American
- ☐ Native Hawaiian or other Pacific Islander
- ☐ White
- ☐ Other/Mixed Race (specify)_____

Phone number:

Email id:

Address:

Medical History (List current or past condition that occurred in the last 5 years)

Duration of chronic rhinosinusitis:

Number of episodes per year:

Frequency of medication use:

Antibiotics:

Oral steroid:

Nasal steroid spray/douching:

Antihistamines:

Others:

Date of last treatment taken:

ODIFIED LUND-KENNEDY SCORING SHEET

Time of assessment:

	Polyp		Discharge		Oedema	
	R	L	R	L	R	L
Day 1						
3 weeks						
6 weeks						

	L		R	
POLYPS				
Ordinal Scale (0-2)	No polyps	0	No polyps	0
	Polyps in middle meatus only	1	Polyps in middle meatus only	1
	Beyond middle meatus	2	Beyond middle meatus	2
DISCHARGE				
Ordinal Scale (0-2)	No discharge	0	No discharge	0
	Clear, thin discharge	1	Clear, thin discharge	1

	Thick, purulent discharge	2	Thick, purulent discharge	2
MUCOSAL OEDEMA				
Ordinal scale (0-2)	Absent	0	Absent	0
	Mild	1	Mild	1
	Severe	2	Severe	2

SNOT-22

Time of assessment: Day 1 Pre-treatment, 3-week, 6-week

Considering how severe the problem is when you experience it and how frequently it happens, please rate each item below on how 'bad' it is by *circling the number* that corresponds with how you feel using this scale →	No Problem	Very Mild Problem	Mild or slight Problem	Moderate Problem	Severe Problem	Problem as bad as it can be	5 Most Important Items
1. Need to blow nose	0	1	2	3	4	5	☐
2. Sneezing	0	1	2	3	4	5	☐
3. Runny nose	0	1	2	3	4	5	☐
4. Cough	0	1	2	3	4	5	☐
5. Nasal obstruction	0	1	2	3	4	5	☐
6. Loss of smell or taste	0	1	2	3	4	5	☐
7. Post-nasal discharge	0	1	2	3	4	5	☐
8. Thick nasal discharge	0	1	2	3	4	5	☐
9. Ear fullness	0	1	2	3	4	5	☐
10. Dizziness	0	1	2	3	4	5	☐
11. Ear pain	0	1	2	3	4	5	☐
12. Facial pain/pressure	0	1	2	3	4	5	☐
13. Difficulty falling asleep	0	1	2	3	4	5	☐
14. Wake up at night	0	1	2	3	4	5	☐
15. Lack of good night's sleep	0	1	2	3	4	5	☐
16. Wake up tired	0	1	2	3	4	5	☐
17. Fatigue	0	1	2	3	4	5	☐
18. Reduced productivity	0	1	2	3	4	5	☐
19. Reduced concentration	0	1	2	3	4	5	☐

20. Frustrated/restless/irritable	0	1	2	3	4	5	□
21. Sad	0	1	2	3	4	5	□
22. Embarrassed	0	1	2	3	4	5	□

5 Please mark the most important items affecting your health (maximum of 5 items) ____

↑

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