The Environment and the Host in Chronic Rhinosinusitis

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Submitted for the title of Doctor of Philosophy

May 2012

This thesis is dedicated to those who have sacrificed the most during my scientific endeavors

My amazing family

Julia, Thomas & Will

Financial Support

The work throughout this thesis would not have been possible without the assistance of The Garnett Passe & Rodney Williams Memorial Foundation. The Foundation's generous support of Otolaryngology research in Australia ensures we continue to advance the specialty for the benefit of our patients.

Thank you

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Thesis declaration

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Dr Sam Boase

Acknowledgements

Nothing has been more satisfying throughout my journey back into research life than sitting with my colleagues and friends discussing a research dilemma, and synergistically finding a solution. The dynamic group of people at the ENT Department, Queen Elizabeth Hospital have contributed significantly to my scientific knowledge, presentation skills, and critical thinking. The fantastic camaraderie made the difficult days bearable, and the good days memorable. Professor Wormald has fostered this environment in the department that we all enjoy. The opportunity to work under his supervision has been phenomenal. His commitment to absolute excellence both surgically, and academically is inspirational. I thank him for offering me this opportunity, his guidance and friendship, and for his unwavering passion for academic surgery.

To my surgeon-scientist colleagues, my friends, who have shared with me the trials and tribulations of combining two simultaneous apprenticeships - the clinical and the academic. Dr Andrew Foreman, Dr Rowan Valentine, Dr Josh Jervis-Bardy, Dr Ed Cleland and Dr Camille Jardeleza. On so many levels you have helped bring this work to fruition. I cannot thank you enough for your support.

To my friends in the laboratory, Leonie Baker, Damien Jones, and Dijana Miljkovic thank you for your patience as I learnt to be a research scientist again. You have taught me so much – I could not have done it without you.

To Dr Lorwai Tan, my co-supervisor. Your regular ‘consultations’ to keep my research on track were invaluable. Thanks for believing it would be OK, sometimes against all odds.

To Dr Harshita Pant, my co-supervisor. Thank you for your guidance and invaluable expertise, helping to keep my research relevant. Your advice and encouragement were always appreciated. Thank you also for your constructive criticism – helping me to be better.

Thanks to Matthew Smith & Michelle Slawinski, of The Animal House, The Queen Elizabeth Hospital, Adelaide, South Australia. Your assistance, and patience was always appreciated. Thanks to Lyn Waterhouse of Adelaide Microscopy, who taught me so much about the intricacies of biological microscopy, which formed an integral component of this work. Dr John Field, thank you for passing on so much of your statistical knowledge, and invaluable advice in planning and publishing research works.

Special thanks to the staff of The Memorial Hospital who have consistently supported ENT research, especially Dr Graham van Renen and Kathy Jarman.

To my great friend Ben van den Akker, your friendship & scientific expertise have helped me keep it together throughout this project. Always on the end of the phone for statistical advice or a chat – thanks mate. To my family, Matt, Zoe, Ben, Dale, Jason, Laura, Ron, Airlie, Sarah & Paul – thanks for your lifelong support –

this is what I have been up to for the last few years. To my Parents Deb & Leon, thank you for giving me the gift of education. You have given me the confidence to follow my ambitions wherever they take me, without ever applying pressure or criticism – thank you. To my Grandfather Bevan, thank you for quietly helping out in the pursuit of education.

To my phenomenal, gorgeous wife Julia. Your support is the foundation to my achievements. Without you there would be nothing. You have offered unwavering dedication to my professional interests, often to the detriment of your own. You have raised our beautiful boys, often in my absence and always offered me everything I need to be completely fulfilled in life. I cannot thank you enough. To my phenomenal boys, Thomas & Will. You have been my inspiration to complete this work. The light at the end of the tunnel every day. What you two have learned over the period of this research makes this thesis seem insignificant.

I owe a great debt of gratitude to our patients, who graciously consent to involvement in much of this research, in a hope that we will be able to improve the lives of our future patients. Finally to Serendipity, thank you for your help – your gifts are scattered throughout this thesis.

Publications arising from this thesis

The microbiome of chronic rhinosinusitis: culture, molecular diagnostics and biofilm detection

Boase, S., Foreman, F., Tan, L.W., Melton-Kreft, R., Pant, H., Hu, F.Z., Ehrlich, G.D., Wormald, P.J.

BMC Infectious Diseases (in review)

A sheep model to investigate the role of fungal biofilms in sinusitis: fungal and bacterial synergy

Boase, S., Valentine, R., Singhal, D., Tan, L. W., Wormald, P.J.

International Forum of Allergy & Rhinology 2011, 1 (5): 340-347

Bacterial induced cilia damage promotes fungal biofilm formation in a sheep model of sinusitis.

Boase, S., Jervis-Bardy, S., Cleland, C., Pant, H., Tan, L.W., Wormald, P.J.

International Forum of Allergy & Rhinology (in review)

Microorganisms and host immunoglobulin E responses in chronic rhinosinusitis: *Staphylococcus aureus* potentiates inhalant aeroallergen sensitization.

Boase, S., Baker, L., Foreman, A., Tan, L.W., Pant, H., Wormald, P.J.

Journal of Allergy and Clinical Immunology (in review)

Awards arising from this thesis

Maurice Cottle Award - Best Scientific Manuscript

American Rhinologic Society

Boston, MA, USA. September 2010.

Best oral presentation - Senior PhD Category

The Queen Elizabeth Hospital Research Foundation Research Day

Adelaide, October 2010.

The Ron Gristwood Medal - Best Registrar Presentation

The Australian Society of Otolaryngology Head & Neck Surgery Scientific Meeting Adelaide, Nov 2011.

Presentations arising from this thesis

Developing an animal model of fungal sinusitis: promises and pitfalls

Basil Hetzel Institute Post-Graduate Seminar

Adelaide, July 2010

A sheep model to investigate the role of fungal biofilms in sinusitis: fungal & bacterial synergy

American Rhinologic Society Annual Meeting

Boston, USA, September 2010

A model to investigate the role of fungal biofilms in chronic rhinosinusitis

The Australian Society of Otolaryngology Head & Neck Surgery Scientific Meeting (SA), Adelaide, November 2010.

Fungal biofilm formation in sinusitis: fungal & bacterial interactions in the sheep model of sinusitis.

The Queen Elizabeth Hospital Research Day

Adelaide, October 2010

The aetiopathogenesis of CRS

14th Advanced Functional Endoscopic Sinus Surgery Course

Adelaide, November 2011

CRS: microorganisms and the host

The Australian Society of Otolaryngology Head & Neck Surgery Scientific Meeting (SA), Adelaide, November 2011.

Microorganisms and the host in Chronic Rhinosinusitis: Making the link. The Royal Australian College of Surgeons (SA) Annual Scientific Meeting: The RP Jepson Medal, Adelaide, November 2012.

Abbreviations

A. alternata	*Alternaria alternata*
A. flavus	*Aspergillus flavus*
A. fumigatus	*Aspergillus fumigatus*
Aa	*Alternaria alternata*
ABPA	Allergic bronchopulmonary Aspergillosis
AD	Atopic dermatitis
Af	*Aspergillus fumigatus*
AFRS	Allergic fungal rhinosinusitis
AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cell
AR	Allergic rhinosinusitis
ARS	Acute rhinosinusitis
ATCC	American Type Culture Collection
BAFF	B cell activating factor
C. albicans	*Candida albicans*
cAMP	3'-5'-cyclic adenosine monophosphate
CAZS	Citric acid zwitterionic surfactant
CD	Cluster of differentiation
CF	Cystic fibrosis
CHIPS	Chemotaxis inhibitory protein of *S. aureus*
CNS	Coagulase-negative *staphylococci*
COPD	Chronic obstructive pulmonary disease

CRS	Chronic rhinosinusitis
CRSsNP	Chronic rhinosinusitis without nasal polyps
CRSwNP	Chronic rhinosinusitis with nasal polyps
CSF	Cerebrospinal fluid
CT	Computed Tomography
CVID	Common variable immunodeficiency
DC	Dendritic cell
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
ECP	Eosinophilic cationic protein
EM	Eosinophilic mucus
ESI	Electrospray ionisation
ESS	Endoscopic sinus surgery
FISH	Fluorescence *in situ* hybridisation
GM-CSF	Granulocyte macrophage colony stimulating factor
H. influenzae / HI	*Haemophilus influenzae*
H&E	Haematoxylin and Eosin
HLA	Human leukocyte antigen
IgE	Immunoglobulin E
IL	Interleukin
IQR	Interquartile range
IT	Immunotherapy
L-M	Lund-Mackay
MHC	Major histocompatibility complex
MRI	Magnetic resonance imaging

MS	Mass spectroscopy
OR	Odds Ratio
P. aeruginosa	*Pseudomonas aeruginosa*
P. chrysogenum	*Penicillium chrysogenum*
PA	*Pseudomonas aeruginosa*
PAMPs	Pathogen-associated molecular patterns
PCD	Primary ciliary dyskinesia
PNAG	poly-N-acetylglucosamine
RCT	Randomised controlled trial
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
S. aureus	*Staphylococcus aureus*
S. epidermidis	*Staphylococcus epidermidis*
SA	*Staphylococcus aureus*
SAE	*Staphylococcus aureus* enterotoxin
SAg	Superantigen
SCIN	*Staphylococcal* complement inhibitor
SCV	Small colony variants
SD	Standard deviation
SE	*Staphylococcus epidermidis*
SEA	*Staphylococcus aureus* enterotoxins A
SEB	*Staphylococcus aureus* enterotoxins B
SEC	*Staphylococcus aureus* enterotoxins C
SEM	Scanning electron microscopy

SNOT-20	Sino-nasal Outcomes Test 20
TCR	T cell receptor
TEM	Transmission electron microscopy
Th	T helper lymphocyte
TLRs	Toll-like receptors
TOF	Time of flight
T_{reg}	Regulatory T lymphocyte
TSLP	Thymic stromal lymphopoietin
TSST	Toxic Shock Syndrome Toxin
Vβ	Variable β

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Thesis summary

The research described herein follows an extensive literature review of the role of environmental agents and the host immune system in the manifestation of CRS. There are clear deficiencies in our understanding regarding the microbial flora of CRS patients and non-diseased sinuses. Chapter two describes a detailed study of the fungal and bacterial microbiome of diseased and healthy sinuses and forms a basis on which to build the subsequent research projects. The third and fourth chapters describe the development of two animal models to determine the environmental and host factors, which are associated with sinonasal fungal biofilm formation. The final chapter seeks to determine the relevance of sinonasal microorganisms by detecting them on host surfaces and correlating these with specific host immune responses. The interaction of bacteria and host hypersensitivities to allergens is also explored.

The initial investigation focused on understanding the microbial flora in CRS patients. This study forms a foundation for the thesis, and was critical to address the many deficiencies and contradictions in the published literature regarding the microbiome of CRS patients. We used state of the art microbial detection techniques to determine the presence and abundance of fungi and bacteria on the mucosa of CRS patients, and appropriate healthy control mucosa. This highlighted some cornerstones of microbial variability between healthy and diseased sinuses. We have shown that the healthy sinus is clearly not sterile, and that prevalence, but more importantly, species composition and population

density are critical factors in determining the disease state. Comparisons between various detection techniques such as molecular analysis, Fluorescence *in situ* hybridization (FISH), and conventional culture showed FISH to be highly sensitive and specific, with a detection threshold related to organism abundance, whereas culture has a tendency to select for rapidly growing organisms.

The subsequent study is detailed in chapter three, and addresses two of the most contentious, environment versus host issues in the CRS research community – the interaction between fungal organisms, and the host with type I hypersensitivity to fungi. We developed a large animal (sheep) model of fungal sinusitis to investigate these factors and successfully sensitized 45% of animals to fungal antigens, as evidenced by positive skin prick tests. Despite the presence of fungal hypersensitivity, we were unable to produce fungal biofilms in the occluded frontal sinus. Following our clinical observations of fungi frequently co-habiting with bacteria, particularly *Staphylococcus aureus,* we co-inoculated fungi with this bacterium and florid fungal biofilm formed on sinus mucosa. Type I hypersensitivity to fungi had no correlation with fungal biofilm or inflammation. These results suggested that fungi may not be able to form biofilm on mucosa with intact immune defences and a primary insult from the bacteria was requisite for fungal adhesion and proliferation.

A follow up study addressed the factors, which contribute to fungal biofilm establishment on sinus mucosa. An animal model was again developed to determine if co-inoculation of fungi with other bacterial species would allow fungi to proliferate. Four bacterial species commonly detected in CRS patients were

utilized. We hypothesized that bacterial induced cilial injury may have a role in allowing early fungal adhesion, and a cilia toxin was utilized to assess the effect of isolated cilial impairment on fungal proliferation. Cilia were assessed using transmission electron microscopy. Again, no fungal biofilm formed when fungi was inoculated in isolation. Three of the bacterial species formed bacterial biofilms in >75% of sinuses, and this was associated with significant cilial damage, and fungal biofilm formation. One of the bacterial species did not form biofilm, and no fungal biofilm formed in co-inoculated sinuses. Cilia toxin caused significant cilial injury, and was also associated with fungal proliferation. This study demonstrates the importance of the physiochemical barrier in defence against fungal organisms. This led to the question of the role of fungi in CRS patients – are they contributing to the inflammation or merely saprophytic colonizers of the impaired mucosa?

The final study addressed this question in a human subject cohort. To determine if microorganisms have a role in inflammatory processes, we need to be able to display an organism specific immune response in the host. We measured the organism specific IgE levels in the serum and mucosa of 48 CRS patients and 10 controls. We also determined the presence of these microorganisms on the mucosa using conventional culture, and FISH using specific probes. We showed that in CRSwNP patients, the presence of *S. aureus* and fungi on the mucosa was related to elevated organism specific IgE within the mucosa. This phenomenon was specific to nasal polyp patients, and was not observed in non-polyp CRS or control patients. This demonstrates that these organisms have the capacity to incite specific immune responses in the host, potentially contributing

to mucosal inflammation in CRS. Additionally we determined that the presence of *S. aureus* on the mucosa also exacerbates mucosal fungal allergy, potentially enhancing hypersensitivity to ubiquitous airborne fungal allergens. Although this mechanism has been observed in other atopic diseases, this is the first study to document the phenomenon in CRS. It adds to the mounting evidence that *S. aureus* has an important role in the pathogenesis of CRS.

Systematic review of the literature

chapter 1

1.1 CHRONIC RHINOSINUSITIS - BACKGROUND

1.1.1 The definition of rhinosinusitis

The term 'rhinosinusitis' describes a constellation of disease entities with a common feature - inflammation of the mucosa lining the nasal cavity and the paranasal sinuses[2]. There are multiple subtypes and distinct aetiologies, with varied severity and clinical presentations, which is further complicated by overlapping symptoms and pathology with other medical conditions[3]. For simplicity, the definition has been based on the duration of inflammation and symptoms – see Table 1.1 The classification of rhinosinusitis

Table 1.1 The classification of rhinosinusitis

Classification	Duration
Acute Rhinosinusitis	Up to four weeks
Subacute Rhinosinusitis	Between four & twelve weeks
Recurrent Acute Rhinosinusitis	Four or more episodes per year with complete resolution between episodes. Each episode lasts at least seven days
Chronic Rhinosinusitis	12 weeks or longer

The complexity and controversy surrounding rhinosinusitis is highlighted by the abundance of guidelines, which have been published in the last 10 years. Five separate multi-national expert panels have recently published guidelines addressing the definitions, diagnosis, and management of rhinosinusitis[2,4-7].

These documents have drawn on the evidence base of the published literature, as well as the expert opinions of many of the worlds leading otolaryngologists and immunologists. Whilst many of these guidelines are contradictory and limited in their conclusions[3], they provide clinical guidance, and form the basis for further research, to address many of the unanswered questions in this field.

Acute rhinosinusitis (ARS) is a common disorder affecting approximately 31 million people in the US annually with direct costs estimated at $US 3 billion[8]. It is frequently caused by an acute viral infection associated with the common cold, but bacteria have also been implicated in the primary infection but more commonly in a secondary infection[9]. The principal bacterial pathogens in ARS are *Streptococcus pneumoniae*, *Haemophilus influenzae, Moraxella catarrhalis* and *Streptococcus pyogenes*[10]. Key diagnostic criteria include symptoms following upper respiratory tract infection, purulent nasal discharge, unilateral maxillary sinus tenderness, maxillary tooth or facial pain (especially unilateral), and a history of initial improvement followed by a worsening of symptoms[9,11]. Other non-specific symptoms include malaise, halitosis, nasal congestion, hyposmia / anosmia, fever and cough. Complications are uncommon, occurring in an estimated 1 in 1000 cases, with the majority settling with empirical antibiotic therapy[12].

Whilst many of the symptoms are similar, understanding the distinction between the acute forms of rhinosinusitis and CRS has both clinical and scientific importance. In CRS the duration is greater than 12 weeks, and

whilst disease fluctuations occur, the signs and symptoms of CRS never completely resolve between these, setting CRS apart from ARS, subacute rhinosinusitis, and recurrent ARS. It is believed that CRS is not one disease but a complex of separate, but related entities with differing clinical and pathological manifestations, and applying a definition to these disorders is difficult. However, both clinicians and researchers require a broad definition to enable progress in this field. According to the most recent European position paper on rhinosinusitis and nasal polyps (EP^3OS)[2], the term rhinosinusitis describes inflammation of the nose and paranasal sinuses which is associated with two or more symptoms (see Table 1.2 EP^3OS Criteria for the diagnosis of CRS). These must include at least one of - nasal blockage, obstruction or congestion, or nasal discharge (anterior or posterior), and may include facial pain or pressure, and hyposmia or anosmia. These symptoms must be associated with objective signs of disease; either endoscopic and / or radiological (computed tomography) signs. Endoscopic evidence of polyps, mucopurulent discharge (mostly from the middle meati), mucosal oedema / obstruction are all considered important signs of CRS. Computed tomography may show thickened mucosa within the ostiomeatal complex and / or the paranasal sinuses[2].

Table 1.2 EP^{3}OS Criteria for the diagnosis of CRS

Symptoms Two or more for > 12 weeks	Special Assessment
Nasal blockage / obstruction / congestion*	Endoscopic assessment ? nasal polyposis†
Nasal discharge (anterior / posterior nasal drip)*	CT Sinuses†
Facial pain / pressure	Allergy Testing if history suggestive
Reduction or loss of smell	
* Must include at least one of these symptoms	† Must include at lease one endoscopic or radiological finding

1.1.2 Epidemiology

According to the National Health Interview survey, sinusitis (acute, and chronic types) affects 12.5% of the American population, or 31 million people in the US alone[13-15]. Data from a recent Australian national health survey has shown that 9.0% of Australians suffer from CRS symptoms[16]. Europe has a prevalence of 10.9% overall, but there is significant regional variation. For example, 6.9% of the population suffer from CRS in Brandenburg, Germany, compared to 27.1% in Coimbra, Portugal[17]. This contrasts with a rate of only 1% CRS reported in Korea[18]. These studies relied predominately of self-reporting of CRS symptoms, and the actual prevalence of CRS may be lower. However symptomatic CRS diagnosis has been shown to correlate well with self reported doctors diagnosis[19].

CRS has significant socioeconomic implications, with an estimated annual direct health care cost in the US of $US 5.8 billion, which results predominately from outpatient and emergency department attendances, as well as approximately 500,000 surgical procedures on the paranasal sinuses annually[20]. Indirect costs related to CRS from decreased productivity and work absenteeism are even greater, resulting in an estimated 73 million days of restricted activity annually in the US[7].

Patients with CRS visit primary care clinicians twice as often as those without the disorder, and have five times as many prescriptions filled[7]. As a proportion of all ambulatory care attendances, CRS comprises 1.39%, and ARS 0.3%[21]. CRS is the principal diagnosis in 14 million visits to a health care facility in the US, compared to 3 million for ARS[22]. CRS is therefore, the second most prevalent chronic health condition in the US population[13-15]. It is extremely detrimental to the quality of life of those suffering from it, and quality of life measures are similar or worse than chronic obstructive pulmonary disease (COPD), chronic back pain, and congestive cardiac failure[23].

1.1.3 CRS subclassification

The complexity of CRS is exemplified by the lack of consensus as to the categorization of the disease. The simplest, and most commonly used classification is based on the presence or absence of nasal polyps[5]. 'Polyp' in the context of CRS, describes the macroscopic appearance of a grape-like tissue projection pedicled from a mucosal surface, most often originating from the middle meatus / ostiomeatal complex. They are usually soft and

shiny, somewhat translucent, with a blue-grey appearance. Polyps are composed of benign, non-granulomatous inflammatory tissue, with loose connective tissue, inflammatory cells, oedema, glands and capillaries[24]. They are usually covered with ciliated pseudostratified epithelium with goblet cells. Eosinophils are the most common leukocyte within nasal polyps, however they can also contain neutrophils, mast cells, lymphocytes, monocytes, and fibroblasts. The eosinophil content of nasal polyps is greater in the presence concomitant asthma, aspirin sensitivity, or both[25,26]. Interestingly eosinophil predominant polyps are seen in 80% of CRS patients in Western countries, however Asian patients, and patients with cystic fibrosis with nasal polyposis have a predominantly neutrophilic cellular infiltrate[27]. This may have important implications for treatment, as eosinophilic polyps are thought to be more responsive to corticosteroid treatment than non-eosinophilic polyps[28]. The symptom patterns of CRS can be different between polyp and non-polyp CRS with CRSsNP patients often complaining of anterior and posterior nasal discharge and pain, whereas CRSwNP patients may complain more of nasal obstruction and anosmia, however clearly differentiating the diseases based on symptoms lacks accuracy, and such measures are best used to assess individual response to therapy[28]. The prognostic implications of nasal polyps are conflicting. The presence of nasal polyposis was shown to be the most important predictor of poor outcome following ESS in a 5-year prospective outcomes trial[29]. Another study showed that CRSwNP patients have significant symptomatic improvement following ESS, but again noted a higher revision rate than CRSsNP[30]. Contrary to these findings, another group found that the success rate (based on symptom reduction) was higher in CRSsNP

patients but this was not statistically significant, in a study of 132 patients[31]. Other studies have likewise found no adverse effect of nasal polyps on post-surgical outcomes[32,33].

Apart from nasal polyposis, other important differentiating factors include the presence or absence of eosinophilic mucus, and fungal hyphae or bacteria within sinus mucus[5]. A histological sub-classification based on the presence or absence of eosinophilic mucus (EM) has been proposed[34]. EM consists of necrotic eosinophils, mixed inflammatory infiltrate, and Charcot-Leyden crystals – the byproduct of eosinophils[34]. The presence of EM or peripheral eosinophilia has been shown to be a predictor of the need for revision surgery in a small study of 56 patients[35]. EM-CRS patients are often sub-classified based on the presence or absence of fungal elements in the mucus, and fungal allergy. However a recent study has found no distinction between these subgroups on clinical or immunological grounds[36].

It is clear that accurate subclassification of CRS is clinically important. It will enable us to tailor treatment regimes to our patients, and provide accurate prognostic information. It will also enable the development and utilization of targeted therapeutic strategies. Hopefully, in the future, we will categorize CRS patients according to genotypic or phenotypic characteristics, allowing us to target specific inflammatory pathways with immunomodulatory medications, such as the emerging therapies for treatment of inflammatory bowel disease, and other autoimmune diseases.

Subclassification is also critical to direct further research into the disease, as critical pathogenic differences may be missed if disease subsets are not analyzed separately. Much of the current research effort into CRS may provide pathological characteristics, which can be used to classify patients. These can be broadly classified as environmental or host factors. Environmental factors which may be important include microorganism presence and abundance, including bacteria and fungi, encompassing biofilm phenotypes, and superantigen production and possibly viruses. Other environmental factors may include aeroallergens, air pollution, and cigarette smoking. Host specific factors may include comorbid conditions which interact with, or pre-dispose to CRS including, asthma, aspirin sensitivity, cystic fibrosis, cilia motility disorders, and certain congenital and acquired immunodeficiency states. Other host factors which may form a basis for future disease subcategorization include genetic susceptibility, autoimmune states, including certain human leukocyte antigen (HLA) subtypes, systemic or local immunodeficiency (both innate & adaptive) including defects in inflammatory pathways, defective immune surveillance, or tolerance due to for example, abnormal regulatory T cell function, allergy (type I hypersensitivity) either systemically or locally within the mucosa, and epithelial barrier defects including mucus abnormalities, and cilia dysfunction. The current research examining many of these factors is discussed in 1.3 - Host factors in CRS. Allergic fungal rhinosinusitis (AFRS) is a specific subclassification of CRS based on environmental (presence of fungi) and host (fungal allergy) factors, and will be discussed in section 1.1.5.

1.1.4 Non-surgical management of CRS

Medical treatments for CRS are first line therapies to be considered prior to any surgical intervention. In severe forms of CRS, they are used to reduce symptoms and delay recidivism, however no single treatment modality has been shown to have durable results in preventing disease recurrence and the need for revision surgery. Systemic anti-inflammatory agents such as corticosteroids have been shown to reduce polyp size & decrease intra-operative bleeding[37,38]. Post-operative systemic corticosteroids may also prevent early polyp recurrence[39], however long term use is limited by significant side effects. Despite their widespread use, no ideal treatment dose or duration has been agreed upon. Intranasal corticosteroids (INCS) have minimal side effects and can reduce sinonasal inflammation and can reduce polyp size[40], and are currently considered the medical treatment of choice for nasal polyposis[41]. A pilot uncontrolled study suggested 0.5mg of budesonide in >100mL of saline for twice daily nasal douching can improve symptoms and CT sinus scores in patients with EM CRS[42]. Two large, multicentre placebo controlled trials showed efficacy of mometasone furoate in providing lasting symptom relief compared with placebo for nasal congestion, anterior rhinorrhoea, and post-nasal drip scores in nasal polyposis patients[43,44]. Symptom relief can commence as soon as two to five days after initiation of therapy[45]. However, despite their efficacy, a large proportion of patients with nasal polyposis will continue to have significant symptoms whilst using INCS[46]. Leukotriene receptor antagonists have been employed due to their favourable side effect profile, aiming to reduce systemic steroid use, but efficacy is lacking[46]. Macrolide antibiotics have also

been used for their anti-inflammatory action, with a recent trial comparing methylprednisolone, doxycycline and placebo suggesting both agents reduced polyp size, symptoms, and markers of inflammation[47]. Methylprednisolone had a greater effect, which lasted 8 weeks, whereas doxycyline's benefit lasted for 12 weeks.

1.1.5 Allergic Fungal Rhinosinusitis (AFRS)

1.1.5.1 Epidemiology of AFRS

AFRS is the most common type of fungal rhinosinusitis[48], and accounts for between 7 and 12% of CRS cases which require operative intervention in the US[40,49]. The incidence of AFRS in a similar cohort of Australian CRS patients was 9%[50]. AFRS has significant regional variation with warmer climates having a much higher incidence[48,51]. The incidence is highest in adolescent and young adult males, with a male to female ration of 1.6:1 [38,52]. Affected individuals are immunocompetent and have a history of atopy. By definition[5,53], all patients have fungal allergy by skin prick testing or in vitro testing however, only 2/3 of these will report a history of allergic rhinitis[52].

1.1.5.2 Clinical presentation of AFRS

The symptoms of AFRS are often subtle in the early stages of disease. Patients often delay seeking medical attention for the rhinosinusitis symptoms until the advanced stages, when complete nasal obstruction, visual disturbance or facial distortion are noted[40]. Symptoms are frequently unilateral, and may include the production of nasal casts of dark, thick mucus drainage. The accumulation of inflammatory mucus and polypoid material can lead to sinus obstruction, erosion of bone, rupture of the sinus wall, and

prolapse of sinus contents into the orbit and brain. Proptosis and less commonly, hypertelorism can be seen at initial presentation, especially in younger patients in whom sinus structures may not have fully calcified[54]. Endoscopic examination may reveal extensive polyposis, which is often asymmetric, and mucus is usually evident between the polyps.

The natural history of AFRS suggests a recurrence rate following treatment between 10 and 100%[38]. Universal recurrence was noted in one study following endoscopic sinus surgery where rigorous post-operative medical therapy was not instituted[55]. A longitudinal study over 7 years suggested AFRS patients undergo an average of two surgical procedures, and three courses of systemic corticosteroids per year, and despite being symptom free, endoscopy suggests that ongoing polypoid inflammation persists in many patients[56].

1.1.5.3 Diagnostic criteria for AFRS

Allergic fungal rhinosinusitis (AFRS) is a distinct clinical entity, however much controversy exists over the clinical criteria for its diagnosis[53,57-59]. Whilst no consensus exists, there is general agreement regarding most aspects. A combination of clinical, radiographic microbiological and histopathologic criteria are required, hence it is a diagnosis that can only be made post-operatively. A number of authors have proposed specific diagnostic criteria, the most widely accepted being that of Bent & Kuhn[53] (See Table 1.3).

Table 1.3 Diagnostic criteria for AFRS (Bent & Kuhn, 1994)

1	Type I hypersensitivity to fungi
2	Nasal polyposis
3	Characteristic CT scan findings (double densities)
4	Eosinophilic mucus, Absence of fungal tissue invasion
5	Positive fungal stain/culture of sinus contents

Arguably the most important criterion is the presence of eosinophilic mucus, often termed ‘allergic mucus’ or ‘fungal mucus’. On gross examination it is darkly coloured, thick and tenacious, and can often appear similar to peanut butter, however microscopically they are distinct. It is characterized by laminations of degraded eosinophils on a background of mucus often including the eosinophil breakdown product of Charcot-Leyden crystals (lysophospholipase) which appear as long needle-shaped, or bipyramidal eosinophilic crystals. Fungal hyphae are present but may be scarce, if not seen they should be cultured and may require specific staining for identification. Despite reports of heterogeneity within patient samples, evidence of fungi invading tissue is still considered an exclusion criterion for AFRS. The most disputed diagnostic criterion has been the presence of fungal allergy. Only 2/3 of patients had systemic fungal sensitization to the actual fungal species cultured as reported in a study by DeShazo & Swain, and they concluded that if not universal, they should not be a criterion for diagnosis[57]. In stark contrast another group detected fungi in 97% of CRS patients regardless of atopic status, and determined that all CRS was due to fungi, and hypersensitivity was irrelevant[60]. Despite this controversy, a

collection of international experts formed the Rhinosinusitis Taskforce (American Academy) and concluded that AFRS is defined by the presence of eosinophilic mucus and type I hypersensitivity to fungi[5].

1.1.5.4 Radiologic features of AFRS

The characteristic radiologic findings of AFRS form an important component of the diagnostic criteria[53]. CT scans often show multiple opacified sinuses, often with central hyperattenuation. Sinus expansion, with bony erosion or effacement with a pushing border is commonly seen, especially involving the lamina papyracea or skull base. AFRS tends to cause bone erosion more commonly than other forms of CRS and such radiographic findings along with nasal polyps, should raise suspicion of the diagnosis. In one study, more than 50% of AFRS patients had radiographic evidence of skull base erosion or orbital extension, compared to approximately 5% of other CRS cases[61]. MRI may be indicated when there is concern for intracranial or intraorbital invasion and complications. Eosinophilic mucus has a high protein and low water content, hence involved sinuses have central low signal on gadolinium enhanced T1, and T2 sequences with peripheral high signal corresponding to inflamed mucosa[62].

1.1.5.5 Immunotherapy for AFRS

Allergen immunotherapy (IT) has been used in diseases associated with IgE mediated hypersensitivity such as asthma and allergic rhinitis[63,64]. Immunotherapy appears to be safe in AFRS patients[65], and has been proposed to decrease the reliance on systemic corticosteroids[66]. One study compared IT treated AFRS patients with non-IT treated AFRS patients over

33 months, and showed improved mucosal appearance, lower symptom scores and less reliance on topical and systemic corticosteroids[67]. Clearly a randomized, placebo controlled trial is needed, however these studies suggest an important role for IT in the treatment of AFRS.

1.1.5.6 The universal fungal theory - implications for therapy

Following the finding of fungi in 97% of CRS patients in one study[68], two uncontrolled trials of the topical antimycotic agent, Amphotericin B were undertaken, both showing clear efficacy in symptom reduction and objective improvement in endoscopic and radiological parameters[69,70]. A subsequent randomized, double blinded placebo controlled trial (RCT) showed similar results for the objective measures though no symptomatic improvement[71], and the research group concerned propagated a theory of universal fungi pathogenesis in CRS[72]. A battery of RCTs followed, analyzing the efficacy of topical[73-76] or systemic[77] antifungal therapies in CRS patients. The findings were recently summarized in a Cochrane review which reported there is no evidence of efficacy of anti-fungal therapy in CRS patients[78]. Based on this, many commentators have dismissed fungi as a mere bystander, with no role in the pathogenesis of CRS[79,80]. There are problems with such comprehensive claims, however. The underlying hypothesis for these studies has been that fungi have a universal role, contributing to all CRS, hence the presence of fungi within the sinuses, or indeed the other diagnostic features of allergic fungal sinusitis[53] were not used as inclusion criteria. Importantly, one of the trials specifically excluded AFRS patients[75]. Studies assessing the efficacy of antifungal therapy in appropriately selected patients are few, but suggest subjective and objective benefit[81-83]. However, until larger studies

are performed with rigorous methodology, which include patients with diagnostic features of AFRS, the efficacy of antifungal therapy will remain unknown.

1.1.5.7 Surgical management of AFRS

Surgery is required in the great majority of AFRS cases because whilst intensive medical treatment can reduce polyp volume, high grade, obstructing polyposis and tenacious eosinophilic mucus rarely respond. In times past, external approaches, followed by aggressive mucosal stripping was regularly performed[84]. Contemporary procedures involve mucosal sparing endoscopic approaches, with removal of polypoid mucosa and anatomical obstruction, restoring sinus drainage pathways, followed by complete evacuation of eosinophilic mucus. Care must be taken especially when normal surgical landmarks, and bony barriers have been distorted or destroyed by disease. Incomplete removal of obstructed cells, and mucus is a risk factor for early recurrence[85].

1.2 ENVIRONMENTAL FACTORS IN CRS - MICROBIAL AGENTS

1.2.1 Introduction

The aetiology of chronic rhinosinusitis is complex and multifactorial, and the historical concept of subclinical or chronic infection has been replaced by a concept of chronic mucosal inflammation. In some cases, clear genetic or systemic host disorders such as cystic fibrosis, Wegener's granulomatosis or primary ciliary disorders are identified as causes for persistent mucosal inflammation. However, the overwhelming majority of cases remain idiopathic. A plethora of possible contributory environmental and host factors have been described, including ostial obstruction, impaired mucociliary clearance, genetic susceptibility, osteitis, allergy, airborne irritants including cigarette smoking and air pollution, microbial organisms including biofilms and secreted superantigens[1].

CRS is increasingly recognized as a heterogeneous disease with respect to clinical phenotypes, immunopathology, and response to therapy, which calls for better understanding of the disease and improved treatments. The relative contribution of host factors and environmental triggers is unknown but much research is focusing on this issue. A large body of research has focused on potential external (environmental) triggers, including bacteria, fungi, biofilms, superantigens, and aero-allergens. However it is clear that of

these environmental factors studied, none have been universally attributed to disease causation.

CRS was originally believed to be an infectious disease, primarily caused by pathogenic bacteria. However, it became clear with time, that this was a disease which could not fulfill Koch's postulates[86] for a disease caused by microorganisms and many came to believe it was primarily an inflammatory disorder[5]. Classical methods for the study of bacterial pathogens have proven to be inadequate to inform with respect to chronic diseases, which are increasingly recognized as polymicrobial[87]. The effort to develop antibiotics has been directed solely at the planktonic minority (associated with systemic illness), which explains our inability to eradicate chronic infections[87]. Despite this, CRS patients do experience symptomatic improvement following antibiotic use[88,89], although this result is generally not durable, with recurrence of symptoms following cessation of the agent.

Historically, the role of fungi in CRS pathogenesis has been limited to AFRS, however, this was recently expanded, predominately based on literature that suggested an excessive mucosal inflammatory response to common airborne fungi (specifically *Alternaria alternata)* was the universal cause for all CRS[60,90] (See Section 1.1.5.6). This was supported by experiments demonstrating *A. alternata* as a trigger for cytokine release from peripheral blood mononuclear cells from CRS patients but not controls[90], and others demonstrating *A. alternata* as the target of eosinophil chemotaxis, and degranulation[91], with resultant potential for tissue destruction[92].

The following discussion will examine the current research regarding the role of bacteria and fungi in CRS, including controversy regarding the flora of diseased and non-diseased sinuses, contemporary microbial detection techniques, virulence factors which impede microbial detection and promote persistence and damage to the host.

1.2.2 Microorganisms in disease - historical perspectives

The processing and interpretation of nasal swabs from CRS patients by laboratories tasked with determining the organisms present, has been shaped by the historical development of diagnostic microbiology. Who made the earliest observation of microorganisms is debated today, but the microscope was available in the mid-1600s and Robert Hooke, an English scientist made early observations. In the 1670s, a Dutch student of natural history and maker of microscopes, Anton van Leeuwenhoek, made observations of microscopic organisms and termed them ‘animalcules’, from the Latin meaning little animals. He is regarded as one of the first to accurately describe fungi and bacteria.

In the 1800s, Louis Pasteur performed numerous experiments to discover why dairy products and wine soured, and discovered bacterial contaminants were responsible for this reaction. He wondered similarly if they could cause illness in humans. He pioneered the germ theory of disease, which states that microbes are the cause of infectious disease[93].

The German scientist Robert Koch earned a Nobel Prize in 1905 by providing evidence for the germ theory of disease; cultivating organisms from anthrax affected mice in the laboratory, and injecting them into healthy animals, proving that the organism caused anthrax disease. These experiments were the genesis of Koch's postulates, which provide a set of principles designed to establish a causal relationship between a microorganism and certain diseases[86] (See Table 1.4).

Table 1.4 Koch's Postulates

1	The microorganism must be found in abundance in all organisms suffering from the disease, but should not be found in healthy organisms.
2	The microorganism must be isolated from a diseased organism and grown in pure culture.
3	The cultured microorganism should cause disease when introduced into a healthy organism.
4	The microorganism must be re-isolated from the inoculated, diseased experimental host and identified as being identical to the original specific causative agent.

The philosophy of isolating a single organism and relating it to disease, hence fulfilling Koch's postulates, has shaped microbiological methods throughout their development. This process can be entirely appropriate in many cases, with diseases such as septicaemia or meningitis attributable to a single organism isolated on culture. Increasingly however, we are

discovering that many infections are polymicrobial, and axenic culture has been polarizing our understanding of these[94]. In diseases such as CRS, where the host mucosa is in constant exposure to the environment, polymicrobial flora are common, and fulfilling Koch's postulates, and finding a distinction between pathogen and commensal becomes incredibly complex.

