Nitric Oxide and Staphylococcus aureus Biofilms: Defining their Intricate Relationship in Chronic Rhinosinusitis

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The University of Adelaide
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Dedicated to my parents Ted and Teret
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Statements of Authorship

NOTE: Statements of authorship appear in the print copy of the thesis held in the University of Adelaide Library.
Thesis Abstract

This thesis aims to address the relationship of *Staphylococcus aureus* (*S. aureus*) biofilms to the endogenously produced gas nitric oxide (NO) in chronic rhinosinusitis (CRS). While *S. aureus* biofilms are associated with recalcitrance and high severity in CRS, the naturally elevated NO gas is significantly lower in sinuses of CRS patients. However, the relationship of these 3 important factors in CRS etiopathogenesis is poorly defined. To further clarify this host-microbe-environment (NO) relationship, this thesis first looks into the history of each factor, the roles they play in other disease processes, and the most recent clinical findings and applications in current literature. Building on this foundation, the projects emanating from this thesis hoped to fill in some gaps in knowledge of these 3 components, identifying that all are linked to disease manifestation, and that each can mutually contribute to CRS pathogenesis.

The first project was designed to establish a clearer description of the relationship between NO and *S. aureus* biofilms. Utilizing *S. aureus* strains from CRS patients, these were grown as biofilms and exposed to various NO concentrations mimicking NO levels measured in healthy sinuses vs. CRS patients. We demonstrated the dualistic effects of NO on biofilm growth: increased at lower NO concentrations mimicking diseased sinuses, and anti-biofilm effects at higher concentrations similar to measurements in healthy sinuses. These findings became a stepping stone for the potential design of NO as a therapeutic agent in *S. aureus*-associated CRS.

But first, further characterization of NO’s role on the host immune response was needed. The 2\textsuperscript{nd} and 3\textsuperscript{rd} projects aimed to define the host–NO relationship, focusing on the genes involved in NO regulation within the sinonasal mucosa. Because NO is
considered one of the reactive oxygen species (ROS), major players of the innate immune response, genes involved in ROS/innate immunity were investigated. CRS patients, with or without polyps, were sub-classified as either with or without *S. aureus* biofilms, allowing a separate analysis of the role *S. aureus* biofilms play in the alteration of gene expression. The results showed that *S. aureus* biofilm presence associates with a significant difference in the certain gene expressions which have specific roles in NO regulation. This indicates that the microorganism may alter or contribute to an impaired localized innate immune response in the sinuses, or alternatively favor growth in genetically susceptible individuals. Although the cause-effect timeline was not established, these results will serve as baseline for future gene and protein studies that will further increase our understanding of the NO-CRS pathophysiology.

Lastly, building on the therapeutic potential of NO as an anti-biofilm agent, we aimed to design a suitable NO-based topical agent against *S. aureus* biofilms. The 4th project tested a multitude of liposome-encapsulated NO formulations in-vitro with the best formulation tested for safety and efficacy in a sheep model of rhinosinusitis. These projects were designed with an aim for future clinical trials, to test a novel NO-based topical agent, which can be used as a safe and efficacious topical sinus rinse to benefit CRS patients.
Declarations

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution awarded to Camille Jardeleza. To the best of my knowledge and belief, this work contains no material previously published or written by another person, except where due reference has been made in the text.

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.

I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

I acknowledge that copyright of published works contained within this thesis resides with the copyright holders of those works.

Dr. Camille Jardeleza
Acknowledgements
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Publications arising from this Thesis

The effects of nitric oxide on *Staphylococcus aureus* biofilm growth and its implications in chronic rhinosinusitis
*International Forum of Allergy and Rhinology* 2011, 1(6): 438-44.

Gene expression differences in nitric oxide and reactive oxygen species regulation point to an altered innate immune response in chronic rhinosinusitis

Inflammasome gene expression alterations in *Staphylococcus aureus* biofilm-associated Chronic Rhinosinusitis

Liposome-encapsulated ISMN: A novel nitric oxide-based therapeutic agent against *Staphylococcus aureus* biofilms

Liposome-encapsulated nitric oxide donor: a novel topical treatment for *Staphylococcus aureus* biofilm-associated rhinosinusitis
Prepared for submission
Awards arising from this Thesis

**BEST ORAL PRESENTATION**: 1st Year Higher Degree Student
Research Day, The Queen Elizabeth Hospital / Basil Hetzel Institute, Adelaide SA, Oct 2010

**BASIC RESEARCH SCIENCE AWARD WINNER** for best scientific paper
American Rhinologic Society, San Francisco Ca, USA, Sept 2011

**RONALD GRISTWOOD MEDAL**, Best South Australian ENT Registrar
Presentation for Research, Adelaide, SA, Nov 2012

**NATIONAL HEALTH AND MEDICAL RESEARCH FOUNDATION**, Successful
Presentations arising from this Thesis

The role of nitric oxide in the pathophysiology of *Staphylococcus aureus* biofilm formation in chronic rhinosinusitis
Basil Hetzel Institute Post Graduate Seminar, Adelaide, July 2010

The role of nitric oxide in the pathophysiology of *Staphylococcus aureus* biofilm formation in chronic rhinosinusitis
The Queen Elizabeth Hospital / Basil Hetzel Institute Research day, Adelaide Oct 2010

The Effects of Nitric Oxide on *Staphylococcus aureus* Biofilm Growth and its Implications in Chronic Rhinosinusitis
ASOHNS, Annual Scientific Meeting, Melbourne, April 2011

The role of nitric oxide on *Staphylococcus aureus* biofilm formation in chronic rhinosinusitis
Basil Hetzel Institute Post Graduate Seminar, Adelaide, May 2011

The Effects of Nitric Oxide on *Staphylococcus aureus* Biofilm Growth and its Implications in Chronic Rhinosinusitis
American Rhinologic Society Annual Scientific Meeting, San Francisco Ca, USA September 2011

Gene expression differences in nitric oxide regulation point to an altered innate immune response in chronic rhinosinusitis
The Australian Society for Medical Research SA Scientific Conference, Adelaide, June 2012

The role of nitric oxide on *Staphylococcus aureus* biofilm growth in chronic rhinosinusitis
Endoscopic Sinus and Skull Base Surgery Course August 2-4 2012, St. Vincent’s Hospital, Sydney NSW, August 2012 - Invited speaker

The Efficacy of Liposome-encapsulated Nitric Oxide on *Staphylococcus aureus* biofilms in Chronic Rhinosinusitis
Basil Hetzel Institute Post Graduate Seminar, Adelaide, May 2012

Gene expression difference in nitric oxide and reactive oxygen species regulation point to an altered innate immune response in chronic rhinosinusitis
American Rhinologic Society Annual Scientific Meeting, Washington DC, USA September 2012
The Effects of Nitric Oxide on *Staphylococcus aureus* Biofilm Growth and its Implications in Chronic Rhinosinusitis
Ronald Gristwood Medal, Adelaide, October 2012

**Management of the recalcitrant sinus infection**
With NC Tan, 15th Advanced FESS course, Adelaide, November 2012 – Invited speaker

Gene expression difference in nitric oxide and reactive oxygen species regulation point to an altered innate immune response in chronic rhinosinusitis
ASOHNS, Annual Scientific Meeting, Perth, March 2013

**Liposome-encapsulated Nitric Oxide against *Staphylococcus aureus* biofilms in a rhinosinusitis sheep model**
Basil Hetzel Institute Post Graduate Seminar, Adelaide, June 2013

The Effects of Nitric Oxide on *Staphylococcus aureus* Biofilm Growth and its Implications in Chronic Rhinosinusitis
RP Jepson Medal, Royal Australasian College of Surgeons ASM, August 2013.
## Abbreviations Used in the Thesis

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td><strong>AdSA</strong></td>
<td>Adenosine synthase A</td>
</tr>
<tr>
<td><strong>AERD</strong></td>
<td>Aspirin Exacerbated Respiratory Disease</td>
</tr>
<tr>
<td><strong>AFS</strong></td>
<td>Allergic Fungal Sinusitis</td>
</tr>
<tr>
<td><strong>AIM2</strong></td>
<td>Absent in Melanoma 2</td>
</tr>
<tr>
<td><strong>ASC</strong></td>
<td>Apoptosis-associated Speck-like protein with a CARD</td>
</tr>
<tr>
<td><strong>bNOS</strong></td>
<td>Bacterial Nitric Oxide Synthase</td>
</tr>
<tr>
<td><strong>B– P-</strong></td>
<td>Biofilm negative Polyp negative</td>
</tr>
<tr>
<td><strong>B– P+</strong></td>
<td>Biofilm negative Polyp positive</td>
</tr>
<tr>
<td><strong>B+ P-</strong></td>
<td>Biofilm positive Polyp negative</td>
</tr>
<tr>
<td><strong>B+ P+</strong></td>
<td>Biofilm positive Polyp positive</td>
</tr>
<tr>
<td><strong>CARD</strong></td>
<td>Caspase Recruitment Domain</td>
</tr>
<tr>
<td><strong>CASP5</strong></td>
<td>Caspase-5</td>
</tr>
<tr>
<td><strong>CAT</strong></td>
<td>Catalase</td>
</tr>
<tr>
<td><strong>CCL2</strong></td>
<td>Chemokine Ligand 2</td>
</tr>
<tr>
<td><strong>CCL20</strong></td>
<td>Chemokine Ligand 20</td>
</tr>
<tr>
<td><strong>CF</strong></td>
<td>Cystic Fibrosis</td>
</tr>
<tr>
<td><strong>CFTR</strong></td>
<td>Cystic Fibrosis Transmembrane Conductance Regulator</td>
</tr>
<tr>
<td><strong>CLSM</strong></td>
<td>Confocal Scanning Laser Microscopy</td>
</tr>
<tr>
<td><strong>CRS</strong></td>
<td>Chronic Rhinosinusitis</td>
</tr>
<tr>
<td><strong>CRSsNP</strong></td>
<td>Chronic Rhinosinusitis without Nasal Polyps</td>
</tr>
<tr>
<td><strong>CRSwNP</strong></td>
<td>Chronic Rhinosinusitis with Nasal Polyps</td>
</tr>
<tr>
<td><strong>Cyclic GMP</strong></td>
<td>Cyclic Guanosine Monophosphate</td>
</tr>
<tr>
<td><strong>DAMP</strong></td>
<td>Danger-Associated Molecular Patterns</td>
</tr>
<tr>
<td><strong>DC</strong></td>
<td>Dendritic Cells</td>
</tr>
<tr>
<td><strong>DNA</strong></td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td><strong>DPPG</strong></td>
<td>Dipalmitoylglycero-phosphoglycerol</td>
</tr>
<tr>
<td><strong>dsDNA</strong></td>
<td>Double stranded DNA</td>
</tr>
<tr>
<td><strong>DUOX1</strong></td>
<td>Dual Oxidase 1</td>
</tr>
<tr>
<td><strong>EDRF</strong></td>
<td>Endothelium Derived Relaxing Factor</td>
</tr>
<tr>
<td><strong>EGFR</strong></td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td><strong>eNOS</strong></td>
<td>Endothelial Nitric Oxide Synthase</td>
</tr>
<tr>
<td><strong>EPS</strong></td>
<td>Extracellular Polymeric Substance</td>
</tr>
<tr>
<td><strong>ESS</strong></td>
<td>Endoscopic Sinus Surgery</td>
</tr>
<tr>
<td><strong>FISH</strong></td>
<td>Fluorescence in situ hybridization</td>
</tr>
<tr>
<td><strong>GTN</strong></td>
<td>Glyceril Trinitrate</td>
</tr>
<tr>
<td><strong>H. influenzae</strong></td>
<td>Haemophilus influenzae</td>
</tr>
<tr>
<td><strong>HPLC</strong></td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td><strong>HSP90AA1</strong>: Heat shock Protein 90 KDα</td>
<td><strong>NLR</strong>: NOD-like receptors</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td><strong>IE</strong>: Infective Endocarditis</td>
<td><strong>NLRP3</strong>: NOD-like receptor pyrin</td>
</tr>
<tr>
<td><strong>IgE</strong>: Immunoglobulin E</td>
<td>domain containing protein</td>
</tr>
<tr>
<td><strong>IgG</strong>: Immunoglobulin G</td>
<td><strong>NME5</strong>: Non-metastatic cell 5</td>
</tr>
<tr>
<td><strong>IL-1β</strong>: Interleukin 1-β</td>
<td><strong>NO2</strong>: Nitrite</td>
</tr>
<tr>
<td><strong>IL-18</strong>: Interleukin 18</td>
<td><strong>NO3</strong>: Nitrate</td>
</tr>
<tr>
<td><strong>IFN-γ</strong>: Interferon-γ</td>
<td><strong>NOD</strong>: Nucleotide Oligomerization</td>
</tr>
<tr>
<td><strong>iNOS</strong>: Inducible Nitric Oxide Synthase</td>
<td><strong>Domain</strong></td>
</tr>
<tr>
<td><strong>ISMN</strong>: Isosorbide Mononitrate</td>
<td><strong>nNOS</strong>: neuronal Nitric Oxide Synthase</td>
</tr>
<tr>
<td><strong>LPS</strong>: Lipopolysaccharide</td>
<td><strong>NO</strong>: Nitric Oxide</td>
</tr>
<tr>
<td><strong>LTA</strong>: Lipoteichoic acid</td>
<td><strong>NOS</strong>: Nitric Oxide Synthase</td>
</tr>
<tr>
<td><strong>LFNO</strong>: Liposomal-formulated nitric oxide donor</td>
<td><strong>OME</strong>: Otitis Media with Effusion</td>
</tr>
<tr>
<td><strong>MBEC</strong>: Minimum Biofilm Eradication Concentration</td>
<td><strong>ONOO-</strong>: Peroxynitrite</td>
</tr>
<tr>
<td><strong>M. catarrhalis</strong>: Moraxella catarrhalis</td>
<td><strong>OXR1</strong>: Oxidoreductase 1</td>
</tr>
<tr>
<td><strong>MLV</strong>: Multilamellar vesicles</td>
<td><strong>P. aeruginosa</strong>: Pseudomonas aeruginosa</td>
</tr>
<tr>
<td><strong>MRSA</strong>: Methicillin Resistant</td>
<td><strong>PALS</strong>: Phase analysis light scattering</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td><strong>PAMP</strong>: Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td><strong>N₂O</strong>: Nitrous Oxide</td>
<td><strong>PCD</strong>: Primary Ciliary Dyskinesia</td>
</tr>
<tr>
<td><strong>NFκβ</strong>: Nuclear factor kappa beta</td>
<td><strong>PCR</strong>: Polymerase Chain Reaction</td>
</tr>
<tr>
<td><strong>NADPH</strong>: Nicotinamide adenine dinucleotide phosphate</td>
<td><strong>PRDX2</strong>: Peroxiredoxin 2</td>
</tr>
<tr>
<td><strong>NCF2</strong>: Neutrophil cytosolic factor 2</td>
<td><strong>PRDX5</strong>: Peroxiredoxin 5</td>
</tr>
<tr>
<td><strong>NCF2</strong>: Neutrophil cytosolic factor 2</td>
<td><strong>PRDX6</strong>: Peroxiredoxin 6</td>
</tr>
<tr>
<td><strong>PRNP</strong></td>
<td>Prion protein</td>
</tr>
<tr>
<td>----------</td>
<td>---------------</td>
</tr>
<tr>
<td><strong>PRR</strong></td>
<td>Pattern-recognition receptors</td>
</tr>
<tr>
<td><strong>PSTPIP1</strong></td>
<td>proline-serine- threonine phosphatase interacting protein 1</td>
</tr>
<tr>
<td><strong>PYCARD</strong></td>
<td>PYD and CARD Domain containing gene</td>
</tr>
<tr>
<td><strong>qRT-PCR</strong></td>
<td>Quantitative Real Time PCR</td>
</tr>
<tr>
<td><strong>RANKL</strong></td>
<td>Receptor activator of nuclear factor kappa-β ligand</td>
</tr>
<tr>
<td><strong>RAST</strong></td>
<td>Radioallergosorbent test</td>
</tr>
<tr>
<td><strong>RLH</strong></td>
<td>RNA-sensing RIG-like helicases</td>
</tr>
<tr>
<td><strong>RNS</strong></td>
<td>Reactive Nitrogen Species</td>
</tr>
<tr>
<td><strong>ROS</strong></td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td><strong>S. aureus</strong></td>
<td><em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td><strong>SCV</strong></td>
<td>Small Colony Variants</td>
</tr>
<tr>
<td><strong>S. pneumoniae</strong></td>
<td><em>Streptococcus pneumoniae</em></td>
</tr>
<tr>
<td><strong>TH1</strong></td>
<td>T-Helper 1</td>
</tr>
<tr>
<td><strong>TH2</strong></td>
<td>T-Helper 2</td>
</tr>
<tr>
<td><strong>TLR</strong></td>
<td>Toll-Like Receptor</td>
</tr>
<tr>
<td><strong>TNFSF11</strong></td>
<td>Tumor necrosis factor member 11</td>
</tr>
<tr>
<td><strong>ULV</strong></td>
<td>Unilamellar vesicles</td>
</tr>
<tr>
<td><strong>XIAP</strong></td>
<td>X-linked inhibitor of apoptosis protein</td>
</tr>
<tr>
<td><strong>ZBP1</strong></td>
<td>Z-DNA-binding protein</td>
</tr>
</tbody>
</table>
Tables

Table 1.1 Sub-classification of CRS based on allergy and asthma status.

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Figure 6.3: Average biofilm biomass of control vs. treatment sinus within each sheep group showing only the LFNO group with statistical significance.
Chapter 1: Systematic Review of Literature

1.1 Chronic Rhinosinusitis

A. Definition and Epidemiology

The definition of CRS is best summarized by the individual description of its components. Rhinitis has been defined as inflammation of the nasal cavity while sinusitis refers to the inflammation of the sinuses. Because it has been well documented that the two co-exist, the correct terminology is now known as rhinosinusitis.\textsuperscript{1,2} For a clinical diagnosis, the presence of inflammation of the nose and paranasal sinuses must be accompanied by two or more clinical symptoms as described by the European taskforce of 2012,\textsuperscript{3} one of which should include:

a. nasal blockage, obstruction or congestion or

b. nasal discharge: either anterior rhinorrhea or postnasal drip

+/- the presence of facial pain/pressure and +/- reduction or loss of smell.

These symptoms should be accompanied by clinical or radiologic findings, such as:

a. endoscopic signs of nasal polyps, mucopurulent discharge or oedema/mucosal obstruction, usually in the middle meatus and/or

b. CT scan changes which are mucosal changes usually in the osteomeatal complex or sinuses.

The duration of symptoms separates the definition to acute rhinosinusitis which is <12 weeks with complete resolution, and chronic which is >12 weeks without complete symptom resolution. In essence, CRS is the inflammation of the nose and sinuses characterized by symptoms such as nasal airway obstruction, anterior nasal discharge, post nasal drip, facial pain or pressure and anosmia or hyposmia, accompanied by
radiologic changes and/or endoscopic signs as mentioned above, for a duration of more than 12 weeks.

The incidence of CRS varies across literature from as low as 2% to as high as 16%. In Australia, it affects as many as 1.8 million or 9.2% of the population and comparable to asthma, is one of the most frequently reported health conditions. Despite the localization of signs, the severity of symptoms can greatly affect quality of life and functional capacity. In a study by Bhattacharya et al., it was found that CRS causes an average of 4.8 missed workdays due to sinonasal symptoms, 2.7 episodes of acute exacerbations, and 3.9 physician visits per patient every year.

This high incidence of CRS and its detrimental effects on a patient’s well being correspondingly has a large global socio-economic impact. In the US, it has been observed that CRS results in an estimated 18-22 million physician visits, with direct treatment costs of $8.6 billion per annum. These computed costs not only include missed workdays and physician visits, but also the consumption of a variety of prescription drugs such as intranasal corticosteroids, antihistamines and antibiotics. The approximate yearly economic cost is between $1500-$2500 per patient. Furthermore, when surgery is required, the cost average increases to over $7500 per patient, from the time of surgery to 45 days post-procedure. From these figures, the potential of CRS to consume a vast amount of economic resources cannot be ignored.
B. Classification

The simplest classification of CRS is based on the presence (CRSwNP) or absence (CRSsNP) of nasal polyps. A nasal polyp is an oedematous mucosal membrane which forms a pedunculated mass, with a base or a stalk usually originating from the narrow regions of the nasal cavity, such as the osteomeatal complex or middle meatus. The exact etiology of nasal polyps is still unknown, although the narrow slits of mucous membranes where they often originate is postulated to play a role in its formation. It is said that mechanical stimulation of adjacent epithelial cells on the mucosal surfaces causes a release of pro-inflammatory cytokines inciting or contributing to a localized inflammatory reaction and polyp formation. Most likely nasal polyposis is multifactorial in cause, but despite its unclear pathologic process, many studies have shown CRSwNP to be clinically, histologically and genetically distinct from its non-polyp counterpart. Histologic characteristics of nasal polyps show greater stromal oedema, plasma cells, and eosinophils with a higher degree of inflammation. This reflects greater symptomatology and the need for more aggressive treatment in this patient group. CRSsNP patients on the other hand, tend to have a generally more neutrophilic predominance. Eosinophilia in itself has been strongly associated with polypoid mucosal changes. Certainly, patients with nasal polyps clinically manifest with more severe symptoms, and are more frequently associated with other diseases that can contribute to disease severity such as asthma, cystic fibrosis, aspirin sensitivity and primary ciliary dyskinesia.

With the emergence of new molecular and histologic techniques, discoveries of new disease entities are challenging the traditional classification of CRS with and without nasal polyps. Several studies have emerged describing other potential sub-
Allergic fungal sinusitis (AFS) is said to have its own distinct characteristics, and sometimes thought of to be a separate classification. The presence or absence of eosinophilic mucus can further sub-classify CRS to another 3 distinct histologic entities. For example, in CRSwNP, three distinct histologic features were further discovered based on cell predominance:

a. Eosinophilic
b. Neutrophilic
c. Non-eosinophilic, non-neutrophilic

The eosinophilic type is characterized by a T-helper 2 (TH2) response, while the non-eosinophilic type has a T-helper 1 (TH1) polarization. This becomes clinically important as the eosinophilic phenotype is associated with atopy, more extensive disease and poorer prognosis. Further characterization of different CRS subtypes is important, as they will likely dictate future sub-type specific treatment strategies.

Asthma, allergy and aspirin sensitivity have also been found to play a greater role in the disease process, and thus certain sub-classifications take their presence into consideration. Samter’s triad of aspirin sensitivity, asthma and nasal polyposis is a particular disease entity associated with a more recalcitrant form of CRS. Han et al. proposed a new sub-grouping of CRS that stratifies the disease into 7 separate categories:

a. Allergic fungal sinusitis (AFS)
b. Aspirin triad or Aspirin Exacerbated Respiratory Disease (AERD)
c. Asthmatic sinusitis with allergy (Asthma + Allergy +)
d. Asthmatic sinusitis without allergy (Asthma + Allergy -)
e. Non-asthmatic sinusitis with allergy (Asthma – Allergy+)
f. Non-asthmatic sinusitis without allergy (Asthma - Allergy -)
g. Cystic fibrosis (CF) associated sinusitis

CRS patients with asthma, allergy and aspirin sensitivity (AERD) or Samter’s triad, have been shown to have the worse symptoms and more aggressive nasal polyposis, and are associated with higher degrees of eosinophilia.\textsuperscript{21,22} The pattern often starts with rhinitis symptoms at the 3\textsuperscript{rd} decade, followed by months of nasal congestion, rhinorrhea and eventually a diagnosis of nasal polyposis, followed shortly by asthmatic attacks and aspirin sensitivity.\textsuperscript{23,24} Asthmatic sinusitis with allergy is also associated with a TH2 response, polyposis and allergy, but the asthma component commences during childhood years. Non-asthmatic non-allergic sinusitis is deemed the mildest sub-type, and likely caused by impairments in sinus drainage due to anatomic abnormalities. This type is associated with localized hypoxia in the sinuses and bacterial infection, hence a TH1 response, with non-asthmatic with allergy CRS halfway between the 2 in terms of severity.\textsuperscript{20} Asthmatic CRS without allergy is similar although to a lesser severity than AERD, with asthma developing during the adult years. AFS, although similar to asthma and allergy associated CRS, has a more localized TH2 allergic reaction to fungus, often confined to the sinuses. CF associated CRS is associated with higher polymorphonuclear cell count, and thick mucopus production rather than polyposis, marking an infectious inflammatory process rather than an allergic type of reaction.\textsuperscript{20} Table 1.1 summarizes these findings.
### Table 1.1: Sub-classification of CRS based on allergy and asthma status.

