THE ROLE OF BACTERIAL BIOFILMS IN
CHRONIC RHINOSINUSITIS

A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF ADELAIDE

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Department of Surgery, Faculty of Health Sciences
The Queen Elizabeth Hospital Adelaide, South Australia
October 2008
Dedicated to my wonderful parents Jim and Lela
& to my beautiful wife Angela
“To climb steep hills requires slow pace at first.”
William Shakespeare
DECLARATION

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 and, to the best of my knowledge and belief, contains no material published or written by another person, except where due reference has been made in the text.

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Alkis James Psaltis

1st June 2008
This thesis embodies research investigating the role that bacterial biofilms play in the pathogenesis of chronic rhinosinusitis (CRS). It focuses on their detection on the sinus mucosa of CRS patients and the implications of their presence. Finally, it addresses deficiencies in the innate immune system that may predispose to their development in this condition.

Bacterial biofilms are structural assemblages of microbial cells that encase themselves in a protective self-produced matrix and irreversibly attach to a surface. Their extreme resistance to both the immune system as well as medical therapies has implicated them as playing a potential role in the pathogenesis of many chronic diseases. Although their role in many diseases is now well established, their objective presence and importance in CRS remains largely unknown.

Chapter 1 of this thesis reviews the current literature pertaining to CRS and biofilms and critically evaluates the small body of research relating to this topic.

Chapter 2 describes the development of a sheep model to study the role of bacterial biofilms in rhinosinusitis. It compares the use of traditional electron microscopy (EM) and more recent confocal scanning laser microscopy (CSLM) in the detection of biofilms on the surface of sinus mucosa. The results of this study inferred a causal relationship between biofilms and the macroscopic changes that accompany rhinosinusitis. Furthermore, it illustrated the superiority that CSLM has over EM in the imaging of biofilms on sinus mucosa.

Chapter 3 and 4 outline the results of human studies utilizing the more objective CSLM to evaluate the prevalence of bacterial biofilms on the sinus mucosa of CRS patients and their effect on post-operative mucosal healing. The results of these studies demonstrated a biofilm prevalence of approximately 50% in the CRS population studied and suggested, that biofilm presence may predispose to adverse post-operative outcomes following sinus surgery.

Chapter 5 and 6 describe experiments examining the level of the innate immune system’s anti-biofilm peptide lactoferrin, in patients with CRS. Lactoferrin was found to be down-regulated at both an mRNA and protein level in the majority of CRS patients, with biofilm positive patients demonstrating the most significant reduction.

In summary, this thesis provides further evidence that bacterial biofilms play a major role in the pathogenesis and disease persistence in a subset of CRS patients. Deficiencies in components of the innate immune system, such as lactoferrin, may play an important role in the predisposition of certain individuals to the initial development of bacterial biofilms.
I would like to acknowledge and thank my supervisor Professor Peter-John Wormald for all that he has done for me throughout the three years of my candidature. From the moment I first set foot into “Prof’s” office I felt inspired and have continued to feel so ever since. I wish to thank Professor Wormald for not only providing me with such a life-changing opportunity, but for his guidance, support, belief and above all friendship throughout my PhD.

Special acknowledgement must also be made of my good friends; co researcher Dr Kien Ha, for his contribution to this research, and Mr Cecil “Bo” Lewis, a significant innovator in rhinological-based biofilm research. Mention must also be made of my laboratory supervisor Dr Lor Wai Tan and my laboratory colleagues; Maressa Bruhn, Nick Hatziridos and Eng Ooi, as well as Ms Lyn Martin and all members of the Department of Otorhinolaryngology at the Queen Elizabeth Hospital for their friendship and support over the past three years. Thank you also to the Garnett-Passe and Rodney Williams Memorial Foundation for providing me with the scholarship that has allowed me to pursue my research goals.

On a personal note I wish to thank my Yiayia Athina and my late Papou Alkiviadis for teaching me integrity, honesty and internal strength. Also a special thank you my brother Peter, for always being there for me and for providing me with the benchmark of excellence.

To my darling wife Angela, thank you for your love, devotion and support. You always seem to steady the ship even when the waters appear rough.

Finally, this thesis would not have been possible without the endless sacrifices made by my selfless parents Dr Jim and Mrs Lela Psaltis. Whatever I have achieved has been all thanks to them. Mum and Dad thank you for everything!
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description/Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRS</td>
<td>Chronic Rhinosinusitis</td>
</tr>
<tr>
<td>EM</td>
<td>Electron Microscopy</td>
</tr>
<tr>
<td>CSLM</td>
<td>Confocal Scanning Laser Microscopy</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribosomal Nucleic Acid</td>
</tr>
<tr>
<td>GP</td>
<td>General Practitioner</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomized Control Trial</td>
</tr>
<tr>
<td>FESS</td>
<td>Functional Endoscopic Sinus Surgery</td>
</tr>
<tr>
<td>ESS</td>
<td>Endoscopic Sinus Surgery</td>
</tr>
<tr>
<td>NA</td>
<td>Not available</td>
</tr>
<tr>
<td>CNS</td>
<td>Coagulase Negative Staphylococci</td>
</tr>
<tr>
<td>SA</td>
<td>Staphylococcus aureus</td>
</tr>
<tr>
<td>SP</td>
<td>Streptococcus pneumoniae</td>
</tr>
<tr>
<td>GNR</td>
<td>Gram Negative Rods</td>
</tr>
<tr>
<td>SV</td>
<td>Streptococcus viridans</td>
</tr>
<tr>
<td>PA</td>
<td>Pseudomonas aeruginosa</td>
</tr>
<tr>
<td>H inf</td>
<td>Haemophilus influenza.</td>
</tr>
<tr>
<td>DIC</td>
<td>Differential Interference Contrast</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatability Complex</td>
</tr>
<tr>
<td>Th1</td>
<td>T Helper Cell 1</td>
</tr>
<tr>
<td>Th2</td>
<td>T Helper Cell 2</td>
</tr>
<tr>
<td>SEA</td>
<td>Staphylococcal Enterotoxin A</td>
</tr>
<tr>
<td>SEB</td>
<td>Staphylococcal Enterotoxin B</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>CRS/NP</td>
<td>Chronic Rhinosinusitis with Nasal Polyposis</td>
</tr>
<tr>
<td>TSST-1</td>
<td>Toxic Shock Syndrome Toxin 1</td>
</tr>
<tr>
<td>EPS</td>
<td>Exopolysaccharide matrix</td>
</tr>
<tr>
<td>Bap</td>
<td>Biofilm associated proteins</td>
</tr>
<tr>
<td>PIA</td>
<td>Polysaccharide Inter cellular Adhesin</td>
</tr>
<tr>
<td>MDR</td>
<td>Multidrug efflux pumps</td>
</tr>
<tr>
<td>CAM</td>
<td>Cationic Antimicrobial Peptides</td>
</tr>
<tr>
<td>OME</td>
<td>Otitis Media with Effusion</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>HPRT</td>
<td>Hypoxanthine-guanine phosphoribosyltransferase</td>
</tr>
<tr>
<td>Ct</td>
<td>Cycle threshold</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescent In Situ Hybridization</td>
</tr>
<tr>
<td>OSA</td>
<td>Obstructive Sleep Apnoea</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclearcytes</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes Simplex Virus</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<tr>
<td>HBV</td>
<td>Hepatitis B Virus</td>
</tr>
<tr>
<td>HCV</td>
<td>Hepatitis C Virus</td>
</tr>
<tr>
<td>RSV</td>
<td>Respiratory Synctial Virus</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>TNFa</td>
<td>Tumour Necrosis Factor alpha</td>
</tr>
<tr>
<td>IL8</td>
<td>Interleukin 8</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer Cells</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary strand DNA</td>
</tr>
<tr>
<td>CF</td>
<td>Cystic Fibrosis</td>
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<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>MQ</td>
<td>Milli-Q</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered solution</td>
</tr>
<tr>
<td>RAST</td>
<td>Radioallergosorbent testing</td>
</tr>
<tr>
<td>CT</td>
<td>Computerized tomography</td>
</tr>
<tr>
<td>ICC</td>
<td>Interobserver correlation coefficient</td>
</tr>
<tr>
<td>AFS</td>
<td>Allergic Fungal Sinusitis</td>
</tr>
<tr>
<td>NAFES</td>
<td>Non Allergic Fungal Eosinophilic sinusitis</td>
</tr>
<tr>
<td>NAFES</td>
<td>Non allergic, Non Fungal Eosinophilic sinusitis</td>
</tr>
<tr>
<td>OR</td>
<td>Odds Ratio</td>
</tr>
<tr>
<td>C.I.</td>
<td>Confidence Interval</td>
</tr>
<tr>
<td>VAS</td>
<td>Visual Analogue Scale</td>
</tr>
<tr>
<td>CSS</td>
<td>Chronic sinusitis survey</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real-time reverse-transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>LF</td>
<td>Lactoferrin</td>
</tr>
</tbody>
</table>
AWARDS OBTAINED FOR RESEARCH ASSOCIATED WITH THIS THESIS

The Ronald Gristwood Medal for Best Presentation by a South Australian Ear Nose and Throat Registrar, for the presentation titled “Lactoferrin expression in chronic rhinosinusitis patients” Adelaide September 2005

The Queen Elizabeth Hospital Research Day Presentation Award for the Best Clinical Presentation titled “Lactoferrin expression in Chronic Rhinosinusitis Patients” Adelaide October 2005

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**A sheep model for the study of biofilms in rhinosinusitis.**
Psaltis AJ and Ha KR (Co-first authors), Tan L, Wormald PJ.

**Confocal scanning laser microscopy evidence of biofilms in patients with chronic rhinosinusitis.**
Psaltis AJ, Ha KR, Beule AG, Tan LW, Wormald PJ.

**Nasal mucosa expression of lactoferrin in patients with chronic rhinosinusitis.**
Psaltis AJ, Bruhn MA, Ooi EH, Tan LW, Wormald PJ.

**Reduced Levels of Lactoferrin in Biofilm-Associated Chronic Rhinosinusitis.**
Laryngoscope. 2008 Jan 21; [Epub ahead of print]

**The effect of biofilms on post-sinus surgical outcomes.**
Psaltis AJ, Weitzel E, Wormald P-J

**In Vitro Activity of Mupirocin on Clinical Isolates of Staphylococcus aureus and its Potential Implications in Chronic Rhinosinusitis.**
Laryngoscope. 2007 Dec 3; [Epub ahead of print]
### PRESENTATIONS

