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Characterising host-microbe interactions in chronic rhinosinusitis

Andrew James Wood

A thesis submitted in partial fulfilment of the requirements

for the degree of Doctor of Philosophy in Surgery

The University of Auckland, 2012
Abstract

Chronic rhinosinusitis is a morbid and expensive disorder involving persistent inflammation of the nose and sinuses. Despite advances in medical and surgical treatment it remains a major health burden that in many cases is extremely challenging to treat. This in part arises from the limited understanding of its pathogenesis. The inflammatory response observed is complex but appears to include significant defects in some protective mechanisms. Interest in a putative role for micro-organisms as causative agents waned with bacterial culture data indicating limited differences from the flora seen in health. This thesis aimed to bypass the limitations of bacterial culture and use culture-independent techniques to assess the resident flora of the sinuses and closely correlate the presence of micro-organisms to indices of the inflammatory response that define the disorder.

The presence of persistent respiratory virus infection was sought but not found to be an aetiological factor. Limited fungi were identified. However colonies of bacteria adherent to the mucosal surface were seen in association with a particular immune cell infiltrate. Small colonies of bacteria were also identified within the mucosa and the bone of the paranasal sinuses that appeared to be actively suppressing the host immune response. While the presence of such colonies within the bone appeared to be sporadic and not related to inflammatory disease they were more prevalent within the mucosa of patients with chronic rhinosinusitis with nasal polyps.

*Staphylococcus aureus* was seen to be a common organism within the mucosa and is well recognized *in vitro* to have the capacity to elude immunity. The staphylococcal superantigen-like proteins are one of these mechanisms but they were not identified, possibly due to limitations in the sensitivity of the test used.
By examining bacteria and inflammation *in situ* it has been possible to characterise the host-microbe interface in far greater depth than using bacterial culture techniques. This has provided evidence of mechanisms by which bacteria are able to both persist, evading immune detection but also stimulate an inflammatory response. In combination with other recent studies this thesis advances a hypothesis that incorporates bacteria in the pathogenesis of this challenging disorder.
Dedication

Dedicated to Prabani, Nish, Amba, Mum, Dad and David. Everything that I achieve stems from your support, advice and love.
Acknowledgements

I have been enormously lucky with regards the academic, financial, practical and social support that I have enjoyed throughout my research and wish to acknowledge some of those that have contributed.

I very gratefully acknowledge the supervision provided to me by Dr. Richard Douglas and Prof. John Fraser. Their hard work, passion for scientific research and pursuit of excellence have been inspirational to me. Without their support and guidance I would never have been able to start, let alone finish this thesis.

I am also most grateful to the agencies that have funded my research: The Garnett Passe and Rodney Williams Memorial Foundation, the Green Lane Research and Education Fund Board and the University of Auckland Faculty Research Development Fund.

A large number of other members of Auckland University and Auckland City Hospital have advised and helped me including Dr. Simon Swift, Dr. Satya Amirapu, Dr. Emma Patterson-Emanuelson, Hilary Holloway, Dr. Raymond Kim, Dr. Sally Roberts, Dr. Josh Freeman, Dr. Kitty Croxson, Hanna Antoszewska, Susan Wilkinson, Sharon Waldvogel, Dr. Nick Stow, Dr. Campbell Baguley, Dr. Siouxsie Wiles, Prof. Jill Cornish, Prof. John Windsor and Lois Blackwell.

I wish to also acknowledge those individuals who inspired me to pursue a career in Ear, Nose and Throat surgery and academia: Prof. Randall Morton, Mr. Martin Burton and Prof. Alison Brading.

I am similarly blessed with the support that I enjoy at home. Prabani continues to be my best friend, advisor and counselor.
Finally, I would like to acknowledge the patients who have stimulated my interest in sinus disease and have freely and willingly assisted me in my research by providing tissue samples for me to study.
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ABPA</td>
<td>Allergic bronchopulmonary aspergillosis</td>
</tr>
<tr>
<td>AERD</td>
<td>Aspirin exacerbated respiratory disease</td>
</tr>
<tr>
<td>AFS</td>
<td>Allergic fungal sinusitis</td>
</tr>
<tr>
<td>agr</td>
<td>Accessory gene regulator</td>
</tr>
<tr>
<td>AMPs</td>
<td>Antimicrobial peptides</td>
</tr>
<tr>
<td>AR</td>
<td>Allergic rhinitis</td>
</tr>
<tr>
<td>ARS</td>
<td>Acute rhinosinusitis</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CRS</td>
<td>Chronic rhinosinusitis</td>
</tr>
<tr>
<td>CRSsNP</td>
<td>Chronic rhinosinusitis without nasal polyps</td>
</tr>
<tr>
<td>CRSwNP</td>
<td>Chronic rhinosinusitis with nasal polyps</td>
</tr>
<tr>
<td>CSLM</td>
<td>Confocal scanning laser microscopy</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>DAPI</td>
<td>4,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein Barr virus</td>
</tr>
<tr>
<td>ECP</td>
<td>Eosinophilic cationic protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>EMCRS</td>
<td>Eosinophilic mucin chronic rhinosinusitis</td>
</tr>
<tr>
<td>ESS</td>
<td>Endoscopic sinus surgery</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>FESS</td>
<td>Functional endoscopic sinus surgery</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridisation</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FOXP3</td>
<td>Forkhead box P3</td>
</tr>
<tr>
<td>HHV-6</td>
<td>Human herpes virus-6</td>
</tr>
<tr>
<td>HU</td>
<td>Hounsfield unit</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>INS</td>
<td>Intranasal steroids</td>
</tr>
<tr>
<td>LRT</td>
<td>Lower respiratory tract</td>
</tr>
<tr>
<td>NAFES</td>
<td>Nonallergic fungal eosinophilic sinusitis</td>
</tr>
<tr>
<td>NANFES</td>
<td>Nonallergic nonfungal eosinophilic sinusitis</td>
</tr>
<tr>
<td>NF-κβ</td>
<td>Nuclear factor-kappa beta</td>
</tr>
<tr>
<td>NLR</td>
<td>Nucleotide-binding oligomerisation domain (NOD)-like receptors</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>Non-steroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>OMC</td>
<td>Osteomeatal complex</td>
</tr>
<tr>
<td>PBMCs</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PNA-FISH</td>
<td>Peptide nucleic acid fluorescence in situ hybridization</td>
</tr>
<tr>
<td>PRRs</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RSV</td>
<td>Respiratory syncytial virus</td>
</tr>
<tr>
<td>SCV</td>
<td>Small colony variant</td>
</tr>
<tr>
<td>SEB</td>
<td>Staphylococcal enterotoxin B</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SSL</td>
<td>Staphylococcal superantigen-like protein</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-beta</td>
</tr>
<tr>
<td>TH1</td>
<td>T helper cells type-1</td>
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<tr>
<td>TH2</td>
<td>T helper cells type-2</td>
</tr>
<tr>
<td>TH17</td>
<td>IL-17 producing T-helper cells</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-alpha</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>URT</td>
<td>Upper respiratory tract</td>
</tr>
<tr>
<td>US</td>
<td>United States of America</td>
</tr>
<tr>
<td>VBNC</td>
<td>Viable but non-culturable</td>
</tr>
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</table>
Chapter 1. Introduction

1.1 Chronic rhinosinusitis

1.1.1 Clinical context

Chronic rhinosinusitis (CRS) is a unifying term for a clinical syndrome characterised by the presence of persistent, symptomatic inflammation of the sinonasal mucosa. Although the subclassification of CRS remains a matter of some debate it is clear that CRS is an extremely variable condition with regards its pathophysiology and is most likely a collection of distinct disorders (Payne, Borish et al. 2011).

Until quite recently there has not been a clear consensus on the definition of CRS. Published CRS studies used definitions as loose as patients recalling being told by a health professional that they had sinusitis (Lethbridge-Cejku, Schiller et al. 2004) or self-reported “sinus trouble” (Collins 1997). An early attempt at diagnostic criteria was published in 1997 (Lanza and Kennedy 1997) but when compared to a more objective assessment of sinus pathology it was found that these criteria had deficiencies, and in particular a poor specificity (Hwang, Irwin et al. 2003). Subsequently a more robust consensus document was published in 2004 by a working party drawn from five different North American Societies with an interest in CRS (Meltzer, Hamilos et al. 2004). In trying to define CRS they focused heavily on recent data on the relative prevalence of various rhinologic symptoms (Bhattacharyya 2003) to generate a largely symptom-based definition. The group agreed that symptoms must be present continuously for more than 12 weeks and include at least two out of the following four symptoms: anterior rhinorrhoea, posterior rhinorrhoea, nasal obstruction and mid face congestion. Critically however, objective evidence such as
positive endoscopic or radiological findings was also required.

Several subsequent guidelines and consensus statements have been published. In 2007 the United States of America (US) guidelines included hyposmia as one of the possible symptoms (Rosenfeld, Andes et al. 2007) and both 2007 European (Fokkens, Lund et al. 2007) and 2008 British guidelines (Scadding, Durham et al. 2008) concluded that either nasal obstruction or rhinorrhoea must be present to merit the diagnosis of CRS. In essence there has been a degree of consistency since 2004 with persistent symptoms and objective evidence being required to secure the diagnosis. The implication however is that for any data on CRS that has been published, particularly prior to 2004, the diagnostic criteria must be closely scrutinised.

There is reasonable agreement that paediatric CRS is different from CRS occurring in adults. Clear histopathological distinctions have been observed (Berger, Kogan et al. 2011) and the clinical management of the two patient groups is very different (Ramadan 2012). This thesis will focus exclusively on adult CRS.

A large study of over 300 000 US patients spanning a 10 year period published in 2009 reported a 12-month prevalence of sinusitis of 15.2%, although the diagnosis was not rigorously defined (Bhattacharyya 2009). More recently a multi-centred study from Europe has been published using symptom-based criteria (Hastan, Fokkens et al. 2011). There was however no objective diagnostic criteria and so it may be expected that some over-reporting occurred. After collecting data from a total of 57 128 individuals this study reported a prevalence of CRS in Europe of 10.9%. A group of 4098 individuals from Korea were also considered but in this study all individuals
underwent assessment by a physician and endoscopic examination as well as reporting on sinonasal symptoms (Kim, Kim et al. 2011). All patients that had had prior nasal or sinus surgery were excluded which may be expected to result in a falsely low prevalence figure, but despite this the authors reported a total prevalence of CRS of 6.95%. Given the variability in these studies and the likely geographic variation it is difficult to estimate the exact prevalence of CRS. It is however clear that it is an extremely common condition across the world.

CRS produces an enormous health burden being common, morbid and expensive. In a US study of health-related quality of life 232 patients booked for surgical treatment of CRS were questioned about their health state utility values. Although this group represents a group of CRS patients with disease at the severe end of the spectrum it was notable that the mean utility score was well below the normal value for people living in the US and below those reported for Parkinson’s disease, coronary artery disease requiring interventional treatment and moderate chronic obstructive pulmonary disease (COPD) (Soler, Wittenberg et al. 2011).

Examining data from more than 4.4 million US patients it was found that 46.2% of CRS patients undergo surgical treatment and over the study period 8.7% underwent revision surgery. The average per patient costs related to functional endoscopic sinus surgery (FESS) were US$7726 with an average of more than US$1000 per patient per year for non-surgical care during the year prior and 2 years after FESS (Bhattacharyya, Orlandi et al. 2011). An estimated total of US$8.6 billion is spent each year on treating CRS in the US (Bhattacharyya 2011). Beyond the immediate medical costs of treating CRS there are far wider financial implications of the disease
such as loss of workplace productivity and absenteeism, both of which are prevalent in this patient group (Sahlstrand-Johnson, Ohlsson et al. 2011).

It is notable that the disease often co-exists with asthma with significant overlap between the two disorders in terms of their pathogenesis (Bachert, Claeys et al. 2010). It is likely that CRS exacerbates symptoms of asthma and as such the clinical impact of CRS may well extend beyond the confines of the upper respiratory tract (URT) (Fasano 2010).

As a general algorithm CRS patients are initially treated with oral antibiotics and corticosteroids as well as saline lavage and intranasal steroids (INS). In those that remain symptomatic FESS is typically performed (Wood and Douglas 2010). Despite the proven efficacy of these treatments there is a significant minority of patients that are left with recalcitrant symptoms (Hopkins, Slack et al. 2009). Increasingly clinical research efforts are directed at this important subgroup (Woodbury and Ferguson 2011; Bassiouni, Naidoo et al. 2012) and the existence of these challenging patients highlights the need for improvements in understanding of the pathogenesis of this complex disorder.

1.1.2 Disease subtypes

There is general acceptance that CRS is not a single disease entity and there is a need to subclassify the disorder. As nasal polyps are easy to identify endoscopically, their presence or absence is often used for classifying the disorder into those with (CRSwNP) or without (CRSsNP) polyps (Meltzer, Hamilos et al. 2004; Fokkens, Lund et al. 2007).

There is clearly some merit as well as convenience to this distinction with demonstrable differences in the inflammatory profile between these subgroups
(Polzehl, Moeller et al. 2006). However this subclassification is not perfect. The mucosa of patients with CRS often has an irregular “polypoid” appearance without frankly pedunculated lesions and while the guidelines categorise these individuals as CRSsNP there is little if any basis on which to make this distinction. Furthermore there is ongoing debate as to whether the groups with and without polyps are discreet disease processes (Van Cauwenberge, Van Zele et al. 2008) or whether the two groups are merely at different points on the same disease spectrum (Kern, Conley et al. 2008).

The classically described distinction between the two subgroups based primarily on patients of European descent is that CRSwNP is characterised by an eosinophilic infiltrate (Polzehl, Moeller et al. 2006) and an inflammatory profile reflecting this with interleukin-5 (IL-5), eotaxin and eosinophilic cationic protein (ECP) being dominant (Bachert, Gevaert et al. 2002). However this is not a universal feature in CRSwNP. CRSwNP in the Chinese population has now been studied particularly closely and in this patient group an alternative inflammatory pattern skewed more towards a neutrophilic pattern is dominant (Ba, Zhang et al. 2011). Furthermore it has now been proposed that CRSwNP should be subclassified on the presence or absence of eosinophilic inflammation (Payne, Early et al. 2011). Some authors have suggested that the presence or absence of eosinophils is more significant with regards classification than the presence or absence of nasal polyps (Nakayama, Yoshikawa et al. 2011).

In 1968 Samter and Beers reported a cohort of patients with an adult-onset intolerance to aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) (Samter and Beers 1968). A clinical triad of nasal polyposis, asthma and a characteristic and often marked airway hyper-responsiveness to aspirin was described. From this initial
description the terms “Samter’s triad” and “aspirin-exacerbated respiratory disease” (AERD) originated. The disorder is caused by alterations in leukotriene and prostaglandin metabolism (Szczeklik, Sladek et al. 1996). Aspirin desensitization offers a viable and important treatment option (Pleskow, Stevenson et al. 1982). It is therefore clear that the AERD subgroup of CRS is an important group to recognise both in the clinical care of these patients but also when researching CRS due to the distinct pathophysiology.

In a perhaps similar fashion it has recently been proposed that IgG4-related disease should be considered as a distinct subgroup of CRS but this is yet to reach popular acceptance (Moteki, Yasuo et al. 2011).

The “united-airways hypothesis” arose from the clear overlap between allergic rhinitis (AR) and asthma in relation to epidemiology, pathogenesis and treatment (Passalacqua, Ciprandi et al. 2001). It has however become increasingly clear that there is a similar overlap between CRSwNP and asthma. Recent epidemiological evidence clearly associates the two disorders (Jarvis, Newson et al. 2012) and common pathophysiological mechanisms and treatment options exist such as the central role for eosinophilic inflammation, IL-5 and potentially even anti-IL-5 targeted treatments (Nair, Pizzichini et al. 2009; Gevaert, Van Bruaene et al. 2011). Further to this there is now some evidence that CRSwNP with asthma should be considered as a distinct group from CRSwNP without asthma (Bachert, Zhang et al. 2010).

A subset of CRS patients have refractory disease with distinctive rubbery, eosinophilic mucin from which fungi can often be identified. In parallel with the well substantiated diagnosis of allergic bronchopulmonary aspergillosis (ABPA), this was termed
allergic fungal sinusitis (AFS) implying a central role for immunoglobulin E (IgE) mediated allergy to fungi in its pathogenesis. Criteria for the diagnosis of AFS were defined by Bent and Kuhn (Bent and Kuhn 1994). After studying 15 cases they concluded that five features must be present, namely type 1 hypersensitivity to fungal allergens, nasal polyposis, characteristic CT features, eosinophilic mucin and identifiable fungi. Originally this was considered to be a rare disease entity but it has been suggested that a low rate of diagnosis merely reflected a lack of sensitivity of the laboratory tests used (Ponikau, Sherris et al. 1999). After conducting a rigorous search for fungi in 210 consecutive patients with CRS at least one species of fungi was identified using novel techniques in 96% of study patients. Specific IgE to fungal allergens were identified in 28% of those CRS patients who were tested. The authors went on to conclude that the majority of CRS patients previously considered to be idiopathic were in fact caused by allergy to fungi. However the authors also reported the findings from normal control subjects who exhibited a similar high frequency for detection of fungal species and of specific IgE to fungi. Subsequently the complexity of classification in this subgroup of patients with eosinophilic mucin, termed eosinophilic mucin CRS (EMCRS) has increased: laboratory tests to identify allergy and fungi have generated further subgroups such as allergic fungal sinusitis-like (AFS-like), nonallergic fungal eosinophilic sinusitis (NAFES) and nonallergic nonfungal eosinophilic sinusitis (NANFES) (Pant, Kette et al. 2005). It remains unclear whether these groups are distinct clinical entities or simply reflect the imperfect sensitivity and specificity of laboratory tests used to define them (Pant, Kette et al. 2006). It has subsequently been found that bacteria are commonly identifiable within the mucin of AFS patients casting further doubt on whether fungi
are truly central to the pathogenesis in those CRS patients with eosinophilic mucin (Ferguson, Seethala et al. 2007).

1.1.3 Risk factors

In recent years there has been a refinement in understanding of the risk factors for the development of idiopathic CRS. Longstanding hypotheses suggesting an aetiological role for atopy and variations in sinonasal anatomy have not been confirmed by more recent studies (Robinson, Douglas et al. 2006; Holbrook, Brown et al. 2005). A suggested role for reflux of gastric contents into the nose and nasopharynx as a contributing factor remains unproven (Flook and Kumar 2011). However, very few of these questions on CRS pathogenesis have been answered definitively and further studies are required.

There are studies associating cigarette smoking with CRS (Lieu and Feinstein 2000; Hastan, Fokkens et al. 2011). It has also been shown that the outcome after FESS is worse in current smokers (Krzeski, Galewicz et al. 2011). There are several in vitro studies that explore possible mechanisms by which smoking may interact with other proposed aetiological factors in CRS including dysfunction of the innate immune response (Lee, Ramanathan et al. 2007; Kulkarni, Rampersaud et al. 2010) and the promotion of sinonasal biofilm formation (Goldstein-Daruech, Cope et al. 2011). The identified association between CRS and smoking is helpful when counseling CRS patients, but its mechanism remains unclear (Tamashiro, Cohen et al. 2009).

There has been a recent report of occupational exposure to allergic and non-allergic irritants being associated with a worse outcome from FESS (Hox, Delrue et al. 2012). Although the authors do not imply that occupational exposure to irritants is a risk factor for CRS it may be inferred that it is. This is as yet unproven however.
Studies of the genetic component of CRS pathogenesis have evolved significantly from the family concordance studies which suggested little if any role for genetic variation in the development of CRS (Greisner and Settipane 1996). Geneticists have subsequently developed a spectacular array of techniques (Tewfik, Bosse et al. 2010).

The study of the genetics of CRS is hindered by the current limitations of the classification of the disorder and understanding of the influence of geographical and ethnic differences. Other difficulties in interpreting genetic data relate to the high chance of spurious associations occurring due to the enormous number of permutations, the importance of gene-gene and gene-environment interactions and the role of multiple genes in a common pathway (Mfuna-Endam, Zhang et al. 2011).

There has been particular research interest in genes contributing to innate immunity. Given the possibility of spurious associations there is need for replication studies and such studies appear to have confirmed a role for mutations in the IL1A gene (Karjalainen, Joki-Erkkila et al. 2003; Mfuna Endam, Cormier et al. 2010) and the tumour necrosis factor alpha (TNF-α) gene (Erbek, Yurtcu et al. 2007; Bernstein, Anon et al. 2009) in CRS. An as yet unreplicated study has implicated the p73 gene (Tournas, Mfuna et al. 2010) and contradictory results have been found in relation to the IL1B gene (Erbek, Yurtcu et al. 2007; Mfuna Endam, Cormier et al. 2010). The implication of other genes related to innate immunity continue to be reported (Kilty, Bosse et al. 2010; Wang, Chien et al. 2010). It is not clear how many polymorphisms in these genes contribute to the disease pathogenesis.

The possibility of epigenetic changes in disease pathogenesis adds a further layer of complexity to this field. Epigenetics is the study of long-term changes in gene expression as a result of environmental exposures mediated by such processes as DNA methylation. Although data is accumulating on the role of epigenetics in asthma
(Ho 2010) this remains a largely unexplored field in CRS (Cho, Moon et al. 2012; Seiberling, Church et al. 2012).
1.2 Dysfunctional immunity and physiology in chronic rhinosinusitis

1.2.1 Mucociliary function and dysfunction

The paranasal sinuses are lined by ciliated pseudostratified respiratory epithelium (Image 1-1). Within the epithelial layer are goblet cells that produce the major components of the mucus that lines the nose and paranasal sinuses. The nasal cilia function to move nasal mucus posteriorly through the nose and into the pharynx where it is swallowed (Hilding 1931). In a series of observations made in the 1960’s by Messerklinger it was demonstrated that the flow of mucus through the paranasal sinuses occurs in a more complex but stereotyped fashion (Messerklinger 1967). Mucus flows, often against gravity, towards the natural sinus ostia and the concept of “functional” endoscopic sinus surgery involving the augmentation of the natural sinus ostia was developed. This concept differed radically from the previous reliance on earlier surgical approaches that promoted gravity dependent sinus drainage (Stammberger and Posawetz 1990).
Cystic fibrosis (CF) and primary ciliary dyskinesia (PCD) are disorders affecting normal mucociliary function that predispose to CRS implicating malfunction of this process in the disorder. Cystic fibrosis is an autosomal recessive disorder affecting the function of the epithelial chloride channel, resulting in an increase in the viscosity of the secretions from exocrine glands and respiratory epithelium. One consequence of this is an increase in the bacterial and fungal load (Lipuma 2010) and a significant proportion of CF patients develop nasal polyposis (Schraven, Wehrmann et al. 2011). Nasal polyps from CF patients appear to be clinically and immunologically distinct from idiopathic nasal polyps (Van Zele, Claeys et al. 2006). PCD is less common than CF but equally relevant in this context. In this condition dysfunction of ciliary motility predisposes to purulent sinopulmonary disease (Leigh, Pittman et al. 2009).
There is evidence of histopathological and functional changes in mucociliary function in idiopathic CRS (Wilson, Sykes et al. 1986; Al-Rawi, Edelstein et al. 1998). What remains less clear however is whether the observed disruption to mucociliary flow in idiopathic CRS is a cause or an effect of the disorder. The examples of CF and PCD would tend to implicate disordered mucociliary function as a principal cause.

It has been assumed that obstruction of sinus ostia and consequent disruption to the flow of mucus from those sinuses is an index event in the development of CRS and restoration of sinus ventilation and drainage is considered key to the success of FESS (Stammberger and Posawetz 1990). There are results from animal models to support this concept (Ha, Psaltis et al. 2007) and some studies suggest that FESS improves mucociliary function (Bassiouny, Atef et al. 2003). However other studies have demonstrated that CRS can develop without obstruction of the osteomeatal complex (OMC) which is the major drainage site of the paranasal sinuses (Leung, Kern et al. 2011).

**1.2.2 Systemic immune dysfunction**

Systemic immune dysfunction can predispose patients to CRS. Both primary immunodeficiencies and the auto-immune disorders have been clearly associated with CRS. Several inherited disorders that cause antibody deficiencies: X-linked agammaglobulinaemia, common variable immunodeficiency, isolated IgA deficiency and IgG subclass deficiencies are all known to predispose to CRS. The major inflammatory disorders known to predispose to CRS are Wegener’s granulomatosis, Churg-Strauss syndrome and sarcoidosis (Douglas and Wood 2012).

When a patient has no circulating antibodies (as is the case in X-linked agammaglobulinaemia), the diagnosis of hypogammaglobulinaemia is easily
established. However many patients have IgG and IgA levels just below the normal range. The physiological significance of this relative deficiency of serum immunoglobulins can be determined by measuring the antibody response provoked by the administration of vaccines such as tetanus toxoid and *Pneumovax* (Douglas and Wood 2012). Two case series have reported a high prevalence of attenuated vaccine responses in CRS patients (Alqudah, Graham et al. 2010; Carr, Koterba et al. 2011). While these are extremely valuable studies in heightening the index of suspicion for these disorders in clinical practice it should be noted that these two series were skewed to more severe disease, being drawn from selected patients being treated in tertiary rhinology practices. The true prevalence of such deficiencies in unselected CRS patients is probably lower than that reported in these two series.

There are other mechanisms by which systemic disorders may predispose to CRS. Vitamin D is now recognized to have a far-reaching effect on immunity (Akbar and Zacharek 2011) and there is some evidence that Vitamin D may be deficient in some subgroups of CRS when compared to normal controls (Mulligan, Bleier et al. 2011). In an interesting study of auto-immunity in CRS patients significantly higher titres of auto-antibodies were found in nasal polyp tissue than in control samples of mucosa (Tan, Li et al. 2011). Only a proportion of samples exhibited a titre that was out of the normal range however and the effect was much less marked when directly comparable mucosal samples were compared between the CRSwNP and control subjects.

The authors of a recent review of current CRS research have speculated that all CRS patients may have some degree of systemic immunodeficiency (Bachert, Van Bruaene et al. 2009). At present there is a clear but infrequent association of CRS with well-recognised immune dysfunction syndromes (Chandra, Lin et al. 2011). The studies
discussed above however show that at present a contribution from a systemic immune dysfunction looks to be relevant in only a strictly limited number of cases and the majority of CRS cases still merit the term “idiopathic”. Whether idiopathic forms remain as such in the face of continued research efforts remains uncertain.