<table>
<thead>
<tr>
<th>Sub-classification</th>
<th>Severity</th>
<th>T Helper cell polarization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asthma (-) Allergy (-)</td>
<td>Mild</td>
<td>TH1</td>
</tr>
<tr>
<td>Asthma (-) Allergy (+)</td>
<td>Moderate, between AA (-) and AA (+)</td>
<td>TH1 → TH2</td>
</tr>
<tr>
<td>Asthma (+) Allergy (+)</td>
<td>Severe, polyposis</td>
<td>TH2</td>
</tr>
<tr>
<td>Asthma (+) Allergy (-)</td>
<td>Moderate, precursor to AERD</td>
<td>TH2</td>
</tr>
<tr>
<td>AERD</td>
<td>Severe, polyposis</td>
<td>TH2 but lower IgE levels than AA (+)</td>
</tr>
<tr>
<td>AFS</td>
<td>Severe</td>
<td>Localized TH2 response to fungus</td>
</tr>
<tr>
<td>CF</td>
<td>Severe, polyposis, mucopus</td>
<td>Infectious, mast cell predominance but not purely TH1</td>
</tr>
</tbody>
</table>

The importance of this sub-classification, as detailed by Han et al.,\textsuperscript{20} is that it may dictate which topical and systemic therapies will suit best depending on the sub-classification. Bacterial-induced CRS will likely benefit most from oral and topical antibiotics with steroids, while the TH2-driven allergic and asthma-associated CRS will have a better response with oral steroids. Whichever sub-classification is used, it is obvious that the traditional with- or without- polyp classification is no longer sufficient to predict disease progression and success with current treatment strategies. This only further highlights the evolution of the disease definition with emerging discoveries, which will be accompanied by future changes in therapy.
C. Aetiopathogenesis

One of the underlying reasons for the absence of a cure for CRS is due to its complex multifactorial aetiology. The interplay of two main factors, the host and the environment, leads to the manifestation of CRS at varying degrees of severity. Each of these factors will be discussed in detail.

C.1 The Host

In the host, both general and localized immune responses to environmental stimuli have been implicated in the disease process. The role of the general immune response is evident in the association of CRS with immune deficiency and genetic disorders. Immunoglobulin deficiency, the most common of which is an IgG deficiency, has been commonly found in CRS patients, and concluded by some to be the first sign of an immunologic predisposition to persistent sinus infection. The frequency of CRS in genetic diseases such as cystic fibrosis (CF) and primary ciliary dyskinesia (PCD) also implicates the role of a possible genetic component in its etiology. For example, the cause of CF, a Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene mutation, has been found to be higher in patients with CRS compared to non-diseased patients. PCD, an autosomal recessive disorder, is characterized by ciliary dysfunction and impaired mucociliary clearance, with sinusitis being one of its clinical manifestations. Not isolated to a single genetic mutation, PCD is a genetically heterogeneous disorder, implicating 8 genes involved in structural or functional defects of the cilia. Because of the several anatomical and systemic factors involved in the maintenance of normal sinonasal function, it is more likely multiple genes, rather than a solitary gene, will be implicated in disease aetiopathogenesis. This is already evident in
a multitude of genes presented in current literature, most of which have roles in the host immune response.\textsuperscript{28,29}

The localized immune response in the sinonasal tract is greatly regulated by the innate immune system, a first-line of defense against infection. Although a non-specific response, this localized protective mechanism is crucial due to the regular exposure of the sinonasal mucosa to pathogens and allergens from inhaled air. Many steps are involved in the mounting of a successful innate immune response.\textsuperscript{30} Larger inhaled particles, which are trapped in the superficial mucus, are swept away via a coordinated mucociliary system, physically clearing pathogens from the sinonasal tract. For pathogens that have evaded this primary barrier, receptors in the epithelium, called pattern-recognition receptors (PRRs) can recognize certain microbial molecular components called pathogen-associated molecular patterns (PAMPs). The binding of PAMPs to epithelial PRRs can elicit the production of a myriad of antimicrobial enzymes, peptides and small molecules such as NO by epithelial cells in an attempt to neutralize infection.\textsuperscript{31} These secreted molecules may not only function as direct antimicrobials but can also serve as chemoattractants and activators of other effector cells.\textsuperscript{32} Specific PRRs such as toll-like receptors (TLRs), not only play key roles in the initiation and orchestration of the innate immune response, but also activate parts of the more specific adaptive immunity.\textsuperscript{30} TLRs can recognize and bind specific molecules of gram negative and positive bacteria such as lipopolysaccharides (LPS), lipoteichoic acid (LTA) and peptidoglycans and mount an appropriate inflammatory response for bacterial eradication.
Cell surface receptors are also found in circulating inflammatory cells such as macrophages, dendritic cells (DC) and polymorphonuclear cells, all of which are involved in innate immunity. The recognition of non-self elements by these receptors leads to either of the following: engulfment of the antigen and intracellular destruction, engulfment and antigen presentation to another cell to incite either direct antigen destruction or proliferation of other effector cells, and the release of inflammatory mediators and cytokines to attract other effector cells and potentiate further inflammation.30

The adaptive immune response has both general and localized immune functions in CRS and is closely linked to innate immunity. In contrast to innate immunity, adaptive immunity has the ability to mount a specific response to antigens based on memory from a previous exposure to the same microbe. T, B and dendritic cells are the classic cells involved, and although of a slower response, possess specific cell receptors, which recognize particular antigens and propagate a more enhanced effect in pathogen elimination. When the innate immune response fails to clear non-self and other microbial particles, adaptive immunity can potentially be lifesaving as it dominates the body’s attempt to eliminate antigens.

A localized adaptive response is said to occur with the epithelium being a key player in the initiation and mediation of both innate and adaptive immunity. Epithelial cells can trigger and modify the differentiation of T, B and DCs, via the release of sub-type specific chemokines and expression of cell surface molecules which orchestrate the action of nearby T and B cells including proliferation and differentiation.31 Activated epithelial cells can induce dendritic cell migration via the chemokine CCL20 with
secondary antimicrobial properties, the recruitment of which is essential for an effective adaptive immune response against viral and bacterial infection, as well as airway inflammation.\(^\text{33}\) Airway epithelial cells can also chemoattract cells involved in both TH1 and TH2 response, which, although possible to occur sequentially, function as distinct pathways of adaptive immunity.

Apart from CRS, the role of the epithelium in immunity is also seen in closely associated airway diseases such as asthma and rhinitis. This implicates a similarity in the pathophysiology of these diseases, and highlights the importance of the sinonasal epithelium in the analysis of gene regulation and protein expression involved in both innate and adaptive immunity.

\textit{C.2 The Environment}

Postulations have emerged as to the inherent susceptibility of certain individuals in developing CRS. An exaggerated immune response is propagated by certain triggering factors, which can potentiate an uncontrolled inflammatory process. Many environmental factors have been associated with an increased incidence of CRS, including environmental pollution,\(^\text{34}\) allergens and irritants which cause mucosal oedema and narrowing of sinus drainage pathways leading to secondary bacterial infection.\(^\text{35}\) This hyperactive mucosal reaction is more associated with the allergic sub-type of CRS and a TH2 response, hence unlikely to be applicable to the entire spectrum of the disease. The sequence of events of disease development, a concrete description of underlying host susceptibility, and the role of external factors in CRS need to be more clearly defined.
C.3 The Microorganism

The role of microbes in CRS pathogenesis has been given its place as evidenced by the common use of antibiotics in disease treatment. Bacteria however, are not the only pathogens implicated in the disease process. Clinical history of a significant proportion of patients elicited CRS as being preceded by a common cold or flu-like symptoms, pointing to a virus as the primary trigger, although this is not supported by documented literature. Furthermore, attempts to identify any specific virus as the primary cause have also been so far unsuccessful. PCR studies looking at viral genes found in polyp mucosa of CRS patients have isolated a variety of viruses that cause flu-like symptoms in these patients, but the results are inconclusive. Further studies with greater patient numbers encompassing all the subtypes of CRS are needed to better define the role of viruses in the etiology of CRS.

Fungus has been characterized to play a significant role in CRS pathogenesis. Its presence puts patients under a different sub-classification of allergic fungal sinusitis (AFS). This is attributed to a difference in an etiologic mechanism dominated by an IgE mediated response, eosinophilic infiltration, the presence of eosinophilic mucus and Charcot Leyden crystals in the absence of invasive fungal disease or immunodeficiency. The presence of fungal elements or a positive culture may or may not be present, but the importance of this distinction lies in its allergic component, pointing to a distinct clinico-pathologic process and potentially different treatment paradigm.

Several recent research attempts have been made to delineate the differences between AFS and CRS associated with the classical bacterial infection. Bacteria have been
thought of to play a significant role in the initiation and propagation of the disease.\textsuperscript{1}
This has been supported by early theories that normal sinuses are sterile while CRS sinuses are heavily colonized with bacteria. Recently this traditional thinking has changed, with findings of healthy sinuses also colonized by bacteria,\textsuperscript{39} and that it is the microbial type and relative abundance that differs between healthy and diseased subjects. The polymicrobial nature of the infected sinuses and the change in microbial flora as it transitions from an acute to chronic infection, further complicates our understanding of the role of bacteria in the pathogenesis of the disease.\textsuperscript{40} Patient demographics, location and differences in specimen collection, storage and culture techniques may also influence bacterial culture findings, making inter-institutional comparison of data difficult. Furthermore, with the advent of molecular techniques in bacterial identification,\textsuperscript{41} even the minutest of microbial presence can now be identified. Although increasing the possibility of identifying new pathogens, this expansion of microbial knowledge can make interpretation even more difficult. Nonetheless the opportunity to improve our understanding of the bacterial role in CRS and thus modify treatment modalities is greatly enhanced with increasing sensitivity of new molecular techniques.

The treatment of the traditional acute bacterial infection has shifted to the control of a chronic infectious process.\textsuperscript{42} This has been attributed to the discovery of bacterial biofilms in the sinuses of CRS patients. With this discovery, therapeutic strategies have changed, owing to the more resistant nature of biofilms to complete eradication.
1.2 Biofilms

A. Bacterial biofilms: History, Discovery and Definition

How were bacterial biofilms discovered? In the past, bacteria were thought to just exist and flourish in free-floating forms. It was Anton Van Leeuwenhoek, the father of microbiology, who first noticed in the 1600s the presence of aggregates of microorganisms attached to his tooth scrapings. He examined his own dental plaque under a microscope, and thus was attributed as the first to discover bacterial biofilms.\(^{43}\)

With the advent of stronger microscopic techniques such as scanning electron microscopy, bacterial cells were more clearly seen to cluster together, appearing to be encased in a slime-like matrix on surfaces. Studies in the 1940s certainly showed that bacterial growth was increased in the presence of a surface\(^ {44}\) and grew more on surfaces rather than surrounding medium,\(^ {45}\) but it was only in the late 1970s that Costerton described in detail and crystalized the concept of a “biofilm”.\(^ {46}\) Over a short span of time, clearer descriptions of biofilm formation, maturation, and dispersal occurred, revolutionizing the concept of microbial existence and altering the approaches of bacterial eradication.

A biofilm is thus defined as clusters of microbial cells in a structured community surrounded by a self-producing polymeric matrix attaching to an inert or living surface.\(^ {47}\) Biofilm communities may be mono or polymicrobial in nature within the matrix. This extracellular polymeric substance (EPS) matrix was initially thought to comprise only of polysaccharides, but proteins, DNA, lipids and other biopolymers were also found, all of which serve important functions for the enhanced survivability and growth of the bacterial community.\(^ {48}\) Horizontal gene transfer for example, can occur between biofilm cells thru DNA transfer through the matrix, sharing genes of
resistance against antimicrobial agents. The matrix also serves as nutrient source for bacterial cells, facilitates adhesion and contains enzymatic proteins that form or degrade matrix and allow dispersal and spread of bacteria to other sites.

There is still much knowledge to be obtained in the understanding of biofilms, but an abundance of information has been gained in the last decade. Its lifecycle and mechanism of spread is distinctly different to its planktonic counterpart. Attachment of free-floating microbial cells in a nutrient-rich environment on a surface is the initial step in biofilm formation. Generating only weak physical forces, bacterial-surface attachment can be transient, and only bacterial cells that do not immediately separate adhere firmly to the surface. They subsequently undergo a phenotypic change and microcolony aggregation. Biofilm growth occurs with further cell attraction to the remaining surface or on top of the adhering cells through quorum sensing. The surrounding EPS matrix forms and undergoes further phenotypic changes as the biofilm matures. Complex channels within the matrix facilitate growth, the sharing of genetic information, and nutrient and waste management, leading to biofilm maturation. Detachment of bacterial cells from the surface of the mature biofilm is the last step, these new planktonic cells dispersing to find a new surface to attach to. New biofilms then form in new locations, aiding in the spread of microbial infection. In nutrient
deficient states however, bacterial cells in the biofilm can become quiescent and not proceed directly to the detachment stage. It is during these periods where negative culture rates are often observed, leading many to believe that infection has been successfully treated. It is due to these discrepancies that there remains heavy interest in understanding each of these stages of the biofilm lifecycle as attempts at creating novel strategies for biofilm treatment are ongoing.

Figure 1.2: The biofilm lifecycle. Attachment of free-floating planktonic cells to a surface first occurs, followed by aggregation, further EPS matrix formation and biofilm maturation. Detachment of bacterial cells from the top part of the mature biofilm to become planktonic cells completes the cycle.

B. Implications in Diseased States

Biofilms have the potential to grow on any appropriate surface in both the natural environment and human body as long as suitable conditions permit. A phenotypic difference to its planktonic free-floating form has been well established, signifying a more complex and thus more difficult process of eradication. They have host evasive mechanisms, preventing effective phagocytosis. Nutrient gradients form certain pockets of anoxic and acidic regions in the biofilm complex, specifically in deeper recesses. In
these areas of poor nutrient penetration, bacterial cells are in a relatively dormant state and respond poorly to antimicrobial therapy. While destruction of the more superficial active cells occurs, these dormant cells survive, serving as a nidus of infection as they phenotypically change to become active when environmental conditions improve. The EPS matrix also plays a role in slowing antibiotic diffusion, giving bacterial cells time to express genes of resistance through cell-to-cell signaling via quorum-sensing. These and many other factors demonstrate the complexity of biofilm resistance mechanisms, and contribute to its resilience to conventional antibiotic treatment.

In the human body, biofilms seem to prefer inert surfaces and dead tissue, as well as indwelling medical devices such as catheters and implants, tissue fragments such as bone, and live tissue such as heart valves. Its robustness and versatility to adapt to stressful conditions are reflected in the chronicity of the infectious process where they are involved. Osteomyelitis in the bone, infective endocarditis, dental caries, and in the respiratory tract, cystic fibrosis, are some of the many diseases where biofilms have been implicated in causing recurrent and persistent infection. When identified, modifications of standard antibiotic schemes are often required. Ongoing characterization of adaptive mechanisms with new molecular and genetic techniques will hopefully enhance our understanding of biofilms in the near future.

**C. Role in Otolaryngologic Conditions**

In the field of otolaryngology, many chronic infections highlight the significant role of biofilms in causing disease recurrence. The ear, nose and throat are all susceptible to bacterial colonization due to its direct continuity and regular exposure to external elements. Pathogens in the air for example, are inhaled on a regular basis, constantly
exposing the nasal cavity and nasopharynx to airborne pathogens. The oral cavity and oropharynx are a natural home to bacterial oral flora, which can serve as an easy source of biofilms in times of bacterial overgrowth, infection and host susceptibility. Biofilms have been isolated in tonsillar crypts of patients with chronic tonsillitis, infective cholesteatomas of the middle ear, and in in-dwelling devices such as endotracheal tubes, ventilation tubes and voice prostheses, all of which are associated with chronic infection. In recurrent otitis media and otitis media with effusion (OME), a dysfunction of the Eustachian tube connecting the nasopharynx to the middle ear causes an increased negative pressure and poor oxygenation. This, along with ciliary denudation, increased secretory cells, and microbial ascension from the adenoids and nasopharynx, serve as a rich environment for biofilm formation in the middle ear and contributing significantly to many chronic middle ear conditions.

As expected, most biofilm-forming organisms associated with otolaryngologic infections are natural colonizers of the upper respiratory tract. In acute otitis media, the most common causative organisms Streptococcus pneumoniae (S. pneumoniae), Haemophilus influenzae (H. influenzae) and Moraxella catarrhalis (M. catarrhalis) are also the most common organisms seen in biofilm form in the middle ear of those with recurrent otitis media and OME, but were not isolated in mucosa of cochlear implant controls. The same organisms also colonize the nasopharynx and adenoidal pad, a common reservoir of bacteria for otitis media, and also associated with adenoidal hypertrophy. Potentially pathogenic bacteria such as S. aureus and β-hemolytic Streptococcus, which can also colonize the nasal mucosa, have been found in biofilm form in tonsillar tissue of patients with chronic tonsillitis and in tracheostomy tubes. The presence of these biofilm-forming bacteria during diseased state implies its
important role in recalcitrant chronic infections. The polymicrobial nature of the oral and nasal flora is also reflected in the multiple bacterial components found within these biofilms. The involvement of multiple microbes in ear nose and throat infections needs to be taken into consideration when targeted anti-biofilm treatment is used.

D. Role in Chronic Rhinosinusitis

Recent evidence has suggested a major role biofilms play in CRS. Although the sampling site and imaging modality may slightly alter the sensitivity of biofilm pick-up in tissue research, it has been concluded that biofilms are present in sinuses of CRS patients. Studies have shown biofilms to be present in nasal polyps, polypoid mucosa and diseased maxillary sinus mucosa. Although some studies have isolated biofilms in control tissue samples, they are more than often absent in normal healthy sinus mucosa. However, the question still remains on whether biofilms are part of disease causation or simply opportunistic residents favoring growth on inflamed mucosa. Regardless, many studies show symptomatic and clinical improvement with therapeutic goals of biofilm eradication. This points to biofilms having a distinct pathologic role in CRS aetiopathogenesis.

The polymicrobial nature of infection associated with CRS is well documented. Recent gene sequencing techniques demonstrate a vast variety of microbes found in the sinonasal cavity of CRS patients, more than what is isolated using conventional culture techniques. However, microbial DNA is also abundantly found in non-CRS control samples, highlighting the non-sterility of the sinonasal cavity, and the fact that not all microbes necessarily play an important role in disease pathology. It is more likely that microbial abundance and the relative predominance of pathologic bacteria are
associated with disease persistence,\textsuperscript{68} which correlates with the culturability of the microorganism. Of the biofilm-forming bacteria, \textit{H. influenzae, Pseudomonas aeruginosa (P. aeruginosa)}, \textit{S. aureus} and anaerobes are found to be the most common in CRS.\textsuperscript{63} Certain bacterial types are associated with greater disease severity and recalcitrance. In a study by Foreman et al, they showed that biofilms with \textit{H. influenzae} are associated with milder forms of CRS, while those with \textit{S. aureus} had greater disease severity and poorer post-operative outcomes.\textsuperscript{69} \textit{P. aeruginosa} biofilms have also been linked to poor outcomes,\textsuperscript{70} but it is the microorganism \textit{S. aureus} that is repeatedly linked to unfavorable results, despite maximal medical and surgical treatment.\textsuperscript{65,71} Hence there is great focus of attempts to develop novel treatment strategies against this particular biofilm-forming microorganism.\textsuperscript{72-74}

1.3 \textit{Staphylococcus aureus}

A. Historical facts

There is probably no other microorganism as well-known and as extensively studied as \textit{Staphylococcus aureus}. It was first discovered in 1880 by a Scottish surgeon Alexander Ongston, who found clusters of “micrococcus” in pus from one of his patient’s wounds.\textsuperscript{75} He demonstrated that pus from wound abscesses have the ability to form further abscesses and cause septicemia when injected into guinea pigs and mice, exhibiting a microbial causation of this infectious spread. In 1882, he later coined the term “staphylococci”, meaning “bunch of grapes”, describing the morphologic clusters of spherical bacteria.
It was the German surgeon, Anton Rosenbach who further described 2 types of Staphylococci: “aureus” meaning gold as depicted by gold-colored bacterial colonies, and “albus” (now epidermidis) for white.\textsuperscript{75,76} In the pre-antibiotic era, \textit{S. aureus} was found to cause severe infection and sepsis, with a mortality rate of as high as 80\%.\textsuperscript{77} But perhaps the most important discovery was that of a cure, found in the 1920s when Alexander Fleming accidentally noticed the inhibited growth of \textit{S. aureus} when grown with the mold \textit{Penicillium notatum}. The eventual isolation of this particular substance in the mold was called Penicillin, the antibiotic which drastically improved the outcome of patients with \textit{Staphylococcus} infections in the 1940s.\textsuperscript{78} Pivotal in the treatment of infection during World War II, the discovery of Penicillin paved the way for treatment of \textit{Streptococcus} and other gram-positive bacteria, and the synthesis of other Penicillin-derived antibiotics.

The success of anti-\textit{Staphylococcus} therapy however, was not long term for Penicillin. Over a short period of time, resistant strains were already emerging, and by 1944, the first Penicillin-resistant \textit{Staphylococcus} was identified, a Penicillinase-producing \textit{S. aureus} strain.\textsuperscript{79} Even in the early course of history, this demonstrates the remarkable
ability of *S. aureus* to mount an effective response against antibiotic therapy. With the discovery of new antibiotics, the emergence of drug-resistant strains seemed to inevitably follow. Up to 90% of *S. aureus* strains are now resistant to Penicillin, and reports as high as 50% in certain communities have developed Methicillin-resistance.80-82

**B. Role in Human Disease**

Surprisingly *S. aureus* is considered a commensal microorganism existing in a non-infectious state in several parts of the body. With humans serving as natural reservoirs,83 its isolation in healthy skin and mucosa demonstrates its existence as part of the normal flora. However, overgrowth can lead to significant disease burden both locally and systemically, necessitating prompt treatment and eradication. Up to 60% of individuals can be colonized by *S. aureus*, placing them at higher risk of obtaining *S. aureus*-associated infection.84 Its ability to cause significant morbidity and propensity for resistance still makes it one of the most important pathogens to date. These abilities can be explained by several unique bacterial components and mechanisms of host evasion.

**B. 1 Components, products and host evasive mechanisms**

The bacterial cell wall of gram-positive microbes such as *S. aureus* serves not only as a protective mechanical and osmotic barrier, but also comprises and binds surface molecules which are involved in host evasion and bacterial survival.85 A specific polysaccharide microcapsule for example, inhibits phagocytosis, promoting mucosal persistence and enhancing microbial virulence.86 Surface proteins such as Protein A can
bind the Fc portion of immunoglobulins and also prevent phagocytosis. Other related proteins can bind extracellular matrix molecules, which enhance host colonization.\textsuperscript{83,87} Most recently, another \textit{S. aureus} surface protein, adenosine synthase A (AdsA) catalyzes the production of adenosine, which has anti-inflammatory effects via down-regulation of the innate immune response and evading host defenses.\textsuperscript{88}

Certain \textit{S. aureus} strains also produce various toxins with specific virulence activities enhancing the ability to cause significant morbidity and mortality. Pro-inflammatory cytotoxins, pyrogenic toxin superantigens, and toxins responsible for toxic shock and Staphylococcal scalded skin syndrome, are some of the toxins produced by certain \textit{S. aureus} strains,\textsuperscript{83} distinguishing their infectious features from other Staphylococcal species. This highlights the variability of \textit{S. aureus} manifestations, making them unique from other bacterial strains.