1.2.3 Microbial detection in CRS – culture based techniques

Accurate information regarding the presence of organisms in our patients has important implications for clinical practice, as well as for research, where we endeavor to determine the pathogenic importance of organisms in CRS. Traditional culture dependent techniques have been the workhorse of microbial diagnostics in CRS. However, despite decades of CRS research using clinical microbiological culture techniques, the role of microbes in this disease is incompletely understood. Between 10 and 53% of all CRS specimens sent for routine microbiological culture result in no growth of organisms[95,96]. Additionally, samples of overt purulence are frequently reported as sterile following culture, prompting questions over the sensitivity of these techniques in CRS[97,98].

The process of isolating an organism for culture detection necessitates that it be separated from the polymicrobial milieu using a process of selection using enrichment culture – providing conditions favourable for growth, whilst excluding other species. This principle forms the basis of traditional culture based microbial detection, and persists today in modern diagnostic microbiological laboratories. By it's very nature, culture based diagnosis of

aetiological agents relies on our ability to provide the appropriate conditions for *in vitro* growth of the organism, including specific oxygen tension, energy sources, major and trace elements, pH and temperature. This implies a degree of selection bias, as one must choose which media and conditions to use, and therefore which organism(s) to select for. Clearly to identify all of the organisms in a CRS sample using these techniques would be exhaustive. Most clinical laboratories use guidelines, which determine how a sample will be processed, based on the most 'pathogenic' organisms to be detected,[99]. The choice of target 'pathogen' organisms which were used for the current guidelines is based on a single microbiological study of CRS, in which, over 50% of the patients had been using antimicrobial therapy in the month preceding sample collection[100]. Whilst guidelines are essential to ensure laboratories can perform diagnostic analysis within reasonable time and cost constraints, the predetermination of organisms may be significantly skewing our understanding of the diversity of microbial flora in our patients, and controls.

Many organisms are unable to thrive on nutrient media once removed from the native environment, and the advantages of polymicrobial communities, and mutualistic relationships are lost. Environmental changes experienced by organisms being transferred to growth media include significant alterations in temperature, pH, and nutrient sources, which may significantly impact the capacity to thrive, and hence reduce detection sensitivity. The reliance of cultivation on nutrient media can also result in 'enrichment bias' with detection of a narrow range of microbes which is not representative of the

diversity present[101]. The competition between organisms, using such methods can result in dominance of the fastest growing organisms, further polarizing the results[101]. In a study to culture the complete diversity of a microbial community from an oral site using multiple chemically diverse media types, and aerobic and anaerobic conditions, only a maximum of 50% were recovered despite exhaustive efforts [102].

1.2.4 Microbial detection in CRS – molecular techniques

The paradigm of organism cultivability has been central to the practice of microbiology since it's inception. However, in this molecular age it is now possible, and indeed necessary to move beyond this traditional methodology and attempt to unravel the complexity of chronic polymicrobial infections[103]. Additionally, the long-standing use of culture–dependant techniques for microbial characterization, has led to considerable confusion regarding the prevalence, and importance of organisms in CRS. The occurrence of sterile results from a swab of mucopurulence from the sinuses is a common occurrence in clinical practice[98]. There has been an increasing awareness of the role of complex polymicrobial communities in chronic diseases, which occur at the interface between the host and the environment, such as mucosa, and the majority of these species are refractory to culture[104]. Microbial biofilms, were recognized as a common cause of persistent infections, and were detected in CRS, and many of these organisms were resistant to culture[105]. Fortunately, the growing impetus for more accurate characterization of these fastidious organisms in CRS has been paralleled by

advances in molecular biological detection techniques. Detection methods that target microbial nucleic acids have revolutionized modern microbiology. Such techniques rely on the specificity of nucleic acid sequences, which act as a fingerprint for any given organism.

The advent of PCR (polymerase chain reaction) amplification has provided a basis for the development of assays to exploit differences in DNA sequences. Two different approaches depend on the specificity of the technique. Broad focused approaches amplify kingdom specific DNA sequences, and then certain methods are employed to determine which species the sequences correspond to. Narrow focused methods use primers, which are specific for certain species to determine the presence or absence of that organism in a sample. The fundamental difference between these techniques, is broad focused techniques do not require *a priori* selection of microbial targets[106]. Both narrow focused molecular methods, and conventional culture require pre-analysis decisions regarding which species to search for.

The initial process therefore involves extraction of DNA / RNA from the organisms. This is most efficiently performed, when preceded by a physical disruption phase, by bead beating and use of specialized buffers and proteases to lyse the cells[106]. The nucleic acids are purified, followed by amplification using PCR.

Narrow focused molecular methods use oligonucleotide primer sets, which match a target region within the DNA of the organism in question. In other words, one first decides which organism (s) to target, and if present, the specific primers will bind to, and amplify the sequence, thus identifying the presence of the organism. This technique is extremely sensitive to small numbers of organisms, including within the context of a large population of non-targeted organisms[106]. This is particularly useful if only searching for one or two organisms within a polymicrobial population, and provides a much higher resolution of bacterial detection and identification than that provided by culture[103]. Thus whilst these culture independent approaches provide significant improvements in accuracy, the use of species specific PCR techniques is equivalent to the use of selective media in culture dependent approaches. They still require a prediction to be made as to which agent is likely to be associated with a particular sample, and have a practical limit as to the number of species specific assays that can be performed[94,106].

Broad focused molecular methods use specific oligonucleotide primers to amplify specific regions of DNA which are used as a phylogenetic fingerprint, most commonly those which encode for - 16S rRNA for bacteria[106], and 18S, 5.8S and 28S rRNA for fungi[107]. Initial broad focused techniques for microbial identification following DNA extraction & purification, used denaturing gradient gel electrophoresis (DGGE) which separated the DNA from a sample based on its ability to move through an acrylamide gel containing a denaturing agent which causes the DNA strands to melt (split). The resulting strands travel different distances through the gel based on the length,

nucleotide ratios, and charge of the DNA sequence. This is a laborious process but results in relatively high separation of DNA sequences to a resolution of 200-700 base pairs. The distinct bands within the gels can then be removed and analyzed by 454 sequencing[106]. Such sequences can be compared to known species, using tools such as the BLAST database[108].

A more recent development in broad focused molecular diagnostics has been the Ibis T5000 Biosensor. Following imminent threats of bioterrorism, The US Department of Defense commissioned the search for new methods for the rapid detection and identification of microbial species, as the molecular methods at the time were seen as too time consuming and cumbersome[106]. Ibis developed a novel strategy whereby amplicons, which were amplified by PCR, would be weighted by mass spectroscopy and their precise mass would be used to calculate the nucleotide base composition. Electrospray ionization (ESI) is used to gently separate DNA strands as they enter the time of flight mass spectrometer. The calculated nucleotide base composition is then compared with a database of known base compositions to determine the identities of any microorganisms that are present[109]. The Ibis biosensor has been shown to be extremely sensitive and specific for organisms from different kingdoms including bacteria, fungi, and viruses[109,110]. Additionally, the Ibis T5000 biosensor can provide crucial information on relative abundance of organisms, as well as antimicrobial resistance, such as the *mecA* gene for MRSA[109]. Additionally, such molecular based methods confer a much greater level of sensitivity of detection compared to conventional culture[111]. This can be particularly critical

when attempting to detect uncommon or fastidious bacteria[112]. This increased sensitivity can also be crucial when specimens have been collected after antibiotic administration, or transportation conditions have been sub-optimal[113]. Another significant advantage of broad based molecular detection techniques such as the Ibis T5000 has been in relation to the analysis of samples regarded as culture negative. Multiple studies have shown that such techniques can secure a diagnosis where traditional culture has failed[106,114]. Broad focused molecular methods offer great potential for analysis of microbial diversity in CRS, which is untempered by the limitations of conventional culture. Access to molecular detection instrumentation and expertise may limit it's utility in the clinical setting, but this is rapidly improving.

1.2.5 The bacteriology of chronic rhinosinusitis

The medical management of CRS usually involves the use of antimicrobial therapy, often empirically. The basis for antibiotic selection is often extrapolated from ARS, a disease which has well defined microbial aetiology[115]. However, applying these treatment protocols to CRS may be flawed. The relationship between microbial agents and disease is not clearly established in CRS. ARS is often treated successfully with empiric antimicrobial treatment directed towards the three most commonly cultured organisms, *S. pneumoniae, M. catarrhalis*, and *H. influenzae,* however, many CRS patients continue to have symptomatic disease despite repeated administration of antimicrobial agents[116]. Characterizing the microbial flora

common to CRS patients was determined to be of high importance to improve antimicrobial therapy in CRS[117].Unfortunately, despite extensive research involving predominately microbiological culture methods, the bacteriology of CRS remains uncertain[118].

It has been suggested that the microbiological dynamics of CRS change over time, with a predominance of aerobic organisms in the first few months, with gradual replacement by anaerobes in the longer term, as sinuses become more obstructed and oxygen levels and pH fall[119]. Selective pressure from antibiotics directed predominately at aerobic species may also have a role[100].

Some studies have stressed the predominance of anaerobic species in CRS[120-124] whilst others have found little or no role[118,125]. More recent studies highlight the importance of both anaerobes and aerobes in CRS[126]. Anaerobic bacteria possess many virulence factors, which may be important in chronic infectious states. These include the production of catalases, immunoglobulin proteases, superoxide dismutase, collagenase and fibrinolysin. Encapsulation of anaerobes is common, and can also confer a survival advantage by increasing adhesion, providing oxygen tolerance, and resisting opsonization and phagocytosis[127].

The use of antimicrobial therapy in these patients, particularly those with recalcitrant disease who may have multiple courses, may also impact on the flora of the sinuses. Some authors propose therapy for up to 10 weeks

continuously[119]. Antibiotic resistance rates as high as 21% have been reported in CRS patients[118]

The heterogeneity of results from microbiological studies may be explained partly by variations in disease type and severity, revision surgery rate, sample type, site, contamination by skin and vestibule flora, the uncontrolled use of medications including antibiotics and corticosteroids, transportation, and processing methods in the laboratory[128]. Some studies have examined mucus samples via swabs[129], others have used mucosal biopsies in an attempt to reduce contamination[115,126], whilst others have used inferior antrostomy [100] or canine fossa approaches to sample maxillary sinus contents[130]. One study in children showed a good correlation between culture results from the maxillary sinus and the middle meatus[125].

Atypical bacteria such as *Mycoplasma pneumoniae, Chlamydia pneumoniae,* and *Legionella pneumophila,* are resistant to culture and have been associated with chronic infection of the lower respiratory tract and chronic asthma[131,132], and a potential role for such organisms in CRS is compelling. However, these bacteria have been investigated in a small number of CRS patients using PCR techniques, and were not detected[133].

1.2.6 The bacteriology of non-diseased sinuses

Knowledge of the composition of the normal sinonasal flora in non-disease states is critical to understanding the importance of microbial populations in disease. Whilst normal flora can potentially be hazardous, they can also be

beneficial partners, by protecting the host from colonization, or subsequent invasion by potentially virulent bacteria[117]. The literature again, is ambiguous in this regard. Many reports of the bacteriology of CRS have not included control tissue, which limits the capacity to interpret them accurately. Many investigators have reported that all sinuses of healthy controls are completely sterile[134-137], whilst others have suggested that the majority are sterile[138]. Others reported that aerobic species existed as normal flora in non-diseased sinuses, but anaerobic organisms were only present in chronically inflamed sinuses, concluding therefore that anaerobes were the hallmark of disease[124]. One critical limitation of many of these studies is the definition of control tissue. Most samples were taken as part of a diagnostic or therapeutic procedure in symptomatic patients. Radiology and symptom scores have not been routinely reported. One study used canine fossa puncture during a diagnostic procedure and 'normal' mucosa was based on the lack of mucus, and patent ostia, even if patients had a history of CRS[130]. The use of such patients in studies examining the flora of normal sinuses is clearly misleading. Valid control tissue must satisfy these criteria:

Table 1.5 Criteria for control sinonasal tissue

1	Absence of rhinological symptoms as assessed by a validated rhinological symptom battery
2	Absence of radiological (CT) evidence of sinus disease (Lund-Mackay Score = 0)
3	Absence of endoscopic evidence of sinusitis / mucosal inflammation (Lund-Kennedy Score = 0)
4	Absence of previous nasal surgery
5	Absence of co-morbidities which may affect the sinonasal environment (e.g. Diabetes mellitus, cystic fibrosis, Kartagener's syndrome, Asthma)
6	Absence of antimicrobial therapy for preceding 3 weeks

Brook reported the first study of control patients, who were undergoing nasal septal surgery, reporting the presence of aerobes and anaerobes in the maxillary aspirates. However controls were recruited based on the absence of symptoms only, and sinus disease was not excluded endoscopically or radiologically[139]. Additionally, those with a septal deviation symptomatic enough to require surgery arguably should be excluded as the symptoms of nasal obstruction and CRS can overlap. Additionally some septal deflections, depending on location, may efface the ostia. A recent study enrolled patients undergoing Le-Fort I osteotomies for orthognathic surgery, and used strict exclusion criteria similar to Table 1.5.

Using rigorous asepsis, and aerobic and anaerobic culture methods, more than 80% of maxillary aspirates were found to be sterile[140]. Organisms recovered included coagulase negative *Staphylococci,* and *Citrobacter fundii.* A study employing both culture and PCR methods detected organisms in most control sinuses, including aerobic and anaerobic species[141]. Unfortunately endoscopy, symptoms scores, and co-morbidities were not recorded.

1.2.7 The mycology of chronic rhinosinusitis

The first published record of fungal involvement in sinonasal disease was in 1965 when Hora identified two different clinical presentations in patients who cultured fungi from the mucus; one had symptoms indistinguishable from chronic bacterial sinusitis, whilst the other had a fungal mass which eroded bone and spread like malignancy into adjacent tissues[142]. The invasive capacity of sinonasal fungi was further described following histopathological analysis[143,144]. In 1971, researchers discovered that 10% of their allergic bronchopulmonary aspergillosis (ABPA) patients suffered from nasal polyps, and were productive of nasal plugs similar to those expectorated from the lung[145]. Culture of these nasal plugs grew *A. fumigatus*. Additionally, 41% of the ABPA patient group had partial or complete maxillary opacification on plain X-ray[145]. The first description of the symptoms of rhinitis in ABPA patients was reported in 1975[146], and the following year, a patient with ABPA, who suffered from severe nasal obstruction, improved dramatically following a course of oral corticosteroids[147]. Two years later, Young et al, reported a

case study, describing a young patient with sinusitis, nasal polyposis, and proptosis due to extension of fungal material into the orbit[148]. A third manifestation of fungal disease, with a rapid and malignant course in an immunodeficient patient, was described in 1980[149]. In 1983, the first series of patients suffering from CRS and having fungi isolated from the mucus was reported[58,150]. A sinonasal disease with distinct similarities to ABPA was described in 1981 by Miller et al[151], and again in 1983 by Katzenstein et al[58,150], who coined the term 'allergic *Aspergillus* sinusitis' when describing the thick, tenacious, eosinophil rich mucus filling the sinuses, along with dense polyposis. Following the discovery of other fungal organisms in this disease it was renamed allergic fungal rhinosinusitis (AFRS)[53,152-154]. In an attempt to provide clarity for clinicians and researchers, the International Society for Human and Animal Mycology convened a working group to attempt consensus on terminology and disease classification of fungal rhinosinusitis[155]. Though much confusion still exists, this classification describes six forms of fungal involvement in sinonasal disease, based on histopathological evidence of invasion, and the condition of the immune defences (See Table 1.6).

Table 1.6 The classification of fungal rhinosinusitis

Disease Type	Tissue Invasion	Immune Competence
Acute invasive (fulminant) FRS	Yes	Immunodeficient
Granulomatous invasive FRS	Yes	Immunocompetent
Chronic invasive FRS	Yes	Immunodeficient
Saprophytic fungal infestation	No	Immunocompetent
Fungal Ball	No	Immunocompetent
Fungal-related eosinophilic FRS including AFRS	No	Immunocompetent

Acute invasive (fulminant) fungal rhinosinusitis is a disease characterized by a rapid time course (<4 weeks) with histopathological evidence of vascular invasion by fungal hyphae. Vasculitis with haemorrhage, thrombosis, necrosis and neutrophillic infiltrates are characteristic[156]. The most susceptible populations are those with neutropenia, such as those with haematological malignancies, aplastic anemia, uncontrolled diabetes mellitus, those undergoing chemotherapy for malignancy, or immunosuppression for transplantation [156-158].

Granulomatous invasive fungal rhinosinusitis has a longer time course of >12 weeks and is characterized by an enlarging mass in the orbit, nose or sinuses in the immunocompetent host, with proptosis as the most common clinical feature[155]. It is seldom seen outside of Sudan, India, Pakistan, and Saudi Arabia[159,160]. Histopathological analysis shows fibrosis, and a non-

caseating granuloma with giant cell formation, vasculitis, and angiogenesis. *Aspergillus flavus* is the fungal agent commonly associated with this disease, but is rarely present in great quantity[161].

Chronic invasive fungal rhinosinusitis is a destructive disease which occurs slowly over a period of >12 weeks. It involves predominately the sphenoid and ethmoid sinuses, and in contrast to the granulomatous form, fungal hyphae (predominately *A. fumigatus*) tend to be dense[160]. Vascular invasion is seen occasionally, and the inflammatory reaction is limited. Immunodeficiency is requisite, and is usually related to AIDS, poorly controlled diabetes mellitus, or chronic corticosteroid use[156].

Saprophytic fungal infestation describes the asymptomatic colonization of the nasal cavity with fungi, often associated with mucus crusts following endoscopic sinus surgery. It has been proposed that this condition may related to the early stages of fungal ball formation[162].

Fungal ball describes a relatively common condition where a conglomeration of fungal debris is present, usually within an isolated sinus. The maxillary sinus is the most commonly affected, but it has occurred in others[163,164]. Other terms have been used including aspergilloma and mycetoma[162]. The treatment involves endoscopic extirpation which is curative[163,164]. This disease is clearly differentiated from AFRS, and is characterized by the following criteria[155]:

1. Radiological evidence of sinus opacification +/- heterogeneity
2. Mucopurulent or cheesy / clay-like material within sinus
3. A dense conglomeration of fungal hyphae separate from mucosa
4. Non-specific mucosal inflammation
5. No predominance of eosinophils / granuloma / allergic mucin
6. Absence of tissue invasion

There are some caveats to these criteria however. Tissue invasion has been reported following immunosuppression for organ transplantation in a patient with a fungal ball[165]. Additionally, allergic mucous has been reported in patients with fungal balls when corticosteroid dosages have been reduced[162,166]. There have also been case reports of AFRS and chronic invasive fungal rhinosinusitis in the same patient specimen on histopathological analysis, with speculation that they are part of a disease spectrum rather than distinct entities[48,162].

1.2.8 Bacterial biofilms in chronic rhinosinusitis

Biofilm infections were defined in 1999, and have revolutionized Medicine, and our understanding of chronic diseases[105], and an estimated 99% of all bacteria preferentially form biofilms[167], therefore they constitute the ubiquitous and natural phenotype of bacteria. The characterization of a biofilm infection requires the demonstration of a matrix enclosed microbial community within or upon the affected tissue[106]. The matrix is self-produced, and the organisms are irreversibly attached to the surface. The biofilm mode

of growth affords the microorganisms considerable protection from antibiotics[168-170], host defenses[106], and other environments considered hostile to planktonic organisms[105]. Microorganisms embedded in these complex structures are under altered transcriptional regulation, and are thus phenotypically different to their planktonic counterparts, resulting in reduced metabolism and slower growth[167]. They are often slow to produce symptoms, often with an insidious onset[171]. Antibiotic therapy may ameliorate symptoms in the short term (likely due to eradication of planktonic clones) but the symptoms recur until the biofilm is physically removed[105].

Many biofilm organisms are resistant to culture[172], or have variable culture rates[173], and their detection requires specialized techniques[174]. Phenotypic differences which occur between planktonic and biofilm based organisms, may contribute to the relative incapacity of biofilm associated organisms to grow on nutrient media[105,106].

1.2.8.1 Detection of microbial biofilms in CRS

Since the discovery in 2004, of biofilms on the sinonasal mucosa of CRS patients[175], multiple investigators have used various imaging modalities to characterize the occurrence, microbial diversity, and clinical relevance, of mucosal biofilms in CRS. Initial studies used scanning electron microscopy (SEM)[176] to detect and characterize the nature and structure of biofilms in CRS patients[175], which was confirmed by a number of other SEM based studies[177], and in an animal model[178]. Transmission electron microscopy (TEM) was also employed to examine biofilms in CRS patients with similar findings[176,179]. An animal model of sinus biofilm formation examined the

sensitivity and specificity of SEM, TEM, and confocal scanning laser microscopy (CSLM) for detecting mucosal biofilms, confirming the former two modalities to have inherent flaws for the accurate detection and description of biofilms, recommending CSLM as the modality of choice for this purpose[180]. CSLM allows critical assessment of the three-dimensional structure of the biofilm, and resident microorganisms thus reducing false positive results[180].

1.2.8.2 Biofilms in CRS – organism specificity

The overriding limitation of all of the biofilm detection methodologies described above, is lack of organism specificity. Characterization of relevant biofilm forming organisms was based on the detection of biofilm using microscopy, and relating this to conventional laboratory culture results[175,177,179]. However, this approach is clearly limited, as biofilms are known to be particularly resistant to conventional culture techniques[106,172,181]. More recently investigators have used organism specific FISH probes to examine the biofilms in CRS patients. When combined with CSLM detection, FISH has enabled investigators to characterize the relevant microorganisms and relate these to clinical outcomes. Despite it's utility in biofilm research, FISH/CSLM techniques still require investigators to pre-select organism specific probes. In other words, one must choose which organisms to detect, and due to the limitations of the technique, only a small number of probes can be used on a piece of mucosal tissue. Molecular detection techniques such as 16S rRNA PCR and sequencing, and Ibis T5000 biosensor detection, afford the opportunity to detect all organisms on the sinonasal mucosa without *a priori* selection of organisms, and without the limitation of

culture techniques. They are described above in Section 1.2.4 - Microbial detection in CRS – molecular techniques.

1.2.8.3 Clinical relevance of microbial biofilms in CRS

The discovery of biofilms in CRS patients but not controls, was a pivotal finding in a number of independent studies, which highlighted the association of biofilms with the disease state[182-186]. However, two studies have detected *H. influenzae* biofilms in a proportion of control patients, casting some doubt on this dictum[187,188].

The prognostic implications of biofilms in CRS patients have been examined in both a retrospective[189] and a prospective trial[182]. The presence of mucosal biofilm was associated with more severe disease pre-operatively, more post-operative visits to the surgeon, and persistent CRS symptoms and mucosal inflammation on endoscopy following ESS[182]. SEM based studies have shown that biofilms also impact negatively on the physiochemical barrier of the epithelium (see Section 1.3.8). The presence of biofilm was associated with significant destruction of the mucosal epithelium, with destruction of cilial elements[177,185]. Additionally, biofilms have shown to significantly impair wound healing in an animal model[190].

The use of organism specific biofilm detection techniques, such as FISH, has furthered our understanding of the capacity of certain microbial biofilms to impact on disease. *S. aureus* was identified as the most common biofilm forming organism in CRS patients[186]. *H. influenzae* was identified as the

second most common organism within mucosal biofilms in CRS patients. A follow-up study compared the clinical outcomes of CRS patients with either *H. influenzae* or *S. aureus* biofilms, finding that the latter, was associated with more severe disease pre-operatively, and more protracted post-operative course, marked by higher complication rates[191]. A prospective study of CRS patients confirmed *S. aureus* biofilms as the 'nemesis of the endoscopic sinus surgeon'[192].

1.2.9 Fungal biofilms in chronic rhinosinusitis

The study of medical mycology has observed a paradigm shift recently, with the emerging appreciation that clinically important fungi are capable of forming biofilms on host surfaces[193,194]. Industrial mycologists have known of, and exploited the beneficial aspects of *Aspergillus* biofilms for some time[195]. Despite this there is still debate on what constitutes a fungal biofilm, however the basic criteria – that the fungi must be irreversibly attached to a surface and / or one another, and surrounded by an exopolymeric substance are generally accepted[196]. Fungal biofilms, like bacterial biofilms, have defined developmental phases that include the initial physical contact with an appropriate substrate, adhesion, colonization, matrix production, and biofilm maturation and dispersal[197-199].

Filamentous growth is a fundamental feature of fungal proliferation, however germination of conidia is required, and conidial adhesion is essential to trigger germination[196,200]. Fungal spores adhere to surfaces through several

mechanisms. Physical properties including hydrophobicity, electrostatic charge, and the roughness of the surface are important in initial conidial adhesion[201,202]. Fungi also produce a number of proteins, which stabilize the adhesion of spores to natural surfaces[203].

Healy *et al,* discovered the presence of fungal biofilms using epifluorescent microscopy, whilst investigating microbial biofilms in CRS patients [187]. These fungi were noted to be physically associated with bacterial biofilms, and were more prevalent in those with more severe disease – eosinophilic mucus chronic rhinosinusitis (EMCRS) patients. More recently, Foreman *et al,* detected fungal biofilms in 11/50 (22 %) CRS patients using fluorescence *in-situ* hybridization (FISH)[186]. Interestingly, 7 / 11 (64%) of these patients also had evidence of *S. aureus* biofilms highlighting a potential cross-kingdom synergy. This is also supported by another study which found evidence of fungal hyphae in eosinophilic mucus coincident with positive culture of *S. aureus*[204].

1.2.10 Polymicrobial flora in CRS

The role of polymicrobial flora in disease pathogenesis is being increasingly recognized. The nature of interspecies interaction can significantly alter the way microorganisms interact with the host, and can mean the difference between disease and health. Certain bacterial species may not be able to maintain chronic infections on their own, but if these species co-occur in appropriate mixtures, they can act symbiotically to successfully establish a pathogenic biofilm which contributes to inflammation[103]. Mixed fungal-

bacterial biofilms have been reported in CRS patients[186,205], as described above, however why these develop, and their clinical significance is not yet known. The complex structure of biofilms allows stratification into spatially organized populations of microorganisms that can be of mixed species, where interspecies cooperation can develop[206]. Multiple metabolic interactions have been observed in mixed biofilms, including mutualistic and commensal relationships[207]. For example, within polymicrobial biofilms, oxygen only penetrates a few μm into the surface[208], and aerobic species may consume oxygen, creating anaerobic niches, allowing obligate anaerobes to gain an advantage when in close proximity to their oxygen consuming counterparts[103]. Co-aggregation is seen in oral biofilms, and is another example of a mutually beneficial interaction, where distinct species increase adherence to mucosal surfaces by specific cell – cell recognition.

1.2.11 Functional equivalence in polymicrobial biofilms

The concept of functional equivalence has importance as it may significantly alter the single-pathogen paradigm of chronic infections[103]. This concept is based on the requirement of organisms to perform a number of roles in order to persist within, and interact with the host. A group of otherwise innocuous organisms may together, have the required virulence factors to cause harm to the host. These diverse consortia may possess functional equivalence to organisms such as *P. aeruginosa* and *S. aureus*, which may be capable of performing these essential function in isolation[103]. Furthermore, diverse pathogenic biofilms are more stable than less diverse biofilms[87], which may

explain the recalcitrance when single treatment modalities, especially narrow-spectrum antimicrobials, are employed[119].

1.3 HOST FACTORS IN CRS

1.3.1 Introduction

The sinonasal cavity, like the gastrointestinal system and lower respiratory tract, is an area of constant interaction between the host and the environment. Here, the host becomes in intimate contact with a plethora of inhaled and implanted organisms, which have potential to harm the host, and contribute to disease. The host has a repertoire of physical and immunologic defenses, which, in normal circumstances prevent the environmental agents from inciting disease. Ciliated epithelium prevents colonization by microorganisms in three ways[209]:

1. The physical removal by ciliary action and cough
2. The presence of broad spectrum innate antimicrobial agents in the mucus
3. The recruitment of phagocytic cells and an immune response (innate and adaptive)

A number of host factors may be important in CRS pathogenesis, including sinonasal anatomical abnormalities, impairment of mucociliary function, and a number of co-morbidities including allergy, asthma, immunocompromise, and aspirin sensitivity.

1.3.2 Anatomic abnormalities

Many anatomical variants can contribute to obstruction of the outflow tracts of the paranasal sinuses, including deviation of the nasal septum, concha bullosa, Haller cells, and paradoxical curvature of the middle turbinate.

However it is unlikely that these play a significant role in most cases of CRS, as they are present in similar frequencies in CRS patients and controls[210,211]. However, a number of anatomical factors have been associated with disease recalcitrance, including middle meatal stenosis, middle turbinate lateralization, scarred frontal recess[212], and residual ethmoid cells[212,213].

1.3.3 Ciliary impairment

As will be discussed in further detail in Section 1.3.8 - The physiochemical barrier, cilia play an important role in the clearance of organisms, debris, allergens and pollutants from the nose and paranasal sinuses through their movement of sinonasal mucus. Primary cilia dyskinesias (PCD) are a group of autosomal recessive disorders, which are characterized by inherited defects in cilia function, which results in inefficient, uncoordinated cilial movements. Kartagener's syndrome is a type of PCD, which is also characterized by situs inversus, due to the critical role of monocilia in embryonic development. These conditions result in mucus stasis, and affected patients are particularly pre-disposed to develop CRS, and often suffer from chronic upper and lower respiratory illness[214]. As genetic markers for these diseases are discovered, subclinical cilial dysfunction may be found to predispose to CRS[215].

1.3.4 Sinonasal mucus abnormalities

Cystic fibrosis is one of the most common autosomal recessive disorders, affecting approximately 1 in 2500 live births, and 1 in 25 Caucasians are heterozygote carriers[216]. The most common mutation (in more than 2/3 of sufferers) is Δ508, which results in an abnormal chloride transport protein –

the cystic fibrosis transmembrane conductance regulator (CFTR). As a result, the surface liquid of upper and lower airways suffers major modifications with high concentrations of chlorine, mucus thickness and reduction of mucociliary clearance. 25-40% CF patients suffer from CRS with nasal polyposis[217], and some authors suggest that carrier status may also be associated with an increased prevalence of nasal polyposis[218]. Others have recommended genetic screening of nasal polyp patients due to the overrepresentation of CFTR mutations in this population[219].

1.3.5 Immunocompromise

Although most patients with sinus disease are not overtly immunodeficient, a significant proportion of patients with recalcitrant CRS may have a systemic immunodeficiency[220]. Recurrent ARS and CRS are the most common manifestation of common variable immunodeficiency (CVID) and many patients are only diagnosed as immunodeficient following multiple sinus procedures[220]. Patients with global immunoglobulin deficiency and CRS clearly benefit from immunoglobulin replacement therapy, and there may also be a role for CRS patients with more selective deficiencies[220]. In a review of recalcitrant CRS patients, 55% had abnormal results for *in vitro* T lymphocyte testing, 5 – 18% had low immunoglobulin isotype titres, and CVID was diagnosed in 10% of patients. Another study showed similar levels of systemic immunoglobulin deficiency[221].

Acquired immunodeficiency has also been related to CRS manifestation. One study reported a prevalence of 35% CRS in HIV patients. The $CD4^+$ cell

count was an independent predictor of CRS manifestation[222]. In another study, 12% of HIV infected patients were diagnosed with CRS[223].

1.3.6 Aspirin sensitivity

Aberrant arachidonic acid metabolism, and impaired eicosanoid production may contribute to the pathogenesis of CRS with nasal polyps, however the underlying mechanisms remain to be elucidated[224]. 36 – 96% of aspirin sensitive patients have nasal polyps, and up to 96% have radiographic evidence of mucosal abnormalities consistent with CRS[225]. A recent study of aspirin tolerant and aspirin sensitive CRS patients showed significant differences in the expression of enzymes involved in the arachidonic acid cascade[226]. Aspirin sensitive patients with nasal polyposis have a high rate of recurrence, requiring revision surgery[227]. The diagnosis of aspirin sensitivity has important implications for research, as well as clinical outcomes for the patient, who may benefit from desensitization[228].

1.3.7 Asthma

There is growing evidence of a unified airway phenomenon, linking the upper and lower airways. Nasal polyposis has a prevalence of 2 – 4% in the general population, approximately 5% in atopic asthmatics, and 13% in non-atopic asthmatics[2,229]. Asthmatics generally have more severe disease, with a higher proportion of nasal polyps, and higher rates of surgical revision than non asthmatics[230]. The clinical severity of asthma may also be important - in a cross sectional analysis of 187 patients, asthma severity was associated with a greater incidence of nasal polyposis, and severity of sinus CT Lund-Mackay scores[231].

1.3.8 The physiochemical barrier

The initial line of defense for the sinonasal cavity is the barrier function of the epithelium, which effectively separates the luminal surface from the basolateral surface. Structurally, the sinonasal mucosa has many characteristics, which resist, and remove microorganisms. An epithelial layer of ciliated, pseudostratified, columnar cells, which are joined by tight junctions, rest on a collagenous basement membrane, and provides a physical barrier to prevent microbial invasion and adherence[232].

The mucociliary escalator is important for the clearance and prevention of bacterial colonization[233,234]. Each epithelial cell is covered by 50 – 200 cilia; membrane-bounded microtubules, which are approximately 10-15μm long and each, contain an outer and inner dynein arm, which are the force producing molecules, which respond to cAMP and Ca^{2+} flux[235]. The cilia extend into the overlying mucus layer and propel mucus and any trapped foreign matter and microbes from the sinuses into the nasal cavity towards the pharynx for swallowing. Primary ciliary dyskinesia (PCD) is a disease, which is characterized by genetic mutations resulting in dynein arm defects, and impaired ciliary beat frequency[236]. Clinical features of the disease are indications of the processes in which ciliary motility are essential. Some of the strongest phenotypic markers of PCD are chronic rhinosinusitis, as well as otitis media with effusion. It is of great interest that both of these diseases have been independently associated with microbial biofilms[186,237-240], even when PCD criteria are not met, highlighting the intimate relationship between ciliary clearance and microbial colonization. Many bacteria are known to

secrete toxins, which impair cilia motility[241]. Additionally, certain inflammatory cytokines have been shown to impair cilia beat frequency, including IL_8[242], IL_{13}[243], IL_6[244] and tumor necrosis factor-α[245]. Modulation of cilia physiology by cytokines, which are often elevated in CRS, is a potential mechanism for decreased mucociliary clearance in this disease.

Mucus production and composition is critically important to the function of mucociliary clearance. Cystic fibrosis provides an instruction into the profound effects of abnormal mucus composition even when cilia motility is normal[236]. The sinonasal epithelial surface is covered by mucus - a thicker (gel) mucus layer which rides along the tips of the cilia, overlying a thinner peri-ciliary (sol) layer[246], produced by submucous glands, goblet cells, ciliated epithelial cells, Clara cells, bloods vessels, and secretory cells resident within the mucosa[246]. The nature of the mucus is critical to respiratory tract health and it serves many critical functions, including protection of the mucosa and essential host-defense roles. The sol phase is a solution of water and electrolytes (Na^+, K^+, Ca^{++}, Cl^-). In health, the mucus layer is completely replaced every 10-20 minutes[247]. The mucus layer travels at approximately 1cm per minute, removing microbes and particulate matter.

In addition to the physical trapping and removal of foreign material and microorganisms, mucus is also a complex, immunologically active substance made of carbohydrates, enzymes, proteins, immunoglobulins, and other active molecules. The most abundant compound is mucus glycoproteins (mucins), which provide viscoelastic properties[248]. Whilst not offering

antimicrobial properties *per se,* the mucins can provide host defense by physical protection of the tissue, and segregation of inhaled particles and microorganisms[248]. Fleming discovered the first of the antimicrobial agents of nasal secretions in lysozyme[249]. Since discovered are many other peptides and enzymes with antibacterial and antifungal activity, including lactoferrin, immunoglobulins, defensins, uric acid, nitric oxide, peroxidases, β-defensins[209,246,250]. Lactoferrin in particular, possesses a variety of functions, including antimicrobial and antibiofilm activity[251].

1.3.9 Innate immunity

Faced with constant immunological stimulation, the unique challenge for the mucosa is to find homeostatic balance between tolerance and immunity. A number of protective mechanisms operate at mucosal level to prevent adherence, invasion of microorganisms, and subsequent induction of cell damage. The innate immune system of the sinonasal cavity provides the first defense against potential pathogens at this host – environment interface. It consists of multiple immunologic components, with complex functions, which act to reduce the microbial load within the sinuses, and reduce antigenic stimulation of immune cells residing within the mucosa. It also has critical functions in differentiating self and non-self, and in the early detection of pathogens.

Innate immune responses include a universal and ancient form of host defense, which relies upon a limited number of germ line encoded receptors.

These receptors have evolved to recognize well conserved pathogen-associated molecular patterns (PAMPs) found in bacteria, fungi and viruses[250]. The specificity of these receptors forms a critical, early self vs. non-self discrimination step in pathogen recognition. Principal functions of these receptors include opsonization, activation of complement, phagocytosis, and activation of pro-inflammatory signaling pathways[250]. Toll-like receptors (TLRs) are a family of membrane bound receptors, which have a role in detecting PAMPs and performing early pro-inflammatory functions. They are expressed on many cell types including dendritic cells and sinonasal epithelial cells[209,252].

Innate immune system cells consist of dendritic cells, macrophages, natural killer cells, mast cells, basophils, eosinophils, and $\gamma\delta$ T cells[253]. Dendritic cells are pivotally positioned at the interface of the innate and adaptive immune systems[254]. They are the most efficient antigen-presenting cells in the immune system, and have emerged as key players in initiating and regulating adaptive immune responses[255]. Dendritic cells reside in the peripheral tissues, where they sample antigens from the environment. TLRs on the surface of DCs recognize PAMPs and activate dendritic cell maturation via intracellular signaling pathways, leading to antigen processing, and co-stimulation of lymphocytes[254].

Glucocorticoids have been traditionally believed to have immunosuppressive effects, but recent evidence suggests they actually augment many aspects of innate immunity. These include enhanced mucociliary function, neutrophil

survival and phagocytosis, and increased epithelial production of antimicrobial molecules such as collectins, complement, and pentraxins[256]. The positive effects of glucocorticoids on innate immunity mirror the therapeutic effects, with clinical improvement of diseases, which are subject to infective exacerbations, including asthma, CRS, and COPD.

1.3.10 Adaptive immunity

Many researchers have subclassified CRS according to the predominant cytokines detected within the tissue often termed the 'cytokine profile'. These are generally grouped into those associated with T helper 1 (Th_1) or Th_2 cytokine profiles[257]. Most have shown that CRSsNP is generally associated with a Th_1 profile, whilst CRSwNP is associated with Th_2 cytokines. This has recently been called into question however, as Asian CRSwNP patients tend to have Th_1 / Th_{17} cytokine predominance, compared to European polyp patients who have Th_2 predominance[27]. CRSwNP patients with co-morbid cystic fibrosis (CF) also have Th_1 associated cytokines within nasal tissues[257]. Furthermore, the polyps of Asian CRS, and CF patients, show a predominance of neutrophils, rather than eosinophils[257].