1.2.3 Local immune dysfunction

CRS is a disorder characterized by the presence of inflammation in the nose and paranasal sinuses. As the complexities of human immunity are unraveled the inflammatory mechanisms at play in CRS are also understood better. Fundamental to our understanding of the immune response is the distinction between innate and adaptive immune responses. An innate immune response can be mounted on initial recognition of non-self molecular patterns producing a non-specific response that is neither dependent on nor affected by prior exposure to these molecular patterns. The adaptive response involves the generation of a targeted response to a specific antigen of a pathogen that is orchestrated by B and T lymphocytes (Ooi, Wormald et al. 2008).

Corticosteroids are well recognized to have broad anti-inflammatory effects (Fauci 1978) and have become an established part of medical therapy for CRS (Wood and Douglas 2010). There are now randomized controlled trials showing the efficacy of both systemic steroids (Hissaria, Smith et al. 2006; Van Zele, Gevaert et al. 2010) and intranasal steroids (Small, Hernandez et al. 2005; Jankowski, Klossek et al. 2009) in CRSwNP. Somewhat surprisingly there is a dearth of a similar study of systemic steroids in CRSsNP (Lal and Hwang 2011). In 2009 a meta-analysis of INS in CRSsNP did not confirm efficacy (Kalish, Arends ts et al. 2009) but a subsequent randomized trial has suggested that there is a clinically significant effect (Mosges, Bachert et al. 2011). There are study results that support the use of INS following
FESS (Jorissen and Bachert 2009). The specific effects of corticosteroids are numerous and the exact mechanism by which corticosteroids effect a reduction in mucosal inflammation is unclear (Pujols, Benitez et al. 2009). It is very interesting to note however that some components of the innate immune response in the upper airway may in fact be spared this suppressor effect or even boosted by corticosteroids (Zhang, Truong-Tran et al. 2007; Schleimer, Kato et al. 2009).

As well as the apparent bimodal effects of corticosteroids, appearing to boost some components of immunity while suppressing others, there is also not simply an overactivity of all inflammatory pathways in CRS. When compared to control subjects some deficiencies in the local immune response are noted in CRS, particularly in components of innate immunity. This corresponds to the predisposing conditions described above which include syndromes characterized by both pro-inflammatory states and immune deficiencies.

Epithelial cells, their cilia and associated secretions make up the physical interface between the sinonasal mucosa and the cocktail of irritants and antigens contained in inhaled air. The mucociliary system, discussed above, is recognised as an important physical barrier but recent research has demonstrated that there is a far broader array of mechanisms by which epithelial cells contribute to and control innate immunity (Vroling, Fokkens et al. 2008). Epithelial cells secrete into nasal mucus a wide variety of antimicrobial peptides (AMPs) (Laudien, Dressel et al. 2011) and other non-peptide mediators such as nitric oxide (Guida, Rolla et al. 2010) as well as expressing cytoplasmic and cell surface receptors termed pattern recognition receptors (PRRs) that recognise stereotyped components of pathogens (van Drunen, Mjosberg et al. 2012). In response to stimulation of PRRs epithelial cells produce cytokines and chemokines that augment the inflammatory response (Vroling, Fokkens et al. 2008).
Surfactant protein D provides particular defense against fungal pathogens and its expression and role was considered in CRS including various EMCRS subgroups (Ooi, Wormald et al. 2007). While it was found to be present in CRS and control subjects and production could be induced in most subgroups in vitro on exposure to fungal extracts, baseline levels were lower in the various subgroups of CRS when compared to controls. Lactoferrin is a further secreted protein with more broad antimicrobial activity and in a similar study was seen to be present in reduced levels in all subgroups of CRS when compared with control subjects (Psaltis, Bruhn et al. 2007). The S100 family of AMPs have been studied in some depth and reduced levels of psoriasin and calprotectin have been observed in both nasal lavage fluid and epithelial cells in CRS (Tieu, Peters et al. 2010). Changes in cathelicidin antimicrobial peptide levels in CRS have been more difficult to characterize (Ooi, Wormald et al. 2007). Recent studies have implied that the microflora as well as the presence of inflammatory disease may impact on AMP production with S.aureus colonisation in CRSwNP being associated with lower levels of LL-37 (Thienhaus, Wohlers et al. 2011). The profile of AMPs in the nose is large (Laudien, Dressel et al. 2011) and it seems inevitable that further advances will be made in the understanding of the role of AMPs in CRS in the near future.

Complement factors may also be important. In a study of complement gene expression, most factors were seen to be upregulated in CRS although interestingly the C7 component exhibited reduced expression in the AFS group (Schlosser, Mulligan et al. 2010). A possibly equally relevant protein is the Centrosomal protein 110. This is thought to have a negative impact on ciliogenesis but was found to be overexpressed in CRS despite the obvious need for regeneration of cilia (Lai, Chen et al. 2011).
The PRRs that have attracted the most attention are the Toll-like receptors (TLRs) and the Nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs).

Reduced expression of TLR2 was described in CRS compared to controls (Lane, Truong-Tran et al. 2006) although subsequent studies have failed to further implicate TLR2 (Tewfik, Bosse et al. 2008; Sachse, Becker et al. 2010). Initial data relating to NLRs have suggested that expression of these genes is increased in nasal polyps when compared to healthy inferior turbinate mucosa (Mansson, Bogefors et al. 2011).

Interleukin-6 (IL-6) is one cytokine produced by epithelial cells and increased levels of both IL-6 and of IL-6 receptors have been observed in CRSwNP (Peters, Kato et al. 2010). Interestingly however the possibility was raised of reduced downstream effects of IL-6 as in the same study lower levels were observed of signal transducer and activator of transcription 3 (STAT3), an IL6 dependent transcription factor. Thymic stromal lymphopoietin (TSLP) is another epithelial cell derived cytokine that has attracted interest and is secreted in response to stimulation of TLR3 receptors and rhinovirus infection (Kato, Favoreto et al. 2007). Greater expression of TSLP has been observed in epithelial cells in CRSwNP compared to controls as well as a higher expression of TSLP receptors (Liu, Li et al. 2011).

When the data on mucociliary dysfunction and epithelial cell abnormalities in CRS are taken together it has been argued in a widely cited review that the initial step of the inflammatory process lies with deficiencies in the mechanical barrier and innate immune functions of the epithelial cell layer (Kern, Conley et al. 2008). In advancing the so called “immune barrier hypothesis” there was little specific known about what the underlying cause of these defects might be. It was suggested that these barrier defects may arise from genetic, epigenetic or environmental influences. It is not clear whether all immune defects can be attributed to the epithelial cells and it seems highly
likely that to some extent they are a part of a more complex change involving an interplay between several components of the immune response (Basinski, Holzmann et al. 2009). For instance, interleukin-22 (IL-22) is secreted by T lymphocytes and has a non-specific function in boosting innate immunity and deficient IL22 receptor numbers have been described on epithelial cells in recalcitrant CRSwNP (Ramanathan, Spannhake et al. 2007).

The phenotypic characterization of T lymphocytes has moved beyond the initial division into CD8 positive cytotoxic T cells and CD4 positive T helper cells with the TH1 and TH2 subtypes to include other subtypes such as T Regulatory (TReg) cells and TH17 cells. Such developments have led to the identification of further derangements in cellular immunity, remote from the epithelial cells. For example, an increased proportion of TH17 cells and decreased proportion of TReg cells has been noted in PBMCs of CRSwNP patients (Shen, Tang et al. 2011) and a decreased expression of forkhead box P3 (FOXP3), a TReg marker in nasal polyp tissue when compared to control mucosa (Van Bruaene, Perez-Novio et al. 2008). Furthermore in an in vitro study of TRegs isolated from peripheral blood mononuclear cells (PBMCs), a deficiency in migratory capacity was identified in CRS (Kim, Munoz et al. 2010). In further in vitro studies of isolated immune cells, macrophages derived from the nasal mucosa of CRSwNP patients were found to be deficient in their ability to phagocytose bacteria when compared to controls (Krysko, Holtappels et al. 2011).

The activity of immune pathways related to B cell function have been shown to be increased in CRSwNP (Kato, Peters et al. 2008).

An exhaustive review of the described changes in immune cells, receptors and mediators in CRS is beyond the scope of this thesis. However it is noted that while a variety of key inflammatory processes are upregulated there are significant
demonstrable gaps in some of the processes, particularly surrounding innate immunity. The study of the subtleties of these changes is hampered by our limited understanding of the role of racial, geographic and other phenotypic changes in CRS. The “immune barrier” defects have however been considered to be central to the pathogenesis but the underlying cause of these defects remains elusive.

1.2.4 Mucosal remodeling

Remodeling has been defined as a temporary or permanent change in tissue structure due to degradation of and subsequent production of new extracellular matrix components (Bousquet, Chanez et al. 1992; Pawankar and Nonaka 2007). Increased deposition of subepithelial collagen has been described in association with the inflammatory infiltrate in CRS (Sobol, Fukakusa et al. 2003).

Matrix metalloproteinases (MMPs) are a family of endogenous proteinases that act to degrade ECM (Shapiro and Senior 1999) and are thought to be key mediators in remodeling. In a study of transgenic mice upregulation of MMP-2, MMP-9 and MMP-12 was observed and was found to be correlated with various indices of lower airway tissue destruction (Wert, Yoshida et al. 2000). A further family of proteins, the tissue inhibitors of metalloproteinase (TIMPs) exist which have a variety of functions as well as the ability to inhibit active MMPs (Brew, Dinakarpandian et al. 2000). The profile of changes in these proteins in CRS is complex. In CRSwNP levels of MMP-7 and MMP-9 were found to be elevated without significant changes in TIMP-1. In CRSsNP levels of MMP-9 and TIMP-1 but not MMP-7 were elevated (Watelet, Bachert et al. 2004).

New collagen is produced by fibroblasts and myofibroblasts and when fibroblasts were extracted from CRSwNP patients they were seen to be stimulated to proliferate
by isoforms of transforming growth factor beta (TGF-β) even at low titres (Serpero, Petecchia et al. 2006). This proliferation was inhibited by corticosteroids but in another study corticosteroids were not seen to be capable of reversing the downstream collagen deposition (Molet, Hamid et al. 2003). Production of TGF-β and of collagen appears to be of more significance in CRSsNP than in CRSwNP (Watelet, Claeys et al. 2004; Van Bruaene, Derycke et al. 2009).

It is not entirely clear whether remodeling constitutes an adaptive or a maladaptive response in CRS although most authors tend to imply that it is the latter, possibly representing an “end-stage” of the disease at which point it becomes irreversible (Van Bruaene and Bachert 2011; Bassiouni, Naidoo et al. 2012). Interestingly however, higher ratios of MMP-8 : TIMP-1 and MMP-9 : TIMP-1 (implying a response that favours remodeling) was seen to be associated with a better outcome from surgical treatment in CRSwNP (Kostamo, Tervahartiala et al. 2007).
1.3 Micro-organisms and chronic rhinosinusitis

1.3.1 Classical models of infection

In the late 19th Century Robert Koch developed the criteria that he considered needed to be fulfilled to confirm a role for an organism in the causation of disease. Originally presented in German in relation to tuberculosis these have been translated and popularised as “Koch’s postulates” and are considered to be applicable for any disease caused by micro-organisms (Rivers 1937). In essence these criteria were that the organism had to be identifiable in all cases of the disease and only that disease, and that the disease is caused when the organism is introduced to naïve subjects. It is highly relevant that Koch’s description focused heavily on the need to isolate and study the organism using culture techniques. The discipline of diagnostic microbiology has come to be largely based on microbial culture and despite the enormous number of factors that affect the ability of different micro-organisms to grow in vitro (Monod 1949) Koch’s postulates have come to be interpreted as meaning that only organisms grown from clinical samples can be considered as relevant to any particular disease. This assumption is not supported by an increasing recognition that bacteria can exist in a “viable but non-culturable state” (VBNC) (Nilsson, Oliver et al. 1991). The convenience of inserting a microbiology swab into the anterior nares or middle meatus has meant however that much of what has been written on the microbiology of the nasal and sinus mucosa has been based on swab culture results. Such studies comparing the culture results from nasal and sinus mucus have not yet identified any organism that is present in CRS but not in health, leading many to conclude in accordance with Koch’s postulates that micro-organisms cannot be the cause of CRS (Nadel, Lanza et al. 1998; Bhattacharyya 2005). There are studies of culture results from whole tissue samples in CRS, which bypass the
assumption that the mucus represents the entire sinus flora but not the assumption that all relevant organisms will be grown (Ramadan 1995).

Antibiotics whose efficacy in the treatment of CRS have been studied to date have yielded largely disappointing results. A study comparing ciprofloxacin and amoxicillin/clavulanic acid reported clinically meaningful improvements in both groups but the study was greatly limited by the lack of a placebo group (Legent, Bordure et al. 1994). A study of “culture-directed” antibiotics reported resolution of CRS after treatment in 133 of 172 patients (77%). Although there was again no placebo group, more than half had failed previous empiric antibiotic treatment (Chin, Yeak et al. 2010). There are however published randomized double-blind placebo controlled trials reporting efficacy of oral antibiotics in CRS using roxithromycin (Wallwork, Coman et al. 2006) and doxycycline (Van Zele, Gevaert et al. 2010) respectively. Of note however both studies looked at multiple endpoints and both reported a significant improvement in only some of these. It is also noteworthy that macrolide antibiotics (Wallwork, Coman et al. 2004) and doxycycline (Hanemaaijer, Visser et al. 1998) have been reported to have immunomodulatory as well as antimicrobial activity raising questions regarding the mechanism by which their efficacy is derived.

In a similar fashion to the role of bacteria, there has been active debate around the role of fungi in CRS. The findings of Ponikau et al (Ponikau, Sherris et al. 1999) provoked much debate about the presence of fungal material in the sinuses and its role, and have subsequently been repeated (Bassiouny, Ragab et al. 2010). It is now generally accepted that fungi are ubiquitous within the nose although there may be a greater fungal load in the sinuses in CRSwNP (Rao, Mathers et al. 2006).
Oral and topical anti-fungal agents have been studied in CRS. A recent systematic review of systemic antifungal treatments in CRS concluded that there was the possibility of efficacy in some subgroups but also that there was a paucity of high quality data (Thanasumpun and Batra 2011). After identifying 356 published abstracts they found only one study that was considered Level 1 evidence and that reported no efficacy of terbinafine (Kennedy, Kuhn et al. 2005). Similarly a recent meta-analysis of topical amphotericin B in CRS reported no evidence of efficacy (Isaacs, Fakhrri et al. 2011). The difficulty associated with the topical administration of agents to the paranasal sinuses is noted (Harvey and Schlosser 2009) but antifungal agents in any form are not currently considered part of routine therapy for CRS (Fokkens, Lund et al. 2007; Wood and Douglas 2010).

Despite the disappointing results from treatment and fungal culture studies a number of researchers have continued to attempt to define the aetiological role of fungi. A series of in vitro experiments have been performed on cells isolated from CRS patients that have implied a role for a dysfunctional immune response to fungi. Shin et al isolated PBMCs from CRS and non-CRS subjects and showed a relative increase in the immune response of PBMCs from CRS patients when challenged with fungal antigens (Shin, Ponikau et al. 2004). Subsequently epithelial cells from nasal polyps were isolated and stimulated with increasing titres of different fungal species and a protease-dependent stimulation of cytokine production was observed (Shin and Lee 2010). These studies have been criticised however. In adopting this approach it is impossible to know whether the concentration and duration of exposure to the antigen were reflective of that occurring in vivo (Ling and Hamilos 2011). It has also been reported that the cell culture technique used can impact significantly on the production of inflammatory mediators (Fernandez-Bertolin, Mullol et al. 2011). It was
also noted that in the 2004 study 78% of the CRS patients had co-morbid asthma (Shin, Ponikau et al. 2004) and the exaggerated response of PBMCs that was observed may merely have represented a pre-existing state of systemic immune activation (Kern, Conley et al. 2008).

Some of these issues were addressed by other similar studies that stimulated isolated cells with other antigens in parallel with fungal antigens. However it remains unknown whether the concentration of antigens used were directly comparable. In one study lymphocytes were isolated from CRS and control subjects and stimulated with either fungal antigens or staphylococcal exotoxin B (SEB) (Douglas, Bruhn et al. 2007). The immune response to SEB was significantly greater than the response to fungal antigens. Dispersed nasal polyp cells have also been studied following stimulation with fungal antigens and SEB (Okano, Fujiwara et al. 2011). In the same fashion, a significantly more pronounced effect was seen with SEB than fungi. Furthermore in a small study cytokine production following the exposure of PBMCs to fungi was not observed to be universally associated with CRS (Orlandi, Marple et al. 2009).

The “fungal hypothesis” has polarized some researchers. Fungi are still postulated by some to be the unifying immunological factor in CRS and asthma (Pakdaman, Corry et al. 2011) while a recent review article from another group that have researched the role of fungi extensively concluded that it is time to abandon the fungal hypothesis (Fokkens, van Drunen et al. 2012). As will be discussed later it may well not be as simple as a straight yes or no and there may be some form of synergistic effect between fungi and other factors (Boase, Valentine et al. 2011).
1.3.2 Viral infection

Episodes of acute viral respiratory infections are almost universal in the human population (Monto 2002). Acute viral infection commonly extends to include the mucosa of the paranasal sinuses (Gwaltney, Phillips et al. 1994) and acute rhinosinusitis can ensue. Despite the certainty around the role of viruses in acute rhinologic infections much less is known about their role in CRS. There has been surprisingly little study of the presence of respiratory virus infection in CRS and only limited numbers or such viruses have been sought (Ramadan, Farr et al. 1997; Jang, Kwon et al. 2006).

Much about the pathogenesis of CRS can be inferred from the study of viral infections of the lower respiratory tract (LRT). Viral infections have been implicated in the development in acute exacerbations of symptoms in established asthma (Papadopoulos, Christodoulou et al. 2011). Some studies suggest a similar effect in CRS with a seasonal variety in the frequency of consultations for CRS that required an escalation of care (Rank, Wollan et al. 2010). Of particular interest however is that there are epidemiological data linking viral infection in early childhood and an increased risk of developing asthma in later life (Sigurs 2001; Ruotsalainen, Pippop-Savolainen et al. 2010). It is not known if this effect occurs due to a transient infection that mediates a long-lasting, perhaps epigenetic, change or whether there is persistence of virus particles within the tissue. In chapter 2 this question is explored further in the context of CRS.

1.3.3 Biofilms

After an initial study showing the presence of microbial biofilms on the surface of paranasal sinus mucosal specimens from subjects with recalcitrant CRS the “biofilm hypothesis” was born (Cryer, Schipor et al. 2004). By this point research into the
physiology of biofilms was already advanced (Costerton, Stewart et al. 1999) and it quickly became apparent that the features of biofilms such as the non-culturable state (Hall-Stoodley, Hu et al. 2006) and adaptations for survival despite host immunity and antibiotics (Stewart and Costerton 2001) made them potential candidates as a causative factor in CRS.

Although there have been some detractors (Mladina, Skitarelic et al. 2010) a number of publications have ensued whose results promote the hypothesis that biofilms have a causative role in CRS. Initial work primarily focused on determining the prevalence of biofilms in the paranasal sinuses in health and disease. There has been extensive discussion on the optimum method for biofilm detection, with transmission electron microscopy (TEM), scanning electron microscopy (SEM), bacterial culture and confocal scanning laser microscopy (CSLM) all being used (Ferguson, Stolz et al. 2005; Ramadan, Sanclement et al. 2005; Sanclement, Webster et al. 2005; Bendouah, Barbeau et al. 2006; Psaltis, Ha et al. 2007). Although none of the larger studies reported a prevalence in CRS of 100% there was a repeated finding of a greater prevalence in CRS than health. The issues of sensitivity and specificity in biofilm detection persist and so the possibility remains that biofilms are universal in CRS.

Given the dormant state in which bacteria exist within a biofilm it is difficult to successfully apply culture techniques to study the species of organisms contained within them. The use of fluorescence in situ hybridisation (FISH) was popularised in order to further characterise the organisms present without need for culture techniques. By using a complimentary nucleotide sequence to the bacterial genetic material contained within the ribonucleic acid (RNA) of the 16s subunit of ribosomal bacteria it is possible to identify any bacteria for which sequencing data are available by attaching a fluorescent marker to the complimentary nucleotide sequence (Kempf,
The existence of multiple ribosomes within any bacterial cell provides a convenient amplification of this fluorescent signal. The major drawbacks of FISH as a technique however are that successful hybridisation is extremely sensitive to experimental conditions (Pernthaler, Glockner et al. 2001) and only bacteria which are directly sought can be identified. At this point therefore only a limited number of organisms have been identified within sinus biofilms but it has quickly become clear that multi-kingdom biofilms containing both bacteria and fungi, as well as multi-species biofilms exist (Sanderson, Leid et al. 2006; Healy, Leid et al. 2008; Foreman, Psaltis et al. 2009).

The next landmark in the development of the biofilm hypothesis was the identification that CRS patients who had demonstrable biofilms within their sinuses at the time of FESS have a worse outcome from surgical treatment suggesting that they represent a worse disease phenotype. This observation has now been made in both retrospective (Psaltis, Weitzel et al. 2008) and prospective studies (Hochstim, Masood et al. 2010; Singhal, Psaltis et al. 2010).

Understandably research in this field has quickly moved to a point at which therapies specifically directed towards the disruption of biofilms are being trialled. By targeting biofilms specifically, options for treatment are broadened beyond antibiotics to include agents that disrupt the biofilm extracellular matrix or other molecular pathways critical to biofilm survival. A study investigating the regular addition of 1% baby shampoo to saline lavage fluid was conducted in the expectation that the surfactant component might help to breakdown the biofilm matrix (Chiu, Palmer et al. 2008). While the study did, in the absence of a placebo group, demonstrate significant improvements in symptoms the use of baby shampoo has fallen out of favour due to potential toxicity to cilia (Isaacs, Fakhri et al. 2011). Citric acid/zwitterionic
surfactant has also been trialled with similar intent but again there are studies that demonstrate ciliary toxicity (Valentine, Jervis-Bardy et al. 2011). Despite this however an in vitro study concluded that antibiotics in a dose achievable topically were capable of disrupting Staphylococcus aureus biofilms (Desrosiers, Bendouah et al. 2007). In a whole animal setting further data have supported the use of topical antibiotics with mupirocin appearing to have better efficacy than vancomycin or ciprofloxacin (Le, Psaltis et al. 2008).

The successful delivery of topical medications to sinus mucosa is challenging (Harvey and Schlosser 2009). A review of nebulised antibiotic therapy in CRS did not find evidence for clinically meaningful efficacy but reported minimal adverse effects (Woodhouse and Cleveland 2011). While the absence of adverse effects on an individual level may be true in the short-term at least, there is a significant potential for the development of antibiotic resistance when inadequate, sub-therapeutic doses of antibiotics are delivered and there are implications of this both for future treatment of that patient and the population in general. In summary, treatments directed specifically at biofilms have to this point not added significantly to routine care but to date this principally relates to the difficulties in administering safe and efficacious agents rather than serving as proof that biofilms are not relevant to CRS pathogenesis.

In the recent past the literature in this field has moved towards attempts to correlate the presence of biofilms more specifically with the inflammatory process that defines CRS. An interesting study using SEM showed some degree of correlation between the presence of surface biofilm in CRS and ciliary disruption (Galli, Calo et al. 2008). Hekiert et al examined mucosal samples from CRS patients for the presence of biofilm using SEM and then homogenized other samples from the same patients to examine the cellular profile and cytokine levels. Those patients that were biofilm-
positive were seen to exhibit a profile skewed more towards a local TH1 profile (Hekiert, Kofonow et al. 2009). Foreman et al followed a similar approach of examining samples for the presence of biofilms and the immunological profile in separate samples but reported the contrary finding of S.aureus biofilms being associated with a TH2 response, although in a very different study population (Foreman, Holtappels et al. 2011). Sun et al have also followed a similar approach and showed that the presence of biofilms on one sample is associated with more transcription of and expression of TLR2, TLR4 and nuclear factor-kappa beta (NF-κβ) on other samples (Sun, Zhou et al. 2012).

In Chapter 3 the relationship between surface biofilm and the underlying immune response is explored further, with particular focus on the interface of the biofilm with the epithelial layer that separates the two. By sectioning through the tissue and hence the biofilm these associations were directly observed rather than consideration being made of biofilm and inflammatory response on separate samples. Since this chapter was published a further study has employed a very similar method assessing serial tissue sections for the presence of biofilm on the mucosal surface correlating this with the epithelial integrity, the underlying cellular infiltrate and the expression of tumour necrosis factor-α (TNF-α) receptors within the tissue (Karosi, Csomor et al. 2012).

1.3.4 Intramucosal bacteria

Although there has been focus on bacteria which are easily able to be sampled from the surface of the mucosa with a swab, and subsequently on biofilms attached to the surface of the mucosa, very little is known about bacteria existing within the mucosa itself. A report of intramucosal bacteria in maxillary sinusitis was made in 1984 and
occasional observations had been published before this date (Lundberg and Engquist 1984).

It is clear that pathogens do indeed have the ability to exist within mucosa. *Haemophilus influenzae* was observed within samples of adenoid tissue nearly 20 years ago (Forsgren, Samuelson et al. 1994). An important study in this field was published in 2005 in which it was reported in a small series of patients with recalcitrant CRS that *S.aureus* was present in the intracellular space within epithelial, glandular and myofibroblastic cells (Clement, Vaudaux et al. 2005). As a follow-up to this study 27 CRS patients were followed and the presence of intracellular *S.aureus* at the time of surgery considered with respect to their post-operative course (Plouin-Gaudon, Clement et al. 2006). Although there was no statistical analysis presented there appeared to be a clear skew towards a greater frequency of post-operative recurrence in those that exhibited intracellular *S.aureus* at the time of surgery. These observations correlate with evidence demonstrating that *S.aureus* and its small colony variant (SCV) are capable of evading host immunity and establishing long-term intracellular residency (Tuchscherr, Heitmann et al. 2010). There exists a potential role for persistent *S.aureus* intracellular residency in CRS pathogenesis, particularly in regard recalcitrance in the face of antibiotic therapy.