<table>
<thead>
<tr>
<th>Title</th>
<th>Location and Details</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lactoferrin And Biofilms</strong></td>
<td>Biofilm Roundtable Discussion, Sydney Australia, October 2005.</td>
</tr>
<tr>
<td><strong>The Heterogeneity of Lactoferrin Expression in Patients with Chronic Rhinosinusitis</strong></td>
<td>Queen Elizabeth Hospital Research Day, Adelaide Australia, October 2005.</td>
</tr>
<tr>
<td><strong>The Expression of Lactoferrin in Patients with Chronic Rhinosinusitis.</strong></td>
<td>International Rhinological Society Meeting, Sydney Australia, October 2005.</td>
</tr>
<tr>
<td><strong>Biofilms and the nose</strong></td>
<td>Australasian Rhinological Society Annual Meeting, Barossa, South Australia, October 2006</td>
</tr>
<tr>
<td><strong>A sheep model for the study of biofilms in Chronic Sinusitis</strong></td>
<td>Annual Meeting of the American Rhinologic Society, Toronto, Canada September 2006</td>
</tr>
<tr>
<td><strong>A new technique for the study of biofilms in sinusitis</strong></td>
<td>The Cabrini Institute, Monash University, Melbourne Victoria October 2006</td>
</tr>
<tr>
<td><strong>An animal model to study biofilms in chronic rhinosinusitis</strong></td>
<td>Queen Elizabeth Hospital Research Day, Adelaide, Australia, October 2006</td>
</tr>
<tr>
<td><strong>CSLM study of biofilms in human CRS patients</strong></td>
<td>ASOHNS Scientific Meeting, Adelaide, Australia, March 2007</td>
</tr>
<tr>
<td><strong>Biofilms and Chronic Rhinosinusitis</strong></td>
<td>Invited Speaker Divisions of Surgery Departmental Meeting, Adelaide, Australia June 2007</td>
</tr>
<tr>
<td><strong>The effect of biofilms on post-sinus surgical outcomes.</strong></td>
<td>Annual Scientific Meeting at the Royal Australian College of Surgeons, Adelaide, Aug 2007</td>
</tr>
<tr>
<td><strong>The effect of biofilms on post-sinus surgical outcomes.</strong></td>
<td>Queen Elizabeth Hospital Research Day, Adelaide, Australia October 2007</td>
</tr>
<tr>
<td><strong>A sheep model for study of biofilm in rhinosinusitis.</strong></td>
<td>Research Expo of the Faculty of Health Sciences, University of Adelaide, Australia Oct 2007</td>
</tr>
<tr>
<td><strong>The role of biofilms in CRS.</strong></td>
<td>Centre for Genomic Studies, Allegheny General Hospital Pittsburgh, PA. USA April 2008</td>
</tr>
<tr>
<td><strong>A sheep model investigating several potential antibiofilm treatments.</strong></td>
<td>The American Triological Society of Otorhinolaryngology (COSM), Spring Meeting, Orlando Florida, USA May 2008</td>
</tr>
<tr>
<td><strong>Biofilms and Chronic Rhinosinusitis</strong></td>
<td>American Academy of Head and Neck Surgeons and Otolaryngologists, Annual Scientific Meeting, Chicago, Illinois, USA September 2008</td>
</tr>
</tbody>
</table>
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEDICATION</td>
<td>ii</td>
</tr>
<tr>
<td>DECLARATION</td>
<td>iv</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>v</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>vi</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>vii</td>
</tr>
<tr>
<td>ACCOMPLISHMENTS</td>
<td>viii</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>xi</td>
</tr>
<tr>
<td><strong>CHAPTER 1 LITERATURE REVIEW</strong></td>
<td>1</td>
</tr>
<tr>
<td>1.1 Chronic Rhinosinusitis</td>
<td>2</td>
</tr>
<tr>
<td>1.1.1 Research Definition</td>
<td>2</td>
</tr>
<tr>
<td>1.1.2 Epidemiology and Socio-Economic Implications</td>
<td>2</td>
</tr>
<tr>
<td>1.1.3 Aetiological/Pathogenic Factors</td>
<td>4</td>
</tr>
<tr>
<td>1.1.4 Treatments for Chronic Rhinosinusitis</td>
<td>4</td>
</tr>
<tr>
<td>1.2 Bacteria and Chronic Rhinosinusitis</td>
<td>8</td>
</tr>
<tr>
<td>1.2.1 Controversy Regarding the Role of Bacteria in CRS</td>
<td>8</td>
</tr>
<tr>
<td>1.2.2 Bacterial Super-Antigens</td>
<td>11</td>
</tr>
<tr>
<td>1.2.3 Bacterial Mediated Osteitis</td>
<td>12</td>
</tr>
<tr>
<td>1.3 Bacterial Biofilms</td>
<td>14</td>
</tr>
<tr>
<td>1.3.1 Historical Perspectives</td>
<td>14</td>
</tr>
<tr>
<td>1.3.2 Definition</td>
<td>14</td>
</tr>
<tr>
<td>1.3.3 Biofilm Ultrastructure</td>
<td>15</td>
</tr>
<tr>
<td>1.3.4 Biofilm Composition</td>
<td>17</td>
</tr>
<tr>
<td>1.3.5 Biofilm Formation</td>
<td>18</td>
</tr>
<tr>
<td>1.3.6 Biofilm Lifecycle</td>
<td>19</td>
</tr>
<tr>
<td>1.3.7 Biofilms and Antibiotic Resistance</td>
<td>21</td>
</tr>
<tr>
<td>1.3.8 Biofilms and the Immune System</td>
<td>24</td>
</tr>
<tr>
<td>1.3.9 Biofilms and Chronic Diseases</td>
<td>25</td>
</tr>
<tr>
<td>1.3.10 Biofilms and Otolaryngology</td>
<td>26</td>
</tr>
<tr>
<td>1.3.11 Biofilms and Rhinology</td>
<td>29</td>
</tr>
<tr>
<td>1.4 Lactoferrin</td>
<td>34</td>
</tr>
<tr>
<td>1.4.1 Introduction</td>
<td>34</td>
</tr>
<tr>
<td>1.4.2 Structure</td>
<td>34</td>
</tr>
<tr>
<td>1.4.3 Lactoferrin’s Antimicrobial Action</td>
<td>36</td>
</tr>
<tr>
<td>1.4.4 Immune Regulation by Lactoferrin</td>
<td>37</td>
</tr>
<tr>
<td>1.4.5 Anti-Biofilm Action</td>
<td>38</td>
</tr>
<tr>
<td>1.4.6 Lactoferrin and CRS</td>
<td>39</td>
</tr>
<tr>
<td>1.5 Conclusions From Review Of The Literature</td>
<td>40</td>
</tr>
<tr>
<td>1.6 Studies To Be Conducted</td>
<td>41</td>
</tr>
</tbody>
</table>

xii
CHAPTER 1

LITERATURE REVIEW
1.1 CHRONIC RHINOSINUSITIS

1.1.1 RESEARCH DEFINITION
Chronic rhinosinusitis (CRS) can be considered a group of disorders characterised by inflammation of the mucosal lining of the nasal cavity and para-nasal sinuses lasting for at least 12 weeks. Historically the diagnosis of CRS was largely a clinical one based on the presence of major and minor symptoms (see table 1); however, due to the broad spectrum of diseases that CRS is now thought to represent reliance purely on a clinical diagnosis may not always be accurate [1]. In 2004, 30 physicians from 5 national American societies, representing the fields of otorhinolaryngology, allergy-immunology, respiratory medicine, infectious diseases and radiology convened to develop new research definition criteria for the diagnosis of rhinosinusitis. According to the criteria set out by this task force, in order for a diagnosis of CRS to be made, patients are required to have not only have \( \geq 2 \) of the following symptoms for at least 12 consecutive weeks: (1) anterior and/or posterior mucopurulent drainage (2) nasal obstruction and (3) hyposmia or anosmia but also objective evidence of sino-nasal inflammation on both endoscopy and radiological imaging with computerised tomography [2].

1.1.2 EPIDEMIOLOGY AND SOCIO-ECONOMIC IMPLICATIONS
CRS is a highly common condition affecting up to 16% of the US population. Its prevalence resembles that of hypertension and non-specific lower back pain and it remains the single most common self-reported chronic health condition affecting adults in the western world[3]. The financial burden of this condition is far reaching, with direct annual US health care expenditure in excess of $ 5.8 million US [4]. Estimates of restricted activity days in the USA exceed 73 million days/year making the true financial costs of CRS to society significantly
higher [5]. In 2004 the National Ambulatory Medical Care Survey estimated that 12.5 million office-based doctor visits resulted in a diagnosis of CRS, with hospital out-patient visits reaching more than 1.1 million [6]. Patients with CRS were shown to visit their GP 2 times more often than non sufferers and had 5 times as many prescriptions filled [7]. Aside from the enormous economic implications of CRS, numerous quality life and disability index studies have repeatedly demonstrated the significant negative psycho-social impact that this condition has on the sufferer. The disability and discomfort caused by CRS has been shown to be comparable to that of other chronic diseases such as asthma, angina and lower back pain [8].

### TABLE 1    Clinical, Radiological and Endoscopic Features associated with CRS

<table>
<thead>
<tr>
<th>Major Symptoms*</th>
<th>Minor Symptoms</th>
<th>Symptom duration for a duration of at least 12 consecutive weeks</th>
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<tbody>
<tr>
<td>Purulent anterior nasal discharge</td>
<td>Headache</td>
<td></td>
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<tr>
<td>Purulent posterior nasal discharge</td>
<td>Otalgia/aural fullness</td>
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<tr>
<td>Nasal obstruction/blockage</td>
<td>Halitosis</td>
<td></td>
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<tr>
<td>Hyposmia/anosmia</td>
<td>Cough</td>
<td></td>
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<tr>
<td>Facial pain/pressure/fullness</td>
<td>Dental pain</td>
<td></td>
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<tr>
<td></td>
<td>Fatigue</td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>CT Findings</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isolated or diffuse mucosal thickening</td>
<td>Supporting radiological evidence of CRS required for diagnosis to be made</td>
<td></td>
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<tr>
<td>Complete/Partial Opacification</td>
<td></td>
<td></td>
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<tr>
<td>Air-fluid levels</td>
<td></td>
<td></td>
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<tr>
<td>Bony changes</td>
<td></td>
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<tr>
<td>Nasal Endoscopy</td>
<td></td>
<td></td>
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<tr>
<td>1. Generalised or localized erythema, oedema, or granulation tissue</td>
<td>At least one of these signs of inflammation must be present for a diagnosis of CRS to be made</td>
<td></td>
</tr>
<tr>
<td>2. Discoloured nasal drainage arising from the nasal passages, nasal polyps, or polypoid swelling</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Oedema or erythema of the middle meatus or ethmoid bulla as identified by nasal endoscopy</td>
<td></td>
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</tbody>
</table>

* Facial pain/pressure/fullness alone does not constitute a suggestive history in the absence of another major nasal symptom or sign.
1.1.3 AETIOLOGICAL/PATHOGENIC FACTORS

Despite increasing research into the pathophysiology of CRS over the last two decades, the exact aetiology and pathogenic mechanisms still remain unclear. Without the identification of a single unifying cause for this condition, CRS is now considered a multi-factorial disease with varying levels of evidence for certain risk factors. These factors have been broadly categorised into extrinsic or non-host related factors and intrinsic or host related factors. Extrinsic factors found to be associated with CRS include environmental factors such as air pollution [9], smoking [10, 11] and exposure to allergens [12, 13] as well as microbial infections (bacterial and fungal) and their associated pathogenicity (biofilms, superantigens, osteitis and non-IgE mediated eosinophilic inflammation). Intrinsic factors predisposing to the development of CRS are thought to include anatomic/structural abnormalities, genetic abnormalities such as cystic fibrosis [14], Young’s disease [15] or primary ciliary dyskinesia [16], and disorders of innate and cell mediated immune system. The focus of this thesis will be the role that bacteria and in particular bacterial biofilms have in the pathogenesis and recalcitrant nature of CRS.

1.1.4 TREATMENTS FOR CHRONIC RHINOSINUSITIS

The treatment of CRS involves both medical and surgical interventions. Two recent surveys of US otolaryngologists found oral antibiotics and intranasal corticosteroids the most commonly employed first line agents in the management of CRS [17, 18]. Other agents also used, although less frequently include nasal douches and additional oral medications such as corticosteroids, decongestants, mucolytics and antihistamines. Although widely and liberally used, the level of evidence supporting the effectiveness of different medical therapies in the treatment of CRS is variable and often tenuous. Despite considerable disagreement
surrounding the role of bacteria in CRS, two prospective randomised control trials (RCT) have demonstrated the efficacy of long term antibiotic treatment (>12 weeks) in the management of CRS. Wallwork et al [19] showed a statistically significant improvement in symptom, endoscopic and rhinometric parameters in patients treated with long term macrolide antibiotics compared to those in the placebo arm. A similar improvement in objective and subjective outcome measures was also found by Ragab et al [20], in patients medically treated with long-term erythromycin, intra-nasal corticosteroids and alkaline douches compared to controls. As well as the numerous studies of Level III evidence, demonstrating clinical and radiological improvement in CRS patients after long term antibiotic therapy [21-25], multiple cohort studies have also shown clinical improvement in patients treated with shorter 4-6 week antibiotic courses with or without intra-nasal steroids [26-29]. The use of topical/aerosolized antibiotic treatments has also been investigated and although three cohort studies have demonstrated some benefit in CRS patients experiencing frequent acute exacerbations [30-32], the only prospective double blind RCT concerning topical antibiotic use, showed no additional benefit in the addition of tobramycin to nebulised saline in the treatment of CRS [33].

To date three RCTs investigating the use of topical steroids [34-36] and two RCTs evaluating the efficacy of intra-sinus instilled steroids [37, 38] in CRS have been published. Four of the five trials demonstrated a significant improvement in symptoms with no evidence of increased infection rates. Although the role of systemic steroids in the management of CRS has not been as extensively evaluated, a double-blind RCT exists demonstrating a clinically significant improvement in symptoms and pathology of nasal polyposis patients treated with a 14 day course of oral prednisolone [39]. Other lower evidence studies also suggest that oral steroids may be particularly useful in the management of certain subtypes of CRS, particularly allergic fungal sinusitis [40],[41].
A systematic review of the literature by the Cochrane Collaboration, identified a number of randomised controlled trials evaluating the use of nasal saline irrigation for the symptoms of CRS [42]. Meta-analysis of three studies comparing the effect of saline vs no treatment [43-45], revealed a statistically significant improvement in symptoms and disease-specific quality of life scores in the saline arm. Although an improvement was also seen in both saline treatment arms in the RCT published by Heatley et al [46], this was not significantly better than the placebo arm. Two RCTs comparing hypertonic vs isotonic solutions have revealed conflicting results. Cordray et al [47], showed a significant improvement in the symptoms of CRS patients using hypertonic saline but not in those using isotonic saline, while Bachmann et al [48] showed both saline solutions improved symptom scores relative to baseline, with no significant difference between the two.