Enzyme secretion is another pathogenic mechanism used by \textit{S. aureus} to promote tissue invasion. These include proteases, nucleases, lipases, hyaluronate lyase and staphylokinase, causing tissue lysis and spread of infection.\textsuperscript{89} β-lactamase or penicillinase is the enzyme responsible for Penicillin resistance, hydrolyzing the β-lactam ring of the drug and leading to inactivation. Up to 90\% of staphylococcal isolates now produce Penicillinase,\textsuperscript{78} making \textit{S. aureus} relatively resistant to this first line and popular antibiotic.

\textbf{B. 2 Role in major organ system infection}

Because of its ubiquitous nature, \textit{S. aureus} can cause a multitude of severe infections throughout the body. It can cause skin infections from as simple as furuncles and
carbuncles, to as life threatening as Staphylococcal scalded skin syndrome. It is also the second most commonly isolated microorganism in burn wounds, next only to *P. aeruginosa*. A great percentage of bone infections also involves *S. aureus*, with an 80% cause of osteomyelitis, the infectious process originating either from hematogenous spread, direct inoculation through trauma or from an adjacent site, or from surgical implantation such as prosthesis insertions. Without treatment, this can lead to severe bony destruction, loss of limb function and death from sepsis and shock.

Implantation of other medical devices such as prosthetic heart valves, hemodialysis catheters and pacemakers also place patients at risk of *S. aureus* contamination and bacteremia with serious sequelae. *S. aureus* infective endocarditis (IE) is one major complication, and although in the past was not the major cause, is now in recent years, the most frequent cause of IE. Initially more commonly found in intravenous drug users, *S. aureus*-associated IE is increasing in incidence with a change in demographic prevalence. This is likely due to a greater number of those exposed and susceptible to the infection. Factors such as spread via the community or via health care contact, along with more frequent use of indwelling medical devices has increased the carrier rate and the risk of infection.

But perhaps the greatest global impact involving *S. aureus* infection to date is the development of methicillin resistant *S. aureus* (MRSA) strains. Created in 1959, Methicillin was developed to address the emerging Penicillin-resistant *S. aureus* strains. In a short span of two years however, MRSA-resistant strains were already isolated in the United Kingdom, with other European countries, Australia and the United states.
soon following. Currently representing a major healthcare concern, risk factors for MRSA colonization are increasing globally. The traditional risk factor for MRSA exposure in health care institutions have now been joined by an increasing incidence of risk factors in the community setting such as repeated antibiotic use, surgery and exposure to MRSA colonizers. Methicillin resistance is explained by the presence of the methicillin resistance gene (mecA), carried on a mobile genetic element and encoding a methicillin-resistance Penicillin binding protein. Although the true origin is still poorly understood, the initial theory was of MRSA resistance originating from a single S. aureus strain that acquired the mecA gene. This theory though has been recently disputed, with new suggestions that the mecA gene was transferred between S. aureus lineages, implying a highly diversified organism with great ability for environmental adaptation. MRSA infection complicates patient treatment and limits appropriate antibiotic options. This creates a greater burden to both patient and the health care professional in obtaining successful treatment outcomes.

**B. 3 Role in Chronic Rhinosinusitis**

Although CRS is polymicrobial in nature, only recently have organisms playing a more pathogenic role been more clearly identified. Certainly S. aureus has been repeatedly linked to disease recalcitrance in CRS. A study by Singhal et al. showed that the presence of S. aureus in CRS sinuses, whether alone or co-inhabiting with other bacteria, have worse symptom scores, quality of life and post-operative outcomes. Its presence in the CRS sinonasal cavity has also been positively linked to the presence of nasal polyps. S. aureus enterotoxins can act as superantigens which incite a hyper-reactive IgE mediated response and eosinophilic inflammation, the classical immune-
mediated response found in patients with nasal polyposis.\textsuperscript{100} This indicates a role for \textit{S. aureus} in polyp formation. Although \textit{S. aureus} enterotoxins may not be the only element involved in polyp formation, its recent link to the subset of CRS patients with the most severe disease type points to an important role in CRSwNP manifestation and pathogenesis.

Demonstrations on the capability of \textit{S. aureus} to reside intracellularly and within the mucosal epithelium open another possible explanation of its recalcitrance and high recurrence rates despite antibiotic treatment. When inside the cell, \textit{S. aureus} is described to be in a “small colony variant” (SCV) form, phenotypically distinct from its extracellular counterpart. SCVs are metabolically less active and do not incite a host response, are one tenth the size of the parent strain, and resist intracellular host defenses.\textsuperscript{101} Furthermore, \textit{S. aureus} seems to have the ability to reside in non-phagocytic cells such as epithelial cells, avoiding host defenses successfully and possibly serving as reservoirs of infection. This characteristic of \textit{S. aureus} further adds to its ability to persist within previously infected sinuses, serving as a nidus of infection and thus a failure of complete bacterial eradication.

Antimicrobial resistance in CRS is also becoming a major concern as new topical therapies emerge in attempts to lessen oral antibiotic use. Adding to the risk of resistance is the vast array of physicians involved in the medical management of CRS. From general practitioners, to allergy specialists and otolaryngologists, these patients have often had a history of multiple or extended antibiotic use, putting them at greater risk of developing drug resistant strains. The prevalence of MRSA for example, approaches a 9% incidence in CRS,\textsuperscript{102} with up to 19% of isolated \textit{S. aureus} species, and
more commonly seen in individuals who have had previous antimicrobial therapy.\textsuperscript{103} Erythromycin-resistant \textit{S. aureus} strains are also increasing, with higher rates in MRSA than non-MRSA strains.\textsuperscript{104} The emergence of multi-drug resistant strains, more so with the commonly used antibiotics, strengthens the argument for the practice of culture-directed antibiotic therapy. Despite this, ongoing surveillance of drug-resistant \textit{S. aureus} strains must continue, to allow us to tailor our treatment strategies and increase the likelihood of a successful outcome.

\textbf{C. Recalcitrance in biofilm form}

The existence of polymicrobial biofilms in the sinuses of CRS patients is well documented,\textsuperscript{65,66} and associations between the biofilm type and clinical outcomes have been clearly demonstrated. This points to an essential role biofilms play in the pathophysiology of CRS. \textit{H. influenzae}, \textit{P. aeruginosa} and \textit{S. aureus} are some of the more common organisms found, with evidence of fungal elements in some mucosal samples indicating a co-existence of fungus within the biofilm matrix in some cases.\textsuperscript{105}

However, it is the presence of \textit{S. aureus} biofilms, which seems to be linked to more unfavorable outcomes.\textsuperscript{70,71} Whether isolated alone or in combination with other microbes at the time of surgery, the poorer patient outcomes in those with \textit{S. aureus} biofilms are consistently demonstrated via a higher rate of re-infection and more severe patient symptoms. Reduced quality of life and greater symptom scores are reflected by greater frequency of outpatient visits,\textsuperscript{65} long standing antibiotic use,\textsuperscript{73} and subsequently a greater chance of requiring revision surgery. The high propensity to re-culture \textit{S. aureus} at the post-operative period despite a culture-directed 2 week oral antibiotic regimen,\textsuperscript{71} demonstrates its extreme recalcitrance, and the insufficient ability of current
treatment paradigms to successfully eradicate the microorganism. This is further reflected in our tertiary hospital clinical practice, where patients with *S. aureus* biofilms often present with recurrence within weeks of completing antibiotic treatment.

Repeated antibiotic use will thus increase the risk of developing drug resistance. MRSA strains in CRS are also more commonly encountered. With these concerns and obvious limitations seen with oral antibiotic use, a visible shift in treatment schemes to include topical antimicrobial therapy is emerging. Thus there is a resulting increase in attempts to discover novel topical therapies or re-visit classical antimicrobial agents to address these issues. Direct application of an antimicrobial agent theoretically improves the chance of successful treatment due to exposure of bacteria to higher concentrations with lesser chance of systemic side effects.

Several topical therapies are available off-prescription to treat bacterial exacerbations of CRS. Chemical surfactants such as baby shampoo have been shown to not only lessen thickened secretions and symptoms of post-nasal discharge, but also have potential biocidal effects with prevention of biofilm formation. They however, failed to have anti-biofilm effects against established *Pseudomonas* biofilms. Topical antibiotics such as Moxifloxacin and Mupirocin have been shown to be effective in established *S. aureus* biofilms as topical agents, but the risk of developing drug resistance still remains, as well as the recurrence of infection when treatment is ceased.

A shift to the use of more natural products with antimicrobial properties will attempt to attenuate the concerns of drug resistance development. Silver, a well-known antimicrobial, appears to have significant anti-biofilm effects on *S. aureus* in vitro.
Although showing great promise as an alternative topical agent, a safety profile still needs to be established,\textsuperscript{108} and further studies are needed before its clinical use in the sinuses. Manuka honey has also been shown to have anti-\textit{S. aureus} biofilm effects when topically applied.\textsuperscript{72} Its relatively inexpensive cost and availability makes it an attractive antimicrobial agent, as evidenced by a multitude of research looking into its potential clinical application in CRS,\textsuperscript{109,110} but certain higher concentrations may cause ciliary denudation and squamous metaplasia,\textsuperscript{110} limiting the safe dose range for topical use and necessitating the need for monitoring of surface mucosal effects.

Thus, the quest for an ideal topical agent with anti-\textit{S. aureus} biofilm effects continues. A natural compound, existing normally in healthy sinuses, and locally safe at high concentrations, with the capacity to penetrate the biofilm matrix and avoid systemic effects is what is needed. This will allow regular topical use with minimal risk of side effects and emergence of drug resistant strains. Ultimately this research on the role of nitric oxide in the sinuses led to further exploration on its potential use as a novel topical therapy for \textit{S. aureus} biofilms in CRS.

1.4 Nitric Oxide

Nitric Oxide is a naturally occurring free radical with several, but often conflicting roles within living organ systems. Its dualistic nature, but also precise functions in specific physiologic processes within the human body, creates a mysterious aura with much yet to be explored in its vast array of functions and potential applications. This review attempts to highlight NOs physiologic role in the sinuses, its role in \textit{S. aureus} bacterial physiology, and lastly, as a potential therapeutic agent in CRS against \textit{S. aureus} biofilm infection.
A. Discovery and History

Nitric oxide was discovered by a chemist and philosopher Joseph Priestley in 1772, along with his discoveries of oxygen and nitrous oxide (N\textsubscript{2}O).\textsuperscript{111} Whereas N\textsubscript{2}O became known as “laughing gas” and went on to be used in the fields of anesthetics,\textsuperscript{112} NO was described further in 1979 for its vascular smooth muscle relaxant effects, demonstrated first in bovine coronary arteries.\textsuperscript{113} In the 1980s the beginning of NOs rise to scientific fame truly started when a potent vasodilating substance named endothelium derived relaxation factor (EDRF) was found to be produced by intact endothelial cells in response to acetylcholine and other agonists, causing vascular smooth muscle relaxation.\textsuperscript{114,115} In 1987, EDRF was proven to be NO, synthesized from a protein precursor L-arginine via an oxygen-dependent enzymatic pathway. This led to the establishment of NO as an important signaling molecule in the cardiovascular system.

The discovery of NO as the active substance in nitrovasodilators was well after the start of their therapeutic use in the treatment of chest pain and hypertension. Amyl nitrite and nitroglycerin (GTN) have been used to treat angina chest pain as early as in the 1800s, but it was only in the 1970s where nitrate compounds and NO were both shown to have a common pathway of inducing smooth muscle relaxation, through activating guanylate cyclase and increasing cyclic GMP. Subsequently it was proven that NO was generated by these organic nitrates and is the key component of their vasodilating properties.\textsuperscript{116} This led to a massive range of therapeutic applications of nitrovasodilators in the field of cardiology on conditions such as angina, hypertension, congestive cardiac failure, pulmonary hypertension among others.
Over the years, the discovery of NO’s functions have broadened beyond the cardiovascular system to encompass roles in both normal and pathologic processes, implicated in both essential physiologic functions and diseased states. In 1992 it was named molecule of the year and by the 20th century, an average of 6000 publications annually are all in some form pertaining to NO.\textsuperscript{114} A reflection of newly described roles within the human body, NO has now been shown to play key roles in neurotransmission, melanogenesis,\textsuperscript{117} immunity,\textsuperscript{118} respiration and wound healing,\textsuperscript{119} as well as implicated in conditions such as diabetes, cancer and recently in CRS.

B. The role of nitric oxide in human and bacterial physiology

NO is an unstable diatomic molecule containing an unpaired electron, which is responsible for many of its functions. Its gaseous form and high reactivity give it a very short half-life, thereby needing a constant source of production within requiring physiologic systems. In humans, NO is mainly formed via a 2-step reaction from a natural, semi-essential amino acid L-arginine, utilizing oxygen and NADPH to produce NO and L-citrulline.

\begin{align*}
\text{L-Arginine} & \rightarrow \text{\textsuperscript{N\textdegree} OH- L-Arginine} & \rightarrow \text{NO} \\
\text{NADPH} & \rightarrow \text{NADP+} & \rightarrow \text{NADPH} \text{ and H2O} & \rightarrow \text{NADP+} \\
\text{O2} & \rightarrow \text{H2O} & \rightarrow \text{O2} & \rightarrow \text{H2O}
\end{align*}

\textbf{Figure 1.4: Pathway of NO synthesis in oxygen available states. L-arginine is the main substrate for NO generation.}
This pathway is regulated by an enzyme nitric oxide synthase (NOS), coming in 3 isoforms: endothelial (eNOS, NOS-1), inducible (iNOS, NOS-2) and neuronal (nNOS, NOS-3), named after their primary location. While eNOS and nNOS are constitutively expressed and are calcium-calmodulin dependent on activation (cNOS), iNOS was found to be expressed only in activated cells. Calcium-calmodulin independent, iNOS is produced in response to inflammatory stimulants such as pro-inflammatory cytokines and endotoxins. The iNOS–induced NO production also differs from its cNOS counterparts, with NO produced in sustained and more elevated levels. So as long as iNOS is expressed NO production will continue, in contrast to eNOS and nNOS – produced NO which is generated in a pulsatile manner at significantly lower levels. In essence, iNOS expression is induced in response to infection. Phagocytic cells such as macrophages and their monocyte precursors express iNOS, with NO generation being one of their major antimicrobial mechanisms. NO’s oxidation to reactive nitrogen species (RNS) within macrophages also adds to these desired effects, and in concert with ROS, can kill or prevent intracellular bacterial replication, thus displaying both bactericidal and bacteriostatic effects. NO also plays an essential role in wound healing, as evidenced by delayed wound closure in eNOS and iNOS knock-out mice. iNOS expression in wound macrophages during the initial inflammatory phase of wound healing implicates NO as an essential molecule for wound healing. Whether eNOS or iNOS-derived, NO also has important roles in fibroblast collagen synthesis, angiogenesis and keratinocyte proliferation. Conversely, uncontrolled inflammation has also implicated iNOS-produced NO in cellular damage. Via its conversion to peroxynitrite (ONOO') and generation of other ROS, NO-mediated effects causing cytotoxicity and tissue damage have also been reported.
Due to its important and multifaceted physiologic roles, it is not surprising that there is an alternative source of NO.\textsuperscript{125} In the absence of oxygen, NOS-independent NO generation can occur from nitrate (NO3) and nitrite (NO2). Initially thought to only be end-products of endogenous NO consumption, NO3 and NO2 can be an essential source of NO in the absence of oxygen, serving as a back-up source during hypoxic states. Exogenous sources of NO3 such as in food can also supplement NO requirements within the body. The recycling of these by-products ensures another pathway of NO generation to maintain the body’s physiologic functions despite diseased states which cause or are characterized by low oxygen tensions.\textsuperscript{125}

In contrast to its functions in cytoprotection and against microbial invasion in humans, NO is also produced by bacteria as part of their protective mechanism against antimicrobial destruction. Using bacterial NOS (bNOS), bacteria produce NO in a manner similar to the eukaryotic NOS, synthesizing NO from the oxidation of L-arginine to L-citrulline. In gram-positive bacteria, NO protects them against oxidative stress generated by certain antibiotics whose bactericidal function are due in part to forming ROS.\textsuperscript{126} In a study by Gusarov et al., it was demonstrated that NO produced by bNOS can directly alter the active chemical structure of certain antibiotics, making them less bactericidal and also directly protect bacteria from ROS-mediated effects of the antibiotic. Two independent mechanisms were found to be used by bNOS-expressing bacteria: by the suppression of free cysteine reduction which subsequently suppresses the cytotoxic Fenton reaction, and by the reactivation of catalase, a strong antioxidant.\textsuperscript{127} As bNOS is found only in gram-positive bacteria, there is additional evidence that \textit{S. aureus} can utilize NO as a form of adaptation to oxidative stress induced by antibiotics and the host immune system. This further highlights the dualistic
nature of NO as having both pro- and anti-bacterial effects, most likely dependent on specific NO concentrations. Furthermore, *S. aureus* has the ability to mount a strong resistance to high levels of NO by altering the expression of an array of genes to battle the host’s innate immune response.\(^{128}\) These must be taken into consideration when attempts at using NO as an antimicrobial agent are made.

### C. Nitric oxide in the Nose and Paranasal Sinuses

In comparison to other organ systems, a significant concentration and difference in the mode of NO production was found in the nose and paranasal sinuses. It was Alving et. al. who discovered that high concentrations of NO were measurable in exhaled air, more when exhaled from the nasal cavity than from the mouth.\(^{129}\) Further experiments showed that although the mucosal lining of the nasal cavity also produced NO, the paranasal sinuses were the major source, enzymatically produced by an inducible-NOS in the apex of the epithelial cells. A slight difference in this region is that iNOS is also constitutively expressed, constantly generating high levels of NO within the sinuses. Concentrations as high as maximum allowable air pollutant levels were measured in the sinuses of healthy individuals,\(^{130}\) indicating that specific roles for NO should exist in such confined spaces. Although the exact physiologic role of NO in the sinuses still needs to be elucidated, studies have demonstrated possible roles in host defenses. Anti-viral and anti-bacterial properties are depicted in the ability of macrophages to produce NO to destroy invading microbes. These effects are further strengthened by the demonstration of genetically engineered mice lacking iNOS being more susceptible to bacterial and viral infections.\(^{131,132}\) Certainly diseases associated with low sinonasal NO levels such as Kartagener’s syndrome and cystic fibrosis are more prone to recurrent...
sinus infection, the symptoms so severe that sinusitis is classified as part of the spectrum of these disorders.

Another potential function of NO is its inhalation from the upper airways to reach the lungs where it exerts its desired effects of broncho- and vasodilation to improve ventilation-perfusion. Localized regulation of blood flow and mucociliary activity by NO is also another important role, demonstrated in both animal and human studies. An increase in ciliary beat frequency with the administration of a NO donor in a study by Runer et al. strongly suggests that NO enhances mucociliary clearance, supplementing its antimicrobial properties in keeping the sinonasal cavity relatively clean.

It is only recently that the sinuses have been shown to lack complete sterility with molecular techniques identifying a multitude of microbial DNA in healthy sinus tissue. It is more likely that a balance in microbial flora exists in normal sinuses, and so as long there is no shift to allow a pathogenic organism to dominate and overgrow, then infection is prevented. With NO seeming to have both pro- and anti-bacterial functions, it is necessary to determine what specific concentration range prevents pathogen overgrowth prior to therapeutic utilization. Measurable NO concentrations in patients with acute and chronic rhinosinusitis are significantly lower than those of healthy individuals, strengthening the theory behind NOs antimicrobial effects. Although it is still unknown if the low NO levels are the cause or consequence of the disease process within the sinuses, there remains a steady increase of explorative attempts to use NO as a potential treatment for sinus infection.
D. Nitric oxide as a Therapeutic Agent for CRS

It is well documented that NO levels in the sinuses of CRS patients are significantly lower than those of healthy subjects.\textsuperscript{136,137} Postulations on the causes of low NO levels have been made. Although not yet established, ostial blockage from mucosal oedema and inflammation may decrease the amount of NO emerging from the sinuses to the nasal cavity, thus affecting NO measurements. The paradoxical increase in iNOS levels in the sinonasal mucosa may signify localized attempts to increase nasal NO levels during this inflammatory state. The lower NO levels in patients with nasal polyposis than non-polyp CRS supports this hypothesis, further strengthened with a noticeable increase in nasal NO after medical and surgical treatment.\textsuperscript{138}

Highlighting all the physiologic functions of NO, its high concentrations in healthy sinuses, and the lack of its protective mechanisms in CRS, it is not unreasonable to determine if NO has potential therapeutic applications for CRS. Normal sinus NO levels exceed that which is bacteriostatic to \textit{S. aureus} growth,\textsuperscript{139} and NO’s anti-biofilm effects have been demonstrated in the past, with metabolites having inhibitory effects on biofilm growth.\textsuperscript{140} Enhanced \textit{Pseudomonas} biofilm dispersal by NO has also been previously shown,\textsuperscript{141} but there is a paucity of studies looking at the effect of NO on established biofilms, more so with \textit{S. aureus}, which is arguably the most pathogenic organism involved in CRS recalcitrance. NOs anti-viral and anti-bacterial effects, its enhancement of mucociliary clearance and wound healing, and its endogenous production in the sinuses, give it great potential as a topical therapy in the context of CRS. The appropriate anti-biofilm dose using a practical mode of delivery to the sinuses is however required, if it is to be used as a topical anti-biofilm agent. Being
aware of its contradictory properties also requires the strict establishment of a safety profile prior to clinical use.

**E. Nitric oxide delivery mechanisms**

The challenge of delivering NO in sustained amounts at correct concentrations is a justifiable concern when planning a topical application to the sinuses. This is due to the irregular configurations and variability in sinus anatomy. To ensure therapeutic success, obtaining the desired anti-biofilm effects of NO at a specific duration and dose are needed. Because of factors such as NO’s gaseous state and short half-life, mucus accumulation and oedema associated with CRS, and the thick biofilm matrix impeding drug penetration, adequate NO delivery on its own will be difficult to achieve for effective biofilm treatment and eradication.

Delivery of NO into aqueous solutions has been investigated using devices that incorporate a predictable concentration of NO into liquids,\textsuperscript{142} but these were designed more for investigating its biological effects rather than a therapeutic application. In vitro chambers delivering gaseous NO have also been used to demonstrate antibacterial effects against more common pathogenic organisms including *S. aureus*,\textsuperscript{143} and attempts to deliver at intermittent short term exposure for pulmonary infections have been made to decrease risk of side effects from continuous exposure.\textsuperscript{144} These devices however, will not be feasible for use in patients with CRS given their size and cost, and if used, patients will most likely need to return to hospital for drug administration. A more portable device using an aqueous solution similar to the topical washes currently being used by CRS patients is needed.
NO was not spared in the potential applications of nanotechnology in the medical field. NO-releasing silica nanoparticles have been recently used against a variety of biofilms in vitro, including *S. aureus*, *P. aeruginosa* and *Escherichia coli (E.coli)*. The anti-biofilm eradication rate of over 99% for these organisms was reported, showing great promise for these nanoparticles as anti-biofilm agents. Although the fibroblast toxicity of these nanoparticles was comparable to more commonly used antiseptics, nanoparticle application is not yet approved for clinical use, limiting the current applications to in-vitro and animal experiments until a clearer safety profile is defined.

