BACTERIAL & FUNGAL BIOFILMS IN CHRONIC RHINOSINUSITIS

A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF ADELAIDE

Deepti Singhal

Discipline of Surgery, School of Medicine, Faculty of Health Sciences

The Queen Elizabeth Hospital, South Australia

March 2011

ॐ

त्वमेव माता च पिता त्वमेव
त्वमेव बंधू च सखा त्वमेव
त्वमेव विद्या च द्रविणं त्वमेव
त्वमेव सर्व मम देव देव ॥

"O Lord, You Are My Mother, Father, Kinsman And Friend.

You Are My Wealth Of Knowledge, Strength, Velour And Power.

You Are My All God Of Gods".

Dedicated to the anchor of my life, my dear husband
Nimit

And my adorable children
Gunin *&* **Niya**

TABLE OF CONTENTS

ABSTRACT

BACTERIAL-FUNGAL BIOFILMS IN CHRONIC RHINOSINUSITIS

Chronic Rhinosinusitis (CRS) is a recalcitrant disease, characterized by headache, nasal discharge / blockage, which substantially impairs daily functioning and negatively affect quality of life. Endoscopic Sinus Surgery (ESS) is an important treatment option for CRS, but has variable success rates. Biofilms are well organised heterogeneous communities of microbes embedded in a mosaic of extracellular matrix, adherent to biotic / abiotic surfaces. As they are resistant to host defences and medical treatments, they have been touted as possible pathogenic factors in CRS, which may perpetuate the recurrent and recalcitrant character of the disease and negatively affect treatment outcomes.

This thesis encompasses research undertaken to enhance our understanding about the effect that presence and types of biofilms have on the clinical profile and treatment outcomes of patients suffering with chronic rhinosinusitis. An in-vitro model of fungal biofilms and a potential tool to assay in-vivo mucosal biofilms on sinonasal tissues has also been described.

Chapter 1 of the thesis comprehensively reviews the scientific literature pertaining to biofilms and CRS, and exhaustively evaluates the evidence present in relation to bacterial and fungal biofilms in CRS.

Chapter 2 describes a study to investigate the effect of biofilms on outcomes following ESS in CRS patients using internationally accepted standardised symptom scores, quality of life measures and endoscopy scores to assess the disease. It showed that patients with biofilms presented with more severe disease before surgery, and after surgery had persistent symptoms, ongoing mucosal inflammation and infections necessitating extra post-operative visits and multiple antibiotic treatments. This study thus strengthened the evidence for the role that biofilms may play in recalcitrant CRS.

Chapter 3 describes a further subgroup analysis of the above patients in whom the specific organisms forming the biofilms were identified and how patients with specific biofilm types progressed after surgery was studied. Patients with polymicrobial biofilms suffered more severe disease and had worse post-surgery mucosal outcomes requiring more post–operative visits. *S.aureus* biofilms played a dominant role in negatively affecting outcomes of ESS with persisting post-operative symptoms, ongoing mucosal inflammation and infections.

Chapter 4 describes an in-vitro model characterizing *A. fumigatus* biofilm formation on primary human sinonasal epithelium cultures under different growth conditions. 3-dimensional biofilm structures with parallel-packed and cross-linked hyphae, channels/passages, extracellular matrix (ECM) encasing the hyphae, were formed. Biofilms formed under flow conditions displayed more robust and faster growth kinetics as compared to those under static conditions, with extensive ECM production.

Chapter 5 investigates application of an analysis program 'COMSTAT 2' for assaying & quantitatively describing the 3-dimensional in-vivo biofilm structures observed via confocal scanning microscopy on sino-nasal mucosal samples. This can be used for temporal analysis of biofilm development, comparison of different types of biofilms formed under controlled conditions, analysis of influence of varying environmental factors on biofilms and the efficacy of different antibiofilm treatments.

Chapter 6 summarises and discusses the salient features of the studies included in this thesis which has attempted to characterize fungal and bacterial biofilms and the impact they may have in CRS patients.

THESIS DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Deepti Singhal and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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

The author acknowledges that copyright of published works contained within this thesis (as listed below) resides with the copyright holder(s) of those works.

- The impact of biofilms on outcomes after endoscopic sinus surgery

  Singhal D, Psaltis A.J, Foreman A, Wormald P.J.
  American Journal of Rhinology & Allergy, 2010 May; **24** (3): p. 169-74.

- *Staphylococcus Aureus* Biofilms: Nemesis of Endoscopic Sinus Surgery

  Singhal D, Bardy J.J, Foreman A, Wormald PJ,
  Laryngoscope, 2011 (Under publication)

- *Aspergillus fumigatus* biofilm on primary human sinonasal epithelial culture

  Singhal D, Baker L, Wormald PJ, Tan LW
  American Journal of Rhinology & Allergy, 2011 (Under publication)

- Quantitative analysis of mucosal bacterial biofilms

  Singhal D, Field J, Boase S, Jardeleza C, Foreman A, Wormald PJ
  American Journal of Rhinology & Allergy (Under review)

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library catalogue, the Australasian Digital Theses Program (ADTP) and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Deepti Singhal
Date: ....