Intramucosal *S.aureus* and SCVs were sought in one study from Denmark. No SCVs were cultured, no difference in *S.aureus* detection was identified between diseased and control mucosa and intramucosal *S.aureus* was identified using FISH in only one out of 51 patients studied (Niederfuhr, Kirsche et al. 2008). Criticisms have however been leveled at this study in regard both the methodology used and the data interpretation (Corriveau, Zhang et al. 2009).
Further studies have confirmed the presence of _S.aureus_ both within the epithelial cell layer and in the subepithelial layer. Although attempts have been made to correlate the presence of intramucosal _S.aureus_ with inflammatory mediators in one study (Corriveau, Zhang et al. 2009) and results were combined with _in vitro_ cell culture data in another (Sachse, Becker et al. 2010), the _ex vivo_ data from these studies principally reported on prevalence. Taken together they indicated a particular prevalence of intramucosal _S.aureus_ in CRSwNP compared to controls, particularly in those with associated AERD. Lesser numbers were found in CRSsNP and control samples of inferior turbinate mucosa. Neither study looked for species other than _S.aureus_ or yielded particular information on how the intramucosal _S.aureus_ might contribute to disease pathogenesis.

In the light of these previous observations, the study presented in Chapter 4 was designed to examine samples of mucosa using non-species specific and species specific techniques and to begin to define the relationship between the presence of intramucosal bacteria and the host inflammatory response.

### 1.3.5 Intraosseous bacteria

With computed tomography (CT) imaging allowing evaluation of the paranasal sinuses in great detail it became clear that a proportion of patients demonstrate abnormalities in the bones of the paranasal sinuses (Zinreich 1993). Given that the observed population is skewed towards patients with more severe disease (Telmesani and Al-Shawarby 2010) this phenomenon is common in patients presenting to the tertiary academic units that typically research this disorder. Subsequently it has achieved prominence in the literature despite being a finding in only a minority of unselected CRS patients.
Definitions and categorisation of changes in the parasinus bones is not yet clear. It is uncertain whether CT or histopathology is the better investigation, with there being a lack of correlation between the findings of the two modalities (Lee, Kennedy et al. 2006). It has been proposed that an increase in bone thickness to more than 3mm is required for a diagnosis of “osteitis” to be secured (Lee, Kennedy et al. 2006) while another recent classification also included abnormal appearing bone of less than 3mm diameter (Georgalas, Videler et al. 2010).

The consistent histopathological finding in these individuals is of increased bone turnover - both rates of bone resorption and neoosteogenesis are increased (Kennedy, Senior et al. 1998; Giacchi, Lebowitz et al. 2001). The term “osteitis” has been used to describe these changes, but this term implies the presence of inflammation, presumed to be as a consequence of bone infection. A series of studies utilising an animal model of sinusitis have suggested a role for bone infection in the pathogenesis of CRS (Perloff, Gannon et al. 2000; Khalid, Hunt et al. 2002).

There are few sound observations made in CRS patients which suggest that these bone changes are caused by infection. In 1994 a case series of four patients was presented with each considered to have “osteoblastic osteitis of the maxillary sinus” (Tovi, Benharroch et al. 1992). In one of those four cases colonies of Actinomyces were described within bone samples collected intra-operatively. It was noted in the Discussion section from that paper that there is some overlap between chronic and acute rhinosinusitis (ARS) but the presence of lymphadenopathy, facial tenderness and leukocytosis in that particular case would tend to imply an acute inflammatory process. Given that infection is known to be able to extend beyond the confines of the sinus cavities in ARS (Kastner, Taudy et al. 2010) the presence of intraosseous bacteria in ARS is not surprising. If this case from 1994 is indeed taken as being a
case of ARS there is not a single report of intraosseous bacteria in CRS to date (Videler, Georgalas et al. 2011).

The study presented in Chapter 5 was designed to test the hypothesis that the bone changes observed in CRS relate to the presence of bacteria within the bone with a consequent inflammatory response.

### 1.3.6 Staphylococcal virulence factors

It is becoming clear that a wide spectrum of micro-organisms are capable of suppressing and modulating the human immune response (Davies, Sheil et al. 2009). It is most likely that this active control exerted by secreted bacterial proteins contributes to the dense bacterial colonisation of the skin and mucous membranes that exists in health without inducing an inflammatory response (Peacock, de Silva et al. 2001; Round and Mazmanian 2010).

Based on bacterial culture data it has been recognized that *S. aureus* is prevalent in CRSwNP patients with one study using swabs of the middle meatus reporting *S. aureus* prevalence in CRSwNP of 63.6%, significantly higher than controls (33.3%) and CRSsNP (27.3%). In the same study, even higher rates of *S. aureus* detection were reported in CRSwNP patients with comorbid asthma and AERD (Van Zele, Gevaert et al. 2004). The prevalence of *S. aureus* in combination with the array of virulence factors that it is known to produce (Chavakis, Preissner et al. 2007) makes it an ideal candidate for the study of secreted virulence factors in CRS. Somewhat surprisingly given the number of possible relevant virulence factors, the study of staphylococcal superantigens in CRS has almost completely dominated this field.

Superantigens are a family of proteins produced by a number of microorganisms including Gram positive and Gram negative bacteria and viruses. They are capable of
binding to the Vβ region of the T cell receptor, away from the antigen-specific region, inducing a non-antigen specific activation of large populations of T lymphocytes. They also have other pro-inflammatory effects including an interaction with B cells that is also not antigen specific. They are therefore capable of inducing a vigorous inflammatory response in the human host of far greater magnitude than conventional antigens (Stow, Douglas et al. 2010). A variety of techniques have been employed to attempt to clarify their role in CRS including animal models, mucosal explant studies, assessment of *S.aureus* isolates for the presence of superantigen genes and an immunoassay for the expressed proteins in clinical specimens. Some research groups have favoured an immunoassay of host IgE to the superantigens in the majority of their studies (Bachert, Zhang et al. 2008). It is perhaps relevant to note that not all authors have supported this approach with some studies suggesting that IgE antibodies in sinus mucosa are not antigen specific (Pratt, Collins et al. 2010).

Determining the presence or absence of superantigen genes in *S.aureus* cultured from CRS and control subjects has not lead to clear results. This may in part be because the presence of genes does not predict the gene expression or the nature of the response to that gene expression. Furthermore this technique is based on the assumption that the *S.aureus* that is cultured is representative of *S.aureus* present *in vivo*. In one study of 40 *S.aureus* strains derived from CRSwNP and control subjects no difference was found in the frequency of detection of superantigen genes between the two groups with surprisingly a trend to more in the control group (Van Zele, Vaneechoutte et al. 2008). In a multi-centre study employing similar methodology, 93 *S.aureus* strains were assessed and a similar frequency of superantigen gene detection was observed between the subgroups of CRSsNP, CRSwNP and control samples (Heymans, Fischer
et al. 2010). Interestingly they did detect genes for enterotoxins P and Q, proteins that have not been studied in great detail, in the CRSwNP and not in the control group.

Assessment of the downstream expression of superantigens identifies that they are indeed produced in sinus mucosa. Superantigens were detected in 63% of CRSwNP patients in a Chinese series (Wang, Shi et al. 2011) and 48% in a US series (Seiberling, Conley et al. 2005). Neither study reported the detection of superantigens in their control subjects. However as raised by Claus Bachert’s group in advocating assays of host antibodies, the presence of *S. aureus*, or for that matter its superantigens does not necessarily imply anything about their effect on the host (Corriveau, Zhang et al. 2009).

It is not yet clear what the net effect of superantigens is on sinus mucosa. It was identified at an early stage that the detection of specific IgE to superantigens is not only more prevalent in CRSwNP but also correlates with a skew of the inflammatory response to a TH2 profile (Bachert, Gevaert et al. 2001). The presence of specific IgE would imply that a conventional immune response is being mounted rather than the non-specific response characteristic of superantigens. Other data exist which do however demonstrate the particular prevalence of individual Vβ domains in T cell populations with the implication being that this expansion is a true superantigen effect (Conley, Tripathi et al. 2006). Other studies have shown an association between the detection of superantigens and a shift in the predominant host glucocorticoid receptor phenotype with the suggestion made that superantigens contribute to glucocorticoid resistance (Wang, Shi et al. 2011). An animal model also exists implying that SEB promotes the development of nasal polyps (Kim, Khalmuratova et al. 2011). Most authors would consider superantigens to not be a universal feature of CRS but that
they most likely act as a disease-modifier principally or even exclusively in CRSwNP (Kern, Conley et al. 2008; Tomassen, Van Zele et al. 2011).

Not all studies support a role for superantigens in the stimulation of inflammation in CRS. In a Chinese study population no association was found between the presence of IgE to superantigens and T cell phenotypes (Shi, Fan et al. 2009). It is also not clear what the survival advantage for *S. aureus* is in evolutionary terms from a family of proteins promoting a strongly immunogenic response from its host. It has been found that low concentrations of superantigens (picogram/millilitre or nanogram/millilitre range) can actually induce anergy in the host (Taylor and Llewelyn 2010). Given that it is unknown what the concentration of superantigens are in sinus mucosa (assays of the proteins discussed above report superantigens as detectable or not detectable) it may be that the clear pro-inflammatory effect reported at concentrations in the microgram/millilitre range are not representative of what exists *in vivo* (Patou, Gevaert et al. 2008).

*S. aureus* probably evolved to exist in the human host evading rather than stimulating inflammation. It is noticeable that there are numerous *in vitro* mechanisms by which this may be achieved. Anti-inflammatory mechanisms exhibited by *S. aureus* include microbial surface components recognizing adhesive matrix molecules, secreted expanded repertoire adhesive molecules, extracellular enzymes, non-proteinaceous factors, toxins, anti-inflammatory peptides, capsular polysaccharides, cell wall components and wall component-modifying factors (Chavakis, Preissner et al. 2007). Within that group of “toxins” are some of the staphylococcal superantigen-like proteins (SSLs). Although there is an expanding body of evidence showing their anti-inflammatory function *in vitro* (Fraser and Proft 2008; Langley, Patel et al. 2010) very little is known of what their *in vivo* effect is in the human host. It is not known for
instance whether the anti-inflammatory effects of the SSLs are at work in the maintenance of normal *S.aureus* colonisation or in the genesis of staphylococcal diseases or both. SSLs have not been studied in the context of CRS. It is notable that SSLs disrupt components of the innate immune system including the complement pathway (Laursen, Gordon et al. 2010), matrix metalloproteinase (MMP) 9 (Itoh, Hamada et al. 2010) and neutrophil diapedesis (Bestebroe, Poppelier et al. 2007). This is an important distinction from the superantigens that target the adaptive immune response and is particularly relevant in the context of the observed deficiencies in the innate immune response in CRS.

The study presented in Chapter 7 was designed therefore to explore the possibility that SSLs exist within the mucosa of the paranasal sinuses and to assess if they may contribute to the development or perpetuation of CRS.
1.4 Summary

1.4.1 Conclusions from the literature review

CRS is prevalent, morbid and expensive to treat. While there are treatments whose efficacy has been proven in randomised placebo controlled trials there is a clear need to improve understanding of the disease to direct further advances in therapy.

The study of the microbiology of CRS has previously been limited by techniques of bacterial and fungal culture. Parallel studies of the immunology of the disorder have indicated that there is not simply an over-activity of inflammatory mechanisms. Demonstrable defects in both physical mucociliary barriers and local immune function exist. The incorporation of culture-independent techniques for examining the sinus flora as well as the evolution of understanding of microbial biology have opened several new avenues from which hypotheses can be formed about how microorganisms may be responsible for the mucosal changes in CRS.

1.4.2 Hypotheses

This thesis will address a number of hypotheses related to the microbiology and pathophysiology of CRS:

- Chapter 2: that CRS is caused by persistent respiratory viral infection.
- Chapter 3: that adherent bacterial biofilms are associated with a more vigorous inflammatory response in CRS.
- Chapter 4: that intramucosal microcolonies stimulate inflammation in CRS. The study also attempts to describe some of the species of intramucosal bacteria present in CRS.
- Chapter 5: that thickening of the bones around the paranasal sinuses is caused by bacterial infection that induces an inflammatory response.
• Chapter 6 discusses some important methodological issues identified during the development of the methodology for Chapter 7.

• Chapter 7: that SSLs are present within sinus mucosa and that the presence of SSLs is skewed towards those with inflammatory disease.

1.4.3 Study design

There are factors that facilitate and others that are a challenge in the study of the pathogenesis of CRS. In comparison to other related diseases such as asthma, fresh CRS tissue samples are readily accessible due to the frequency with which FESS is performed (Bhattacharyya, Orlandi et al. 2011). However the study of diagnosis, disease severity and treatment outcomes is principally defined by subjective symptoms that tend to correlate only poorly to objective measures (Wabnitz, Nair et al. 2005).

Classification of CRS is not well understood but for ease of comparison with the literature, it remains reasonable to follow the published guidelines of CRSsNP and CRSwNP, while noting co-morbid asthma, the presence of eosinophilic mucus and other specific sub-categories such as AERD and cystic fibrosis. Paediatric patients should be excluded in the study of adult CRS (Berger, Kogan et al. 2011) as the disease pathogenesis and prognosis may well be different. Local unpublished data exist which show a significant association between sinonasal anatomical variants and limited mucosal disease around the OMC (but not for more extensive mucosal disease). Only patients with extensive mucosal disease that had been selected for a complete sphenoethmoidectomy and frontal recess dissection were included in these studies therefore, as these are the ones less likely to have sinusitis secondary to local anatomical abnormalities. Patients were all prospectively recruited.
There has been an assumption that normal sinus mucosa is sterile (Larson and Han 2011). However there have been reports of a low prevalence of bacterial biofilms on the surface of normal sinus mucosa (Sanderson, Leid et al. 2006; Healy, Leid et al. 2008). Intramucosal bacteria have also been found in control samples although like many studies these control samples were of inferior turbinate rather than directly comparable sinus mucosa (Corriveau, Zhang et al. 2009; Sachse, Becker et al. 2010). These findings emphasise the importance of proper control samples for direct comparison with test samples and given that it is not known whether inferior turbinate mucosa is directly equivalent to sinus mucosa the collection of normal sinus mucosa was favoured. Samples were then analysed blinded to clinical details. Scientific credibility is only gained by the use of a full battery of appropriately designed experimental controls (Lawson, Connally et al. 2011) and so care was taken in designing appropriate positive and negative controls. This point is particularly emphasised in Chapter 6.
Chapter 2. Is chronic rhinosinusitis caused by persistent respiratory virus infection?

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2.1 Abstract

2.1.1 Background
Many chronic rhinosinusitis (CRS) patients recall an upper respiratory tract infection as the inciting event of their chronic illness. Viral infections have been shown to cause obstruction of the osteo-meatal complex, which is likely to be a critical step in the development of CRS. There is clear overlap between the pathogenesis of CRS and asthma. Infections with respiratory viruses in childhood increase the risk of subsequently developing asthma. Viral infections in established asthmatics are associated with acute exacerbations.

2.1.2 Objective
We sought to determine whether respiratory viruses could be detected within the sinonasal mucosa of CRS patients using polymerase chain reaction (PCR) techniques.

2.1.3 Methods
Sinus mucosa was sampled from 13 patients with CRS and 2 patients with normal sinuses. PCR was used to look for common respiratory viruses (parainfluenza 1, 2 and 3, respiratory syncytial virus, human metapneumovirus, adenovirus, rhinovirus, coronavirus, bocavirus, cytomegalovirus and influenza A and B).

2.1.4 Results
No respiratory viruses were detected in any of the samples.
2.1.5 Conclusion

Persistence of respiratory viruses within the sinonasal mucosa is unlikely to be a cause of ongoing inflammation in CRS. The possibility remains that a transient viral infection provides the initial inflammatory stimulus.
2.2 Introduction

Despite being one of the most common chronic diseases in the Western world the pathogenesis of chronic rhinosinusitis remains poorly understood. As a consequence of this, the significant morbidity and financial burden associated with this disease persist (Wood and Douglas 2010). A wide variety of pathogenic mechanisms, mostly related to micro-organisms, have been investigated extensively, including bacterial and fungal biofilms (Psaltis, Ha et al. 2007; Healy, Leid et al. 2008), intracellular bacteria (Clement, Vaudaux et al. 2005) and aberrant immune responses to both fungal allergens (Shin, Ponikau et al. 2004) and staphylococcal superantigens (Bachert, Zhang et al. 2008).

Two of the key events in the development of CRS are thought to be obstruction of the osteomeatal complex (Ha, Psaltis et al. 2007) and the development of epithelial cell dysfunction (Tieu, Kern et al. 2009). It is common for CRS patients to report that their symptoms initially developed after a viral infection and it is noteworthy that viral infections have been shown to cause obstruction of sinus ostia (Gwaltney, Phillips et al. 1994), production of inflammatory mediators by nasal epithelial cells (Wang, Watanabe et al. 2009) and damage to epithelial cells and cilia (Pedersen, Sakakura et al. 1983). Rhinovirus has also been shown to induce persistent changes in the local cytokine milieu (Kato, Favoreto et al. 2007) and to increase bacterial adhesion to nasal epithelial cells (Wang, Kwon et al. 2009) with both mechanisms potentially providing a lasting effect following transient infection.

The link between asthma and CRS, particularly in those with nasal polyps, is well established (Bachert, Patou et al. 2006) and potential parallels in pathogenesis of these conditions exist. Childhood infection with respiratory syncytial virus (RSV)
increases the probability of an affected child developing asthma for at least a decade afterward (Sigurs 2001). It may be that the virus induces a persisting change in the mucosa before it is cleared or that viral particles persist within the mucosa, their being clear evidence that respiratory viruses are capable of establishing latent infections in human tissue (Neumann, Genersch et al. 1987). It has also been demonstrated that many acute exacerbations of asthma relate to infection with respiratory viruses (Johnston, Pattemore et al. 1995).

Several studies have used polymerase chain reaction (PCR) techniques to look for the presence of respiratory viruses in samples from CRS patients. Ramadan et al reported that 20% of patients had evidence of RSV in their mucosa but no patients had evidence of adenovirus (Ramadan, Farr et al. 1997). They did not however have a control group nor report whether samples were collected from patients during the winter months when a significant proportion of the general population are affected by respiratory viruses (Sullivan, Monto et al. 1993). Jang et al published a similar study that avoided these problems, finding rhinovirus in 21% of epithelial cell samples from CRS patients and none in controls during the summer months (Jang, Kwon et al. 2006). However the number of virus species sought was limited and samples were collected from the inferior turbinates rather than sinus mucosa.

Hypotheses concerning a role for viruses in the pathogenesis of CRS appear to fall into three groups. Viruses have been considered as potentially causative in the initial development of inflammation, the ongoing stimulus of inflammation or the cause of acute exacerbations in symptoms.

This study was designed to evaluate whether evidence could be found to implicate respiratory virus persistence in the ongoing inflammation seen in CRS.
2.3 Methods

2.3.1 Patients

Fifteen adult patients who were undergoing endoscopic sinus surgery in the tertiary practice of the senior author (RGD) either for CRS or for access to skull base lesions were prospectively recruited. CRS patients fulfilled agreed diagnostic criteria for CRS (Fokkens, Lund et al. 2007) and had failed a prolonged trial of medical therapy (Wood and Douglas 2010). All had sufficiently extensive disease to merit dissection of all their paranasal sinuses and there were no exclusion criteria. Normal sinonasal mucosa was sampled from one patient with a non-functioning pituitary adenoma and one patient with a malignant lesion of the pterygopalatine fossa. Neither of these patients had symptoms of CRS or evidence of CRS on endoscopy or imaging. Patients were recruited during the southern hemisphere summer and early autumn months (February to April 2010). Recruitment was ceased at an agreed time when in previous years the rate of detection of respiratory viruses in the general population had been seen to rise. The regional ethics committee and the hospitals involved gave prior approval of the study and informed written consent was given by all patients.

2.3.2 Clinical data

Patient demographics as well as relevant past medical and surgical history were recorded. Patients were also asked to quantify how long they had had symptoms from their nose and sinuses (Table 2-1). Patients were classified on the basis of the presence (CRSwNP) or absence (CRSsNP) of nasal polyps as per published guidelines (Fokkens, Lund et al. 2007). No patients had aspirin exacerbated respiratory disease. Subjective and objective measures of disease severity in the form of the Lund-MacKay score (Lund and Mackay 1993) and pre-operative symptom scores
(Wabnitz, Nair et al. 2005) were recorded. Our practice is to ask patients to give the 5 main symptoms of CRS (obstruction, anterior rhinorrhoea, posterior rhinorrhoea, hyposmia, midface congestion) a score from 0 to 5 based on their severity in the preceding 2 weeks.

<table>
<thead>
<tr>
<th>No.</th>
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<th>Age</th>
<th>Sex</th>
<th>Ethnicity</th>
<th>Comorbidities</th>
<th>Duration of symptoms</th>
<th>Revision surgery</th>
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<td></td>
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<td>N/A</td>
</tr>
</tbody>
</table>

Table 2-1 Patient details
2.3.3 Sample collection

Representative mucosal samples were collected from the ethmoid or sphenoid sinuses and immediately placed into sterile normal saline. They were then transferred to the laboratory where analysis was undertaken by a technician blinded to clinical details.

2.3.4 Nucleic acid extraction

Tissue pieces approximately 5 mm in diameter were pre-digested in 50 µl proteinase K 20 mg/µl and 150 µl of tissue lysis buffer at 55°C until completely dissolved (Roche High Pure PCR Template Preparation kit. Mannheim, Germany), then extracted using MagNA Pure LC automatic extractor and Total Nucleic acid High Performance kit according to the manufacturer’s recommendations. Total nucleic acid was eluted with 100µl elution buffer. Extracted samples yielded on average 50-200 ng/µl nucleic acids.

For each patient, between 2-6 tissue pieces were processed.

2.3.5 Respiratory Panel

Fifteen independent PCR assays were designed with identical assay protocols and PCR platforms. These included influenza A, influenza B, parainfluenza 1, multiplex parainfluenza 2 and 3, multiplex RSV A and B, human metapneumovirus (hMPV), adenovirus (ADV), cytomegalovirus (CMV), bocavirus (BoV), multiplex rhinovirus (RV)1 and 2, coronavirus OC43 and HKU1, coronavirus NL63, coronavirus 229E and human RnaseP (which acted as the extraction control).

Sequences were adapted from the Centres for Disease Control and Prevention Protocol for Detection and Characterization of Influenza (www.cdc.gov) and from published reports (Heim, Ebnet et al. 2003; Deffernez, Wunderli et al. 2004; Kuypers, Wright et al. 2004; Maertzdorf, Wang et al. 2004; Watzinger, Suda et al. 2004;
Gunson, Collins et al. 2005; Lu, Chittaganpitch et al. 2006; Kuypers, Martin et al. 2007). The CMV assay was designed in house. All assays used TaqMan hydrolysis probes labelled with fluorophore FAM or CAL Fluor Orange 560 at 5’ end and no fluorescent Black Hole Quencher 1 (BHQ1) at 3’ end. All primers and probes were synthesized by Biosearch Technologies, Novato CA and sequences are listed in Table 2-2.

<table>
<thead>
<tr>
<th>Primers and Probes</th>
<th>Oligonucleotide sequence (5’-3’)</th>
<th>Target gene</th>
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<tr>
<td>Inf A R</td>
<td>AGG GCA TTY TGG ACA AAK CGT CTA</td>
<td></td>
</tr>
<tr>
<td>Inf A P</td>
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<tr>
<td>Inf B F</td>
<td>TCC TCA AYT CAC TCT TCG AGC G</td>
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</tr>
<tr>
<td>Inf B P</td>
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<tr>
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<td>Hemagglutinin-neuramidase (HN)</td>
</tr>
<tr>
<td>PIV1 R</td>
<td>GTA GCC TMC CTT CGG CAC CTA A</td>
<td></td>
</tr>
<tr>
<td>PIV1 P</td>
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<td></td>
</tr>
<tr>
<td>PIV2 F</td>
<td>GCA TTT CCA ATC TTC AGG ACT ATG A</td>
<td>Hemagglutinin-neuramidase (HN)</td>
</tr>
<tr>
<td>PIV2 R</td>
<td>ACC TCC TGG TAT AGC AGT GAC TGA AC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CAL FO560-CCA TTT ACC TAA GTG ATG GAA TCA ATC</td>
<td></td>
</tr>
<tr>
<td>Gene</td>
<td>Forward Primer</td>
<td>Reverse Primer</td>
</tr>
<tr>
<td>--------</td>
<td>----------------</td>
<td>----------------</td>
</tr>
<tr>
<td>PIV 2 P</td>
<td>GCA AA-BH</td>
<td></td>
</tr>
<tr>
<td>PIV 3 F</td>
<td>CCA GGG ATA TAY TAY AAA GGC AAA A</td>
<td>Hemagglutinin-neuramidase (HN)</td>
</tr>
<tr>
<td>PIV 3 R</td>
<td>CCG GGR CAC CCA GTT GTG</td>
<td></td>
</tr>
<tr>
<td>PIV 3 P</td>
<td>FAM-TGG RTG TTC AAG ACC TCC ATA YCC GAG AAA-BHQ1</td>
<td></td>
</tr>
<tr>
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<td>GCC CCA GTG GTC TTA CAT GCA CAT C</td>
<td>Hexon</td>
</tr>
<tr>
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<td>GCC ACG GTG GGG TTT CTA AAC TT</td>
<td></td>
</tr>
<tr>
<td>ADV P</td>
<td>FAM-TGC ACC AGA CCC GGG CTC AGG TAC TCC GA-BHQ1</td>
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<tr>
<td>RSV F</td>
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<tr>
<td>RSV R</td>
<td>GCC AAG GAA GCA TGC AAT AAA</td>
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<td>CAL FO560-CAC TAT TCC TTA CTA AAG ATG TC-BHQ1</td>
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<td>Nonstructural gene (NS)</td>
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<td>hMPV P</td>
<td>FAM-TGY AAT GAT GAG GGT GTC ACT GCG TGG G-BHQ1</td>
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<td>CMV F</td>
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<td>FAM-CCG CAA CCC TTC AT-BHQ1</td>
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</tr>
<tr>
<td>RV F</td>
<td>GCA CTT CTG TTT CCC C</td>
<td>5’Non-coding region</td>
</tr>
<tr>
<td>RV R</td>
<td>GGC AGC CAC GCA GGC T</td>
<td></td>
</tr>
<tr>
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<td>Primer 2</td>
</tr>
<tr>
<td>---------</td>
<td>----------</td>
<td>----------</td>
</tr>
<tr>
<td>RV P1</td>
<td>FAM-AGC CTC ATC TGC CAG GTC TA-BHQ1</td>
<td>CAL FO560-AGC CTC ATC CAC CAA ACT A-BHQ1</td>
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<td>hBoV P</td>
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<td></td>
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<td>OC43+HKU1</td>
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<td>Polymerase 1b</td>
</tr>
<tr>
<td>CoV F1</td>
<td>GGC ATA GCA CGA TCA CAC TTA GG</td>
<td></td>
</tr>
<tr>
<td>CoV R1</td>
<td>6-FAM-ATA ATC CCA ACC CAT RAG-BHQ1</td>
<td></td>
</tr>
<tr>
<td>CoV P1</td>
<td>FAM-ATA ATC CCA ACC CAT RAG-BHQ1</td>
<td></td>
</tr>
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<td>NL63</td>
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<td></td>
</tr>
<tr>
<td>CoV R2</td>
<td>FAM-ATA ATC CCA ACC CAT RAG-BHQ1</td>
<td></td>
</tr>
<tr>
<td>CoV P1</td>
<td>FAM-ATA ATC CCA ACC CAT RAG-BHQ1</td>
<td></td>
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<td>229E</td>
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</tr>
<tr>
<td>CoV R3</td>
<td>CAL FO560-ATA GTC CCA TCC CAT CAA-BHQ1</td>
<td></td>
</tr>
<tr>
<td>CoV P2</td>
<td>AGA TTT GGA CCT GCG AGC G</td>
<td></td>
</tr>
<tr>
<td>RnaseP F</td>
<td>GAG CGG CTG TCT CCA CAA GT</td>
<td>Human</td>
</tr>
<tr>
<td>RnaseP R</td>
<td>FAM-GAG CGG CTG TCT CCA CAA GT-BHQ1</td>
<td>Ribonuclease P</td>
</tr>
</tbody>
</table>

Table 2-2. Primers and Probes. F= Forward, R= Reverse, P= Probe
2.3.6 Reagents

Reactions were carried out in 25µl reaction mixtures containing 1x reaction mix (Invitrogen SS III Platinum One-step Quantitative RT-PCR system. Carlsbad, CA, USA), 0.5µl enzyme mix Superscript III / Platinum Taq Polymerase, in case of ADV, CMV and BoV Platinum Taq Polymerase was used, 0.8-0.9 µM forward and reverse primers, 0.2 µM fluorescent probe and 5 µl extracted RNA/DNA.