Functional endoscopic sinus surgery (FESS) has now become well established for the treatment of chronic rhinosinusitis refractory to medical management. A systematic review of the literature by the Cochrane collaboration in 2006 [49], revealed that the vast majority of studies examining the effectiveness of FESS for CRS were either cohort studies or case series of level III evidence at best. Although these studies generally demonstrated high efficacy of FESS [50-54], the 2 RCTs identified in the review [20, 55], did not demonstrate an overall significant difference in the clinical outcomes of FESS compared to medical management in the treatment of CRS. It must be kept in mind however that methodological flaws, ethical issues, differences in the patient populations, inherent difficulties in standardising and blinding surgical procedures as well as the fact that surgery is typically reserved for patients who have failed medical therapy, makes it not only difficult to statistically compare the results of the different studies but also to compare the efficacy of FESS versus medical treatment alone.
Numerous level III evidence studies have also attempted to determine possible factors affecting outcome following FESS. A history of previous polypectomy or sinus surgery [56-59], presence of allergy [57], smoking [10, 59] and more severe initial disease/inflammation on endoscopic or histopathological examination [58, 60] have all been shown to correlate with poorer post-operative outcomes and a more frequent need for revision surgery. Although some studies have also reported that polyposis and more severe radiological disease may also predispose to poorer outcomes [59, 61, 62], these findings have not been universal [20, 54, 63, 64].
1.2 BACTERIA AND CHRONIC RHINOSINUSITIS

1.2.1 CONTROVERSY REGARDING THE ROLE OF BACTERIA IN CRS

The role of bacteria in acute rhinosinusitis is well defined, with *Streptococcus pneumoniae*, *Moraxella catarrhalis* and non typeable *Haemophilus influenza* the most common pathogenic organisms involved [65, 66]. The microbiology and importance of bacteria in the aetiology of CRS remains debated however. Summarising the literature pertaining to the bacteriological evaluation of CRS patients is extremely difficult due to the many methodological differences existing between studies. Such differences include the following: (1) characteristics of patients studied (age, gender, immune state and presence of co-morbidities) (2) duration, extent and severity of disease (3) use of previous or concurrent treatments (antimicrobials and anti-inflammatory agents vs. surgical) (4) site and sinus of sampling (5) sampling methods used (irrigation, aspiration or blind vs. endoscopically guided biopsy/swab) (6) handling and processing of specimens prior to analysis and (7) methods used to detect bacteria and quantitate bacterial load (culture vs. PCR). It is thought that these differences may not only affect the culture yield rate but also the type of organism isolated. Table 2 summarises the most common bacteria isolated from a number of recent studies [67-79]. Despite being among the most frequently cultured bacteria from CRS patients, it is generally agreed that the low virulence of organisms such as Coagulase negative staphylococci (CNS), makes them unlikely to be pathogenic in immune-competent people [80, 81]. Interestingly, there is also an observed shift towards Gram negative organisms such as *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Proteus mirabilis*, *Enterobacter* spp and *Escherichia coli* in the sinus cultures of patients who have undergone previous sinus surgery or medical treatment with steroids, antimicrobial agents and sinus irrigation. [69, 72, 82, 83] The reason for this remains unknown with some researchers believing selection pressure to
play a role [65] while others propose that gram negative bacteria may colonize or secondarily infect because of underlying defects in host defences [84, 85].

Despite the frequent co-occurrence of bacteria with CRS, controversy still exists as to the role that they play in the pathogenesis of this condition. It has been postulated that in many cases, bacteria may simply be present as non-pathogenic bystanders invading already inflamed tissue [86]. This opinion has largely stemmed from the following observations: (1) the finding that the paranasal sinuses are not actually sterile as once thought, with more than half of all healthy sinuses culturing bacteria [87-90]. (2) the relative absence of neutrophils and predominance of eosinophils and mixed mononuclear cells in the inflammatory infiltrate [91, 92] (3) the poor correlation between clinical findings and microbiology [93] and (4) the often short lived or poor response to seemingly appropriate culture-directed antibiotic therapy [29]. Although these observations have provided mounting evidence for the limited role of bacteria in CRS, the recent discovery of bacterial superantigens in patients with CRS with nasal polyposis; the new evidence of underlying bacterially driven osteitis in the bony walls of inflamed sinuses and the re-discovery of an alternative form of bacterial existence, the biofilm, has rekindled the debate of the importance of bacteria in the pathogenesis of CRS.
### TABLE 2

Bacteriological Studies of CRS patients aged 17-79

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Patient No.</th>
<th>Antibiotics &lt;1wk before surgery</th>
<th>Aseptic technique</th>
<th>Sampling Method</th>
<th>Sample site</th>
<th>Sample site</th>
<th>Micro-organisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doyle et al&lt;sup&gt;9&lt;/sup&gt;</td>
<td>1991</td>
<td>59</td>
<td>Yes</td>
<td>Yes</td>
<td>Biopsy</td>
<td>Ethmoid</td>
<td>NA</td>
<td>CNS (73%), SA (34%) GNR</td>
</tr>
<tr>
<td>Hoyt et al&lt;sup&gt;10&lt;/sup&gt;</td>
<td>1992</td>
<td>197</td>
<td>NA</td>
<td>Yes</td>
<td>Aspiration &amp; Biopsy</td>
<td>Maxillary</td>
<td>NA</td>
<td>CNS, SA, GNR</td>
</tr>
<tr>
<td>Hsu et al&lt;sup&gt;11&lt;/sup&gt;</td>
<td>1998</td>
<td>34</td>
<td>NA</td>
<td>Yes</td>
<td>Endoscopic</td>
<td>All sinuses</td>
<td>90%</td>
<td>CNS (28%) PA (17%) SA (13%)</td>
</tr>
<tr>
<td>Biel et al&lt;sup&gt;12&lt;/sup&gt;</td>
<td>1998</td>
<td>174</td>
<td>Yes</td>
<td>Yes</td>
<td>Endoscopic</td>
<td>Maxillary</td>
<td>95%</td>
<td>CNS (36%) SA (25%), SV, Anaerobes</td>
</tr>
<tr>
<td>Brook et al&lt;sup&gt;14&lt;/sup&gt;</td>
<td>2001</td>
<td>108</td>
<td>NA</td>
<td>Yes</td>
<td>Aspiration</td>
<td>Maxillary</td>
<td>NA</td>
<td>SA (16%) SV (13%) PA (11%), Anaerobes</td>
</tr>
<tr>
<td>Jiang et al&lt;sup&gt;13&lt;/sup&gt;</td>
<td>2002</td>
<td>186</td>
<td>NA</td>
<td>Yes</td>
<td>Endoscopic</td>
<td>Middle Meatus Ethmoid</td>
<td>83%</td>
<td>CNS, GNR, SA</td>
</tr>
<tr>
<td>Finegold et al&lt;sup&gt;15&lt;/sup&gt;</td>
<td>2002</td>
<td>150</td>
<td>NA</td>
<td>NA</td>
<td>Aspiration</td>
<td>Maxillary</td>
<td>76%</td>
<td>GNR, ACS, Anaerobes</td>
</tr>
<tr>
<td>Araujo et al&lt;sup&gt;16&lt;/sup&gt;</td>
<td>2003</td>
<td>114</td>
<td>No</td>
<td>Yes</td>
<td>Endoscopic</td>
<td>Middle Meatus</td>
<td>92%</td>
<td>SA (36%) CNS (20%) SP (17%) Anaerobes (8%)</td>
</tr>
<tr>
<td>Kalcioglu et al&lt;sup&gt;17&lt;/sup&gt;</td>
<td>2003</td>
<td>27</td>
<td>Yes</td>
<td>Yes</td>
<td>Aspirate</td>
<td>Maxillary</td>
<td>70%</td>
<td>SA (11%) SP (11%) H Inf (7%) Anaerobes (36%)</td>
</tr>
<tr>
<td>Merino Et al&lt;sup&gt;18&lt;/sup&gt;</td>
<td>2003</td>
<td>510</td>
<td>No</td>
<td>Yes</td>
<td>Aspirates</td>
<td>Maxillary</td>
<td>98%</td>
<td>SV (28%) SP (12%) Coryn (12%) SA (9%) Anaerobes</td>
</tr>
<tr>
<td>Kingdom et al&lt;sup&gt;19&lt;/sup&gt;</td>
<td>2004</td>
<td>101</td>
<td>NA</td>
<td>Yes</td>
<td>Endoscopic Swab and Biopsy</td>
<td>All sinuses</td>
<td>86%</td>
<td>CNS (45%) GNR (25%) SA (24%) PA (9%)</td>
</tr>
<tr>
<td>Yildirim et al&lt;sup&gt;20&lt;/sup&gt;</td>
<td>2004</td>
<td>48</td>
<td>Yes</td>
<td>Yes</td>
<td>Endoscopic Swab</td>
<td>Middle Meatus Sphenethmoidal recess</td>
<td>Not applicable</td>
<td>CNS (46%) SP (17%) GNR (17%) SA (10%) PA (10%)</td>
</tr>
<tr>
<td>Busaba et al&lt;sup&gt;21&lt;/sup&gt;</td>
<td>2004</td>
<td>179</td>
<td>No</td>
<td>Yes</td>
<td>Biopsy</td>
<td>Ethmoid</td>
<td>90%</td>
<td>CNS, SA, Anaerobes</td>
</tr>
<tr>
<td>Aneke et al&lt;sup&gt;22&lt;/sup&gt;</td>
<td>2004</td>
<td>54</td>
<td>NA</td>
<td>NA</td>
<td>Aspirate</td>
<td>Maxillary</td>
<td>57%</td>
<td>SA, PA, GNR</td>
</tr>
</tbody>
</table>

NA – Not available  
CNS- Coagulase Negative Staphylococcus, SA Staphylococcus aureus, SP Streptococcus pneumoniae, GNR Gram Negative Rods, SV Streptococcus viridans, PA Pseudomonas aeruginosa, H inf Haemophilus Influenza.
1.2.2 Bacterial Super-Antigens

The increasing evidence of superantigen mediated inflammation in chronic eosinophilic-lymphocytic inflammatory disorders such as atopic dermatitis [94, 95], allergic rhinitis [96] and asthma [97] has led researchers to believe that they may also play a role in the inflammation associated with CRS. Super-antigens are microbial derived toxins capable of triggering massive polyclonal T cell proliferation and activation. They do so by directly binding to and cross-linking the MHC class II molecule on antigen presenting cells to the variable region β chain of the T cell receptor. This bypasses the conventional MHC restrictions of the immune system, enabling them to activate up to 20-30% of the host T-cell population [98]. In the acute setting superantigens may lead to the sudden and massive release of Th1 and Th2 cytokines as seen in toxic shock syndrome, where as in chronic conditions they may act as allergens, promoting a systemic and local IgE response with histamine release on repeated exposure. In 2001 Bachert et al [99] published the first paper suggesting a possible role for bacterial superantigens in the pathogenesis of CRS with nasal polyposis. This study demonstrated the presence of specific IgE to staphylococcal enterotoxins A and B (SEA and SEB) in the polyp homogenates of CRS patients. The positive correlation they showed between the concentration of total and specific IgE to eosinophilic inflammation in the nasal polyp tissue, further strengthened their argument that the staphylococcal superantigens were inciting and maintaining the cellular inflammation. A similar study examining the serum of 23 CRS patients with nasal polyposis (CRS/NP), demonstrated the presence of IgE to staphylococcal superantigens (SEB and toxic shock syndrome toxin (TSST-1)) in a large proportion of the patients (60.9% and 39.1% respectively) and in none of the controls [100]. Further indirect evidence for the role of superantigens in CRS/NP is demonstrated in two studies which found significant clonal proliferation of specific variable β domains in lymphocytes located within the nasal polyp tissue of CRS patients [101, 102]. The
recent direct detection of superantigens by enzyme-linked immunosorbent assay in the mucus and tissue of CRS/NP but not in healthy controls has now provided direct evidence for the role of these enterotoxins in CRS patients [103].

1.2.3 Bacterial mediated Osteitis

Histological changes of the bone underlying the sinus mucosa were first described in animal studies of bacterial induced CRS [104, 105]. The later findings of inflammatory- mediated bony changes in adjacent and distant non-infected animal sinuses [106, 107], led some authors to compare this inflammatory process with the histological diagnosis of osteomyelitis [2]. The fact that sinus bones are flat bones lacking marrow spaces resulted in the term “osteitis” being used rather than osteomyelitis to describe the pathological process occurring in the bony walls of sinuses. Only a limited number of human clinical studies have been performed in CRS patients to examine the possible role of osteitis in the pathogenesis of this condition. Both Kennedy et al [108] and Giacchi et al [109] have found histomorphometric and histopathological evidence of bone remodelling in human ethmoid sinuses. Using radionucleotides Jang et al [110] also demonstrated a higher uptake of radioisotope in the paranasal sinus bones of patients with poorer outcomes post endoscopic sinus surgery, suggesting that ongoing osteitis may perpetuate mucosal inflammation post operatively. Whether the mechanism of bone remodelling is as consequence of direct infection of the bone or as a result of bacterial-induced release of inflammatory mediators, that in turn stimulate osteoblastic activity, remains unknown [107]. The fact that bacterial organisms have yet to be identified in the bone of either human patients or animal models of CRS had led some researchers to believe the latter is a more likely explanation. It should be noted however that even in the well established bacterial entity of osteomyelitis, the recovery of bacterial organisms is often very
difficult. Nevertheless, it is certainly possible that underlying bony changes may explain the often recalcitrant and difficult to treat nature of CRS. Further human studies are most certainly required.
1.3 Bacterial Biofilms

1.3.1 Historical Perspectives

The first description of bacteria and indeed biofilms was made by a Dutch lens maker, Anton Van Leewenhoek in 1683. Despite his observations of bacteria existing either as individual highly motile organisms or in seemingly stationary clusters, his descriptions of the second “biofilm” form were largely ignored. Scientists became focused with the planktonic or free floating form that became popularised by Robert Koch in his doctrine of bacterial causation of acute diseases. It was not until the emergence of chronic diseases, that the concept of bacteria existing in biofilms. Although it has taken more than two decades since the “re-discovery” of biofilms by Costerton et al in 1978 [111], the veracity and interest in the biofilm world is now overwhelming, with more than 6500 biofilm related articles published since 1990 [112]. Despite this, there is still a paucity of biofilm research in the field of otorhinolaryngology.