Innate and adaptive immunity are often described as separate entities which act sequentially, like lines of defence. However in reality they are intricately linked, working together to maintain homeostatic balance. Adaptive immunity is so named, because it is able to adapt to new microorganisms, and has specificity, amplification, and memory characteristics. As described above, dendritic cells are believed to be a critical link between epithelial surface

based innate mechanisms, and the cellular environment of the submucosa, which forms the cellular and humoral aspects of the adaptive response[256]. Recent research supports this hypothesis, and offers pathways of immune activation, which may be common to many diseases associated with atopy. The activation of epithelial surface TLRs results in secretion of granulocyte macrophage colony stimulating factor (GM-CSF), which is a powerful inducer of dendritic cell formation and recruitment[258]. When TLR_3 is activated, epithelial cells secrete a substance known as thymic stromal lymphopoietin (TSLP) which also stimulates dendritic cells to become activated, and subsequently skews T cells to become Th_2 cells[259]. Interestingly the expression of TSLP was strongly suppressed by topical glucocorticoid application, mirroring the efficacy of these medications clinically. Furthermore, TSLP production was significantly increased in the presence of respiratory viruses, which are known to exacerbate disease clinically. TSLP mRNA is elevated in diseases which may have a related pathophysiology - CRSwNP, asthma, and atopic dermatitis. In addition to activation of T cells through antigen presentation, and cytokine release, superantigens have been shown to activate large populations of T cells in nasal polyps, as evidenced by skewing of the Vβ receptor profile of the lymphocytes[260].

B lymphocytes are actively recruited to mucosal sites where they are activated, undergo class switch recombination, and differentiation into plasma and memory cells[256]. These processes play a role in asthma and allergy, and class switch recombination to IgE is of particular importance, as IgE is significantly elevated in CRSwNP[261]. Certain proteins which are

important in B cell recruitment and class switch recombination to IgE are significantly elevated in CRSwNP. Levels of B cell activating factor (BAFF) were significantly elevated following antigen challenge, and were correlated with B cell activating cytokines and eosinophil numbers, supporting it's role as a critical protein in allergy, and class switch recombination[262].

1.3.11 The role of IgE in chronic inflammation

Classical type I hypersensitivity reactions occur with IgE as a critical factor. In these reactions IgE binds the high affinity $Fc\varepsilon R_I$, which exists on mast cells, basophils and antigen-presenting cells. Multivalent antigen binding to these IgE-$Fc\varepsilon R_I$ complexes on mast cells and basophils leads to the release of histamine, leukotrienes and IL_4, leading to immediate mucosal inflammation. Recent advances in our understanding suggest additional roles for IgE in the late phase response and non-allergic inflammation[263]. Binding of IgE to $Fc\varepsilon R_I$ in the absence of antigen for which the IgE has known specificity, can enhance mast cell survival, signaling and mediator secretion[264]. IgE binding to the low affinity receptor, $Fc\varepsilon R_{II}$, has effects on B cell differentiation and apoptosis, and the regulation of IgE synthesis[265]. Such pathways may underlie novel mechanisms of chronic inflammatory stimulus in CRS.

1.4 HOST - ENVIRONMENT INTERACTIONS IN CRS

1.4.1 Introduction

The interaction of environmental factors and the host immune system is critical in understanding CRS pathogenesis. Clearly, the plethora of microbial and non-microbial factors, their complex inter-relationships, and interactions with a poorly understood immune system, makes this a formidable task. Despite this challenge, much is being discovered about how the host, particularly at the mucosal level, interacts with the environment in health and disease states. The following discussion highlights the literature on this topic, and concludes with a focus on the important and contentious organisms in CRS, namely *S. aureus* and fungi.

1.4.2 Environmental factors and atopic disease

The interaction between environmental agents and atopic disease is intriguing. Allergy involves an abnormal host response to an otherwise innocuous environmental agent, resulting in adverse outcomes for the host. The manifestation of allergic disease therefore, usually occurs at the site of contact with the environment: the host-environmental interface, although systemic manifestations can occur. There is emerging evidence that microorganisms can alter the magnitude, and perhaps the development of these allergic responses, which may have implications in CRS.

The role of environmental agents, such as microorganisms, in other diseases involving the mucosal – environmental interface raises questions of

additional pathogenic mechanisms in CRS. The role of bacteria in modifying allergic responses has particular importance in CRS, as both allergy, and bacteria have been individually linked to the pathophysiology of CRS. Atopic respiratory diseases such as asthma and allergic rhinitis have been classically associated with aeroallergens such as pollens and fungi, but bacterial products have increasingly been implicated[266]. *Staphylococcal* superantigens have been demonstrated to enhance inflammatory airway responses in models of asthma and allergic rhinitis[266,267], and *S. aureus* colonization increases the sensitization to inhaled aero-allergens in asthmatic patients[267]. Additionally, the use of antibiotics in the first year of life increases the risk of developing asthma, allergic rhinitis and atopic eczema at age 6, by 56-82%, which suggests a role for bacterial infection in the development of atopic disease[268].

Atopic dermatitis (AD) is another 'interface' disease in which, IgE mediated hypersensitivity plays an important role, and AD also has a very high rate of *S. aureus* colonization[269]. IgE synthesis in the peripheral blood of these patients is significantly upregulated in the presence of *S. aureus* enterotoxins[270]. Another study showed that pollen specific IgE production was also enhanced in the presence of *staphylococcal* superantigens.[271]

Shiomori et al*,* found that *S. aureus* colonization rates were higher in perennial allergic rhinitis patients (45%) compared with controls (20%)[272]. Furthermore, perennial allergic rhinitis patients have significantly worse nasal symptom scores when *S. aureus* is present[272]. It is possible that *S. aureus*

enhances the mucosal IgE mediated allergy in these patients however this was not specifically addressed. These studies suggest a role for microorganisms as disease modifying agents, with the potential to exacerbate sensitization to inhaled aero-allergens in susceptible individuals.

Medical treatments for CRSwNP are generally directed at either host or environmental mechanisms, and include corticosteroids to reduce mucosal inflammation and exudation, and antibiotics, antimycotics, and antibiofilm treatments to reduce the microbial bioburden. Other immunomodulatory medications are targeted at specific inflammatory pathways such as histamine and leukotriene antagonists[273]. In practice, these treatments play a role in reducing disease burden in the short term, but cessation of these agents often leads to disease recurrence and subsequent surgical intervention. Recent trials of the novel anti-IgE monoclonal antibody, omalizumab have shown promise for nasal polyposis in preliminary studies[274-276], and suggest a critical role of IgE in the underlying pathogenesis of this disease.

1.4.3 Polymicrobial flora and the host - Symbiosis

Following birth, colonization of environmentally exposed surfaces begins, and continues throughout life. Many host – organism associations have evolved into beneficial relationships, creating an environment of mutualism. By adulthood, the human host supports one of the most complex microbial ecosystems known[277]. Symbiosis derives from Ancient Greek, meaning to

'live with'. A common misconception is the assumption that symbiosis is a positive phenomenon for both parties. Three types of symbiosis exist[278].

Table 1.7 The three types of symbiosis

Parasitism	One organism benefits, while the other is harmed
Commensalism	One organism benefits and no harm occurs to the other
Mutualism	Both organisms benefit

In the past certain organisms have been labeled as commensals (such as *S. epidermidis* on the skin) but it is becoming clear that the same organism can take on different roles at different times. The distinction between an organism which benefits the host, and one which harms, may lie in the host's capacity to resist infection, rather than inherent characteristics of the microbe itself[278]. An example is *S. epidermidis*, traditionally considered to be a commensal, but contemporary research suggests it has mutualistic properties, significantly benefiting the host, through secretion of multiple antibacterial peptides, which are toxic to other organisms such as *S. aureus* and *Streptococcus pyogenes*. *S. epidermidis* also produces modified peptide pheromones which interact with quorum sensing mechanisms of bacteria such as *S. aureus*, which ultimately leads to colonization inhibition[279]. However, when the host defenses are compromised, due to, for example, immune barrier breakdown or immunosuppression, *S. epidermidis* can be pathogenic, secreting proteases, collagenases and numerous toxins which cause significant tissue damage[280].

1.4.4 Polymicrobial flora and the host - Dysbiosis

The importance of understanding the complex polymicrobial communities in the sinuses is highlighted by the concept of dysbiosis. In conditions of dysbiosis, there is an unnatural shift in the composition of the microbiota, which leads to immunological dysregulation and inflammation. Several studies have shown that the surface microflora can influence the host immune system, with certain basic developmental features and functions of the human immune system depending on interactions with the human microbiome[281]. Unlike opportunistic pathogens which elicit immune responses that result in inflammation and tissue damage, some symbiotic bacterial species have been shown to prevent inflammatory disease during colonization. Surprisingly the 'normal' microbiome also contains microorganisms that have been shown to induce inflammation under certain conditions[281]. It is possible that alterations in the development or composition of the microbiota, known as dysbiosis, disturbs the partnership between microbes and the human immune system, ultimately leading to altered immune responses that may underlie various human inflammatory disorders[282].

There is evidence to suggest that microbial diversity is important for health[283], and a reduced biodiversity associated with increased abundance, is associated with chronic inflammation and poor healing[284]. There is also literature to suggest that host genetics and immunity strongly influence the composition of the mucosal microflora[285]. Microbial communities inhabiting mucosal surfaces such as the gastrointestinal tract can result in a significant

mutualism, including local immune homeostasis[281,282] and protection from pathogens through processes such as nutrient consumption, occupation of attachment sites, and secretion of antimicrobial substances[286]. *Propionibacterium acnes,* which is commonly found in the sinonasal cavity using molecular detection, has been shown to produce bacteriocins which have antibacterial and antifungal activity which may be protective against pathogens.[287] Competition between microbes on mucosal surfaces can result in selection of virulence factors which can be detrimental to the host[288]. In an elegant model of polymicrobial interactions, Sibley et al, have shown that avirulent organisms can enhance the pathogenicity of other organisms, highlighting the importance of comprehensive microbial community analysis to investigate disease[289].

The use of probiotics in inflammatory bowel disease is an example of microorganisms, which can be beneficial to the host. Initially thought to be a physical phenomena, with mutualistic organisms out-competing pathogens for attachment sites, nutrients etc., it is now understood that probiotic strains modulate intestinal immune responses, by interacting with epithelial cells, dendritic cells, and T lymphocytes to maintain immune homesostasis[282]. Clearly, characterizing the microbial communities which reside on the mucosa of CRS patients, and their interactions with each other, and with the host immune system, is critical to furthering our understanding of this disease

1.4.5 Microbial bioburden

There are two main microbial factors, which can influence whether microbes exist with the host (mutualism or commensalism) or aggravate the host eliciting an immune response and subsequent inflammation (parasitism). The first is the net pathogenic effect, or microbial virulence, which relates to the organisms present[290], as described above. Current CRS research has focused specifically on this, and more recently, on the polymicrobial diversity of the sinuses as described above. The second factor is the contamination level, otherwise known as the disease load, or bioburden. Importantly, the great majority of studies aimed at characterizing the microbial flora of CRS, fail to analyze the abundance of organisms in the sinuses, particularly in reference to controls. One isolated study however, did show the chance of symptomatic recurrence was related to the abundance of anaerobic organisms. To fully understand the potential role of microorganisms in disease the abundance of organisms must be analyzed, as well as the diversity. A novel theory of chronic infection management focuses on reducing the microbial abundance rather than aiming for microbial eradication, the goal being to create a host-manageable bioburden[290].

1.4.6 *Staphylococcus aureus* – host interactions

S. aureus deserves special mention here as it emerging as an important organism in CRS, with potential disease modifying characteristics. It is one of the most commonly detected, and well studied species in the CRS literature. Much can be learned from it's plethora of virulence factors, and the complex interaction of this organism with the host.

S. aureus is a facultative anaerobic Gram-positive spherical (coccal) bacterium approximately 1µm in diameter. It is a common human commensal microorganisms carried chronically in the upper respiratory tract and on the skin of 20% of the population, and intermittently by up to 60% of the population[291]. In healthy individuals, the carrier state for *S. aureus* does not appear to be associated with any immediate complications. Multiple microbial and host factors have been linked to the development and maintenance of *S. aureus* carrier state but the precise mechanism remains unclear[292]. In contrast to it's commensal status, *S. aureus* is capable of causing many human infections ranging from skin and soft tissue, to invasive disease such as pneumonia, osteomyelitis and endocarditis. It is the most commonly isolated microbe from inpatient specimens and the second most common from outpatient specimens[292].

The duality of *S. aureus* interactions with human hosts, from apparent innocuous colonization, to highly pathogenic invasion begs the question of what mechanisms determine the balance between these two phenomena. This question is particularly relevant when considering the many toxins and virulence molecules produced by *S. aureus* and the innate and adaptive mechanisms that these molecules interact with.

A broad array of virulence factors contribute to *S. aureus* pathogenesis including pore-forming toxins (alpha-hemolysin, Panton-Valentine leukocidin), superantigens (enterotoxins A, B, C, and toxic shock syndrome

toxin-1), phagocytosis inhibitors (polysaccharide capsule, protein A), and immune evasion molecules (chemotaxis inhibitory protein, staphylokinase, aureolysin)[293]. The virulence factors of *S. aureus* have significant redundancy in subverting the same host mechanism, which underlies it's importance as a human pathogen.

1.4.6.1 Adhesion

S. aureus produces numerous surface proteins, which enable it to persist in the sinonasal cavity, via enhanced adhesion to host tissues and secretions[294], and reduced mucociliary clearance. It, and other bacteria have been shown to secrete specific toxins to impair nasal ciliary motion and co-ordination[241]. Agents that aid bacterial attachment include collagen binding protein, protein A, fibronectin binding protein, and clumping factor A & B.

S. aureus is also capable of forming biofilms (see Section 1.2.8) which affords the microorganism significant adhesion capabilities[295]. The polysaccharide biofilm matrix is predominately composed of poly-N-acetylglucosamine (PNAG)[295]. Some investigators suggest that 60% of *S. aureus* strains are capable of forming biofilms[296], whilst others suggest that all strains have that capacity[297]. In a recent study, 45% to 75% of clinical isolates of *S. aureus* were reported to be of biofilm forming capacity[298].

1.4.6.2 S. aureus innate immunity evasion

The crucial role of the innate immune system in controlling *S. aureus* is reflected by the abundance of mechanisms that the bacterium employs to evade killing by phagocytosis. *S. aureus* produces a protective

polysaccharide coat to evade phagocytosis, which also aids in surface adherence and biofilm formation[299]. It also secretes proteins, which block phagocyte receptor function. Following ingestion, *S. aureus* counters the antimicrobial mechanisms of phagocytes by secreting proteins such as catalase and superoxide dismutase to neutralize reactive oxygen species (ROS). The characteristic yellow pigment of *S. aureus,* staphyloxanthin also plays a crucial role in protecting the bacterium from ROS[300]. Post-phagocytosis, the bacterium is capable of lysing the phagocyte, using similar killing mechanisms as the phagocyte itself[301]. Other innate immune avoidance strategies employed by *S. aureus* include secretion of CHIPS (chemostaxis inhibitory protein of *S. aureus*) which blocks Toll-like receptor recognition of PAMPs[301,302], and secretion of SCIN (*staphylococcal* complement inhibitor) which is one of many proteins which inhibit host complement function[303]. The immune evasion mechanisms described thus far are passive, enabling the bacterium to evade recognition or elimination. *S. aureus* also produces a battery of toxins which directly harm human cells. A large family of leukocidins and hemolysins exist which destroy white and red blood cells, respectively.

Further highlighting the duality of *S. aureus* – host interactions, recent studies have shown that the bacterium produces multiple ligands for human TLR_2 receptors some of which are associated with a profound pro-inflammatory effect, whilst others are associated with immunomodulation, which may explain the protective immunity afforded *S. aureus* during the carrier state[292,304].

1.4.6.3 S. aureus acquired immunity evasion

In addition to innate immune evasion, *S. aureus* also moderates the acquired immune system of the host to improve its survival. Protein A enables *S. aureus* to sequester antibodies on it's surface, affording protection from attacks of the acquired and innate immune host defenses[305]. Protein A also has a specific role in respiratory infections by interacting with tumor necrosis factor α on respiratory epithelium[306].

1.4.6.4 S. aureus small colony variants

S. aureus is capable of forming small colony variants (SCV), following mutations in metabolic genes, becoming auxotrophic – unable to synthesize essential compounds required for growth[307]. The small colony variant phenotype is associated with biofilm formation, and the phenotypes share many similarities [308]. SCVs are often associated with chronic bacterial infections, and have distinctive phenotypic and pathogenic traits, which afford increased resistance to host defenses. Such factors include hyper-adherence to host cells[309], intracellular persistence[310], serum resistance[311], and also antibiotic resistance[310]. Selection pressure from antibiotic exposure, particularly long term, can promote SCV formation[307]. The reduced metabolism of SCV *S. aureus* results in extremely long generation times, which often results in overgrowth of other competitive flora on nutrient media. When they are able to be grown on nutrient agar, they often lack the characteristic appearance of wild type strains, and biochemical tests used for identification are often non-reactive, making diagnostic detection difficult[307].

Specialized media for *S. aureus* SCVs can be used[312], however, molecular detection techniques are becoming the method of choice[307].

1.4.6.5 Superantigens – modification of the host immune response

Overstimulation of the immune response represents a unique method of interfering with the host defenses. *S. aureus* produces many enterotoxins which have been linked to human disease. Toxic shock syndrome was named in 1978 following the observation that a *staphylococcal* illness in mucosal sites produces systemic illness despite the absence of the organisms systemically, leading to the assumption that a secreted toxin was the cause[313]. These toxins, termed superantigens (SAgs) are usually coded by accessory genetic elements such as plasmids, hence are transferrable between strains. More than twenty SAgs are known to be produced by *S. aureus,* termed TSST-1, SAE-A, SAE-B, SAE-C etc. The potent immunostimulatory capacity of SAgs is based on their capacity to directly bind to specific Vβ regions of the T cell receptor (TCR) and to human leukocyte antigens (HLA) class II molecules outside the peptide binding groove on antigen-presenting cells (APCs), linking them to Th_2 lymphocytes but avoiding the need to go through the processing and loading into HLA molecules required for presentation of conventional antigens to T cells[260,314]. This stimulates potent mitogenic expansion of T lymphocytes and results in massive inflammatory influx, known as a 'cytokine storm'[292]. The hyperactivation of the T cell compartment induced by SAg ultimately leads to

activation induced apoptosis, and the majority of surviving T cells enter a state of anergy[315,316].

As discussed in section 1.4.2, microorganisms may have a role in altering allergic responses in a number of atopic diseases such as asthma[266,267], allergic rhinitis[272], and atopic dermatitis[270,271]. Superantigens secreted by mucosal organisms such as *S. aureus*, may play an important role in the modulation of allergic disease by affecting the activation and proliferation of B lymphocytes. The staphylococcal superantigen TSST-1 has been shown to modulate T cell dependent IgE synthesis by B cells *in vitro* by induction of CD40 ligand expression on T cells, which may be independent of the MHC class II engagement classically attributed to superantigens[317]. The capacity of bacterial superantigens to promote T_h cell dependent B cell activation has also been shown in an animal model[318] Superantigen exposure may also enhance the immune response following conventional fungal antigen presentation, as superantigen mediated T_h cell – B cell interactions have been shown to selectively promote the proliferation of B cells which have encountered antigen[319]. Conventional allergens are phagocytosed by antigen presenting cells, such as dendritic cells, where the antigen is cleaved into small peptides, which bind to MHC class II molecules. Subsequent allergen specific activation of T lymphocytes occurs through specific binding between the T cell receptor (TCR) and the MHC class II / peptide complex. Superantigens stimulate T cells without antigen specific binding, linking the TCR-Vβ with the MHCII β chain. Direct binding of SAE to the MHC class II molecule which is processing conventional fungal antigen may amplify this

reaction[320]. In an animal model, Okano et al, demonstrated significantly increased antigen specific IgE and eosinophillia when conventional antigen was presented to the nasal mucosa in the presence of SAE (SEB)[320]. Finally, superantigen activation of T lymphocytes leads to the production of a significant pro-inflammatory milieu of cytokines and chemokines, which may influence the processing of conventional antigens.

1.4.7 Fungal – host interactions

As discussed in section 1.1.5 and 1.2.7, the role of fungi in CRS is still a topic of active debate in the rhinology literature. Innate and adaptive components collaborate, with dendritic cell input, to resist mycotic infections. The following discussion examines the current research of fungal – host interactions in CRS, and potential immune defects which may lead to fungal disease in CRS.

1.4.7.1 Fungi & the innate immune system

As discussed in 1.3.8, nasal mucus contains many substances with antimicrobial and immunomodulatory actions. These substances are secreted by epithelial cells, submucosal glands, and local inflammatory cells, and many have anti-fungal activity[321,322]. Recent studies have shown differential expression of many of these substances in CRS patients compared to controls[323]. Lactoferrin was shown to be down-regulated in CRS patients with nasal polyps[324], and biofilms[251], however no difference was observed between those with, and without fungi[251]. The cathelicidins are a

family of innate defense proteins with antimicrobial actions. Expression of the cathelicidin peptide LL-37 has been shown to be upregulated in response to fungal antigens in CRS explant tissue, but this was not seen in tissue from EMCRS patients, suggesting a potential innate deficiency in these patients, however no fungal positive EMCRS patients were included[325]. Certain surfactant proteins, believed to have anti-fungal activity, are found in high levels in healthy controls, but are undetectable in AFRS patients, suggesting their absence may be related to poor anti-fungal defense in these patients[326].

Innate immune effectors on the surface of mucosal epithelial cells have a limited number of receptors, which detect common peptides on the surface of microorganisms (see 1.3.9). Toll like receptors are involved in innate defense against fungi, and certain TLR polymorphisms have been associated with susceptibility to fungal infections[327]. Of these, TLR_2, TLR_4 and TLR_6 are believed to be the most important in innate fungal defence[328]. A number of studies have generally found lower TLR_2 and TLR_4 expression in CRS patients compared with controls, positing that this may reduce the ability of the epithelium to resist to fungal organisms[329-331].

1.4.7.2 Fungi and eosinophils

The study of eosinophils in CRS mucosa has become a focus of intense research, as eosinophil induced tissue damage is somewhat of an endpoint of the poorly understood immune dysregulation in CRS, particularly CRSwNP. The interactions of fungi with eosinophils have therefore been of great interest. Eosinophils are prominent in immune reactions to parasites,

and also in the late phase of allergic rhinitis. The concurrent presence of eosinophils and fungi in CRS tissue specimens led to the suggestion of a cause and effect relationship. One study showed that mucus and mucosa from CRS patients resulted in eosinophil chemotaxis[332]. Another showed that peripheral blood cells from CRS patients produced a florid cellular and humoral immune response, whilst control blood cells did not respond[90]. Fungal elements have also been shown to directly cause activation and degranulation of eosinophils[333]. The possibility of a T cell driven antifungal response which results in the accumulation of eosinophils in response to nasal fungi is postulated, but confounding by high numbers of patients with co-morbid asthma, have made these studies difficult to interpret. Furthermore, conflicting studies have since been published examining the critical eosinophil related cytokines, IL_5 and IL_{13}. Peripheral blood cells from CRS patients and controls were exposed to the same fungal elements as the previous studies, but showed heterogeneous secretion of these cytokines, which was unrelated to the presence of CRS[334]. The authors did note however, that IL_5 levels were significantly correlated to levels of fungal specific IgE in the serum, suggesting a possible link between fungal allergy, and eosinophil activation cytokines. Interestingly, these results were also disputed[335], highlighting how far we are from conclusive evidence for either argument.

1.4.7.3 Fungi and adaptive immunity

In human fungal infections, a dominant Th_1 response, driven by IL_{12} is essential for the expression of protective immunity to fungi[336]. Through the production of IFNγ, and opsonizing antibodies, the activation of Th_1

lymphocytes results in optimal activation of phagocytic cells for clearance of fungal elements. Patients with disseminated fungal infections often show defective production of IFNγ, and associated elevations of Th_2 cytokines (IL_4 & IL_5), IgE, and eosinophil levels, which are all markers of poor prognosis in systemic mycoses[337,338]. Applying this paradigm to the mucosal level in CRS, a Th_2 based, hyper IgE response to colonizing fungi may be detrimental to the clearance of the organisms.

The dichotomy of Th_1 / Th_2 inflammation has been challenged in recent years with the discovery of IL_{17} secreting $CD4^+$ T cells known as Th_{17} cells. Th_{17} cells are induced in fungal infections through TLR and non-TLR dependent signaling, and are associated with defective pathogen clearance and failure to resolve inflammation[337]. In situations of high fungal burden, activation of pathogenic Th_{17} cells, and non protective Th_2 cells is seen[336], potentially leading to fungal persistence and ongoing inflammation. Additionally, neutralization of IL_{17} increases fungal clearance, ameliorated inflammatory pathology, and restores protective Th_1 antifungal resistance[339].

To limit the pathologic consequences of an excessive inflammatory cell-mediated reaction, the immune system resorts to a number of protective mechanisms including the actions of regulatory T cells (T_{reg})[337]. The balance between microbial tolerance and resistance is believed to be orchestrated by T_{reg} cells, by acting to restrain exuberant immune activity, which may be inciting tissue damage[337]. T_{reg} cells are believed to be deficient within nasal polyps, and some have suggested that this may account for the more

pronounced Th_2 inflammation seen in these patients[340]. Whilst these populations have been examined in CRS patients, their relationship to disease is poorly understood, and no relationship to mucosal microorganisms have yet been made[340-344].

1.4.8 The role of systemic allergy in CRS

The role of systemic allergy in the pathogenesis of CRS has divided opinion in the rhinology research community. Many have speculated that allergic inflammation in the nose predisposes the atopic patient to the development of CRS through mucosal oedema, which may obstruct sinus drainage and lead to mucus stasis and infection[345,346]. The prevalence of both CRS and atopy is increasing[347]. However many authors disagree primarily due to the large number of CRS patients who do not show systemic sensitivity to commonly tested allergens.

In diseases such as allergic rhinitis, allergen specific IgE in serum correlates well with symptoms[348], however in CRS, some authors advocate that atopic status has no bearing on symptom scores, or surgical revision rate[349]. Others have shown that systemic atopy has no effect on the cellular content of nasal polyps compared to non atopic polyp patients[350]. It has also been argued that nasal polyps are not more frequent in atopic individuals[351,352], and more polyp patients have negative skin prick tests than positive ones[353]. However conversely, there is evidence to suggest that allergy rates are higher in polyp patients than the general population[354,355]. Another study found much higher

radiological evidence of sinus disease in allergic rhinitis patients (67%) compared with a control group (33%)[356].

Analyzing fungal allergy more specifically, some studies have found similar rates of fungal allergy in CRS patients and healthy controls[90], however others have found evidence of higher rates of fungal specific allergy in CRS patients[354,355] than the general population[357]. The prevalence of allergy (any allergen) in the general population is estimated at 20-25%[358]. The reported prevalence of systemic fungal sensitivity (allergy) in CRS patients varies significantly. Collins et al, reported no difference in inhalant allergy between CRSwNP patients and controls, although fungal allergy was significantly higher in the polyp group[351]. Another study showed CRS patients with and without polyps had significantly higher fungal allergy (46%) than healthy volunteers (0%), although there was no difference between eosinophilic CRS patients and allergic rhinitis patients[359]. Corey et al, found that 51.5% of CRS patients undergoing endoscopic sinus surgery had fungal allergy[354], and other studies have had skin prick test positivity rates of 60% in those undergoing polypectomy[360]. Houser et al, found the prevalence of perennial allergy in CRSwNP patients to be 73%, with 29.8% of these having fungal allergy[355]. Additionally, fungi are far more numerous and antigenically variable than other aero-allergens and are impossible to avoid, further complicating allergy management[357]. There is considerable regional variability in the environmental burden of fungal aeroallergens, and fungal allergy rates[361], which complicates the comparisons between studies. Whilst hard epidemiologic evidence for a role of allergy in CRS is still lacking, it is

known that failure to address allergy as a contributing factor to CRS diminishes the chance of success of ESS[362].

1.4.9 Entopy - the concept of local allergy

The concept of localized, mucosal allergy in the absence of systemic allergy, was first proposed in 1975, when specific IgE antibodies were detected in the nasal secretions of allergic rhinitis patients in the absence of positivity of both skin prick, and RAST testing[363]. Others have noted that 50% of allergic individuals have normal serum IgE levels[364], and allergic rhinitis and allergen skin reactivity may occur independently of each other[365]. More recently, researchers found that 30% of 'idiopathic rhinitis' patients who had no evidence of systemic allergy on skin and serum testing, actually had allergen specific IgE within the mucosa, and coined the term entopy to describe the presence of local IgE[366]. Sensi et al, examined the kinetics of IgE production by examining the specific IgE levels in the nose and serum of rhinitis patients during a period of controlled antigen avoidance, noting that nasal IgE dropped rapidly over three days following antigen avoidance, with serum levels trailing by approximately 60 days[367]. This raises the possibility of an overflow phenomenon where surplus IgE from the nasal mucosal sites enters the systemic circulation.

1.4.10 Entopy in chronic rhinosinusitis with nasal polyps

As described above in Section 1.4.8, the role of systemic allergy in CRSwNP is controversial, however it has been proposed that local allergic responses are more important in the aetiology of nasal polyp formation that systemic allergic responses[368]. In 1985, Small et al, were the first to demonstrate

specific IgE production in nasal polyps associated with negative skin prick and serum testing[369]. Several studies have since demonstrated isolated mucosal allergic response to environmental products and allergens, including *staphylococcal* superantigens, within nasal polyps[368,370]. Additionally, Sabirov et al, found no relationship between *Alternaria alternata* specific IgE in serum and mucosa in CRSwNP, further supporting the concept of local IgE production[371]. Interestingly Bachert et al, found evidence of local production of specific IgE in nasal polyps, but they found a good correlation between skin prick tests and specific IgE in inferior turbinate tissue of control patients, suggesting that entopy is associated with the disease state[372].

The definition of AFRS includes systemic fungal allergy, however a number of patients have AFRS like disease in the absence of systemic allergy, and a number of studies have sought local allergy as an explanation. Collins et al, examined 32 CRS patients with eosinophilic mucus containing fungal elements but no systemic fungal allergy. 6/32 (19%) of these had fungal specific IgE within nasal mucus, supporting the argument for entopy[373]. Another study examined 14 'AFRS' patients with allergy to only non-fungal antigens on serum testing. However 12/14 of these patients had mucosal IgE sensitivity to *Aspergillus* species[374]. The concept of entopy may have significant implications for our understanding of the role of allergy in CRS, in particular relating to CRSwNP and AFRS, as the true role of antigen specific IgE sensitization may be significantly underestimated using systemic testing measures.

Summary of the Literature Review

Chronic rhinosinusitis describes a group of related disorders, which have a significant prevalence in society. It results in an enormous financial and social burden. Despite the magnitude of the problem however, the pathogenesis remains obscure. A significant body of research has been completed thus far, examining many potential factors, which may play a role. These can be divided into environmental and host factors.

Of the environmental factors, microorganisms such as bacteria and fungi are of significant interest. Initial concepts of infectious aetiology have been surpassed by a more insidious role of microorganisms. Our understanding of the flora in CRS is still evolving, and the majority of literature in this area is based on outdated scientific methods. The detection of microorganisms in these patients has been impeded by the reliance on conventional culture, which has many limitations in this context. The majority of microorganisms are unculturable by conventional laboratory methods, and remain so even when exhaustive culture techniques are used. Furthermore, biofilm associated microorganisms are also resistant to growth on nutrient media.

We are beginning to understand that polymicrobial flora exist, and interactions between organisms which may be regarded individually as innocuous, or commensal, may be critical in determining disease pathogenesis. Culture based detection methods have distinct limitations in detecting the plethora of microorganisms in a biological sample, further limiting our understanding.

Many studies examining the flora in CRS have also been hampered by a lack of suitable control tissue with which to add context to disease findings. Many have used control tissue based on unacceptable criteria, which may lead to inadvertent labeling of disease tissue as healthy controls.

In addition to understanding which microorganisms can successfully colonize the sinonasal mucosa of diseased and healthy individuals, there is evidence to suggest that abundance can be just as important when determining a pathogenic role. Studies from other specialty areas have suggested that the burden of microorganisms may actually be more important than the species themselves. The majority of microbial studies in CRS have failed to deliver information regarding microbial abundance.

Multidiscilpinary biofilm research provides an added perspective to Koch's postulates and suggests a complexity of host-pathogen interaction that traditional culturing does not reveal. The discovery of bacterial and fungal biofilms in CRS patients, but not controls has been a landmark finding. Subsequently, certain bacterial species within these biofilms, such as *S. aureus,* have been shown to portend a poorer prognosis, compared to other bacterial species. Biofilm related organisms are known to be resistant to culture, further limiting the use of these detection methods in rhinological research.

The finding of co-existing fungal, and *S. aureus* biofilms in a number of disease states, as well as CRS has been of particular interest. The role of fungi in CRS

remains contentious and a universal role for fungi has been refuted. However, a small proportion of CRS patients have fungal biofilms, but the reasons for fungal proliferation in these patients is obscure. Fungi are known to be ubiquitous in the environment, and a number of innate and adaptive immune mechanisms effectively clear fungi from the sinonasal cavity in health, and these may be impaired in disease states.

Of the host factors involved in CRS manifestation, the role of IgE in CRS, particularly CRS with nasal polyps, is a topical issue. Allergy in particular has been a focus of intense research, and the role of systemic sensitivity to antigens has been questioned, particularly in relation to fungal allergy in allergic fungal rhinosinusitis. However, it has recently been discovered that local, mucosal IgE may be more important than systemic IgE, and measures of systemic hypersensitivity may significantly underestimate the role in disease manifestation. Furthermore, microorganisms such as *S. aureus,* have been shown in other disease states, to modulate allergic responses to other antigens, possibly through the actions of secreted superantigens.

A systematic review of the literature raises a number of topical issues, which will be addressed within this thesis:

- The microbial flora, which exists on the sinonasal mucosa of CRS patients and controls has been based on inadequate methodologies, and therefore remains uncertain.
- The relative abundance of these microorganisms compared to controls may be of importance but is unknown.
- Biofilm detection techniques have been employed successfully to analyze the presence and clinical significance of biofilms in CRS patients and controls. However, the sensitivity and specificity of these contemporary detection techniques is unknown.
- Fungal biofilms have been detected in CRS patients, often in the context of bacterial biofilms, but the biological and environmental factors that encourage fungal biofilm formation in the sinuses are unknown.
- The role of local and systemic IgE based hypersensitivity may be important in CRS, but the relationship between them requires further elucidation.
- Both bacterial and fungal organisms have been discovered on the mucosa of CRS patients, but contention exists as to the role of these in inciting inflammation. There is little evidence in the literature that these microorganisms can incite organism specific immune responses in the host.

- *S. aureus* microorganisms have been shown to modulate, and exacerbate allergic immune responses in diseases such as asthma and atopic dermatitis, however a similar role in CRS patients is unknown.

Aims

1. Characterize and compare the diversity and abundance of bacterial and fungal microorganisms, which comprise the sinonasal mucosal environment of CRS patients and controls, using sensitive molecular detection techniques.
2. Determine the sensitivity and specificity of contemporary biofilm detection techniques.
3. Investigate the factors which contribute to the formation of sinus fungal biofilms in a sheep model of sinusitis
4. Investigate four bacterial species commonly detected in CRS, and their capacity to promote sinus fungal biofilm formation by altering sinonasal physiochemical barriers in a sheep model.
5. Investigate the role of mucosal cilia in resisting fungal biofilm proliferation by applying cilia toxin to the sinus in a sheep model.
6. Investigate the relationship between microorganisms and mucosal allergy in CRS patients.
7. Determine a potential pathogenic role of fungi and bacteria in CRS by determining the presence of these microbes on the mucosa, and observing organism-specific immune responses in the host.
8. Examine the capacity of *S. aureus* to modulate allergic responses in CRS

The microbiome of CRS

Culture, molecular diagnostics and biofilm detection

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Financial assistance provided by the Garnett Passe and Rodney Williams Memorial Foundation

chapter 2

Submitted to BMC Infectious Diseases

Under review.

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2.1 ABSTRACT

Introduction: Bacteria and fungi are thought to influence inflammation of sinonasal mucosa in chronic rhinosinusitis. Through recent advances in microbial detection we are beginning to understand the flora of sinuses and the role of biofilms. This study used multiple detection methods to further our understanding of microbial diversity and microbial abundance in healthy and diseased sinuses.

Methods: Sinonasal mucosa was analyzed from 38 CRS and 6 controls. Bacterial and fungal analysis was performed using conventional culture, molecular diagnostics (polymerase chain reaction coupled with electrospray ionization time-of-flight mass spectrometry) and fluorescence *in situ* hybridization (FISH).

Results: Microbes were detected in all samples, including controls, and were often polymicrobial. 33 different bacterial species were detected in CRS, and 5 in control patients, with frequent recovery of anaerobes. *Staphylococcus aureus* and *Propionibacterium acnes* were the most common organisms in CRS and controls, respectively. Using a model organism, FISH had a sensitivity of 78%, and a specificity of 93%.

Conclusion: This study highlights some cornerstones of microbial variations in healthy and diseased paranasal sinuses. Whilst the healthy sinus is clearly not sterile, it appears prevalence and abundance of organisms is critical in determining disease. Evidence from high-sensitivity techniques, limits the role of fungi in CRS to a small group of patients. Comparison with molecular analysis suggests that the detection threshold of FISH and culture is related to organism abundance and, furthermore, culture tends to select for rapidly growing organisms. These findings have implications for our clinical management of CRS and future research.

2.2 INTRODUCTION

Chronic rhinosinusitis is a disease cluster with a significant societal burden, and despite extensive research efforts, has an unknown pathophysiology. There is

emerging evidence however, that microorganisms play an important role in the exacerbation and perpetuation of mucosal inflammation. However, the microbial biodiversity in disease and controls is not well defined. To further our understanding of the role of microorganisms in CRS, it is important to comprehensively characterize the resident microbial community in healthy and diseased tissue and examine the specific host immunological responses to these organisms. Thus, we sought to characterize the microbial populations in CRS and controls to establish a basis for further species directed research into this heterogeneous disease. Only through such a systematic approach can we determine the importance, or otherwise, of these microorganisms in the disease phenotypes. Furthermore, comparative microbiome studies will provide important information for the selection of antimicrobial therapies, and enable the determination of the effectiveness of such treatments.