Nonetheless much research is currently being performed on NO to treat nosocomial infections associated with implantable devices, many of which are in biofilm form. Investigations on slow release NO highlight the importance of continuous exposure to eradicate biofilms on implant surfaces. Slow-release NO designed on gel-coatings for example, has been shown to decrease infection rates of *S. aureus* in a rat model. Utilizing the same principle of slow release in the sinuses, a safe drug delivery of NO at the correct anti-biofilm dose can be achieved.

Drug encapsulation using liposomes has been investigated in the past and used for a wide variety of clinical applications. Defined as “spherical vesicles with a bilayered membrane composed of natural or synthetic amphiphilic molecules”, liposomes have been used for many years in several industries. They have been used to encapsulate substances such as antibiotics with high efficacy, with aims of enhancing drug specificity, lessening side effect risks, prolonging drug release and decreasing the required effective dose. Its potential for use as a topical agent to encapsulate NO for use in CRS has not yet been fully explored.
To our knowledge, no application to date has been designed to use NO as a topical therapeutic agent in the sinuses. With an abundance of literature documenting NO’s antibacterial and anti-biofilm effects on many microorganisms including *S. aureus*, there is great promise for its use as a potent anti-biofilm agent in the sinuses. However, side effects of NO have also been reported,\textsuperscript{149} as well as its potential cytotoxicity.\textsuperscript{150} A clear safety profile should accompany the high efficacy of a NO-based topical solution, should it ever be designed.
Summary of Literature Review

CRS is a debilitating disease with symptoms severely affecting activities of daily living and function. This is reflected in its large socio-economic impact as seen in the high use of out patient services, drug prescription, and amount of time taken off work. Its multifactorial etiology likely involves a complex interplay of host, microorganism and environmental factors, leading to challenges in pinpointing a single etiologic cause. As a result, treatment regimes are also complex and abundant, and no single therapeutic modality is successful. Approaches to control rather than cure the disease is met with some degree of success, as rhinologists, allergy specialists and scientists strive to better understand this complex disease.

With disease control comes the identification of key players which are associated with recalcitrance and symptom severity. Bacterial infection, whether a cause or consequence of CRS, is consistently associated with disease exacerbation and increased symptomatology. *S. aureus* is the microorganism repetitively isolated in this subset of patients who seem to suffer most and have the highest risk of poorer outcomes despite maximal medical and surgical treatment. Its existence in biofilm form makes eradication even more challenging, leading to infection recurrence shortly after cessation of oral or topical antibiotics.

With high NO levels in sinuses of healthy individuals and significantly lower levels in sinuses of CRS patients, the use of NO as a safe and effective antimicrobial agent in the sinuses is promising. Its anti-bacterial, anti-viral effects and enhancement of mucociliary activity make it an attractive replacement to current antibiotics. Its endogenous presence in concentrations as high as maximum allowable air pollutant
levels in normal sinuses points to a safe existence in the sinonasal cavity at high concentrations, theoretically allowing the use of high doses with relative safety.

However, a further understanding of the role of NO in *S. aureus* biofilm growth is needed to establish the correct anti-biofilm concentration. This is essential given NO’s often contradictory functions within physiologic systems. From the host perspective, a clearer description of gene regulation in association with NO in CRS patients is also required. Its role in innate immunity, and the determination of gene expression differences in healthy vs. CRS patients, will indicate if there are any discrepancies in NO or ROS generation between groups. This will hopefully determine if there are any host factors involved in NO dysregulation, which inevitably may make certain individuals more prone to developing CRS.

The key issues that this thesis aims to address are:

1. What is the role of NO on *S. aureus* biofilm growth in the setting of CRS?
2. Are there gene-expression differences in NO and ROS regulation in CRS patients, pointing to a host factor in the dysregulation of NO synthesis and metabolism in the sinuses?
3. Given the deficiencies in effective topical therapies for CRS, is there a role for NO as a therapeutic agent in the context of *S. aureus-* associated CRS?
Figure 1.5: Summary of the aims of the projects of this PhD addressing the host, environment and microbe component of CRS pathophysiology.

**Studies to be performed**

1. Determine the in-vitro effects of NO on established *S. aureus* biofilms isolated from CRS patients mimicking concentrations in healthy and diseased sinuses.

2. Determine the localized expression of genes involved in NO and ROS regulation in CRS patients with *S. aureus* biofilms vs. healthy individuals, including genes involved in innate immunity.

3. Determine if NO can be delivered in an appropriate anti-biofilm concentration effective against *S. aureus* biofilms.

4. To design a safe and effective NO-based therapeutic agent to be used as a topical wash against *S. aureus* biofilm- associated CRS.
Chapter 2: The Effects of Nitric Oxide on *Staphylococcus aureus*

Biofilm Growth and its Implications in Chronic Rhinosinusitis

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Financial assistance from the University of Adelaide, Australia
2.1 Abstract

**Background:** The relationship between sinonasal nitric oxide levels and the pathogenic organism *Staphylococcus aureus* is yet to be established. High nitric oxide levels measured in healthy sinuses likely contribute to maintenance of relative sterility. Lower concentrations such as is found in the sinuses of chronic rhinosinusitis (CRS) patients may decrease this effect. *S. aureus* in biofilm form has recently been implicated in recalcitrant CRS, its isolation predicting a higher risk of post-treatment re-infection. This in-vitro study aims to characterize the changes in *S. aureus* biofilm formation when exposed to different NO levels mimicking the normal and diseased NO sinus concentrations reported in previous literature in an in-vitro setting.

**Methodology:** *S. aureus* ATCC 25923 and seven clinical isolates were cultured in biofilm form using the MBEC device and the established biofilms exposed to 1-1000 micromolar (µM) NO concentrations. Biofilms were visualized using *LiveDead* Baclight stain and confocal scanning laser microscopy, and quantified using Comstat 2, a biofilm quantification software.

**Results:** Biofilm biomass decreases from an average of 0.105 to 0.057 µm³/µm² at higher NO concentrations (125-1000 µM), but is increased to 0.470 µm³/µm² at lower NO concentrations (0.9-2 µM). The average biomass at high vs. low concentrations are statistically significant (p<0.001).

**Conclusion:** *S. aureus* biofilm formation varies across exposure to different NO levels, with anti-biofilm effects at higher concentrations, and enhanced biofilm formation at lower or sub-physiologic concentrations. These results coincide with the often dualistic function of NO, and have implications in its future use in the treatment of CRS.
2.2 Introduction

In a recent study performed on the Hawaiian bobtail squid, nitric oxide (NO) was found to play a pivotal role in the selective colonization of its light organ with *Vibrio fischeri*, the only organism with the ability to cause illumination through beneficial symbiosis. NO was found in high concentrations in parts of the light organ where the bacteria aggregate, and has also been implicated in the maintenance of bacterial colonization. This serves as an essential model for associations of hosts with their bacterial symbionts using nitric oxide as a form of signaling molecule. Certain characteristics unique to *Vibrio fischeri* may allow them to survive the high NO environment and therefore selectively colonize the light organ.

Similarly, high concentrations of NO are present in the sinuses of healthy individuals. Locally it has been shown to exert antimicrobial and antiviral effects, providing the relative sterility of the sinuses and also enhancing mucociliary clearance. Patients with chronic rhinosinusitis (CRS) however, have significantly lower levels of sinonasal NO. Although the exact mechanism is not yet fully understood, this occurrence may potentially explain the higher risk of sinus re-infection in CRS patients, contributing greatly to the severity of symptoms and disease recalcitrance.

The isolation of *Staphylococcus aureus* in the sinuses of CRS patients at the time of endoscopic sinus surgery has shown these patients to have a higher rate of re-infection, persistent evidence of mucosal disease and increased difficulty in treatment eradication, therefore having a predictably poorer post-operative outcome. The relationship between *S. aureus* and NO levels in diseased sinuses need to be further defined, although there have been recent publications showing that *S. aureus* growth is inhibited
These in-vitro studies, however, have been performed on bacteria in their planktonic form. It will be important to characterize the effects NO will have on their biofilm counterparts, as *S. aureus* is known to exist in biofilm form in the sinuses.\(^{52,153}\) A strong structural formation, extracellular polymeric substance (EPS) matrix and distinct phenotypic characteristics provide biofilms greater resistance to antimicrobial therapy. Whether NO will exert its classical antimicrobial function on *S. aureus* biofilms, or act as a signaling molecule in a manner similar to the squid-*Vibrio* model, attracting and enhancing bacterial colonization, remains to be seen. The aim of this study is to therefore determine what effects NO will have on established biofilms of *S. aureus* isolated from CRS patients when tested in an in-vitro setting.

### 2.3 Methodology

**Bacterial strains and culture conditions**

*Staphylococcus aureus* American Type Culture Collection (ATCC) 25923, a known biofilm-forming strain, and seven *S. aureus* clinical strains isolated from CRS patients’ sinuses were used. Each strain was cultured for 18-24 hours in 2 mLs of Cerebrospinal fluid (CSF) broth (Oxoid Australia, Thebarton South Australia) at 37°C with moderate shaking (90-100 rpm). One loop of the CSF culture broth was streaked on horse blood agar plates (Oxoid Australia SA) and allowed to grow at 37°C for 18-24 hours. 1-2 colonies were immersed in 2 mLs of 0.45% Sodium Chloride, adjusted to create a 1 McFarland unit solution (\(3 \times 10^8\) colony forming units/mL). 400 µL of this solution was mixed with 5.6 mLs of CSF broth to make a 1:15 dilution. This serves as the bacterial solution to be used in the formation of biofilms.
**Biofilm formation and exposure to Nitric Oxide**

Bacterial biofilms were grown using the Calgary Biofilm or MBEC device as previously described by the manufacturer (Innovotech Inc., Edmonton, AB Canada). Briefly, the MBEC device contains 96 pegs attached on a lid that is designed to fit into a standard 96 well plate. 150 µL of the bacterial solution was pipetted into each well of the plate allowing the pegs to be immersed in the solution. The device was then incubated at 35°C in a gyratory shaker at maximum speed of 70 rpm (Ratek Instruments, Vic Australia) for 44 hours, to allow adequate biofilm formation on the pegs’ surface.

The pegs were then washed for 1 minute with 1x Phosphate Buffered Saline (PBS) in a new 96 well plate (Greiner Bio-one, Germany) to remove excess planktonic bacteria and immersed into a new plate containing 180 µL/well of DetaNONOate, a nitric oxide donor with a predictable NO molecular release (A.G. Scientific, San Diego CA USA) at serial concentrations of 1-1000 µM, the range chosen as it encompasses the NO concentrations measured in healthy and diseased sinuses based on previous literature. DetaNONOate releases 2 moles of NO per moL of parent compound, allowing computation of NO concentrations at a later stage. This is essential as gaseous NO is generally measured in parts per million (ppm), and it will be necessary to convert the NO donor concentrations from µM to ppm, allowing comparability of NO levels created in-vitro to that measured in the sinuses based on previous literature. As DetaNONOate instantaneously decomposes at an acidic pH, the NO solution was tested to a pH of 7 prior to use to maintain predictability of NO release. To ensure the actual presence of NO, the solution was tested using a Nitric oxide colorimetric assay (Biovision, CA USA) which utilizes the Griess reaction to reflect the amount of NO in
the sample (Figure 2.1: Addendum graph). CSF broth was used as diluent for the solution to ensure biofilm death is caused by NO rather than absence of media nutrient. 20 µL of 0.4 mM L-arginine (Musashi, Vic Australia) was also added per well to mimic a bacterial culture environment\textsuperscript{156} and provide L-arginine for the bacteria to use as a substrate in response to high NO levels.\textsuperscript{157} The pegs were then immersed in the NO solution and incubated for another 24 hours, broken off the lid the following day and washed twice with 0.9% Sodium Chloride at 1 minute and 10 seconds respectively, prior to staining and quantification.

Figure 2.1: Addendum Graph: Comparison of NO concentrations of the NO donor Deta NONOate and its computation using the Greiss reaction. The near identical concentrations demonstrate the accuracy of the Greiss reaction in detecting NO concentrations in the solutions, and the predictability of NO release in Deta NONOate.
Image Acquisition & Quantification of Biofilms

The biofilm-coated pegs were fixed in 5% glutaraldehyde (Sigma-Aldrich MO USA) for 30-60 minutes at room temperature and washed again with 0.9% NaCl for 10 seconds to remove excess fixative. They were immersed in individual tubes containing 1 mL sterile MQ water with aliquots of 1.5 µL each of component A (Syto 9) and B (Propidium Iodide) of LiveDead BacLight stain. (Invitrogen Molecular Probes, Mulgrave, VIC) The tubes were incubated in the dark at room temperature for 15 minutes. The pegs were removed from the tubes and washed with 0.9% NaCl to remove excess stain and mounted on coverslips for viewing under the Leica TCS SP5 confocal scanning laser microscope (Leica Microsystems, Wetzlar Germany). 2 z-stacks, each containing 120 +/- 5 images were captured in the middle 2/3 of each peg and image slices were taken at 0.7 µm apart. The length and width of each z-stack are 775 µm each with a depth of 85 µm. Thus, each z-stack volume is approximately 5.1 x 10^7 µm^3. Total surface area per z-stack is \( \sim 6 \times 10^5 \) µm^2, more than the recommended surface area by Korber et al. to obtain representative data for Pseudomonas biofilms.\(^{158}\) Given a peg’s surface area is 109 mm^2 with 4-5 mm of the peg depth immersed in solution,\(^ {159}\) 2 z-stacks captured for each peg represents \( \sim 1/44^{th} \) of a pegs total surface area. S. aureus biofilms were identified as immotile coccal structures, irreversibly attached and of appropriate size (0.5-2µm diameter) and morphology, existing in characteristic clusters and towers of microcolonies.\(^{160}\) Visualization of the green to orange hazy matrix was often seen surrounding coccal structures. A three-dimensional (3D) projection image, which is a compression of all images in a z-stack to a single picture, was created for each z-stack using the Leica Application Suite Advanced Fluorescence Software (LAS AF). Biofilm biomass, which is the overall volume of the biofilm, was computed by the biofilm quantification software Comstat 2, by obtaining the number of biomass pixels in
all images of the stack multiplied by the voxel size \([(\text{pixel size}_x) \times (\text{pixel size}_y) \times (\text{pixel size}_z)]\) and divided by the substratum area of the stack, resulting in a volume/area ratio \(\left(\mu\text{m}^3/\mu\text{m}^2\right)\).\textsuperscript{161} The biomass of the live or green-staining (Syto-9) biofilms was quantified. Thresholds were set by a single investigator to remove any remaining background noise and artifact. The experiment was replicated in all 7 clinical isolates.

**Planktonic Bacterial Quantification**

After removal of the pegged lid of the MBEC device, planktonic bacteria at the bottom of the 96 well plates were quantified to determine if NO had any effect on planktonic detachment from mature biofilms, or if it caused planktonic death. The solution in each well was collected into Eppendorf tubes labeled according to the corresponding NO concentration in the wells. The tubes were centrifuged at 2000 rpm for 10 minutes, (Heraeus Biofuge Fresco, UK), the supernatant extracted, and the bacterial pellets re-suspended in the original volume of 200 µL using sterile 1x PBS. Serial 10-fold dilutions were performed up to \(10^{-6}\) and 20 µL of the diluted solutions were streaked in triplicate on Horse Blood Agar Plates for colony-forming unit (CFU) counting. All 7 clinical isolates were run in duplicate.

**Statistical Analysis**

GraphPad Prism 5.0 (San Diego, CA USA) was used to convert data to a logarithmic scale to remove differences between isolates and replicates and compute statistical significance between NO levels using one-way analysis of variance (ANOVA).

Genstat 13\textsuperscript{th} edition (VSN International Ltd., UK) was used to compute multi-stratum ANOVA of biomass on a logarithmic scale to again remove differences between
isolates and replicates and allow comparison of biomass readings between at high (125-1000 µM) vs. low (0.975-1.95 µM) NO concentrations.

2.4 Results

*Biofilm Formation of Clinical Isolates*

There was uneven growth of biofilm on the surface of the pegs, with the thickest growth seen at the air-fluid interface or meniscus. This area was avoided when capturing representative z-stacks. When assessing experimental reproducibility of biofilm growth in flow chambers, Heydorn *et al.* recommended acquiring images in the middle two-thirds of the flow channel and avoid the inlet which had typically greater biofilm growth due to structural heterogeneities.\(^{162}\) The z-stacks were thus recorded at areas of highest biofilm growth visualized in the middle 2/3\(^{rd}\) of the pegs. *Figure 2.2* shows scanning electron microscopy images of a peg demonstrating high-powered magnification of the *S. aureus* biofilms.
Figure 2.2: Scanning electron microscopy images of *S. aureus* ATCC 25923 biofilms on an untreated peg. A. 80x magnification B. 3500x, C. 50000X and D. 120000x magnification.

Biofilm forming capacity also differed across clinical isolates, with ATCC 25923 and isolates 1, 4 and 5 being stronger biofilm formers. **Figure 2.3** shows the average biofilm biomass readings of the untreated (control) pegs of ATCC and the different isolates.
Baseline Biofilm Biomass of S. aureus Isolates

![Graph showing biofilm biomass of S. aureus isolates](image)

**Figure 2.3:** Baseline biofilm biomass of ATCC and the 7 clinical isolates showing differences in biofilm formation across strains.

**Effects of Nitric Oxide on Biofilm Formation**

**Figure 2.4** and **Figure 2.5** are representative examples of 3D projection images of a peg surface exposed to different NO concentrations.

![3D projection images](image)

**Figure 2.4:** 3D projection images of Biofilm-forming reference strain *S. aureus* ATCC 25923. A. Un-treated control peg, B. Peg exposed to 0.976 μM NO donor, C. Peg exposed to 15.625μM NO donor & D. Peg exposed to 1000 μM NO donor.
Figure 2.5: Confocal scanning laser microscope 3D projection images of *S. aureus* Clinical Isolate 1. A. Un-treated control peg, B. Peg exposed to 0.976 µM NO donor, C. 15.625 µM NO donor & D. 1000 µM NO donor.

The biofilm growth pattern is similar in all isolates, with an inverse relationship of biofilm biomass to NO concentrations. One-way ANOVA of the logarithmic values showed statistically significant differences between NO concentrations in all 7 clinical isolates. **Figure 2.6** shows the scatter plot graphs of the 7 isolates on a logarithmic scale with the corresponding p values.
Figure 2.6: Scatter plot graphs of the *S. aureus* isolates. Biomass values are plotted on a logarithmic scale. Replicates were performed on the 7 clinical isolates.

Biomass on a logarithmic scale at high (125-1000 µM) vs. low (0.975-1.95 µM) NO concentrations were then compared using multi-stratum ANOVA. The compared concentrations were chosen based on the computed conversion of NO levels in µM NO donor to ppm, measured in healthy vs. diseased sinuses based on previous literature. The normal NO levels in the sinuses range from 6-25 ppm,\(^{130,154}\) equivalent to approximately 100-450 µM of the NO donor concentration, while diseased maxillary sinus levels range from 0.021-0.07 ppm,\(^{137,155}\) equivalent to 0.3-1.2 µM NO donor. The results were statistically significant between the two groups in all isolates. Table 2.1 shows the p-values and average biofilm biomass readings of ATCC & the 7 clinical isolates.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>High NO</th>
<th>Low NO</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC</td>
<td>0.053</td>
<td>3.11</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.081</td>
<td>0.828</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>2</td>
<td>0.029</td>
<td>0.372</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>3</td>
<td>0.014</td>
<td>0.294</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>4</td>
<td>0.038</td>
<td>0.485</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>5</td>
<td>0.184</td>
<td>0.586</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>6</td>
<td>0.006</td>
<td>0.144</td>
<td>0.002</td>
</tr>
<tr>
<td>7</td>
<td>0.048</td>
<td>0.585</td>
<td>0.02</td>
</tr>
<tr>
<td>Ave Biomass 1-7</td>
<td>0.057</td>
<td>0.470</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.1: Average biofilm biomass of ATCC & clinical isolates. The two groups are significantly different. High NO = 125-1000 µM, Low NO= 0.975-1.96 µM.
Colony forming unit counting of Planktonic Bacteria

There was no clear universal pattern in planktonic count between isolates. There were also different trends between replicates of some isolates. The most common pattern shared between isolates 1, 2, 6 and one repeat of isolate 7 was an increase in CFU counts in the middle NO concentrations, with a drop in the higher and lower NO concentrations. However, the opposite pattern was seen in isolates 3, 5 and the 2nd repeat of isolate 7. Other patterns were also present. Figure 2.7 shows the linear graph of the average CFU counts of ATCC & 7 clinical isolates.

Figure 2.7: Line graph of the average planktonic bacterial CFU counts released from the biofilms of S. aureus ATCC 25923 and the 7 clinical isolates exposed to various NO concentrations, showing no general pattern within or between strains, hence no further statistical testing was performed. Results include replicates performed on the 7 clinical isolates.

2.5 Discussion

In this study, we examined the effects NO has on established S. aureus biofilms in an in vitro setting. Altering its concentrations to test for anti-biofilm effects has confirmed
NO’s ability to disrupt *S. aureus* biofilms at high concentrations, while at lower concentrations, enhanced biofilm formation was observed. This highlights the ubiquitous nature of nitric oxide, clearly exhibiting its multiple, often contradicting functions in the human body. From neurotransmission to blood flow, cytoprotection to host defense, this naturally occurring free radical can be found exerting its effects on almost every major organ system. It can act locally, such as in vasodilation and wound healing, or distantly such as in an aerocrine fashion, inhaled from the upper airways to the lungs to improve ventilation-perfusion. Its diverging roles are clearly demonstrated in its pro- and anti-inflammatory properties as it functions as a regulator of the immune response.

The results of this study exemplify this dualistic nature of NO, showing that changes in concentration can alter its effect from anti- to pro-biofilm forming. NO’s anti-biofilm capacity on other organisms has been illustrated in the past, such as in the study by Barraud *et al.* on *Pseudomonas* biofilms using a different NO donor and concentration range. A study by Shlag *et. al.* showed that nitrite, a known end-product of NO metabolism, inhibited *S. aureus* biofilm formation, but it had yet to be determined what effects NO had on mature & established *S. aureus* biofilms, more relevant to its application in CRS. Surprisingly, our study showed that NO’s anti-biofilm range on CRS-isolated *S. aureus* is similar to the NO concentrations measured in sinuses of healthy individuals based on previous literature, while biofilm formation seems to have an affiliation for lower NO concentrations, similar to the range measured in CRS sinuses. Although these findings can in part, potentially explain the greater incidence of sinus infection in CRS patients, who have measurably lower sinus NO levels, the underlying reason for changes in *S. aureus* biofilm formation at varying NO
concentrations still need to be further explored. *S. aureus* itself produces its own NO for metabolic use through the synthesis of bacterial nitric oxide synthase (bNOS) using L-arginine as substrate,\textsuperscript{126} a mechanism similar to human NO production. This endogenous NO was shown to protect *S. aureus* from anti-microbial therapy. Up-regulation of certain genes in *S. aureus* upon NO exposure has also been documented.\textsuperscript{128} These could be a few of potentially several combative mechanisms that *S. aureus* exert when exposed to sub-lethal NO concentrations, hence leading to a flourishing of bacterial biofilm growth in this environment.

Quantitative assessment of biofilms was performed in this study as an objective means to compare biofilm growth across isolates and NO concentrations. The variations in biomass readings between isolates and replicates were noted, but were not surprising. Heydorn *et al* explains that biofilm development is in part a stochastic process, and independent rounds of biofilm experiments will never be exactly the same despite maintaining constant conditions.\textsuperscript{161} Nonetheless, the biofilm growth pattern and trend in response to NO was similar in all isolates. Planktonic growth on the other hand, had no distinct pattern across isolates, with pattern differences noted even between replicates of the same isolate. Because of the absence of a general trend in planktonic growth, no further statistical testing was done. These findings show that NO likely has no associated effect on planktonic growth or release during the biofilm lifecycle.