1.3.2 Definition

The definition of a biofilm is a constantly evolving one, reflecting advances in scientific research and technology. The early definitions focussed entirely on the structural composition of the biofilm, namely the bacterial clusters and their encasing matrix [111],[113]. Soon after, it became evident that biofilms were not static, homogenous structures but rather exhibited spatial and temporal heterogeneity as well as many differences to their planktonic counterparts in terms of growth, metabolic rate and genetic expression [114],[115, 116]. As a consequence the most recent definition put forward by Donlan and Costerton encompasses both the readily observable structural features of a biofilm as well as the specific
physiological features of the organisms existing within these structures. They now define a biofilm as a *microbially derived sessile community, characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of self produced extracellular polymeric substances, and exhibit an altered phenotype in terms of growth rate and genotype* [117]. This definition has important implications with respect to research, as previous bacterial populations classified as biofilms according to early structural definitions, have now been shown to merely be micro-colonies of planktonic bacteria lacking the inherently resistant phenotype of true biofilms.

1.3.3 Biofilm Ultrastructure

The conceptual understanding of biofilm ultrastructure has evolved with the advent of new imaging modalities. From early light and transmission electron microscopy studies, biofilms were viewed as homogenous, unstructured, planar accretions of bacterial cells embedded within the cells’ exopolysaccharide matrices [118]. This misperception of biofilm ultrastructure arose from the inherent flaws associated with these imaging modalities. Light microscopy suffers from out-of-focus effects that are thought to cause distortion of the structure being viewed, while the complete dehydration of specimens required for electron microscopy could theoretically dehydrate and collapse the typically well hydrated biofilm matrix. New imaging technologies allowing more detailed and less invasive biofilm imaging have led to new conceptual models, casting serious doubt on biofilms existing as homogeneous flat structures. Using the differential interference contrast (DIC) microscope to study water-system biofilms, Keevil et al [119] formulated their ‘heterogeneous mosaic model’ of biofilm growth, following their observation of biofilms growing as numerous microcolony stacks within an exopolysaccaride matrix. The application of Confocal Scanning
Laser Microscopy (CSLM) to biofilm research probably represents the most significant advancement in our understanding of biofilms. CSLM circumvents many of the problems of other imaging modalities, by allowing the fresh processing of specimens and by eliminating out-of-focus distortion through the use of optical sectioning. Using CSLM, the current model of biofilms resembling dense, confluent mushroom-type structures penetrated by interstitial voids, has emerged. Although the initial CSLM studies of biofilms were entirely descriptive [120], the use of CSLM has since been expanded to examine the species and chemical composition of biofilms, their physiological properties and their relationship with the substratum. Numerous factors influencing the formation of biofilms at different times have been identified and are summarised in table 3 adapted from Wimmpeny et al.’s review of biofilms’ heterogeneous structure [121].

<table>
<thead>
<tr>
<th>TABLE 3 Summary of the factors influencing the formation of biofilms at different times</th>
</tr>
</thead>
</table>
| **Genotypic factors** | Organisms specific genotype  
Expression of genes encoding surface properties  
Expression of signalling systems  
Formation of EPS  
Organisms growth dynamics; specific growth rate, affinity for substrates, lag periods, yield coefficients  
Expression of genetic factors not directly confined to biofilm formation (i.e. motility and chemotaxis) |
| **Physico-chemical factors** | Phase interface  
Substratum composition and concentration/gradient  
Temperature/pH/water potential/pressure/oxygen supply and demand |
| **Stochastic processes** | Initial colonization: attachment, detachment  
Random changes in abiotic and biotic factors |
| **Deterministic phenomena** | Specific interaction between organisms: competition, neutralism, cooperation and predation |
| **Mechanical processes** | Shear due to laminar flow or turbulent flow; abrasion; logistic restriction |
| **Temporal changes** | Diurnal or annual periodic changes in environment e.g. light, temperature, pH  
Irregular changes due to unforseen events. |
1.3.4 **Biofilm Composition**

Despite their heterogeneity, biofilms are fundamentally composed of two structural features; the microbial communities themselves which can constitute up to 15% of the biofilm volume and the EPS matrix which forms the remaining and majority of the biofilm. Biofilms characteristically contain multiple species of bacteria often co-existing with fungi in a mutually beneficial relationship called co-metabolism. This process facilitates highly efficient use and complete degradation of organic molecules and is advantageous to the entire microbial community, making commensalism a common phenomenon within biofilms [122]. Much of the biofilm’s microbial heterogeneity is a consequence of the diffusion limitation imparted by the biofilm structure. This creates extremely diverse microenvironments in terms of temperature, pH, nutrient availability and oxygen tension [123]. These micro-niches not only determine what organisms can exist and co-exist but also influence their genetic expression [124].

Another important structure adding both organization and fortification to the biofilm is the extracellular matrix, produced by the constituent microbial cells. The composition of the matrix is complex and variable among different bacterial species and even within the same species under different environmental conditions [125]. Despite their heterogeneous composition, exopolysaccharides and proteins are common essential features of most biofilm matrices, providing a scaffold around which the microbial communities arrange themselves. The recent finding of commonality amongst different bacterial biofilms in the presence of certain exopolysaccharides (cellulose and B-1,6-linked N-acetylglucosamine) as well as some secondary signal pathway associated proteins (GGDEF-domain-containing proteins) and surface proteins (Bap-related proteins) has led researchers to believe that common essential elements may exist in the biofilm formation process [126]. Other important
components identified within the matrix albeit to lesser extents include lipids, extracellular DNA, metal ions, divalent cations and other biopolymers [127]. Although the role of these minor compounds remains largely unknown, research has suggested that they may be of critical importance in the establishment of the overall biofilm structure. Extracellular DNA, initially thought to be simply a by-product of cell lysis has now been shown to be actively released through an exocytotic mechanism involving the outer bacterial membrane [128, 129]. A study by Whitchurch et al of P. aeruginosa biofilms demonstrated that in the absence of extracellular DNA, biofilm formation was inhibited [130], and that enzymatic degradation of extracellular DNA could dissolve immature biofilms, implying the importance of DNA in early biofilm establishment.

1.3.5 BIOFILM FORMATION

The evidence of biofilm formation in the early fossil record (more than 3.25 billion years ago) and their commonality throughout a diverse range of organisms has led researchers to hypothesise biofilm formation to be an ancient and integral component of the prokaryotic life cycle[131]. It is now thought that 99.9 % of bacteria adopt this form for the survival benefits it confers. Recently, there is a growing concept that biofilms may in actual fact represent an earlier evolutionary form than the planktonic state, and may indeed be the default form of growth for some microbial species [124, 132]. Although the evolutionary order of bacterial growth forms remains debated, there is a general consensus that bacteria freely interchange between the planktonic and biofilm form depending on the environmental conditions. This inter-form transition is rapid, owing to the enormous plasticity of bacterial genomes and is mediated by either plasmid exchange, chemotactic signalling or through diffusible signals arising from a process called quorum signalling [133-135].
Biofilm formation has been shown to occur by at least three mechanisms: (1) the redistribution of attached cells by surface motility [136, 137], (2) the binary division of attached cells [138] and (3) the recruitment of cells from the bulk fluid to the development of the biofilm [139]. The relative contribution of each mechanism will depend on the interplay between the organism and surface involved as well as environmental physical and chemical properties.

### 1.3.6 Biofilm Lifecycle

From proteomic studies of Pseudomonas sp, five main steps in the biofilm lifecycle have been established [140] and are diagrammatically represented by Stoodley et al in a recent article [141] (See figure 1). The stages are described as follows:

1. **Reversible Attachment.** During this process, individual microbial cells become reversibly associated with a surface and exhibit several species specific behaviours such as rolling, creeping, aggregate formation and “windrow” formation[136]. They are not yet “committed” to the differentiation process that leads to biofilm formation and may detach. Surface contact sensing and inter-cellular signalling or quorum sensing initiate phenotypic changes within the bacteria to irreversibly secure their initial attachment.

2. **Irreversible attachment** employs molecularly mediated binding between specific microbial adhesins and the surface. Bacteria have been shown to produce multiple different adhesins, many of which are regulated at the transcriptional level. This permits the rapid transition between planktonic and sessile forms depending on environmental factors [142]. One such example is the polysaccharide intercellular adhesin (PIA) that mediates the cell-cell interactions in some staphylococcal biofilms [143, 144]. Further consolidation of adhesion in this stage occurs through the microbial production of exopolysaccharides that complex with
surface molecules and/or receptor-specific ligands located on pili, fimbriae, and fibrillae [145]. At the conclusion of this stage, the biofilm’s attachment is considered irreversible making these structures extremely difficult to remove without chemical intervention or considerable mechanical force.

(3) Aggregation and (4) Maturation. During these stages, the surface bound organisms begin to actively replicate increasing the overall density and complexity of the biofilm. Interaction between the microbial colonies and the extracellular substances they produce results in the generation and maturation of the biofilm architecture, and the redistribution of the organisms away from the substratum [134]. DNA and proteomic studies have shown that in this stage, biofilm bacteria have radically different levels of genetic and protein expression compared to their planktonic counterparts [140, 146], with differences in expression observed as early as 15 minutes of initial surface contact by the microbe [116].

(5) Detachment. When biofilms reach their critical mass as determined by numerous environmental conditions, such as the availability and perfusion limitation of nutrients and wastes, the peripheral layer of growth begins to re-differentiate into planktonic organisms which can embolise. This phenomenon is seen commonly in a clinical setting and is thought to explain the periodic spikes in fever associated with device related biofilm infections.

There is recent evidence to suggest that all these stages of biofilm formation and development growth and development may be under the regulation of population density-dependent gene expression mediated by cell-to-cell signalling molecules such as acylated homoserine lactones.[134, 147, 148]
When attached, bacteria show a profound resistance, rendering biofilm cells 10-1000 fold less susceptible to various antimicrobial agents, disinfectants and biocides than the same bacterium grown in planktonic cultures [149-151]. Although this resistance was initially postulated to be mediated by a single generalizable mechanism, recent studies suggest that it more likely to be a multi-factorial process and that the mechanism may vary among different organisms. The main hypotheses have been summarised below.

(a) Delayed antibiotic penetration of biofilms. The presence of the exopolysaccharide matrix of biofilms has long been held to have a role in limiting the penetration of
antimicrobials to deep within biofilms. It was hypothesised that the matrix did this by either physically influencing the rate of transport of the antimicrobial agent or by deactivating it on its passage through the matrix. Recent in vitro studies have disproven this hypothesis for the majority of antimicrobials by documenting unimpaired antimicrobial penetration of the biofilm [152-158]. Three exceptions must be noted however involving aminoglycosides, β-lactams and some glycopeptide antibiotics. There is some evidence suggesting that electrostatic binding of positively charged aminoglycosides to the negatively charged polymers of the biofilm matrix, may retard the penetration of these antimicrobial agents and allow bacteria the necessary time to implement adaptive stress responses [159-162]. Additionally some biofilms such as those produced by *Klebsiella pneumoniae*, accumulate beta-lactamase in the biofilm matrix as a result of secretion or cell lysis and can subsequently deactivate beta-lactam antibiotics in the surface layers more rapidly than they diffuse into the biofilm [152, 160, 163, 164]. Finally it has been noted that slime associated with certain strains of *S. epidermidis* has been shown to physically complex with and antagonise specific glycopeptide antibiotics [165-167].

(b) Altered Microenvironment and Reduced Growth Rate. It is now well established that within biofilms, micro-gradients occur in the concentration of key metabolites and products [168]. These chemical gradients have been shown to directly alter antibiotic potency. Tack and Sabath showed that oxygen availability alone, modulated the action of aminoglycosides, with bacteria in anaerobic environments more resistant to these antibiotics than those in aerobic ones [169]. Similarly, gradients in pH have also been shown to impact negatively on antibiotic efficacy [170, 171]. Additionally, in areas of nutrient depletion, studies using fluorescent probes and reporter genes, have demonstrated that bacterial cells also significantly reduce their growth and metabolic rate [172-174]. As almost all antimicrobial agents are more
effective in killing rapidly growing cells, this slow growth undoubtedly also contributes to biofilm resistance to antimicrobial killing [175].

(c) **Altered Genetic expression.** DNA microarray and proteomic studies have demonstrated differences in gene expression and protein profiles of biofilm and planktonic bacteria. It has been postulated that increased expression of biofilm-specific resistance genes, such as those coding for multidrug efflux (MDR) pumps or periplasmic glucans may also contribute to antimicrobial resistance [176] [177]. The additional finding by a recent study that genetic disruption of expression of MDR pumps in Pseudomonal biofilms, also affected their biofilm attachment, suggests that antibiotic resistance may be under the same regulatory or genetic control as other biofilm associated traits [178].