The study of the impact of microorganisms on human disease rapidly expanded following advances in microscopy, including the work of scientists such as Pasteur and Koch, resulting in a paradigm where aetiological agents were associated with certain diseases, by fulfilling dogmatic criteria. In many cases this still holds true today, particularly for acute diseases in which the bacteria adopt a clonal planktonic lifestyle such as septicaemia or meningitis attributable to a single organism isolated on culture. Increasingly however, we are discovering that complex polymicrobial communities exist, especially at the host-environment interface such as mucosal surfaces, and the majority of these bacterial species are refractory to culture[104]. Many of these organisms are found to be residing in complex communities known as biofilms; communities of organisms surrounded

by a self-produced exopolysaccharide matrix, irreversibly attached to a live or inert surface[105]. There have been recent advances in our understanding of biofilms in CRS, and their importance regarding disease evolution following treatment[182], increased postoperative infection[182], and altered host immune mechanisms[375]. Many biofilm organisms are resistant to culture[172], and their detection requires specialized techniques[174]. Phenotypic differences which occur between planktonic and biofilm based organisms, may contribute to the relative incapacity of biofilm associated organisms to grow on nutrient media[106,181].

Traditional culture-dependent techniques have been the mainstay of microbial diagnostics in CRS. However, the reliance of cultivation on nutrient media often results in 'enrichment bias' with detection of a narrow range of microbes which is not representative of the actual diversity present[101]. Competition between organisms during enrichment often results in dominance of one or two organisms with the fastest growth rates[101]. Selective media techniques use nutrient restriction to enhance or restrict growth of organisms based on inherent microbial characteristics for identification. In a complex microbial community such as the diseased sinonasal mucosa, the identification of every organism using this method would be exhaustive. Additionally, many organisms may not thrive on nutrient media once the advantages of biofilm structures, and symbiotic relationships are lost.

2.3 MATERIALS AND METHODS

This prospective study was undertaken in the tertiary referral rhinology practice of the senior author (PJW), at the academic hospitals, Adelaide, South Australia. The study was approved by the Human Ethics Committee and 44 consecutive patients provided informed consented to involvement in the study. 38 patients met the definition of CRS as defined by the rhinosinusitis taskforce[376] having failed medical therapy necessitating the need for endoscopic sinus surgery (ESS). A control group consisted of 6 patients who had no clinical or radiological evidence of sinus disease. These patients were undergoing transnasal endoscopic procedures including trans-sphenoidal hypophysectomy for non-functioning adenomas (5 patients) or CSF leak repair (1 patient). Patients were excluded if less than 18 years of age, immunocompromised, or had decreased ciliary dysfunction such as cystic fibrosis and Kartagener's syndrome. Other exclusion criteria included inadequate mucosa for analysis, no fungal or bacterial culture taken, and antibiotic or systemic corticosteroid used in the three weeks preceding surgery.

Tissue Collection

CRS patients had sinus mucosal tissue harvested from the ethmoid sinuses during ESS. Control patients had mucosa harvested from the posterior ethmoid and sphenoid as required to access relevant skull base pathology. Tissue was immediately stored in Dulbecco's modified Eagle medium (Gibco, Invitrogen Corp., Grand Island, NY) without antibiotic or antimycotic, and transported on ice to the laboratory for further analysis. Mucus was harvested for histopathological examination, and for routine bacterial and fungal culture. In the absence of mucus, a middle meatal swab was taken for bacterial and fungal culture.

Bacterial & Fungal Culture

Intraoperative swabs were transported to the laboratory (Oxoid Transport Swabs, Thermo-Fischer Scientific, Scoresby, Australia) and were streaked onto Columbia horse blood agar, and Chocolate agar (Thermo-Fisher Scientific). Fungal swabs were inoculated onto Sabouraud agar (Thermo-Fisher Scientific). Further nutrient restriction and testing was performed as required for identification.

Biofilm Analysis

Fluorescence *in-situ* hybridization (FISH) was performed on mucosa that had been stored at -80^{o}C. Cryopreservation prior to FISH analysis of sinus mucosa has been validated in our department[186]. Defrosted samples were washed thoroughly in MilliQ water prior to hybridization to remove planktonic organisms. Two probes were utilized on separate pieces of mucosa - a *S. aureus* specific 16S sequence conjugated to Alexa-488 probe, and a pan-fungal 18S Alexa-488 probe. (AdvanDx, Woburn, MA). The manufacturer's protocol was followed. Briefly, samples were fixed to glass slides, dehydrated in 90 % ethanol, air dried, and hybridized at 55 ^{o}C for 90 minutes. Samples were transported to Adelaide

Microscopy for analysis using the Leica TCS SP5 Confocal Scanning Laser Microscope (Leica Microsystems, Wetzlar, Germany). An excitation of 488nm with emission range of 495 – 540nm was used to detect *S. aureus* and fungus. The entire sample was systematically scanned for biofilm elements. Axial Z stacks (0.5µM) were recorded of representative areas to construct a three dimensional virtual images of the tissue, overlying mucus and biofilm.

DNA extraction

An 8 x 8mm square of mucosa was carefully dissected for each patient using sterile instruments under laminar flow conditions and stored at -80°C prior to DNA extraction. A 1 mm^3 piece of this tissue was placed into a sterile microcentrifuge tube containing 270 µL of ATL Lysis buffer (Qiagen, Germantown, MD, cat# 19076) and 30 µL proteinase K (Qiagen, cat# 19131). Samples were incubated at 56 $^{\circ}$C until lysis of the material was noted by visual inspection, then 100 µL of a mixture containing 50 µL each of 0.1 mm and 0.7 mm Zirconia beads (Biospec cat# 11079101z, 11079107zx respectively) were added to the samples which were then homogenized for 10 min at 25 Hz using a Qiagen Tissuelyser. Nucleic acid from the lysed sample was then extracted using the Qiagen DNeasy Tissue kit (Qiagen cat# 69506). 10 µL of each sample was loaded per well for both the Ibis Bacteria, Antibiotic Resistance, and Candida (BAC) and Fungal detection PCR plates (Abbott Molecular, cat# PN 05N13-01).

Ibis T5000 Analysis - PCR Coupled with Electrospray Ionization Mass Spectrometry

The BAC detection plate contains 16 PCR primer pairs that collectively survey all bacterial organisms by using both omnipresent loci (eg. 16S rDNA sequences), as well as more taxa-specific targets (eg. the *Staphylococcus*-specific *tuf*B gene)

as well as providing coverage for major antibiotic resistance genes and *Candida*. The fungal detection plate also uses 16 PCR primer pairs that collectively survey nearly all pathogenic fungal species. An internal calibrant consisting of synthetic nucleic acid template is also included in each well for both assays which controls for false negatives (eg. from PCR inhibitors) and enables a semi-quantitative analysis of the amount of template DNA present. PCR amplification was carried out as per Jiang and Hofstadtler[377]. The PCR products were then desalted in a 96-well plate format and sequentially electrosprayed into a time-of-flight mass spectrometer. The spectral signals were processed to determine the masses of each of the PCR products present with sufficient accuracy that the base composition of each amplicon could be unambiguously deduced[109]. Using combined base compositions from multiple PCRs, the identities of the pathogens and a semi-quantitative determination of their relative concentrations in the starting sample were established by using a proprietary algorithm to interface with the Ibis database of known organisms.

Statistical analysis

Demographic data and species data where appropriate were reported as the mean +/- interquartile range. The Kruskal-Wallis test was used to compare multiple groups with Dunn's post hoc test for non-parametric data. Sensitivity and specificity are presented with upper and lower 95% confidence intervals (CI). Genomes per sample are presented as mean (lower – upper 95% CI), and analysed using The Mann-Whitney *U* test. GraphPad Prism software (San Diego, CA) was used for statistical analysis, and a p-value of 0.05 was considered significant.

2.4 RESULTS

Table 2.1 Demographic & Clinical Data

	CRS	Controls
Number	38	6
Age*	41 (35-47)	44 (37-54)
Male / Female	22/16	2/4
Nasal polyposis (%)	25 (66%)	0
Lund-MacKay CT Score*	17 (15-20)	0
Revision Surgery (%)	25 (66%)	0
Smoking	2 (5%)	0
Aspirin Sensitivity	3 (8%)	0
Asthma	10 (26%)	1 (17%)

Number of species detected

Molecular organism detection using the Ibis T-5000 was positive in 100% of CRS patients and controls. A total of 33 different bacterial species were identified in CRS patients by the Ibis system, with a mean of 3.0 (2.0-4.0) species detected per patient. In control patients, 5 different organisms were detected with a mean of 2.0 (1.0-3.0) per patient. There was a trend of increasing mean isolates per patient from controls 2.0 (1.0-3.0), CRS without nasal polyposis (CRSsNP) 2.5 (1.0-3.0), to CRS with nasal polyposis (CRSwNP) 3.2 (2.0-4.0) but this was not statistically significant ($p>0.05$, Kruskal-Wallis test, Dunn's post-hoc comparison). Ibis analysis detected fungi in only 4 CRSwNP patients, and no fungi were detected in CRSsNP patients or controls.

Conventional culture was positive in 73% of CRS patients, with an average of 1.3 organisms per patient detected. 12 different organisms were identified. Cultures were positive from 33% of control patients. Only one organism (*Staphylococcus epidermidis*) was cultured from this subject group.

Diversity

30/38 (79%) CRS patients, and 3/6 (50%) of control patients, had more than one bacterial species detected on the mucosa using the Ibis molecular diagnostic. *Staphylococcus aureus* was the most commonly detected organism in CRS patients (23/38, 61%), followed by *Staphylococcus epidermidis* (21/38, 55%), and *Propionibacterium acnes* (14/38, 37%, see Table 2). *Nocardia asteroides* (9/38, 24%), *Haemophilus influenzae* (5/38, 13%) and *Pseudomonas aeruginosa* (3/38, 8%) were detected less commonly. In control patients, *Propionibacterium acnes* was the most commonly detected organism (5/6, 85%), followed by *Staphylococcus epidermidis*, present in 4/6 (67%) of patients. Less commonly detected were *Staphylococcus aureus* 2/6 (33%), *Nocardia asteroides* 1/6 (17%) and *Streptococcus agalactiae* 1/6 (17%). Anaerobic species were detected in 18/38 (47%) of CRS patients, and 5/6 (83%) of control patients.

Conventional cultures produced polymicrobial results in 21/38 (55%) CRS patients, whereas no control patients had polymicrobial flora using this method. CRS patients yielded predominantly *S. aureus* (14/38) and *Staphylococcus epidermidis* / coagulase-negative *Staphylococcus* (CNS) (11/38). Only *Staphylococcus epidermidis* / CNS was cultivated from 2 control patients. No growth was reported for 9/38 CRS patients, and 4/6 control patients.

Fungi were rarely detected in CRSwNP patients regardless of technique used, and were not detected in CRSsNP patients or controls. Analysis with the Ibis biosensor showed only two species - *Aspergillus fumigatus* (3), and *Bipolaris papendorfii* (1). Culture detected only two of the Ibis fungal positive patients (both *Aspergillus fumigatus*) plus one *Penicillium chrysogenum*, and one *Trichosporon* in other patients, which were not detected by the biosensor. Fungal-specific FISH analysis was positive in the three *A. fumigatus* patients detected by the Ibis biosensor, the *P. chrysogenum* culture-positive patient, and two additional patients, which were negative by the other techniques. Regardless of detection technique, all patients in whom fungi was detected had nasal polyposis.

Detection of S. aureus using Ibis biosensor, FISH, and culture

We used *S. aureus* as a model organism for studying the characteristics of the three different detection methods. The biosensor detected *S. aureus* in 61% of CRS patients, biofilm was positive in 50%, and conventional culture was positive in 37%. FISH analysis had a sensitivity of 78% (±18) for the detection of *S. aureus* compared to Ibis, with a specificity of 93% (± 6). In contrast, conventional culture detected *S. aureus* with a sensitivity of 61% (±17), and a specificity of 93% (+16). *S. aureus* was detected in control patients only by molecular detection (2/6, 33%). Neither conventional culture nor FISH analysis was positive for *S. aureus* in these patients.

Relative Microbial Density

We determined the relative numbers of the bacterial species using the Ibis biosensor, which provides a genomes/well measure. Results for the top ten organisms are shown in Table 2. The most commonly detected organisms in

CRS patients, *S. aureus* and *S. epidermidis*, had markedly different microbial densities, with *S. aureus* organisms present in much higher numbers than *S. epidermidis* based on bacterial genomes detected. Some of the less commonly detected organisms such as *Corynebacterium pseudodiphtheriticum, Moraxella catarrhalis, Streptococcus pneumoniae,* and *Pseudomonas aeruginosa,* were found in relatively high numbers when detected on the mucosa. The abundance of *S. aureus* was much greater in CRS patients compared to controls, however other organisms such as *S. epidermidis* and *P. acnes* were detected in similar quantities in both patient groups. When all organisms were considered, CRS patients had significantly greater bacterial genomes per sample than control patients ($p<0.05$, Mann-Whitney *U* test - see Table 2.2).

Table 2.2 Molecular detection top ten organisms: detection frequency and prevalence

	CRS N=38			**Control N = 6**		
	No. Detected (%)	**Total genomes / sample**	**Mean genomes / sample**	**No. Detected (%)**	**Total genomes / sample**	**Mean genomes / sample**
Staphylococcus aureus	23 (61%)	10549	459	2 (33%)	101	51
Staphylococcus epidermidis	21 (55%)	508	24	4 (67%)	21	5
Propionibacterium acnes	14 (37%)	2339	167	5 (83%)	621	124
Nocardia asteroides	9 (24%)	1587	176	1 (17%)	72	72
Haemophilus influenzae	5 (13%)	404	81	-	-	-
Corynebacterium pseudodiphtheriticum	4 (11%)	2121	530	-	-	-
Streptococcus agalactiae	4 (11%)	57	14	1 (17%)	3	3
Moraxella catarrhalis	3 (8%)	2931	977	-	-	-
Pseudomonas aeruginosa	3 (8%)	1852	617	-	-	-
Streptococcus pneumoniae	3 (8%)	1908	636	-	-	-
Mean microbial genomes per patient sample all detected organisms (lower - upper 95% CI)	870 (605 - 1136)			136 (-32 - 303)		

Are FISH and culture detection related to microbial abundance?

We again used the organism *S. aureus* to compare detection techniques based on microbial numbers. The quantitative genomic analyses of the 25 (CRS and control) samples in which *S. aureus* was detected by the Ibis biosensor, were compared to the detection of the organism by FISH and conventional culture to determine if these latter detection methods are dependent on the abundance of organisms to provide a positive finding (see Figure 2-1). The number of *S. aureus* genomes per sample was significantly higher in those specimens that tested positive for *S. aureus* by FISH and conventional culture ($p<0.05$, Kruskal-Wallis test, Dunn's post-hoc comparison). However there was no statistically significant difference between FISH and culture detection sensitivity based on *S. aureus* genomes per sample ($p>0.05$, see Figure 2-1).

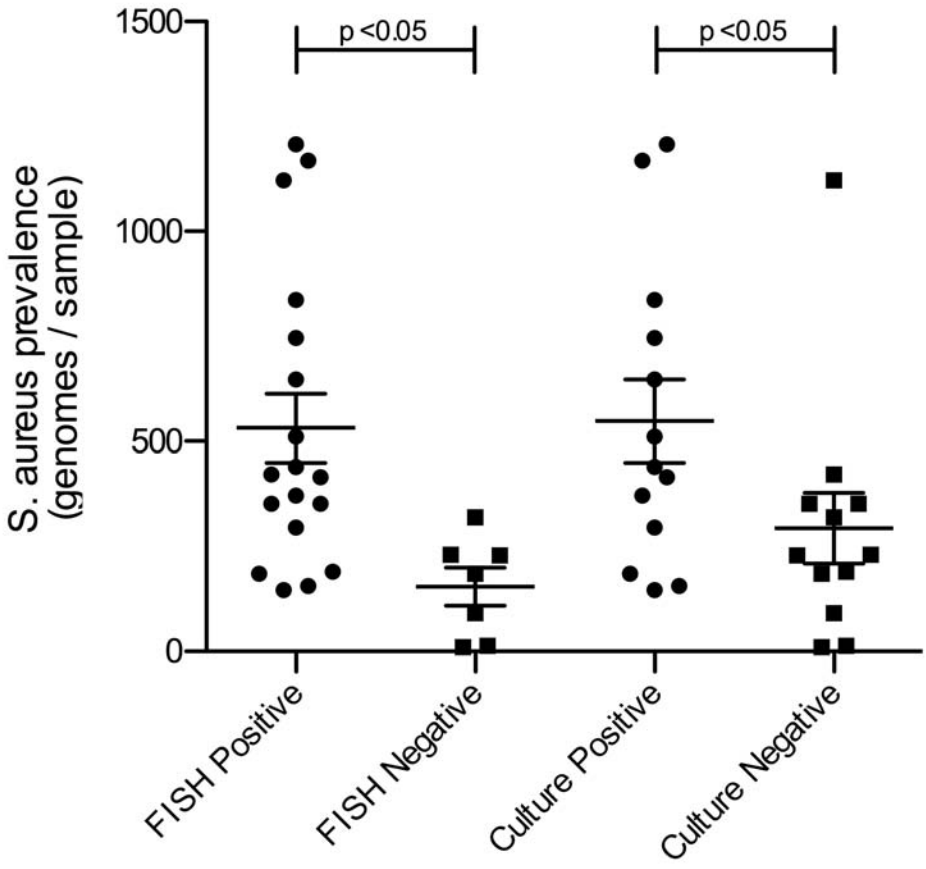

Figure 2-1 The relationship between *S. aureus* abundance, and detection by FISH and culture. The detection of *S. aureus* by FISH and culture is dependent on the abundance of the bacteria as measured by the number of bacterial genomes present in the sample.

2.5 DISCUSSION

This study compares the microbial bioburden in CRS patients, with healthy controls using three markedly different, but complementary detection techniques. We have shown that conventional laboratory culture has a tendency to polarize the detected microbes, selecting for abundant, rapidly growing aerobic organisms with favourable growth characteristics, such as *S. aureus* and *S. epidermidis*. Biosensor molecular detection allows a more comprehensive analysis of the microbial community and has the advantage of not requiring *a priori* knowledge of the flora. *S. aureus* was the most commonly detected organism in CRS patients, and was relatively more abundant in CRS patients compared to controls. It was detected with high sensitivity with FISH compared to the biosensor, but at lower levels by conventional culture. We have shown that molecular quantification can provide additional information with respect to microbial abundance, and comparisons between health and disease may assist our understanding of the role of these organisms in pathogenesis. Additionally we have shown that the detection of organisms using FISH and conventional culture is significantly dependent on microbial abundance as measured using molecular quantification. Fungi were present uncommonly, in a select group of nasal polyp patients.

Staphylococcus aureus was the most prevalent organism in CRS patients using molecular detection, followed by *Staphylococcus epidermidis* and *Propionibacterium acnes*. When the microbial density was compared for these three organisms between CRS and control patients, *S. aureus* was present at

approximately 10 times higher genomes per sample in CRS patients, whereas *S. epidermidis* and *P. acnes* were found in similar abundance between both patient groups. The increased bioburden of *S. aureus* in the disease group is of particular interest, as it is emerging as a prominent disease modifying organism in CRS and it's presence in patients has important clinical implications[192,378]. The capacity of *S. aureus* to exist within dense mucosal biofilms has been documented[186,191], which may explain the variable detection from clinical specimens using traditional culture techniques.

A multitude of other studies have recently reported CRS microbiological data using a variety of techniques, including molecular diagnostics and FISH. Comparison with these studies is of interest to further our understanding of this disease. In the current study, *Haemophilus influenzae* was detected at relatively low levels (13%) in CRS patients, and was not detected in controls. This is in agreement with Stephenson *et al,* who detected *H. influenzae* in 17% of CRS patients[379], but contrasts with earlier FISH based studies which proposed it as the dominant organism in CRS[187,188]. *Pseudomonas aeruginosa* was also found infrequently in the current study (8%), confirming the findings of Stephenson *et al.* These results contrast with a recent molecular study which found *P. aeruginosa* to be the dominant organism in CRS patients, however control patients were not assessed[98]. The disparity in organism profiles between studies may reflect regional variation, patterns of antimicrobial use, methodological differences, or disease severity patterns.

The disease burden in the current study was relatively high as measured by the radiological severity and the rate of non-primary surgery, which were at least twice as high as others[98,379], reflecting the tertiary nature of the practice. Some microbial biofilms have been associated with a more severe disease process than others[191], and the microbial community in this study may reflect some bias towards the more severe end of the CRS spectrum. However, this is arguably the population which is most resistant to current treatment paradigms, and therefore of utmost importance to investigate.

The high prevalence of anaerobes detected in CRS patients is in agreement with previous molecular studies[98,379], however we also found high rates in controls, with similar abundance, casting doubt on a direct pathogenic role. There was poor agreement between the Ibis biosensor and conventional culture data for anaerobes, which reflects the paucity of culture-based studies reporting anaerobic species in CRS. These different detection methodologies seem to have comparable efficacy at detecting abundant, fast growing organisms. The pathologic importance of these species remains to be determined however.

Through the use of molecular detection we are beginning to understand the natural flora of non-diseased sinuses. Contrary to previous reports of sterility[134,140], we have shown that all healthy sinuses in this study are associated with a microbial community, and many of these organisms are also found in diseased sinuses. Nevertheless, we have shown that the abundance of organisms is significantly greater in CRS patients compared to controls - a

phenomenon which requires further investigation with greater numbers of patients.

The cultivation of microorganisms using traditional culture has many pitfalls which may explain it's limited utility in describing polymicrobial communities such as that within diseased sinuses. To be detected, organisms must grow on media after being removed from the native mucosal surface, with significant environmental changes in temperature, pH, nutrient sources, and without the complex dynamics of polymicrobial communities and host immune systems. Molecular methods such as the Ibis biosensor offer great potential for analysis of microbial diversity in CRS, which is untempered by the limitations of conventional culture. Molecular methods are sensitive and accurate and provide a more complete view of the bacterial communities present, and even provide a molecular antibiogram to guide treatment decisions. Access to molecular detection instrumentation and expertise may also limit it's utility in the clinical setting, but this is rapidly improving.

The formation of biofilms also impacts on the culture rates from patients. Organisms that form these immobilized consortia often undergo phenotypic transformations with reduced metabolic activity, which impacts the capacity to grow on selective media[105]. Using the model organism *S. aureus*, we have shown significantly increased detection rates using both FISH and molecular detection compared to cultivation techniques. Similar to previous studies[185,186,240], biofilm was not detected in the control patients, despite detection of *S. aureus* in 2/6 controls by the Ibis biosensor. *In situ* hybridisation techniques have previously

been reported to have reduced sensitivity in detecting low copy number nucleic acids of scarce bacteria[104]. In an attempt to counter this, we have employed protein nucleic acid (PNA) FISH which has higher affinity and stability for microbial DNA sequences than conventional FISH probes[380]. Despite this however, some of the CRS patients, and both controls with *S. aureus* detected at low DNA copy number by Ibis, were not detected by FISH. This limitation not withstanding, we have shown a good correlation between FISH and biosensor detection of *S. aureus* in CRS patients, with high sensitivity and specificity, suggesting it has good clinical and research applicability.

The prevalence of fungi in CRS and healthy control patients has long been debated[79]. Many studies have found a predominance of fungi in both patient groups when the nasal cavity is sampled[60,381,382]. It is possible that these studies are detecting inhaled environmental fungi, which is trapped within nasal mucus en-route to the oropharynx. When sinus mucosa is specifically analysed, studies suggest a lesser prevalence in CRS patients and absence in controls[383,384]. The characterization of fungal species in CRS is far less advanced than for bacteria, and whether or not certain fungal species are more prevalent or important in CRS is still unknown. Therefore we employed a pan-fungal FISH probe to detect all species, in conjunction with culture and molecular detection. We found fungi in a small proportion of the sinus mucosa of CRS patients using all three detection methods, and an absence in control patients. The three methods showed similar sensitivity. Fungi may be playing a role in this small subset of CRS patients, all of whom had nasal polyposis. This study refutes the theory that current culture

methods are insensitive and missing a large proportion of patients in whom fungi are playing a central role.

The importance of understanding the complex polymicrobial communities in the sinuses is highlighted by the concept of dysbiosis, where organisms interact in positive (mutualistic) or negative ways to alter the local community, and interaction with the host. There is evidence to suggest that microbial diversity is important for health[283], and a reduced diversity with increased abundance is associated with chronic inflammation and poor healing[284]. There is also literature to suggest that host genetics and immunity strongly influence the composition of the mucosal microflora[285]. Microbial communities inhabiting mucosal surfaces such as the gastrointestinal tract can result in a significant mutualism including local immune homeostasis[281,282], and protection from pathogens through processes such as nutrient consumption, occupation of attachment sites, and secretion of antimicrobial substances[286]. *Propionibacterium acnes,* which was found in more than 80% of control patients in the current study, has been shown to produce bacteriocins which have antibacterial and antifungal activity which may be protective against pathogens.[287] Competition between microbes on mucosal surfaces can result in selection of virulence factors which can be detrimental to the host[288]. In an elegant model of polymicrobial interactions, Sibley *et al*, have shown that avirulent organisms can enhance the pathogenicity of other organisms, highlighting the importance of comprehensive community analysis to investigate disease[289].

It is possible that disruption of mutualistic relationships, through shifts in mucosal-associated microbial composition, could contribute to the onset, progression, or recalcitrance of CRS. For example, the change in microbial dynamics during a viral upper respiratory tract infection or acute bacterial exacerbation of CRS. The often protracted use of antibiotics in our patients may also have detrimental sequelae on the microbial balance in CRS which requires further substantiation. There is poor understanding of such mechanisms in CRS, but they are an active focus of research at present. The first step in this journey is to greatly improve our knowledge of the mucosal microbial communities in CRS patients and controls.

Future directions for research should examine larger populations of CRS patients to characterize the microbiome of the different CRS phenotypes in comparison with healthy controls. Furthermore, longitudinal molecular studies evaluating the effect of antibiotics, endoscopic sinus surgery, and topical treatments on microbial diversity and abundance in CRS patients would be invaluable. Investigating the relationships within the microbial communities, and their interactions with the host immune system in health and disease, will ultimately lead to a greater understanding of the pathogenesis of chronic rhinosinusitis.

A sheep model to investigate the role of fungal biofilms in sinusitis: Fungal & bacterial synergy

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Financial assistance provided by the Garnett Passe and Rodney Williams Memorial Foundation

chapter 3

Boase, S., Valentine, R., Singhal, D. Tan, L.W. & Wormald, P.J. (2011) A sheep model to investigate the role of fungal biofilms in sinusitis: fungal and bacterial synergy.
International Forum of Allergy & Rhinology, v. 1(5), pp. 340-347

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3.1 ABSTRACT

Background: The role of fungi in the spectrum of chronic rhinosinusitis (CRS) is poorly understood. Fungal biofilms have recently been discovered in CRS patients. We have developed an animal model for the investigation of sinonasal fungal biofilms. The role of type I hypersensitivity, and pathogenic bacteria is presented.

Methods: Thirty sheep were sensitized with fungal antigens – *Aspergillus fumigatus,* and *Alternaria alternata,* or control. Endoscopic surgery was performed to expose both frontal sinus ostia– one was occluded. Fungi with or without *Staphylococcus aureus* were inoculated into the sinus. Skin prick tests assessed for fungal allergy. Fungal and *S. aureus* biofilms, histology, and culture rates were assessed.

Results: 45% of experimental sheep were sensitized to fungal antigen. Only one sinus inoculated with fungus, developed minimal fungal biofilm. 80% developed fungal biofilm when *S. aureus* was co-inoculated. The presence of hypersensitivity to fungus was not related to fungal biofilm development.

Conclusions: Significant fungal biofilm only occurred when *S. aureus* was the co-inoculum. Hypersensitivity was not requisite. The relationship of *S. aureus* to fungal biofilms is of great clinical interest. Fungi may be opportunistic pathogens that simply require inflamed mucosa with weakened innate defenses; alternatively, a cross-kingdom synergy could be contributing to fungal proliferation.

3.2 INTRODUCTION

Chronic rhinosinusitis (CRS) is a heterogeneous group of disorders, characterized by inflammation of the sinonasal mucosa, which is often refractory to medical and surgical treatment. A significant global research effort is currently underway aimed at understanding the underlying pathophysiological mechanisms of these diseases. It is probable that a constellation of factors including host immune mechanisms and environmental triggers such as microorganisms, lead to disease manifestations.

Of the environmental triggers, fungi are perhaps the most controversial. Katzenstein *et al,* first discovered fungus in the sinuses of CRS patients in 1983, describing the thick, tenacious, eosinophil rich mucus filling the sinuses, along with dense polyposis, coining the term “Allergic *Aspergillus* Sinusitis” [150]. Since then it has emerged as a prominent, but contentious etiologic agent in CRS. Whilst fungi are identified in various CRS subgroups, at the most severe end of the spectrum is allergic fungal rhinosinusitis (AFRS) representing some of the most recalcitrant CRS patients. AFRS represents the most robust and accepted involvement of fungi in the pathogenesis of CRS. The diagnostic criteria for AFRS were described by Bent & Kuhn, which include IgE mediated, type I hypersensitivity[385]. It is proposed that IgE mediated hypersensitivity may contribute to the mucosal inflammation in these patients, which may facilitate fungal retention and proliferation in the sinuses. Additionally, numerous other mechanisms may contribute to the development of inflammation, possibly including biofilm formation.

Healy *et al,* discovered the presence of fungal biofilms using epi-fluorescent microscopy, whilst investigating microbial biofilms in CRS patients [187]. These fungi were noted to be physically associated with bacterial biofilms, and were more prevalent in those with more severe disease – eosinophilic mucus chronic rhinosinusitis (EMCRS) patients. More recently, Foreman *et al,* detected fungal biofilms in 11/50 (22 %) CRS patients using fluorescence *in-situ* hybridization (FISH)[186]. Interestingly, 7 of these patients also had evidence of *Staphylococcus aureus* (*S. aureus*) biofilms highlighting a potential cross-kingdom synergy. This is also supported by histological evidence of fungal hyphae in eosinophilic mucus coincident with positive culture of *S. aureus*[204].

Much of the challenge in elucidating the pathophysiology of fungal rhinosinusitis is related to the lack of a reliable animal surrogate[386]. We have developed a novel *in-vivo* model of sinusitis in the aerated frontal sinus of sheep, to investigate the role of systemic type I hypersensitivity to fungi, and the influence of pathogenic bacteria, in fungal biofilm formation.

3.3 MATERIALS AND METHODS

Fungal Sensitization

All protocols were approved by the Animal Ethics Committees of the University of Adelaide and The Institute of Medical & Veterinary Science, South Australia. Thirty male Marino sheep were used in this study. Sensitization commenced at the time of the sinus access procedure (Day 0). 8 sheep were controls, 11 were

sensitized to *Aspergillus fumigatus* antigen, and 11 were sensitized to *Alternaria tenuis (alternata)* antigen (Hollister-Stier Laboratories, LLC, Spokane, WA). Control solution consisted of 50% glycerol (Sigma-Aldrich, St Louis, MO, USA) in 1X phosphate buffered saline (PBS). All solutions were sterile filtered, pooled and stored at -80^{o}C prior to use.

Sheep were immunized intraperitoneally with control or study solution mixed with aluminium hydroxide as adjuvant (1:1), as previously described[387,388]. The immunization protocol involved three injections per week for four weeks (see Figure 3-1).

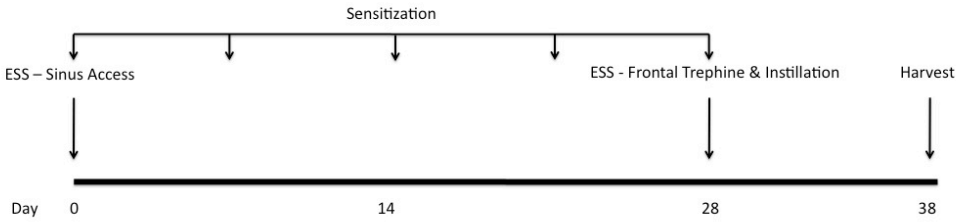

Figure 3-1 Timeline of the experimental protocol. 28 days of sensitization followed by frontal sinus inoculation and 10 day incubation.

Skin Prick Testing

All sheep were skin prick tested prior to initial sensitization, and again at the end of the sensitization period, immediately prior to trephination. Two test antigens were used, *A. tenuis (alternata)(Aa)* and *A. fumigatus (Af)* (Hollister-Stier Laboratories, LLC, Spokane, WA). Negative control was sterile filtered 50 % glycerol in 1X PBS. Histamine phosphate 10mg/mL supplied by the Royal Adelaide Hospital Pharmacy Production Service, was used as a positive control. The animals were restrained in the sitting position, and the non-wool bearing skin of the rear inner thigh was cleaned with ethanol. A single drop of allergen was applied to the skin and plucked with a single use lancet. Allergen solution was blotted at 1 minute. Wheal diameter was recorded at 10 minutes. Results were recorded as non-diagnostic if positive control wheal was <4mm, or negative control was >1mm.

Fungal Inoculum

Pure strains of *A. fumigatus* (Af) and *A. alternata* (Aa) were inoculated onto inhibitory mold agar (Becton-Dickinson, NJ, USA) without antibiotic, and grown to confluence over 5 days, in the dark at room temperature. Fungi were harvested, agitated, and resuspended in cerebrospinal fluid (CSF) broth (Oxoid, Adelaide Australia) and adjusted to 1.5 McFarland units above baseline. Samples were placed on ice until instillation.

Bacterial Inoculum

A pure strain of *S. aureus* was isolated from the sinus of a CRS patient with proven *S. aureus* biofilm, and supplied by the Department of Microbiology, The Queen Elizabeth Hospital, Adelaide, Australia. *S. aureus* was initially grown on

Columbia Horse Blood Agar (Oxoid) overnight at 37^{o}C. A single colony was inoculated into CSF broth (Oxoid), placed on a shaker and incubated overnight at 37^{o}C. The culture was adjusted to 0.5 McFarland units above baseline, and placed on ice prior to instillation.

Anesthetic

All 30 sheep were given a general anesthetic by an experienced animal technician. Intravenous induction with sodium thiopentone (19mg/kg) via the internal jugular vein, followed by endotracheal intubation, and maintenance anesthesia with 1.5 – 2 % inhalational isoflurane. The nasal cavities were topically decongested with 2 sprays of co-phenylcaine forte nasal spray (ENT Technologies, Victoria, Australia).

Endoscopic Sinus Surgery – Sinus Access

Endoscopic access to the frontal sinus was required for the next stage of the protocol. A standard endoscopic procedure to access the frontal ostia in sheep has been developed in our department using custom made endoscopic instruments.[180,389] Briefly, under general anesthesia as described above, the sheep was placed supine on the operating table. A middle turbinectomy was performed to expose the anterior ethmoid complex, which was dissected and removed to reveal the frontal sinus ostia. Following hemostasis, the animal was recovered. During the convalescent phase, the sheep were housed in a paddock to undergo the 4 week sensitization procedure.

Endoscopic Sinus Surgery – Trephination & Occlusion

Following sensitization, all sheep were again skin prick tested. Subsequently, a second general anesthetic was given to permit frontal sinus trephination. The forehead was shorn and landmarks for the frontal trephine made on the skin, 1cm on each side of the midline in line with the mid supra-orbital ridge. Sterile saline was injected, and aspirates of the frontal sinus were taken for mycology and bacteriology. The site of the frontal ostia was confirmed endoscopically following a flush of 1 % fluorescein through the trephines. The left frontal ostium was then occluded with petroleum jelly impregnated gauze until fluorescein was unable to be passed into the nasal cavity. The right frontal ostium was left patent. Any residual fluorescein was removed from the sinuses. 1 mL of control or study inoculum was injected into each sinus via trephine, according to the study protocol. Trephines were capped and left *in-situ*, and the animals were recovered.

Specimen Collection

Sheep were euthanized at day 10 with intravenous pentobarbitone sodium (>100mg/kg). The skin and anterior table of frontal sinus were removed, exposing the sinus mucosa. The sinus mucosa was carefully dissected using sterile instruments. The mucosa was placed in Dulbecco's Modified Eagle's Medium (Gibco, Invitrogen Grand Island, NY) without antibiotic or antimycotic, and transported to the laboratory. Under laminar flow conditions, the sinus tissue was dissected into appropriate sized pieces for the various analytical processes: 10 x 10 mm for fungal biofilm detection, 10 x 10 mm for FISH analysis, and 5 x 5 mm in 10% formalin for histology – with hematoxylin and eosin (H&E) stain. Mucous was scraped from the residual mucosa and sent for mycology and bacteriology.

Fungal Biofilm Determination

Sinus mucosal samples for fungal biofilm analysis were initially washed thoroughly in 3 consecutive flasks of 100mL MilliQ water (Millipore, Billerica, MA) to remove any planktonic organisms. Each tissue sample was processed fresh, and immersed in a solution containing 100 µL of concanavalin A, Alexa Fluor 488 conjugate (5 mg/mL in 0.1 M NaHCO3 pH 8.3, Invitrogen GmbH Karlsruhe, Germany), 5 µL FUN-1 Cell Stain (10 mM solution in DMSO, Invitrogen), and 895 µL of 1X PBS. These were incubated for 1 hour, in the dark at room temperature. Samples were transported to Adelaide Microscopy for analysis using a Leica TCS SP5 Confocal Scanning Laser Microscope (CSLM) (Leica Microsystems, Wetzlar, Germany). Prior to slide mounting, samples were gently rinsed in 1X PBS to remove excess stain. An excitation wavelength of 488 nm, and dual emission detection at 495 – 540nm and 560 – 610nm was employed. A combination of 20x and 63x magnification was used. The entire 10 x 10mm sample was systematically scanned for fungal elements. Axial Z stacks were recorded of representative areas to construct a three dimensional virtual image of the tissue, overlying mucus and biofilm. The scoring system employed was: 0 no fungal elements identified, + infrequent fungal elements found, ++ florid fungal biofilm.

Histopathologic Scoring

A blinded examiner graded inflammation on H&E stained slides on a scale from 0-4. The scoring system has been previously described[386]. 0 - reflecting normal mucosa; 1 – minimal change with rare individual inflammatory cells within mucosa & submucosa; 2 - mild changes with light infiltrate of inflammatory cells; 3 – moderate changes with moderately dense inflammatory cells; 4 – severe changes

with dense inflammatory infiltrate – partially obscuring normal tissue architecture. Secretory hyperplasia was graded based on loss of cilia, and hyperplasia & cytoplasmic blebbing of non- ciliated cells. 0 – no change; 1 – minimal changes; 2 – mild; 3 – moderate, and 4 – severe changes affecting most of the mucosa.

Fluorescence in-situ hybridization

Following our observation that significant fungal biofilm only formed in the presence of *S. aureus* infection we performed FISH to examine the physical relationship between the two biofilms. Additionally, the molecular specificity of the FISH probe ensures the bacterial biofilms are indeed composed of *S. aureus* species. FISH was performed on surplus mucosal samples that had been stored at -80^{o}C. Cryopreservation prior to FISH analysis of sinus mucosa has been validated in our department[186]. Defrosted samples were washed in MilliQ water prior to hybridization. A pan-fungal Alexa-488 probe, and a *S. aureus* - TAMRA probe were utilized (AdvanDx, Woburn, MA). The manufacturer's protocol was followed. Briefly, samples were fixed to glass slides, dehydrated in 90 % ethanol, air dried, and hybridized at 55^{o}C for 90 minutes. Samples were transported to Adelaide Microscopy for analysis using the Leica TCS SP5 Confocal Scanning Laser Microscope (Leica Microsystems, Wetzlar, Germany). Sequential scanning was performed, with scan 1 at an excitation of 488nm, emission range 495 – 540nm, scan 2 at an excitation of 543nm, emission range 550 – 590nm, for pan-fungal and *S. aureus* probes, respectively.