In a study published by Kirihene *et al*, it was shown that enlargement of the maxillary sinus ostial size significantly lowers measurable nasal and sinus NO levels in CRS patients\textsuperscript{163} possibly preventing NO re-accumulation in the sinus cavities. Taking into conjunction the results of this study, this may explain the existence of the subset of
patients who experience recurrent infection with *S. aureus* after endoscopic sinus surgery (ESS) and are extremely difficult to treat. However, a conclusion as to the series of events that lead to recurrent *S. aureus* infection in these patients is far from being reached. It remains uncertain whether the low NO levels is the cause or consequence of bacterial biofilm infection. Nasal NO levels have been demonstrated to increase with symptom score improvement in CRS patients treated either medically or surgically but the role biofilm eradication plays in this correlation remains to be determined. Further investigation is required to better understand this pathophysiologic relationship.

Incorporation of the host response is also crucial in the further elucidation of the NO-*S. aureus* biofilm interaction, a limitation of a pure in-vitro study. Host factors will most likely alter this interaction. Biofilm attachment to sinonasal mucosa, for example may be more difficult to eradicate with NO due to the additional presence of mucus and pus and higher concentrations may be required. Progression into in-vivo studies is needed to further define the host-NO-biofilm triad.

### 2.6 Conclusion

Nitric oxide’s anti-microbial properties and the fact that it is naturally occurring and endogenously produced in the sinuses give it great potential to be used as an efficacious and safe anti-microbial agent. This study showed that NO has anti-biofilm effects on *S. aureus* biofilms in an in-vitro setting, but only at higher concentrations. Enhanced biofilm formation at lower NO concentrations may explain the difficulty in treating recalcitrant CRS patients who are colonized with *S. aureus*. Challenges to attain and sustain the key anti-biofilm concentration range in the sinuses will be expected, as well as determining an effective and feasible delivery system. Further studies are needed to
address these issues, and determine what role host factors will play in this NO-biofilm interaction.
Chapter 3: Gene Expression Differences in Nitric Oxide and Reactive Oxygen Species Regulation Point to an Altered Innate Immune Response in Chronic Rhinosinusitis

Conducted at the Department of Otolaryngology Head and Neck Surgery, University of Adelaide, Adelaide, Australia

Financial assistance from The Garnett Passe and Rodney Williams Memorial Foundation
3.1 Abstract

**Background:** The complex interplay between host, environment and microbe in the aetiopathogenesis of chronic rhinosinusitis (CRS) remains unclear. This study focuses on the host-microbe interaction, specifically the regulation of nitric oxide (NO) and reactive oxygen species (ROS) against the pathogenic organism *Staphylococcus aureus* (*S. aureus*). NO and ROS play crucial roles in innate immunity and in the first line defense against microbial invasion.

**Methodology:** Sinonasal tissue samples were harvested from CRS and control patients during surgery. CRS patients were classified *S. aureus* biofilm positive (B+) or negative (B-) using Fluorescence in situ hybridization and clinically as polyp positive (P+) or negative (P-). Samples were assessed using a Nitric oxide PCR array containing 84 genes involved in NO and ROS regulation and gene expression of all sub-groups were compared to each other.

**Results:** 23 samples were analysed with 31 genes significantly changed, the greatest seen in the B+P+ CRS patients. 4 genes consistently displayed differential expression between the groups including the cytoprotective OXR1 (Oxidation Resistance 1) and PRDX6 (Peroxiredoxin 6), NCF2 (Neutrophil cytosolic factor 2) and the PRNP (Prion Protein) genes.

**Conclusion:** Alteration in gene expression points to impaired innate immune responses differing among CRS sub-groups based on *S. aureus* biofilm and polyp status. The consistent alteration of four genes among distinct groups demonstrates that *S. aureus* biofilms and polyps are associated with specific changes in gene expression. Further studies are required to validate these findings in a wider cohort of patients and correlate this to protein expression and disease manifestation.
3.2 Introduction

The aetiopathogenesis of chronic rhinosinusitis (CRS) has yet to be completely elucidated. As a chronic inflammatory condition, the role of the adaptive immune response in CRS has been abundantly described in the literature.\(^{164,165}\) However, there is increasing interest in investigating the role of innate immunity in this complex disease.\(^{30,32,166}\) The sinonasal tract, due to its location, is exposed to a variety of inhaled pathogens on a daily basis and serves as the primary barrier against noxious substances.\(^{167}\) Its ability to combat and prevent infection from bacteria and viruses, demonstrates an active innate immunosurveillance in normal individuals.\(^{30}\) In CRS patients, where inflammation is continuous and uncontrolled, an impaired first-line defense to pathogens can potentially be one of the first triggering mechanisms in the disease process, inevitably leading to an ongoing cycle of chronic inflammation and infection.

*Staphylococcus aureus* (*S. aureus*) may be the key microorganism in this host-pathogen interaction. *S. aureus* has been associated with more severe and recalcitrant forms of CRS, its isolation predicting poorer patient outcomes despite appropriate medical and surgical therapy.\(^{65,152}\) Its existence in biofilm form has been demonstrated in patient sinuses, which makes successful treatment even more difficult.\(^{64,69}\) Evidence of *S. aureus* biofilms’ role in the alteration of the innate immune response in CRS patients, if present, has yet to be provided.

Nitric oxide (NO) and reactive oxygen species (ROS) are key players in host innate immunity, involved in direct bacterial defense and inflammatory cell regulation.\(^{168}\) However, they can also cause negative effects such as cell death and carcinogenesis.
when dysregulation of production or elimination occurs. With this delicate balance required, it is important to identify and further describe the genes involved in NO and ROS regulation. This will determine if gene expressions are significantly altered in CRS patients, and provide a crucial glimpse of the underlying innate immunity mechanisms that are impaired in CRS.

This study therefore aims to determine if gene expression involved in nitric oxide and reactive oxygen species regulation are altered in CRS patients and whether S. aureus biofilm plays a role in this alteration. This will provide a baseline description of the host-pathogen relationship and important clues to the further understanding this complex disease process.

3.3 Methodology

Patient Clinical Data

After approval from the local Human Research Ethics Committee (Ethics number 2010055), consent for tissue collection was obtained from each patient prior to endoscopic sinus surgery for CRS. Patient demographics including age, allergies, medication and pre-operative symptom scores were recorded. Lund-MacKay scores were recorded from patient sinus CT scans. CRS patients were classified at the time of surgery as either with (CRSwNP) or without (CRSsNP) nasal polyps based on published guidelines with the addition of strict exclusion criteria. Patients who do not fulfill the CRSwNP definition of polyps visible in the middle meatus were excluded from the study. Patients positive for fungus based on microscopy or culture were also excluded from the study.
Patients in the control group were also consented for tissue to be used for research purposes. All were undergoing endoscopic sinus surgery (commonly an endoscopic approach to non-secreting pituitary tumor) for reasons other than CRS. Those with symptoms of CRS or evidence of sinus disease on imaging were excluded from the study.

_Tissue Collection and Storage_

Mucosal tissue samples were harvested from the sinonasal cavity at the time of surgery and placed in Dulbecco's Modified Eagle Medium (Gibco, Invitrogen Life Technologies, VIC, Australia) for transport to the laboratory. Diseased ethmoidal tissue was used from the CRS patients while non-diseased ethmoidal tissue was taken from the control patients. Tissue samples are then removed of bone fragments and 8x8 mm² pieces of tissue were cryofrozen for future biofilm analysis. Remaining tissue was transferred into RNAlater solution (Ambion, Life Technologies, Grand Island, NY). Samples were stored overnight at 4°C to allow complete saturation of tissue in solution, followed by storage in -80°C for future use.

_S. aureus Biofilm Analysis using Fluorescence-in-situ Hybridization_

Tissue samples stored for biofilm assessment were defrosted and analyzed for the presence or absence of _S. aureus_ biofilms using Fluorescence-in-situ-hybridization as per previously published departmental protocol using a _S. aureus_-specific Alexa 488 probe (AdvantDx, Woburn, MA). Briefly, mucosal samples were washed three times in milliQ water to remove any planktonic bacteria. Samples were then air dried at 55°C for 20 minutes on glass microscope slides and immersed in 90% alcohol for 10 minutes. The _S. aureus_ FISH probe was applied and hybridization occurred for 90 minutes at
55°C in a moisture chamber. Samples were then immersed in the prepared wash solution for 30 minutes and again air dried, then mounting medium and coverslips applied. These were then viewed under the Leica TCS SP5 confocal scanning laser microscope (Leica Microsystems, Wetzlar Germany) at 20x and 40x magnification. An excitation wavelength of 488nm with emission range of 495 – 540nm was used to detect S. aureus biofilms with the entire sample systematically scanned. Representative z-stacks were recorded of between 30-40 consecutive images with a slice thickness of 1-2um. Biofilm status was assessed by a blinded investigator who was not aware of the patient’s disease classification (CRSwNP, CRSsNP or control).

**RNA Extraction and Quality Assessment**

One-hundred milligrams (100 mg) of tissue stored in RNAlater was measured into 5 mL tubes and homogenized using the TissueRuptor (Qiagen, VIC, Australia) for a maximum of 20 minutes or until complete homogenization occurred. RNA was then extracted using the RNeasy Lipid tissue Mini Kit-QIAzol method according to the manufacturer's protocol (Qiagen). DNAse treatment was performed on all samples to remove genomic DNA contamination using the RNase-Free DNase Set (Qiagen).

RNA quality was checked using the Experion Electrophoresis Station (BioRad, NSW, Australia). Samples were run through using the Experion RNA StdSens Kit according to manufacturer's instructions. RNA quantification was performed using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE) prior to polymerase chain reaction (PCR) array analysis.
Real-Time PCR Nitric Oxide Array

One microgram (1 µg) of total RNA was reverse transcribed to complementary DNA (cDNA) using the RT2 First Strand Kit (SA Biosciences, Qiagen, VIC, Australia) with the incubation steps performed by the MyCycler Thermal Cycler (BioRad). Synthesized cDNA was then mixed with the RT2 qPCR Master Mix (SA Biosciences) and 25 µL of the mixture aliquoted to each well of a 96-well formatted Nitric Oxide Human PCR Array (SA Biosciences, Qiagen) as per manufacturer’s instructions. DNA amplification was performed using the iCycler with iQ5 Software (BioRad, NSW, Australia).

Statistical Analysis

The CRS patient groups were each compared to the control group and to each other. Data analysis to compare 2 groups was performed using the SA Biosciences RT² Profiler PCR Array Data Analysis Template v3.3 (SABiosciences, Qiagen).

3.4 Results

Patient Demographics and Classification

Twenty-three patients were included in the study; 6 control and 17 CRS patients. Mean age was 41.2 years (range 18-72) with a M:F ratio of 10:13. None of the CRS patients were on antibiotics or oral steroids for at least 2 weeks from the date of surgery. CRS patients were further classified based on polyp and S. aureus biofilm status with 6 patients belonging to the biofilm positive polyp positive (B+P+) group, 6 patients in the biofilm negative polyp negative (B-P) group and 5 patients in the B+P- group. Patients in the B+P+ group have undergone more extensive sinus surgery than the other 2 groups with all undergoing revision surgery. A standardized clinical symptom scoring system was also recorded using a scale of 1-5 for each CRS symptom, including anterior
rhinorrhoea, post nasal drip, facial pain, headache, sense of smell and nasal obstruction. A score of 1 means the absence of symptoms and 5 being the most severe. Table 3.1 lists the patient profiles of the 3 CRS groups. All control patients had primary surgeries for endoscopic resection of pituitary macroadenoma with no evidence of sinus disease, mucosal inflammation or infection at the time of surgery.

<table>
<thead>
<tr>
<th>Patient Group</th>
<th>Mean Pre-op Symptom Score (Total 30)</th>
<th>Number of revision cases</th>
<th>Mean Lund-Mackay Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>B+ P+</td>
<td>18.8</td>
<td>6/6</td>
<td>17.3</td>
</tr>
<tr>
<td>B+ P-</td>
<td>15.4</td>
<td>2/5</td>
<td>8.6</td>
</tr>
<tr>
<td>B- P-</td>
<td>14.8</td>
<td>0/6</td>
<td>8.1</td>
</tr>
</tbody>
</table>

Table 3.1: Patient profile of CRS patients classified base on biofilm and polyp status. B+P+ = Biofilm positive Polyp positive; B+P- = Biofilm positive Polyp negative; B- P- = Biofilm negative Polyp negative.

Patient Biofilm Status

Tissue samples stored for S. aureus biofilm analysis were viewed using FISH by a single blinded investigator and defined as areas of clustered fluorescence with an intense blush surrounding bacterial elements of 0.5-3 µm in size and shape arranged in a characteristic 3 dimensional structure.66 Figure 3.1 shows representative examples of patient biofilm status. Control patient tissue samples were also analyzed for S. aureus biofilms to ensure the absence of incidental S. aureus biofilm presence/colonization. All control patients in this study were biofilm negative.
Figure 3.1: Representative images of *S. aureus* biofilm status using fluorescence-in-situ hybridization. Note the clusters of brightly fluorescent bacterial cocci with a surrounding blush in A. Biofilm positive (B+) vs. B. Biofilm negative (B-) tissue samples.

**Gene Regulation Differences**

A total of 31 genes were significantly altered when comparing 2 different patient groups with the greatest number of changes between the B+P+ and Control groups, having 15 genes significantly altered. *Table 3.2* lists these corresponding genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold Regulation</th>
<th>p-value</th>
<th>Gene Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCF2</td>
<td>+2.8</td>
<td>0.03</td>
<td>Neutrophil Cytosolic Factor 2</td>
</tr>
<tr>
<td>PRNP</td>
<td>-1.6</td>
<td>0.02</td>
<td>Prion Protein gene</td>
</tr>
<tr>
<td>PRDX5</td>
<td>-2.4</td>
<td>0.03</td>
<td>Peroxiredoxin 5</td>
</tr>
<tr>
<td>PRDX2</td>
<td>-2.5</td>
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<td>Peroxiredoxin 2</td>
</tr>
<tr>
<td>PRDX6</td>
<td>-2.7</td>
<td>0.003</td>
<td>Peroxiredoxin 6</td>
</tr>
<tr>
<td>CAT</td>
<td>-2.7</td>
<td>0.003</td>
<td>Catalase</td>
</tr>
<tr>
<td>EGFR</td>
<td>-3.3</td>
<td>0.01</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>DUOXI</td>
<td>-3.7</td>
<td>0.01</td>
<td>Dual Oxidase 1</td>
</tr>
<tr>
<td>OXR1</td>
<td>-4.3</td>
<td>0.0001</td>
<td>Oxidation Resistance 1</td>
</tr>
<tr>
<td>NME5</td>
<td>-6.4</td>
<td>0.02</td>
<td>Non-metastatic cell 5</td>
</tr>
</tbody>
</table>

*Table 3.2*: Selected genes significantly changed in the B+P+ group when compared to controls. Fold regulation: (-) down-regulated; (+) up-regulated.
Of the 84 genes in the array, only 4 genes were consistently changed when comparing the 4 patient groups. Table 3.3 summarizes the group comparisons, the 4 genes and fold-regulation. Oxidation Resistance 1 or OXR1, involved in the protection of cells against oxidative damage\textsuperscript{172} is persistently down-regulated in the B+ groups regardless of polyp status. PRDX6 is also persistently down-regulated in the B+ groups. Coding for Peroxiredoxin 6, this gene is also involved in cellular protection against oxidative stress.\textsuperscript{173}

<table>
<thead>
<tr>
<th>B+P+ vs. Control</th>
<th>B+P- vs. Control</th>
<th>B-P- vs. Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCF2</td>
<td>OXR1</td>
<td>PRDX6</td>
</tr>
<tr>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B+P+ vs. B-P-</th>
<th>B+P+ vs. B+P-</th>
<th>B+P- vs. B-P-</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCF2</td>
<td>OXR1</td>
<td>PRDX6</td>
</tr>
<tr>
<td>↑</td>
<td>↓</td>
<td>↓</td>
</tr>
</tbody>
</table>

Table 3.3: Genes significantly and consistently changed upon comparison of the 4 groups. B+P+ = Biofilm positive Polyp positive; B+P- = Biofilm positive Polyp negative; B- P- = Biofilm negative Polyp negative. ↑: gene up-regulated; ↓: gene down-regulated. P value < 0.05.

Neutrophil Cytosolic Factor 2 or NCF2, a gene coding for part of the NADPH complex and thus involved in the oxidative burst to combat bacterial invasion,\textsuperscript{174} is consistently up-regulated in the P+ group but down-regulated in the P- group. Lastly, PRNP, a gene coding for the prion protein, was slightly but consistently and significantly down-regulated in the B+ group, but becomes up-regulated in the B+P+ group relative to the
B+P-group, thereby showing up-regulation when the polyp status changes in B+ groups.

3.5 Discussion

In this study, we examined the genes involved in NO and ROS regulation in sinonasal tissue samples of CRS patients compared to those without the disease using a NO Human PCR microarray. NO and ROS are key players in the function of a normal innate immune response, responsible for the first line defense in eliminating pathogens and in the destruction of phagocytosed bacteria. As natural by-products of aerobic respiration, they can also cause unwanted cellular damage when production overwhelms the body’s antioxidant response, ultimately leading to cell death via apoptosis and necrosis. This highlights that a delicate regulatory balance is needed to ensure that an appropriately functioning innate immune response exists, and that regulation of pro and anti-oxidant compounds form a critical component of an effective innate immune response.

Surprisingly, out of the 84 genes evaluated by the NO array only 4 genes were differentially expressed when comparing the specific patient groups. However, it must be noted that the B-P+ group is not present for comparison, as the group could not be completed. Only 1 patient fulfilled the category over a span of 2 years of tissue collection. This is likely due to the extremely close association of polyp patients with S. aureus biofilms in our patient population. In a recently published departmental study by Foreman et. al., 17 out of the 22 CRSwNP patients (77%) were positive for bacterial biofilms, 50% of which were S. aureus. Despite these supporting results, further studies with greater patient numbers are needed to truly determine if S. aureus biofilms are
more prevalent in nasal polyposis, and what the clinical implications would be in terms of patient management.

Two genes, OXR1 and PRDX6 were consistently down-regulated in *S. aureus* biofilm positive patients. OXR1, a gene present in all eukaryote genomes, has been shown to exhibit a response to oxidative stress in human cells with its corresponding protein providing protection from oxidative damage. In a study by Oliver et al, it was demonstrated that a lack of OXR1 increased neurodegeneration noting significant DNA damage in a mouse model, while overexpression confers cytoprotection. PRDX6, which codes for a protein found in all major mammalian organs, has been shown to have highest level of expression in epithelial layers such as the olfactory epithelium and lungs. Apart from functioning as an antioxidant enzyme, it also aids in the repair of damaged cell membranes by reducing phospholipid peroxidation. PRDX6 knock-out mice for instance, exhibited a greater sensitivity to oxidative stress with reduced survival time upon exposure to 100% oxygen. This was accompanied with greater lung injury, inflammation and oedema.

A significant down-regulation of OXR1 and PRDX6 in B+ patients regardless of polyp status may be linked to an inherently impaired host innate immunity with low antioxidant gene expression, thereby causing an increase in cellular death and apoptosis at the sinonasal mucosal interface of these patients. This breach of mucosal barrier function may serve as a rich surface for *S. aureus* biofilm formation, further promoting pathological immune cell activation. Alternatively, the biofilms themselves may affect the innate immune response directly, decreasing OXR1 and PRDX6 expression leading to an increase in ROS to combat bacterial infection. Whichever the scenario, these
results point to the fact that *S. aureus* B+ CRS patients have significantly altered gene expressions pertaining to ROS detoxification and cellular protection compared to B- and control patients. This implies that *S. aureus* biofilms in CRS are playing a role in triggering the host immune response which cannot effectively combat its presence. This may potentially help explain why this subset of patients with *S. aureus* biofilms continues to do poorly despite maximum medical or surgical therapy.

NCF2 is another gene identified in the NO array, to show a significant up-regulation in patients who are both B+P+ while being down-regulated in B-P- patients compared to control. NCF2 is one of the 4 genes coding for a subunit of NADPH oxidase, an essential component of the innate immune response responsible for effective superoxide production. Gene defects in NADPH oxidase lead to recurrent bacterial and fungal infection and granulomatous inflammation, a condition called chronic granulomatous disease. An up-regulation of NCF2 mRNA in B+P+ patients, and a down-regulation of NCF2 in B-P- patients compared to controls, indicates that the presence of biofilms and polyps in these patients is associated with an increased mRNA expression of NCF2. This suggests that B+P+ patients are attempting an appropriately directed, albeit ineffective innate immune response against *S. aureus* biofilms. The up-regulation in B+P+ patients but lack of significant difference when comparing expression of B+P- to B-P- and controls may indicate that the presence of polyps is needed to elicit NCF2 up-regulation in B+ patients. Alternatively, it may imply that the NCF2 mRNA up-regulation correlates with the development of nasal polyps. Studies looking into the association of biofilm status and nasal polyps have so far concluded that biofilm presence is independent of nasal polyposis, but further studies are needed to define this pathophysiologic relationship.
PRNP, coding for the prion protein, is found to be slightly but consistently down-regulated in B+ patients but up-regulated in P+ vs P- patients. The prion protein, through a still unknown mechanism, is associated with the development of neurodegenerative disorders when the human-encoded form is converted to its disease isoform.\textsuperscript{181,182} Prion diseases also correlate with greater inflammation as evidenced by the higher expression of pro-inflammatory cytokines.\textsuperscript{183,184} The role of PRNP gene expression alterations in CRS has yet to be fully explored and needs to be clarified by further research.

Surprisingly, gene expression of the NO synthase (NOS) genes, involved in NO production, was not significantly changed in this study. This goes against the findings of previous reports which show that the inducible NOS form is increased in CRS patients especially in those with polyp disease.\textsuperscript{185,186} This may be either due to a difference of patient classification in this study, which incorporates \textit{S. aureus} biofilm status, or the small patient numbers. Future studies with greater patient numbers are needed to investigate and confirm these findings.

### 3.6 Conclusion

Alterations of gene expression involved in ROS regulation are present in CRS patients, with the greatest changes seen in those with the presence of \textit{S. aureus} biofilms. This may explain why the clinical observation of greater disease severity and poorer outcomes are seen in this subset of patients, although the conclusion of whether \textit{S. aureus} biofilm is the cause or consequence of greater gene changes cannot be made. Nasal polyposis also seems to play a role in gene regulation, associated with certain gene expressions as evidenced in changes to NCF2 and PRNP. This implies that it may
be an independent factor affecting ROS regulation. 4 genes of interest have been identified: OXR1, PRDX6, NCF2, and PRNP which play different, but important roles in the innate immune response. Validation of this array data with greater patient numbers using quantitative real-time (qRT)-PCR coupled with corresponding protein analysis of these genes need to be performed to obtain a clearer picture of their role in CRS.
Chapter 4: Inflammasome Gene Expression Alterations in *Staphylococcus aureus* Biofilm-associated Chronic Rhinosinusitis

Conducted at the Department of Otolaryngology Head and Neck Surgery, University of Adelaide, Adelaide, Australia

Financial assistance from The Garnett Passe and Rodney Williams Memorial Foundation and The Hospital Research Foundation, Basil Hetzel Institute

**NOTE:**
This publication is included on pages 81-100 in the print copy of the thesis held in the University of Adelaide Library.
Chapter 5: Liposome-encapsulated ISMN: a Novel Nitric Oxide-Based Therapeutic Agent against *Staphylococcus aureus* Biofilms

Conducted at the Department of Otolaryngology Head and Neck Surgery, University of Adelaide, and The Ian Wark Institute, University of South Australia, Adelaide, Australia

Financial assistance from The Garnett Passe and Rodney Williams Memorial Foundation and The National Health and Medical Research Foundation (Application 1047576)
5.1 Abstract

**Background:** *Staphylococcus aureus* in its biofilm form has been associated with recalcitrant chronic rhinosinusitis with significant resistance to conventional therapies. This study aims to determine if liposomal-encapsulation of a precursor of the naturally occurring antimicrobial nitric oxide (NO) enhances its desired anti-biofilm effects against *S. aureus*, in the hope that improving its efficacy can provide an effective topical agent for future clinical use.