(c) **Persisters.** It has been proposed that within the heterogeneous population of biofilm microbial cells, a small sub-population of cells, referred to as persisters, may exist. It is thought that these cells adopt a unique and highly protected, phenotypic state, akin to spore formation and are thought to serve as a nidus for biofilm regeneration following antimicrobial treatment[179-182]. Data in support of this persister hypothesis includes measurements of biphasic biofilm killing in which the majority of the cells are killed but a fraction remain unaffected despite prolonged antibiotic treatment [176, 183] Multiple specific genes that contribute to the persister state have now been isolated, an example of which is the high level persistence gene (hip) described in E.coli [181, 184, 185]
Although biofilms are known to be less susceptible to antimicrobial drugs, less is known about their susceptibility to the innate immune system. The innate immune system represents the first line of defence against bacterial colonization and infection. It provides humans with antigen-independent mechanisms of coping with infectious challenges in the absence of pre-existing adaptive immunity and has been shown to be of critical importance in the early stages of infection [186]. This system is multi-tiered and encompasses factors that prevent bacterial adherence to mucous membranes (e.g. mucus and ciliary movement), limit bacterial growth and replication (e.g. antimicrobial peptides) and direct host immune cell responses through specific recognition (Toll-Like receptor activation). Through these mechanisms it is thought that the innate immune system may prevent pathogens gaining a foothold and may also provide the host with the time needed to mobilize the more slowly developing mechanisms of adaptive immunity. It is becoming increasingly evident that pathogenic bacteria use very efficient strategies to circumvent and misguide these host defences in order to colonise and invade human tissues [187]. One such strategy is through the formation of thick biofilms that may prevent the recognition and/or inactivation by antimicrobial molecules and phagocytes [188, 189]. Research examining pseudomonal biofilms have shown that the exopolysaccharide matrix may afford protection against human neutrophils and interferon-\(\gamma\) mediated macrophage killing [190],[191]. Although not well characterised, several mechanisms have been proposed for this increased biofilm resistance to human leukocyte killing; (1) the inactivation or suppression of leukocyte-specific proteases by the biofilm matrix or bacterial components; (2) the decreased ability of leukocytes to phagocytise biofilm bacteria (3) the presence of global response regulators and quorum sensing that increase resistance to leukocytes in biofilms and/or (4) specific genetic switches that lead to increased resistance to components of the human innate system. Although it was initially thought that
limited biofilm penetration of leukocytes and their antimicrobial products may play a role, Leid et al [192] have recently shown that in some biofilms leukocytes may penetrate the matrix. Interestingly, Walker et al [193] have also shown that when the host fails to eradicate an infection, neutrophils can undergo necrosis and serve as a biological matrix that may further facilitate microbial biofilm formation.

Research, although limited, has also been conducted on the activity of antimicrobial peptides on biofilms. Several studies have shown that polysaccharide intracellular adhesin (PIA), an important polymer required in the EPS matrix formation of Staphylococcal and some Escherichia coli biofilms, significantly reduces the ability of cationic antimicrobial peptides (CAM) to inactivate S. epidermidis [194, 195]. Studies examining the fungal biofilms of Cryptococcus neoforms, have also demonstrated that fungal cells in biofilms are less susceptible to certain defensin antimicrobial peptides than planktonic cells [196, 197]. Despite the obvious decreased activity of some antimicrobial peptides against bacterial biofilms, a recent study by Singh et al[198] has demonstrated that lactoferrin, the second most common antimicrobial peptide after lyzosyme may prevent the initial development of bacterial biofilms. A review of the structure and functions of lactoferrin is included in section 1.4.

### 1.3.9 Biofilms and ChroniC Diseases

Progress in microbiology and the development of antibiotics and vaccines has led to the successful treatment and in some cases almost complete eradication of many acute epidemic bacterial diseases. With these advances also have emerged the less aggressive and more persistent chronic diseases. Until the rediscovery of biofilms, many chronic diseases were thought to be sterile inflammatory conditions that persisted after the eradication of all microorganisms. This belief stemmed from the following: (1) it was often difficult to successfully
apply Koch’s postulates, the long standing paradigm of bacterial causation of disease, to many chronic conditions, (2) in many chronic diseases, bacteria recovery using conventional culturing methods was not possible, (3) on the occasions when bacteria were isolated and found to be sensitive to antibiotics in laboratory cultures, use of these antibiotics yielded little or no benefit in the treatment of the chronic disease and (4) unlike acute infections which generally involved either host immune-suppression or highly virulent pathogenic organisms, many of the pathogens isolated from chronic diseases were common environmental organisms with seemingly low virulence and poorly defined pathogenic mechanisms. The unequivocal demonstration, using molecular diagnostics, of the presence and metabolic activity of bacteria within many of these “sterile” conditions, accompanied by the increasing environmental and industrial evidence of bacteria existing in an alternate biofilm form, led researchers to revisit the role that bacteria may play in chronic disease. Based on direct examinations of material from device related and other chronic infections, and on patterns of inherent resistance to antibiotics, and to host clearance mechanism, the US Centre for disease control and prevention (CDC) estimated in 1999 that biofilms may be responsible for in excess of 65% of all infections in the developed world [199]. A partial list of common biofilm mediated diseases is shown in table 4 taken from reference [199].

1.3.10 Biofilms and Otolaryngology

Research in the field of otorhinolaryngology has demonstrated bacterial biofilms in a variety of common ENT conditions previously thought to have non bacterial aetiologies. For many years the belief that Otitis media with effusion (OME) was an inflammatory condition, largely stemmed from the often sterile cultures obtained from middle ear aspirates of patients suffering from this condition. With the advent of newer technologies such as the polymerase-
chain reaction (PCR) based assay system, bacterial DNA has been found in a significant percentage of middle ear effusions, sterile by culture [200, 201]. This coupled with the large number of genomic equivalents present in surgically treated cases of OME and the presence of bacterially produce endotoxins within the effusion fluid has led researchers to believe that viable metabolically active bacteria may be contributing to the pathogenesis of OME [200, 202, 203]. The biofilm paradigm of live yet difficult to culture and antibiotic resistant bacteria seemed to offer a plausible explanation for the clinical and microbiological findings of OME. Further evidence supporting the role of biofilms in this condition was provided by the demonstration of mucosal biofilms on the middle ear mucosa in an experimentally infected chinchilla model for OME [204]. Since then CSLM, FISH and immuno-staining examination have provided direct evidence of biofilm presence on the middle ear mucosa of children with OME, further strengthening the biofilm hypothesis of disease causation/persistence [205]. Biofilms have also been demonstrated on tonsillar and adenoidal tissue. Galli et al [206], used scanning electron microscopy to show structures resembling biofilms in 21/25 patients with adenotonsilitis. Furthermore they isolated Haemophilus influenza, with a high in vitro biofilm forming capacity, in large proportion of the adenoidal and tonsillar specimens taken from these patients. Also using SEM Zuliani et al [207], demonstrated almost universal mucosal coverage (mean biofilm coverage 94.9%) of adenoidal tissue in all 7 CRS patients included in their study while only 4 of 9 children with OSA and no CRS showed biofilm structures (mean biofilm coverage of 1.9%). These findings coupled with the recent CSLM evidence of biofilms on the adenoidal tissue of 54% of children with chronic or recurrent otitis media, suggests that biofilms in the nasopharynx and in particular the adenoidal pad, may serve as a reservoir for recurrent and persistent infection of neighbouring anatomical structures [208]. This may also explain why mechanical removal of these biofilms via adenoidectomy is often accompanied by marked clinical improvement in the chronic sinus and ear related conditions suffered by children.
Aside from the above conditions, biofilms have also been demonstrated in the keratin accumulations of patients with cholesteatoma [209], in the tonsillar crypts of patients with chronic tonsillitis [210, 211], and on otolaryngological-related prosthetic devices such as tracheostomy tubes [212], endotracheal tubes [213, 214], cochlear implants [215-217] voice prostheses [218-220] and tympanostomy tubes [221, 222].

### Table 4 Chronic diseases thought to be mediated by biofilms

<table>
<thead>
<tr>
<th>Nosocomial infections:</th>
<th>Common biofilm bacterial species</th>
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<tbody>
<tr>
<td>ICU pneumonia</td>
<td>Gram-negative rods</td>
</tr>
<tr>
<td>Sutures</td>
<td><em>Staphylococcus epidermidis</em> and <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>Exit sites</td>
<td><em>S. epidermidis</em> and <em>S. aureus</em></td>
</tr>
<tr>
<td>Arteriovenous shunts</td>
<td><em>S. epidermidis</em> and <em>S. aureus</em></td>
</tr>
<tr>
<td>Schieral buckles</td>
<td>Gram-positive cocci</td>
</tr>
<tr>
<td>Contact lens</td>
<td><em>Pseudomonas aeruginosa</em> and Gram-positive cocci</td>
</tr>
<tr>
<td>Urinary catheter cystitis</td>
<td><em>Escherichia coli</em> and other Gram-negative rods</td>
</tr>
<tr>
<td>Peritoneal dialysis (CAPD) peritonitis</td>
<td>A variety of bacteria and fungi</td>
</tr>
<tr>
<td>IUDs</td>
<td><em>Actinomyces israelii</em> and many others</td>
</tr>
<tr>
<td>Endotracheal tubes</td>
<td>A variety of bacteria and fungi</td>
</tr>
<tr>
<td>Hickman catheters</td>
<td><em>S. epidermidis</em> and <em>Candida albicans</em></td>
</tr>
<tr>
<td>Central venous catheters</td>
<td><em>S. epidermidis</em> and others</td>
</tr>
<tr>
<td>Mechanical heart valves</td>
<td><em>S. aureus</em> and <em>S. epidermidis</em></td>
</tr>
<tr>
<td>Vascular grafts</td>
<td>Gram-positive cocci</td>
</tr>
<tr>
<td>Biliary stent blockage</td>
<td>A variety of enteric bacteria and fungi</td>
</tr>
<tr>
<td>Orthopedic devices</td>
<td><em>S. aureus</em> and <em>S. epidermidis</em></td>
</tr>
<tr>
<td>Penile prostheses</td>
<td><em>S. aureus</em> and <em>S. epidermidis</em></td>
</tr>
<tr>
<td>Dental caries</td>
<td>Acidogenic Gram-positive cocci (e.g. <em>Streptococcus</em>)</td>
</tr>
<tr>
<td>Periodontitis</td>
<td>Gram-negative anaerobic oral bacteria</td>
</tr>
<tr>
<td>Otitis media</td>
<td>Non-typable strains of <em>Haemophilus influenzae</em></td>
</tr>
<tr>
<td>Musculoskeletal infections</td>
<td>Gram-positive cocci (e.g. staphylococci)</td>
</tr>
<tr>
<td>Necrotizing fasciitis</td>
<td>Group A streptococci</td>
</tr>
<tr>
<td>Biliary tract infection</td>
<td>Enteric bacteria (e.g. <em>E. coli</em>)</td>
</tr>
<tr>
<td>Osteomyelitis</td>
<td>Various bacteria and fungal species – often mixed</td>
</tr>
<tr>
<td>Bacterial prostatitis</td>
<td><em>E. coli</em> and other Gram-negative bacteria</td>
</tr>
<tr>
<td>Native valve endocarditis</td>
<td>Viridans group streptococci</td>
</tr>
<tr>
<td>Cystic fibrosis pneumonia</td>
<td><em>P. aeruginosa</em> and <em>Burkholderia cepacia</em></td>
</tr>
</tbody>
</table>

*Abbreviations: CAPD, continuous ambulatory peritoneal dialysis; ICU, intensive care unit; IUD, intrauterine device.*

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Despite the exponential increase in biofilm research in the wider scientific community, there has been limited research concerning the role of these structures in chronic sino-nasal inflammatory conditions, such as CRS. Prior to the commencement of this PhD, in 2005, there were a total of only 7 publications (5 human studies, 1 animal model and 1 review) concerning biofilms and chronic rhinosinusitis. Although all of these studies had demonstrated biofilms on the sino-nasal mucosa of CRS patients, concerns existed as to the detection modalities used. Furthermore, these studies were mainly descriptive and did not appear to examine the clinical significance of biofilm presence. This early literature will be reviewed now, with more recent studies, published after 2005, addressed in the final discussion chapter.

The first study suggesting biofilm presence on sinus mucosa of CRS patients was a descriptive paper published by Cryer et al in 2004 [223]. This small study of 16 CRS patients, who had failed maximal medical and surgical treatment of their condition, utilised SEM to analyse sinus mucosal specimens. Their findings of near-total surface coverage of four specimens by a coating thicker than the normal mucocilliary blanket, led the authors to speculate biofilm presence in these patients. It is important to note however, that structures resembling actual bacterial elements were only seen in one of these four specimens. Despite the lack of conclusive evidence, the similarity between the SEM appearances of this single specimen to that of previously published images of biofilms, led the authors to conclude that there was “compelling” evidence of bacterial biofilms in patients with recalcitrant CRS. They further hypothesized that these structures may explain the treatment resistant nature of this disease.
Similar pilot studies using electron microscopy were published the following year in 2005. Ramadam et al [224] reported staphylococcal-type biofilm in all 5 CRS patients they examined using SEM. According to the authors, biofilm presence was determined by using strict SEM morphologic criteria as described in the referenced literature, as well as by comparing the images they obtained with “hundreds of biofilm photographs”. Unfortunately, of the ten papers referenced by the authors, no evidence of specific SEM morphologic criteria for biofilm detection could be found in any of them. Furthermore, two of the included references made no mention of the structure of biofilms at all [225, 226], and only two used CSLM or epifluorescent microscopy as part of their methodology [127, 226]. Additionally, the sources of the comparative photographs used, were not provided by the authors. Importantly, in their discussion, the authors do address some of the possible limitations of using SEM to visualise biofilms. They acknowledge that artefacts arising from the dehydration and protein cross-linking that accompany standard SEM preparation, may be confused with biofilms and raise concerns of inadvertent biofilm removal by the harsh SEM preparation process.