PCR mixes without enzyme were prepared in large volumes and stored in single use aliquots at –20°C. Before use, aliquots for each PCR were thawed, mixed with enzyme and aliquoted onto reaction plate. RNA/DNA was then added. Positive controls were aliquoted last to minimize possible contamination.

2.3.7 Controls

Each run contained a No template control (NTC) water, extraction blank controls and positive controls.

Positive controls consisted of RNA extracted from confirmed tissue culture isolates. ADV type 5 (Ad-5) strain and CMV AD169 were obtained commercially from Advanced Biotechnologies, Maryland, USA. Boca virus control consisted of a cloned amplified isolate, confirmed by sequencing.

All these assays (except BoV) are subjected to annual quality control programmes as distributed by Quality Control for Molecular Diagnostics (Glasgow, Scotland) and RCPA Australia.

2.3.8 Amplification

Assays were carried out on the Roche Light Cycler 480 using 96 well microplate format. Amplification conditions were 50°C for 20 min (reverse transcription), 95°C 2
min (initial DNA Polymerase activation), then 45 cycles of denaturation at 95°C for 15 seconds followed by 45 seconds at 55°C (annealing/extension).

Results were analyzed in FAM channel and Cal Orange 560 separately. Assays were considered valid if RnaseP amplification was positive.
2.4 Results

In the CRS patients, the median duration of symptoms reported was 4 years (range 2 to 35 years). The median pre-operative symptom score was 17/25 (range 10 to 21). The median Lund-MacKay score was 16/24 (range 10 to 22).

No respiratory viruses were detected in any of the samples.

Since conventional respiratory viruses were not found, further assays were performed looking for viruses known to be capable of establishing persistent infection, namely human herpes-6, (HHV-6) and Epstein-Barr virus (EBV) using PCR techniques with published primers (Gautheret-Dejean, Manichanh et al. 2002; Croxson, Taylor et al. 2006). Low titre HHV-6 was found in samples from 3 / 8 CRSsNP patients, 4 / 5 CRSwNP patients and 1 / 2 normal subjects. Low titre EBV was found in 1/8 CRSsNP patients, 4 / 5 CRSwNP patients and 0/2 normal subjects. The low titres of virus present suggest latent rather than active infection.
2.5 Conclusions

It seems likely that CRS has a multifactorial pathogenesis, and that the inflammatory stimuli differ at different stages of the disease and in different subgroups. Respiratory viruses can however cause florid sinonasal symptoms and may generate a long lasting effect on respiratory mucosa. Respiratory viruses have also been shown to be capable of establishing latency in human tissue (Neumann, Genersch et al. 1987). The role of respiratory viruses in the ongoing inflammation seen in CRS therefore warrants further investigation.

In this series of patients all CRS patients had active inflammation and despite using sensitive tests for an extensive panel of respiratory viruses we did not identify any evidence of respiratory viral presence in our patients. It may be that CRS is a spectrum of disease and our small sample size may not therefore include all variants but our clearly negative results do suggest that persistent respiratory virus infection is not responsible for the chronic inflammation seen in the major variants of this disorder. Evidence of latent EBV infection was seen in CRS mucosa in 42% of cases but in the small number of patients examined, EBV was not found in the controls. The significance of this is unclear.

Despite not finding respiratory viruses in our series it is noted that animal and in vitro models have shown that the deleterious effect of respiratory virus infection may persist well beyond the time when the virus particles have been cleared (Klemens, Thompson et al. 2006; Kato, Favoreto et al. 2007).

Our rate of detection of viruses in CRS is lower than in other published series. This may in part be due to methodological differences such as the collection of sinus mucosa rather than inferior turbinate samples (Jang, Kwon et al. 2006) and collection...
of specimens during the summer months when respiratory viruses are far less prevalent.

A study of whether viral infections are responsible for acute exacerbations in CRS symptoms could be designed in a similar fashion but undertaken during winter months. A large group of negative control patients would be required to establish what the background rate of viral infection is at that time of year.

The chronic nature of CRS implies that presentation to specialist care is remote from the development of the condition, which makes study of the initiating factors more difficult. Transient respiratory virus infection causing damage to the epithelial surface could be the process that allows bacterial biofilms to form and adhere to the mucosal surface or could cause long-lasting changes in the inflammatory milieu to occur that are critical to the subsequent development of CRS. We found however no evidence of persisting respiratory virus infection in diseased sinus mucosa.
Chapter 3. Are biofilms associated with an inflammatory response in chronic rhinosinusitis?

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3.1 Abstract

3.1.1 Background
Bacterial biofilms have been identified on the sinonasal mucosa of patients with chronic rhinosinusitis (CRS) but also on control samples. Their role in the disease pathogenesis is unproven.

3.1.2 Objectives
To further evaluate the role of biofilms in CRS by assessing whether they are associated with an inflammatory response.

3.1.3 Methods
Mucosal samples were collected from 18 patients with CRS and 7 normal subjects. Bacteria on the mucosal surface were identified by Gram stain. Immune cells were identified by Giemsa stain and immunohistochemistry. The number of local immune cells was recorded beneath areas of the mucosal surface both colonized with and free from bacteria.

3.1.4 Results
In CRS patients, biofilms which were directly opposed to a disrupted epithelial layer were associated with more T lymphocytes (p=0.01), and more macrophages (p=0.003) than areas of mucosa without bacteria present. Biofilms associated with but
not directly opposed to the epithelium were not associated with raised numbers of immune cells.

3.1.5 Conclusion

Not all surface bacterial colonies are associated with a particular inflammatory response in CRS. Biofilms adherent to a disrupted epithelial layer are associated with higher numbers of immune cells and therefore appear to have a role in the pathogenesis of CRS.
3.2 Introduction

Before the recent interest in biofilms, several authors had expressed skepticism about the role of microorganisms in the aetiology of chronic rhinosinusitis (CRS). Observations based on laboratory culture data comparing CRS patients to normal controls (Bhattacharyya 2005) and the relatively poor efficacy of antibiotics in the treatment of the condition (Legent, Bordure et al. 1994) were variously cited as evidence that bacteria were not central to the pathogenesis of CRS.

However, the concept of biofilms in which colonies of bacteria can reside within a matrix on the mucosal surface, protected from antibiotics and host immunity (Parsek and Singh 2003) reignited interest in a possible role for bacteria in the pathogenesis of this condition. It is now acknowledged that the laboratory culture techniques previously used in the investigation of CRS not only fail to model the biofilm phenotype but are also poor at detecting biofilm microorganisms when compared with methods detecting bacteria in situ (Hall-Stoodley, Hu et al. 2006). Several published articles have reported the presence of biofilms on the mucosal surface in CRS using in situ techniques (Cryer, Schipor et al. 2004; Sanclement, Webster et al. 2005; Sanderson, Leid et al. 2006; Psaltis, Ha et al. 2007; Healy, Leid et al. 2008; Psaltis, Weitzel et al. 2008; Foreman, Singhal et al. 2010). There is some evidence that the presence of biofilm-like structures may be associated with a worse patient outcome from sinus surgery (Psaltis, Weitzel et al. 2008).

A critical analysis of the investigation of biofilms in CRS highlights a number of difficulties and contradictions. In part, this stems from the fact that the normal nose has a resident microflora contained within an organic matrix (normal mucus) that is associated with the mucosal surface, leading to criticisms of some of the published
research due to the presence of normal mucus creating potential for overestimation of biofilm prevalence (Psaltis, Ha et al. 2007). Additionally, there has been a lack of consensus regarding the optimum method for examining biofilms in CRS with scanning electron microscopy (SEM) (Cryer, Schipor et al. 2004; Sanclement, Webster et al. 2005) transmission electron microscopy (TEM) (Sanclement, Webster et al. 2005) and confocal scanning laser microscopy (CSLM) using either a Live/Dead stain (Psaltis, Ha et al. 2007; Psaltis, Weitzel et al. 2008; Foreman, Singhal et al. 2010) or fluorescence in situ hybridisation (FISH) (Sanderson, Leid et al. 2006; Healy, Leid et al. 2008; Foreman, Singhal et al. 2010) all having being used to detect biofilms on sinus mucosa. There is a lack of consensus on the definition of a biofilm. Biofilms have been defined as being “a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert of living surface” (Costerton, Stewart et al. 1999). Since the publication of this definition in 1999 it has been suggested that not only bacteria but also fungi can exist within biofilms (Healy, Leid et al. 2008). Furthermore it has been questioned whether the matrix must be “self-produced” (Parsek and Singh 2003) and whether biofilms must be “adherent to” or merely “associated with” a surface (Hall-Stoodley and Stoodley 2009).

One of the defining factors for a biofilm in a clinical context is that it provokes an inflammatory response by the host (Hall-Stoodley and Stoodley 2009). Indeed, if biofilms are not associated with inflammation their eradication will not likely yield therapeutic benefit. Interestingly some studies have described the presence of biofilms in normal subjects, leading to speculation that biofilms may not by themselves be directly responsible for inflammation (Sanderson, Leid et al. 2006; Healy, Leid et al. 2008). The majority of techniques used to detect biofilms look at surface structures
from above, and so are not well suited to visualise the inflammatory response below the mucosal surface.

The aim of this study was to observe bacterial flora on the mucosal surface, and relate the presence of biofilms to inflammation in the mucosa below the epithelial surface.
3.3 Method

3.3.1 Patients and clinical data

Twenty-five adult patients who were undergoing endoscopic sinus surgery in the practice of a single surgeon (RGD) either for CRS or for access to the pituitary fossa were prospectively recruited. The CRS patients fulfilled diagnostic criteria for this condition (Fokkens, Lund et al. 2007) and had failed a prolonged trial of medical therapy (Wood and Douglas 2010). Patients were excluded if they had used a course of antibiotics or systemic corticosteroids in the four weeks prior to sample collection but pre-operative intra-nasal steroids or antihistamines were permitted. Patients with a predisposing condition such as cystic fibrosis or Kartagener’s syndrome were excluded. For comparison normal sinus mucosa was sampled from patients with non-functioning pituitary adenomas who were undergoing an endoscopic approach to their pituitary fossa, providing they had neither symptoms nor radiological or endoscopic evidence of CRS. As outlined later however areas of mucosa from CRS patients that were free from bacteria were used as the controls. Prior approval of the study was given by the regional ethics committee and the hospitals involved and written informed consent was given by all patients.

Patients were classified on the basis of the presence or absence of nasal polyps as per published guidelines (Fokkens, Lund et al. 2007). Relevant medical and surgical history was recorded as well as the Lund-MacKay score (Lund and Mackay 1993) and patient demographics.

The study group was composed of 9 patients with nasal polyps (CRSwNP), 9 patients with CRS without nasal polyps (CRSsNP) and 7 normal subjects. The relevant clinical and demographic details are presented (Table 3-1). The CRSwNP group had a
higher rate of co-morbid asthma and higher Lund-MacKay scores despite more
commonly undergoing revision surgery than the CRSsNP group. There were no cases
of aspirin exacerbated respiratory disease in the CRSwNP group.

<table>
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<th></th>
<th>Age: Median (Range)</th>
<th>Sex</th>
<th>Asthma</th>
<th>Revision</th>
<th>Duration: Median (Range)</th>
<th>Lund-MacKay</th>
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<td>9M:0F</td>
<td>6/9</td>
<td>7/9</td>
<td>4ys (2-30yrs)</td>
<td>19</td>
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<tr>
<td>CRSsNP (n=9)</td>
<td>53 (19-63)</td>
<td>5M:4F</td>
<td>1/9</td>
<td>0/9</td>
<td>16months (4m-50y)</td>
<td>12</td>
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<td>3M:4F</td>
<td>0/7</td>
<td>N/A</td>
<td>N/A</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3.1 Clinical and demographic details

3.3.2 Initial studies

We found much superior preservation of surface material when specimens were fixed
in Carnoy’s fixative rather than formalin (Swidsinski, Weber et al. 2005; Winther,
Gross et al. 2009). The morphological appearance of the epithelium was noted to be
variable with epithelial cell disruption not directly correlated to the presence or
absence of bacterial colonies on the mucosal surface. It was also noted that the
arrangement of surface colonies was variable with some directly opposed to the
mucosal surface, considered to be adherent and some less closely opposed, and
considered to be merely associated.
None of our patients had positive results for fungal culture and only occasional, isolated fungal hyphae were seen on the mucosal surface when samples from 17 patients were assessed using a panfungal FISH probe (AdvanDx, Woburn, USA) (unpublished data, 2010). This is consistent with our clinical experience of only occasional cases of overt fungal disease in the local population. We therefore elected to solely pursue bacterial biofilms in this study.

Various methods were trialed for the identification of bacteria on the mucosal surface including Gram stain, Giemsa and a eubacterial FISH probe. Whilst they all demonstrated the presence of bacteria Gram stain was considered to be the optimum method for detection of bacteria in this context.

3.3.3 Samples

At the time of recruitment patients were allocated a reference number and all mucosal specimens were processed anonymously allowing analysis to be conducted blinded to clinical details. Two representative mucosal samples were collected from the ethmoid or sphenoid sinuses from each patient with one undergoing serial washing in three baths of normal saline, with the intention of removing non-adherent planktonic bacteria (Psaltis, Ha et al. 2007; Foreman, Singhal et al. 2010). Samples were fixed in Carnoy’s fixative for 24-72 hours and then processed for conventional histology. Mucosal samples were embedded on their side in paraffin so that sectioning could proceed approximately perpendicular to the mucosal surface.

Serial 5 µm sections were cut from each block and mounted on Superfrost® Plus Positively Charged Microscope slides (Thermo Fisher Scientific New Zealand Ltd,
Auckland, New Zealand). Immediately adjacent sections were mounted on two separate slides for assessment using Gram stain and immunohistochemistry (IHC) techniques. A minimum of four paired Gram and IHC slides were processed per patient.

3.3.4 Histology and Immunohistochemistry

A routine Gram stain protocol was followed for the first slide and a safranin counterstain applied.

The next 5 immediately adjacent sections were mounted on the second slide for assessment using IHC. The NovoLink™ Polymer Detection System (Leica Microsystems, Wetzlar, Germany) was used and recommended protocols were followed. Antigen retrieval was not required with Carnoy’s fixed tissue. Primary antibodies used were murine monoclonal antibodies to the following antigens: CD3 (pan-T cell), CD20 (pan B-cell), CD68 (macrophages) and Human Neutrophil Defensin (neutrophils). The final section had phosphate-buffered saline applied in place of a primary antibody and served as a negative control. IHC sections were counterstained with Giemsa which stains eosinophils red/pink and bacteria blue/black, allowing eosinophil numbers to be quantified. We found however that Gram stain was far superior to Giemsa for identifying bacteria on the mucosal surface due to the dense staining of the matrix seen with Giemsa (Image 3-2).

Samples of lamb’s liver incubated for 24 hours in pure broth cultures and processed in an identical fashion were used as positive controls for the Gram stain. Slides mounted with 5 sections of human spleen and stained in the same fashion as the mucosal IHC slides served as positive controls for the immune cell IHC.
Slides were examined using a Leica DMR upright microscope and photographed with a Nikon Digital Sight cooled colour camera (Nikon Corporation, Tokyo, Japan) using EclipseNet software (Nikon Corporation, Tokyo, Japan). All microscopy was undertaken by 1 author (A.J.W.)

For purposes of this study a biofilm was defined as a colony of bacteria demonstrated by Gram stain, embedded in a matrix and associated with (in the same high powered field as) the mucosal surface. No other described features of a biofilm such as tower formation and water channels were sought (Sanderson, Leid et al. 2006; Psaltis, Ha et al. 2007). Biofilms were recorded as present or absent at any one site and where present were noted to be adherent or merely associated with the epithelial surface dependent on whether the bulk of the biofilm was directly opposed to the mucosal surface or not.

It was assumed that the serial sections mounted on consecutive slides could be considered as superimposable. Areas of mucosal surface colonised by biofilms were identified at high power on the Gram stain section (Image 3-1). Subsequently, areas of epithelium free from bacteria were randomly selected at low power where the distribution of inflammatory cells was not discernible. The state of the epithelium at each site was recorded based on the preservation or loss of cell to cell integrity. Once the areas of interest on the mucosal surface had been selected on the Gram stain slide, the sections on the IHC slide were reviewed and counts of each of the immune cell types were then made per medium powered field (x63) immediately deep to the mucosal surface at each of the selected points (Image 3-2).
Image 3-1. 5\(\mu\)m section of a mucosal sample from a patient with CRSwNP stained with Gram stain and counterstained with safranin. Note the clusters of Gram positive organisms (arrows) contained within a matrix and directly opposed to a disrupted epithelial surface.
Image 3-2. 5µm section of a mucosal sample from a patient with CRSwNP with CD3 positive cells identified by DAB chromagen (brown/black) and counterstained with Giemsa. This is the same point on the immediately adjacent section to image 1 and shows T lymphocytes deep to the previously identified biofilm. Note also the dense staining of the biofilm matrix by Giemsa providing poor discrimination of the bacteria within it.
3.3.5 Statistics

For every patient a control count was determined for each immune cell type as the mean number of immune cells in areas free from bacteria. The number of immune cells deep to each biofilm was then compared to the control counts for that patient. The data sets generated failed the D’Agostino and Pearson omnibus normality test and so a one-tailed Wilcoxon matched pairs test was applied, the data being analysed using Prism software (Prism Software Corp., Irvine, CA).
3.4 Results

3.4.1 Biofilm prevalence

Biofilms were found in 7/9 (78%) of CRSwNP, 7/9 (78%) of CRSsNP patients and 3/7 (43%) of normals. In normal subjects, only one biofilm was considered to be adherent to a disrupted epithelial layer, compared to 18 in the CRS groups.

3.4.2 Immune cell numbers

T lymphocytes and macrophages were the most frequently observed immune cells, with the numbers of B lymphocytes, neutrophils and eosinophils being relatively lower. We were particularly surprised that eosinophil numbers were low even in CRSwNP samples although this result correlated with local unpublished whole tissue flow cytometry data.

Biofilms that were directly opposed to a disrupted epithelial layer in CRS patients were associated with a raised number of some immune cells (Figure 3-1). Significantly more T lymphocytes were seen associated with such colonies than areas of the mucosa without colonies from the same patients (p=0.01). Similarly more macrophages were seen associated with these colonies than the control counts (p=0.003). There was however no significant elevation in numbers of B lymphocytes (p=0.41), neutrophils (p=0.11) or eosinophils (p=0.50).
In the subgroup of CRS patients in which the surface colony was not directly opposed to the epithelial layer and therefore considered to be non-adherent no significant association with raised numbers of immune cells was noted (Figure 3-2).
Figure 3-2. Number of immune cells in areas of intact epithelium colonised by non-adherent biofilm compared to control counts. Data shown is for CRS patients only. Plotted is the Mean ± 95% Confidence interval.

Those colonies that were seen persisting on the surface of mucosal samples that had been washed prior to fixation also showed no significant association with raised numbers of immune cells.
3.5 Conclusion

The notion that biofilms induce inflammation remains attractive given the prevalence of biofilms in CRS (Cryer, Schipor et al. 2004; Sanclement, Webster et al. 2005; Sanderson, Leid et al. 2006; Psaltis, Ha et al. 2007; Healy, Leid et al. 2008; Psaltis, Weitzel et al. 2008; Foreman, Singhal et al. 2010), again demonstrated in this study, and the known ability of biofilms to cause chronic inflammation in some situations (Hall-Stoodley and Stoodley 2009).

Our study shows that bacterial colonisation on the mucosal surface does not consistently result in locally inflamed, diseased mucosa. This may well explain how it can be that biofilms are present on the surface of normal as well as diseased mucosa as is our finding and the finding of others (Sanderson, Leid et al. 2006; Healy, Leid et al. 2008).

Given the apparent significance of the adhesion of biofilms to the mucosa it might be assumed that washing samples prior to fixation would remove insignificant non-adherent biofilms and select out those that are virulent. This was not the case in this study. This may be because there are other factors which also determine the virulence of a biofilm such as the species of bacteria within it (Foreman and Wormald 2010).

This study demonstrates a clear association between elevated numbers of some types of immune cells and biofilms that are adherent to an abnormal epithelial layer but this association has not been proven to be causal. An alternative explanation would be that biofilms tend to form and adhere to epithelium that is already inflamed, potentially with invasive infection occurring subsequent to that (Clement, Vaudaux et al. 2005; Corriveau, Zhang et al. 2009).
The majority of investigation into biofilms in CRS has been undertaken using either SEM or CSLM, techniques which do not clearly demonstrate the interface between biofilms and the mucosa (Cryer, Schipor et al. 2004; Sanclement, Webster et al. 2005; Sanderson, Leid et al. 2006; Psaltis, Ha et al. 2007; Healy, Leid et al. 2008; Psaltis, Weitzel et al. 2008; Foreman, Singhal et al. 2010). While our technique is labour intensive we believe it offers significant advantages given the capacity to assess in situ the biofilm, the inflamed mucosa and the interface between the two. Further characterising the composition of the biofilms and the subclasses and activation state of the immune cells, could extend the findings of this study further.

In summary, biofilms not directly opposed to the epithelium are not associated with raised numbers of immune cells. Biofilms which are directly opposed to disrupted epithelium are associated with raised numbers of some types of immune cells. It remains to be seen whether the association that has been identified is a causal one.
Chapter 4. Intramucosal bacterial microcolonies exist in chronic rhinosinusitis without inducing a local immune response

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4.1 Abstract

4.1.1 Background
Although chronic rhinosinusitis (CRS) causes very significant morbidity much about its pathogenesis remains uncertain. Recent studies have identified polymicrobial biofilms on the surface of sinus mucosa and Staphylococcus aureus within the sinus mucosa of patients with CRS, both with and without nasal polyps. The pathogenic implications of intramucosal bacteria in CRS are unknown.

4.1.2 Objective
To determine the prevalence and species of bacterial colonies within the sinus mucosa of adult patients with and without CRS and to describe the relationship of these bacterial colonies to the host immune response.

4.1.3 Methods
Sinus mucosa from patients with and without CRS was examined using Gram and Giemsa staining, immunohistochemistry, bacterial culture and fluorescence in situ hybridization techniques.
4.1.4 Results

Bacterial microcolonies were observed within the mucosa in 14 of 18 patients with CRS. In 10 of these patients colonies were positively identified as *Staphylococcus aureus*. Staphylococcal microcolonies were observed at a lower level (1 of 8 patients) in normal sinus mucosa. There was no correlation between detection of *Staphylococcus aureus* on the mucosal surface and microcolonization of the mucosa. Surprisingly there was no evidence of an immune reaction to microcolonies. Indeed fewer T lymphocytes (p=0.03) and eosinophils (p=0.03) were counted immediately surrounding the microcolonies compared to uninfected areas of the same tissue.

4.1.5 Conclusion

Bacterial microcolonies are prevalent within paranasal sinus mucosa and are commonly *Staphylococcus aureus*. These microcolonies do not provoke immune detection and may represent a phenotype that actively evades host immunity. This may underpin the recalcitrance of CRS to antibiotic therapy. These findings challenge classical views of both infection and mucosal immunity in human chronic disease. The presence of intramucosal bacteria in samples of normal sinus mucosa also questions the sensitivity of detecting nasal carriage of pathogens by swabbing the surface of the anterior nares.
4.2 Introduction

Intramucosal bacteria have been demonstrated in diseased tissue from patients with tonsillitis, gastritis, colitis, cystitis and otitis media (Semino-Mora, Doi et al. 2003; Darfeuille-Michaud, Boudeau et al. 2004; Swidsinski, Goktas et al. 2007; Coates, Thornton et al. 2008; Anderson, Goller et al. 2010). There has been considerable interest in surface biofilms that are prevalent in many patients with CRS (Cryer, Schipor et al. 2004; Sanclement, Webster et al. 2005; Sanderson, Leid et al. 2006; Psaltis, Ha et al. 2007; Foreman, Psaltis et al. 2009) but little is known about those bacteria that inhabit the underlying sinus mucosa.