**Methodology:** *S. aureus* ATCC 25923 biofilms were grown *in-vitro* using the Minimum Biofilm Eradication Concentration (MBEC) device and exposed to 3 and 60 mg/mL of the NO donor isosorbide mononitrate (ISMN) encapsulated into different anionic liposomal formulations based on particle size (unilamellar ULV, multilamellar MLV) and lipid content (5 and 25 mM) at 24 h and 5 min exposure times. Biofilms were viewed using *Live-Dead* Baclight stain and confocal scanning laser microscopy and quantified using the software COMSTAT2.

**Results:** At 3 and 60 mg/mL, ISMN-ULV liposomes had comparable and significant anti-biofilm effects compared to untreated control at 24 h exposure (p=0.012 and 0.02 respectively). ULV blanks also had significant anti-biofilm effects at both 24 h and 5 min exposure (p=0.02 and 0.047 respectively). At 5 min exposure, 60 mg/mL ISMN-MLV liposomes appeared to have greater anti-biofilm effects compared to pure ISMN or ULV particles. Increasing liposomal lipid content improved the anti-biofilm efficacy of both MLV and ULVs at 5 min exposure.

**Conclusion:** Liposome-encapsulated “nitric oxide” is highly effective in eradicating *S. aureus* biofilms *in-vitro*, giving great promise for use in the clinical setting to treat this burdensome infection. Further studies however are needed to assess its safety and efficacy *in-vivo* before clinical translation is attempted.
5.2 Introduction

The battle against bacterial infections persists despite discoveries of new and broader spectrum antibiotics. Over use of antibiotics promotes the emergence of drug-resistant strains which makes successful treatment even more challenging. Thus the global quest for better and more efficacious antimicrobial agents continues.

*Staphylococcus aureus* (*S. aureus*) remains one of the most commonly known opportunistic pathogens colonizing up to 2 billion people worldwide. Ranging from chronic skin infections\textsuperscript{216} and allergic dermatitis,\textsuperscript{217} to osteomyelitis\textsuperscript{218} and sepsis,\textsuperscript{83} *S. aureus* has great versatility in contributing to diseased states in both healthy and immunocompromised individuals.\textsuperscript{219} Its existence in biofilm form has been well documented,\textsuperscript{65,220} partially explaining the difficulty in eradication and its ability to cause a repeated cycle of infection. Encased in an extra-polymeric substance matrix which enhances microbial survival and impairs antimicrobial penetration,\textsuperscript{47} bacterial biofilms require up to 1000x greater antibiotic dose for effective treatment compared to their planktonic counterparts.\textsuperscript{221} This contributes to treatment failure and emphasizes the need for the development of alternative antimicrobial treatment strategies.

In particular, the isolation of *S. aureus* biofilms in the sinuses of patients with chronic rhinosinusitis (CRS) has been linked to poorer clinical outcomes and disease prognosis\textsuperscript{66,69,152}, further defining the importance of this microorganism in disease severity. Although antibiotics initially improve patients’ signs and symptoms, recurrence after treatment cessation is often the case, demonstrating the limitations of the use of antimicrobial compounds.
In the sinuses of healthy individuals, the endogenous gas nitric oxide (NO) has been documented to be highly concentrated, sometimes reaching maximum allowable air pollutant levels\textsuperscript{130} while the levels found in CRS patients are significantly lower.\textsuperscript{135,137,155} NO has demonstrated antibacterial and antiviral properties\textsuperscript{154}, plays a role in innate immunity and mucociliary clearance\textsuperscript{133} and is thought to provide a significant contribution to maintaining the balance of normal flora in non-diseased sinuses. Recent evidence has shown NO to also have anti-biofilm effects against a wide range of organisms\textsuperscript{222}, including \textit{S. aureus}\textsuperscript{145}. Its natural occurrence in the body in conjunction with its antimicrobial properties gives it great potential for topical application against \textit{S. aureus} biofilm-associated infections, both within and outside the confines of the paranasal sinuses.

Whereas high NO levels have been shown to have anti-biofilm effects on established \textit{S. aureus} biofilms, we have demonstrated that low NO levels increase the quantity of a \textit{S. aureus} biofilm biomass.\textsuperscript{223} The dualistic effect of NO on \textit{S. aureus} biofilms indicates that the desired anti-biofilm effects are critically dependent on the NO concentration and exposure time.\textsuperscript{223} Therefore there is a need to improve NO’s formulation prior to clinical application as a topical therapy. Isosorbide mononitrate (ISMN), a clinically approved NO-donor, has a well-established safety profile in a range of applications.\textsuperscript{224,225} Conversely, a number of liposome-based drugs are already clinically approved thereby demonstrating safety for clinical use.\textsuperscript{226,227} Liposomes typically increase drug specificity, lessen the risk of adverse drug reactions, decrease the required dose and prolong drug-release, all ideal properties of a suitable topical anti-biofilm agent. In addition, liposomes have been found to be effective in delivering antibiotics
and other therapeutics to various biofilms. Importantly, the inherent bactericidal and anti-biofilm effects of cationic and anionic liposomes are well demonstrated.

This motivated the design of a novel ISMN liposomal formulation towards the development of a novel topical treatment against *S. aureus* biofilms with potential clinical applications in *S. aureus* biofilm-associated diseases such as CRS. We specifically aimed to determine the synergistic anti-biofilm effects obtained with unilamellar and multilamellar anionic liposomal (ULV and MLV respectively) formulations of ISMN. To this end, anionic ULV and MLV liposomes were prepared and their efficacy tested *in vitro.*

### 5.3 Methodology

*Nitric oxide donor*

99% pure grade ISMN (Bosche Sci, NB, New Jersey) was used as the NO donor. ISMN was chosen for liposomal encapsulation due to its well-documented side effect profile and its established safety for human use in the field of cardiology.

Characterization of the anti-biofilm and anti-planktonic effects of the free drug form was first performed. Since the maximum aqueous solubility of ISMN was at 60 mg/mL, serial dilutions down to concentrations of 3.75 mg/mL were tested for anti-biofilm effects. CSF broth was used as the diluent to ensure that it was not the absence of bacterial culture media that was causing the desired effect. Anti-planktonic properties were obtained by reading the optical density (OD) at 540 nm at the bottom of each well of the challenge plate.
Culture and formation of S. aureus biofilms

A biofilm forming reference strain *S. aureus* American Type Culture Collection (ATCC) 25923 was used to test against the different liposomal-NO formulations. *S. aureus* culture and biofilm formation was performed as previously described. Briefly, a single loop of *S. aureus* glycerol stock was defrosted at 37°C in 2 mL of cerebrospinal fluid (CSF) broth (Oxoid, Australia) for 18-24 hours under agitation. 1 loop of culture was then plated onto a blood agar plate (Oxoid, Australia) and incubated for 18-24 hours at 37°C, following which 1-2 bacterial colonies were immersed in 0.45% saline to create a 1 McFarland unit (MFU) solution (3 x 10^8 colony forming units/mL). This solution was then diluted to 1:15 in CSF broth, 150 µL of which was pipetted into each well of a 96-well plate of the Minimum Biofilm Eradication Concentration (MBEC) biofilm-forming device (Edmonton, Canada).

The MBEC device was used as per manufacturer’s instructions. The pegs suspended on the lid were immersed into the 96-well plate containing the bacterial solution and incubated for 44 hours at 35°C on a gyrorotary shaker (Ratek, Vic, Aus) at 70 rpm, allowing the biofilms to form on each peg’s surface.

Liposomal preparation

Anionic liposomes were prepared containing egg lecithin:dipalmitoylglycero-phosphoglycerol (DPPG) at 4:1 mol ratio. The required amount of lipids was weighed into a 25 ml round bottom flask and dissolved in 5 ml of chloroform. The chloroform was slowly removed under reduced pressure using a Buchi rotary evaporator (Buchi, Germany) to deposit a thin film of dry lipid on the inner wall of the flask. The dry lipid film was hydrated with 5 ml of blank phosphate-buffered saline (PBS) solution or PBS solution containing either 3 mg/ml or 60 mg/ml of ISMN for at least 1.5 hours at a
temperature of 5°C above the phase transition temperature of the main lipid to obtain the MLV vesicles. ULV were produced from MLVs by extrusion through 800 nm, 400 nm and 200 nm pore size polycarbonate membranes in a Lipex 10 ml Thermobarrel Extruder (Burnaby, BC Canada). In the anti-biofilm activity studies, freshly prepared ULV and MLV ISMN liposomal formulations were used without purification. The latter approach was selected to increase the translational potential of the proposed ISMN liposomal formulations.

**Liposome Characterization**

**Particle Size Analysis**

The particle size of the blank liposomes and ISMN-loaded liposomes were characterised using a dynamic light scattering (DLS) technique which has a size detection range of 0.6 nm to 6 μm (Malvern Zetasizer Nano ZS, UK). Liposomes were diluted 100-fold with 10 mM sodium chloride (NaCl) aqueous solution prior to measurement at 25°C. Water (refractive index = 1.33) was used as the dispersant in the DLS analysis. A typical liposome refractive index of 1.45 was used.\(^{233}\) Size distribution results are expressed as the z-average diameter (i.e. the intensity-weighted mean hydrodynamic diameter) together with the polydispersity index (PDI) indicating the width of the size distribution.

**Determination of Zeta-potential**

Liposomes were diluted 100-fold with 10 mM NaCl aqueous solution prior to the measurement of zeta potentials. Zeta potentials were determined by a using phase analysis light scattering (PALS) technique (Malvern Zetasizer Nano ZS, UK) at 25 °C, with the detection limit of 5 nm to 10 nm particles. The mean zeta potential was
computed based on the electrophoretic mobility (\textit{i.e.} the ratio of the velocity of particles to the field strength) by applying the Smoluchowski or Huckel theories.

Determination of drug encapsulation efficacy
Liposomes were ultra-centrifuged at 30,000 rpm at 4°C for one hour. The supernatant was taken and diluted with mobile phase and analysed by high performance liquid chromatography (HPLC) to determine the amount of free drug ($C_{\text{free}}$). The pellet phase was rehydrated in 1 mM PBS. 0.2% Triton X-100 was added to break the phospholipid structure and free the entrapped drug. The mixture was sonicated (30 min) and centrifuged at 20,000 rpm for 30 min. The supernatant was taken, diluted and analysed by HPLC to determine the amount of encapsulated drug ($C_{\text{encapsulated}}$). The encapsulation efficiency ($En\%$) was calculated using the following equation:

$$En\% = \frac{C_{\text{encapsulated}}}{(C_{\text{free}} + C_{\text{encapsulated}})} \times 100$$

\textit{HPLC Analysis of ISMN}
An HPLC method employing UV detection was used for quantification of ISMN-containing samples (Shimadzu, Japan). Chromatographic separation was performed on a LiChrospher RP C$_{18}$ column (5 μm, 4.6 mm ID x 150 mm, Grace Davison Discovery Science, Rowville, VIC) at a detection wavelength of 196 nm. The mobile phase was a mixture of methanol and water (20:80 v/v), eluted at a flow rate of 1.5 ml/min. The sample was injected at a volume of 50 μl at 40°C. The average retention time was 3.4 min; detection limit of the method was 15 ng/ml. The linearity range of the method used was 0.1–10 μg/ml with an $R^2$ (correlation coefficient) value of 0.998. Within-day precision was < 3% and between-day precision was < 4%.
Exposure of S. aureus biofilms to the Liposome-encapsulated ISMN

All liposomal experiments were repeated twice. The S. aureus biofilm-coated pegs prepared using the MBEC device were washed for 1 minute in 1x PBS to remove planktonic bacteria and exposed to a challenge plate containing 180 µL of the liposomal test agent added with 20 µL of 0.4 mM L-Arginine (Musashi, Vic Aus) to mimic a bacterial culture state for 24 hours at 37°C. Based on these results, the liposomal formulations with the best anti-biofilm effects were selected and tested at a shorter exposure time of 5 minutes, chosen to better simulate the rapid exposure time of topical douching into the sinuses. After 5 minutes of exposure to the challenge plate, the pegs were re-immersed into a new 96-well plate containing 180 µL of CSF broth with 20 µL of 0.4 mM L-Arginine and incubated for 24h.

Biofilm Imaging and Quantification

After treatment exposure, the pegs were washed twice in 0.9% NaCl for 1 minute and 10 seconds respectively to remove planktonic bacteria as per manufacturer’s instructions. These were then fixed in 5% glutaraldehyde (Sigma Aldrich, St Louis MO) for 45 minutes, followed by a repeat wash in 0.9% NaCl for 10 seconds to remove excess fixative. The pegs were then individually placed in 1 mL sterile milliQ water (Millipore, Billerica, MA) containing 1.5 µL each of the LiveDead Baclight stains (Invitrogen Molecular Probes, Vic Aus) Syto 9 and propidium iodide and incubated on a rotating mixer at 10 rpm (Pelco, CA USA) in the dark at room temperature for 15 minutes. The pegs were again rinsed in 0.9% NaCl for 10 seconds and individually mounted on cover slips for viewing under the Leica TCS SP5 confocal scanning laser microscope (Leica Microsystems, Wetzlar, Germany).
2 representative z-stacks each containing 120 +/- 5 serial images set at 0.7 μm distance between 2 images were obtained per peg. Biofilm quantification of each z-stack was then calculated using the COMSTAT 2 software.\textsuperscript{161}

**Statistical Analysis**

Graphpad Prism 5.0 (San Diego Ca) was used to calculate one-way analysis of variance (ANOVA) when comparing biofilm biomass of more than 2 treatment groups with Bonferroni multiple comparisons (95% confidence interval) as post hoc test using the R statistical software (R Foundation for statistical computing, Vienna, Austria). Unpaired t-test was used to compare 2 groups for the anti-biofilm effects of the pure drug ISMN. A p value of < 0.05 was considered statistically significant.

**5.4 Results**

*Characterization of Liposomes*

Anionic liposomes were prepared from Egg lecithin and DPPG (4:1 molar ratio) using the standard thin film hydration method. ULV liposomes were prepared from MLV’s using standard extrusion. The physicochemical properties of the samples, namely hydrodynamic diameter and zeta-potential, were obtained using dynamic light scattering and are presented in Table 5.1. The average hydrodynamic diameter of the as prepared MLV was 692 nm (PdI= 0.7). After membrane extrusion, ULV liposomes were obtained with an average hydrodynamic diameter of 351 nm (PdI= 0.6). The incorporation of ISMN during the hydration step had little effect on the size of the liposomes, although a small reduction was observed for the ISMN loaded MLV liposomes (536 nm, PdI= 0.8) and a small increase was observed for the ISMN loaded
ULV liposomes (384 nm, PDI= 0.2). The zeta potential was measured in $10^{-2}$ M NaCl and as expected all liposomal preparations displayed negative potentials. The zeta potential values have been shown to be sufficient with excellent colloidal stability.

<table>
<thead>
<tr>
<th>Liposome</th>
<th>Lipid Composition</th>
<th>Hydrodynamic diameter (nm)</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>Egg lecithin : DPPG 4 : 1</td>
<td>692</td>
<td>351</td>
</tr>
<tr>
<td>ISMN</td>
<td>ISMN</td>
<td>536</td>
<td>384</td>
</tr>
</tbody>
</table>

Table 5.1: Particle size and zeta-potential of the prepared anionic liposomes. ISMN: isosorbide mononitrate; MLV: multilamellar; ULV: unilamellar.

Next the ISMN encapsulation efficiency was determined using HPLC at two lipid concentrations, 5 mM and 25 mM. As expected, the encapsulation efficiency was higher at higher lipid concentrations for both ULV (1.3% at 5 mM vs. 6.3% at 25 mM) and MLV (1.5% at 5 mM vs. 10.7% at 25 mM) liposomes (Table 5.2), while the increase in the lipid concentration had no significant impact on the particle size.

<table>
<thead>
<tr>
<th>Liposome</th>
<th>En%</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>MLV</td>
</tr>
<tr>
<td>Ani-ISMN-5mM</td>
<td>1.47 ± 0.32</td>
</tr>
<tr>
<td>Ani-ISMN-25mM</td>
<td>10.73 ± 1.51</td>
</tr>
</tbody>
</table>

Table 5.2: Drug encapsulation efficiency of anionic liposomes loaded with 60 mg/mL of isosorbide mononitrate. MLV: multilamellar; ULV: unilamellar; En%: percentage of drug encapsulated.
Anti-biofilm and anti-planktonic effects of ISMN (free-drug)

There was variability of biofilm growth of the untreated control pegs across all experimental runs. These findings are similar to our previous in-vitro biofilm studies, and is attributed in part to the stochastic process of biofilm development despite maintaining constant growth conditions. One-way ANOVA failed to show statistical significance when comparing all treatment groups. A global overview however, shows that biofilm growth at the lowest ISMN concentration of 3.75 mg/mL was increased, compared to the untreated control peg. (Figure 5.1) The unpaired t-test was then used to determine if statistical significance was present when comparing 2 groups. There was still no statistical significance between the 3.75 mg/mL ISMN vs. untreated control (unpaired t-test, p = 0.21). However, subsequent and increasing concentrations of ISMN showed anti-biofilm effects, resulting in an almost complete eradication at 60 mg/mL ISMN dose with statistical significance (unpaired t-test vs. control, p = 0.024). This paradoxical pattern of enhanced biofilm growth at low NO concentrations and anti-biofilm effects at higher concentrations is consistent with findings from our previous studies using the NO donor DetaNONOate. The lowest ISMN concentration with statistically significant anti-biofilm effects compared to the untreated control was at 15 mg/mL (unpaired t-test vs. control, p = 0.025).

The anti-planktonic effects were also tested in the free-drug form to completely characterize ISMN’s antibacterial effects in order to obtain the best possible dose for liposomal encapsulation. Planktonic growth is shown to be inversely proportional to ISMN concentrations with the greatest anti-planktonic effect at 60 mg/mL (One way ANOVA, p = <0.0001). Figure 5.1 summarizes these findings.
Figure 5.1: Dose-response of pure ISMN on *S. aureus* biofilm biomass (A) and planktonic cell growth (B). 4.64 is the biofilm biomass at 3.75 mg/mL ISMN concentration. ISMN: Isosorbide mononitrate. Data represents Mean +/- SD of a duplicate experiment. *, P < 0.05; **, P < 0.01, ***, P < 0.001 compared to untreated control; Two-tailed t test.
Anti-biofilm efficacy of ISMN liposomal formulations at 24-hour exposure

To establish the anti-biofilm potential of the ISMN-Liposomal formulations, anionic ULV liposomes prepared with different ISMN doses (3 mg/mL and 60 mg/mL) were tested using initially a contact time of 24 hours. Their anti-biofilm activity was compared to untreated control pegs (S. aureus biofilms grown for 44 hours on pegs immersed in pure CSF broth), corresponding ULV blanks, and liposome-free treated control of pure drug 60 mg/mL ISMN.

The results are summarized in Figure 5.2. One-way ANOVA using Bonferroni comparisons test showed that there was a significant decrease in biofilm biomass in pegs treated with both the 3 mg/mL ISMN and 60 mg/mL ISMN ULV liposomal formulations when compared to their corresponding untreated control (respectively 190 fold decrease- 0.024 µm³/µm² vs. untreated control 4.56 µm³/µm² p= 0.0013; and 2126 fold decrease- 0.005 µm³/µm² vs. untreated control of 10.63 µm³/µm², p= 0.003). Blank ULV liposomes also resulted into a significant decrease in the biofilm biomass (152 fold decrease; 0.03 µm³/µm² for 3 mg/mL ISMN p= 0.0013; and 2650 fold decrease; 0.004 µm³/µm² for 60 mg/mL ISMN, p= 0.003 respectively) compared to the untreated control. Comparing ULVs incorporating either 3 or 60 mg/mL ISMN vs. their corresponding blanks however showed no statistical significance using unpaired t-test (p> 0.05). In agreement with our mechanistic study, ISMN alone had a very strong negative effect on the biofilm biomass at a dose of 60 mg/mL (26,575 fold decrease; 0.0004 µm³/µm² p= 0.003). There was no significant difference in anti-biofilm effects of ISMN alone compared to ULV at 3 or 60 mg/mL ISMN and their blanks (p> 0.05). These results indicate that both ISMN alone, ULV alone and both compounds combined diminish S. aureus biofilms in vitro after 24 hours.
Figure 5.2: Effects of ISMN, ULV Blank and ISMN-ULV liposomes on *S. aureus* biofilm biomass after 24 hours. The numbers above the bar represents the biofilm biomass of the untreated controls. ULV: Unilamellar liposome; ISMN: Isosorbide mononitrate. Data represents Mean +/- SD of a duplicate experiment. A replicate experiment showed the same trend. *, P < 0.05; **, P < 0.01, ***, P < 0.001 vs untreated control; Bonferroni’s multiple comparisons test.
Effects of ISMN-liposome formulations in short term (5 minutes) exposure

Based on these results, we wanted to see if the anti-biofilm effects of the above formulations were maintained at a time of 5 min incubation mimicking the shorter contact time of a topical wash into the nose and paranasal sinuses. One-way ANOVA showed a significant decrease in biofilm biomass at 3 mg and 60 mg/mL ISMN ULV liposomes compared to untreated control (respectively 33 fold at 0.25 µm³/µm², p=0.008; and 922 fold at 0.009 µm³/µm², p=0.007 vs. untreated control of 8.3 µm³/µm²).

Blank ULV liposomes also significantly reduced the *S. aureus* biofilm biomass compared to the untreated control (15 fold, 0.55 µm³/µm² vs. 8.3 µm³/µm², p=0.009). Although the results did not reach statistical significance, ISMN-liposomal formulations showed stronger anti-biofilm effects than blank liposomes, especially at the higher ISMN dose. The 60 mg/mL liposomal formulation had a greater anti-biofilm effect than the liposome-free ISMN at the same dose (922 fold; 0.009 µm³/µm² for 60 mg/ml ISMN ULV vs. 69 fold; 0.12 µm³/µm² for 60 mg/ml ISMN alone, p>0.05). (Figure 5.3) Together, these results demonstrate the anti-biofilm effects of ULV liposomes, ISMN free drug and ISMN-ULV liposomes also at short (5 minutes) exposure times, in particular for higher (60 mg/ml) ISMN concentrations.
Building on the above results, 60 mg/mL ISMN liposomal formulations were now selected to investigate the anti biofilm efficacy of this approach at a 5 min incubation duration altering lipid composition. A non-statistically significant decrease was observed in the biofilm biomass when treated with the 60 mg/mL ISMN ULV liposomal formulation at a 5 mM lipid concentration compared to the blank ULV liposome at the same lipid concentration (1.1 $\mu$m$^3$/µm$^2$ vs. 1.7 $\mu$m$^3$/µm$^2$, p> 0.05). However, both groups showed a significantly reduced biomass compared to the untreated control (4.4 $\mu$m$^3$/µm$^2$, p= 0.01 for ULV loaded and p= 0.04 for ULV Blank) but no significant difference compared to the ISMN treated group (1.1 $\mu$m$^3$/µm$^2$). Next a 60 mg/ml ISMN-ULV liposomal formulation at 25 mM lipid concentration was tested. Unlike the lower lipid concentrations, both blank ULV and ISMN ULV
liposomal formulations significantly reduced the biofilm biomass (1.1 µm³/µm², p= 0.01 for Blank and 0.6 µm³/µm² for loaded, p= 0.004) compared to the untreated control. No significant inhibiting effects were however observed compared to the pure-drug ISMN control. Although no statistical difference was attained between the ISMN liposomal formulations at 5 mM and 25 mM, the higher lipid concentration tended to reduce the biofilm biomass to a greater extent than the lower concentration counterpart (0.6 µm³/µm² for 25 mM vs. 1.1 µm³/µm² for 5 mM p > 0.5).