Other early electron microscopy research also revealed that biofilms were not only limited to the sinus mucosa. Perloff et al [227] visualised multicellular syncytia coated with extracellular matrix on all frontal stents removed post-operatively from 6 CRS patients. The similarity of these structures to known images of biofilms as well as to structures visible on sterile stents cultured in vitro with known biofilm forming organisms, suggested, that they may represent biofilms. The absence of these structures on stents that did not undergo in vitro culture with bacteria, supported the author’s hypothesis that frontal sinus stents may serve as a reservoir for biofilms. Ferguson and Stolz [228] visualised biofilms within the amorphous material overlying the mucosa in 2 of the 4 CRS patients they analysed with TEM. In the two patients without biofilms, a non-bacterial aetiology was discovered. Interestingly, both patients with bacterial biofilms had failed medical management with culture-directed antibiotics,
topical steroids, and nasal lavage, further supporting Cryer et al’s [223] initial hypothesis that biofilms may not only be involved in the pathogenesis of CRS but more importantly may explain its recalcitrant and often resistant nature. Although these early studies represented significant advances in biofilm research in the field of rhinology, their small sample size and lack of healthy control specimens, made it difficult to draw definitive conclusion about the role of biofilms in the pathogenesis of CRS. To address this, Perloff and Palmer created a biofilm model in NZ white rabbits [229]. In this study they consistently created sinusitis in each of the 22 maxillary sinuses inoculated with *Pseudomonas aeruginosa*. Mucosa from the middle turbinate and maxillary sinus of the contra-lateral uninoculated side was used as the control. The absolute correlation they observed between the macroscopic features of sinusitis and the presence of biofilms under SEM, and the absence of any biofilms in the controls further strengthened the possible causal relationship of bacterial biofilms and CRS. Furthermore they noted that mature biofilms at day 5 underwent little morphological change at day 10 or 20, suggesting a plateau stage of biofilm development. Although their rabbit model provided further evidence of biofilm involvement in CRS, methodological flaws exist in this model. Firstly, although rabbit models have been used extensively in sinusitis research since Hilding first introduced their use in 1941 [230-238], the universal colonization of rabbits with *Pasteurella multilocoda*, a known biofilm forming organism, may render their use limited in biofilm research. Furthermore, a study by Juan et al in 1995, showed that despite the macroscopic normal clinical appearance of healthy New Zealand white rabbit sinuses, 70% demonstrated histological evidence of sinusitis and positive bacterial cultures [239]. A second problem identified with Perloff’s model pertains to the external surgical technique used to achieve ostial obstruction. This technique which relies upon surgical violation of the sinus cavity, may not only introduce external bacterial contamination but is also to thought to alter the normal physiology of the
sinus mucosa by interrupting normal sinus capillary flow and pre-existing mucociliary flow patterns [240, 241]. Extrapolation of the findings of this non-physiologic “sinogenic” model of sinusitis to that seen in humans with CRS may be erroneous. Other additional specific problems with their proposed model involve: (1) the lack of consistent control tissue, with middle turbinate tissue often used instead of sinus tissue (2) the lack of a study arm to examined the contribution of the surgical procedure itself to the formation of sinusitis and (3) the use of cyanomethacrylate glue to ensure maintenance of the ostial plug. Research has shown that shorter-chain derivatives (methyl- and ethyl) of cyanoacrylate are histotoxic to sinus mucosa causing acute inflammation, tissue necrosis, and a chronic foreign body giant cell reaction [242]. Finally the over-riding flaw of this study was the reliance on SEM alone to document biofilm presence, despite previously raised concerns of its subjectivity, and potential problems associated with dehydration, distortion, and artefact introduction.

In an attempt to address many of the theoretical concerns of using SEM in biofilm research, Sanclement et al conducted a human study of 30 CRS patients [243]. In this study, 24 CRS specimens and all 4 controls were processed using standard SEM techniques while 6 CRS specimens were prepared with advanced cryofixation methods for SEM and TEM analysis. According to the authors, the purpose of this smaller experimental group was to examine (a) whether standard SEM preparation caused dehydration or artefactual error and (b) whether structures resembling bacterial biofilms on surface analysis, demonstrated bacterial structures on cross-sectional analysis with TEM. This study reported an absence of biofilms on control specimens and 80% prevalence in CRS patients. The authors also concluded that standard SEM preparation did not introduce significant artefacts from protein cross linking and that although it caused some reduction in biofilm size from dehydration, biofilms were still easily recognizable. They also reported an absolute correlation between structures deemed biofilms on SEM and TEM. When critically reviewing this study however, it is important to note the
following; (1) the very small number of controls (n=4) included in the study, (2) the fact that the majority of specimens (24 of 30) were only analysed with SEM, (3) specimens from the same patient were not used in the comparison of standard SEM preparation and cryofixation and (4) that no control or non-biofilm patients were included in the group analysed with both SEM and TEM. Such methodological flaws, not only throw into question the high biofilm prevalence rate observed in this study, but also make it difficult to substantiate many of the authors’ conclusions.
1.4 LACTOFERRIN

1.4.1 INTRODUCTION

Although initially identified as an abundant milk protein[244], lactoferrin has since been found to be predominantly expressed by surface epithelia and secreted into the mucosal environment along with many other antimicrobial substances. Its production is particularly high in upper airway, gastric, genital and ophthalmic secretions and has been found to be strongly upregulated during periods of environmental and physiological stress [245-247]. Lactoferrin is also present in much lower concentrations in the nuclei and secondary granules of neutrophils which provide the only circulating form of this peptide [248, 249]. It is for this reason that many researchers believe that lactoferrin levels may also serve as a good marker of both inflammation and the acute phase response [250, 251].

1.4.2 STRUCTURE

The complete amino acid sequence of human serum lactoferrin was first determined in 1984 by Metz-Boutigue [252]. He not only demonstrated its 60% sequence identity with serum transferrin, another iron binding protein, but also showed 40% internal homology of its two domains, the N- and C-terminals. In addition approximately 70% amino acid sequence identity has since been demonstrated between different species (human, bovine, buffalo, camel, goat, horse, mouse, pig), suggesting early evolutionary importance of this antimicrobial peptide [253]. In 1990, the complete cDNA sequence was isolated and found to encode a signal peptide of 19 amino acids followed by a mature protein, 692 amino acids in length [254],[255].
Using crystallographic analysis with a resolution of 2.8 Å, the tertiary structure of lactoferrin, has been well characterized [256]. The polypeptide chain of human lactoferrin has been shown to be folded into two almost homologous globular lobes each with the same fold, reflecting their previously eluded to sequence identity. These lobes consist of an N terminal half of amino acid residues 1-333 and a C terminal half of amino acid residues 345-691 linked together by a 10 residue peptide chain (residues 334-344) that forms an extended 3-turn alpha-helix [257]. Within each lobe 2 alpha/beta domains exist that enclose a deep cleft surrounding the iron binding site. The iron binding sites are chemically and geometrically ideal for high affinity but reversible iron binding, and any mutagenesis of these ligands substantially weakens their iron binding ability [258],[259],[260]. See figure 2 for the diagrammatic representation of lactoferrin’s structure. At least four isoforms of lactoferrin, α, β,γ,δ are now known to exist. Although they share very similar chemical compositions, they have been shown to differ in their iron binding activity (only possessed by the α isoform), and enzymatic activity ( with β and γ having RNase activity but not α). It is thought that these differences may explain the many diverse functions that have been attributed to lactoferrin.

<table>
<thead>
<tr>
<th>Figure 2</th>
<th>Lactoferrin’s three dimensional structure</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Iron Bound" /></td>
<td><img src="image2" alt="Iron Free" /></td>
</tr>
</tbody>
</table>

Iron Bound | Iron Free
1.4.3 LACTOFERRIN’S ANTIMICROBIAL ACTION

Lactoferrin has been shown to possess diverse antimicrobial actions against a wide range of bacteria, fungi, viruses and protozoa. Much of this activity was initially thought to be mediated by lactoferrin’s ability to sequester iron, an important nutrient for microbial growth. Although lactoferrin’s bacteriostatic properties have been shown to be iron dependent, recent studies have demonstrated iron-independent microbicidal activity of this peptide [261]. Studies of cationic peptides obtained from the-N-terminus of either human or bovine lactoferrin have shown them to be significantly more active than the parent protein towards bacteria, suggesting this terminus as the location for lactoferrin’s microbicidal activity [262-264]. This anti-pathogenic activity of lactoferrin and indeed its active cleavage product lactoferricin, is thought to be similar to that of other amphipathic cationic peptides that can bind directly to and disrupt the membranes and ultra-structure of gram negative and positive bacteria and some candidal fungi. [265-268] Aside from this mechanism of action, lactoferrin has also been shown to alter the virulence and pathogenicity of bacteria through its (1) antibiofilm action which will be reviewed in more detail later, (2) its inhibition of bacterial attachment to host surfaces as seen with H pylori [269] (3) its direct cleavage and removal of critical colonization factors, attenuating the pathogenicity of organisms such as H influenza [270] (4) its ability to amplify the apoptotic signals within infected cells [271] and (5) its direct intracellular interaction and enhancement of bactericidal activity within of PMN [272].

Lactoferrin and lactoferricin have also been shown to have antiviral activity against both enveloped viruses (HSV 1and 2 CMV, HIV, HBV, HCV, RSV) as well as naked viruses (rotavirus, poliovirus, adenovirus and enterovirus) [273]. This action is again thought to be iron independent and is postulated to either be mediated by lactoferrin directly binding to viral
particles before they infect host cells, or by it binding to and altering host cell molecules which viruses ordinarily use as receptors to enter cells [274]. This direct viral binding capacity of lactoferrin has led some researchers to explore the use of this anti-microbial peptide as a selective delivery system for antiviral medications.

1.4.4 IMMUNE REGULATION BY LACTOFERRIN

Multiple studies exist examining the effect of lactoferrin on the immune system. A recent study by Ishikado et al, demonstrated a sustained significant increase in the production of the anti-inflammatory cytokine, interferon-alpha (IFN-alpha) in healthy volunteers given oral liposomal lactoferrin [275]. Further evidence of lactoferrin’s regulation of the immune system is provided by in vivo models of LPS-induced sepsis. These models demonstrate that lactoferrin is capable of differentially suppressing the production of specific inflammatory cytokines, by not only binding to and inactivating LPS, but also through competitive as well as direct receptor-mediated interaction with inflammatory and endothelial cells [276-278].

Other reported actions of lactoferrin on the immune cells include (1) in vitro evidence of its receptor-mediated regulatory effects on T cell maturation and activation [279], (2) its enhancement of polymorphonuclearcyte killing by promoting motility, superoxide production and release of pro-inflammatory molecules such as NO, TNFa and IL8 [272, 280] and (3) its ability to increase the number of Natural Killer (NK) cells and enhance their killing action through direct modulation of the NK cell cytotoxicity and increased sensitivity of target cells to lysis [281, 282].
1.4.5 Anti-biofilm action

The ubiquitous nature of lactoferrin and the apparent lack of biofilms on healthy mucosal surfaces led many researchers to postulate that this anti-microbial peptide may also play a role in the prevention of biofilm formation. Early studies demonstrating lactoferrin’s iron independent inhibition of bacterial adhesion to surfaces support this hypothesis [270, 283, 284]. In 2002 Singh et al [198] showed that P aeruginosa, existing in biofilms, exhibited 100 times more resistance to lactoferrin-mediated killing than the same bacteria grown in the planktonic state. Further in vitro studies by Singh, elegantly demonstrated that in the absence of lactoferrin, the prototypical stages of biofilm development occurred, whilst in sub-inhibitory concentrations of lactoferrin (20ug/ml) bacteria attached and multiplied but failed to form micro-colonies or differentiated biofilm structures [285]. Using time lapse-microscopy he discovered that lactoferrin stimulated a form of bacterial locomotion called twitching, which caused the micro-organisms to move away from the point of parental cell division. In the absence of lactoferrin he noted that the daughter cells and their progeny remained aggregated near the original locus of parental cell division and began forming micro-colonies, the precursor to biofilms. This anti-biofilm action was determined to be primarily related to lactoferrin’s iron sequestering ability and did not appear to affect mature-well established biofilms.