In 2005 *Staphylococcus aureus* was reported within epithelial and myofibroblastic cells in three patients with recalcitrant CRS (Clement, Vaudaux et al. 2005). Corriveau and co-workers subsequently reported finding intramucosal *S.aureus* in 10/31 CRS patients and 3/9 controls using a specific peptide nucleic acid fluorescence in situ hybridization (PNA-FISH) probe, although others have failed to observe such a high prevalence of intramucosal *S.aureus* using bacterial culture, FISH and polymerase chain reaction (PCR) techniques (Niederfuhr, Kirsche et al. 2008; Corriveau, Zhang et al. 2009). Different methodologies were used which may account for at least some of the variation in the reported results (Corriveau, Zhang et al. 2009). Subsequently, another group has again used PNA-FISH and reported the presence of intraepithelial *S.aureus* in 17/25 patients with nasal polyps (CRSwNP), 0/5 CRS patients without nasal polyps (CRSsNP) and in 1/10 control samples. The same study also reported the presence of submucosal *S.aureus* in 11/25 of the CRSwNP patients, 0/5 CRSsNP patients and 1/10 control samples (Sachse, Becker et al. 2010). The
identification of intracellular *S. aureus* in CRS has been associated with a worse outcome after surgical treatment (Plouin-Gaudon, Clement et al. 2006).

None of these studies of sinus mucosa looked for species of intramucosal bacteria other than *S. aureus*. It would be surprising given the diversity of microorganisms present in biofilms on the surface of sinus mucosa in CRS (Sanderson, Leid et al. 2006; Foreman, Psaltis et al. 2009) and given the assumption that invasive infections occur due to release of planktonic bacteria into the mucosa from biofilms (Cohen, Kofonow et al. 2009; Singhal, Psaltis et al. 2010) if other bacterial species could not be detected within the mucosa. Accordingly our aim was to perform a rigorous examination of microorganisms resident both on the surface of and within the mucosa in CRS, and compare these to results from mucosa from normal controls. We also wished to determine the relationship between the intramucosal bacteria and the local inflammatory response that their presence invoked.
4.3 Materials and Methods

The New Zealand Northern Regional Ethics committee and the participating hospitals approved the study. Written informed consent was obtained from participants.

4.3.1 Patients and recruitment

Twenty-six adult patients who were undergoing endoscopic sinus surgery in the tertiary practice of a single surgeon were prospectively recruited. Eighteen of these patients were undergoing surgical treatment of CRS and eight were undergoing a trans-sphenoidal approach to non-functioning pituitary adenomas. Patients were excluded if they had undergone treatment with systemic steroids or antibiotics in the four weeks prior to recruitment or if they had a predisposing condition such as cystic fibrosis or hypogammaglobulinaemia. Mucosa was only sampled from pituitary adenoma patients if they had no evidence of CRS on clinical history, endoscopy or radiology.

All CRS patients fulfilled agreed diagnostic criteria and were categorized on the basis of the presence or absence of nasal polyps as per published guidelines (Fokkens, Lund et al. 2007). Relevant medical and demographic details were collected and are summarized in Table 4-1. All CRS patients had failed treatment with what we consider to be maximal medical therapy (Wood and Douglas 2010). Mucosal specimens were identified solely with reference numbers allowing analysis to be undertaken blinded to clinical details.
### Table 4.1. Clinical and demographic details

<table>
<thead>
<tr>
<th></th>
<th>Age: Median (Range)</th>
<th>Sex Male:Female</th>
<th>Asthma</th>
<th>Revision surgery</th>
<th>Duration: Median (Range)</th>
<th>Lund-MacKay: Median (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CRSwNP</strong></td>
<td>43 (22-74)</td>
<td>9:0</td>
<td>6/9</td>
<td>7/9</td>
<td>4 years (2-30 years)</td>
<td>19 (10-24)</td>
</tr>
<tr>
<td><strong>(n=9)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CRSsNP</strong></td>
<td>53 (19-63)</td>
<td>5:4</td>
<td>1/9</td>
<td>0/9</td>
<td>16 months (4 months – 50 years)</td>
<td>12 (9-16)</td>
</tr>
<tr>
<td><strong>(n=9)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Normal</strong></td>
<td>69 (33-75)</td>
<td>4:4</td>
<td>0/8</td>
<td>N/A</td>
<td>N/A</td>
<td>0</td>
</tr>
<tr>
<td><strong>(n=8)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

At the time of induction of anaesthetic a sample of middle meatal secretions was taken using a culture swab (Medical Wire Co, Corsham, England) with care taken to avoid contamination by the anterior nares. This was processed by the hospital laboratory and any pathogenic flora that were reported were recorded.

#### 4.3.2 Specimen collection

It has been shown that Carnoy’s fixative provides superior preservation of mucosal biofilms when compared to formalin (Swidsinski, Weber et al. 2005; Winther, Gross et al. 2009; Wood, Fraser et al. 2011). Given that it has also previously been reported
that fixation of Gram positive organisms for FISH may be optimized by using an alcohol based rather than an aldehyde based fixative (Roller, Wagner et al. 1994) we wished to investigate the effect of fixation in this context.

Samples of lamb’s liver were incubated in pure cultures of *S.aureus, Streptococcus pneumoniae* and *Haemophilus influenzae* for 24 hours. Samples were then fixed in either Carnoy’s fixative or formalin and processed using our standard protocols (described below). Comparable fixation for FISH of *H.influenzae* with the two fixatives was found but there was far superior hybridization of the probes with *S.aureus* and *S.pneumoniae* following fixation in Carnoy’s fixative.

Representative full-thickness mucosal samples that had been collected during surgery from either the ethmoid or sphenoid sinuses were therefore fixed in Carnoy’s fixative for 24-72 hours (Puchtler, Waldrop et al. 1968). Samples then underwent routine histology processing and were embedded in paraffin. Five micron sections were cut for analysis and mounted on Superfrost® Plus Positively Charged Microscope slides (Thermo Fisher Scientific New Zealand Ltd, Auckland, New Zealand) before being assessed using bacterial stains, IHC and FISH. A minimum of four slides were prepared from each patient for each technique.

**4.3.3 Light microscopy**

Paired Gram stained and IHC slides were prepared with care taken to ensure that the six sections mounted on each pair of slides were immediately adjacent so that they could be considered as superimposable during the analysis.

On the first slide a routine Gram stain was performed with a safranin counterstain. On the second slide five sections were mounted and assessed using the NovoLink™ Polymer Detection System (Leica Microsystems, Wetzlar, Germany). Murine
monoclonal antibodies to the following antigens were used: CD3 (pan-T cell), CD20 (pan B-cell), CD68 (macrophages) and Human Neutrophil Defensin (HND) (neutrophils) (Leica Microsystems, Wetzlar, Germany). The optimal antibody dilutions (1: 400 for CD3, 1: 100 for CD20, CD68 and HND) were determined prior to the study. These antibodies were applied in turn to the first four sections and the final section had phosphate-buffered saline applied in place of a primary antibody and served as a negative control. IHC sections were counterstained with Giemsa that allows identification of both eosinophils and bacteria.

Sections of lamb’s liver infected with \textit{S.aureus} were used as positive controls for the bacterial stains. Slides with five sections of human spleen mounted on them were used as positive controls for the immune cell IHC.

4.3.4 Fluorescence in situ hybridization

A commercially available fluorescein isothiocyanate (FITC) labeled \textit{S.aureus} PNA-FISH probe (AdvanDx, Woburn, USA) was used. Using published sequences a FITC labeled eubacterial probe (Eub338), Cy3 labeled \textit{Streptococcus pneumoniae} (Spn) and \textit{Haemophilus influenzae} (Haeinf) probes and a FITC labeled nonsense (NonEub) probe were produced (Thermo Scientific, Ulm, Germany), diluted in deionized water to 50pg/ul and stored at -20°C until use (Wallner, Amann et al. 1993; Hogardt, Trebesius et al. 2000; Kempf, Trebesius et al. 2000; Trebesius, Leitritz et al. 2000; Hall-Stoodley, Hu et al. 2006; Swidsinski, Goktas et al. 2007; Hoa, Tomovic et al. 2009). The \textit{S.aureus} probe is supplied in a buffer containing a 30% formamide solution but for the other probes a 30% formamide buffer solution was prepared and used as per established protocols (Pernthaler, Glockner et al. 2001).
Again using deliberately infected liver tissue, probes were tested on a range of related bacteria including *Staphylococcus epidermidis, Staphylococcus saprophyticus, Staphylococcus sciuri, Streptococcus pyogenes* and *Moraxella catarrhalis* and the specificity of the probes were confirmed.

Five tissue sections were mounted onto each slide. Slides were deparaffinized and dehydrated, air-dried and then the sections were demarcated. Slides were pre-incubated in a humidification chamber containing a 30% formamide buffer solution at 55°C. To the first section the FITC labeled *S.aureus* PNA-FISH probe was applied. To the remaining sections the buffer solution was applied. Then on the second section the eubacterial FISH probe and the *S.pneumoniae* probe were applied. The *H.influenzae* probe was applied to the third section. The fourth section had the nonsense probe applied and the fifth section no probe, with the final 2 sections acting as negative controls.

Slides were incubated for 90 minutes at 55°C and then immediately transferred to pre-warmed wash solution (AdvanDx, Woburn, USA) for a further 30 minutes at 55°C. Slides were then air dried and mounted with ProLong Gold antifade reagent with 4,6-diamidino-2-phenylindole (DAPI) (Invitrogen, Mulgrave, Australia) and coverslips were applied.

**4.3.5 Microscopy**

Slides were examined using a Leica DMR upright fluorescence microscope (Leica Microsystems, Wetzlar, Germany) and photographed with a Nikon Digital Sight cooled color camera (Nikon Corporation, Tokyo, Japan) using EclipseNet software (Nikon Corporation, Tokyo, Japan). The FISH signals were detected using standard DAPI, FITC and tetramethyl rhodamine isothiocyanate filter sets with a mercury
vapor lamp as the excitation source. All microscopy was undertaken by a single author.

Where bacteria were identified on either of the light microscopy slides that same area of the mucosa on each of the IHC sections was photographed using the medium powered (x63) lens and the number of each of the immune cells around that colony were counted on each IHC section. Areas of mucosa without colonies present were randomly selected at low power where the distribution of immune cells was not discernible and in the same fashion the number of immune cells were quantified under the medium powered lens. The mean number of each of the immune cells in the absence of colonies was calculated for each patient allowing paired analysis to be undertaken comparing areas of the same tissue sections where bacterial colonies were and were not present.

When the negative control FISH sections were reviewed both tissue autofluorescence and occasional artefact due to incomplete washing of the NonEub probe were noted. Bacterial colonies were therefore reported positive only if they demonstrated appropriate staining with DAPI as well as typical morphology and evidence of appropriate hybridization. The counterstains for both fluorescence and light microscopy allowed identification of the tissue morphology and so where identified, bacterial colonies were recorded as present on the mucosal surface, within the epithelial layer or subepithelial.

4.3.6 Statistics

Given the absence of data available on effect size no attempt was made at power calculations.
The data showing the prevalence of intramucosal colonies across the 3 groups were analyzed using the Kruskal-Wallis Test.

Data correlating the identification of *S. aureus* on the mucosal surface to the identification of intramucosal *S. aureus* were formulated into a contingency table and assessed using the Fisher’s exact test.

The data sets generated for the immune cell IHC failed the D’Agostino and Pearson omnibus normality test and so a one-tailed Wilcoxon matched pairs test was applied.

A p value of <0.05 was used to determine significance. Data are presented as mean (± standard error of the mean).
4.4 Results

4.4.1 Prevalence of intramucosal colonies

A total of 28 intramucosal colonies were identified across all techniques as represented in Image 4-1. Of these 3 were in normal mucosa from non-CRS patients (2/8 patients, 25%), 18 were in CRSwNP mucosa (8/9 patients, 89%) and 7 were in CRSsNP mucosa (6/9 patients, 67%). All of those identified by light microscopy were subepithelial but using FISH 3 colonies of intraepithelial *S. aureus* were seen, 2 of which were in CRSwNP mucosa and 1 in CRSsNP mucosa. As shown in Figure 4-1, the mean number of colonies seen in CRSwNP mucosa was significantly more than the mean number seen in normal mucosa (p=0.007). There was no statistically significant difference when the mean number of colonies seen in mucosa from CRSsNP patients was compared to either normal mucosa or CRSwNP patients.

Colonies would often span adjacent sections confirming that they were not artifacts of the slide preparation or staining.
Image 4-1. High power (x100) images showing five micron sections of tissue

Image 4-1a. Sheep liver infected with *S.aureus* for use as a positive control. Fluorescence in situ hybridisation has been used with a fluorescein-labelled *S.aureus* specific probe. Note the fluorescent labeling of the bacteria but also the background fluorescence from the tissue.
Image 4-1b. Sinus mucosa from a patient with chronic rhinosinusitis with nasal polyps.

Immunohistochemistry has been used to identify CD68-positive cells and the section has been counterstained with Giemsa. Note the microcolony existing in an oedematous interstitial space. Note also that the tissue is inflamed but there is no recruitment of immune cells to the immediate region of the microcolony.
Image 4-1c. Sinus mucosa from a patient with chronic rhinosinusitis with nasal polyps. Fluorescence in situ hybridisation has been used with a fluorescein-labelled Eub338 probe. Note the bacterial microcolony present within the mucosa.
Image 4-1d. Sinus mucosa from a patient with chronic rhinosinusitis without nasal polyps. Fluorescence in situ hybridisation has been used with a fluorescein-labelled *S. aureus* specific probe. Note the intraepithelial colony of *S. aureus*
Image 4-1e. Sinus mucosa from a patient with chronic rhinosinusitis without nasal polyps. Fluorescence in situ hybridisation has been used with a fluorescein-labelled $S. aureus$ specific probe. Note the subepithelial colony of $S. aureus$
Mean number of intramucosal microcolonies identified per patient

Figure 4-1. Chart illustrating the mean number of intramucosal microcolonies identified per patient divided up by disease subgroup. * signifies p<0.05

4.4.2 Species data

The results for the species of bacteria that were positively identified either within the mucosa by FISH or on the surface of the mucosa by FISH or bacterial culture is represented in Table 4-2. There was no *S.pneumoniae* identified and intramucosal *H.influenzae* was identified in only 1 CRS case and 1 non-CRS case. *S.aureus* was found within the mucosa in 6/9 (67%) of CRSwNP patients and 4/9 (44%) of CRSsNP patients compared to 1/8 (13%) of patients with normal mucosa.
<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Diagnosis</th>
<th>Intramucosal microcolony (all techniques)</th>
<th>Intraepithelial organism (FISH)</th>
<th>Surface (FISH)</th>
<th>Surface (MC&amp;S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CRSwNP</td>
<td>+</td>
<td>SA</td>
<td>SA</td>
<td>EC</td>
</tr>
<tr>
<td>2</td>
<td>CRSwNP</td>
<td>+</td>
<td></td>
<td>SA</td>
<td>HI</td>
</tr>
<tr>
<td>3</td>
<td>CRSwNP</td>
<td>+</td>
<td></td>
<td>SA</td>
<td>SA</td>
</tr>
<tr>
<td>4</td>
<td>Normal</td>
<td></td>
<td></td>
<td>SA</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Normal</td>
<td></td>
<td></td>
<td>SA</td>
<td>HI</td>
</tr>
<tr>
<td>6</td>
<td>CRSwNP</td>
<td></td>
<td></td>
<td>SA</td>
<td>SA</td>
</tr>
<tr>
<td>7</td>
<td>CRSwNP</td>
<td></td>
<td></td>
<td>SA</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>CRSwNP</td>
<td></td>
<td>SA, HI</td>
<td>SA</td>
<td>SA</td>
</tr>
<tr>
<td>9</td>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>CRSsNP</td>
<td>+</td>
<td></td>
<td>SA</td>
<td>EC</td>
</tr>
<tr>
<td>11</td>
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<td>+</td>
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</tr>
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<td></td>
</tr>
<tr>
<td>13</td>
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<td>+</td>
<td></td>
<td>SA</td>
<td>HI</td>
</tr>
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<td></td>
</tr>
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<tr>
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<td>SA</td>
<td>HI</td>
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</tr>
<tr>
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<td>SA</td>
<td></td>
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<tr>
<td>19</td>
<td>CRSsNP</td>
<td></td>
<td></td>
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<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>+</td>
<td></td>
<td>SA</td>
<td>SA</td>
</tr>
<tr>
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<td>CRSsNP</td>
<td>+</td>
<td></td>
<td>SA</td>
<td>SA</td>
</tr>
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</tr>
<tr>
<td>26</td>
<td>Normal</td>
<td>+</td>
<td></td>
<td></td>
<td>HI</td>
</tr>
</tbody>
</table>

Table 4-2. Organisms for which bacterial species were positively identified broken down by patient, location and technique. + = Positive; CRSwNP = chronic rhinosinusitis with nasal polyps; CRSsNP = chronic rhinosinusitis without nasal polyps; SA = *Staphylococcus aureus*; HI = *Haemophilus influenzae*; EC = *Escherichia coli*; FISH = Fluorescence in situ hybridization; MC&S = Microscopy culture and sensitivity
Combining data for all patients 11/28 had *S.aureus* within the mucosa and 12/28 had *S.aureus* on the surface of the mucosa. However only five patients were common to both groups with no correlation seen between the surface and intramucosal flora (p=1.00).

**4.4.3 Intramucosal colonies and the inflammatory response**

All intramucosal colonies identified by light microscopy were in CRS cases and so no evaluation of the effect of intramucosal bacteria on immune cells in normal mucosa was possible. Although the mucosa from CRS patients contained significant inflammatory infiltrates it was clear that the microcolonies were not microabscesses, having no walled off aggregate of inflammatory cells around them (Image 4-1b). This finding was borne out by the semi-quantitative IHC data with no significant recruitment of B cells (p=0.1), macrophages (p=0.4) or neutrophils (p=0.6). Remarkably there were fewer T lymphocytes (p=0.03) and fewer eosinophils (p=0.03) around the microcolonies compared to the control counts (Figure 4-2).
Figure 4-2. Chart illustrating the number of immune cells in the region of and away from intramucosal microcolonies. * signifies p<0.05

Although our protocol did not closely define the relationship of these colonies to the host cell membranes it was clear on light microscopy that the subepithelial colonies that were identified existed in the extracellular, interstitial space (see Image 4-1b).
4.5 Discussion

The inflammation seen in infection arises from a complex interplay between bacteria and the host immune response. *S.aureus* particularly possesses an array of virulence mechanisms well defined *in vitro* that provides it with an impressive ability to both modulate and evade human immunity (Langley, Wines et al. 2005; Anwar, Prince et al. 2009; Sendi and Proctor 2009; Taylor and Llewelyn 2010). In this study the intramucosal microcolonies identified did not appear to attract myeloid or lymphoid cells. T lymphocyte and eosinophil numbers around microcolonies were in fact reduced compared to non-colonized areas of tissue, raising the possibility that this is an active process instigated by these bacterial microcolonies.

Although there was no quantification of colony size in this study one notable feature is that all the colonies were small and relatively uniform in size. The concept of quorum sensing, whereby bacteria communicate with one another to regulate the size of and promote survival of that colony is an accepted part of biofilm physiology (Atkinson and Williams 2009). We propose that in the same way bacteria sense their intramucosal niche and actively regulate and limit their colony size to avoid immune detection while actively suppressing immunity. These microcolonies may provide reservoirs of organisms evading immune detection, in part explaining the recalcitrance of CRS and the recurrence of infections even after apparently appropriate treatment (Jervis-Bardy, Foreman et al. 2009). There is increasing recognition of a locally dysfunctional immune response in CRS (Schleimer, Kato et al. 2009). We hypothesize that overgrowth of intramucosal bacteria, actively secreting virulence factors and modulating host immunity may initiate this local immune dysfunction in CRS (El Fiky, Khamis et al. 2009; Wang, Kwon et al. 2010).
The methods employed in this study have inherent limitations. IHC is a semi-quantitative technique and given the protocol employed to quantify immune cell numbers on adjacent sections the area considered to be surrounding a microcolony may have been up to 5 tissue sections (25 microns) away. It is technically difficult to determine the true prevalence of intramucosal microcolonies in sinus mucosa. We do not regard the methodological approach used in this study and by others of examining tissue sections cut from biopsy samples to be highly sensitive because only a strictly limited amount of mucosa is assessed. The prevalence of intramucosal microcolonies both in health and disease may well be higher than has been reported here and elsewhere (Plouin-Gaudon, Clement et al. 2006; Niederfuhr, Kirsche et al. 2008; Corriiveau, Zhang et al. 2009; Sachse, Becker et al. 2010). There are other limitations to the prevalence data, particularly with regard to the comparison between subgroups, as the area of tissue sections prepared was not controlled. However our finding of a greater bacterial load in CRSwNP mucosa than normal mucosa is consistent with previous studies (Corriiveau, Zhang et al. 2009; Sachse, Becker et al. 2010).

The presence of intramucosal bacteria in normal mucosa suggests an alternative explanation for the findings of this study. It may be that non-immunogenic, small colonies of intramucosal bacteria represent an extension of the normal host-pathogen interaction. Rates of nasal colonization may therefore be better determined by assessing whole tissue samples rather than simply swabbing the mucosal surface (Sachse, Becker et al. 2010). In CRS, intramucosal colonies may be released from their normal growth restraints as a consequence of rather than as a cause of local dysfunction in mucosal immunity.

*S. aureus* was the most frequently found intramucosal pathogen in our study population. However the presence of *S. aureus* on the mucosal surface was not
predictive of the presence of *S.aureus* within the tissue. This observation is consistent observations of the transit of allergens, which may be inhibited by mucosal inflammation (Hens, Bobic et al. 2007). It may be that bacteria from the surface biofilm may not pass easily through a disrupted epithelial layer into the mucosa.

In summary *S.aureus* was found to be prevalent within the mucosa of the paranasal sinuses, but intramucosal colonies of *H.influenzae* were also observed. Evaluation of the surface bacteria was not seen to be predictive of the bacteria within the tissue. This observation may have important implications for CRS treatments based on sampling and eradicating surface bacteria. The colonies of bacteria seen in the tissue did not appear to stimulate a local immune reaction, and may have been actively suppressing local immunity.
Chapter 5. Bacterial microcolonies exist within the sphenoid bone in chronic rhinosinusitis and healthy controls

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5.1 Abstract

5.1.1 Background

Some patients with chronic rhinosinusitis (CRS) exhibit thickening of the sinus bones that has been termed osteitis. The histopathology and microbiology of these changes have not been fully described. The aim of this study was to look for the presence of bacteria and immune cells within samples of bone from patients with and without CRS and correlate these findings to radiological findings.

5.1.2 Methods

Bone of the anterior face of the sphenoid was examined radiologically and histologically in 8 patients with CRS with nasal polyposis, 8 patients with CRS without polyposis and 6 control patients with pituitary adenomas and normal sinuses. Bone thickness and density were measured by CT scanning. Bone samples were collected intra-operatively and 20 tissue sections were analysed for each patient. Bacteria were identified by Giemsa and Gram stains. Immune cells were identified by conventional histology and immunohistochemistry.

5.1.3 Results

Small colonies of bacteria were identified within the bone in 3/16 CRS patients and 2/6 control subjects (p=0.6). Isolated immune cells were identified within the bone in 3/16 CRS patients and 2/6 control subjects (p=0.6) but in only 1 case did both bacteria
and immune cells occur together. The presence of bacteria or immune cells within bone samples did not correlate with either bone thickness or bone density.

5.1.4 Conclusion

This study describes the presence of bacteria and immune cells within a minority of CRS patients and normal controls. The bacterial microcolonies identified do not appear to be the cause of the bone changes seen in many CRS patients.
5.2 Introduction

Abnormalities of the bones of the paranasal sinuses observed radiologically in some patients with chronic rhinosinusitis (CRS) has been labeled “osteitis”, a term that implies the presence of inflammation. The histology and microbiology of this bony reaction have not however been fully described, and its pathogenesis is incompletely understood (Videler, Georgalas et al. 2011). There are no agreed criteria for defining these changes. It has been proposed that radiologically, bone must be seen to be more than 3mm in thickness (Lee, Kennedy et al. 2006) but a recently published grading scale from another group includes abnormal bone of less than 3mm thickness within both “Grade 1 osteitis” and “Grade 3 ostieitis” (Georgalas, Videler et al. 2010).

Abnormalities of the bones of the paranasal sinuses have been observed in up to 88% of selected CRS patients (Lee, Kennedy et al. 2006; Georgalas, Videler et al. 2010; Telmesani and Al-Shawarby 2010). However, as the majority of published series reporting the prevalence of bony reaction in CRS are derived from tertiary rhinology clinics the prevalence of this observation in CRS patients across the general population is not known.

In a series of studies performed using a rabbit model of sinusitis, persistent and extensive ipsilateral and contralateral bone changes were seen following experimentally induced sinus infections (Bolger, Leonard et al. 1997; Perloff, Gannon et al. 2000; Khalid, Hunt et al. 2002). Despite the inherent limitations of an animal model with acute infection, these studies have provided the basis for a presumed infective cause of the bony changes in CRS.

The clinical implications of these bony changes remain unclear. Their presence has been associated with worse clinical indices of CRS severity and a worse outcome.
from surgical treatment (Park, Kim et al. 2005; Kim, Dhong et al. 2006; Bhandarkar, Mace et al. 2011). Bone thickening has been associated with previous surgery, and some authors have speculated that debridement of abnormal bone would improve treatment outcome (Biedlingmaier, Whelan et al. 1996; Kennedy, Senior et al. 1998).

It is likely that the bone changes described in CRS represent an increase in the turnover of the bone as there is evidence of fibrosis, bone resorption and new bone formation. Some of these histological features parallel the better described process of long bone osteomyelitis but evidence of active inflammation and direct infection of the bone in CRS is limited (Kennedy, Senior et al. 1998). Unfortunately the situation is further complicated by historical reports showing bone infection (Tovi, Benharroch et al. 1992) in patients who would most likely be defined as acute or recurrent acute rhinosinusitis rather than CRS by modern criteria (Meltzer, Hamilos et al. 2006; Fokkens, Lund et al. 2007). Recognizing the uncertainty surrounding what pathological process the bone changes represent, various authors have used other terminology such as hyperostosis (Kim, Dhong et al. 2006; Georgalas, Videler et al. 2010), neo-osteogenesis (Kennedy, Senior et al. 1998), new bone formation (Cho, Kim et al. 2007) and bone remodeling (Park, Park et al. 2007) to describe the bone changes seen in CRS.