3.4 RESULTS

Skin Prick Test Responses to Fungal Immunizations

Sheep were inoculated with fungal antigen (Af or Aa) or control, mixed with alum adjuvant over a period of four weeks via the intraperitoneal route. Immediately preceding the sensitization protocol, no sheep (0/30) had recordable skin reactions to either Af or Aa, or control. At the conclusion of the protocol, 0/8 control sheep, 7/11 Af sheep, and 3/11 Aa sheep had a positive skin prick test to the respective fungal antigen (see Figure 3-2). Combined, 10/22 (45%) of experimental sheep were sensitized to fungal antigen. No adverse local or systemic effects from intraperitoneal inoculation were noted, however there was frequently some deep tissue induration at the injection site.

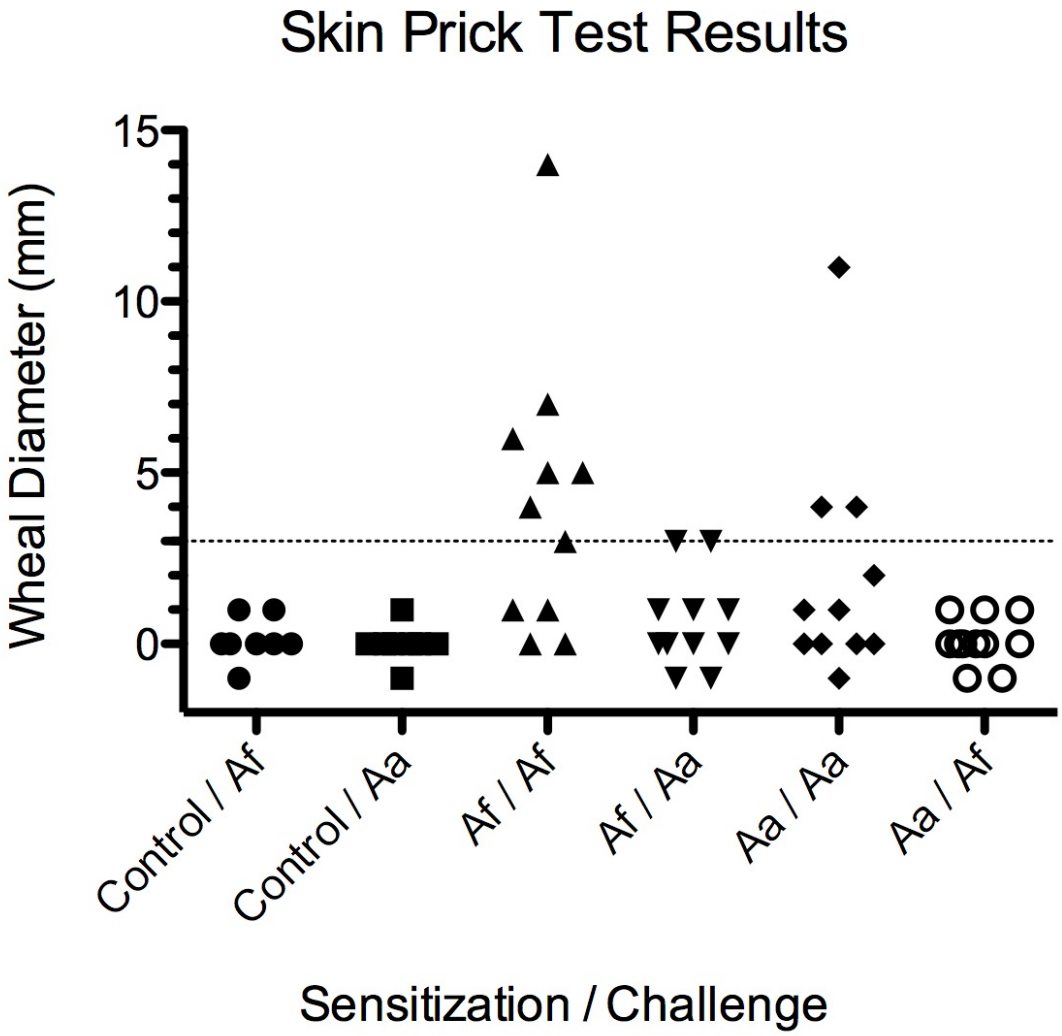

Figure 3-2 Skin Prick Test Results. X-axis denotes the sensitization antigen followed by the skin prick challenge antigen. Dotted line at 3mm, ≥3mm was taken as a positive result. Af = *A. fumigatus* antigen, Aa = *A. alternata* antigen.

Histopathologic Analysis

A moderate to severe mucosal inflammatory infiltrate, with predominant neutrophils and eosinophils was noted following *S. aureus* inoculation. Similar infiltrates were seen in sinus mucosa following fungal inoculation alone, but to a lesser degree. Some of the control mucosa showed low levels of inflammation also, likely secondary to nasal packing and post-operative change (see Figure 3-3 & Figure 3-4). Histopathological scores were analyzed using 1 way ANOVA and Tukey *post-hoc* test. Inflammation scores were significantly greater when *S. aureus* was inoculated, compared to fungal inoculations, and controls ($P<0.01$). However, there was no statistical difference in mucosal inflammation between *S. aureus* inoculation alone, and *S. aureus* and fungus together ($P>0.05$). Additionally, there was no statistical difference in inflammatory scores between fungal inoculation and control sinuses ($P>0.05$,) This suggests the inflammatory mucosal responses were primarily due to the presence of *S. aureus* (see Figure 3-5).

Histopathological scoring of secretory hyperplasia showed a trend of higher scores when *S. aureus* was inoculated compared to fungus and controls, however the results were not statistically significant (ANOVA, $P>0.05$). Similar to inflammation scores, fungal inoculation did not significantly affect mucosal secretory hyperplasia compared to controls ($P>0.05$, see Figure 3-6).

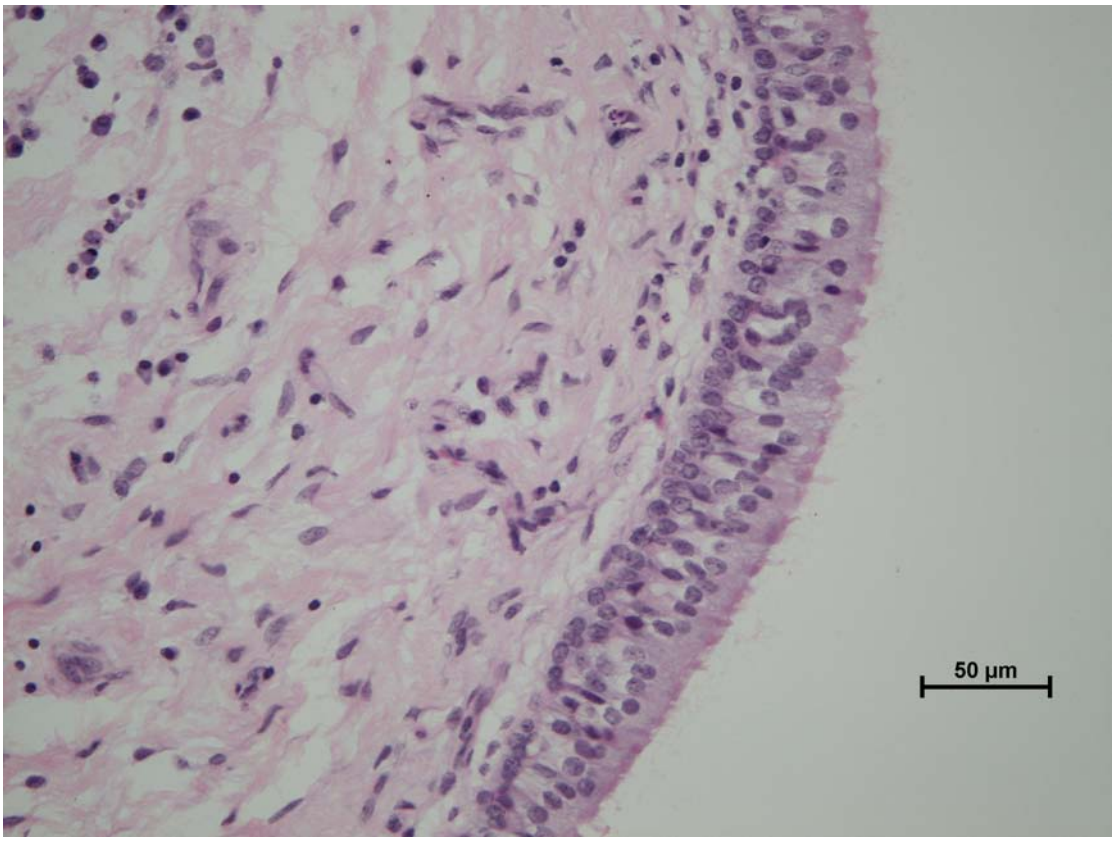

Figure 3-3 H&E stained sinus tissue, 20x micrograph – Control tissue

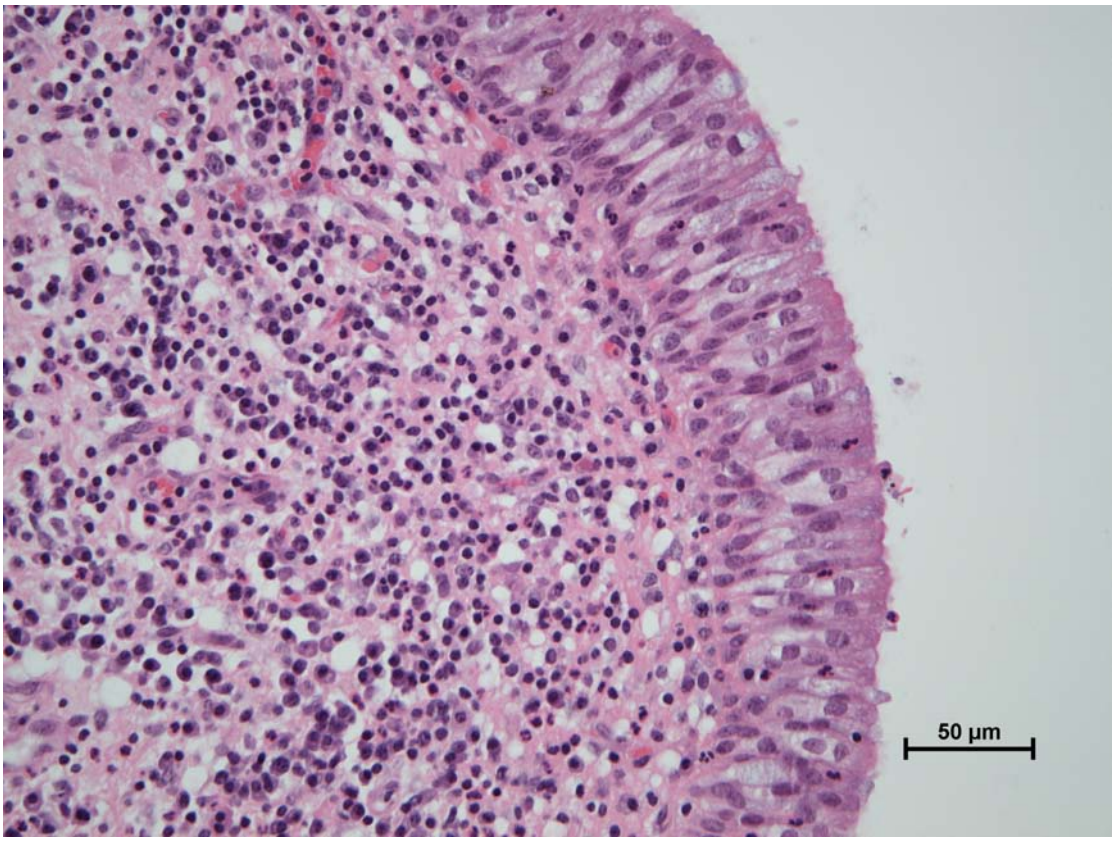

Figure 3-4 *A. alternata / S. aureus* inoculation. Note the influx of lymphocytes, neutrophils and eosinophils, epithelial hyperplasia.

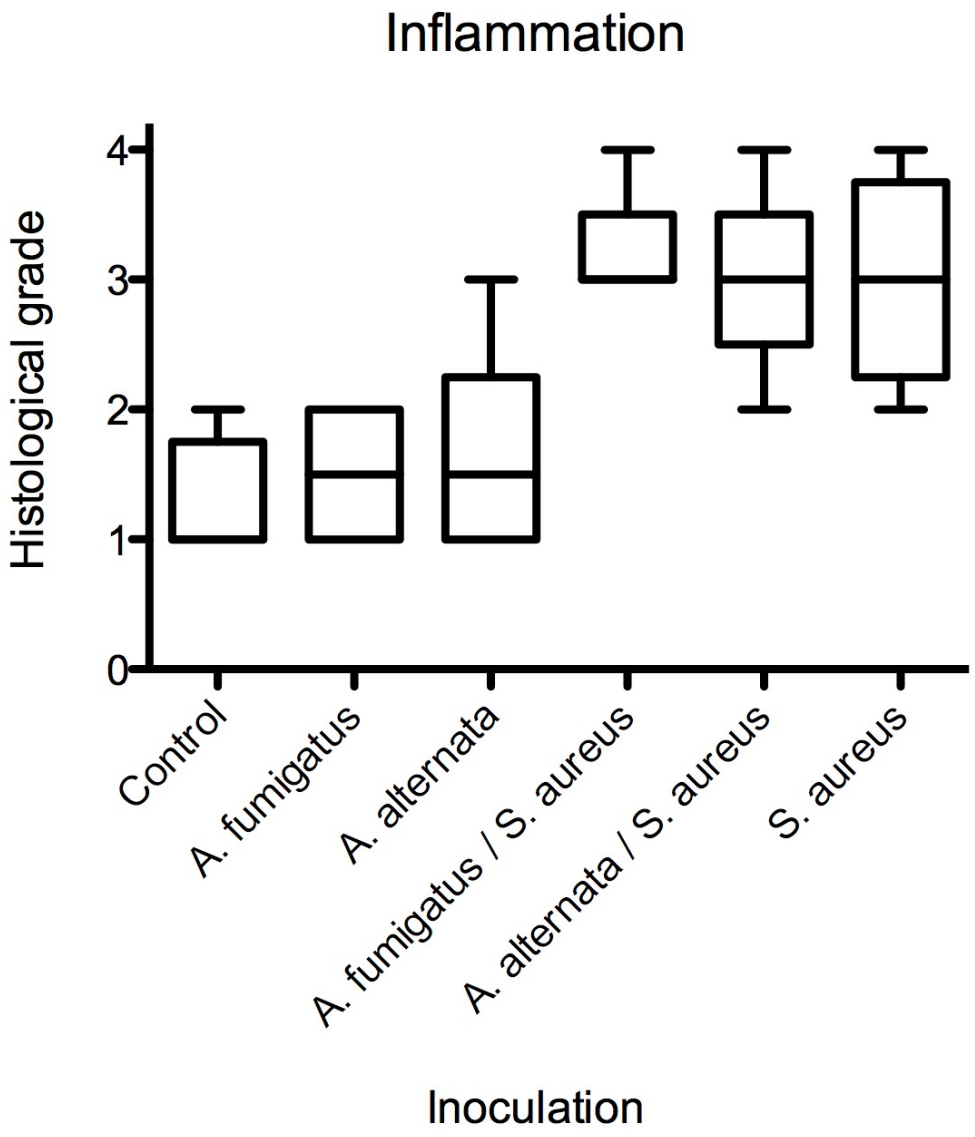

Figure 3-5 Histology scoring: Inflammation compared to frontal sinus inoculum. The 'box' represents the interquartile data range, the horizontal bar shows the median value and the 'whiskers' represent the 5th and 95th percentile values.

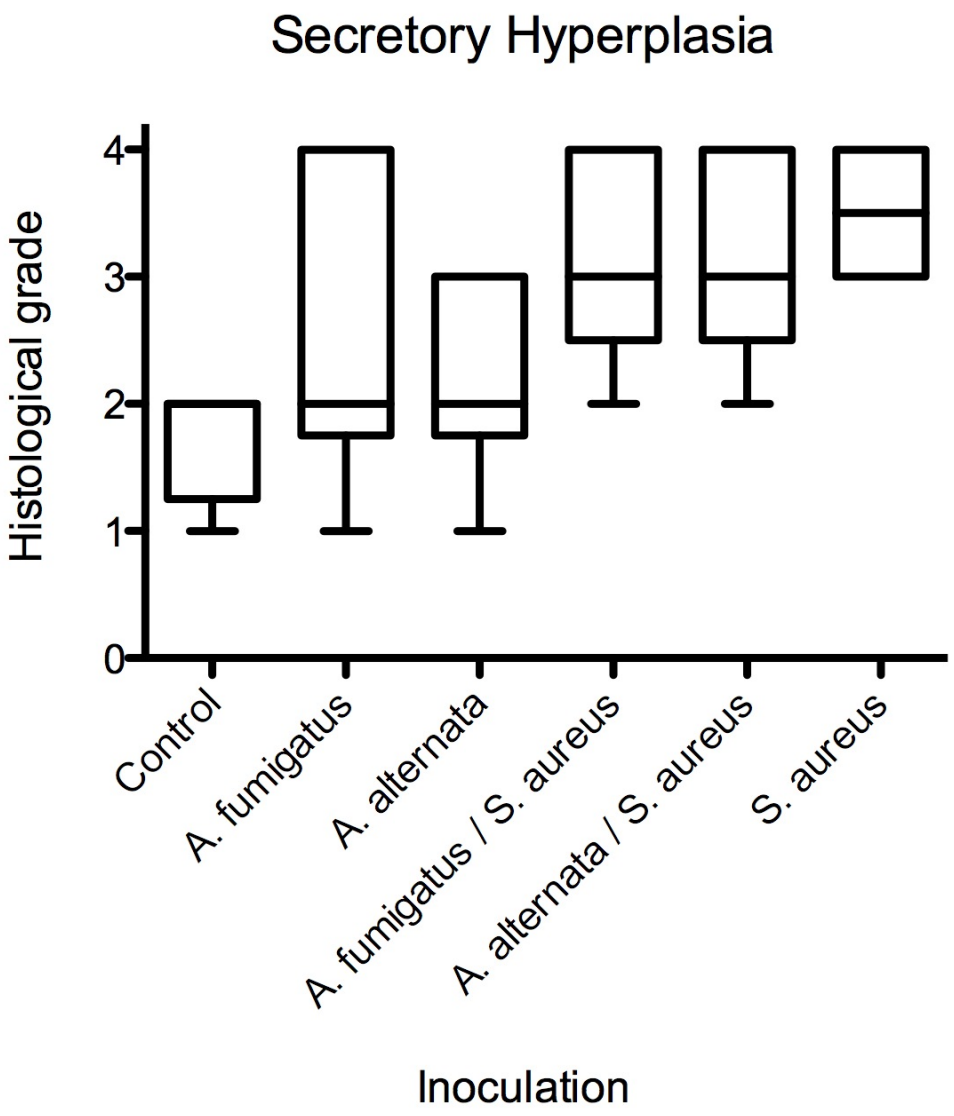

Figure 3-6 - Histology scoring: Secretory hyperplasia compared to frontal sinus inoculum.

Fungal Sensitization and Histopathological Change

Fungal sensitization as measured by positive skin prick test was compared to histological scores. The degree of inflammation and secretory hyperplasia was not statistically different between animals based on skin prick test (Mann Whitney *U* test, P = 0.556 & 1, respectively).

Fungal Biofilm Analysis

Confocal scanning laser microscopy was used to assess for fungal biofilm formation. There was no significant growth of fungus, bacteria, or biofilm formation in the non-occluded sinuses. The following data are from the left (occluded) frontal sinus. No fungal biofilm was detected in any of the control sinuses (see Figure 3-7). There were a small number of scattered hyphae detected in 1 of 6 sinuses inoculated with *A. fumigatus* alone (16.7%). No fungal biofilm was detected in the sinuses inoculated with *A. alternaria* alone. However, when either fungal species was co-inoculated with *S. aureus,* 80% produced fungal biofilm. 2/10 showed occasional fungal elements (see Figure 3-9), whilst 6/10 developed florid fungal biofilm (see Figure 3-10). The co-innoculation of fungal species with *S. aureus* produced significantly more frontal sinus fungal biofilm than fungus inoculation alone (Chi-square, $p<0.001$ - see Table 3.1).

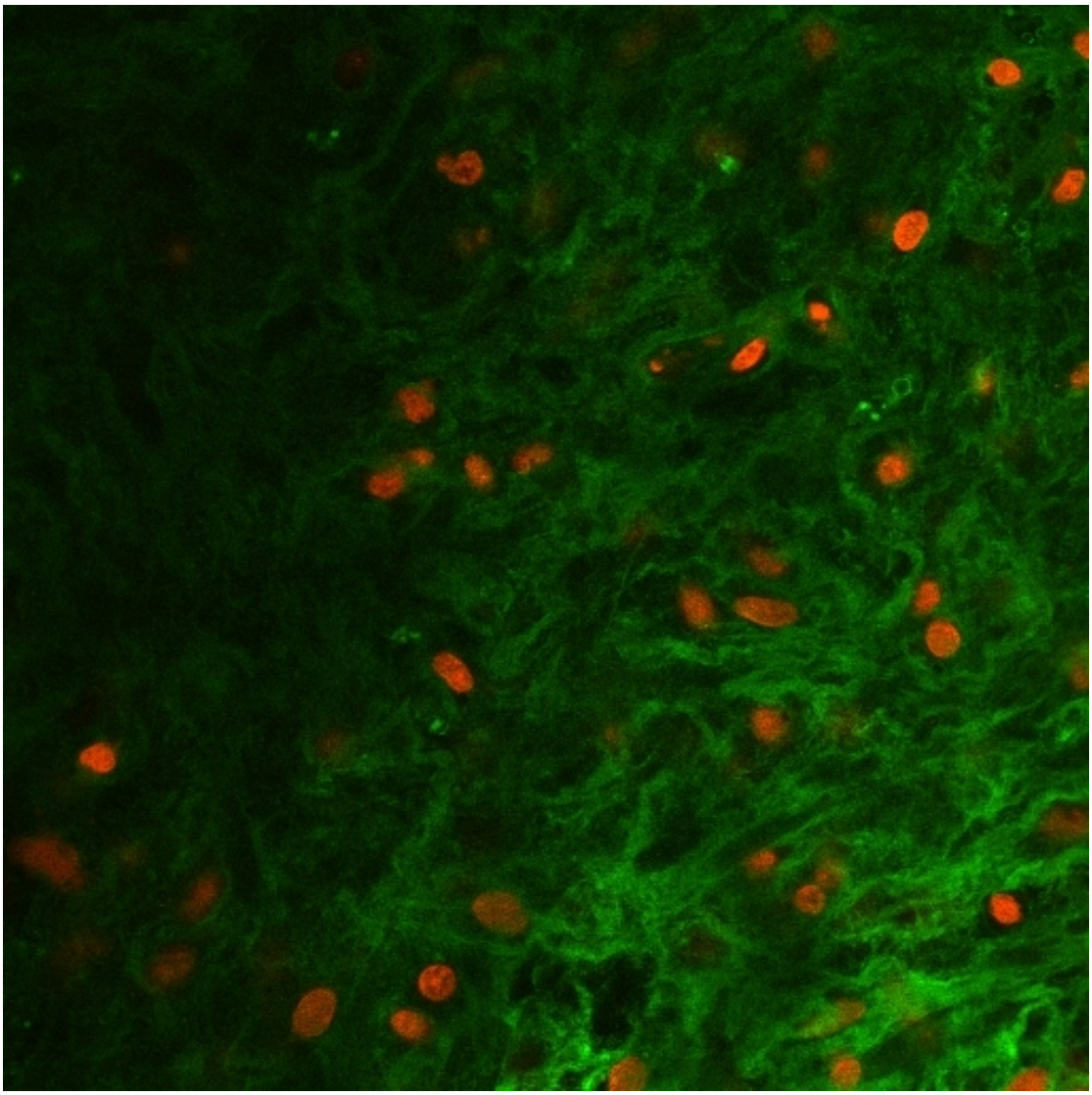

Figure 3-7 Fungal Biofilm Analysis CSLM – Control mucosa 20x

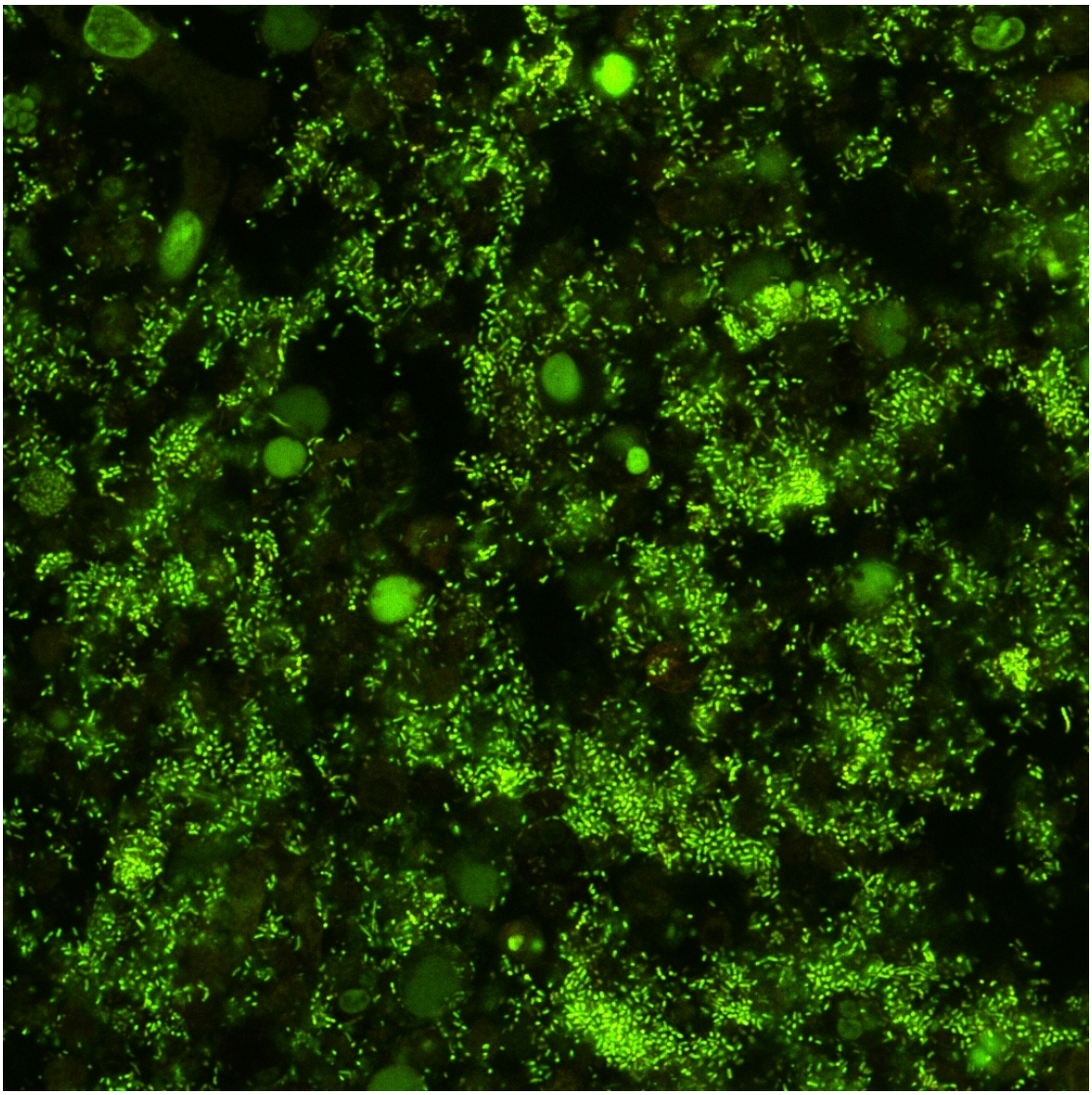

Figure 3-8 Fungal Biofilm Analysis CSLM – *S. aureus* inoculation 63x

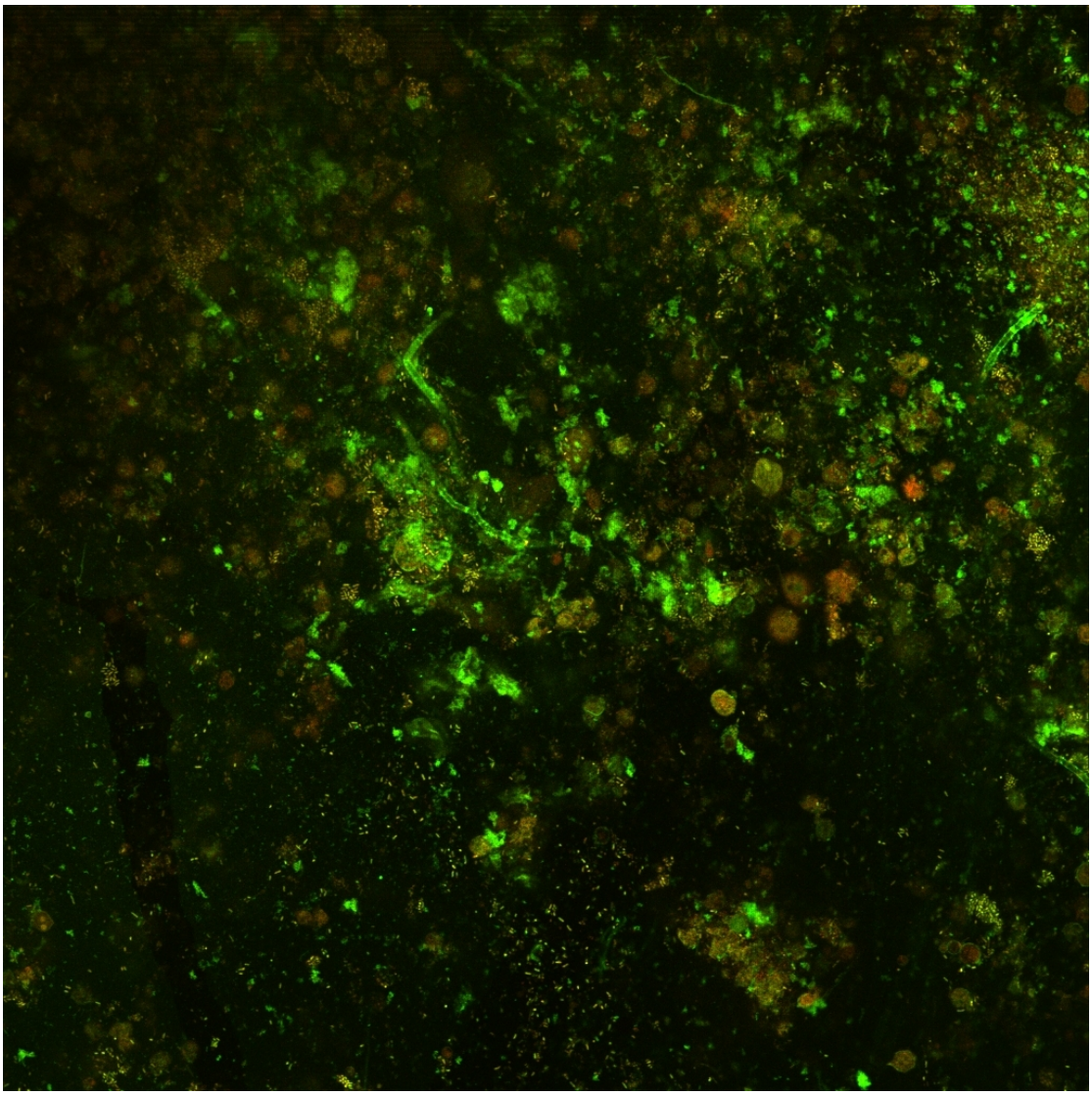

Figure 3-9 Fungal Biofilm Analysis CSLM – *A. fumigatus* / *S. aureus* - Occasional fungal biofilm (+)20x

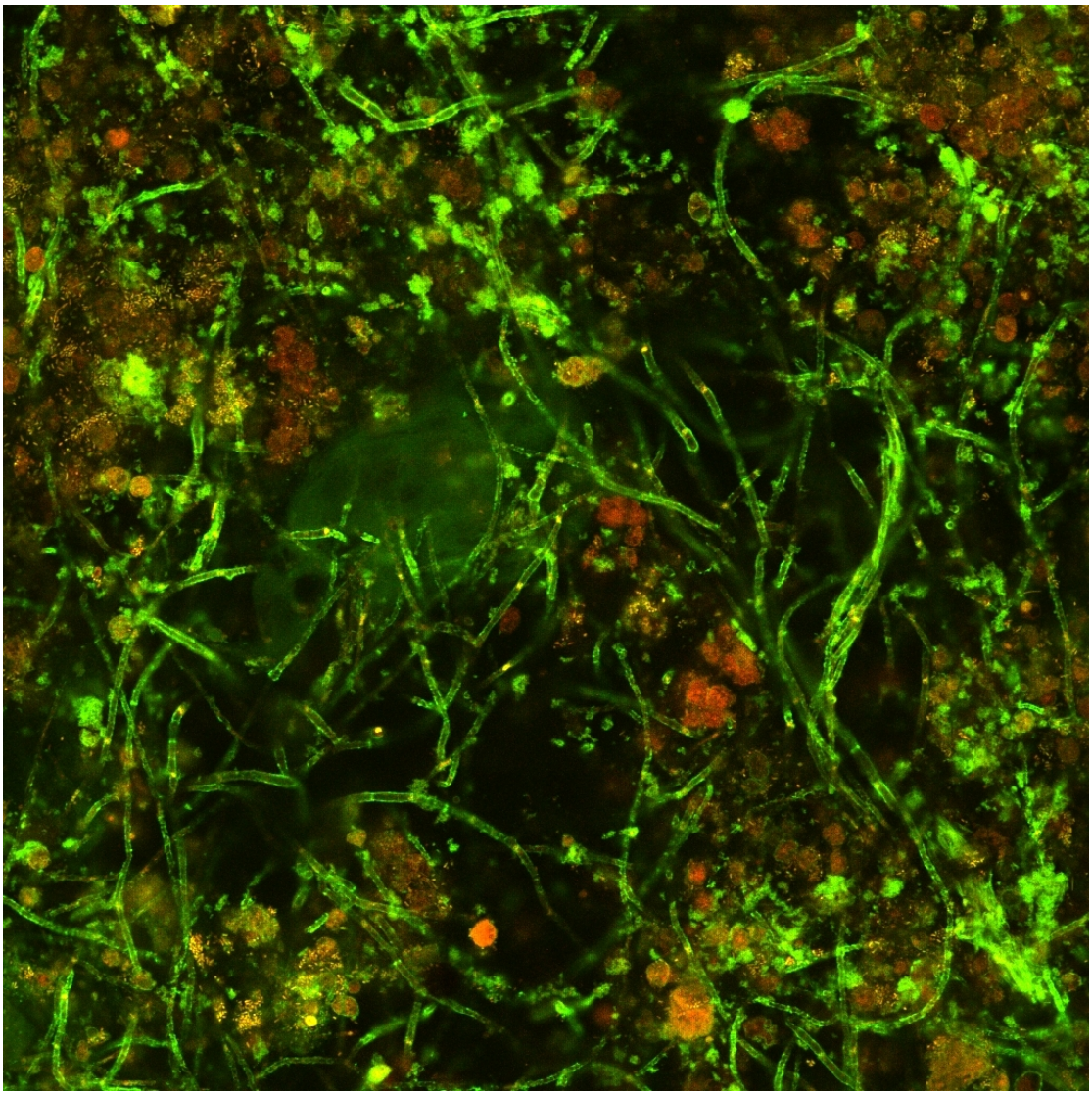

Figure 3-10 Fungal Biofilm Analysis CSLM – *A. fumigatus* / *S. aureus* Florid fungal biofilm (++) 20x

Table 3.1 Frontal sinus fungal biofilm formation results

	Fungal Biofilm formation					
Sinus inoculation	**0** **No fungal biofilm**		**+** **Occasional fungal biofilm**		**++** **Florid fungal biofilm**	
Control (n=4)	4	(100%)	0	(0%)	0	(0%)
A. fumigatus (n=6)	5	(83.3%)	1	(16.7%)	0	(0%)
A. alternata (n=6)	6	(100%)	0	(0%)	0	(0%)
A. fumigatus / S .aureus (n=5)	0	(0%)	1	(20%)	4	(80%)
A. alternata / S. aureus (n=5)	2	(40%)	1	(20%)	2	(40%)
S. aureus (n=4)	4	(100%)	0	(0%)	0	(0%)

Fungal Biofilm and Systemic Type I Hypersensitivity

The presence of type I fungal hypersensitivity measured by skin prick test, was compared to fungal biofilm formation for both species of fungi. There was no significant relationship between skin prick results and the propensity to form fungal biofilm. (Fisher's exact test, $P = 0.467$)

Fungal & Bacterial Culture

Bacterial and fungal cultures from the sinuses were compared at day 28, prior to sinus inoculation, and at day 38 at euthanasia. Prior to inoculation the most commonly cultured sinus organisms were coliforms (8/30). Fungi were less commonly cultured from the sinuses pre-inoculation, the most prevalent species was *Candida* sp. (not *albicans*) (2/30). There was no significant difference between the culture rates of fungi or bacteria at day 28 between the treatment groups. Importantly, neither *A. fumigatus*, *A. alternata,* nor *S. aureus* were cultured from the sinuses prior to sinus inoculation.

At day 38 (euthanasia), *A. fumigatus* was cultured from 2/6 (33.3%) sinuses following *A. fumigatus* inoculation alone, and from 4/5 (90%) sinuses that were co-inoculated with *A. fumigatus* and *S. aureus*. Similarly, *A. alternata* was cultured from 1/6 (16.6%) sinuses inoculated with *A. alternata* alone, and 3/5 (60%) of sinuses co-inoculated with *A. alternata* and *S. aureus.* Neither fungus was cultured from control sinuses, or *S. aureus* inoculated sinuses.

Fluorescence in-situ Hybridisation

FISH was performed to investigate the co-localisation of fungi and *S. aureus*. The fungal hyphae were often found around areas of dense *S. aureus* biofilm

(see Figure 3-11). Additionally, this analysis confirmed the species of bacterial biofilms formed following *S. aureus* inoculation. The molecular specificity of FISH probes for fungus, and *S. aureus* assists in the correct identification of these organisms, confirming the fungal biofilm analysis results.