Next a 25 mM lipid MLV formulation loaded with 60 mg/mL ISMN was tested and compared to the ULV formulations and untreated and pure-drug ISMN controls. As shown in Figure 5.4, the 60 mg/mL ISMN loaded MLV formulation resulted into a significant decrease in the biofilm biomass in comparison to the untreated control (0.003 µm³/µm² vs. 4.4 µm³/µm², p= 0.002). Although the MLV formulation had stronger anti-biofilm effects compared to its ULV counterpart at similar ISMN and lipid concentration (0.003 µm³/µm² for MLV vs. 0.6 µm³/µm² for ULV) the results did not reach statistical significance. This was the same when comparing the MLV formulation with the pure-drug ISMN (0.003 µm³/µm² vs. 1.14 µm³/µm²). The blank MLV also resulted in a biofilm biomass decrease when compared to the untreated control (1.4 µm³/µm² vs. 4.4 µm³/µm², p= 0.02). Although there was a decrease in biofilm biomass with the encapsulated 60 mg/mL ISMN MLV formulation compared to its blank (0.002 µm³/µm² vs. 1.4 µm³/µm²), the results did not reach statistical significance with the Bonferroni test. Figure 5.5 shows representative 3D projection images of S. aureus biofilms exposed to the MLV liposomes.
Figure 5.4: Biofilm biomass of *S. aureus* ATCC 25923 exposed for 5 minutes to MLV liposomes of 25 mM lipid composition. Data shows MLV Blank and MLV liposomes encapsulating 60 mg/mL of the NO donor ISMN. MLV: Multilamellar liposome, ISMN: Isosorbide mononitrate. Data represents Mean +/- SD of a duplicate experiment. *, P < 0.05; **, P < 0.01, ***, P < 0.001; Bonferroni multiple comparisons test.

Figure 5.5: 3D projections of *S. aureus* biofilms on pegs exposed for 5 minutes to MLV liposomes with a 25 mM lipid composition. A. Untreated control peg, B. MLV blank liposomes, C. MLV 60 mg/mL ISMN loaded liposomes, and D. 60 mg/mL pure drug ISMN.


5.5 Discussion

In this study, the anti-biofilm effect of liposomal-formulations of ISMN against *S. aureus* was demonstrated. *In vitro* experiments indeed showed that short exposure times mimicking nasal flush induced a strong reduction in the biofilm biomass. These also showed that modification of liposome size and lipid content dramatically alters its efficacy. Our results may guide which liposomal characteristics are critical in the formulation of liposomes encapsulating compounds for optimal topical delivery to *S. aureus* biofilms.

There is a vast existence of *S. aureus*-related infections where effective anti-biofilm therapy is needed. Infections of the skin, bone, heart (endocarditis), sinuses, and device-related infections (catheters, implantable prosthetics) could be targeted with specifically designed liposomes encapsulating the appropriate drug dose. Liposomes, with their phospholipid bilayer, can be modified to deliver drugs to specific physiologic targets to obtain the desired therapeutic efficiency. Alteration of the lipid composition and concentration in the bilayer have been demonstrated in past studies to have significant effects on the extent of adsorption into *S. aureus* biofilms. These liposomal properties thus change the effectiveness of the encapsulated drugs in performing their action. Given the degree of difficulty of biofilm matrix penetration by topical antimicrobials, which originates in a combination of physical and metabolic barriers, ensuring that highly efficient drug doses diffuse into and reach embedded bacterial cells, is key for effective biofilm eradication. Bacterial properties such as cell wall hydrophobicity have also been shown to affect liposome penetration and hence drug mobility through the biofilm matrix. In this study, the *S. aureus* biofilm-
liposome interaction is yet to be explored and further characterization of this relationship is required.

We chose to use anionic liposomes by virtue of their well-documented anti-biofilm properties against *S. aureus*,\(^{240,241}\) with an aim to obtain a synergistic effect with the NO donor ISMN and improve overall efficacy. Furthermore, a comparison of ULV and MLV formulations was carried out. ULV liposomes were demonstrated to have anti-biofilm effects even without the encapsulated NO donor and these effects were more pronounced compared to their MLV counterparts. Although beyond the scope of this study, many factors may alter this antimicrobial effect of bare liposomes. A direct interaction of the liposome with the bacterial cell wall may play a role in better biofilm penetration.\(^{242}\) Bacterial cell wall properties, different for gram positive and negative bacteria, have been shown to be an important factor in nanoparticle penetration of biofilm matrix.\(^{243}\) Changing expression of cell-wall proteins can completely switch bacterial surface properties from hydrophilic to hydrophobic without altering biofilm structure, thus significantly altering susceptibility of bacteria to nanoparticles\(^{244}\) such as liposomes. Thus, characterization of the physicochemical properties of the targeted bacteria is needed to develop specifically designed liposomes in order to produce desired anti-biofilm results. Although the liposomal-bacterial interaction was not characterized in detail in this in-vitro study, a clearer description of this interaction is therefore warranted prior to in-vivo and clinical application.

When comparing the ISMN liposomal formulations of 5 mM and 25 mM lipids, liposomes of higher lipid concentration tended to be superior in reducing the biofilm
biomass in comparison to the lower concentration counterpart. In addition, according to
the encapsulation study, the drug encapsulation efficiency in liposomes of high lipid
concentration was at least 5-fold higher than that of the lower concentration counterpart.

It is likely that the encapsulation efficiency plays a significant role in defining the extent
of drug delivery to the biofilm. In this study, it was deliberately chosen not to purify the
ISMN liposomal formulations towards facilitating their translational uses against CRS.
However, this approach further increased the complexity of the system from a structure-
activity point of view since both free ISMN and encapsulated drug is present in the
formulation. Further mechanistic understanding of how the liposomal formulations
affect the delivery of the NO donor to the biofilm is required, as well as elucidating the
how the synergistic role of liposomes and ISMN in reducing the biofilm biomass.

Although ULV liposomes have been to date the preferred clinical option considering
their optimal pharmacokinetics in blood, in the context of topical treatment such as in
CRS, MLV liposomes present many advantages over their ULV counterparts. They are
indeed easier to manufacture, and are more stable with longer storage in liquid form.
MLV liposomes will thus most likely offer a more straightforward translational path to
the creation of a topical sinus rinse. In this study, it was demonstrated that MLV
liposomes with greater lipid composition are comparable to ULV liposomes in terms of
anti-\textit{S. aureus} biofilm effects encapsulating the same ISMN concentration of 60
mg/mL. Thus in future \textit{in vivo} studies, MLV liposomes will most likely be the approach
in the conversion to clinical application in the context of CRS.
Providing alternative antimicrobial agents that are safe and effective, will hopefully contribute to decreasing antibiotic use, and consequently reduce the risk of developing drug resistance. With MRSA emerging as a global concern and a shift in pharmaceutical interest away from antibiotic therapy due to lesser profitability, the development of novel antimicrobials is urgently needed. Despite the necessity to further improve the proposed liposomal ISMN formulations and characterize their mechanisms of action, the identification of the efficacy of high-lipid anionic MLV liposomes proves an important first step in the successful topical utilization of liposomal-encapsulated nitric oxide to treat *S. aureus* biofilm infection in CRS.

5.6 Conclusion

Liposomal formulation of ISMN has significant anti-biofilm effects against *S. aureus*, showing greatest efficacy with higher lipid content in both ULV and MLV systems. Future in-vivo studies are required however, to determine their safety prior to attempts at a topical clinical application.
Chapter 6: Liposomal Formulation of Nitric Oxide Donor: A Novel Topical Treatment for *Staphylococcus aureus* Biofilm-associated Rhinosinusitis

Conducted at the Department of Otolaryngology Head and Neck Surgery, University of Adelaide, and The Ian Wark Institute, University of South Australia, Adelaide, Australia

Financial assistance from The Garnett Passe and Rodney Williams Memorial Foundation and The National Health and Medical Research Foundation (Application 1047576)
6.1 Abstract

Background:

Refractory chronic rhinosinusitis (CRS) has been repeatedly associated with *Staphylococcus aureus* biofilm infection. The endogenous anti-microbial agent nitric oxide (NO) is found at extremely low levels in CRS sinuses and at high levels in healthy sinuses. As a novel treatment modality, we have designed a liposomal-formulation of nitric oxide (NO) donor (LFNO) using isosorbide mononitrate (ISMN) as a topical sinus wash, and we report its safety and efficacy against *S. aureus* biofilms in a rhinosinusitis sheep model.

Methodology:

24 sheep were included: 8 safety sheep: 4 saline controls, and 4 treatment flushed with LFNO twice daily for 7 days. Heart rate (HR), Blood Pressure (BP) and mean arterial pressure (MAP) were monitored, and tissue samples harvested for histology and ciliary analysis. 16 sheep had frontal sinus *S. aureus* biofilms grown and treated with different agents: 4 Saline, 4 LFNO, 4 pure drug ISMN and 4 sheep with liposomal blanks. Tissue samples were harvested after 3 days and *S. aureus* biofilms quantified using Fluorescent In Situ Hybridisation (FISH) and Comstat 2. Biofilm growth was compared using a two-tailed unpaired t-test.

Results:

A transient increase in HR and decrease in MAP 10 minutes after flushing with LFNO (p<0.001) was seen, with return to baseline levels in between flushing times. No associated clinical side effects were observed. Greater inflammation was seen in the control group (p=0.01) and greater epithelial thickness in the treatment group (p=0.021), but no significant difference in ciliary preservation. 3 out of 4 sheep had significantly
lower biofilm biomass in the LFNO group, which was only seen in 1 other sheep in the other treatment arms using ISMN solution.

**Conclusion:**

Liposomal formulation of ISMN appears to be a safe and effective topical therapy against *S. aureus* biofilms in CRS, although improvements in formulation may be needed to decrease the known risk of NO side effects such as tachycardia and hypotension. Proceeding to well-controlled clinical trials will be the next crucial step.

**6.2 Introduction**

With the focus of current medical therapy on the treatment of widespread infections and systemic diseases, it is not surprising that localized chronic conditions are seldom prioritized. Chronic rhinosinusitis (CRS) is one of those debilitating long-standing diseases with no cure even though it has significant prevalence and is associated with poor quality of life. Patients suffer from persistent nasal congestion, nasal discharge and post nasal drip, but loss of sense of smell and pain such as frontal headaches and facial pain can also be a feature. Failure of treatment can result in complications, of which acute exacerbations can lead to spread of infection to the orbit and brain, causing blindness, meningitis and a high risk of mortality. Up to 16% of the western population is affected, leading to an average of 18-22 million patient visits to a physician a year in the United States (USA). Its protracted course means ongoing treatment, and whether requiring medical or surgical therapy, puts significant burden to both patient and society.

Recalcitrant sinus infection does not in most cases respond to normal therapies, and recently, a substantial research effort has been directed to fill this gap. The emergence
of new topical washes such as manuka honey, chemical surfactants such as baby shampoo, and topical antibiotics such as Moxifloxacin and Mupirocin, have attempted to address infection associated with CRS with the aim to provide an antimicrobial component to topical therapy. Research has found *Staphylococcus aureus* (*S. aureus*) to be associated with greater disease severity and more unfavorable clinical outcomes, equating to a higher frequency of post-operative visits and lower quality of life scores. *S. aureus* existence in biofilm form in infected sinuses enhances its survivability to antimicrobial therapy, making eradication even more challenging.

In healthy human sinuses, nitric oxide (NO) is an endogenous molecule that exists in extremely high concentrations. Proven to have anti-microbial, anti-viral, and anti-fungal effects, and to enhance mucociliary clearance, NO is typically found at very low levels in sinuses of CRS patients in comparison to healthy sinuses. A number of diseases associated with the more severe forms of CRS such as cystic fibrosis and primary ciliary dyskinesia, have been observed to have very low to almost negligible levels of sinonasal NO. Both planktonic and biofilm forms of *S. aureus* have been shown to be susceptible to NO, but to our knowledge, no studies to date have completely explored NO’s therapeutic use within the sinuses, and no NO-based topical therapy exists that is designed specifically for use in CRS. Therapy utilizing endogenous compounds such as NO to combat infection in the sinuses may aid in the development of safe and effective topical antimicrobials whilst minimizing localized and systemic adverse effects.

To overcome the challenge of topical delivery of NO, we have recently developed a novel liposomal formulation of the NO-donor isosorbide mononitrate (ISMN).
Liposomes are currently used in drug delivery for a variety of existing therapeutic agents in the fields of cosmetics, cancer, gene therapy, as well as antibiotics. On the other hand, ISMN, is a NO-donor already clinically approved for use in the fields of Cardiology and Internal Medicine. Building on our successful in vitro studies which have demonstrated the anti-biofilm potency of the ISMN liposomal formulation, this study aims to determine the safety and the anti-biofilm effects of this novel formulation in an established animal model of rhinosinusitis.

### 6.3 Methodology

*Preparation of the different treatment agents*

Three formulations were developed for the different treatment groups for the in vivo comparison of anti-biofilm efficacy. ISMN solution (Pure ISMN) was prepared by dissolving 60 mg/mL of 99% pure grade ISMN (Bosche Sci, NB, New Jersey USA) in 1x phosphate-buffered saline (PBS). Liposomes formulation of the NO donor ISMN (LFNO) were prepared based on a previous in-vitro study aimed at screening liposomal formulations using the Minimum Biofilm Eradication Concentration (MBEC) device. Briefly, LFNO was prepared with 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC): dipalmitoylglycerol-phosphoglycerol (DPPG) at an 8:2 mol ratio. The required amount of lipids was weighed into a 500ml round bottom flask and dissolved in 80ml of chloroform. The chloroform was slowly removed under reduced pressure using a rotary evaporator (Buchi, Germany) to deposit a thin film of dry lipid on the inner wall of the flask. The dry lipid film was hydrated with 80ml of PBS solution containing 60mg/ml of ISMN for at least 1.5 hours at a temperature of 5°C above the phase transition temperature of the main lipid to obtain the LFNO. The blank liposomes (LB) were prepared in a similar manner as the LFNO, but the dry lipid film was hydrated with pure...
PBS solution. Both LFNO and LB appeared visually clear. Particle size analysis using dynamic light scattering suggested the LFNO has a mean particle size of 969.6nm (PdI=0.7), and the LB has a mean particle size of 1026nm (PdI=0.6). The ISMN encapsulation efficiency as determined using HPLC suggested a 7.24% (w/w) drug encapsulation in the LFNO.

**Safety Study**

*Sheep telemetry implantation*

Eight sheep were used in the safety component of the study. Each sheep was implanted with the TL11M2-D70-PCT implant (DSI, MN, USA), which serves as the internal device and monitors the sheep’s heart rate (HR) and blood pressure (BP). This was placed surgically between the sheep’s upper lateral thigh and lower ribs on the flank in a subcutaneous pocket. A pressure probe connected to the device was fed into the femoral artery, which directly monitored the HR and systolic and diastolic pressures. The sheep were then allowed to recover from the procedure for a minimum of 2 weeks in single pens each with an attached transmitter to its wall to capture signals from the internal device. Each transmitter was connected to a central computer located in a separate room, equipped with a specific software (Ponemah Physiology Platform, DSI, OH, USA) designed to record HR and BP readings continuously. The pens had a distance of 6 meters from each other to prevent cross transmission of signals.

*Sheep sinus minitrephination and instillation of agents*

After the 2-week convalescence period, the sheep underwent a 2nd procedure to access each frontal sinus. Each frontal sinus was accessed via a 1mm drill hole on the anterior table of the forehead, using a minitrephination set from Medtronic, MN USA. A
cannula was inserted into the hole (minitrephination) to allow the instillation of agents. This was capped in between flushes to prevent external contamination.

After 2 days of recovery and monitoring of baseline BP and HR, each sinus was flushed with 20 mLs of the assigned agent twice daily for 7 days, with a minimum of 5 hours between each flush.

Both frontal sinuses of sheep 1 to 4 were flushed with 20 mLs each of 0.9% NaCl (Control), while the frontal sinuses of sheep 5 to 8 were flushed with 20 mLs in each sinus of LFNO at a dose of 60 mg/mL (Treatment). Sheep were grouped into pairs during monitoring (Sheep 1 and 2, 3 and 4, 5 and 6, 7 and 8). During this time, continuous monitoring of vital signs via the transmitter occurred. The sheep were then euthanized on the 8th day, when the frontal sinus mucosa were harvested and assessed for inflammation, ciliary preservation and histological characteristics.

**Observation of sheep and Heart rate and Blood pressure recording**

Baseline heart rate (HR) in beats per minute (bpm), blood pressure (BP) and mean arterial pressure (MAP) readings were recorded within the 2 days of the rest period post-minitrephine insertion at a minimum of 2 hours of recordings. Subsequent readings were obtained continuously for the 7 day flushing period of the paired sheep, recording specifically the following time points clustered during flushing times:

- Time point 1: 10 minutes before flushing 1st sheep
- Time point 2: flushing of 1st sheep
- Time point 3: flushing of 2nd sheep and
- Time point 4: 10 minutes after flushing 2nd sheep
Time point 5: 2x 30 minute random readings taken after the morning and afternoon flushing periods

An average of 2 random readings recorded between flushes within a day were also recorded. Because sheep have been documented to react to NO in a similar manner to humans such as a drop in BP and increase in HR,\textsuperscript{258} it would be expected that they would show clinical and objective signs similar to humans should side effects develop. Sheep were monitored for clinical signs of hypotension or general discomfort for at least 1 hour after the last flush looking for: knee bending, fainting or teeth grinding. Within the control and treatment groups, HR, BP and MAP readings were compared between time points.

\textit{Ciliary count}

A 1 x 1 cm representative piece of mucosa from each frontal sinus was taken and processed in preparation for scanning electron microscopy (SEM). Briefly, samples were placed in 1mL 1x PBS and placed in a water sonicator (Soniclean, SA Australia) on high for 20 minutes twice to remove the surface mucous, changing the PBS in between sonication times. The samples were then placed in 1 mL of electron microscopy fixative (4% paraformaldehyde/1.25% glutaraldehyde in PBS with 4% sucrose) for at least overnight. They were then washed in washing buffer, fixed in osmium and dehydrated in 70%, 90% and 100% ethanol. Samples were then placed in a critical point dryer (Tousimis Autosamdr\textsuperscript{\textregistered} 931 Series Critical Point Dryer) and the prepared samples mounted and coated with carbon. Specimens were observed using a SEM (XL30, Philips, Eindhoven, Netherlands).
Four images at 2500x magnification were taken randomly from each sinus tissue sample, and each image divided with gridlines into 100 equal squares using Adobe Photoshop CC (version 14.0). Each square was given a grade of either 0, 0.5 or 1 based on the amount of ciliary coverage in each square, with 0 virtually having less than 25% ciliary coverage, 0.5 having 25-75% coverage, and 1 having >75% coverage. The total highest score obtainable per image is 100. Four images were captured per sinus tissue and each image scored over 100 based on cilia coverage of an image’s surface area.

**Histology**

Mucosal samples from each frontal sinus were stored immediately into 10% formalin (Sigma-Aldrich, MO USA) and sent to an accredited pathology laboratory (Adelaide Pathology Partners, SA Australia) for processing. A clinical pathologist blinded to the sheep groups then characterized and compared each sample looking specifically at: degree of inflammation, inflammatory cell predominance, goblet cell hyperplasia, epithelial thickness and fibrosis.

The degree of inflammation was semi-quantified, given a score of 1-3, 1 being mild: <30% of the superficial half of the stroma is occupied by inflammatory cells; a score of 2 for moderate: 30-60% occupied by inflammatory cells and 3 for severe: >60% occupied by inflammatory cells. Both acute (neutrophils) and chronic inflammatory cells (lymphocytes, histiocytes, plasma cells and mast cells) were assessed in the degree of inflammation. Epithelial thickness was measured at an area of maximum thickness for every sheep sinus sample under 10x magnification in millimeters (mm). Fibrosis was also assessed using a scale of 0 to 3, with 0 having no fibrosis to 3 having severe fibrosis.
**Efficacy Study**

*S. aureus biofilm growth and sheep bacterial inoculation*

The natural frontal sinus drainage ostium was accessed as per previously published departmental studies.\(^{256,257}\) Firstly, the sheep underwent minitrephination of the frontal sinus with identification of the natural opening by flushing 0.2% fluorescein dye (Phebra, NSW Australia) through the frontal sinuses. This identified the frontal sinus opening from within the nasal cavity which was then sealed with Vaseline ribbon gauze.

*S. aureus* ATCC 25923 (American Type Culture Collection) was used to inoculate the sheep frontal sinuses. One milliliter of 0.5 MFU (1 MFU= 3 x 10^8 colony forming units/mL) of *S. aureus* solution was used to inoculate each frontal sinus. *S. aureus* biofilms were allowed to flourish in the frontal sinuses of the live sheep for 7 days ensuring adequate biofilm growth as per previous established protocols.\(^{256}\)

**Treatment of sheep**

Sixteen sheep were classified into the different treatment groups with 4 sheep in each group: Normal Saline (Control), Pure ISMN, LB, and LFNO. The Vaseline ribbon gauze used to plug the natural frontal ostium was removed in all sheep sinuses prior to treatment. Both sinuses of the control group were flushed with normal saline. In the remaining three groups, one sinus was flushed with the agent, and one sinus flushed with normal saline. 20 mLs of the assigned agent was flushed in each sinus through the minitrephine, twice daily for 3 days, with a minimum of 5 hours in between flushes per day. The sheep were euthanized on day 4 with harvesting of sinus tissue.
**Imaging of S. aureus biofilms and quantification**

A 1 x 1 cm sample from each sinus was taken adjacent to the natural frontal ostium. This was to ensure that the flushed treatment contacted surface biofilm in every flush. Each sample was assessed for *S. aureus* biofilms using fluorescence in situ hybridization (FISH), in which a *S. aureus* specific probe Alexa 488 (AdvanDx, Woburn, MA) was used as per manufacturer’s instructions. Biofilm identification using this protocol has been performed and validated on our previous sheep studies.66,255

After processing, the samples were mounted on coverslips and viewed using a Leica TCS SP5 confocal scanning laser microscope (Leica Microsystems, Wetzlar Germany) using 20x magnification at an excitation wavelength of 488nm with an emission range of 495 – 540nm. A systematic scanning of each sample was performed and 3 areas with greatest biofilm growth were chosen. A z-stack of 100 sequential images with a slice thickness of 0.7 µm was captured per area. Z-stacks were then inputted into the biofilm quantification software, Comstat 2, assigning a threshold per z-stack to remove any background artifact, and obtain biofilm biomass readings. This was performed by a blinded investigator who was unaware of the treatment groups (AD).

**Statistical Analysis**

For ciliary preservation, data were initially summarized using means and medians stratified by treatment group. Group differences were then assessed using a linear generalized estimating equation (GEE) model to account for clustering of ciliary score by sinus and sheep. All analyses were performed using SAS 9.3 (SAS Institute Inc., Cary, NC, USA).
HR, BP and MAP readings were analyzed using a mixed effects model with treatment treated as a fixed effect and sheep, day and time within day (AM v PM) as random factors. The treating sheep, day and time of day as random factors removes all variability that is due to differences between sheep, differences due to day and differences due to time of day (AM and PM). All analyses were completed using SAS V9.3 (SAS Institute Inc., Cary, NC, USA).