We have recently described the presence of bacteria existing both on the surface of and within sinus mucosa, and using immunohistochemistry (IHC) have correlated the presence of bacteria to the mucosal inflammatory response (Wood, Fraser et al. 2011). The purpose of this study was to see if bacteria and immune cells could be identified histologically within samples of bone from the paranasal sinuses of patients both with and without CRS and to correlate their presence to indices of bone changes and disease.
5.3 Materials and Methods

The study was approved by the New Zealand Northern Regional Ethics Committee and written permission provided by the hospitals involved. Written informed consent was obtained from all patients at the time of recruitment.

5.3.1 Patients

A total of 22 adult patients undergoing endoscopic sinus surgery in the tertiary practice of a single surgeon (RGD) were prospectively recruited between August 2010 and April 2011. Of these 16 were undergoing surgical treatment of CRS having failed a course of medical therapy (Wood and Douglas 2010). CRS patients were diagnosed and categorized into the subgroups of CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP) in line with international guidelines (Fokkens, Lund et al. 2007). The remaining 6 patients were enrolled as control subjects and were undergoing surgery using a transsphenoidal approach to a non-functioning pituitary adenoma with no evidence of CRS on clinical history, computed tomography (CT) scan or endoscopy. Patients were excluded from either group if they had undergone a course of systemic antibiotics or corticosteroids in the 4 weeks prior to surgery. Low dose hydrocortisone replacement therapy was permitted when clinically indicated in pituitary patients. Demographic and clinical data including Lund-Mackay scores (Lund and Mackay 1993) and relevant medical and surgical history were collected for all patients. The sphenoid sinus specific Lund-Mackay (marked out of 4) was recorded as well as the total Lund-Mackay score (marked out of 24).
5.3.2 Radiologic assessment of bone

The CT scan images for all patients were collected and analyzed using WEB1000 software (Agfa HealthCare, Mortsel, Belgium). For the purposes of this study the axial images were reviewed and 5 separate points on the anterior wall of the sphenoid bone were randomly selected by a single author (AJW) away from the sphenoid rostrum and inter-sinus septum. These were then marked manually and at each point the bone density was measured in Hounsfield units (HU) and the bone thickness in millimeters (mm) by the software. The mean bone density and bone thickness of the front wall of the sphenoid bone was then calculated for each patient.

5.3.3 Sample collection and analysis

At the time of patient recruitment all patients were allocated a reference number that was used to identify specimens allowing samples to be analyzed in a blinded manner. Samples of bone from the anterior wall of the sphenoid were removed using a 4mm Hajek-Koffler punch and any loosely attached mucosa was removed. Samples were then immediately placed in Carnoy’s fixative for 24 to 72 hours. Bone samples were then decalcified for 8 to 16 hours in 10% formic acid before undergoing routine histology processing and embedding in paraffin. Five micron tissue sections were cut and mounted on Superfrost® Plus Positively Charged Microscope slides (Thermo Fisher Scientific New Zealand Ltd, Auckland, New Zealand).

Tissue sections were handled in groups of five serial sections. The first tissue section was mounted on one glass slide and the remaining four sections on a second glass slide. The first section underwent a routine Gram stain and was counterstained with safranin. The four sections on the second slide were stained by hand using immunohistochemistry (IHC). The NovoLink™ Polymer Detection System (Leica Microsystems, Wetzlar, Germany) was used and recommended protocols were
followed. Murine monoclonal antibodies were used with specificity for the following
antigens: CD3 (T lymphocytes), CD20 (B lymphocytes), CD68 (macrophages). These
were applied in turn to the first 3 sections on the slide and the fourth section
underwent the same protocol but with phosphate buffered saline used in place of a
primary antibody as a negative control. The IHC slide was then counterstained with
Giemsa. This protocol allowed bacteria and neutrophils to be identified on any of the
tissue sections, eosinophils to be identified on any of the IHC sections with the
Giemsa counterstain and the other immune cells on their specific IHC sections. For
each patient four pairs of slides were prepared and examined.

As a positive control for the bacterial stains, a small piece of bovine vertebral bone
was incubated in a pure culture of *Staphylococcus aureus* for 24 hours and then
processed and stained. Slides mounted with 4 sections of human spleen served as
positive controls for the IHC staining.

Slides were examined using a Leica DMR upright microscope and photographed with
a Nikon Digital Sight cooled camera (Nikon Corporation, Tokyo, Japan) using
EclipseNet software (Nikon Corporation, Tokyo, Japan). All microscopy was
undertaken by a single author (AJW)

**5.3.4 Statistics**

Data sets were compared using a two-tailed Mann Whitney test. The presence or
absence of both immune cells and bacteria within the sections of bone were
formulated into a contingency table and assessed using the Fisher’s exact test. All
data were analyzed using Prism software (Prism Software Corp., Irvine, CA).

A p value of <0.05 was used to determine statistical significance. Data are presented
as mean (± standard error of the mean).
5.4 Results

A summary of the clinical and demographic data is shown in Table 5-1. No patients had aspirin exacerbated respiratory disease or allergic fungal sinusitis. Both the CRSwNP group (0.66mm ± 0.05, p=0.01) and the CRSsNP group (0.61mm ± 0.03, p=0.002) exhibited thicker bone than the control patients (0.44mm ± 0.02). However in neither of the CRS subgroups was the bone significantly denser than in the control patients. Only one CRS patient (from the CRSwNP group) had no radiological evidence of mucosal thickening within the sphenoid sinuses.

<table>
<thead>
<tr>
<th></th>
<th>Normal (n=6)</th>
<th>CRSsNP (n=8)</th>
<th>CRSwNP (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>53 ± 8</td>
<td>42 ± 7</td>
<td>41 ± 3</td>
</tr>
<tr>
<td>Sex (M:F)</td>
<td>2:4</td>
<td>2:6</td>
<td>7:1</td>
</tr>
<tr>
<td>Comorbidities</td>
<td></td>
<td>1 asthma,</td>
<td>3 asthma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 cystic fibrosis</td>
<td></td>
</tr>
<tr>
<td>Revision surgery</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Lund-Mackay score</td>
<td>0</td>
<td>13.9 ± 1.2</td>
<td>16.3 ± 1.7</td>
</tr>
<tr>
<td>Sphenoid specific Lund-Mackay score /4</td>
<td>0</td>
<td>2.25 ± 0.25</td>
<td>2.375 ± 0.5</td>
</tr>
<tr>
<td>Bone thickness (mm)</td>
<td>0.44 ± 0.02</td>
<td>0.61 ± 0.03</td>
<td>0.66 ± 0.05</td>
</tr>
<tr>
<td>Bone density (HU)</td>
<td>485 ± 43</td>
<td>662 ± 73</td>
<td>568 ± 42</td>
</tr>
</tbody>
</table>

Table 5-1. Clinical and demographic data

5.4.1 Prevalence of bacteria and immune cells

In the CRS cases any mucosa left on the surface overlying undisrupted bone was markedly inflamed (Image 5-1). However, within the underlying bone, only isolated immune cells were identified in 3/16 of the CRS cases and 2/6 of the control samples.
(p=0.6) (Figure 5-1). Isolated T lymphocytes were found within the bone of two CRSwNP patients and a single neutrophil in one CRSsNP patient. One of the control subjects had a T lymphocyte and a neutrophil within the bone sample and one other control sample contained a macrophage and a B cell.

Bacterial microcolonies were identified within the bone sections (Image 5-1) in 3/16 of the CRS cases (1 CRSsNP and 2 CRSwNP cases) and 2/6 of the control cases (p=0.6) (Figure 5-1). The bacteria that were identified within the bone samples were all cocci and the three from CRS cases were identified on Gram stained sections on which they were found to be Gram positive. None of the colonies identified in control samples extended onto Gram stained sections.
Image 5-1. Photomicrographs of 5 micrometer sections of bone collected from the anterior wall of the sphenoid sinus. (a) Low power (x25) view of a tissue section from a patient with CRSwNP. This has been stained using IHC with an anti-CD68 primary antibody and counterstained with Giemsa. Note the macrophages present within the mucosa (arrows) but not the underlying bone. (b) High power view (x100) of a sample of normal bone stained using IHC with an anti-CD3 primary antibody and counterstained with Giemsa. Note the intraosseous bacterial microcolony (arrow). (c) High power view (x100) of a sample of bone from a patient with CRSsNP. This has been stained with a Gram stain and counterstained with safranin. Note the intraosseous neutrophil (arrow).
Figure 5-1. Frequency of (a) intraosseous immune cells and (b) intraosseous bacteria by subgroup

Mean number of intraosseous immune cells identified per patient

n.s.

Figure 5-1a
In only one patient, a control subject, was there both a bacterial colony and immune cells identified within the bone (Table 5-2).

<table>
<thead>
<tr>
<th></th>
<th>Bacteria present</th>
<th>Bacteria not found</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>present</td>
<td>1</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Immune cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>not found</td>
<td>4</td>
<td>13</td>
<td>17</td>
</tr>
<tr>
<td>Total</td>
<td>5</td>
<td>17</td>
<td>22</td>
</tr>
</tbody>
</table>

Table 5-2. Contingency table showing presence or absence of immune cells and bacteria (p=1.00)
In two cases, the microcolonies were sufficiently large to span more than one tissue section and so were clearly not artefacts of slide preparation or staining.

5.4.2 Correlation of radiological and histological data

There was no correlation between the presence of intraosseous immune cells and either bone thickness or density (Figure 5-2). The same was true of those patients in whom intraosseous bacterial microcolonies were found (Figure 5-3). There was no significant correlation between either the total Lund-Mackay score or the sphenoid sinus specific Lund-Mackay score and the presence or absence of either intraosseous immune cells or intraosseous bacterial microcolonies.
Figure 5-2. Comparison of those subjects with and without intraosseous immune cells (a) bone density (b) bone thickness

Density of front face of sphenoid bone from subjects with and without intraosseous immune cells

p=0.34

n=5  n=17

Figure 5-2a
Thickness of front face of sphenoid bone from subjects with and without intraosseous immune cells

\[ p = 0.81 \]

- Immune cells present, \( n=5 \)
- Immune cells absent, \( n=17 \)

Figure 5-2b
Figure 5-3. Comparison of those subjects with and without intraosseous bacteria (a) bone density (b) bone thickness

Density of front face of sphenoid bone from subjects with and without intraosseous bacteria

![Graph showing bone density comparison](image)

- **P-value:** p=0.53
- **Sample sizes:** n=5 (Intraosseous bacteria), n=17 (No intraosseous bacteria)
Figure 5-3b

Thickness of front face of sphenoid bone from subjects with and without intraosseous bacteria

Bone thickness / mm

<table>
<thead>
<tr>
<th></th>
<th>n=5</th>
<th>n=17</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intraosseous</td>
<td>0.4</td>
<td>0.6</td>
</tr>
<tr>
<td>bacteria</td>
<td>(0.3-0.5)</td>
<td>(0.5-0.7)</td>
</tr>
<tr>
<td>No intraosseous</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>bacteria</td>
<td>(0.05-0.15)</td>
<td>(0.25-0.4)</td>
</tr>
</tbody>
</table>

p=1.00
5.5 Discussion

In this study bacterial microcolonies and isolated immune cells have been identified within samples of sphenoid bone in a minority of CRS cases. This study compares samples of normal bone removed from the same anatomical site as bone specimens harvested from CRS patients. Of note, immune cells and bacterial microcolonies were observed in control bone samples with the same prevalence as in CRS samples. The presence of bacteria or immune cells was not associated with increased bone thickness or density or the extent of mucosal disease evident radiologically.

There are limitations to this study. The technique of using histology to detect bacteria is time consuming and consequently the volume of tissue and number of patients that can reasonably be assessed is limited. Accordingly, the prevalence data presented in this study may be significantly underestimating the prevalence of intraosseous microcolonies. In this study Gram staining was used but not fluorescence in situ hybridization (FISH) techniques that could identify the species of bacteria from samples of tissue (Foreman, Psaltis et al. 2009; Feazel, Frank et al. 2011). However, the culture and FISH results of our previous studies on mucosa and those of others suggest the strong likelihood that the observed Gram positive cocci are *Staphylococcus aureus* (Corriveau, Zhang et al. 2009; Sachse, Becker et al. 2010).

For this study we chose to focus on a reproducible site within the sinuses, (the anterior wall of the sphenoid bone) rather than directing collection specifically towards areas of abnormality detected on the pre-operative imaging. Unfortunately this approach did not yield any bone thicker than 3mm which some authors consider to be the cut-off for “osteitis” (Lee, Kennedy et al. 2006). It could be debated therefore how much can be inferred as to the cause of the bone abnormalities that are
seen in a proportion of CRS cases. It is interesting to note however that in this study the bone was significantly thicker in the CRS subgroups implying that, consistent with a recently published study, bone may be abnormal even when less markedly thickened (Georgalas, Videler et al. 2010).

There are a number of ways in which this study could be extended. Sampling could be limited to patients and sites with radiologically abnormal bone. Both the radiological assessment and the assessment of inflammation could be enhanced by incorporating techniques such as the assay of a broad range of inflammatory mediators or the use of more sophisticated radiological analysis (Genant, Engelke et al. 2008).

Given the equal prevalence of intraosseous immune cells and bacterial microcolonies and the lack of correlation between these observations and bone thickening these data however do not support the hypothesis that bone changes in CRS relate to infiltration of immune cells or bacteria. An alternative explanation consistent with our results is that the bone changes may be a manifestation of a generalized remodelling of upper airway tissue seen in CRS, which may be a result of the action of cytokines released by the inflamed mucosa overlying the bone (Daines, Wang et al. 2011; Van Bruaene and Bachert 2011).

Whether the microcolonies have a role in the recalcitrance of CRS to treatment is a matter for conjecture. We have recently observed microcolonies within the mucosa of CRS patients that do not appear to provoke an inflammatory response. Similarly, the presence of bacterial microcolonies within the bone did not appear to elicit an infiltration of immune cells. It is possible that these colonies may have a phenotype that is not dissimilar to small colony variants, and as such have a high resistance to some antibiotics (Tuchscherr, Heitmann et al. 2010). It remains unclear however
whether these colonies that we have observed, in normal bone as well as bone from
CRS patients, contribute in some way to the pathogenesis of CRS or to the bone
changes that a subset of patients exhibit.
5.6 Conclusion

Microcolonies of bacteria and isolated immune cells are present within the sphenoid bone in both health and disease. In this study there was no correlation between these findings and indices of CRS.
Chapter 6. The importance of fixation and controls in immunohistochemistry

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We share with others an interest in the production of virulence factors by *Staphylococcus aureus* in chronic rhinosinusitis (CRS) (Bachert, Zhang et al. 2008). We therefore read with interest the paper by Kim et al (Kim, Chung et al. 2011). The study reported the detection using immunohistochemistry (IHC) of Staphylococcus Enterotoxin A and Toxic Shock Syndrome Toxin 1 in frozen sections of mucosa from a proportion of nasal polyposis patients. The published image of positive staining showed a diffuse homogeneous staining across the tissue section with relative sparing of the epithelial layer. Negative control sections in which either the primary antibody step was omitted altogether or replaced with a control antibody (Bussolati and Leonardo 2008; Schlosser, Mulligan et al. 2010) were not presented.

We have also employed similar techniques on frozen sections of sinus mucosa, applying affinity purified rabbit primary antibodies with specificity for staphylococcal virulence factors as well as control rabbit antibodies raised to irrelevant protein antigens. Our sections were initially fixed by dipping the slides in specially denatured alcohol once all the slides had been prepared. In so doing we identified a pattern of dense staining essentially identical to that reported by Kim et al, predominantly in sinus mucosa from patients with nasal polyposis. However this was observed with both test and control antibodies (see Figure 6-1). When a polymer-based secondary antibody detection method was used to amplify staining (Leica Microsystems,
Then a similar but less dense pattern of staining was observed even when the primary antibody was replaced with phosphate buffered saline (PBS). This confirmed that the observed dense staining was non-specific.

Figure 6. High power (x100) photomicrographs of frozen sections of sinus mucosa from a single patient with nasal polyposis. Immunohistochemistry has been used with 3,3′-Diaminobenzidine (DAB) as the chromagen resulting in positive staining being brown. Sections have been counterstained with haematoxylin. (a) anti-streptococcal mitogenic exotoxin z rabbit polyclonal primary antibody and (b) anti-staphylococcal superantigen-like protein 10 rabbit polyclonal primary antibody. Note the intense homogeneous staining largely limited to the subepithelial, extracellular compartment.
Notably, the cellular epithelial layer was largely spared from this artifactual staining and it was also much less pronounced in sections of normal sinus mucosa collected from patients with non-functioning pituitary adenomas without CRS. Given the ability of collagen to non-specifically bind antibodies (Bussolati and Leonardo 2008) we believe that this effect is caused by the non-specific binding of antibodies to the collagen and possibly other interstitial matrix proteins that are in dense abundance in nasal polyposis.

We were puzzled by how we had not identified this effect during previous studies using IHC performed on paraffin embedded sinus mucosa fixed with Carnoy’s fixative (Wood, Fraser et al. 2011). We therefore followed the same IHC protocol in parallel on tissue samples from the same patients fixed using these two different methods and confirmed that this effect was a simple product of the fixation technique (see Figure 2). As we wished to pursue our investigation of staphylococcal virulence...
factors we experimented with different ways of fixing frozen sections and found that this artifactual staining could be substantially reduced simply by fixing sections immediately in absolute analytical grade ethanol for 5 minutes and then washing the sections in PBS prior to performing IHC. This did not affect the successful staining of positive controls.

Figure 6-2. High power (x100) photomicrographs of sections of sinus mucosa from the same nasal polyposis patient as Figure 6-1. This tissue sample was fixed in Carnoy’s fixative and embedded in paraffin but then the same staining and counterstaining protocol was used as in Figure 6-1. (a) anti-streptococcal mitogenic exotoxin z rabbit polyclonal primary antibody and (b) anti-staphylococcal superantigen-like protein 10 rabbit polyclonal primary antibody. Note the absence of artifact.
Our findings highlight the critical importance both of fixation in IHC and more broadly of including appropriate negative controls to validate results.
Chapter 7. Are staphylococcal superantigen-like proteins produced in the mucosa of the paranasal sinuses?

7.1 Abstract

7.1.1 Introduction
There is increasing recognition of local immune dysfunction within the sinus mucosa of patients with chronic rhinosinusitis (CRS). We have recently described the presence of small intramucosal colonies of bacteria in CRS patients that appear capable of evading host immunity. *Staphylococcus aureus* was found to be the predominant bacterial species. *S. aureus* is capable of producing a family of proteins termed staphylococcal superantigen-like proteins (SSLs) that are capable of disrupting human immunity. The aim of this study was to determine whether SSLs could be detected in sinus mucosa.

7.1.2 Methods
Adult patients with CRS undergoing surgical treatment and patients without CRS undergoing a trans-sphenoidal approach to non-functioning pituitary adenomas were prospectively recruited and intraoperative mucosal samples were collected. Immunohistochemistry was used for the detection of SSLs and Gram staining for identification of Gram positive cocci.

7.1.3 Results
Colonies of intramucosal Gram positive organisms were found in 6/14 CRS patients and in 1 out of 9 samples of normal mucosa. The presence of SSL 1,10 or 11 was not detected.
7.1.4 Conclusion

A role for SSLs in either persistence of *S. aureus* within sinus mucosa or in active induction of local immune dysfunction in CRS remains unproven. This may be because they are not present or because this technique was not sensitive enough to detect them.
7.2 Introduction

A central role for *Staphylococcus aureus* in the pathogenesis of chronic rhinosinusitis (CRS) has been suggested by a number of authors (Ramadan 1995). The staphylococcal superantigens, a family of proteins capable of potent stimulation of T cell mediated inflammation, have generated particular interest (Stow, Douglas et al. 2010). In the recent past there has been recognition however that CRS is characterized not only by excessive inflammation but also by immune dysfunction, as deficits in mucosal immunity have been described (Psaltis, Bruhn et al. 2007; Ramanathan, Spannhake et al. 2007).

We have recently identified small colonies of bacteria within sinus mucosa, predominantly *S.aureus*, which are more prevalent in patients with nasal polyps (CRSwNP) (Wood, Fraser et al. 2012). These exist in an immune tolerant environment and as there is some evidence that the bacteria may actively induce immunoparesis we speculated that bacteria may induce local immune dysfunction in CRS. *S.aureus* possesses a multitude of potential mechanisms that can induce suppression of mucosal immunity (Chavakis, Preissner et al. 2007).

The staphylococcal superantigen-like proteins (SSLs), formerly known as the staphylococcal enterotoxin-like or exotoxin-like (SET) proteins (Lina, Bohach et al. 2004) are one such mechanism. The SSLs are a family of 11 virulence factors produced by *S. aureus* that were first identified in 2000 (Williams, Ward et al. 2000). In an early study of these proteins the production of SET3 was noted in all *S.aureus* strains tested and seroconversion was identified in the serum of all 15 normal human subjects tested implying that they had all had prior exposure to the antigen (Arcus, Langley et al. 2002). These findings imply a major role in host-pathogen interactions.
and over the last decade the actions of these highly conserved proteins (Smyth, Meaney et al. 2007) have been investigated, revealing functions that are clearly distinct from the staphylococcal superantigens with which they share significant structural homology (Al-Shangiti, Naylor et al. 2004).

SSLs have been investigated to varying extents. Initial investigation of SSL7 and SSL9 showed that they are capable of binding monocytes (Al-Shangiti, Naylor et al. 2004). Further studies have shown that across the group of SSLs the capability exists to impair the function of the complement pathway (Bestebroer, Aerts et al. 2010), IgA (Wines, Ramsland et al. 2011), IgG (Patel, Wines et al. 2010), neutrophils (Bestebroer, Poppelier et al. 2007) and matrix metalloproteinase 9 (Itoh, Hamada et al. 2010).

Staphylococcal superantigens have proven to be difficult to detect in sinus mucosa and much of the research in this field has involved the quantification of antibodies to the superantigens (Bachert, Zhang et al. 2010) or enterotoxin DNA (Heymans, Fischer et al. 2010) rather the antigens themselves. The limited number of studies in which the superantigens themselves have been assayed have suggested that they are present in low concentrations and have typically been reported as detectable or not detectable (Seiberling, Conley et al. 2005; Wang, Shi et al. 2011). Given that it is possible however to identify colonies of *S. aureus* in tissue sections we wondered whether there may be a concentration gradient of staphylococcal virulence proteins with higher concentrations around these colonies allowing the antigens to be detected *in situ* in tissue sections.
For this study SSL 1, 10 and 11 were chosen as targets. This is because they are known, under the right conditions, to be produced in large quantities and exhibit limited allelic variation (Patel, Wines et al. 2010; Benson, Lilo et al. 2011).

The aims of this study were therefore two-fold: to see if evidence of SSL expression could be identified in sinonasal mucosa around *S.aureus* microcolonies and if so whether there was any relationship between the presence of SSLs and the inflammatory response.
7.3 Methods

The study was approved by the New Zealand Northern X Regional Ethics Committee and written informed consent was obtained from all participants.

7.3.1 Patients and samples

Twenty-three adult patients undergoing endoscopic sinus surgery (ESS) in the tertiary practice of a single surgeon (Dr. Richard Douglas) were prospectively recruited. Fourteen had been diagnosed with CRS and classified in accordance with published guidelines (Fokkens, Lund et al. 2007) and were undergoing ESS after failing a course of medical therapy (Wood and Douglas 2010). The remaining nine patients were undergoing a trans-sphenoidal approach to non-functioning pituitary adenomas. The pituitary adenoma patients were excluded if they had a history consistent with CRS or evidence of mucosal disease on either endoscopy or pre-operative imaging. All patients who had taken a therapeutic course of corticosteroids in the previous 4 weeks were excluded although low-dose hydrocortisone as replacement therapy was permitted in the pituitary adenoma patients. A summary of the patient demographics is shown in Table 7-1.
<table>
<thead>
<tr>
<th></th>
<th>CRSsNP (n=6)</th>
<th>CRSwNP (n=8)</th>
<th>Normal (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>44.7 ± 6.4</td>
<td>42.4 ± 4.7</td>
<td>47.4 ± 5.1</td>
</tr>
<tr>
<td>Sex</td>
<td>3F : 3M</td>
<td>2F : 6M</td>
<td>5F : 4M</td>
</tr>
<tr>
<td>Lund Mackay score</td>
<td>12.3 ± 1.6</td>
<td>15.9 ± 2.2</td>
<td>0</td>
</tr>
<tr>
<td>Revision</td>
<td>2/6</td>
<td>5/8</td>
<td>N/A</td>
</tr>
<tr>
<td>Co-morbidities</td>
<td>1 CF, 1 asthma</td>
<td>2 CF, 2 asthma</td>
<td>nil</td>
</tr>
</tbody>
</table>

Table 7-1. Patient Demographics. Age and Lund MacKay score are presented as Mean +/- Standard error of the mean. CRSsNP = chronic rhinosinusitis without nasal polyps, CRSwNP = chronic rhinosinusitis with nasal polyps, CF = cystic fibrosis.

A representative sample of mucosa was collected intra-operatively from the ethmoid or sphenoid sinuses and transported to the laboratory in an ice box wrapped in gauze soaked in phosphate buffered saline (PBS). Detection of soluble antigen is optimal in fresh frozen tissue (Norrby-Teglund, Thulin et al. 2001) and so the mucosal sample was then submerged in Optimum cutting temperature (OCT) cryomatrix (Medica Pacifica Ltd, Auckland, New Zealand), frozen in liquid nitrogen and stored at -20°C. Patients and mucosal samples were identified with a reference number allowing samples to be analyzed blinded to clinical details.

7.3.2 Positive controls

Solutions of SSL1, SSL10 and SSL11 (courtesy of Prof. John Fraser) were diluted to concentrations of 100 µg/ml, 10 µg/ml, 1 µg/ml and 100 ng/ml. Cubes of fresh sheep liver approximately 3 mm in diameter were then incubated in 1ml of each of the solutions at 4°C for 24 hours. These were then frozen and processed in the same manner as the mucosal samples.
A further cube of sheep liver was incubated for 24 hours at 37°C in a pure broth culture of \textit{S.aureus}. This was then processed in the same manner as the other tissue samples and served as a positive control for the bacterial staining.