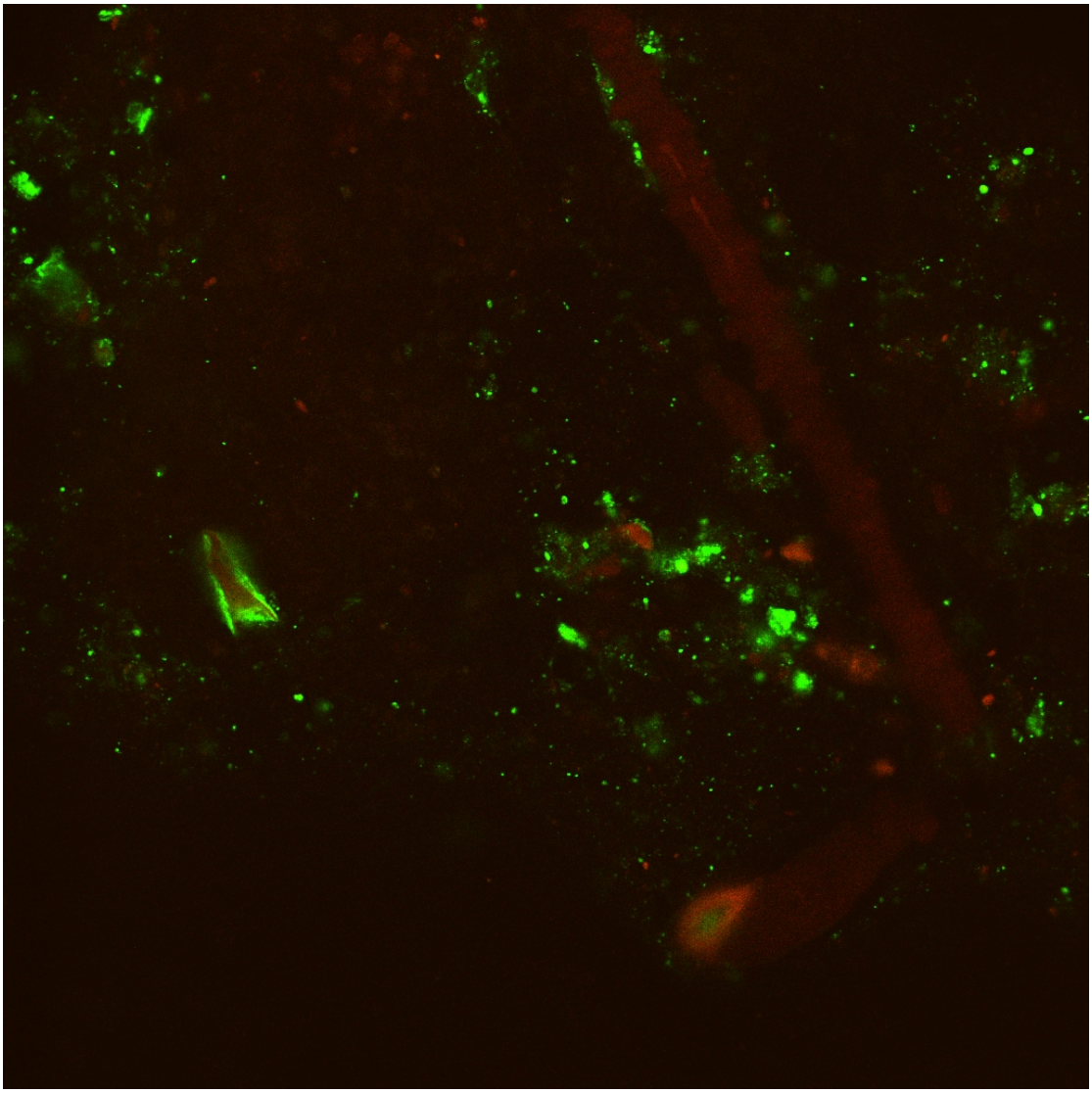

Figure 3-11 Fluorescence *in situ* hybridisation CSLM 63x. *S. aureus* – green; Fungi – red. Note the adherence of *S. aureus* to the upper portion of the hyphae

3.5 DISCUSSION

Fungi are associated with some of the most refractory CRS patients, with AFRS at the most severe end of the disease spectrum. The exact pathological mechanisms are as yet, elusive. Fungal hypersensitivity and biofilms may play a role, and an animal model presents an ideal opportunity to study these *in-situ.* We report that fungi alone do not readily form biofilm structures in otherwise non-inflamed sinuses. *S. aureus* was identified as an important co-factor for fungal persistence and proliferation in the sinuses. There is increasing evidence that cross-kingdom biofilms are prevalent in CRS patients[186,187]. The interactions between, often polymicrobial, flora and the host are highly complex. The type of interaction is dependent on a range of environmental, pathogen, and host factors. One such factor may be type I hypersensitivity to fungi. Our study examined the role of systemic fungal allergy, and it's relationship to fungal biofilm development. We successfully sensitized 45% of animals to fungal antigen. In these animals, there was no relationship between fungal allergy and inflammation, or propensity to form fungal biofilm. This study has provided many insights into the pathogenisis of fungal associated CRS. The role of *S. aureus,* and perhaps more generally, mucosal inflammation in fungal growth and proliferation is of great clinical importance.

IgE mediated hypersensitivity to fungi is one of the five postulates, described by Bent & Kuhn, as diagnostic criteria for allergic fungal rhinosinusitis (AFRS)[53]. Therefore, fungal sensitization was an important factor to include in an animal

model of fungal sinusitis. *A. fumigatus* and *A. alternata* were chosen for this study as they are two of the most commonly identified species from the sinuses of CRS patients[390], and antigenic solutions of these species are commercially available for sensitization and skin prick testing. We successfully induced type I hypersensitivity[385] to *A. alternata* and *A. fumigatus* antigens according to skin prick test results in 10/22 (45%) inoculated animals. This result is comparable to other sheep models of allergy induction[388].

It is theorized that IgE mediated hypersensitivity contributes to the inflammation in CRS / AFRS as resident fungi act to continually stimulate the mucosal immune defenses, leading to IgE crosslinking, mast cell degranulation, and pro-inflammatory mediator release. Additionally, IgE may play a pro-inflammatory role through non-allergic mechanisms [391]. With this in mind, our animal model provided the opportunity to examine the sinonasal response to fungi in allergic, and non-allergic animals. We observed no relationship between fungal culture rates, fungal biofilm status, or histological inflammation, with fungal specific allergy. There is increasing evidence that local mucosal IgE production is more important in the pathogenesis of fungal sinus inflammation than systemic allergy, which could not be assessed in the current study[391-393]. Furthermore, the induced fungal sensitivity in this study is clearly an oversimplification of the immune mechanisms underlying hypersensitivity to fungus, which may be multifactorial, with potential genetic pre-disposition. These limitations prevent further speculation on the role of fungal allergy in sinonasal fungal biofilm formation with this model.

It is intriguing that we were unable to stimulate fungal proliferation in the sheep sinus using fungal inoculation alone. The two fungal species employed are ubiquitous in the environment and their growth was presumably impeded by the host immune response. Mucosal defences such as mucociliary clearance, secretion of antifungal proteins and other actions of the innate immune system, likely prevented fungal adherence and proliferation. It is from our clinical and research experience[186], observing the occurrence of *S. aureus* and fungi together in CRS mucosa, especially in EMCRS patients, that we chose to co-inoculate fungi with *S. aureus.*

The results of fungal – *Staphylococcal* co-inoculation were striking. 80 % of these sinuses showed evidence of fungal biofilm formation. *A. fumigatus* showed particularly florid biofilm structure. This observation may be a specific feature of the species, implying a greater synergy with *S. aureus*. Importantly, *A. fumigatus* has more rapid growth kinetics than *A. alternata*, as well as a more favorable ideal growth temperature (37^oC vs 28^oC, respectively)[394], which may contribute to the differential growth patterns seen between fungal species.

Our unexpected discovery that fungal biofilms only manifest in the presence of S. aureus infection has important clinical implications. In this model, it is possible that the mucosal reaction to *S. aureus*, with the associated inflammatory milieu, results in an environment where fungi can proliferate. Such a reaction may include mucosal disruption, interfering with delicate innate immune defences, such as mucosal integrity, cilia & mucus motility, secretion of antifungal enzymes by host tissue, and toll-like receptor signaling. Applying this mucosal disruption

paradigm to the clinical picture of AFRS patients may, in part, explain the recalcitrant nature of this disease. Surgery itself significantly alters mucosal integrity, with cilia taking up to three months to regain normal function post-operatively. Such an environment in the early post-operative period may provide suitable conditions for rapid re-colonization with fungus, leading to disease recurrence.

The current literature suggests that the relationship between bacteria and fungi is more complex than the bacteria simply attenuating host immune defences, permitting fungal proliferation. Interactions between bacteria and fungi can have profound effects on the virulence, survival and pathogenesis of these organisms[395] There are instances when bacteria produce compounds which enhance the production of fungal virulence determinants. Also, there are occasions when bacteria secrete factors which inhibit fungal pathogenesis, for example, by inhibiting fungal filamentation[395]. The mechanisms of these interactions are undoubtedly diverse. These may include:

1. Environmental modification – pH, nutrient availability.
2. Attachment, co-aggregation, complex biofilm formation
3. Secretion of growth factors, quorum sensing agents
4. Effects on fungal virulence

The majority of published research on bacterial-fungal interactions has focused on *Candida albicans*. A study of the pathogenesis of stomatitis in 50 patients found a significant correlation between *C. albicans* and *S. aureus.* 78 % of patients had co-colonization with these two organisms, probably existing as a

mixed species biofilm[396]. They also showed that a lower pH environment was conducive to fungal biofilm formation. Such environmental modification by the bacterial biofilm may be one method of improving host conditions for fungal proliferation. Previous research on implant related infections has shown the frequent incidence of mixed species biofilms on indwelling catheters[397,398]. It has been proposed that such biofilms are more resistant to antibiotic and antifungal therapy due to more complex matrix composition[396]. El-Azizi *et al,* examined the physical interactions between *C. albicans* and a selection of biofilm forming bacterial pathogens. They showed polysaccharide matrix plays an important role in the colonization of bacterial biofilms by *C. albicans*[399]. Specifically, bacteria which produce glycocalyx, such as *S. aureus*, were better able to adhere to *Candida* biofilms[399]. The results of this study suggest *S. aureus* may interact with other fungal species in CRS in a similar way to the *candida*-bacterial interactions observed in other disease processes.

3.6 CONCLUSION

This study has provided strong evidence of a synergy between fungi and bacteria when forming biofilms on sinonasal mucosa. No role for systemic type I hypersensitivity was identified. It is intriguing that we were unable to form fungal biofilm without co-inoculation with *S. aureus*. It is possible that a cross-kingdom interaction exists between these organisms that permits fungi to adhere and proliferate in an otherwise hostile host environment. Such complex biofilm systems are known to have greater resistance to antibiotic and antifungal

treatments than single species biofilms, which may have important clinical implications. Loss of innate mucosal defences due to *S. aureus* infection may be conducive to fungal growth, analogous to the mucosal disruption in the post-operative period, which may explain the rapid re-colonization seen in AFRS patients following endoscopic sinus surgery. Further studies will investigate the role of other pathogenic bacteria in this relationship as well as the effect of cilia toxins on fungal biofilm formation. The aim will be to determine if this is a *S. aureus* specific phenomenon, or evidence of a more general abrogation of the innate immune response, permitting fungal proliferation.

Bacterial induced cilia damage promotes fungal biofilm formation in a sheep model of sinusitis.

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

Financial assistance provided by the Garnett Passe and Rodney Williams Memorial Foundation

chapter 4

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Submitted to The International Forum of Allergy & Rhinology

Under review.

4.1 ABSTRACT

Introduction: Fungal biofilms have been discovered in CRS patients, but factors contributing to their establishment are obscure. A recent animal study showed bacterial co-inoculation was required to establish sinus fungal biofilms. We examine the role of four bacterial species and a cilia toxin on fungal biofilm formation in a sheep model of sinusitis. The importance of cilial integrity on fungal biofilm formation is also examined.

Methods: 24 sheep had *Aspergillus fumigatus* inoculated into the occluded frontal sinus alone, or with one of four bacteria commonly detected in CRS patients; *Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa and Haemophilus influenzae*. A cilia toxin was also co-inoculated. Bacterial and fungal biofilm was determined using confocal scanning laser microscopy, as well as histological inflammation scores and cilia grading using transmission electron microscopy.

Results: No fungal biofilm formed when fungus was inoculated alone. Florid fungal biofilm developed in more than 75% of sinuses in association with bacterial biofilm of all species, except *Haemophilus influenzae*, which failed to establish bacterial or fungal biofilm. Fungal biofilm also established in association with cilia toxin. Significant cilial damage was incited by all bacterial biofilms, and cilia toxin, and was associated with fungal proliferation. Fungal biofilm formation did not significantly increase mucosal inflammation or cilial damage over that caused by the bacteria or cilia toxin alone.

Conclusion: Bacterial biofilms cause sinonasal mucosal inflammation and cilial injury, which provides conditions appropriate for fungal biofilm proliferation. The role of cilia in sinonasal mucosal defence against fungal organisms has been demonstrated, and without such an insult, fungal biofilms fail to proliferate in the occluded sinus. Improving cilial recovery post-operatively, and treating bacterial biofilms may be key factors in reducing recalcitrance in allergic fungal rhinosinusitis patients.

4.2 INTRODUCTION

Chronic rhinosinusitis is a prominent focus of rhinology research because despite its prevalence, the etiology and pathogenesis is poorly understood. The role of microorganisms and biofilms in CRS is still debated, however it is likely that the chronic mucosal inflammation that is a hallmark of the disease, results, at least partly, from an aberrant mucosal response to agents such as bacteria and fungi. Fungi were first discovered within the sinuses in 1976[147], and in 1981, allergic aspergillosis of the maxillary sinus was described as a distinct clinical entity[151]. Allergic fungal rhinosinusitis (AFRS) as it is now known, is characterized by thick, tenacious mucus containing fungal elements, abundant eosinophils and their breakdown products, nasal polyposis, and fungal allergy[53]. It is marked by a high rate of disease recalcitrance following surgery. Why some patients develop florid fungal sinusitis is unknown.

The formation of fungal biofilms in humans has classically been associated with the abiotic surfaces of indwelling medical devices such as central venous catheters, urinary catheters, and cochlear implants[400,401]. However, there is a growing body of evidence suggesting that fungal biofilm colonization of host surfaces may be an important factor in chronic disease in the absence of foreign bodies[400], and fungal biofilms have been discovered on the sinonasal mucosa of chronic rhinosinusitis patients[186,187]. Recently we developed an animal model of fungal sinusitis, to investigate the factors associated with disease establishment. However, we were unable to produce fungal biofilms in sinuses with intact innate immune defenses. Subsequently, co-inoculation with *Staphylococcus aureus*

resulted in florid fungal biofilm formation. *S. aureus* was associated with greater inflammation than fungal inoculation alone, and controls, and we postulated that *S. aureus* alters the local environment, through mucosal injury, providing conditions suitable for fungal biofilm proliferation[402]. The current study examines the capacity of bacterial species commonly found in CRS patients, to induced cilia damage permitting fungal biofilm formation in a sheep model of sinusitis. Additionally the effect of a cilia toxin on cilial integrity, mucosal inflammation, and fungal biofilm formation was assessed.

4.3 MATERIALS AND METHODS

Fungal Inoculum

Aspergillus fumigatus (Af) American Type Culture Collection (ATCC) reference strain 204305 was inoculated onto inhibitory mold agar (Becton-Dickinson, NJ, USA) without antibiotic, and grown to confluence over 5 days, in the dark at room temperature. Fungi were harvested, agitated, and resuspended in cerebrospinal fluid (CSF) broth (Oxoid, Adelaide Australia) and adjusted to 1.5 McFarland units above baseline. Samples were placed on ice until instillation.

Bacterial Inoculum

Four different bacterial species were utilised; *Staphylococcus aureus* (SA) (ATCC 25923), *Pseudomonas aeruginosa* (PA) (ATCC 27855), *Haemophilus influenzae* (HI) (ATCC 49247), and *Staphylococcus epidermidis* (SE) (ATCC 14990). All organisms were initially grown on Columbia Horse Blood Agar (Oxoid) overnight at 37oC, except for HI, which was grown on Chocolate agar (Oxoid). A single

colony of each was inoculated into CSF broth (Oxoid), placed on a shaker and incubated overnight at 37oC. The culture was adjusted to 0.5 McFarland units above baseline, and placed on ice prior to instillation.

Cilia toxin

CAZS solution, comprising a sodium citrate-buffered citric acid solution with a zwitterionic surfactant (caprylyl sulfobetaine), was supplied by Medtronic ENT, Jacksonville, FL. This agent was shown in previous studies to be highly cilia toxic.

Frontal Ostia Access

All 24 sheep were given a general anesthetic by an experienced animal technician. The first stage endoscopic sinus surgery was performed using custom made instruments as previously described[402], to enable access to the frontal ostia. Animals were recovered for a minimum of two weeks prior to the second procedure.

Frontal sinus trephination & inoculation

A second general anesthetic was given to permit frontal sinus trephination. Frontal trephines were inserted as described. The site of the frontal ostia was confirmed endoscopically using a flush of 1 % fluorescein. Both frontal ostia were occluded with petroleum jelly impregnated gauze until fluorescein was unable to be passed into the nasal cavity. Residual fluid was aspirated from the sinuses. 1 mL of bacterial inoculum was injected into each sinus via trephine. 1ml of fungal inoculum was injected into the left sinus, whilst 1ml of sterile CSF broth was

injected into the right sinus. 4 animals had 10mL of CAZS solution instilled into both sinuses, waiting 10 minutes followed by aspiration of the sinus. 1mL of fungal solution was inoculated into the left sinus plus 1mL sterile CSF broth, the right sinus received 2mL sterile CSF broth. Control animals had 2mL of fungal solution inoculated into the left sinus plus 1mL of sterile CSF broth, with the right sinus treated with 2mL sterile CSF broth. Trephines were capped and left in-situ, and the animals were recovered.

Specimen Collection

Sheep were euthanized on day 8 and the sinuses tissue was removed as previously described[402], and transported in Dulbecco's Modified Eagle's Medium (Gibco, Invitrogen Grand Island, NY) without antibiotic or antimycotic to the laboratory. Sinus tissue was dissected under laminar flow conditions, into appropriate sized pieces for the various analytical processes: 10 x 10 mm for fungal biofilm detection, 10 x 10 mm for FISH analysis, 5 x 5 mm in 10% buffered formalin for histology – with hematoxylin and eosin (H&E) stain, and 3 x 3mm for TEM. Electron microscopy samples were subject to 1 min of ultrasonication to remove mucus to allow assessment of epithelial structures.

Fungal biofilm determination

The analysis of fungal biofilm on sinonasal mucosa has been previously described[402,403]. Briefly, 10x10mm sinus mucosal samples were processed fresh, with dual staining with concanavalin A - Alexa Fluor 488 conjugate (Invitrogen GmbH Karlsruhe, Germany), and FUN-1 Cell Stain (Invitrogen). Following a one

hour incubation these were washed to remove excess stain and were analyzed using the Leica TCS SP5 Confocal Scanning Laser Microscope (CSLM) (Leica Microsystems, Wetzlar, Germany). An excitation wavelength of 488 nm, and dual emission detection at 495 – 540nm and 560 – 610nm was employed. A combination of 20x and 63x magnification was used. The entire sample was systematically in the X and Y planes scanned for fungal elements. Axial Z stacks were recorded of representative areas to construct a three dimensional virtual image of the tissue, overlying mucus and biofilm.

Histopathologic scoring

Two blinded examiners (JJB and EC) graded inflammation on H&E stained slides on a scale from 0-4. The mean results are shown. The scoring system has been previously described[386]. 0 - reflecting normal mucosa; 1 – minimal change with rare individual inflammatory cells within mucosa & submucosa; 2 - mild changes with light infiltrate of inflammatory cells; 3 – moderate changes with moderately dense inflammatory cells; 4 – severe changes with dense inflammatory infiltrate – partially obscuring normal tissue architecture.

Electron Microscopy

Sinus tissue were fixed in a solution of 4% paraformaldehyde / 1.25% glutaraldehyde in phosphate-buffered solution (PBS) with 4% sucrose, pH 7.2, and stored at 4°C overnight. Samples were postfixed with 2% osmium tetroxide (OsO4) for 1 hour on a rotator, and subsequently dehydrated with a series of 70–100% ethanol. Dehydration was continued in 100% propylene oxide for 20 minutes and then infiltrated in a 1:1 mixture propylene oxide/resin overnight. Over the next 24 hours, several changes of 100% resin were performed. Next, the specimens were embedded in fresh resin and polymerized at 70°C for 24 hours. Ultrathin sections, 70 nm in thickness, were collected onto copper grids, which then were stained with 2% uranyl acetate in 50% methanol for 15 minutes, followed by 1% lead citrate for 5 minutes. Sections then were examined with a Transmission Electron Microscope (CM 100, Philips Eindhoven, The Netherlands).

Cilia Grading

Cilia were graded on TEM images by two blinded observers (JJB and EC) using an arbitrary scale – 0 = normal cilia structure, 1 = mild cilial shortening / abnormality, 2 = moderate cilial shortening / abnormality, 3 = severe cilial shortening / abnormality, 4 = complete cilial loss. The mean of the observer results are shown.

Biofilm Analysis

Fluorescence in-situ hybridization was performed on mucosa that had been stored at -80oC. Cryopreservation prior to FISH analysis of sinus mucosa has been validated in our department[186]. Defrosted samples were washed in MilliQ water prior to hybridization to remove planktonic organisms. Species-specific PNA-FISH probes were used (AdvanDx, Woburn, MA), *S. aureus* - Alexa-488 (KT001 – custom probe), *S. epidermidis* (CNS) – Texas Red (KT005), *P. aeruginosa* – Alexa-488 (KT001 – custom probe). A universal bacteria probe (BacUni PNA Alexa 488 – (CP0054) was used to determine total bacterial biofilm formation (AdvanDx). As no *H. influenzae* probe was available, a novel probe conjugated to Cy3 was developed in our laboratory from published sequences[404] and we have used this probe previously[186]. The probe was manufactured by GeneWorks, Thebarton, Australia. The manufacturer's protocol was followed. Briefly, samples were fixed to glass slides, dehydrated in 90 % ethanol, air dried, and hybridized at 55oC for 90 minutes. In addition, the HI mucosa was pre-hybridized with BET-42 (Sigma-Aldrich, St. Louis, MO) for 30 minutes to reduce nonspecific binding of the probe. Plated pure cultures of each bacterium were used as positive controls. Samples were transported to Adelaide Microscopy for analysis using the Leica TCS SP5 CSLM. An excitation of 488nm with emission range of 495 – 540nm was used to detect SA & PA & universal bacteria. An excitation of 561nm with emission range of 570 – 600nm was used to detect HI. An excitation of 594nm with emission range of 610 – 630nm was used to detect SE. A combination of 20x and 63x magnification was used. The entire sample was systematically scanned for biofilm elements. Axial Z stacks (0.5µM) were

recorded of representative areas to construct a three dimensional virtual image of the tissue, overlying mucus and biofilm.

Statistical analysis

The one-way ANOVA test was used to compare multiple groups with Tukey's post hoc multiple comparison test. GraphPad Prism software (San Diego, CA) was used for statistical analysis, and a p-value of 0.05 was considered significant.

4.4 RESULTS

Fungal & Bacterial Biofilm Formation

The formation of bacterial biofilms was determined for all mucosal samples. Control sinuses and *A. fumigatus* alone inoculated sinuses showed no evidence of bacterial biofilm formation. A minimum of 75% of sinuses inoculated with bacteria alone (*S. aureus, S. epidermidis*, and *P. aeruginosa*), or these bacteria co-inoculated with fungi, showed species-specific bacterial biofilm formation. 4/8 (50%) of the CAZS and bacteria inoculated sinuses also showed evidence of bacterial biofilm formation using the universal bacterial probe (see Figure 4-1). Species-specific analysis showed *P. aeruginosa* to be the dominant bacterial organism in CAZS treated sinuses. However, the 4 sinuses inoculated with *H. influenzae* alone, and the 4 sinuses inoculated with *H. influenzae* & *A. fumigatus* showed no evidence of biofilm formation. These results were confirmed with a *H. influenzae* FISH probe, as well as a universal pan-bacterial probe.

Fungal analysis showed no fungal biofilm formation in control sinuses, those inoculated with *A. fumigatus* alone, or those sinuses inoculated with bacteria alone or CAZS alone (see Figure 4-8). 3/4 (75%) of sinuses inoculated with fungi and either *S. aureus, S. epidermidis*, or CAZS solution, formed fungal biofilms. Co-inoculation of *A. fumigatus* and *P. aeruginosa* produced fungal biofilms in all sinuses (see Figure 4-9). Co-inoculation of fungi and *H. influenzae* produced no fungal biofilms (see Figure 4-1).

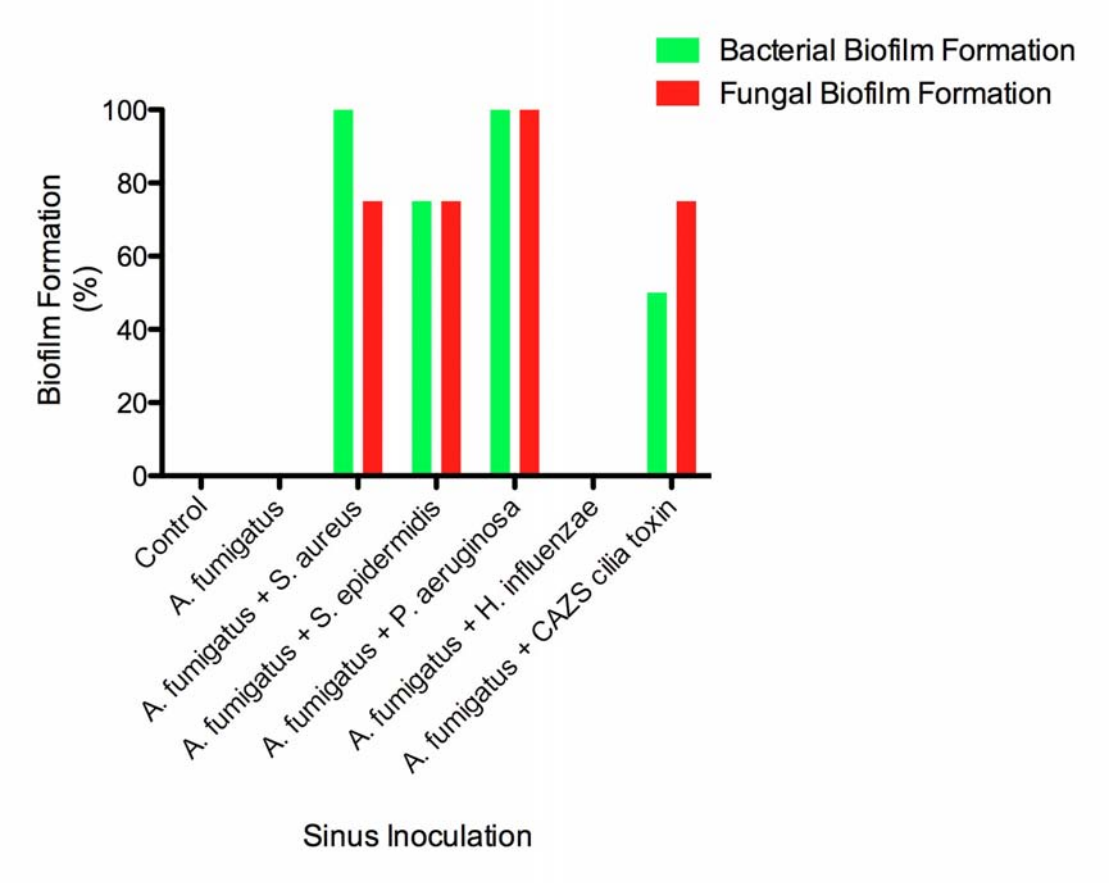

Figure 4-1 Fungal and bacterial biofilm formation according to frontal sinus inoculum.

Table 4.1 Fungal and bacterial biofilm formation according to frontal sinus inoculum.

Sinus Inoculation	Bacterial Biofilm n (%)	Fungal Biofilm n (%)
Control (n=4)	0	0
A. fumigatus (n=4)	0	0
S. aureus (n=4)	3 (75)	0
A. fumigatus + *S. aureus* (n=4)	4 (100)	3 (75)
S. epidermidis (n=4)	3 (75)	0
A. fumigatus + *S. epidermidis* (n=4)	3 (75)	3 (75)
P. aeruginosa (n=4)	4 (100)	0
A. fumigatus + *P. aeruginosa* (n=4)	4 (100)	4 (100)
H. influenzae (n=4)	0	0
A. fumigatus + *H. influenzae* (n=4)	0	0
CAZS cilia toxin (n=4)	2 (50)	0
A. fumigatus + CAZS cilia toxin (n=4)	2 (50)	3 (75)

Cilia integrity grading

Sinus mucosal tissue was processed and analysed using TEM to determine the effect of the interventions on cilia structure. There was a statistically significant reduction in cilia length in the sinuses inoculated with *S. epidermidis*, *S. aureus*, *P. aeruginosa* compared to control ($p < 0.05$, 1 way ANOVA, Tukey, see Figure 4-2 & Figure 4-5). CAZS treatment was also associated with a significant reduction in cilia compared to control sinuses ($p < 0.05$). However, fungal inoculation alone, and *H. influenzae* inoculation did not result in a significant alteration in cilia structure ($p>0.05$, see Figure 4-4 & Figure 4-2). The addition of *A. fumigatus* to sinuses did not lead to a significant difference in cilia damage ($p>0.05$, see Figure 4-2)

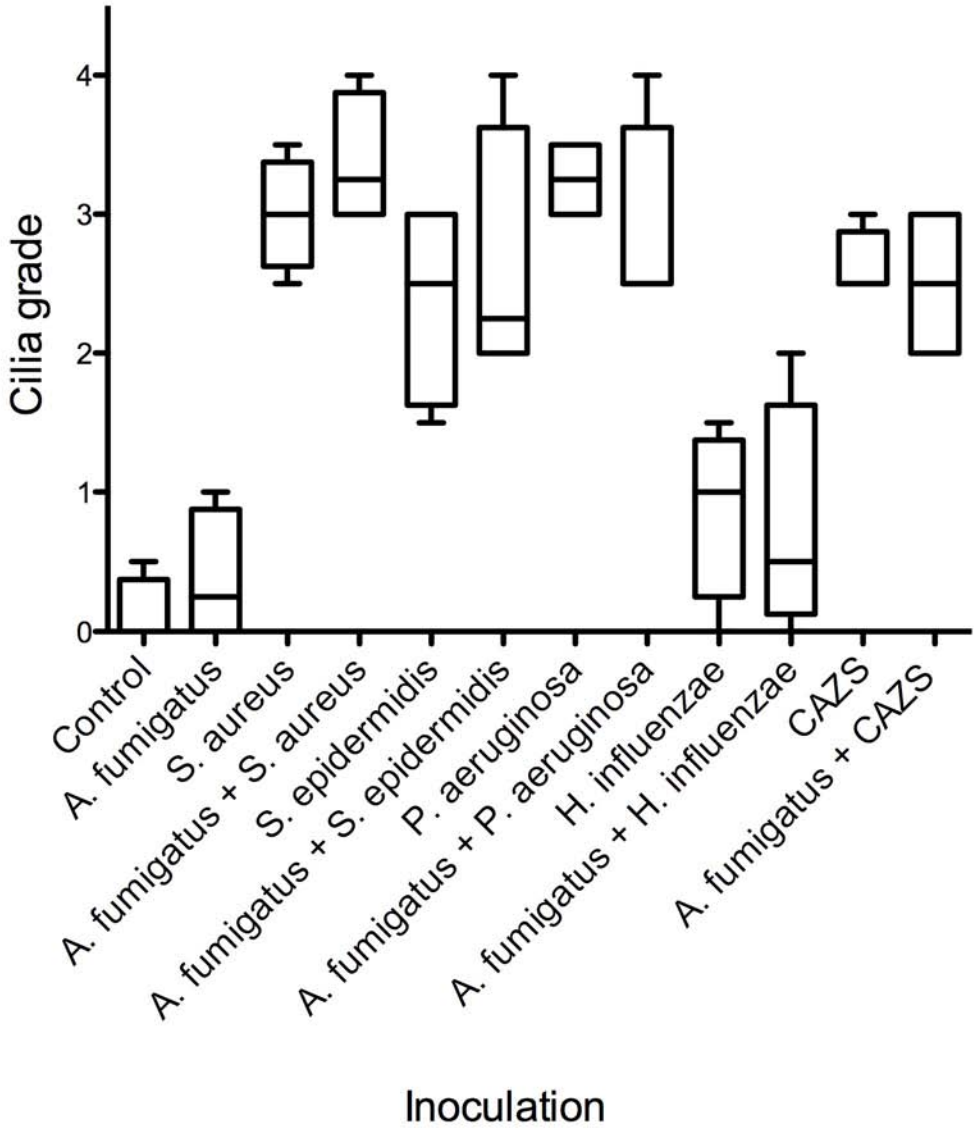

Figure 4-2 Cilia grading according to sinus inoculum. The 'box' represents the interquartile data range; the horizontal bar shows the median value and the 'whiskers' represent the 5th and 95th percentile values.

Inflammation grading

Histological preparations were analysed to determine the degree of inflammation in the sinonasal mucosa. There was no significant difference in inflammation severity between control tissue and those inoculated with fungi alone, HI alone, HI / fungi, CAZS or CAZS / fungi ($p > 0.05$, 1 way ANOVA, Tukey post-hoc multiple comparison test – see Figure 4-3). Inflammation severity was significantly greater in those sinuses inoculated with bacteria (SA, SE, PA) compared to control ($p < 0.05$, 1 way ANOVA, Tukey, see Figure 4-6 & Figure 4-7). Similar to the cilia grading, there was no significant difference in inflammation severity for any inoculum following the addition of fungi ($p>0.05$, see Figure 4-3).

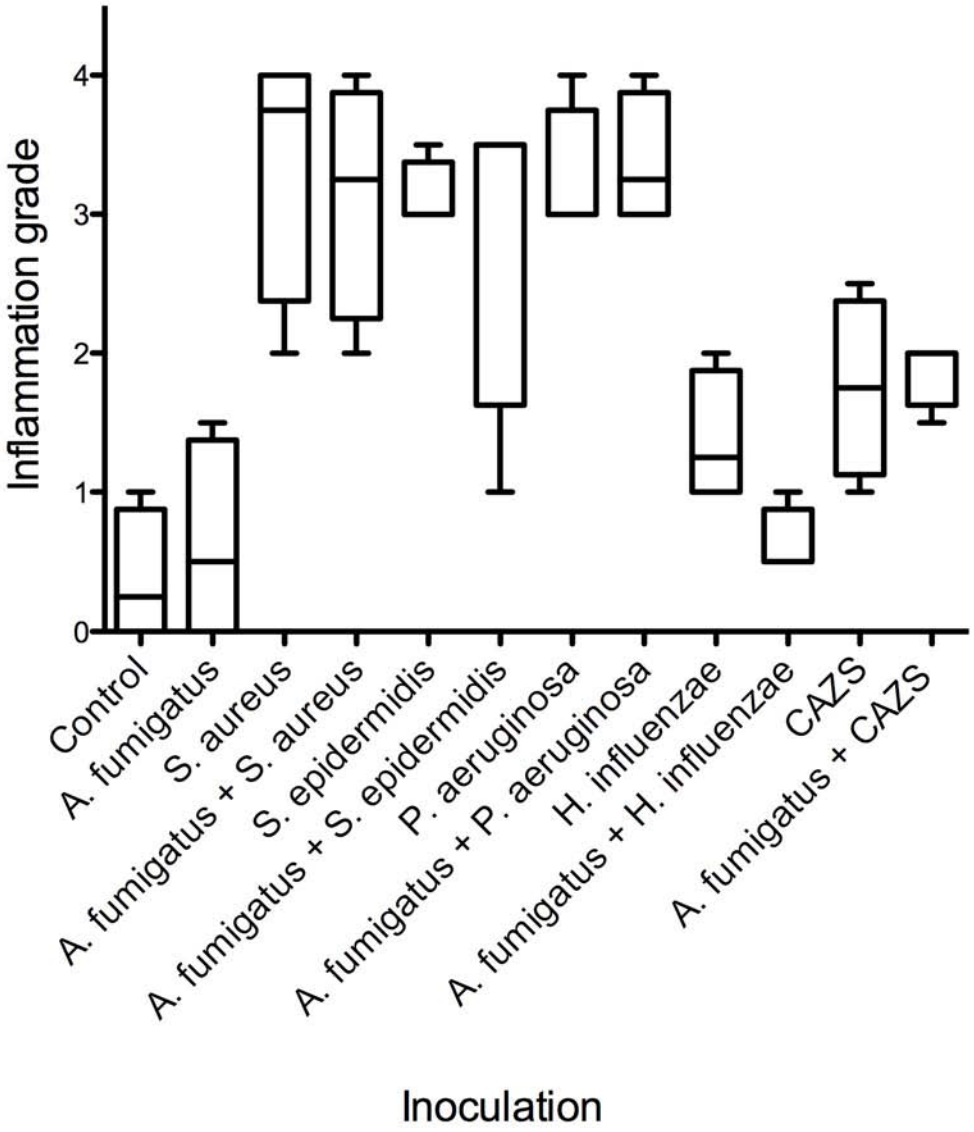

Figure 4-3 Histological grading according to sinus inoculum.