Deficiencies in the level or activity of lactoferrin are thought to partly explain the propensity of biofilm formation in chronic diseases such as cystic fibrosis. In 1993 Britigan et al [286] showed that lactoferrin was proteolytically degraded in the lungs of cystic fibrosis patients infected with Pseudomonas aeruginosa by proteases such as neutrophil elastase and Pseudomonas elastase. Later in 2004, Rogan et al [287] established that this failure to achieve the expected increase in levels of lactoferrin despite large amounts of neutrophil degradation...
was due to the action of another elastolytic protease called cathepsin. They showed that
cathepsins, which cleave and inactivate lactoferrin, reducing its antimicrobial and anti-biofilm
actions, had higher activity in CF patients with pseudomonas positive sputum. Although the
trigger for the increase in cathepsin activity is unknown, studies suggest that bacterial LPS
and cytokines (IFN gamma) may be involved [288, 289].

1.4.6 LACTOFERRIN AND CRS

Despite lactoferrin’s abundance in airway secretions and its presumed importance as a first
line defence against a broad spectrum of invading pathogens, there is little known about the
role it plays in rhinosinusitis. Analysis of nasal secretions of patients with acute or recurrent
rhinosinusitis suggests that lactoferrin secretion may be increased in this acute inflammatory
condition [290, 291]. A further three immunohistochemistry studies have subjectively
demonstrated more intense staining of submucosal glands and goblet cells in the sino-nasal
mucosa of patients with chronic rhinosinusitis, suggesting increased lactoferrin production in
CRS as well[292-294]. No studies exist however that use objective measures to document the
level of lactoferrin mRNA and protein in the sinus mucosa of CRS patients.
1.5 CONCLUSIONS FROM REVIEW OF THE LITERATURE.

The importance of bacterial biofilms in the pathogenesis of many chronic diseases and device related infections is now well established. The role of these structures in the pathogenesis of CRS however still remains unclear, owing to the paucity of research and inherent methodological flaws of the few studies that do exist. Furthermore objective research is required to not only document the existence of bacterial biofilms on the sinus mucosa of CRS patients but also to clearly establish the clinical significance of their presence in this condition.

Objective documentation of the levels of anti-biofilm peptides such as lactoferrin in chronic conditions such as CRS, may provide further insight into the role that deficiencies in the innate immune system may play in the pathogenesis of biofilm-mediated chronic condition.
1.6 STUDIES TO BE CONDUCTED

- The development of a sheep model to examine the role of biofilms in CRS and compare
  the different microscopic modalities used to detect such structures

- The detection of biofilms on the sino-nasal mucosa of CRS patients using Confocal
  Scanning Laser Microscopy.

- Assessment of the clinical importance of biofilms on CRS by examining the impact of
  biofilms on post-operative evolution of patients undergoing surgery for CRS

- Evaluation of the degree of lactoferrin expression in patients with CRS compared to
  healthy controls

- Evaluation of the relationship between the presence of biofilms and the degree of
  lactoferrin expression in CRS patients
CHAPTER 2

A SHEEP MODEL FOR THE STUDY OF BIOFILM IN SINUSITIS

NOTE: This publication is included on pages 43 - 68 in the print copy of the thesis held in the University of Adelaide Library.
CHAPTER 3

CONFOCAL SCANNING LASER MICROSCOPY EVIDENCE OF BACTERIAL BIOFILMS IN PATIENTS WITH CHRONIC RHINOSINUSITIS

NOTE: This publication is included on pages 70 - 89 in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

[http://dx.doi.org/10.1097/MLG.0b013e31806009b0](http://dx.doi.org/10.1097/MLG.0b013e31806009b0)
CHAPTER 4

THE EFFECT OF BACTERIAL BIOFILMS ON POST SINUS SURGICAL OUTCOMES

NOTE: This publication is included on pages 91 - 110 in the print copy of the thesis held in the University of Adelaide Library.
CHAPTER 5

NASAL MUCOSA EXPRESSION OF LACTOFERRIN IN PATIENTS WITH CHRONIC RHINOSINUSITIS

NOTE: This publication is included on pages 112 - 132 in the print copy of the thesis held in the University of Adelaide Library.
CHAPTER 6

REDUCED LEVELS OF LACTOFERRIN IN

BIOFILM-ASSOCIATED CHRONIC RHINOSINUSITIS

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CHAPTER 7

DISCUSSION AND CONCLUSIONS
7.1. OVERVIEW

Biofilm research in the field of rhinology is still in its infancy. Despite the significant advances in the imaging and study of biofilms in the wider scientific community, the limited research investigating the presence of biofilms in CRS patients has largely utilised the more subjective and older imaging modality of electron microscopy. This coupled with the apparent methodological flaws in the studies performed thus far, has resulted in ongoing uncertainty as to the exact role and importance of biofilms in the pathogenesis of CRS.

The main purpose of the research discussed in this thesis was to investigate the presence and significance of these structures in CRS through the creation of a more suitable animal model and the use of an alternative, possibly less subjective imaging modality. Having established CSLM as an objective and non-destructive imaging tool for the visualisation of biofilms on sinus mucosa, we re-examined the prevalence and clinical relevance of bacterial biofilm in CRS. In addition, we investigated possible differences in the innate immune system of CRS patients with and without biofilms. The main conclusions reached from the five studies performed as a part of this research are summarised below.
7.1.1 The ovine model is a suitable model for the investigation bacterial biofilms in rhinosinusitis.

Animal models using mice, rabbits, sheep and pigs have all been described in the investigation of possible aetiological factors of CRS [354-357]. Furthermore, New-Zealand white rabbits have been used to evaluate the role of bacterial biofilms in this condition[229]. As detailed in chapter 2, we demonstrated the suitability and possible superiority of the sheep model for biofilm-related rhinosinusitis research.

Our department has had significant experience with the sheep rhinologic model [301, 318, 357-366]. In Australia sheep are relatively inexpensive and easily obtainable. Their large size and blood volume allow them to withstand multiple long surgical procedures requiring general anaesthesia. Studies have also demonstrated sheep to possess an almost identical sinus mucosa histology and analogous sinus anatomy to humans [308, 309]. In addition, the sheep’s large nasal cavity makes it superior to smaller animals in surgical models trying to replicate endoscopic procedures conducted in humans. Ostial obstruction can be achieved entirely endoscopically, avoiding the need for large externally created surgical windows that could potentially disrupt the normal mucociliary and capillary blood flow patterns within the sinuses. In the model described in this thesis, the frontal sinus was selected to obstruct and inoculate with bacteria. Preceding cadaveric studies of sheep heads showed that as in humans, the frontal sinus is superficially located with an anterior table thickness of 5–12mm. The thin anterior table allows for mini-trephination that not only assists in the confirmation of the frontal sinus’ drainage tract but also provides a minimally traumatic portal for intra-sinus bacterial instillation. The site for safe and effective trephination was determined to be located at the bisection of a perpendicular line drawn from the midpoint of the bony orbit and a para-
sagittal line 1cm lateral to the midline. Using these landmarks, mini-trephination was successfully performed in all 48 sheep frontal sinuses without adverse event. The additional advantage of the mini-trephine is that it can be left in post-operatively and potentially used in treatment extensions of the model we have developed.

A criticism of the previously published rabbit model was the use of *Pseudomonas aeruginosa* as the bacterial inoculum [227]. Although commonly isolated from patients refractory to surgery, the overall prevalence of *P. aeruginosa* in CRS is low. Furthermore, specific strains of *P. aeruginosa*, particularly the mucoid variants, are extremely potent biofilm forming organisms, capable of forming bacterial biofilms in almost any environment. The use of such a strain would no doubt bias an in vivo model. For these reasons, the more commonly isolated pathogenic organism *S. aureus* was chosen for our sheep model. Specifically, American Tissue Type and Culture reference strain 25923 was used for its published biofilm forming ability which was confirmed by our own in vitro experiments [300, 367].

Using this model we were able to successfully and consistently create an inflammatory reaction consistent with sinusitis in the frontal sinuses of sheep. Through the use of different study groups, the relative and independent contribution of both anatomical ostial obstruction and bacterial load was assessed. As demonstrated by our findings; to create consistent sinusitis involving the entire sinus cavity, both obstruction and bacterial instillation were required. Without both of these interventions, the degree of mucosal inflammation was significantly reduced, less generalised and in some cases absent. Also of significance was the finding of largely localised inflammation around the cotton wool pledget in the sinus group randomised to ostial obstruction only. Such a localised inflammatory process would suggest a foreign body reaction rather than sinusitis and so this unrepresentative area of mucosa was excluded from microscopic examination for biofilms.
In addition to its demonstrated usefulness in the study of rhinosinusitis, the sheep model was also shown to be of use in the investigation of the role that biofilms play in this condition. The findings of an absence of biofilms in the control sinuses and an almost absolute correlation of their presence with macroscopic evidence rhinosinusitis in the remaining sinus groups, added support to earlier research suggesting their possible importance in the pathogenesis of CRS.

7.1.2 CSLM PROVIDES AN OBJECTIVE AND LESS INVASIVE MODALITY FOR IMAGING BIOFILMS BIOFILMS ON SINUS MUCOSA

CSLM is generally accepted by the scientific community to be the gold standard imaging modality for biofilms. Despite this, biofilm research in the field of rhinology has relied solely upon electron microscopy until recently. To address this, the sheep model we developed was also used to evaluate and compare CSLM to traditional electron microscopy in the imaging of biofilms on sinus mucosa. In this study, CSLM was found to be the most reliable modality for the detection of biofilms, demonstrating the highest concordance between macroscopic and microscopic findings. SEM, although a seemingly very sensitive modality, tended to overestimate biofilm presence, while the converse was found with TEM. In our study we experienced first-hand many of the previously postulated theoretical problems associated with EM. Dehydration and distortion of specimens during their preparation, was evident both macroscopically and microscopically. Several specimens confirmed as containing biofilms on both CSLM and TEM, lacked the typical three dimensional structures usually seen on SEM imaging. Rather biofilms appeared as collapsed and unorganised amorphous masses. Correct orientation of EM specimens after critical point drying was extremely difficult and inconsistent, making the incorrect mounting of samples for sputter coating highly likely. We
believe that this poses a real problem for new researchers who may make erroneous conclusions regarding biofilm presence, following inadvertent imaging of the non-mucosal surface of the specimen. From this study, and later from the findings of the study in chapter 6, it became evident that under SEM, mucus and the biofilm matrix, both composed of long chain polysaccharides, appear almost identical. Previous SEM sheep studies from our department have shown that the mucus layer which typically obstructs visualisation of the underlying ciliated mucosa is very difficult to remove. These studies required the use of ultrasound energy to dislodge the mucus [359, 365]. Unfortunately ultrasound could not be used in our study as it has also been shown to be an effective method of removing biofilms [305].

From close examination of sheep and later human SEM specimens, we observed that the only distinguishing feature between the biofilm matrix and mucus under SEM was the presence of clusters of bacterial elements. Although not mentioned as an important criterion for biofilm detection by previous SEM studies, we found the visualisation of such structures critical for the definitive documentation of biofilms. This is further highlighted by the findings of the study in chapter 6, which showed that when this criterion was adhered to, the SEM biofilm prevalence rate decreased significantly from 73% to 34% and more closely resembled that seen with CSLM. Unfortunately it must be noted however, that even though such strict criteria may improve the specificity of SEM, its sensitivity may suffer. This is because SEM only allows visualisation of surface structures and bacteria residing deep with the biofilm matrix will not be seen. Although our sheep study showed that TEM may improve the visualisation of these internal bacteria, its requirement for ultra-thin sectioning of specimens, also decreases the sensitivity of this modality. From these findings we concluded that when investigating biofilm prevalence, TEM and SEM should not be used alone.
CSLM seemed to circumvent many of the problems associated with EM. The larger specimen size accommodated by the CSLM’s stage meant that specimen orientation was much easier. Furthermore, it allowed a much larger surface area of mucosa to be imaged quickly, thereby reducing the chance of sampling error created by a biofilm’s typical patchy mucosal distribution. Unlike SEM and TEM, specimens were processed fresh avoiding aldehyde or ethanol-related dehydration. In addition the avoidance of formalin fixatives removed any possibility of artefactual error related to protein cross-linking. We found that through the use of nucleic acid probes and optical sectioning, CSLM allowed the entire three dimensional biofilm structure to be visualised and mucus could be easily distinguished from the biofilm matrix. In addition when combined with FISH, it has also been shown to be useful in the precise identification of the specific bacterial species within the biofilm. It is for these reasons that we believe CSLM has emerged as the imaging modality of choice for biofilm research in the wider scientific community and should be used in future CRS studies.

7.1.3 Objective evidence of the presence of biofilms in CRS patients.

Although biofilms have been previously documented on the mucosa of CRS patients, it was not until 2006 that the first CSLM study was published. In this study, biofilms were demonstrated using fluorescent in situ hybridization (FISH) in 14 of 18 CRS patients and in 2 of 5 healthy control specimens. Using specific radio-fluorescent labelled probes, bacterial identification was possible with *H. influenza* being found to be most common organism isolated within the biofilms (identified in 14/18 CRS specimens). Although *S. pneumoniae* and *S. aureus* were also detected their presence was far less common. Importantly, this study was also the first to demonstrate bacterial biofilms on control specimens leading the
authors to postulate a possible commensal or non-pathogenic role of biofilms in CRS. Although this conclusion may prove to hold true, planktonic contamination as a consequence of methodological deficiencies such as delayed processing time, absence of specimen washing and lengthy specimen preparation times cannot be excluded and may explain this finding. Such contamination may also explain the high biofilm prevalence rate observed in this study. Nevertheless, the use of FISH with CSLM in this study represented a significant advance in rhinological related biofilm research.