\subsection*{7.3.3 Staining}

Tissue sections of 10 \(\mu\)m thickness were cut with a cryostat and immediately mounted onto \textit{Superfrost® Plus} positively charged microscope slides (Thermo Fisher Scientific New Zealand Ltd, Auckland, New Zealand). Four serial mucosal sections were mounted per slide and four separate slides were prepared for each patient. Sections were immediately fixed in 95% absolute analytical grade ethanol and then held in PBS until all slides were cut (Wood, Fraser et al. 2012). Sections were demarcated with a Pap pen prior to staining.

The immunohistochemistry protocol was based on the usage of the \textit{NovoLink}\textsuperscript{TM} Polymer Detection System (Leica Microsystems, Wetzlar, Germany) with some modifications. All staining was undertaken at room temperature. The peroxidase block was applied for a prolonged period (15 minutes) to reduce background staining. After the protein block a further blocking step was incorporated with 10\(\mu\)g/ml of sheep IgG (Prof John Fraser) applied for 60 minutes to eliminate any non-specific IgG binding. The following primary antibodies (Prof John Faser) were then applied to the mucosal sections for 60 minutes after having been optimized in initial experiments:

- Section 1: rabbit polyclonal anti-SSL1 at 10\(\mu\)g/ml
- Section 2: rabbit polyclonal anti-SSL10 at 10\(\mu\)g/ml
- Section 3: rabbit polyclonal anti-SSL11 at 10\(\mu\)g/ml
- Section 4: rabbit purified IgG at 10\(\mu\)g/ml (negative control)
The post primary block was then applied as per the instructions but a horseradish peroxidase-linked goat anti-rabbit secondary antibody (BD Biosciences, Auckland, New Zealand) was then applied at a 1:100 dilution for 60 minutes in place of the secondary antibodies provided. The 2,4 Diaminobutyric acid (DAB) from the kit was then used to produce brown coloured positive staining. Sections then underwent a routine Gram stain and were then counterstained for 3 minutes with haematoxylin.

Sections of the positive control samples were then tested in the same fashion with the various primary antibodies to identify positive staining for SSLs.

7.3.4 Microscopy

Slides were examined using a Leica DMR upright microscope and photographed with a Nikon Digital Sight cooled colour camera (Nikon Corporation, Tokyo, Japan) using EclipseNet software (Nikon Corporation, Tokyo, Japan).

Positive control sections were reviewed for evidence of positive staining. The first three mucosal sections on each slide were reviewed for evidence of bacterial colonies or positive staining for SSLs. Any bacterial colonies identified as being intramucosal were recorded and scrutinized for local positive IHC staining. Any apparent positive IHC staining was checked against the fourth, negative control section.
7.4 Results

7.4.1 Control tissues

Staining of the liver sections pre-incubated in the SSLs revealed that it was not possible to detect SSL1 in the control tissue at any of the concentrations used (Image 7-1). SSL10 (Image 7-2) and SSL11 (Image 7-3) were detectable at 10µg/ml but not at 1µg/ml. Gram positive organisms were easily recognizable in the infected liver tissue (Image 7-4).

Image 7-1. High power (x100) photomicrograph of a 10 µm section of sheep liver which had been pre-incubated in SSL1 at 10µg/ml and stained using immunohistochemistry with an anti-SSL1 primary antibody and counterstained with a Gram stain and haematoxylin. Note the absent immunohistochemical staining.
Image 7-2. High power (x100) photomicrograph of a 10 µm section of sheep liver which had been pre-incubated in SSL10 at 10µg/ml and stained using immunohistochemistry with an anti-SSL10 primary antibody and counterstained with a Gram stain and haematoxylin. The immunohistochemical staining was clearly positive.
Image 7-3. High power (x100) photomicrograph of a 10 µm section of sheep liver which had been pre-incubated in SSL11 at 10µg/ml and stained using immunohistochemistry with an anti-SSL11 primary antibody and counterstained with a Gram stain and haematoxylin. The immunohistochemical staining was clearly positive.
Image 7-4. High power (x100) photomicrograph of a 10 µm section of sheep liver which had been pre-incubated in a broth of *S. aureus* and stained using immunohistochemistry with an anti-SSL10 primary antibody and counterstained with a Gram stain and haematoxylin. Note the easily identifiable Gram positive organisms.
7.4.2 Mucosal samples

Intramucosal colonies of Gram positive organisms were detected in samples of normal mucosa from 1/9 patients, 3/9 CRSwNP patients and 3/5 CRSsNP patients. No positive staining was detected for SSL1, SSL10 or SSL11 (Images 7-5 and 7-6).
Image 7-6. High power (x100) photomicrograph of a 10 µm section of sinus mucosa from a patient with chronic rhinosinusitis with nasal polyps that had been stained using immunohistochemistry with an anti-SSL10 primary antibody and counterstained with a Gram stain and haematoxylin. Note the intramucosal Gram positive organisms (arrows) without any positive immunohistochemical staining.
7.5 Discussion

*S.aureus* is capable of colonising healthy individuals and also of causing life-threatening sepsis. While host factors (such as the presence or absence of systemic immunosuppression) have some role in dictating the clinical outcome, alterations in bacterial gene expression also controls the behaviour of the organism with the accessory gene regulator (agr) system having a central role in controlling the virulence of *S.aureus* (Benson, Lilo et al. 2011). One group of proteins that *S.aureus* is capable of producing is the SSLs and these have been shown *in vitro* to produce a suppressive effect on human immunity (Fraser and Proft 2008).

We have recently observed colonies of *S.aureus* within sinus mucosa in an immune tolerant environment that were over-represented in diseased tissue (Wood, Fraser et al. 2012). We hypothesized that the SSL proteins may in part mediate this immune tolerance and postulated that simultaneous *in situ* detection of both the bacterial colonies and SSLs may be successful.

Unfortunately the use of IHC to detect SSLs presented technical difficulties. Artefactual staining required significant alterations to the protocol (Wood, Fraser et al. 2012). We attempted to calibrate the sensitivity of IHC in this study although the standardized positive control solutions would have been diluted to some extent by interstitial fluid. SSL1 was not detected at any point but we chose to proceed with its use as SSLs are known to bind to carbohydrates and cell surface glycoproteins (Fraser and Proft 2008) and so it could have been that the epitope was bound to liver tissue. SSL10 and 11 were only detectable at concentrations above approximately 10µg/ml. The colonies observed within the mucosa were however small and presumably therefore capable of only low concentrations of virulence factors compared to more
overt infections. Unfortunately it is not known what the tissue concentrations of SSLs are in a human host. Thus the negative result may reflect either insufficient sensitivity of the technique or that the proteins were not present. There is also the possibility that the Gram positive organisms observed were not *S.aureus* although our previous FISH data would imply that the majority were likely to have been (Wood, Fraser et al. 2012). In one case in which dense colonisation with Gram positive cocci had been observed, pyrosequencing had shown that 84% of the bacteria were *S.aureus* (data not presented). This study has not definitively answered the first hypothesis regarding whether SSLs are produced within sinus mucosa.

There is indirect evidence that SSLs are expressed in sinonasal mucosa. Serological data indicates that most, if not all, human subjects have been exposed to SSLs at some stage in their life (Arcus, Langley et al. 2002) and the nose is one of the most common sites in which *S.aureus* resides (Burian, Wolz et al. 2010). The pursuit of SSLs in sinonasal mucosa may be advanced by drawing parallels from the superantigen literature and using an immunoassay for the detection of the SSLs (Seiberling, Conley et al. 2005) or of antibodies to the SSLs (Bachert, Zhang et al. 2010). However we suspect that given the scattered colonisation by *S.aureus* that the homogenization of tissue required for these techniques would dilute the antigen concentration to below detectable. Interpretation of an immunoassay for the detection of antibodies to SSLs would be complicated by the presence of blood within the tissue samples, with blood already known to contain antibodies to the SSLs (Arcus, Langley et al. 2002).

A secondary hypothesis was that the expression of SSLs may contribute to the development of inflammatory sinus disease. Given we could detect no SSL in the tissue specimens examined, this remains highly speculative.
In summary, this study has attempted to test hypotheses regarding the production of SSLs by *S. aureus* in sinus mucosa and what role they might play. It has however served to reinforce the notion that the study of the role of bacterial virulence proteins in human disease is difficult with the currently available laboratory techniques.
Chapter 8. Conclusion

8.1 Major findings

8.1.1 Immune evasion

The most significant novel finding of this thesis is the presence of microcolonies of bacteria within the mucosa and the bones of the paranasal sinuses which fail to incite an inflammatory response. A defect in local immunity would conceivably reduce the inflammatory response. However, these microcolonies were surrounded by fewer immune cells than areas of mucosa without bacteria, suggesting that the microcolonies actively subvert the mucosal reaction to their presence.

As bacterial microcolonies were found to be present in lower frequency in mucosal specimens from healthy control patients, it remains unclear what is the pathogenic significance of these colonies. It maybe that these microcolonies are part of the frequently observed colonisation of the nasal mucosa by *S.aureus* and of little significance to CRS. However, microcolonies were far more prevalent in the mucosal samples in CRSwNP than in controls, suggesting they are relevant to the disease process. An explanation that may explain these observations would be that normal mucosa (and to a much lesser extent bone) are colonised with small numbers of bacterial colonies which actively evade provocation of an inflammatory response. In CRS the number of these colonies is greatly expanded to such a level that they have a clinical impact on host immunity.

The immune barrier hypothesis which has been recently reviewed (Kern, Conley et al. 2008) suggests that defects in innate immunity are at the centre of the pathogenesis of CRS. It provides little information however on what may be the underlying cause of
those defects: the data presented here suggest that release of bacterial immunosuppressant factors may play a role. The observations and inferences suggesting active immunosuppression by intramucosal bacteria do not conflict with the immune barrier hypothesis but may help explain it. Despite an exhaustive search, the study presented in Chapter 7 failed to identify evidence of the production of SSLs by microcolonies. However the SSLs may in fact be produced in low concentrations and are only one of a number of factors released by *S.aureus* that may dampen local immunity.

On an evolutionary level there may be a selective advantage for bacteria to exploit a niche where it gains warmth and nutrition from a host without stimulating an aggressive response from that host. When considered from that perspective, any active stimulation of inflammation initiated in response to the bacteria may be disadvantageous to the bacteria, and contrary to the state of avoidance of a response it would like to achieve. Clinicians and microbiologists have focused their thoughts on bacteria which provoke inflammation and consequently illness – this state may be an aberration for the bacteria itself. There are now *in vitro* observations of the actions of isolated bacterial virulence factors which show that they have an immunosuppressive function in the human host (Chavakis, Preissner et al. 2007; Taylor and Llewelyn 2010). Animal models of *S.aureus* infections show that the effect of infection can be failure of the host immune response. In one study, bacteraemia was induced in pathogen-free mice by intravenous administration of *S.aureus*. Mice were then sacrificed after various time periods and it was seen that after 14 days T cells isolated from the spleen became unresponsive to antigenic stimulation (Ziegler, Goldmann et al. 2011). A further study utilizing a mouse model compared inflammatory indices between *S.aureus* infection and a sterile wound (Thurlow, Hanke et al. 2011). In the
infected mice attenuation of chemokine and cytokine production was observed along with impaired macrophage recruitment and a shift to an “M2” macrophage phenotype, a phenotype that is immunomodulatory with poor bactericidal activity (Benoit, Desnues et al. 2008).

One possible mechanism by which \textit{S.aureus} appears to be capable of surviving in a human host and evading human immunity is by existing in the intracellular compartment. Studies have shown that \textit{S.aureus} is capable of surviving for prolonged periods within a variety of cell types including endothelial cells, epithelial cells, fibroblasts, osteoblasts, keratinocytes, macrophages and neutrophils (Garzoni and Kelley 2009). As well as being capable of establishing persistence, mechanisms exist to allow release of \textit{S.aureus} from human cells (Giese, Glowinski et al. 2011). In Chapter 3 \textit{S.aureus} cocci were seen to be within cells of the epithelial layer (as had been described previously) (Clement, Vaudaux et al. 2005). However the microcolonies of bacteria in the subepithelial mucosa and within the bone appeared to be in the extracellular space rather than within cells.

The microcolonies of bacteria observed within the sinus mucosa were of a uniform small size. This observation has been made by others in CRS (Lundberg and Engquist 1984) and also in a mouse model of nasal colonisation (Margolis, Yates et al. 2010). Quorum sensing, in which bacteria within a biofilm communicate with each other to regulate their proliferation in response to the environment is a well recognised phenomenon (Atkinson and Williams 2009). It would seem possible that in the same manner bacteria within the tissue use this or a similar mechanism to regulate the colony size, limiting the degree of immune stimulation while maintaining viability.
8.1.2 Role of viruses in chronic rhinosinusitis

The study presented in Chapter 2 did not identify evidence of respiratory virus infection in samples of sinus mucosa from patients with and without CRS. This was an important study to undertake as previous studies have found some evidence of respiratory virus infection in CRS (Ramadan, Farr et al. 1997; Jang, Kwon et al. 2006). Our study was undertaken during summer months in order to avoid the autumn and winter peak in respiratory virus infections. This may in part explain why in our study we were not able to replicate the positive findings of others, because we avoided short-term seasonal carriage. The separate hypotheses advanced are particularly significant given the clinical context of patients being recruited for studies at times remote from their disease onset. The hypothesis that transient respiratory viral infection is an instigating factor in CRS was not tested and remains possible.

Some evidence supports a pathogenic role for respiratory virus infection in CRS. The central role of mucociliary function and epithelial barrier function in the development of CRS has already been discussed. In a study of morphological and functional changes in nasal cilia following an acute upper respiratory tract infection, marked changes were observed with impairment in ciliary function still demonstrable 32 days following the initial infection (Pedersen, Sakakura et al. 1983). A similar study reported changes in ciliary ultrastructure being evident for 2 to 10 weeks following infection (Carson, Collier et al. 1985). In vitro it has been shown that adhesion of *S.aureus, H.influenzae* and *S.pneumoniae* to human nasal epithelial cells is increased by co-existent rhinovirus infection (Wang, Kwon et al. 2009). More widespread effects on immunity are also possible. Infection with rhinovirus upregulates the expression of costimulatory molecules on epithelial cells that interact with T cells. The presence of influenza virus has also been shown to inhibit the function of
macrophages, reducing their ability to phagocytose S. aureus (Hang do, Choi et al. 2011). The net effect of the changes induced in the immune response by respiratory virus infection is a promotion of overt S. aureus infection (Iverson, Boyd et al. 2011).

Taken together, an hypothesis could be advanced that is consistent with the reports from patients that a ‘heavy cold’ was the initial event of their CRS. This viral infection may then alter the relatively harmonious host-pathogen interaction involved in normal colonisation to a state where micro-organisms are over-represented and able to induce a local immune dysfunction. The findings in Chapter 2 suggest that any respiratory virus infection is subsequently cleared during the evolution of the disease. A study designed to specifically look at the role of viruses in the initial development of CRS would be difficult to perform but extremely valuable in this regard.

Some non-respiratory viruses were sought in the study described in Chapter 2, (EBV and HHV-6). These were selected as they are viruses that have a well-described ability to induce latent infection. In the small population studied EBV did appear to be over-represented in the CRSwNP group. The significance of this is unclear but in a separate unpublished study not presented in this thesis the titres of serum antibodies specific for EBV virus were not raised in the CRSwNP patient group compared to controls. In a large Chinese study human papillomaviruses (HPVs) were found in 40% of nasal polyp samples but not in control mucosal samples (Pei, Chen et al. 2011). There was however a heterogeneous collection of HPV subtypes found with no correlation to any clinicopathological characteristics and such heterogeneity in detectable HPV subtypes has been interpreted in the context of cutaneous HPV to mean that they are commensals (Antonsson, Forslund et al. 2000). A further local study we have performed in collaboration with the clinical virologists, not presented in this thesis, sought to determine the prevalence of HPV viruses in CRS tissue and in
a small study of 5 CRSwNP, 6 CRSsNP and 2 control subjects no HPV viruses were detected (manuscript in preparation).

**8.1.3 Role of biofilms in chronic rhinosinusitis**

The study of bacterial biofilms presented in Chapter 3 sought to determine the relationship between biofilms and mucosal inflammation. The pathogenic role of biofilms in CRS remains unknown. Most of the published literature in this field utilizes either SEM (Cryer, Schipor et al. 2004; Sanclement, Webster et al. 2005) or CSLM (Sanderson, Leid et al. 2006; Psaltis, Ha et al. 2007) to analyse whole mucosal samples with the imaging modality examining the mucosal surface and any biofilm present rather than the interface between the two. The identification that Carnoy’s fixative provides the optimal preservation of matrix and flora on the mucosal surface (Ota and Katsuyama 1992; Swidsinski, Weber et al. 2005) greatly facilitated this study.

In this study it was shown that colonies of bacteria directly opposed to a disrupted epithelial layer were associated with higher numbers of T cells and macrophages. It is possible that if this study were to be repeated with a larger number of patients, samples or tissue sections then a significant elevation in the numbers of other immune cells would be noted too. A recent review of biofilms in human disease (Hall-Stoodley and Stoodley 2009) included that to be considered a “biofilm infection” bacterial colonies must be “associated with host inflammatory cells”. Using their definition the study in Chapter 3 implies that CRS is a “biofilm infection” although it would seem that while an association with inflammation has been shown this does not prove a causal link between the two. If one assumes briefly that this is indeed a causal relationship that has been observed then an attractive hypothesis continues to be advanced that the irritant effect of biofilms stimulates inflammation. It is unclear...
whether biofilms are initiators of the disease or whether another insult to the epithelium is required first to allow them to become established (Foreman, Jervis-Bardy et al. 2011). It is noted that while the biofilms identified may be the cause of the observed inflammation, alternatively they may be simply adhering to sites that are already inflamed.

The relationship between the mucosa and the biofilms may be more complex however. In a study of human tissue sampled from chronic wounds it was found that the immune cells did not penetrate bacterial biofilms (Kirketerp-Moller, Jensen et al. 2008) and a similar observation was made in our study. If immune cells are not penetrating the surface then it may be inferred that the antigenicity of a biofilm is low. Biofilms are however capable of releasing planktonic organisms from their surface (Kaplan 2010) and planktonic organisms released into the tissue would be expected to be antigenic. Despite the different distribution in immune cells that has been described it could be that both the surface and intramucosal colonies principally evade human immunity but then the planktonic organisms released from the colonies are stimulatory. In support of this suggestion that biofilms may exhibit an active immune evasive phenotype there is some evidence that the presence of bacterial biofilms correlates with reduced expression of the mucin glycoprotein MUC7 which is known to be a component of innate immunity, having a bacteriocidal effect (Tan, Psaltis et al. 2010).

While there is an increasing understanding of both the epithelial changes in CRS and of microbial biofilms, little is known about the interface between the two. In one study using SEM (Galli, Calo et al. 2008), most colonies of bacteria on the mucosal surface were directly opposed to a disrupted epithelial layer. As with all studies of human sinus mucosa it is possible that at least some of those loosely associated
colonies represented non-virulent, colonised nasal mucus. The absence of immune cell recruitment towards bacterial colonies more loosely associated with the mucosal surface suggests however that defining the interface between the epithelial layer and bacterial colony is critical in the study of biofilms in CRS and more work is required to define the exact nature of this interaction.

8.1.4 Role of bone in chronic rhinosinusitis

In Chapter 5 the presence of intraosseous bacteria and immune cells was described in samples of bone from the anterior face of the sphenoid bone. There was no association between these observations and the presence of CRS or the presence of bone changes that were evident radiologically. The radiological data in isolation are harder to interpret. No patients exhibited bone thickening of more than 3mm which would lead some (Lee, Kennedy et al. 2006) but not all (Georgalas, Videler et al. 2010) authors to consider the bone in all cases to not be “osteitic” and thus by implication be “normal”. If the bone samples were all truly “normal” then it is unsurprising that no differences were observed between the patient groups. If the bone samples from CRS patients were truly abnormal (and the fact that they were significantly thicker than the controls implies that they were) then one can conclude that immune cells and small colonies of bacteria are merely sporadic findings.

The study was not designed to examine other possible aetiological factors in the development of bone changes in CRS. There is increasing evidence of mucosal remodeling in CRS (Van Bruaene and Bachert 2011). Bone changes are predominant in those with prolonged or severe disease (Bhandarkar, Mace et al. 2011). The absence of correlation to the presence of bacteria and immune cells described here and the established evidence of new bone formation (Kennedy, Senior et al. 1998) contributes to an attractive hypothesis that bone changes in CRS are due to a similar
remodeling process as a consequence of persistent inflammation of the overlying mucosa.

8.1.5 Challenging established microbiological definitions

The primary goal of this thesis was to investigate the role of sinus microflora in the pathogenesis of CRS. Some of the observations made however challenge traditional microbiological perspectives. It is well known that the warm, moist environment of the nasal mucus allows bacterial colonisation of the nose in the absence of disease and *S. aureus* can be grown from samples collected from the anterior nares of approximately 1 in 3 healthy subjects (Peacock, de Silva et al. 2001). Local data suggest a slightly lower figure of 19% in the Auckland region (Best, Fraser et al. 2011). Colonisation of other cutaneous and mucosal surfaces is also compatible with health (Weir, Berg et al. 2011). There has been a general assumption that swabbing the surface of the anterior nares detects most colonising strains. This assumption implies that normal colonisation involves the mucosal surface only and is confined to the nose with the normal paranasal sinuses considered to be sterile (Larson and Han 2011). The presence of intramucosal and intraosseous bacteria in the samples of normal sinus tissue in this thesis contradicts these assumptions. It is noted that intramucosal bacteria have been identified in small numbers in control samples taken from the inferior turbinate (Corriveau, Zhang et al. 2009; Sachse, Becker et al. 2010) but not before in the sinuses. It is important to explore how pathogens such as *S. aureus* can exist within the tissue of the normal human host without inducing disease and how this might impact on the study of CRS.

As described briefly in Chapter 7 the clinical phenotype of *S. aureus* varies enormously between that observed during normal colonisation and invasive infection and the *agr* system may in part mediate this. Dysfunction of the *agr* regulator tends to
cause upregulation of immunoregulatory virulence factors (Benson, Lilo et al. 2011), the expression of which are more consistent with colonisation than invasion (Muthukrishnan, Quinn et al. 2011). Interestingly however a common observation is that in subjects colonised with S.aureus that go on to develop invasive infection, it is the same strain that causes disease (Wertheim, Vos et al. 2004). Furthermore otherwise genetically identical strains of S.aureus that are both agr positive and agr negative have been observed in the same individuals suggesting that altered function of agr may occur during the course of an infection (Traber, Lee et al. 2008). It is unclear however whether there are predominantly two phenotypes of S.aureus controlled by the agr regulator, namely evasive (favouring colonisation) or invasive or whether there are more intermediate phenotypes controlled by other global regulators (Herbert, Ziebandt et al. 2010). The observations presented in Chapter 3 correlated surface bacterial colonies with an upregulated inflammatory response and those in Chapter 4 indicated immune evasion - it may be that immunoevasive and more aggressive phenotypes of S.aureus co-exist in CRS. Indeed there is clear in vitro evidence that S.aureus strains are far from being equal in terms of their impact on human inflammation (Grundmeier, Tuchscherr et al. 2010).

Some studies have considered the agr regulator in samples of S.aureus grown from patients with and without CRS (Van Zele, Vaneechoutte et al. 2008; Heymans, Fischer et al. 2010). These studies are inherently limited by the removal of the organism from its surroundings in the human host and its growth on nutrient medium which is likely to not only select out those organisms that are metabolically active but also drastically alters the environment to which the organism is responding. If there are different phenotypes of S.aureus co-existent in CRS then this variability will also not be represented. In order to make progress in this area it is clear however that more
needs to be understood about the mechanisms behind normal colonisation of the sinuses for more to be understood about *S.aureus* phenotypic variation in CRS.

As well as questioning exactly what colonisation entails and what is normal with regards the flora of the paranasal sinuses there must also be some doubt around how to define infection. A clinical definition may seem best which would include the presence of symptoms due to the effect of micro-organisms but with the current level of knowledge it would not be possible to classify CRS as an infection or otherwise on these grounds. Other possible definitions may include a demonstrable inflammatory response in association with micro-organisms (in which case the adherent biofilms described are an infection but the intramucosal bacteria described are not) or the presence of invasion into tissues (in which case the intramucosal bacteria are an infection but the surface biofilms are not). A notion of “critical colonization” has been advanced in the context of the microflora of chronic wounds but the use of bacterial load to produce a cut off between normal colonisation and infection has been criticized (White and Cutting 2006). Although the definition of infection is perhaps a peripheral issue in this context it is an interesting debate well highlighted by the data presented here.
8.2 Implications of these findings for the treatment of chronic rhinosinusitis

8.2.1 Antibiotics

Previous investigations of the microbiology of CRS using culture-based techniques have identified limited differences between the resident microflora present when compared to healthy controls. Studies presented in this thesis have identified a greater prevalence of bacterial colonies adherent to a disrupted epithelial cell layer in CRS and a greater prevalence of intramucosal bacteria in CRSwNP. These data in combination with the work of others in this field, outlined in Chapter 1, have started to implicate bacteria once more in the pathogenesis CRS. This raises the question of how to normalise the paranasal sinus flora and furthermore whether such normalisation will lead to meaningful clinical improvements. This final point identifies a limitation in the two major randomized trials of antibiotics in CRS that show antibiotic efficacy (Wallwork, Coman et al. 2006; Van Zele, Gevaert et al. 2010). While both looked at a panel of subjective and objective measures to confirm the efficacy neither looked at any microbiological endpoints. It is therefore unknown whether the limited observed efficacy is because the antibiotics do not alter the resident microflora sufficiently or because alteration to the microflora does not translate to meaningful changes in the clinical course of CRS. A further randomized trial has been undertaken which showed no significant efficacy of azithromycin in treating CRS (Videler, Badia et al. 2011). In this study swabs of the middle meatus were taken pre and post treatment and a significant reduction in the rate of growth of S. pneumoniae was reported in the antibiotic group. Given the limited utility of culture swabs and the fact that there was no significant difference in the other 7 groups of
organisms in the study group it is difficult to know how to interpret these data in this context.

For the purposes of this part of the discussion it will be assumed that both surface biofilms and immune evasive intramucosal bacteria are relevant to the disease pathogenesis in line with the evidence that has been discussed above.