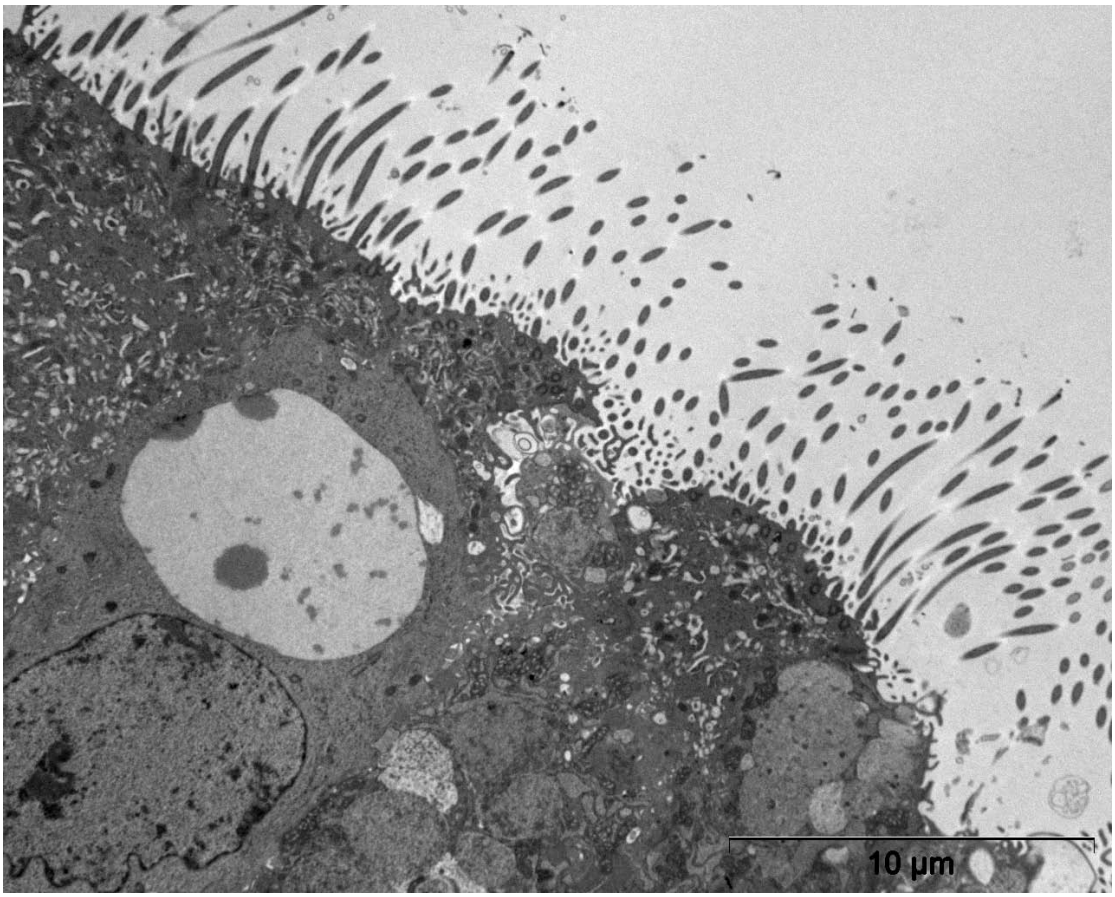

Figure 4-4 *A. fumigatus* inoculation - TEM 3400x showing normal cilial integrity

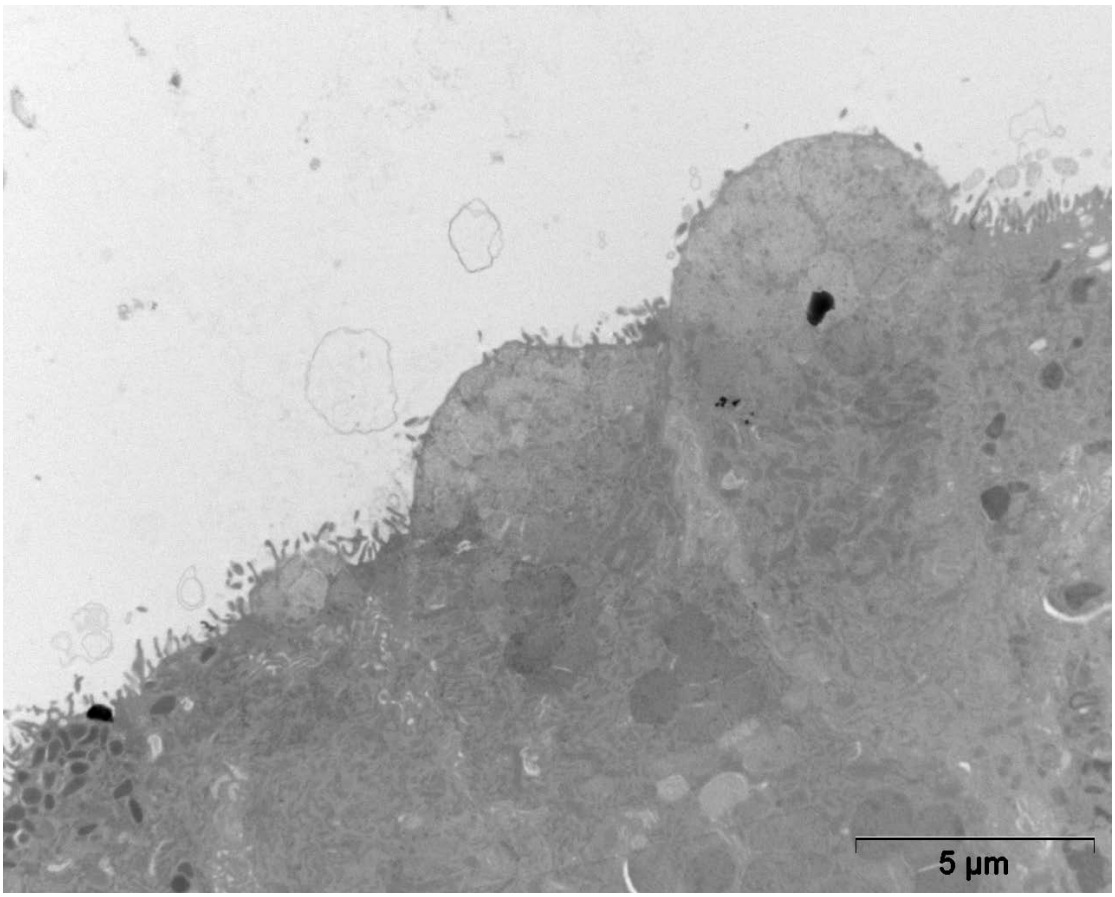

Figure 4-5 *A. fumigatus* / *P. aeruginosa* co-inoculation - TEM 3400x showing severely damaged cilia.

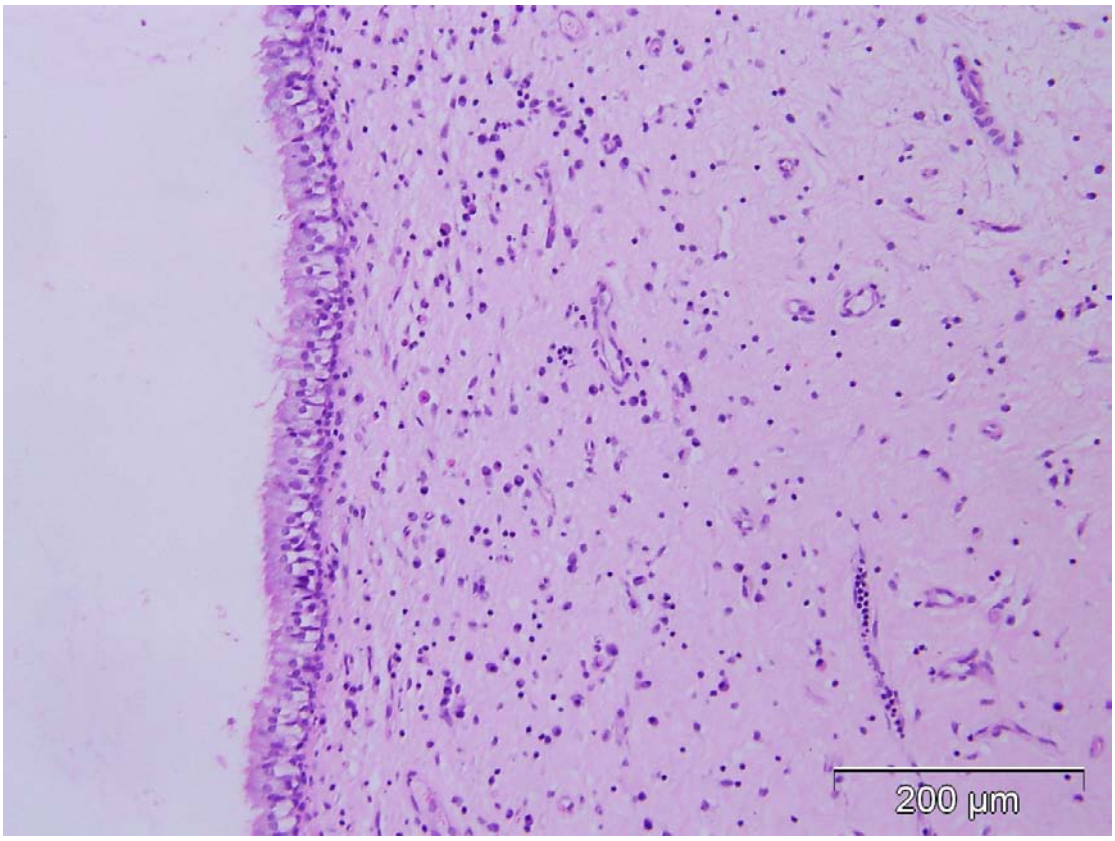

Figure 4-6 *A. fumigatus* inoculation - H&E stain 20x showing minimal mucosal inflammation.

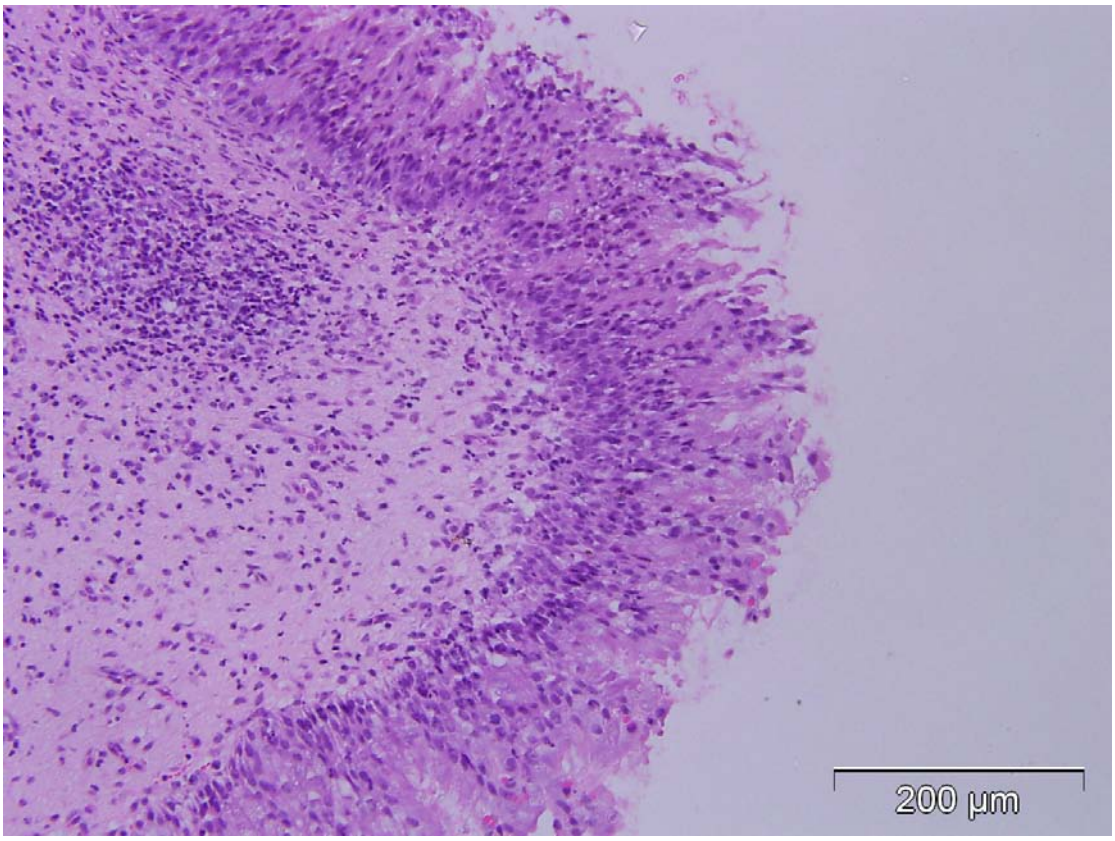

Figure 4-7 *A. fumigatus* / *P. aeruginosa* co-inoculation - H&E stain showing severe inflammation with thickened epithelium and influx of inflammatory cells.

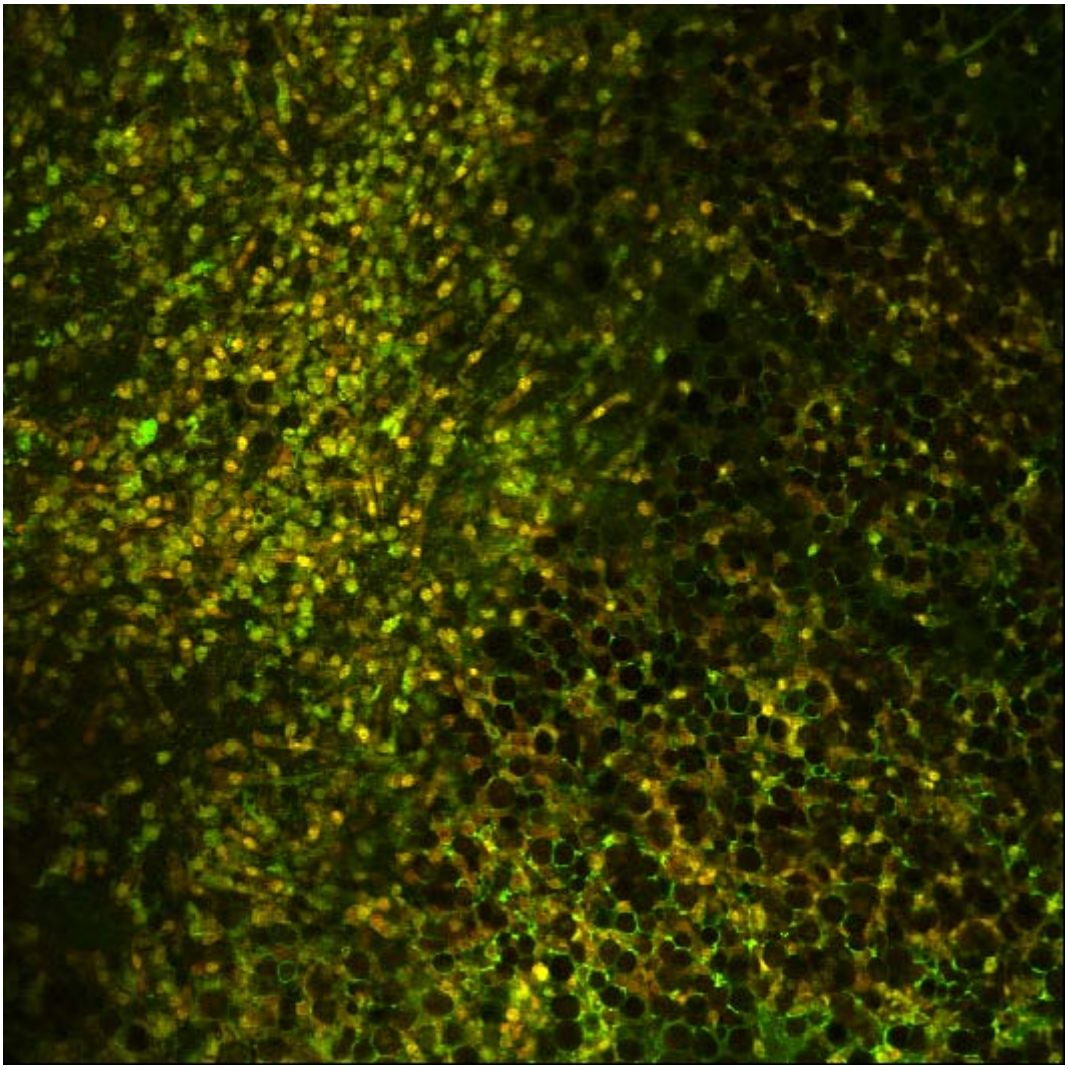

Figure 4-8 *A. fumigatus* inoculation CSLM - showing no fungal biofilm formation.

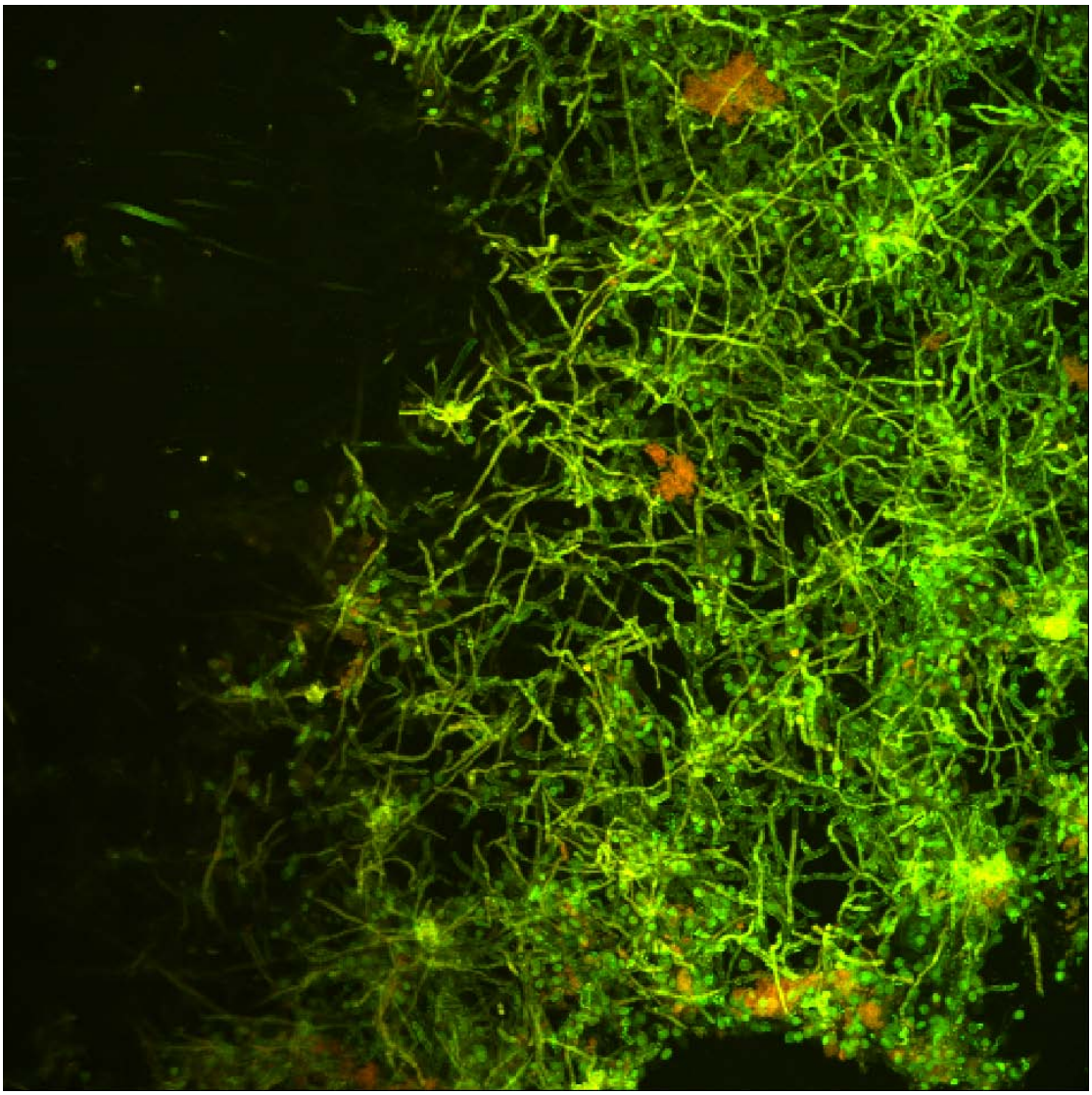

Figure 4-9 *A. fumigatus* / *P. aeruginosa* co-inoculation, CSLM showing florid fungal biofilm formation.

4.5 DISCUSSION

This study examines the effect of induced cilial damage on the formation of sinonasal fungal biofilms. We have shown that bacterial biofilms of multiple species have the capacity to damage epithelia sufficiently to allow fungal biofilms to adhere and propagate. In the absence of cilia damage by cilia toxin or bacterial injury, fungal biofilms do not form in this model. This has significant implications for our understanding of potential mutualistic relationships between bacterial and fungal species in CRS. Controlling the factors, which lead to fungal biofilm formation, may allow us to better manage these difficult patients.

The role of fungi in CRS is still a subject of vigorous debate in rhinology[79,405]. The complete spectrum of opinion exists; from a universal role of fungi in the pathogenesis of CRS[60,90], to no role at all[80,406]. Despite this disparity, most rhinologists are familiar with a subset of their CRS patients, with medically and surgically recalcitrant disease, who have polyps, eosinophilic mucus and fungal elements filling the sinuses. These are some of the most symptomatic, and difficult to treat patients[38,56], and can have debilitating consequences from their disease[40].

Studies reporting the presence of fungal biofilms on sinonasal mucosa from CRS patients show a prevalence of 22% in unselected CRS patients[186], up to 100% in EMCRS patients[187]. Critically, both of these studies found a high correlation

between fungal and bacterial biofilms. Potential mutualistic relationships between bacteria and fungi may contribute to their persistence on sinonasal mucosa.

We have shown that bacterial biofilms have the capacity to significantly damage sinonasal cilia, confirming the findings demonstrated in a recent study[185]. Multiple different bacterial species have been implicated, however the results of the latter study were based on conventional culture rather than organism specific biofilm detection techniques. Others have suggested that bacteria exhibiting the biofilm phenotype, damage or impair the function of cilia more than microbes in the planktonic phenotype[407], although this requires further substantiation. *S. aureus* and *P. aeruginosa* have been implicated in CRS[191,192,408] however, *S. epidermidis* is generally considered to be an organism associated with a favourable outcome in CRS[408]. Despite this, in the current study *S. epidermidis* biofilm was associated with similar degrees of cilia damage and fungal biofilm formation as the other species. In addition to cilial destruction, it is known that bacterial toxins from *S. aureus* and *P. aeruginosa* are capable of significantly impeding cilia beat frequency[409,410], an additional factor which may promote both bacterial and fungal biofilm formation.

In addition to bacterial co-inoculation we have employed a cilia toxin to examine the hypothesis that impaired cilia function promotes fungal biofilm formation. CAZS solution has been previously shown to significantly damage ciliated mucosal surfaces[411,412], and similar results were observed in the current study. It was instructive that the addition of this solution to the sinus was sufficient to allow fungal biofilm proliferation, and suggests that the observed effect of bacteria on

fungal biofilm formation is, at least partly, due to cilial impairment. However, 50% of the CAZS treated sinuses also had bacterial biofilm formation, presumably seeded from endogenous organisms.

The formation of fungal biofilms in the current study was observed in the majority of sinuses, which were co-inoculated with bacteria, except *H. influenzae* species. Examination for *H. influenzae* biofilm on these sinuses was also negative. Why we were unable to produce *H. influenzae* biofilms in the sheep model is obscure. It is known to be a fastidious organism with specific growth factor and environmental requirements[413]. It is also highly adapted to the human environment, and has no non-human hosts[414]. Despite this however, animal models of *H. influenzae* infection[415] and biofilm formation[416,417] have been reported. We were particularly interested in the effect of *H. influenzae* biofilms on fungal biofilm formation, as the species has been shown to portend a favourable clinical picture compared to *S. aureus* biofilms, particularly regarding pre-operative disease severity, and post-operative course[191]. Despite it's relatively benign status in CRS, three studies have shown significant impairment in cilia structure and function following infection with *H. influenzae* on primary respiratory epithelial culture[418], and explant models[419,420]. This species has also been shown to elaborate certain factors, which rapidly inhibit ciliary activity in vitro[409,419]. Additionally, strains expressing certain surface proteins may have a more severe impact on cilia structure and function[421].

Molecular & proteomics research has provided evidence that fungal organisms assuming the biofilm phenotype exhibit altered gene expression[422,423], and protein

production[424], as part of a complex, highly regulated process[194]. Fungal biofilms, like bacterial biofilms, have defined developmental phases that include arrival at an appropriate substrate, adhesion, colonization, matrix production, biofilm maturation and dispersal[197-199]. Filamentous growth is a fundamental feature of fungal proliferation, however germination of conidia (spores) is required, and conidial adhesion is essential to trigger germination[196,200]. Considered to be ubiquitous, the outdoor concentration of fungal spores can be as high as 1×10^6 spores per m^3, depending on ambient conditions, which is up to 1000x higher than the mean pollen concentration[425]. Clearly, the exposure of the sinonasal mucosa to fungal spores is great, however local immune defences, such mucociliary clearance, prevent adhesion and germination of inhaled fungal material. As we have shown, bacterial biofilms have the capacity to impair these local defenses, affording fungal material greater opportunity to adhere to mucosal surfaces and propagate.

The interaction between fungal virulence factors (including biofilm formation), and host defense mechanisms plays a crucial role in determining if fungi are cleared from mucosal surfaces, or cause infection and inflammation. The high density of fungal organisms within a biofilm may present a challenge for the host. Furthermore, fungal biofilms have increased resistance to antifungal medications[426]. Clearly the role of cilia in mucosal defense is critical to maintaining a healthy sinus environment. Endoscopic sinus surgery is known to improve cilia structure and function in CRS patients[427-429], however complete recovery may take between 3 and 6 months following mucosal injury or ESS[430-432]. This may be a period of relative susceptibility to fungal biofilm formation, and

may explain early surgical failures in AFRS patients. Human epithelial defenses against fungal biofilms are poorly understood at present. In addition to mucociliary clearance, recent studies have implicated TLR2 and 4 mediated signaling as important innate anti-fungal defense mechanisms[433,434].

4.6 CONCLUSION

This study has shown that bacterial biofilms can induce damage to the epithelial barrier, resulting in significant cilial destruction. A delicate balance exists at the level of the mucosa, which prevents significant fungal colonization of host surfaces. Innate host defenses, including the epithelial barrier and mucociliary clearance are important mechanisms. However, when these conditions get out of balance due to bacterial biofilm formation, heavy fungal colonization can occur. The importance of cilial function in antifungal defense, may explain the recalcitrance of fungal associated CRS to conventional medical and surgical treatments. It remains unknown if fungal biofilms are pathologically involved in rhinosinusitis, or whether they are saprophytic bystanders, which only colonize the sinuses of those with the most severe inflammatory disease, and mucociliary impairment. Further research into the complex interplay between sinonasal microorganisms, and their interactions with host immune mechanisms is critical to our understanding of this disease.

Microorganisms and host immunoglobulin E responses in CRS: *Staphylococcus aureus* potentiates inhalant aeroallergen sensitization

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Financial assistance provided by the Garnett Passe and Rodney Williams Memorial Foundation

chapter 5

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Submitted to The Journal of Allergy & Clinical Immunology

Under review.

5.1 ABSTRACT

Introduction: The importance of bacteria and fungi in CRS is increasingly recognized, but determining how the host interacts with these organisms is critical to our understanding. We have determined the presence of microorganisms using culture and biofilm detection, and compared this to organism specific immune responses, by measuring immunoglobulin E within the mucosa and serum of CRS patients and controls.

Methods: The presence of *Staphylococcus aureus,* and four fungal species was determined by culture and a validated biofilm detection method in 48 CRS patients and 10 controls. Serum and mucosal tissue were collected for organism specific immunoglobulin E analysis. Correlations between microorganisms and specific host responses were examined.

Results: Mucosal IgE levels to *S. aureus* and fungi were significantly higher in mucosa than serum in all patients. When fungi and *S. aureus* organisms were present on the mucosa in CRS patients with nasal polyps, specific IgE levels were significantly elevated. Interestingly, the presence of *S. aureus* on the mucosa also resulted in higher anti-fungal IgE levels. This relationship was only observed in nasal polyp patients.

Discussion: This study has shown, using sensitive microbial detection techniques, the presence of certain microorganisms in nasal polyposis patients can result in higher mucosal IgE levels, suggesting these organisms have the capacity to interact with the host immune system, with potential to exacerbate mucosal inflammation in CRS. Mucosally based *S. aureus* organisms may also exacerbate disease by enhancing mucosal sensitization to inhaled fungal aeroallergens.

5.2 INTRODUCTION

Chronic rhinosinusitis (CRS) is a heterogenous group of disorders commonly classified according to the presence or absence of polyposis. CRS with nasal polyps (CRSwNP) affects approximately 4% of the population[435], and is characterized by complex inflammatory reactions including high levels of immunoglobulin E (IgE), predominance of T helper 2 (Th2) cytokines including interleukins 3, 5 and 13, and mucosal eosinophillia. The stimuli for ongoing mucosal inflammation are still unknown, however it is becoming clear that environmental exposure to microorganisms such as bacteria and fungi, and inhaled aeroallergens, engaged by a dysregulated host immune response, may perpetuate the pathological inflammation. Recent trials of the novel anti-IgE monoclonal antibody, omalizumab have shown promise for nasal polyposis in preliminary studies[274-276], and suggest a critical role of IgE in the underlying pathogenesis of this disease.

There is an emerging body of evidence implicating *S. aureus* as a prominent disease modifying agent in CRS[191]. In certain geographical regions, it is the most commonly cultured bacteria in CRSwNP patients[436], and has been detected in biofilm form in 50 – 70% of CRS patients[174,186,192]. *S. aureus* is known to produce enterotoxins (SAE) - a class of secreted proteins capable of stimulating potent mitogenic expansion of T lymphocytes[260,314]. It is recognized that bacterial products, such as superantigens, can activate the adaptive immune system via Th2 predominant pathways, with B cell activation and class switching, resulting in

IgE production[315,316,437]. Additionally, the presence of *S. aureus* biofilm has been associated with Th2 biased inflammation, independent of superantigen presence[375]. Additionally, SAE specific IgE in nasal polyps has been associated with a significant increase in eosinophil-related markers such as IL-5 and ECP[372,436]. SAE can also enhance inflammatory airway responses in models of asthma and allergic rhinitis[320,438]. *S. aureus* colonization also increases the sensitization to inhaled aero-allergens in asthmatic patients[267]. These studies suggest a role for *S. aureus* as a disease-modifying agent, with the potential to exacerbate IgE mediated sensitization to inhaled aero-allergens in susceptible individuals. This mechanism has not been explored in CRS to date and is addressed in the current study.

Unequivocal evidence for a role of fungi in CRS pathogenesis has remained debatable[79,80]. The reported prevalence of systemic fungal sensitivity (allergy) in CRS patients varies significantly. Collins reported no difference in inhalant allergy between CRSwNP patients and controls, although fungal allergy was significantly higher in the polyp group[351]. In the current study we examine the prevalence of fungal allergy systemically, and locally within the mucosa, and examine the relationship between these. We also investigate the capacity of colonizing fungi to stimulate the production of specific IgE within the sinonasal mucosa.

An integral step in understanding the pathogenesis of CRS and the relative contribution of microorganisms, is to scrutinize the host and it's relationship with the environment. Specifically, by analyzing the microorganisms which reside at the mucosal interface, and correlating these to organism specific immune

responses in the host. We describe the first quantitative analysis of *S. aureus* enterotoxin and fungal IgE within the mucosa and serum of polypoid and non-polypoid CRS patients and controls. It is also the first study to examine for the presence of these organisms on the sinonasal mucosa, using conventional culture and biofilm analysis, to determine if colonizing organisms can act as antigen sources for IgE production. Additionally, we examine the impact of *S. aureus* organisms on mucosal sensitization to fungal aero-allergens.

5.3 MATERIALS AND METHODS

Patient Selection

This prospective study was undertaken in the tertiary referral rhinology practice of the senior author (PJW), at the academic hospitals, Adelaide, South Australia. The study was approved by the Human Ethics Committee and all patients provided informed consent to participate in the study. The cohort consisted of 58 consecutive CRS patients who met the definition of CRS as defined by the rhinosinusitis taskforce[376] and have failed medical therapy necessitating the need for endoscopic sinus surgery (ESS). A control group consisted of 15 patients who had no clinical or radiological evidence of sinus disease. These patients were undergoing transnasal endoscopic procedures including trans-sphenoidal hypophysectomy for non- functioning adenomas, optic nerve decompression, and CSF leak repair. Patients were excluded if less than 18 years of age, were immunocompromised, or had mucociliary dysfunction due to disorders such as cystic fibrosis and Kartagener's syndrome. Other exclusion criteria included inadequate mucosa for analysis, no culture taken, and antibiotic or systemic corticosteroid used in the three weeks preceding surgery. 10 CRS and 5 controls patients were excluded due to either insufficient mucosal tissue available for analysis, or bacterial and fungal cultures were not performed during surgery.

All patients had serum collected for allergy testing to moulds, grasses, house dust mite & animal dander, as well as total IgE using an automated fluorescent enzyme immunoassay (FEIA) on a Phadia 250 Immunocap system (Phadia AB,

Uppsala, Sweden). Pre-operative data was collected including medical history, allergies, aspirin sensitivity, cigarette smoking, asthma, and sinonasal symptom scoring. CT scans were scored using the Lund-Mackay[439] staging system. All patients had blood drawn before anesthetic induction, and collected in a lithium-heparin tube. Blood was centrifuged at 3500 rpm for 10 minutes. Serum was collected and stored at -80^{o}C to be used for specific IgE analysis.

CRS patients had sinus mucosal tissue harvested from the maxillary or ethmoid sinuses during routine sinus procedure. Control patients had mucosa harvested from the posterior ethmoid and sphenoid required to access relevant skull base pathology. Tissue was immediately stored in Dulbecco's modified Eagle medium (Gibco, Invitrogen Corp., Grand Island, NY) without antibiotic or antimycotic, and transported on ice to the laboratory for further analysis. Mucus was harvested for histopathological examination, and for routine bacterial and fungal culture. In the absence of mucus, a middle meatal swab was taken for bacterial and fungal culture.

Biofilm Analysis

Fluorescence in-situ hybridization was performed as previously described[186]. Two probes were utilized on separate pieces of mucosa - a S. aureus specific sequence conjugated to Alexa-488 probe, and a pan-fungal Alexa-488 probe. (AdvanDx, Woburn, MA). The manufacturer's protocol was followed. Samples were analyzed on a Leica TCS SP5 Confocal Scanning Laser Microscope (Leica Microsystems, Wetzlar, Germany). An excitation of 488nm with emission range of 495 – 540nm was used to detect S. aureus and fungus. The entire sample was systematically scanned for biofilm elements. Axial Z stacks (0.5µM) were

recorded of representative areas to construct a three dimensional virtual image of the tissue, overlying mucus and biofilm.

Mucosal Lysate Preparation

Mucosal lysates for immunoglobulin analysis were prepared from 200mg of snap frozen mucosa using the Bio-Plex Cell Lysis Kit according to the manufacturer's directions (Bio-Rad Laboratories, Hercules, CA). The supernatant was collected and stored at -20^{o}C.

Immunoglobulin E Analysis

Analysis of specific IgE in mucosal lysates and serum was performed using an ImmunoCAP 100 instrument and reagents (Phadia AB, Uppsala, Sweden). Mucosal lysates and serum were centrifuged at 1500rpm prior to undiluted supernatant analysis. Curve controls and calibrators were used for the assay as per the manufacturer's instructions. Fluorescence signals emitted from the sample were compared to known standards, and expressed as the concentration of specific IgE in kUnit/L. Seven serum and lysate samples were analyzed in duplicate immediately following collection (fresh) and subsequently following storage at -80^{o}C and -20^{o}C respectively, to determine inter-assay variability and confirm the stability of the cryopreservation protocol. In addition, each immunoCAP assay included an internal control of pooled sera from a volunteer with known allergy to domestic cats, which was analyzed for specific IgE to Felis domesticus (e1). All experimental sera and mucosal lysate samples were analysed for the following organism specific immunoglobulin E: Alternaria alternata (m6), Aspergillus fumigatus (m3), Penicillium chrysogenum (m1), Aspergillus flavus (m228), Staphylococcus aureus enterotoxin (SAE) A (m80),

SAE-B (m81), SAE-C (m223), and SAE-TSST(m226). Data were analysed using ImmunoCAP Information Data Manager (IDM) software (Phadia AB, Uppsala, Sweden).

STATISTICS

The Kruskal-Wallis test was used to compare multiple groups with non-parametric data with Dunn's post hoc test. The Mann-Whitney U test was used for continuous data. Fisher's exact test and chi-square test for independence were used for dichotomous data. Pearson product-moment correlation coefficient (r) was used as a measure of the strength of linear dependence between two variables. GraphPad Prism software (San Diego, CA) was used for statistical analysis, and a p-value of < 0.05 was considered significant.

5.4 RESULTS

Table 5.1 Patient characteristics

	CRSwNP	CRSsNP	Controls
Number	33	15	10
Age*	46 (37-55)	44 (35-51)	42 (32-52)
Male / Female	18/15	5/10	3/7
Lund-MacKay CT Score*	18 (15-22)	16 (15-20)	0
Symptom Scores*	18 (16-20)	18 (16-19)	0
Revision Surgery (%)	21 (64%)	9 (60%)	0
Smoking	2 (6%)	2 (13%)	1 (10%)
Aspirin Sensitivity	3 (9%)	0	0
Asthma	9 (27%)	3 (20%)	0
Co-existent Medical Conditions	DM (2) HT(4) HC(1), GERD (2), RA (1)	DM (1), GERD(1), IBS (1)	DM(1) RA(1)
Serum Allergy Screening			
House Dust Mix	15 (45%)	4 (27%)	2 (20%)
Mold Mix	20 (61%)	2 (13%)	0
Grasses	18 (55%)	3 (20%)	1 (10%)
Animal Dander	5 (15%)	0	1 (10%)
Pollen mix	9 (27%)	1 (7%)	0
Total – any allergen†	24 (73%)	7 (47%)	2 (20%)

DM = diabetes mellitus, RA = rheumatoid arthritis, GERD = gastro-esophageal reflux disease, IBS = irritable bowel syndrome, HT = hypertension, HC = hypercholesterolemia. * = mean (interquartile range – 25 – 75%). † Denotes total number of patients with positive serum allergy test to 1 or more tested allergens.

Aspirin sensitivity and asthma were both more common in CRSwNP patients than the other groups but this was not statistically significant (χ^2, P>0.05). Age and gender were not significantly different between groups (χ^2, P>0.05, Mann-Whitney *U* test P>0.05, respectively). Both CRS groups were similar with respect to symptom and radiological severity (Mann-Whitney *U* test P>0.05). The high revision rate for both CRS groups indicates the tertiary referral nature of the practice. The rate of systemic allergy (all tested antigens), displayed no significant difference between polyp and non-polyp patients, however there was a significantly higher rate of systemic allergy in polyp patients vs controls (Fisher's exact test, p<0.05). Examining systemic fungal allergy specifically, the CRSwNP group had significantly higher rates of fungal allergy (61%) compared to CRSsNP patients (13%) and controls (0%) (Fisher's exact test, p<0.05). Based on this systemic IgE analysis, patients are herein referred to 'fungal allergic' or non-fungal allergic'

Comparison of systemic and mucosal IgE to fungi & SAE

We analyzed specific IgE levels to 4 individual fungal antigens (*Alternaria alternata, Aspergillus fumigatus*, *Penicillium chrysogenum, and Aspergillus flavus*) in both serum and mucosa of all patient groups. The choice of fungal species was based on the published frequency of culture from CRS patients[60], and the concentration of fungal spore counts from worldwide aero-biologic surveys[357]. There was a significant correlation between serum and mucosal anti-fungal IgE in CRSwNP patients, which was not observed in CRSsNP patients, or controls. (Pearson correlation, r = 0.79, see Figure 5-1). The level of mucosal IgE in fungal allergic patients was frequently elevated to all 4 fungal antigens tested.