Using the CSLM protocol developed in the previously described sheep model, we examined a group of 38 CRS patients and 9 controls for evidence of biofilms. Unlike Sanderson el al’s study, specimens were processed fresh within three hours of collection and samples were subjected to three stringent wash steps to limit planktonic carry-over. 12 independent blinded observers analysed the samples with high concordance demonstrated amongst them. Evidence of bacterial biofilms was observed in 45% (17 of 38) of CRS specimens and in none of the controls. Importantly, two of the later studies we performed, summarised in chapter 4 and 6, showed similar CSLM biofilm prevalence rates of 50% and 41% respectively. We believe that the discrepancy between our lower rate of biofilm detection in CRS patients compared to that seen in previously published studies[243, 314] reflects (a) the stringency employed in our washing protocol to remove planktonic contamination and (b) the heightened specificity and objectivity of CSLM over EM. It should be noted however, that differences in the populations examined and the intra-operative sampling techniques, cannot be excluded and may also contribute to the apparent discrepancy. Despite the inconsistency in prevalence rates, the consistent and objective demonstration of biofilms on the mucosa of CRS patients and relative absence in controls further strengthens the hypothesis that these structures may play an important role in the pathogenesis of CRS.
7.1.4 Clinical features of biofilm patients

In the study performed in chapter 4, an attempt was made to elicit any differences in the clinical characteristics of biofilm patients compared to those in whom biofilms were not detected. Demographically no difference was found with respects to age or gender distribution. Interestingly, although no statistical difference was seen in the symptom scores reported by the two groups prior to surgery (median biofilm symptom score 17 with inter-quartile range 13.5-18.5 compared to median non-biofilm symptom score 16 (13.5-18.0), patients with biofilms had significantly higher Lund-Mackay scores on pre-op CT imaging (median score biofilm 18.5 with inter-quartile range 17.0-22.0 compared to median score non-biofilm 14.5 with inter-quartile range 14.0-18.0). This perhaps reflects a more severe and diffuse disease process in this sub-group of patients. It is important to note that the lack of correlation between CRS symptom scores and the overall radiological score observed in this study, is in concordance with the majority of previously published studies concerning this matter [322].

Due to the tertiary referral nature of the practice from which the patients were recruited, there were a high percentage of revision FESS cases. Worthy of note however, was the particularly high proportion of patients with biofilms requiring revision surgery compared to those without (75% vs 45%) and the trend for them having undergone a higher number of previous surgical procedures (average number 2.7 vs 1.8 previous FESS operations). This findings provides further support to the speculation by earlier researchers that biofilms may predispose to a persistent and recalcitrant sinus disease process, often refractory to surgical intervention [223, 228, 311].
Another important finding of our CSLM study was the lack of statistical relationship of positive planktonic bacterial culture rates and the detection of biofilms. This finding is consistent with that of Sanderson et al [314] and is in accordance with the proposed biofilm paradigm; that bacteria existing within biofilms exhibit much slower growth and metabolic rates than their planktonic counterparts, making them extremely difficult to culture using standard techniques.

7.1.5 The Effect of Biofilms on Post Endoscopic Sinus Surgical Outcomes

The success rate of FESS is extremely variable. Although some studies claim resolution or improvement of symptoms in up to 83-92% of patients [57, 319], the majority of this research is of low level evidence [53]. A recent systematic review of the literature by the Cochrane Collaboration Group concluded that based on the evidence available FESS does not confer any additional benefits to that obtained by medical treatments of CRS [49]. To date multiple studies have been performed addressing possible outcome predictors following FESS. These include demographic differences, smoking, presence of nasal polyposis and disease severity [54, 57, 61, 320, 321]. The first paper to address the possible effect of biofilms on post-operative outcomes was published by Bendouah et al in 2006 [311]. This study examined the relationship between the in vitro biofilm forming capacity of bacteria recovered from CRS patients and the evolution of their disease post-operatively. A significant correlation was reported between the biofilm-producing capacity of clinically isolated *Pseudomonas aeruginosa* and *Staphylococcus aureus* and an unfavourable evolution after FESS. The authors concluded that this provided further evidence for the role of biofilms in CRS.
In the study outlined in chapter 4, we retrospectively analysed prospectively collected data from 40 CRS patients who underwent ESS for symptoms of CRS. Of all the clinical and histological features evaluated, only the presence of biofilms and/or fungus were statistically related to a worse post-operative outcome in terms of ongoing symptoms and evidence of mucosal inflammation. The documented extreme difficulty in completely removing both biofilms and fungus from mucosal surfaces may partly explain this, with residual colonies of micro-organisms potentially serving as a nidus for re-infection. Interestingly, the presence of pus, polyps or eosinophilic mucus, all factors also believed to be associated with poorer post-operative outcomes, were not shown to have an adverse influence on ESS outcomes in this study. The reason for this is unclear but may be in part due to the fact that the scoring of post-operative outcomes in this study was nominal rather than ordinal, with any evidence of mucosal inflammation recorded as unfavourable. To address this, a blinded prospective study with endoscopic grading of post-operative mucosal outcomes is currently being undertaken in our department.

7.1.6 REDUCED LEVELS OF LACTOFERRIN EXPRESSION IN THE NASAL MUCOSA CRS PATIENTS

Despite lactoferrin’s abundance in airway secretions and its presumed importance as a first line of defence against a broad spectrum of invading pathogens, there is little known about the role it plays in CRS. The recent identification of lactoferrin’s anti-biofilm activity provided the motivation for the studies outlined in chapter 5 and 6. To our knowledge, these are the first two published studies to objectively quantify the expression of this antimicrobial peptide in the sino-nasal mucosa of CRS patients. All previous studies had relied upon the subjective and at best semi-quantitative methodology of Immunohistochemistry (IHC) [292-294].
In the study summarised in chapter 5, we observed an overall decrease in the mRNA and protein expression of lactoferrin in a large cohort of 85 CRS patients. These findings were contrary to the results of previous immunohistochemical studies that observed a stronger positive staining reaction for lactoferrin in the nasal mucosa and submucosa of patients with rhinitis, rhinosinusitis or nasal polyposis. The reason for this discrepancy is unknown but may be explained by differences in the sample size of the different studies and the methodology used. Of the three immunohistochemical studies published, two had very small sample sizes consisting of 10 and 19 CRS patients respectively [293, 294]. This in itself increases the possibility of sampling error which when formally calculated for these studies would be in the range of +/-31%, compared to +/-11% sampling error observed in our much larger study. The need for a large sample size is further reinforced by the observation in our own study of a large range of lactoferrin expression within both the CRS and control patients (range of lactoferrin protein expression; CRS 0–287ng/ml and control 30.5-373ng/ml). Although the third study published by Zhang et al [292] cannot be criticized for its sample size, it like the other two studies relied on immunohistochemistry to quantify the degree of lactoferrin expression. Although immunohistochemistry is a valuable research tool that allows both semi-quantification and localisation of protein expression, it does suffer from a degree of subjectivity. This is particularly the case with earlier forms of IHC such as the ABC (avidin-binding complex) method used in all three referenced studies, and is thought to be related to the high background signal produced from both electrostatic and non-specific binding of avidins to lectins in the tissue. As a consequence of this, the ABC technique is being largely replaced by newer more specific techniques [337].

Our study also demonstrated that although all sub-groups of patients with chronic rhinosinusitis seem to have a down regulation of lactoferrin in their nasal mucosa compared to
healthy controls, this reduction only achieved statistical significance in the CRS sub-group. The reason for this is unclear and may either be due to the much smaller sample sizes of the other sub-groups or alternatively may reflect differences in the aetiological, precipitating or perpetuating factors contributing to the rhinosinusitis in each group. The former explanation seems more likely due the fact that when all four subgroups were directly compared against each other, no statistically significant difference in the level of lactoferrin expression was observed between any of them. Another interesting finding of this study was the greater reduction of lactoferrin seen in polyposis patients. It is highly possible that the loss or reduction of the well documented anti-inflammatory and immuno-modulating functions of lactoferrin may predispose to polyp formation, therefore explaining this finding.

7.1.7 REDUCED LEVELS OF LACTOFERRIN EXPRESSION IN BIOFILM ASSOCIATED RHINOSINUSITIS

Having identified a reduction in lactoferrin expression in a subset of CRS patients, we then conducted the study outlined in chapter 6. The purpose of this study was to observe if any correlation existed between reduced levels of lactoferrin and the presence of mucosal biofilms in CRS patients. Given the absolute absence of such structures in the controls of our previous studies, and from the published in vitro evidence of inhibition of biofilm formation by lactoferrin, we hypothesized that an inverse correlation between the two may exist. This hypothesis was upheld with the findings of this study showing that CRS patients with biofilms had statistically significant reductions in lactoferrin mRNA and protein expression compared to non-biofilm patients and controls.

It has already been documented in cystic fibrosis patients that a reduction in the antimicrobial action or gene expression of innate peptides such as lactoferrin may predispose individuals to
recurrent infections and inflammation [287, 339]. Given its diverse anti-pathogenic activity, it also seems plausible that a decrease in the synthesis and production of lactoferrin in certain people may also predispose them to chronic infections like CRS. It must be noted however, that although an association between reduced lactoferrin levels and biofilm presence was evident in this study, a cause and effect relationship could not be deduced. As a consequence the possibility of impaired lactoferrin production by the biofilm itself or through a secondary mediator produced by the biofilm cannot be excluded and further investigation is warranted.
7.2 Treatment of bacterial biofilms

The identification of bacterial biofilms as important in the pathogenesis of CRS selects them as a potential therapeutic target. The development of anti-biofilm treatments is still in its infancy, and although a detailed discussion of such treatments is not part of the scope of this thesis, a brief summary of the more promising treatments will be discussed.

a) Surfactant based treatments – The amphipathic nature of surfactants has caused heightened interest in their possible role as antibiofilm agents. It is thought that by disrupting the protective biofilm matrix, surfactants may render the underlying bacteria more susceptible to antibiotic treatment. Two in vitro studies have investigated the use of surfactants on biofilms grown from clinical isolates. The first study examined the role of baby shampoo on \( P. \) aeruginosa biofilms [368] and although they found no effect on well established biofilms, they showed that at 1% concentration, baby shampoo reliably inhibited new biofilm formation. The second study by Desroisiers et et al [369], showed that the calcium targeting, citric acid zwitterionic surfactant, was capable of causing a significant log reduction in the number of viable bacteria of invito grown \( P. \) aeruginosa and \( S. \) aureus biofilms.

b) Antibiotic based treatments – Although biofilm bacteria have known resistance against antimicrobials at standard concentrations, in vitro evidence now exists that when used at very high concentrations, antibiotics may be effective in reducing biofilm load. Moxifloxacin and Mupirocin have both shown in vitro efficacy against biofilms when used at concentrations easily attainable in topical solution [370, 371]. Such findings have rekindled interest in the use of topical therapies in the treatment of CRS. In
addition to these studies, novel therapies have also shown improved antibiotic susceptibility of biofilm bacteria in both electric fields and from ultrasonic stimulation [372, 373].

c) Mechanically based treatments – The high affinity of biofilms to surfaces as well as their extremely resilient three dimensional structures, has led most researchers to believe that some form of mechanical disruption of the biofilm will be needed to ultimately eradicate them. Delivery of such force through ultra-sonification and high pressured lavage have been investigated with both showing promising results [369, 374, 375]. Indeed it has been proposed that the current use of saline irrigation may have a role in the mechanical removal of biofilms in CRS patients [376].

Other potential treatments may involve manipulating the composition of the biofilm matrix, inhibiting the intercellular signalling between biofilm bacteria or altering the host response to biofilms. As our understanding of biofilms increases no doubt will the development of new therapies emerge.
7.3 CONCLUSION

Although the precise role of biofilms in CRS remains unclear, the objective demonstration of such structures on the surface mucosa of CRS patients, and their consistent absence in control specimens, implies a pathogenic role for bacterial biofilms in CRS. Furthermore, in accordance with the current scientific biofilm literature, our studies suggest that CSLM offers a superior modality to traditional EM in the imaging of biofilms on mucosal surfaces. With this in mind, the observations and conclusions of previous EM studies examining the prevalence of biofilms on sinus mucosa need to be interpreted with caution. Having confirmed their presence in CRS patients with CSLM, we also established an important role that biofilms may play in disease persistence. We found that CRS patients with biofilms were more likely to have post-operative evidence of ongoing symptoms and inflammation than patients without these structures. This supports the findings of a previously published in vitro study [311] and may explain the commonly noted recalcitrant and treatment resistant course that many CRS patients experience. Although further extensive research is needed to identify what factors predispose certain individuals to biofilm formation, our research suggest that deficiencies or abnormalities in components of the innate immune system may play some role in biofilm-mediated CRS. Addressing such deficiencies may aid in the development of treatments for this historically difficult to manage condition.


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