Graphpad Prism 5 version 5 (San Diego, Ca) was used to compare biofilm biomass readings of the control and treatment sinus for each sheep using a two-tailed unpaired t-test. Mann-Whitney test was used to assess the degree of inflammation and epithelial thickness between the control and treatment group. A p value of <0.05 was considered statistically significant.

6.4 Results

Safety: Analysis of systemic effects

Observation of sheep

There were no clinical signs of hypotension or differences in behavior seen in the control or treatment sheep during or after flushing for 30 minutes after the last sinus flush.

Heart rate, blood pressure, and mean arterial pressure

The average HR, systolic and diastolic BP, and MAP were monitored. Because there were significant differences of baseline readings of the same time points between Control and Treatment groups (e.g. MAP at time point 1 was significantly different between the control and treatment sheep), comparisons between these 2 groups could
not be made. Thus we could only compare between 2 time points within the same group and not between groups. The time points listed above were compared within the control and within the treatment group: Time points: 1 vs. 2, 1 vs. 3, 1 vs. 4, 1 vs. 5, 2 vs. 3, 2 vs. 4, 2 vs. 5, 3 vs. 4, 3 vs. 5 and 4 vs. 5.

Systolic and diastolic BP changes were found to be reflective of the MAP changes using the mixed effects model and thus only the HR and MAP were compared. In the control group, the HR showed a significant increase from time point 1 (85 beats per minute or bpm) to point 2 (98 bpm, \( p=0.0002 \)) during the flushing of sheep sinuses. Although the HR at time point 3 was also increased, (91 bpm) it was not significantly different to time point 1 (\( p=0.099 \)). At time point 4 (10 minutes post-flushing, 85 bpm), the HR returned to its pre-flush levels comparable to time point 1 (\( p=0.97 \)). Although time point 4 had lower HR levels compared to time point 5 (92 bpm), the results were not statistically significant (\( p=0.056 \)).

Treatment sheep HR also showed an increase from time point 1 to time point 2 during the flushing of the 1st of the sheep pair, the results also statistically significant (80 vs 93 bpm, \( p=0.0002 \)). There was also an increase in HR at time point 3, but in contrast to the control group, this was statistically significant compared to time point 1 (106 bpm, \( p<0.0001 \)). HR slightly dropped as it approached time point 4 (103 bpm), but was still significantly higher compared to time point 1 (\( p<0.0001 \)). HR at time point 5 (79 bpm) was comparable to pre-flush levels of time point 1 (\( p=0.68 \)). These measurements demonstrate that a transient increase of HR immediately after treatment occurred but that the HR returned to pre-flush level before the start of the next flushing. Figure 6.1 highlights this trend.
Because the baseline MAP of time point 1 of the treatment was greater than the control sheep as also shown in Figure 6.1 (110 mmHg vs. 80 mmHg p <0.0001), inter-group comparisons could not be performed. The control group during the flushing time points of 2 and 3 showed a significant increase in MAP when compared to the pre-flush levels (90 and 93, p <0.0001), and a return to comparable levels by time point 4 (82, p = 0.33). There was no significant difference in MAP between time point 5 vs. 1 and 4 (p = 0.9 and 0.27 respectively).

In the treatment group, an increase in MAP was also seen from time point 1 to time point 2 (110 vs. 121, p<0.0001), but returned to almost pre-flush levels by time point 3 (113, p=0.16) and a decrease in MAP by an average of 10 at time point 4 (99, p<0.0001). Time point 5 had a comparable MAP to time point 4 (97, p=0.44).
Figure 6.1: Average heart rate (HR) and Mean arterial pressure (MAP) of control vs. treatment sheep. Note the increase in heart rate in time points 3 and 4 and a higher baseline MAP of the treatment group. * indicates statistical significance compared to Time point 1 within the group (p < 0.05). Time point 1: 10 minutes before start of flush; Time point 2: Flushing of the 1st of paired sheep; Time point 3: Flushing of the 2nd of the paired sheep; Time point 4: 10 minutes after the 2nd flush; Time point 5: 1-1.5 hours after the last flush.
**Analysis of localized effects on sheep sinus mucosa**

**Ciliary preservation**

The cilia scores over a total of 100, ranged between 24 to 96 with a mean of 62.6 in the control and 64.3 in the treatment group. The mean ciliary score was higher in the 4 sheep treated with LFNO compared to the Control sheep (Table 6.1) however, the difference was not statistically significant (Table 6.2: Mean Difference = 1.69; p = 0.86).

<table>
<thead>
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<th>n</th>
<th>Min</th>
<th>Max</th>
<th>Mean</th>
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<td>33.5</td>
<td>96</td>
<td>64.27</td>
<td>67.25</td>
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**Table 6.1: Mean, median and range (maximum – minimum) of ciliary preservation using Scanning electron microscopy**

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<th>Parameter</th>
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<th>Estimate</th>
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<th>Upper Confidence Limit</th>
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<tr>
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<td>Treatment</td>
<td>1.69*</td>
<td>9.37</td>
<td>-16.68</td>
<td>20.06</td>
<td>0.18</td>
<td>0.86</td>
</tr>
</tbody>
</table>

*Mean difference between the Treatment and Control group

**Table 6.2: Parameter estimates for the Generalized Estimating Equation model of ciliary preservation comparing control (saline) and treatment (LFNO) group. LFNO: liposomal-formulated nitric oxide.**

**Histology**

All of the control and treatment sheep samples had background inflammatory changes which has been noted in all our previously published trials using this animal model.\textsuperscript{255}
with all showing degrees of acute and chronic inflammation comprising of mostly lymphocytes, plasma cells and neutrophils. The degree of inflammation was found to be higher in the control than the treatment group (2.65 vs. 1.75, Mann-Whitney p = 0.01). Although the percentage of acute inflammatory cells was higher in the control group, the results were not statistically significant (16.25% vs. 9.375%, Mann-Whitney test p = 0.42).

All sheep sinuses had oedematous changes on histology, and all but sheep 8 had evidence of goblet cell hyperplasia. The degree of fibrosis was comparable in both groups, with only one sinus in each group showing mild fibrosis. Epithelial thickness was found to be greater in the treatment than control group (0.0625mm vs. 0.0425mm, Mann-Whitney test p = 0.021). Figure 6.2 summarizes these findings.

There was reactive atypia seen in one Control and two Treatment sinuses, but none of the samples showed any evidence of dysplasia, malignancy, granuloma, prominent scarring, mucosal ulcerations, polypoid or foreign body giant cell changes.
Figure 6.2: Histologic analysis of the control vs. treatment sheep sinus. Note the control group had significantly higher inflammation scores while the treatment group had significantly higher epithelial thickness. The percentage of acute inflammation was comparable between the 2 groups.

Anti-biofilm effects of treatment agents

Next a biofilm sheep model was used to determine the efficacy of the LFNO formulation. There was no statistical significance in biofilm growth when comparing each sinus within the four saline control sheep. In the LFNO group, three out of four sheep had significantly lesser biofilm growth on the treated vs. saline flushed sinus. Of the sheep treated with ISMN alone, one out of four had significantly lesser biomass in the treated vs. saline sinus, while in the LB group, three sheep had greater biofilm biomass on treated sinus, but none of them were statistically significant when compared
to the control side. **Figure 6.3** gives the average biofilm biomass for the treated vs. control sinus of each sheep group. **Table 6.3** gives the p-values for comparisons of treated vs. control sinus of each sheep within a group.

**Figure 6.3:** Average biofilm biomass of control vs. treatment sinus within each sheep group. Only the LFNO group showed statistical significance using two-tailed unpaired t-test (P=0.044). LFNO: Liposomal-formulated ISMN; ISMN: pure drug Isosorbide mononitrate; LB: Liposome blank. * p <0.05.
<table>
<thead>
<tr>
<th>Sheep Group</th>
<th>Control Sinus</th>
<th>Treated Sinus</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control S1</td>
<td>0.043</td>
<td>0.042</td>
<td>0.92</td>
</tr>
<tr>
<td>S2</td>
<td>0.035</td>
<td>0.068</td>
<td>0.14</td>
</tr>
<tr>
<td>S3</td>
<td>0.034</td>
<td>0.051</td>
<td>0.28</td>
</tr>
<tr>
<td>S4</td>
<td>0.083</td>
<td>0.068</td>
<td>0.6</td>
</tr>
<tr>
<td>LFNO S5</td>
<td>0.04</td>
<td>0.028</td>
<td>0.35</td>
</tr>
<tr>
<td>S6</td>
<td>0.081</td>
<td>0.02</td>
<td>0.038*</td>
</tr>
<tr>
<td>S7</td>
<td>0.054</td>
<td>0.019</td>
<td>0.044*</td>
</tr>
<tr>
<td>S8</td>
<td>0.027</td>
<td>0.014</td>
<td>0.018*</td>
</tr>
<tr>
<td>ISMN S9</td>
<td>0.017</td>
<td>0.015</td>
<td>0.27</td>
</tr>
<tr>
<td>S10</td>
<td>0.04</td>
<td>0.021</td>
<td>0.004*</td>
</tr>
<tr>
<td>S11</td>
<td>0.024</td>
<td>0.022</td>
<td>0.75</td>
</tr>
<tr>
<td>S12</td>
<td>0.015</td>
<td>0.016</td>
<td>0.88</td>
</tr>
<tr>
<td>LB S13</td>
<td>0.02</td>
<td>0.175</td>
<td>0.26</td>
</tr>
<tr>
<td>S14</td>
<td>0.032</td>
<td>0.037</td>
<td>0.56</td>
</tr>
<tr>
<td>S15</td>
<td>0.009</td>
<td>0.013</td>
<td>0.27</td>
</tr>
<tr>
<td>S16</td>
<td>0.022</td>
<td>0.036</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Table 6.3: Average biofilm biomass of control vs. treated sinus for each sheep classified to their specific treatment groups. The average is taken from 3 readings per sheep sinus. LFNO: Liposomal-formulated ISMN; ISMN: pure drug Isosorbide mononitrate; LB: Liposome blank.

6.5 Discussion

In this study, we determined the safety and efficacy of a liposomal formulation of the NO donor ISMN against *S. aureus* biofilms in an established rhinosinusitis sheep model. The formulation chosen was based on a recently concluded in-vitro project comparing the anti-biofilm effects of anionic liposomes with varying lipid content and ISMN doses. Based on this in vitro study, it was determined that a maximum dissolvable dose of 60 mg/mL ISMN encapsulated into anionic unilamellar (ULV) and MLV liposomes with a higher lipid content showed the best efficacy. In view of its
greater translational capabilities to a topical sinus wash, MLV liposomes were chosen based on their greater stability in liquid form.

For any prospect of clinical application, the safety profile of this novel anti-biofilm formulation needed to be thoroughly examined in a suitable animal model. Local mucosal effects as well as systemic side effects were both assessed in a sheep model. The comparability of mucosal ciliary and inflammatory cell count relative to the saline control demonstrates the localized safe characteristics of the ISMN liposomal formulation. The observed presence of inflammatory cells in both treatment and control mucosa is well recognized in this animal model, and thought to be due to the high prevalence of oestrus ovis infection. Although a histologic standardization of sheep sinus mucosa should be performed in the future, a lesser degree of inflammation compared to the control group demonstrates that LFNO has no significant localized pro-inflammatory effects.

The greater degree of epithelial thickness in the treatment group did not correspond to an increase in inflammatory cells. On the contrary, the inflammatory cell counts were higher in the control arm. In addition, the increase in epithelial thickness may reflect an epithelial hyperplastic response to bacterial eradication associated with decreased inflammation in the treatment group. The safety of LFNO is further strengthened by the absence of squamous metaplasia or dysplastic changes in the treatment specimens. Nonetheless close monitoring of potential subtle histologic changes should be performed in subsequent animal studies and clinical trials.
Vital signs were continuously monitored for 7 days in all safety sheep including direct observation for at least 1 hour after sinus irrigation. The increase in HR and BP during flushing times is expected given the increased animal anxiety associated with holding and positioning of the head for sinus access. However, the noticeable increase in HR and decrease in MAP observed ten minutes after flushing of the treatment sheep is likely due to the systemic effects of ISMN. This however was not accompanied by any observed common side effects such as headache, dizziness and syncope, which can only be indirectly assessed in sheep such as teeth grinding when they are in pain, or knee bending when feeling lightheaded. ISMN is a known cardiac drug, which acts as a vasodilator to treat angina chest pain in patients with ischemic heart disease. The vasodilatory effects can lead to a compensatory tachycardia in an effort to maintain cardiac output. With a return to baseline in the random readings taken between the flushing times, these effects on the treated sheep were noticeably transient. These results are promising for the use of ISMN as a safe topical sinus wash given the trialed maximum dissolvable dose of 60 mg/mL produces only small and transient changes in HR. MAP in contrast, remained at an average of 10 mmHg less at the random readings vs. ten minutes before flushing times. Although the MAP levels after flushing were still within acceptable levels by human standards, and not accompanied by observable clinical effects of hypotension on the sheep, it indicates that LFNO may have longer effects of MAP compared to HR. Further improvement of the formulation aimed at decreasing the overall ISMN dose while increasing encapsulation efficiency in the liposome formulations will likely reduce these effects. However, as with any drug with documented side effects, caution should be used in applying LFNO to patients with a history of cardiac disease. Aims at reducing the ISMN concentration prior to clinical trials should be performed in an attempt to further reduce the risk of these effects.
Anti-\textit{S. aureus} anti-biofilm properties were only statistically significant in the LFNO group and not in the liposomal blanks or pure-drug ISMN group. This indicates that the formulation of ISMN into liposomes enhances the potency of the drug against \textit{S. aureus} biofilms. Further mechanistic studies to characterize the degree of interactions with the biofilms are necessary.

Biofilms are known to be more difficult to treat compared to their planktonic counterparts due to the protective extracellular polymeric substance matrix and alterations in metabolic rate to enhance bacterial survivability.\textsuperscript{50-52} Phenotypic changes of some bacteria within the biofilm render them more resistant to antibiotics.\textsuperscript{262} It is therefore important that a designed topical anti-biofilm agent has the greatest efficacy with a well-documented mechanism of action. Furthermore, potential improvements of the liposomal formulation can be achieved once a better understanding of the biofilm-liposomal interaction occurs.

The additional burden of drug resistance emerges in the wake of chronic and repeated antibiotic use. The rapid ability of microorganisms to adapt and resist antibiotic therapy is exemplified in \textit{S. aureus}, its enhanced survivability well chronicled over the course of history. Penicillin-resistant \textit{S. aureus} emerged shortly after its popular use in the 1940’s, with up to 90% of strains now being Penicillin resistant.\textsuperscript{90,81} Current global concerns of Methicillin resistance are also emerging, with up to 50% resistance reported in certain communities.\textsuperscript{82} This underpins the importance of discovering alternatives to current available antibiotic regimens. In addition, limiting the use of antibiotics with alternative topical antimicrobials can aid in preventing drug resistance. Liposomes have been
demonstrated in the past to be effective in delivering antibiotics to a variety of biofilms, and have many essential characteristics to enhance a drug’s efficacy. The ability to alter its encapsulation efficiency, lipid content and surface charge also gives liposomes the flexibility to be designed based on the biological target. With ISMN also currently approved for human use, there is great capacity for liposomal-formulation of ISMN for clinical translation to a safe and effective sinus topical agent.

6.6 Conclusion

Liposomal formulation of ISMN is a promising anti- S. aureus biofilm agent in the setting of CRS. Its portrayal as a relatively safe drug with no observable side effects at the maximum dissolvable ISMN dose shows great promise towards the development of a safe topical agent for clinical use. Further studies however are needed to determine its mechanism of action against S. aureus biofilms and once established, further refinements in the formulation can be made to create the optimal topical sinus irrigation for clinical application.
Thesis Synopsis

There is still much to be learned about the intricate role of NO in the sinuses. With its multiple functions within human physiologic processes and its successful therapeutic applications in other medical fields, there is great opportunity to explore its potential uses in the sinonasal cavity. Its natural effects within the sinuses have been described to some degree in literature, with antibacterial, anti-viral and mucociliary properties, but a clear link to its effects on CRS pathogenesis and the pathogenic organism *S. aureus* have not yet been clearly defined. To date, there remains a paucity of studies describing its effects on *S. aureus*-infected CRS patients, a subset with arguably the worst disease outcomes. This thesis was created on the basic knowledge that extremely high levels of NO are present in healthy human sinuses, while low to almost negligible levels exist in CRS sinuses. We provided through the results of the first study, a simple but distinct relationship of NO levels with *S. aureus* biofilm growth. Using NO concentrations mimicking healthy and diseased sinus levels against established *S. aureus* biofilms, we demonstrated a pro-biofilm effect of NO at diseased NO levels, vs. anti-biofilm effects at healthy NO concentrations. The study design aimed to create, in a laboratory setting, similarities to a sinus environment by using physiologic and pathologic NO levels, and bacterial isolates from CRS patients who were documented to have *S. aureus* biofilms at the time of surgery.

The results of the first study gave important insights on the relationship of NO and *S. aureus* biofilm growth in the setting of CRS. Although we still do not know which event supersedes the other, our findings imply that low NO levels may provide a suitable environment for biofilm formation within CRS sinuses, while physiologically high NO levels play a role in preventing *S. aureus* biofilm overgrowth. This certainly
fits into the pattern of NOs contradictory functions. The results of this study will hopefully serve as a strong baseline for future research on NO and *S. aureus* biofilm overgrowth, with focus on extracting the sequence of events that lead to their current state in the disease process, and their exact roles in CRS pathogenesis and persistence.

After detailing NO’s relationship to the key microbe *S. aureus*, the second study aimed to explore further the host-NO relationship. With CRS being a chronic debilitating condition with predominantly inflammatory and infectious components, it is possible that NO, an important molecule involved in innate immunity and cell signaling, will also have key roles in the localized inflammatory response within the sinonasal cavity. Taking into consideration that the classifications of CRS may have differences in disease etiopathogenesis, patients were meticulously grouped based on the presence of nasal polyposis or *S. aureus* biofilms, and along with a control group, utilized strict criteria in patient selection. Hoping to obtain an overview of genetic expressions across mucosal samples, genes involved in NO and ROS regulation were chosen and compared between the different groups. The greatest number of gene expression changes was found in mucosal samples positive for *S. aureus* biofilm growth. Although we could not conclude whether the presence of *S. aureus* biofilms was the cause or effect of these changes, the results indicate that there is a significant association of *S. aureus* biofilm presence in altered gene expressions involved in innate immune regulation. Either one of two potential scenarios can exist, requiring further investigations in future studies. *S. aureus* may have an inherent ability to cause corruption of innate immune responses via the alteration of NO and ROS regulation, thereby leading to a persistent inflammatory response which is ineffective in microbial eradication. Alternatively their persistence may be due to an inherently impaired innate immunity, as reflected by altered gene
expressions, allowing biofilms to grow and flourish in an environment with inadequate microbial combative mechanisms. The augmentation of these gene expression findings in the presence of nasal polyposis further strengthens the link between S. aureus biofilms, impaired innate immunity via the degree of gene expression alterations, and disease severity.

Delving further into the host component of the disease process, the third study looked briefly into the role of the inflammasomes in CRS, a group of intracellular protein complexes involved in innate immunity and the first response attempt at control of microbial invasion. Keeping to the theme of defining the role of S. aureus biofilms in CRS, our findings again showed that the greatest number of differentially expressed genes were in the presence of polyps and S. aureus biofilms. Furthermore the gene AIM2 was increased in expression in S. aureus biofilm positive patients, insinuating that microbial DNA enters the host cells to incite this intracellular response. This supports the theory of a major role S. aureus biofilms play in the dysregulation of innate immunity and the propagation of CRS pathogenesis.

Lastly, building on the foundation of a better understanding of NO-S. aureus biofilm-host relationship, we now aimed to translate our findings to a clinical application. The first requirement was to overcome the limitations of NOs gaseous state as a possible therapeutic antimicrobial agent. After an exhaustive literature review, the use of liposomes to encapsulate a NO donor was chosen. With liposomes’ current use in the effective encapsulation of many drugs including antibiotics and its approval for human use, we have hoped to address the most of the obvious barriers of the clinical application of NO. The fourth study aimed at identifying the best liposomal formulation
to effectively encapsulate the NO donor ISMN. This study served as proof of principle that liposomal-encapsulated ISMN can be designed to have significant anti-biofilm effects on established *S. aureus* biofilms. This in-vitro study showed that altering liposome charges and lipid content can change the drug encapsulation efficacy of ISMN, and that increasing its concentration and encapsulation enhances its anti-biofilm effects. Although the smaller ULV liposomes generally had a better anti-biofilm performance than the MLV liposomes, increasing the lipid composition of the MLV improved the efficacy to comparable rates with its ULV counterparts. This was essential to achieve early in the course of the agent’s design. MLVs will be predictably easier to create into a topical solution due to its greater stability in long-term aqueous form.

An additional goal of this in-vitro study was to simulate the exposure time of a topical solution onto *S. aureus* biofilms within the sinuses. The designed anti-biofilm agent needed to exert its antimicrobial effects despite a short exposure time, mimicking similar circumstances of an ejected topical wash from a squeeze bottle into the nose and sinuses. Thus the liposomal formulation of ISMN was chosen based on its retained anti-biofilm efficacy despite a short exposure time of 5 minutes.

In the realm of rhinology, much has yet to be explored in the successful management of CRS. Discussions of significant improvement in therapeutic strategies are ongoing, and needed in order to provide the best treatment for our patients. With an abundance of new topical agents to combat mostly bacterial-associated infections, the risk of adverse localized changes to the sensitive sinonasal surface and systemic side effects with mucosal absorption increases. Thus the establishment of safety is an essential requirement of every novel topical therapy. In the last project of this thesis, we aimed to
determine the most important characteristic of a novel topical solution: the establishment of a clear safety profile to accompany the efficacy of our liposomal-encapsulated NO donor.

Utilizing the departmental sheep model of biofilm-associated rhinosinusitis, this in vivo study incorporated the monitoring of well-known systemic side effects of the NO donor ISMN such as hypotension and tachycardia. Furthermore, mucosal samples were harvested and assessed for histological mucosal changes and ciliary denudation. The results of the study confirmed that the designed liposomal formulation has significant anti-biofilm effects against *S. aureus* biofilms grown within sheep frontal sinuses. The absence of significant differences in localized mucosal effects compared to saline controls portrayed a safe agent within the confines of the sinuses. There were however, slight changes in heart rate and blood pressure readings on sheep exposed to the treatment agent, and although transient in nature, warrants further characterization in subsequent animal studies prior to clinical application. More ideal would be further refinement of the current liposomal formulation to enhance encapsulation efficacy, so that a lower overall ISMN dose can be used. This will theoretically reduce the risk of systemic effects without compromising the agent’s desired anti-biofilm properties.

Nonetheless, this fifth study proves that liposomal encapsulation of NO via the use of a clinically approved NO donor such as ISMN can be a viable and suitable anti-biofilm agent against *S. aureus* - associated CRS. Its promise of an efficacious and safe topical agent for regular use is exemplified in the results of this last project, but haste should not deter any attempts on the further improvement of this formulation. More animal studies are recommended prior to clinical trials.
In summary, this thesis encompassed the three core components of the long-standing debilitating disease of chronic rhinosinusitis. The host (patient), environment (NO) and microbe (S. aureus) in one aspect or another, form the foundation of this disease’s aetiopathogenesis. The complex interplay of all key elements has yet to be completely described, attributable to the likely multiple players contributing to the disease process. Yet the underlying goal of understanding such a complex disease is so that successful treatment strategies can be provided, a gap quite evident in current treatment paradigms. Perhaps in the near future, improved topical therapies to complement surgical intervention will be made available to patients, propelled by ongoing research into this difficult disease. It is with great hope that the result of this thesis has shined a brighter light into the understanding and treatment of CRS, more so in those groups of patients with the most burdensome of symptoms.
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