However, fungal specific IgE in serum was significantly higher for *A. fumigatus* and *A. alternata*, than for *P. chrysogenum* and *A. flavus* (Kruskall-Wallis test, Dunns pos-hoc comparison: $p<0.05$). In contrast to fungal IgE, there was no relationship between systemic and mucosal levels in any of the subgroups for the staphylococcal enterotoxin IgE (Pearson correlation, see Figure 5-2). Regardless of the presence of a statistically significant correlation between them, both anti-SAE and anti-fungal IgE levels were significantly higher in the mucosa than serum across all patient groups (Mann-Whitney *U* test, $p<0.05$, see Figure 5-1 & Figure 5-2).

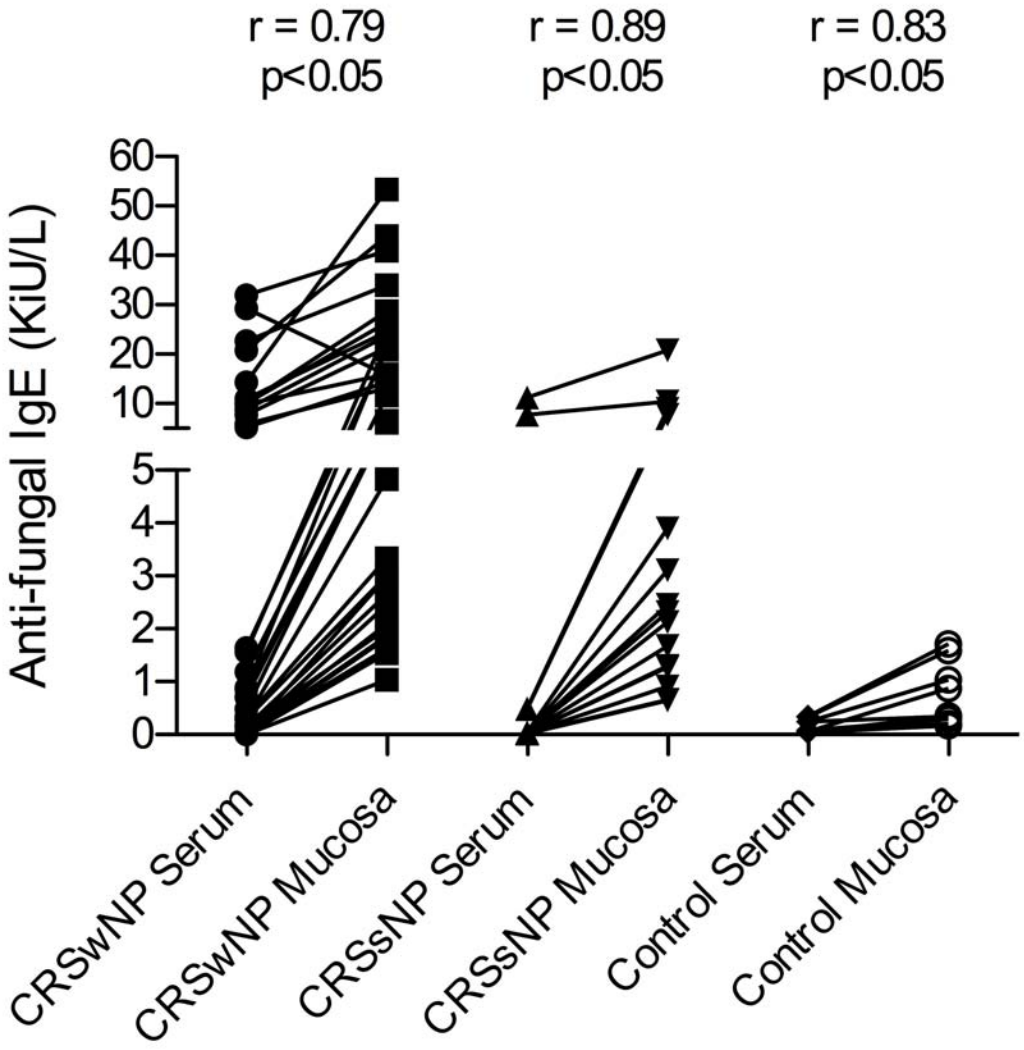

Figure 5-1 Correlation between serum and mucosal anti-fungal IgE levels

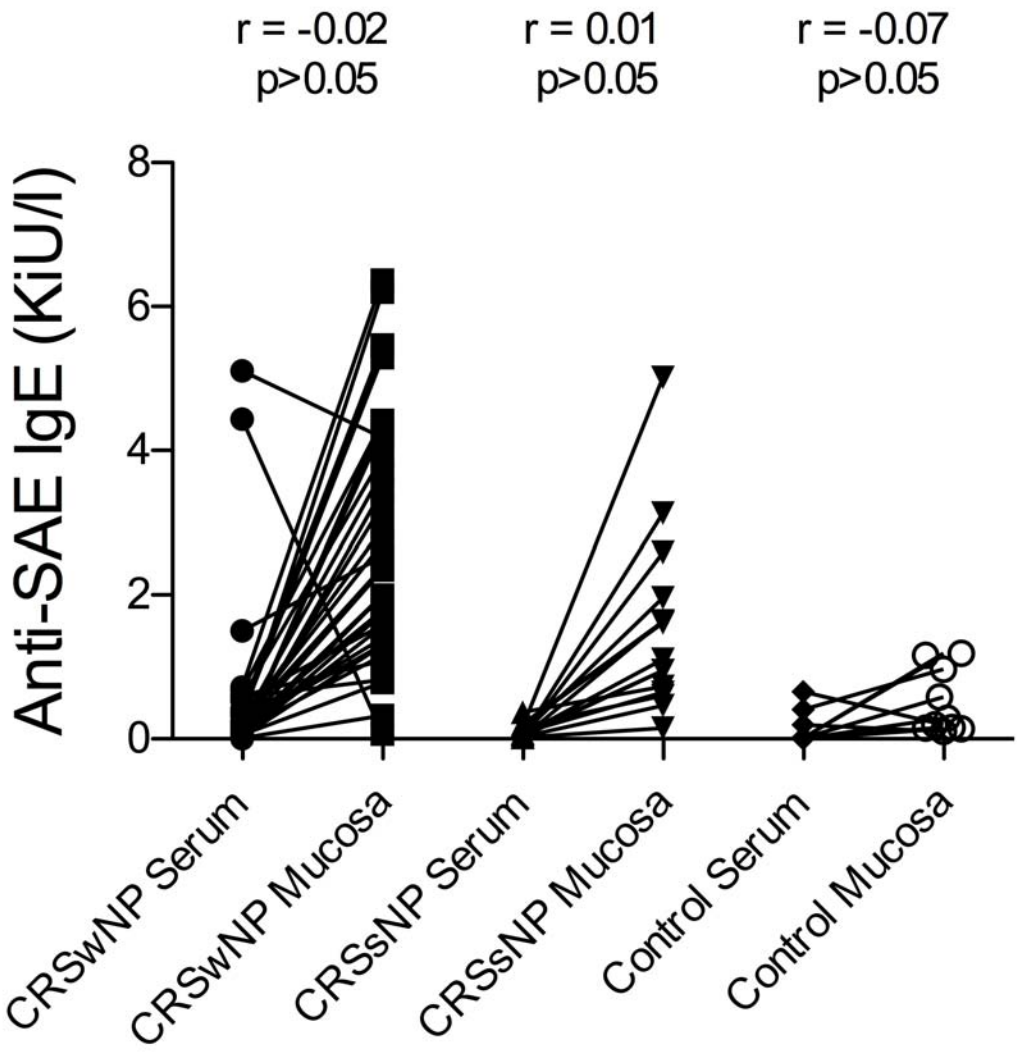

Figure 5-2 Correlation between serum and mucosal anti-SAE IgE levels

Specific Immunoglobulin E responses to microorganisms

The level of organism specific IgE in the mucosa was measured in all patients and compared to the presence of specific microorganisms. “Presence” of an organism was defined as either positive biofilm (FISH) analysis, or identification using conventional bacterial and fungal culture techniques (see Table 5.2). CRSwNP patients had *S. aureus* present more commonly than CRSsNP patients but the difference was not significant (Fisher’ exact test p>0.05).

Table 5.2 Biofilm & Culture Results

	CRSwNP N=33	**CRSsNP N=15**	**Controls N=10**
Total Mucosal *S. aureus* present	22 (67%)	7(47%)	1(10%)
S. aureus Biofilm	18 (55%)	7(47%)	0
S. aureus Culture	11(33%)	3(20%)	1(10%)
Total Mucosal Fungi Present	8(24%)	0	0
Fungal Biofilm	6(18%)	0	0
Fungal Culture	7(21%)	0	0

The level of mucosal SAE IgE was significantly higher in CRSwNP patients when *S. aureus* was isolated on the sinus mucosa (Mann-Whitney *U* test, P<0.05 -see Figure 5-3). Interestingly, this phenomenon was only observed in CRSwNP patients – with no association between *S. aureus* organisms and mucosal SAE IgE levels in CRSsNP patients or controls.

8 patients had evidence of fungi present on their sinonasal mucosa (see table 3). 7 of these patients were identified using standard laboratory culture techniques hence species information was available. *Aspergillus fumigatus* was the most common (3/7) followed by *A. alternata* (1/7) and *P. chrysogenum*(1/7), Trichosporon(1/7), Candida (1/7). 6 patients were positive based on fungal biofilm identification by CSLM. One patient was positive for fungi only on microscopic analysis of histological preparations (Gomori methenamine silver stain). Interestingly, all specimens classified as fungal positive were from CRSwNP patients, and had histological evidence of eosinophilic mucus. Additionally, 7/8 (88%) of these fungal positive patients had systemic fungal allergy. Further examination of the five patients who grew fungal species for which specific IgE analysis was available (*A. fumigatus, A. Alternata and P. chrysogenum*), showed that the species specific IgE was elevated, but not significantly more so than the IgE levels for the other antigens tested (Kruskall-Wallis test, Dunns pos-hoc comparison: $p>0.05$). Of the patients who had fungi in the sinuses, 5/8 (63%) also had allergy to non fungal aero-allergens by FEIA analysis. 5 of the 8 (63%) had concurrent *S. aureus* biofilm present. An additional 12 CRSwNP patients had serum evidence of fungal allergy without detectable fungi in the sinuses.

When analyzing mucosal anti-fungal IgE levels to assess if these correlated with the presence of fungal organisms on the mucosa, we compared this group to the group of fungal organism negative patients who had systemic fungal allergy, because of the observed correlation between systemic and mucosal anti-fungal IgE levels, which may have been a potential confounding factor. Similar to *S. aureus*, there was a significant association between the presence of mucosal

fungi, and anti-fungal IgE levels in the mucosa of CRSwNP patients. (Mann-Whitney *U* test, $P<0.05$ – see Figure 5-4).

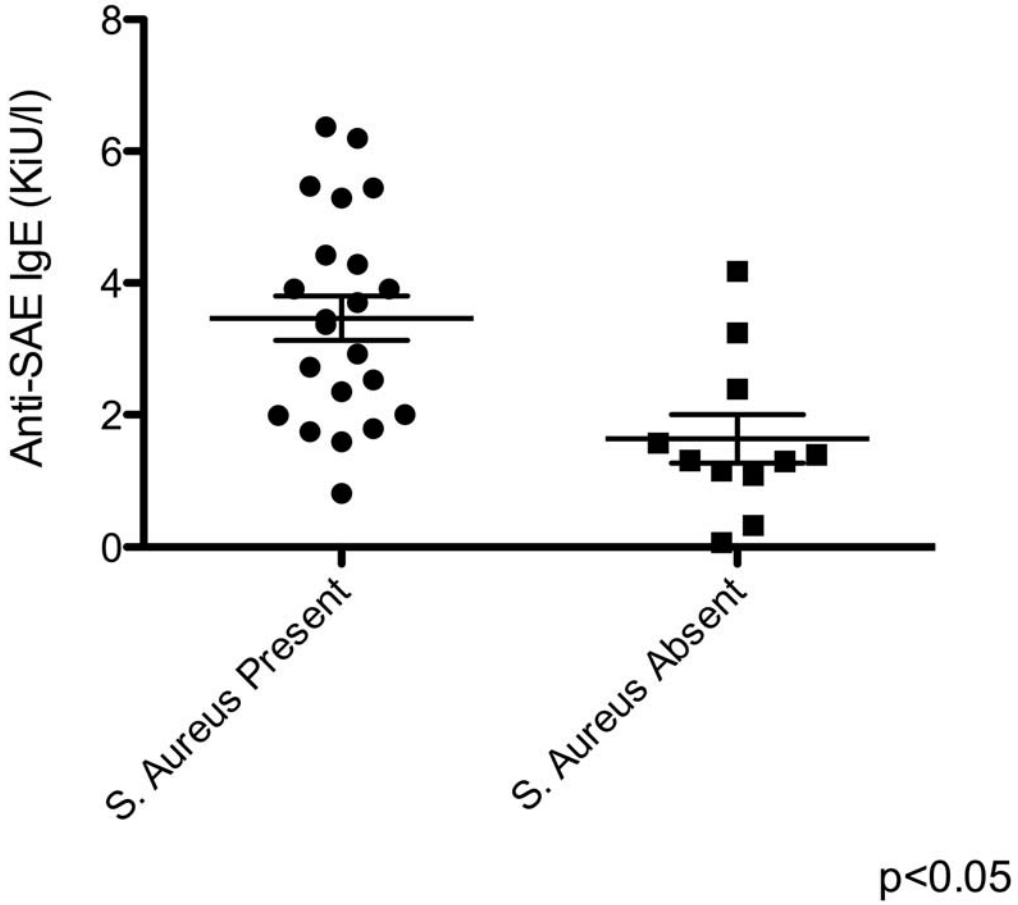

Figure 5-3 The relationship between the presence of *S. aureus* and mucosal anti-SAE IgE in CRSwNP patients

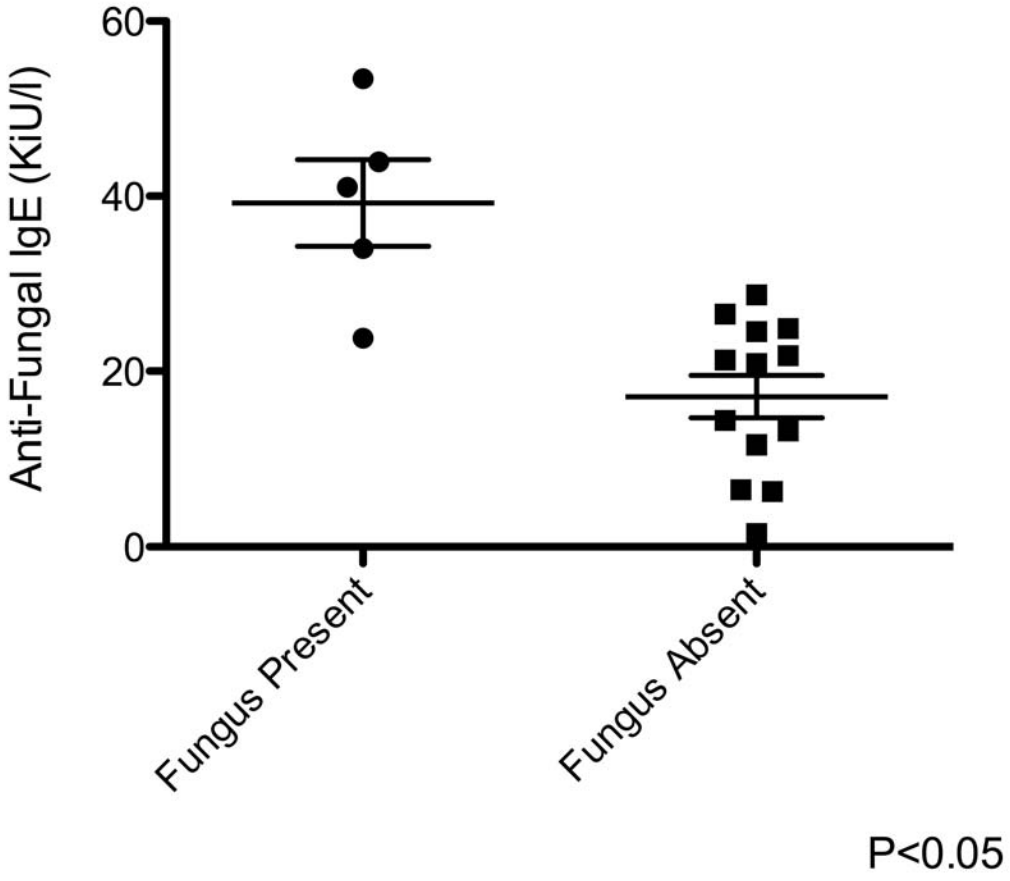

Figure 5-4 The relationship between the presence of fungal organisms and mucosal anti-fungal IgE in fungal allergic CRSwNP patients

The effect of sinonasal *S. aureus* organisms on mucosal fungal allergy

Fungal organisms are often detected on mucosal surfaces associated with *S. aureus*[440], including in CRS patients[186]. An animal study has suggested a synergy between sinonasal fungal and *S. aureus* biofilms[402]. Furthermore, there is evidence in some atopic diseases that the presence of *S. aureus* exacerbates disease severity and potentiates allergen sensitivity. We sought to determine the effect of *S. aureus* organisms on the level of anti-fungal IgE in the mucosa. Due to the observed correlation between systemic and local fungal IgE levels, patient groups were analyzed separately according to presence or absence of systemic fungal allergy. In CRSwNP patients, we observed the presence of *S. aureus* on the sinonasal mucosa resulted in significantly greater levels of anti-fungal IgE in the mucosa (Mann-Whitney *U* test, $P<0.05$). This relationship was found in both fungal allergic, and non allergic patients (see Figure 5-5 & Figure 5-6). However, this relationship was not seen in non-polyp patients.

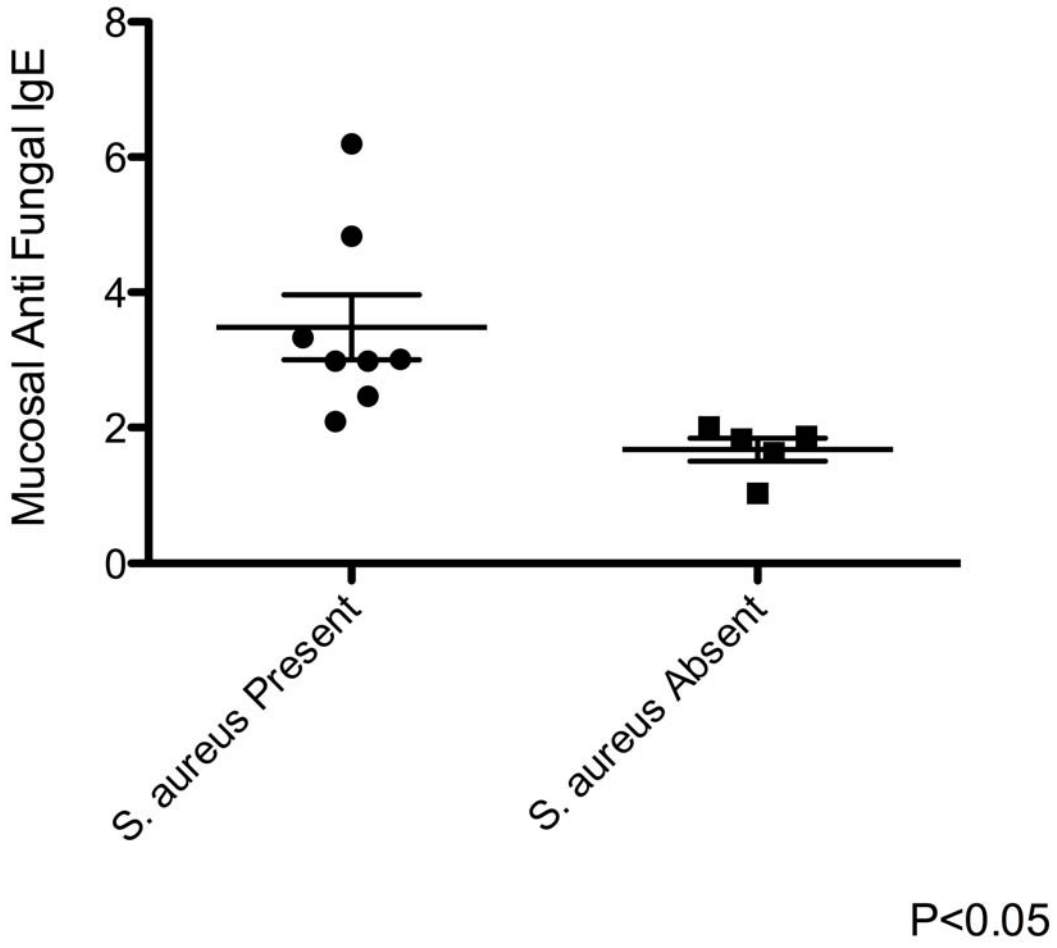

Figure 5-5 The relationship between *S. aureus* organisms and mucosal anti-fungal IgE levels in non fungal allergic CRSwNP patients

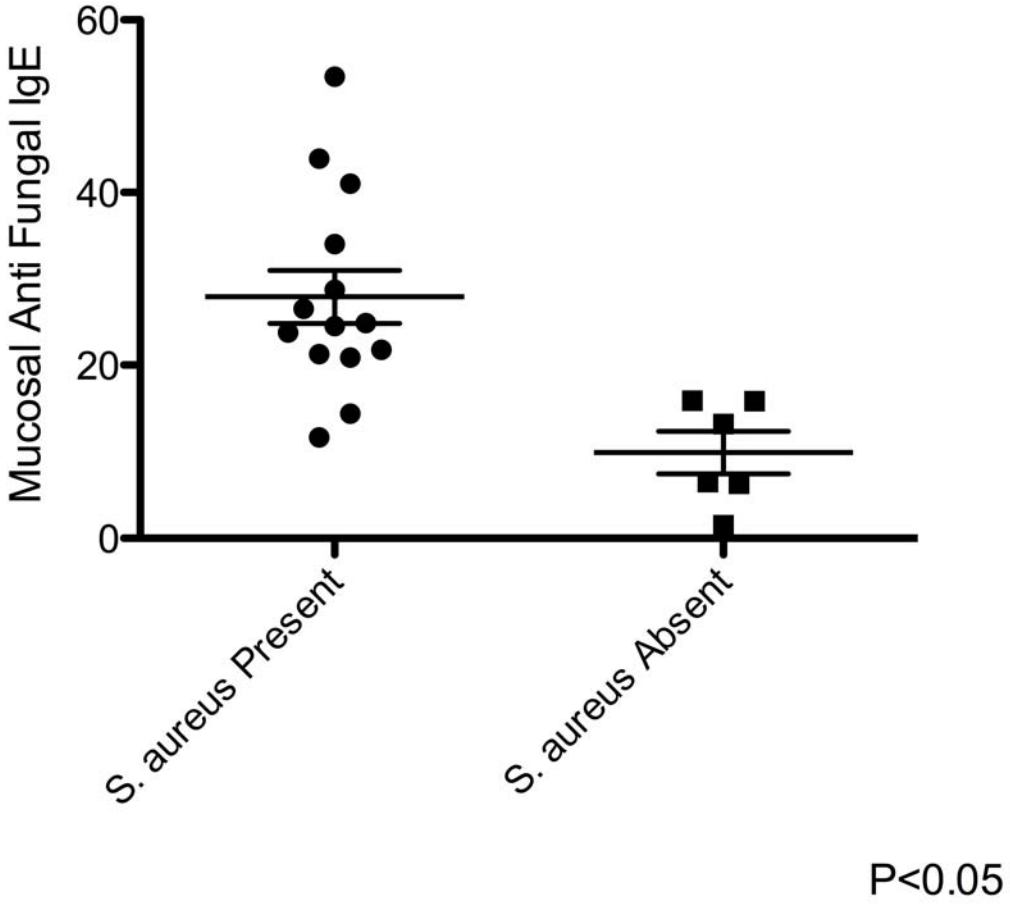

Figure 5-6 The relationship between *S. aureus* organisms and mucosal anti-fungal IgE levels

5.5 DISCUSSION

S. aureus and fungi are often implicated in the pathogenesis of CRS but their role remains incompletely understood. The interaction of resident microorganisms with the host immune system has important implications for our understanding of CRS, and the clinical management of these patients. This study is the first of its kind to investigate the relationship between both *S. aureus* and fungi with organism specific host immune responses. We have shown that the presence of *S. aureus* and fungi on sinonasal mucosa is associated with a greater organism specific IgE response in CRSwNP patients. This is one potential mechanism through which these organisms can contribute to the chronic mucosal inflammation seen in these patients. Additionally we have shown that CRSwNP patients who have systemic fungal allergy, are likely to have greater anti-fungal IgE in the mucosa, regardless of the presence of viable fungi in the sinuses. Exposure to colonizing fungi, or inhalation of ubiquitous fungal aero-allergens may contribute to mucosal inflammation in these patients. Finally, the presence of *S. aureus* on the mucosa is associated with increased anti-fungal IgE in CRSwNP patients, suggesting that the bacteria can act to potentiate inhalant allergy. *S. aureus* may modulate the local response to fungal antigens, which highlights the important role of *S. aureus* as a disease modifying agent in CRS.

Systemic IgE in CRS

The role of systemic allergy in the pathogenesis of CRS has divided opinion in the rhinology research community primarily due to the large number of CRS patients who do not show systemic sensitivity to commonly tested allergens. In

diseases such as allergic rhinitis, allergen specific IgE in serum correlates well with symptoms[348], however in CRS, some authors advocate that atopic status has no bearing on symptom scores, or surgical revision rate[349]. Others have shown that systemic atopy has no effect on the cellular content of nasal polyps compared to non atopic polyp patients[350]. It has also been argued that nasal polyps are not more frequent in atopic individuals[351,352]. On the contrary however, there is evidence to suggest that allergy rates are higher in polyp patients than the general population[354,355]. Analyzing fungal allergy more specifically, some studies have found similar rates of fungal allergy in CRS patients and healthy controls[90], however others have found evidence of higher rates of fungal specific allergy in CRS patients[354,355] than the general population[357]. In the current study we found significantly higher rates of allergy in CRSwNP patients compared to controls. Allergy to fungus was the most common of the allergen groups tested.

Mucosal IgE in CRS

The role of local IgE production in the mucosa, in the absence of elevated systemic IgE has been postulated as a potential mechanism for inflammation in CRS[366,441]. We observed significantly higher mucosal levels of IgE specific for both fungi and SAE in CRSwNP patients compared to non-polyp patients and controls, consistent with other studies. Anti-fungal IgE levels in polyp patients were frequently 10 times that of the anti-SAE levels within the mucosa. We subsequently analyzed the local mucosal levels of IgE with respect to the serum levels. We observed a significant correlation between systemic and local anti-fungal IgE in CRSwNP patients, but not in CRSsNP or controls. Wise *et al*, investigated local production of IgE in allergic fungal rhinosinusitis (AFRS – a subset of CRSwNP) compared to CRSsNP and controls, finding elevated levels

of IgE to fungi and other non fungal antigens in ARFS patients[392]. AFRS patients have, by definition, systemic fungal allergy[53], and the high mucosal levels of anti-fungal IgE in this group is in agreement with our findings. The results from the current study suggest that examining for systemic fungal allergies in CRSwNP patients, through serum testing, is a useful measure of mucosal IgE based sensitivities, as is the practice in allergic rhinitis management. Those patients who test positive for allergy are likely to have elevated mucosal anti-fungal IgE – a potential mechanism for perpetuating mucosal inflammation. This may be an important mechanism in AFRS patients where fungal antigens are produced locally by colonizing fungi, however these patients formed a small proportion of CRSwNP patients in this study. More importantly, CRSwNP patients with systemic fungal allergy who do not have fungi in the sinuses (non-AFRS) may have exacerbated mucosal responses to inhaled fungal aero-allergens which are ubiquitous[425].

The relationship between sinonasal organisms and local IgE production

Infecting or colonizing organisms may provide antigenic sources for the local production of IgE in the sinonasal mucosa. When the presence of organisms (biofilm or culture) was related to the production of specific IgE in the mucosa of CRSwNP patients, both *S. aureus* and fungi showed a statistically significant relationship. These important findings support a link between mucosal-based organisms and specific host immune responses, suggesting they may contribute to inflammatory processes within the mucosa. Interestingly, there was no such relationship in non-polyp patients, despite detecting *S. aureus* organisms in 47%.

In these patients there may be non-IgE immune responses to SAE, which were not analyzed in this study, or perhaps mucosal immune tolerance to these organisms. The overall rate of fungal culture and biofilm positivity was low, and it is unlikely that fungi contribute to a significant proportion of CRS through colonization or infection. Fungal organisms were only detected in patients at the more severe of the CRS disease spectrum, with nasal polyps and eosinophilic mucus.

Staphylococcus aureus organisms exacerbate mucosal fungal sensitization

In this study we have shown that the presence of *S. aureus* organisms on the mucosa of CRSwNP patients is associated with an increased mucosal anti-fungal IgE response. This observation may be due to immunomodulatory effects of the bacterium itself, or a response to secreted superantigen (SAE). Whilst this is the first CRS study to report this phenomenon, it has been described in other diseases with atopic associations. In a study of atopic dermatitis patients, a disease with greater than 90% colonization rates with *S. aureus*[269], *S. aureus* enterotoxins have been shown to upregulate IgE synthesis in the peripheral blood[270]. Pollen specific IgE production has also been significantly enhanced in the presence of *S. aureus* enterotoxins in sera of atopic dermatitis patients[271]. Furthermore, *S. aureus* has been shown to potentiate the sensitization to inhaled aero-allergens in bronchial asthma[267]

5.6 CONCLUSION

A significant research effort is underway worldwide to uncover the pathogenesis of CRS. Many different potential mechanisms for the perpetuation of inflammation have been discovered, highlighting the complex interplay between environment and host in this disease cluster. We have shown that both fungi and *S. aureus* are capable of inciting specific, humoral immune stimulation in the sinonasal mucosa, which may contribute to local inflammation in nasal polyposis. Additionally, we have discovered a novel role for *S. aureus* organisms in nasal polyposis, by exacerbating mucosal sensitization to fungal antigens. This may enhance the mucosal inflammation to viable fungi within the sinuses, where *S. aureus* is often co-habiting. Additionally, the enhanced sensitivity to ubiquitous fungal aero-allergens may contribute to mucosal inflammatory processes in those patients without viable fungi within the sinuses. This additional pro-inflammatory function of *S. aureus* highlights the emerging role of this organism as a prominent disease modifying agent in CRS.

Thesis Synopsis

This thesis has examined many aspects of CRS pathogenesis following an extensive examination of the literature. CRS is a complex disease, with a multiplicity of factors, which may contribute to produce symptomatic disease, but no single factor has been conclusively attributed. Indeed it may be that inherited or acquired defects in host defense mechanisms lead to exaggerated inflammatory responses to otherwise innocuous environmental factors. However, there is also evidence to suggest that microorganisms may play an active role in exacerbating inflammation in the susceptible host. It is the interplay between the environment and the host, which forms the central theme of this thesis. This work is based on the hypothesis that some of the inflammation observed in CRS results from an aberrant immune response to microorganisms such as host and fungi. Microorganisms may interact with the host at many levels of the immune defense, from the epithelial barrier, and innate immune functions, to the humoral immune response and alteration of hypersensitivities. Essential to any study of organism-host interactions is the accurate characterization of the organisms.

Contemporary understanding of polymicrobial populations and their interactions with host surfaces in other diseases at the host-environment interface includes novel concepts such as mutualism and functional equivalence. We know that abiding by single organism paradigms have limited our understanding of disease. To completely understand the role of microbes in pathogenesis we must characterize the flora completely so the complex inter-relationships and symbiotic

associations can be studied. Despite this impetus, there is much confusion and contention in the rhinological literature regarding the microbial flora of CRS. The majority of studies are based on cultivation-based techniques, which have a myriad of limitations when characterizing complex, polymicrobial populations. Not only do such techniques polarize results, selecting for rapidly growing organisms at the expense of those which are slower growing, or more fastidious. But they also require that the investigator pre-select which media to use to cultivate the appropriate microbes. In the polymicrobial environments of mucosal surfaces, which occur at the host-environment interface, there are a plethora of microorganisms, each with specific growth requirements. Removing these from their niche results in dramatic environmental changes, which inevitably limits their capacity to thrive, and hence be detected. To provide ideal conditions for all of the microorganisms using culture based techniques would be exhaustive, if not impossible. Adding to this dilemma, bacterial and fungal biofilms have been discovered on sinonasal mucosa of CRS patients, which are notoriously difficult to culture using conventional techniques. Polymerase chain reaction techniques have been developed to overcome some of the limitations of culture detection, and have great utility when detecting two or three microbes in a mixed population. However these techniques still require *a priori* selection of microbial targets, and can only detect a limited number of species per sample. Many of the CRS microbial studies that have been performed using these detection techniques have either not assessed control tissue, or have selected control tissue using criteria, which does adequately exclude diseased tissue.

In an attempt to overcome these limitations, we have employed a sensitive, broad based molecular detection method for the characterization of fungal and bacterial populations on sinonasal mucosa. The Ibis T5000 biosensor uses broad based PCR, followed by electrospray ionization to divide DNA strands and inject them into a mass spectrometer, which accurately determines the base pair composition and compares this to a proprietary database to determine species present. In this way we can determine the entire cohort of microbes without having to pre-select microbial targets. Importantly, we have also determined the relative abundance of the detected microorganisms. We have shown that *S. aureus* is the most common bacterium on sinonasal mucosa of our CRS patient cohort. It is also frequently present on control tissue but in reduced abundance. *S. epidermidis* was also frequently detected on both diseased and control mucosa, but the relative abundances were similar. Clearly, not only presence, but also their specific population densities are essential when comparing disease and control flora. We have shown that fungi are present in a small proportion of CRS cases, but are found in the more severe disease states with eosinophilic mucus and nasal polyps. In addition to molecular analysis, we also performed conventional culture and fluorescence *in situ* hybridization (FISH) to determine the specificity and sensitivity of these techniques. We have shown that conventional culture is adequate at detecting the majority of rapidly growing organisms such as *Staphylococci* and *Pseudomonas* species, however the detection of more fastidious species, and anaerobes was poor. FISH has greater sensitivity and specificity for microbial detection than culture, and is particularly effective at detecting microorganisms in the biofilm form. Both culture and FISH detection

thresholds were related to microbial abundance as determined by the Ibis biosensor.

We were particularly interested in the role of fungi in CRS as it appears to be related to the severe disease spectrum, and had recently been discovered in biofilm form in CRS patients. However, very little was known about the factors involved in fungal proliferation in sinuses. Type I hypersensitivity to fungi had been proposed as an important aspect of allergic fungal rhinosinusitis, but the exact role was obscure. We developed an animal model of sinusitis in sheep. Using fungal antigens we successfully sensitized 45% of sheep, evidenced by positive skin prick testing. We then inoculated the occluded frontal sinuses of the sheep with the same fungal species to which they were sensitized. We analyzed the mucosa using specialized staining and microscopy techniques but were unable to establish fungal biofilms in the sinuses. A review of our clinical experience, and the common occurrence of fungi and *S. aureus* together in human CRS patients, directed us to co-inoculate the two organisms into the same sinuses. The result was significant fungal biofilm formation. Hypersensitivity to the fungi had no bearing on inflammation, secretory hyperplasia, or fungal biofilm formation in this model. We observed that the inflammation was greater when *S. aureus* was inoculated, however this was no greater when fungi was co-inoclated. There are many reasons why *S. aureus* may enhance fungal biofilm formation, including synergistic actions relating to adhesion and attachment sites, metabolic assistance, and environmental modification. We hypothesized that cilial damage may be contributing and designed the subsequent project to investigate this.

In the third project we again used the sheep model, and chose four bacterial species commonly detected in CRS patients to co-inoculate with fungi. To determine the effect of cilia on fungal proliferation in isolation, we also co-inoculated fungi with a cilia toxin. We used transmission electron microscopy to grade cilia damage. All bacterial species formed bacterial biofilms within the sinuses except *H. influenzae*. Bacterial biofilm formation was significantly associated with cilial destruction. Sinuses with highly damaged cilia showed florid fungal biofilm development. Cilia toxin treated sinuses also formed fungal biofilms, however 50% of these sinuses also formed bacterial biofilms from endogenous seeding. It is likely that the impaired epithelial defenses allow slow growing fungi to adhere, germinate and proliferate. Relating these findings to the clinical forum, the impaired cilial function, which is seen following endoscopic surgery, may provide ideal conditions for fungal proliferation, and may explain early surgical failure in AFRS patients. The relationship between bacterial and fungal virulence also has implications for our understanding of medical treatments, and may help to explain the limited role of antifungal therapies in these patients. Improving cilial function may be a critical target to improve treatment outcomes.

The final step in this research was to analyze these microorganisms in the context of CRS, and examine host immune responses to these. In order to determine if the host response was directed towards these microbes, we needed to measure a host immune molecule with organism specificity. We chose to measure microorganism specific IgE in serum and mucosa as it fulfilled this

criterion, and also allowed us to analyze hypersensitivity responses in the human host, following our interesting results from the first animal study.

We performed a prospective study of 48 CRS patients and 10 controls, analyzing sinus mucosa for the presence of *S. aureus* and fungi using conventional culture and FISH. We also measured organism specific IgE in the serum and mucosa of these patients and correlated these with organism presence to determine relationships. We established that the presence of both *S. aureus* and fungi on the mucosa was associated with elevated mucosal IgE levels in CRSwNP patients. This is one of the only published studies, which demonstrates that these microorganisms are actively interacting with the host, and not merely colonizing the surface. By interacting with mucosal IgE, these microbes may be contributing to the mucosal inflammation evident in CRS patients.

Finally we showed evidence for a novel role of *S. aureus* in CRS pathogenesis. We demonstrated that mucosal fungal hypersensitivity, was significantly increased in the presence of *S. aureus* organisms on the mucosa. This phenomenon has been reported in other allergy-associated diseases such as asthma and atopic dermatitis but this is the first description in CRS patients. This has important implications in CRSwNP patients, as the antigenic stimuli for such exaggerated mucosal reactions are inhaled with every breath. We speculated that this may be a function of *staphylococcal* superantigen activation of allergic pathways, but further research is required to substantiate this. It is clear that management of *Staphylococcal* colonization, and allergy management may be critical to benefit these patients.

This thesis has made a significant contribution to our understanding about the relevant environmental and host factors in CRS patients, and how they may interact to impact disease. However, the more we learn, the more we realize how much farther we have to go, to gain enough insight into CRS pathogenesis to provide sustained relief to our patients.

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