Topical agents are known to be poor at achieving penetration of the sinuses (Harvey and Schlosser 2009) and so penetration of topical antimicrobial agents into the sinus mucosa to reach the intramucosal colonies would be expected to be negligible. This may in part underpin the limited clinical efficacy of topical antibiotics (Lim, Citardi et al. 2008). A systemic agent would appear therefore to be implicated and such a systemic agent would need to be able to reach the known bacterial niches in appropriate concentrations. Therefore penetration of the sinus mucosa is required with secretion from the mucosal surface into the region of the surface biofilms. Penetration into the intracellular compartment (Clement, Vaudaux et al. 2005) would also be required.

Many common antibiotics are bacteriostatic in their function, acting to prevent the growth of bacterial populations rather than actively killing them. This is of little consequence in the majority of infections where rapid expansion of bacterial populations is occurring and preventing this expansion is sufficient with the host immune system then clearing the remaining micro-organisms (Pankey and Sabath 2004). However bacteria existing in a biofilm are known to show slower growth rates (Walters, Roe et al. 2003) and to have reduced susceptibility to immune attack (Stewart and Costerton 2001). The observed immune evasive phenotype of intramucosal colonies along with the small size of the colonies would tend to imply
that they are also growing at a reduced rate. Due to these reductions in both the growth rate of the resident bacterial colonies and their susceptibility to human immunity the optimal agent in CRS would therefore be expected to be bactericidal in its effect rather than bacteriostatic.

Many studies of CRS microflora use the technique of FISH which may not detect all species of bacteria, particularly those in the mucosa. The study presented in Chapter 4 examined only a small number of species but implicated *S. aureus* particularly. This is consistent with the findings of others, with a recent study strongly linking the perioperative identification of *S. aureus* with a worse long-term prognosis (Jervis-Bardy, Foreman et al. 2009). Appropriate activity of any antibiotic agent against *S. aureus* therefore seems to be required although activity against a broad spectrum of upper respiratory tract (URT) pathogens would be favoured.

In summary, the appropriate therapy based on the results of this thesis would appear to be a systemic antibiotic that exhibits excellent mucosal penetration including particular penetration onto the mucosal surface and into the intracellular compartment, anti-biofilm efficacy and particular bacteriocidal anti-*S. aureus* activity. It would also need to be safe, well-tolerated, not associated with particular antibiotic resistance and ideally, inexpensive. This antibiotic would then need to be studied in a way that documents not only subjective and objective markers of disease improvement but also determines appropriate microbiological endpoints.

The pathway from systemic administration of antibiotics to the target site at a sufficient concentration for sufficient time is complex and dependent on a wide range of factors (Matson and Fallon 2009). Antibiotic delivery to the lower respiratory tract (LRT) has been studied more than to the paranasal sinuses and some clues about
penetration of respiratory mucosa can be gained. Some antibiotics, the macrolides being particular examples, are capable of achieving significantly higher concentrations in respiratory mucosa than the serum concentration due to intracellular accumulation (Honeybourne 1994). Relative to penetration of the mucosa itself the concentration of antibiotics in respiratory secretions following systemic administration is however poor. A total of 14 antibiotics were considered in parallel and a wide spectrum was reported with only 3-6% of the serum concentration of amoxicillin reaching respiratory secretions compared to 67% of the serum concentration of tobramycin (Pennington 1981).

Co-amoxiclav is a combination antibiotic/β-lactam agent that is commonly used for CRS (Legent, Bordure et al. 1994) but while there is good penetration of the amoxicillin component into the mucosa of the paranasal sinuses it has been reported that the clavulanate component is commonly undetectable (Dinis, Monteiro et al. 2000). Quinolones (Pea, Marioni et al. 2007), macrolides (Fang, Palmer et al. 2009), and cephalosporins (Dinis, Monteiro et al. 1999) have all been observed to penetrate sinus mucosa at concentrations at least equivalent to if not higher than serum concentrations. None of the cited articles however examined the concentration of antibiotics in the mucosal secretions of the sinuses.

Sandberg et al used a mouse model of S.aureus peritonitis and then determined the ability of various antibiotics to eradicate infection. When intracellular activity was considered dicloxacillin, cefuroxime and rifampicin exhibited similar reductions in colony counts and were seen to be superior to gentamicin and azithromycin. When the extracellular effect was considered rifampicin was the most effective out of the five antibiotics (Sandberg, Hessler et al. 2009). In a study looking at the effect of antibiotics in a model of S.aureus infections using human macrophages, combinations
involving rifampicin were seen to have the greatest intracellular potency (Baltch, Ritz et al. 2008).

Of all possible antibiotics rifampicin would seem to be a valid choice. It is known to penetrate human mucosal tissue and mucosal secretions (Kenny and Strates 1981) and in a more recent study excellent penetration was seen of respiratory mucosa, epithelial lining fluid and macrophages in the LRT (Ziglam, Baldwin et al. 2002). Rifampicin has bactericidal activity against staphylococci (Atlas and Turck 1968) and in a study of minimum biofilm eradication concentrations on clinical isolates of \textit{S.aureus} only rifampicin was thought to have clinical utility in biofilm eradication (Kotulova and Slobodnikova 2010).

Perhaps the best model available for studying the efficacy of rifampicin is in the protocols for eradication of chronic \textit{S.aureus} nasal carriage. In this context rifampicin has significant clinical efficacy, indicating that it penetrates human sinonasal mucosa and the associated secretions in sufficient concentrations to have a good anti-staphylococcal effect and is regarded as the systemic agent of choice (Falagas, Bliziotis et al. 2007). This does not necessarily translate to efficacy in CRS due to some important differences between CRS and nasal carriage. In CRS the tissues are inflamed, potentially affecting drug distribution (Matson and Fallon 2009) although it has been reported that for cefuroxime that this is not of importance in affecting tissue penetration in the sinuses. Furthermore, \textit{S.aureus} probably does not exist in a biofilm phenotype when colonising the normal nose (Krismer and Peschel 2011). The results in this thesis also suggest that the intramucosal bacterial load is likely to be lower in the colonised nose.
Given the complexities of drug delivery to tissue compartments it is not surprising that no efficacy was reported in a small study in which anti-staphylococcal antibiotics were selected on the basis of the minimum inhibitory concentration determined \textit{in vitro} (Schalek, Petras et al. 2009). However rifampicin would appear to be an ideal agent to trial in CRS and a randomized trial is currently underway in our unit. There are various practical considerations including the need to use dual antibiotic therapy as high rates of antibiotic resistance are reported when rifampicin is used as monotherapy (Atlas and Turck 1968; Falagas, Bliziotis et al. 2007).

\subsection*{8.2.2 Other medical therapies}

As discussed in the previous section, the trials evaluating antibiotics in CRS have suggested only limited efficacy. This may be in part because the strategy of eradicating a proportion of the resident flora over-simplifies the microbiological correction required to ameliorate symptoms of CRS. There is increasing evidence showing that the presence of the normal colonising microbiota protects mucosal surfaces from inflammatory diseases (Endt, Stecher et al. 2010; Herbst, Sichelstiel et al. 2011).

In a rabbit model of \textit{S.aureus} colitis it has recently been shown that \textit{Lactobacillus paracasei}, an organism derived from the stool of breastfed newborns, can protect the colon from injury (Bendali, Madi et al. 2011). More recently still it has been shown \textit{in vitro} and \textit{in vivo} that a protein produced by \textit{S.epidermidis}, an organism considered to be a commensal rather than a pathogen, can disrupt biofilm formation by \textit{S.aureus} and protect the human host against \textit{S.aureus} colonisation (Iwase, Uehara et al. 2010).

Probiotics have typically been administered orally and championed for their widespread effect on the health of the gastrointestinal tract (Oelschlaeger 2010). It remains to be seen if they would be considered acceptable to patients but the
possibility exists that probiotics administered to the nose and sinuses may be a viable treatment option in CRS.

A variety of other more selective targets are being investigated, as opposed to the relatively non-specific action of antibiotics. The prospect of a vaccine against \textit{S.aureus} has received much attention and if developed could be of benefit in CRS. At this point however significant challenges need to be overcome in the development of a suitable vaccine (Broughan, Anderson et al. 2011). There is a possibility that if key bacterial virulence factors that tend to be active in CRS can be identified then they could be selectively targeted (Wang, Mattis et al. 2010). It may also be possible to trial agents that focus more on targeting downstream effects such as IL-5 or IL-6 which appear to have major roles in mediating the effects of staphylococcal superantigens on T cell populations (Xu, Xia et al. 2009; Langier, Landsberg et al. 2011).

Ironically, potential antimicrobial effects may be possessed by established agents not generally recognised as having them. Although speculative, some studies have suggested that corticosteroids may mediate their therapeutic effect in CRS by boosting innate immunity (Schleimer, Kato et al. 2009), implying that corticosteroids aid in the clearance of pathogens. This would be consistent with the observation from a multi-centre study that intranasal corticosteroid use reduces the rate at which bacteria can be cultured from CRS patients by almost 50% (Desrosiers, Hussain et al. 2007).
8.3 Future studies

8.3.1 Pathogenesis of chronic rhinosinusitis

The study and interpretation of mucosal samples from CRS patients is greatly facilitated when performed in parallel with control samples of normal sinus mucosa. The data presented here have demonstrated that the paranasal sinuses are not sterile in the way that has been suggested by some (Larson and Han 2011). Without a complete picture of the microbiology of the normal sinuses it is difficult to make deductions about normal sinus host-pathogen interactions and mucosal immunology. Normal sinus mucosa contains numerous immune cells. It may be assumed that given the absence of symptoms they are in a less active state but the immunology of the normal sinuses and how it compares with CRS requires further study. Given the significant difference in airflow across the surface of and therefore immunological challenge to the inferior turbinates the study of normal mucosa from the sinuses rather than inferior turbinate mucosa is advocated.

The existence of different macrophage subtypes was referred to in Section 8.1.1. Macrophages are considered to exist in M1 and M2 phenotypes with the M1 phenotype being more bactericidal but tending to induce tissue injury and the M2 phenotype being less bactericidal but promoting immune modulation (Benoit, Desnues et al. 2008). There has been limited study of these subtypes in the context of CRS but an initial study has shown a higher rate of macrophages expressing the M2 markers CD163 and CD206 in CRSwNP tissue than in CRSsNP or control samples (Krysko, Holtappels et al. 2011). There was however no difference in the rate of macrophages that were considered to be in the M1 phenotype between CRSsNP and CRSwNP. This increased prevalence of M2 macrophages in CRSwNP has been
observed in a further study but somewhat paradoxically, immune modulating M2 macrophages have been seen to be one of the major sites of production of CC chemokine ligand 18 (CCL18) which is known to induce chemotaxis for a wide variety of pro-inflammatory cell types (Peterson, Poposki et al. 2012). Further to the discussion of active immune modulation in Chapter 4 it would be fascinating to explore whether the increases in M2 macrophages are a cause or effect of the higher rates of bacterial colonisation in CRSwNP. In a similar fashion inroads are being made in further defining T cell populations including the expression of FOXP3, CD25, CD69 and IL17 (Ryan and Brooks 2010). The demonstration of particular clusters of M2 macrophages or TReg cells around intramucosal colonies of *S. aureus* would add further support to the concept that the observed immune tolerance is induced by the bacteria.

The use of swabs to collect purulent secretions for culture and sensitivity is central to the care of patients with overt invasive infection. The techniques involved are inexpensive, objective, relatively easy to perform, well-established and easy for clinicians to interpret. It is now recognised however that in the majority of cases CRS is not an infection in the manner seen in abscesses, pneumonia, cellulitis or ARS whereby a single or a small number of organisms are plentiful and therefore easy to detect. A swab taken of the nasal mucus does not adequately sample the relevant microbiology in CRS. Culturing whole tissue samples in CRS avoids some of these issues, but still relies on the assumption that all viable organisms will grow on the culture medium used.

For some time now the viable but non-culturable state (VBNC) as a bacterial phenotype has been recognized (Nilsson, Oliver et al. 1991). Although it is not entirely clear how this phenomenon overlaps with the well described dormancy of TB
(Kana, Gordhan et al. 2008) it is clear that some pathogens have the ability to enter a state that is undetectable by routine laboratory culture techniques yet are still capable of causing disease. Although the term VBNC has not been applied to many of the pathogens that exist in the nose and sinuses (Oliver 2010) it is clear that regardless of terminology, organisms such as *S. aureus* have potential phenotypes that are difficult to grow in conventional bacterial media (Tuchscherr, Heitmann et al. 2010). While *in vitro* culture has its place in the clinical context non-culture dependent techniques are likely to become increasingly important techniques when researching the microbiology of CRS.

A variety of culture-independent techniques are in current use for the detection of resident microflora. Similar to FISH, these techniques typically target the 16s ribosomal RNA of bacteria as it is constrained to but ubiquitous within prokaryotes (Case, Boucher et al. 2007). The first described use of such a technique in CRS was published in 2010 and this initial report described a mean of 10 bacterial isolates per mucosal specimen using molecular techniques compared to a mean of 1.4 bacterial isolates per patient using conventional culture techniques (Stephenson, Mfuna et al. 2010). Consistent with the results reported in this thesis, the normal sinus mucosa sampled was not sterile and the detection of *S. aureus* using molecular techniques was considerably higher than using culture techniques. A further recent study compared the results from conventional culture to a 16s ribosomal RNA molecular technique on samples of mucus collected from patients with and without CRS (Feazel, Robertson et al. 2012). Despite not sampling the mucosal tissue and presumably only collecting surface material that was non-adherent, a particular diversity of organisms was reported that was under-represented by culture results. *S. aureus* in particular was detected more frequently by molecular techniques than in culture. In summary, the
use of culture-independent molecular techniques for bacterial identification has great potential and if combined with imaging modalities for localization of bacteria in or on the surface of mucosal samples (De Baere, Hollants et al. 2009) could greatly advance our understanding of sinus microbiology in both health and disease.

In much the same way that a variety of pathogens can cause a particular type of infection (e.g., pneumonia) it seems highly likely that even with more sensitive molecular methods no single organism will be found universally in CRS. Although the potential for one organism to displace or suppress the growth of another was discussed above it is also quite possible that different species of micro-organisms are able to exist in a symbiotic fashion and that such a combination of micro-organisms are relevant to disease pathogenesis in any one individual. The in vivo interaction of bacteria in the URT has attracted some attention but is poorly understood (Benninger, Brook et al. 2011) and understanding of the role of the co-existence of micro-organisms in CRS particularly is at an early stage. It has already been noted that polymicrobial biofilms exist in CRS (Sanderson, Leid et al. 2006) and furthermore that symbiosis even at a cross-kingdom level may be significant (Boase, Valentine et al. 2011). It is also recognised that mechanisms exist whereby bacteria of different species can communicate and co-ordinate in a sophisticated fashion (Armbruster, Hong et al. 2010).

Examining the literature on paediatric URT infections and the particular role of *H. influenzae* and *S. pneumoniae* highlights both the complexity of inter-species effects in human disease but also the difficulties in their study. It has been reported that when grown in vitro, the presence of *S. pneumoniae* inhibits the growth of *H. influenzae* via the effect of hydrogen peroxide but *H. influenzae* does not exert any reciprocal effect (Pericone, Overweg et al. 2000). However, in a mouse model where these organisms
were introduced to the URT, both were capable of establishing colonisation in isolation but when co-existent the opposite effect to the *in vitro* finding was seen with *H.influenzae* establishing colonisation and *S.pneumoniae* being rapidly cleared (Lysenko, Ratner et al. 2005). To complicate matters further, a Chinchilla model of otitis media produced different findings (Weimer, Armbruster et al. 2010). In this study it was reported that the addition of *H.influenzae* actually augmented the development of *S.pneumoniae* biofilms but paradoxically reduced markers of systemic disease. In the study of human disease it is this final point that is of greatest significance - the impact of the micro-organisms on the disease process. The use of animal models of disease can generate inferences about the clinical consequence but the extension of those techniques advanced in this thesis with the simultaneous *in situ* detection of micro-organisms and the host response may advance understanding of this complex issue. *S.pneumoniae* and *H.influenzae* do appear to co-exist in the URT of the human host but the relevance of their co-existence to human disease requires further study (Nistico, Kreft et al. 2011).

The study of the pathogenesis of CRS can be illuminated by the consideration of other mucosal inflammatory disorders such as the discussion of adenoid and middle ear pathogens, both in terms of the laboratory techniques used and the pathophysiological mechanisms identified. There are significant parallels between CRS and inflammatory bowel disease (IBD) being a chronic, multifactorial mucosal inflammatory condition characterised by demonstrable defects in host barrier functions and aberrant responses to the resident microflora (Indriolo, Greco et al. 2011). One of the most comprehensive studies using FISH to detect mucosal biofilms was performed in IBD and this study using 38 separate FISH probes highlights the scope for further similar work in CRS (Swidsinski, Weber et al. 2005). The findings and methodology behind
that particular study were advanced further using a technique called laser capture microdissection (LCM) to selectively isolate and evaluate bacterial populations in discreet anatomical regions of the mucosa (Rowan, Docherty et al. 2010). That study generated the interesting observation that the bacterial load within the colonic crypts varies in different phases of IBD with a load both greater than and less than normal subjects at different times. The utility of LCM in studying bacteria in different anatomical subsites in CRS is obvious in the context of the findings presented in this thesis. Other potentially relevant insights from the IBD literature include the notion of normal colonising bacteria inducing a phenotype that promotes their survival and therefore a tolerant host-microbe interface with \textit{Bacteroides fragilis} being shown to induce an upregulation of immune regulatory TReg cells (Round and Mazmanian 2010).

Currently there are many ‘-omic’ techniques available. This suffix implies the identification of the totality of things present and includes proteomics (Farajzadeh Deroee, Oweinah et al. 2009; Upton, Welham et al. 2011), genomics (Garzoni, Francois et al. 2007), transcriptomics (van Drunen, Mjosberg et al. 2012) and infectogenomics (Nibali, Donos et al. 2009) along with others. More than ever the challenge in researching disease is to know where and how best to look rather than being constrained by the techniques available for looking (van Drunen, Vroeling et al. 2008).

\section*{8.3.2 Microbiology}

In 2003 Parsek and Singh published their paper defining the role of biofilms in human disease (Parsek and Singh 2003). They noted that the definition used had limitations and an attempt has been made by others to further refine these criteria (Hall-Stoodley and Stoodley 2009). Despite these refinements there is persisting uncertainty about
how best to define a biofilm and the observations presented here challenge some established thinking on this subject. Traditionally it has been considered that the bacterial colony must be adherent to a surface of some form, be it organic or non-organic. This is a concept supported by work showing that specific processes exist within the formation of a biofilm to promote adhesion (Absalon, Van Dellen et al. 2011). Refined criteria subsequently published loosened this requirement to an association rather than adherence, although it was noted that those biofilms described in the literature as non-adherent may have been artefactually disrupted from a surface during processing. The study presented in Chapter 3 has served to reinforce the concept that adherence is relevant, especially in the clinical context.

Several references have been made in this thesis to how intramucosal bacterial populations exhibit some features of biofilms, such as resistance to both immune attack and antibiotics and possibly also quorum sensing. This prompts the question – have the intramucosal colonies adopted a biofilm phenotype? This is not an entirely novel suggestion: in a mouse model of urinary tract infection it was seen that intracellular communities of Escherichia coli formed were resistant to immune attack and these communities surrounded themselves with a polysaccharide matrix with the authors noting the “biofilm-like” characteristics (Anderson, Goller et al. 2010). Clearly if such colonies are to be considered as biofilms then the requirement of a relationship with a surface should be abandoned. The overlap between these intramucosal colonies and the biofilm phenotype could be further investigated by staining for common components of a biofilm matrix such as polysaccharides (Kirketerp-Moller, Jensen et al. 2008) or nucleic acids (Das, Sharma et al. 2010) or seeking evidence of quorum sensing (Atkinson and Williams 2009).
Besides the biofilm phenotype there are other described bacterial mechanisms that may be active in CRS. Small colony variants (SCVs) of bacteria were first described over a hundred years ago and are defined by the characteristic colony size, being approximately 10 times smaller than those of comparable wild-type organisms when grown in vitro. S. aureus SCVs have demonstrable biochemical abnormalities, most commonly in the electron transport chain (ETC) or in thymidine biosynthesis (Proctor, von Eiff et al. 2006). What makes SCVs so relevant in this context is that their in vivo phenotype affords them particular ability to establish long-term persistence, potentially becoming a source of relapsing infections in the human host. (Tuchscherr, Heitmann et al. 2010). It is not known whether the small colonies identified in vitro correspond to their size in vivo but the parallel with the observations in this thesis is clear. Of relevance to their study however is that the described biochemical defects cause SCVs to grow very slowly in vitro and reversion to a non-SCV phenotype is common, making them resistant to detection (Melter and Radojevic 2010). Others have tried unsuccessfully to culture S. aureus SCVs from clinical samples in CRS (Niederfuhr, Kirsche et al. 2008) and to date there is only a single report of an S. aureus SCV being grown from a sinus isolate (Proctor, van Langevelde et al. 1995). In a separate study not presented in this thesis we collaborated with clinical microbiologists who cultured SCVs from other staphylococcal species from some of our CRS mucosal specimens but were unable to culture S. aureus SCVs from samples of normal and diseased sinus mucosa (manuscript in preparation). This may mean that SCVs are not present in CRS or that they there were not detectable with the culture techniques used.

Although we could not detect in vivo evidence of SSL release in sinus mucosa, we wonder if this was due to their being present in very low concentrations. It is known
that when the expression of *agr* becomes dysfunctional then the production of the immunoregulatory SSLs is upregulated (Pantrangi, Singh et al. 2010; Benson, Lilo et al. 2011). Interestingly *S.aureus* is not only well adapted for intracellular survival but it also has defined mechanisms for triggering release from the intracellular compartment and this is also under the control of *agr* (Giese, Glowinski et al. 2011).

There has to date only been limited study of *agr* in CRS with one study reporting an association between *agr* subtypes and CRSwNP (Van Zele, Vaneechoutte et al. 2008) but not the other (Heymans, Fischer et al. 2010). Both of these studies looked at the presence rather than expression of *agr* and examined isolates of *S.aureus* grown from middle meatal swabs, one step removed from the disease process under consideration. The study of normal and diseased mucosa from the sinuses (potentially including samples of infected material from ARS patients) could greatly advance knowledge of the *in vivo* expression of virulence genes and their regulatory systems (Herbert, Ziebandt et al. 2010). Using a technique based on transcriptomics or proteomics would give particular focus on the function rather than the presence of these genes.

Much of the discussion has been around *S.aureus* given the focus of this thesis on its study but it is noted that *S.aureus* is one of many organisms found in the URT in CRS (Feazel, Robertson et al. 2012). In the same manner many of these different organisms possess mechanisms for eluding and modulating host immunity (Erwin and Smith 2007; Kadioglu, Weiser et al. 2008; Guenther, Stroh et al. 2009).

It is unclear what the implications of this thesis are for the study of the colonisation of the healthy human nose, most commonly considered in relation to *S.aureus*. This is an important field of research given the greatly increased risk of nosocomial infection in individuals with *S.aureus* detectable in their anterior nares and the need to limit this risk (Bode, Kluytmans et al. 2010). At this point there is already debate as to the
optimum detection method for examining the anterior nares with studies showing variability in the detection rate with different swabs (Jones, Matthews et al. 2011) and a greatly increased rate of detection using a culture-independent method (Cho, Jung et al. 2011). It would be highly relevant to know if a swab of the anterior nares with subsequent culture reflects the rate of carriage of \textit{S.aureus} on or within the sinonasal mucosa and a local study is currently underway using culture-independent techniques to determine a variety of mechanisms for \textit{S.aureus} detection including the assessment of whole samples of sinus mucosa with both culture-dependent and independent techniques.

\textbf{8.4 Summary}

Many questions remain unanswered about the pathogenesis of CRS. The use of culture-independent and \textit{in situ} techniques for detection of bacteria has significantly altered our perspective of the resident microflora and its pathogenic role. The microflora of normal sinuses is more complicated than previously thought.

Our understanding of the interaction between mucosal microflora and host immune response is also evolving. While micro-organisms are clearly capable of stimulating an inflammatory response this thesis adds to previous \textit{in vitro} reports of bacteria failing to incite an immune response. Microcolonies growing commensally within the mucosa may be the default phenotype of this species, with more virulent planktonic forms associated with inflammation and disease being the exception. At this time the principle mechanism by which this is mediated and the role of this phenomenon in CRS pathogenesis is unknown but increasingly researchers are trying to relate the pathogen load with the inflammatory profile (Ba, Zhang et al. 2011).
Substantial reservoirs of immune evasive bacteria appear to exist both on the surface of and within sinus mucosa in CRS providing the potential to shed immunogenic planktonic bacteria into the surrounding tissue providing a chronic inflammatory stimulus. It may well be that a transient event such as a respiratory virus infection, in an individual predisposed by genetic and environmental factors, is required to disrupt the normal host-microbe interface allowing these colonies to develop. The role of variation in pathogens and their niches, disease phenotypes and ethnic and geographical groupings remains to be clarified.
Appendix 1. List of publications relating to this thesis


Appendix 2. List of National and International Presentations relating to this thesis


5. Bacterial microcolonies exist within the mucosa of patients with chronic rhinosinusitis and exert an immunoparetic effect. Wood AJ, Fraser JD, Patterson E, Amirapu S, Swift S, Douglas RG. New Zealand Microbiological Society Annual Meeting, Auckland. 2010

6. Intramucosal Staphylococcus aureus in chronic rhinosinusitis. Wood AJ. Maurice Wilkins Centre “Superbug symposium”, Auckland. 2010

8. Do viruses have a role in the pathogenesis of chronic rhinosinusitis? Wood AJ, Antoszewska H, Fraser J, Douglas RG. American Rhinologic Society Meeting, Boston, MA. 2010

Appendix 3. List of prizes relating to this thesis

1. Surgical Research Society of Australasia Annual Scientific Meeting Oral Presentation Travel grant prize 2011

2. New Zealand Microbiological Society Annual Meeting Student Oral Presentation First Prize 2010

3. Auckland District Health Board Young Investigator of the Year Award 2010

4. Exposure (University of Auckland Post Graduate research Expo) Winner Oral Category 2010

5. HealtheX (Faculty-wide student conference), University of Auckland 2nd prize Oral Clinical Category 2010
References


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media and impedes the progression of pneumococcal disease." J Infect Dis 202(7):1068-75.


Am J Rhinol Allergy (in press).