ACKNOWLEDGMENTS

CHAPTER 1

LITERATURE REVIEW

1.1 CHRONIC RHINOSINUSITIS

1.1.1 REASEARCH DEFINITION

Rhinosinusitis has been accepted by rhinologists, immunologists, pulmonary physicians, general practitioners across the world to describe the heterogenous group of disorders characterized by inflammation of the sinus and nasal mucosa. Based on the duration of the disease it is classified as acute or chronic, with chronic rhinosinusitis (CRS) diagnosed when symptoms fail to resolve for more than 12 weeks. A European evidence based paper on rhinosinusitis [1] has defined CRS with or without nasal polyposis as presence of two or more symptoms, one of which is nasal blockage/obstruction/congestion or nasal discharge (anterior/posterior nasal drip); ± facial pain/pressure; ±reduction or loss of smell for > 12 weeks with validation by telephone or interview. As per the American rhinosinusitis consensus research definitions and guidelines for CRS [2] described in Table 1, objective documentation of the disease via nasendoscopy and radiology is required along with the presence of symptoms for more than 12 weeks. CRS can be further subclassified on the basis of presence or absence of nasal polyps on nasendoscopy into CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP).

Table 1:RESEARCH DEFINITIONS AND GUIDELINES FOR CRS

	European Position Paper on Rhinosinusitis[1]	**American rhinosinusitis consensus research definitions[2]**
Pattern of disease	Symptoms > 12 weeks, without complete resolution	Symptoms > 12 weeks

Symptoms	≥ 2 symptoms, one of which should be –
	 Nasal Blockage / obstruction / congestion
	 Or
	 Nasal discharge (anterior / posterior nasal drip):
	    ± facial pain/ pressure
	    ± anosmia / hyposmia
		Requires ≥ 2 of the following symptoms
		 • Anterior &/or Posterior mucopurulent discharge
		 • Nasal Obstruction
		 • Facial pain-pressure-fullness
		 • Decreased sense of smell

Objective documentation		• Anterior Rhinoscopy / Nasal Endoscopy
		    o Discolored mucus
		    o Edema of middle meatus / ethmoid area
		    o ± nasal polyps
		 • Radiology – Evidence on CT scan

1.1.2 EPIDEMIOLOGY

Though CRS is being increasingly recognised as a widespread disease cluster across the continents, the heterogenous spectrum of the disease and the diagnostic variability seen in various surveys and publications has made it difficult to provide precise epidemiologic data regarding the condition. As per a National Health Survey conducted in 2004 by the Australian Bureau of Statistics, chronic sinusitis was reported to affect more than 16% of the Australian population. In another survey of physicians and patients, physician-diagnosed rhinosinusitis was reported by over 10% of the population in Europe, Japan, and the USA [3]. A comparitive study of prevalence of the disease in 2 ORL clinics across the Atlantic has shown similar prevalence rates of approximately 9% [4]. Patient based surveys of the US population have shown that CRS affects 16% of the adult US population[5], making it the second most prevalent among all chronic conditions[6]. Prevalence rate of CRS has been shown to be substantially higher in females with a female to male ratio of approximately 6:4 [6, 7] being reported from different population groups. Canadian data also shows that prevalence increased with age, and plateaued after 60 yrs of age[7].

More data from the US has indicated that the annual economic burden of rhinosinusitis is on the rise and has recently been estimated at $6 billion[8]. Patients with CRS have been shown to have twice more visits to a general practise and more than 5 times pharmacy scripts filled as compared to a patient without CRS[9]. The majority of the economic cost related to CRS is a direct result of medical expenditures, but indirect costs including work absence and disability are significant too. It is known to cause significant physical symptoms, negatively affect quality of life and can substantially impair daily functioning. In comparison to other chronic conditions like congestive heart failure, angina, COPD and back pain, patients with CRS have significantly poorer pain and social functioning scores[10].

1.1.3 AETIOPATHOGENESIS

Various intrinsic factors like ciliary impairement[1] (cystic fibrosis[11], Kartagner's Syndrome, primary ciliary dyskinesia-), allergy [12], asthma [13], aspirin sensitivity [14], immune dysfunction [15], nasal anatomical variations (concha bullosa, deviated nasal septum etc.), gastroesophageal reflux[16] and extrinsic factors like micro-organisms (bacteria,viruses, fungi), osteitis[17], cigarette smoking[7] have been touted as possible etiologic points of interest in the multifactorial causation for CRS. Bacterial superantigens and biofilm forms have recently been added as the possible mechanisms via which the microbial community participates in the pathogenesis of CRS. This thesis aims to better understand and characterise the role that microbial biofilms may play in perpetuating the sinonasal mucosal inflammation associated with CRS.

1.1.4 TREATMENTS AND OUTCOMES

Despite the multiple causes attributed towards the aetiopathogenesis of chronic rhinosinusitus, currently there is a singular management approach practised worldwide i.e initial medical management, followed by surgical intervention after maximal medical treatment [18-20]. Medical management options range from antimicrobials, nasal lavage, corticosteroids, antihistamines. However there is little agreement regarding a standardised universal medical therapy. Various types of antibiotics via topical or systemic routes have been trialled in the past. Different studies applying varied inclusion criteria, different antimicrobials with different durations of therapy, and using various outcome measures of success have reported 50-80% success rates after antibiotic treatments. Although β-lactam group of antibiotics has been conventionally used, macrolide antibiotics have now been shown to have immunomodulatory actions along with the antibacterial action [21-23].

Evidence from randomized controlled trials investigating nasal saline irrigation to control symptoms of CRS has been compiled in a systematic review by the cochrane collaboration [24] and it has recommended saline lavage as a beneficial treatment for the symptoms of chronic rhinosinusitis. Corticosteroids have been used intranasally and systemically for their anti-inflammatory action on the sino-nasal tissues. Once again good quality evidence from RCT and controlled trials [25-27] exists supporting their use across the wide spectrum of patients with CRS.

Over the years many studies have supported the use of ESS in CRS [28] by showing improvement in patient symptoms, quality of life, and endoscopic examination. But majority of them have represented level 4 or 5 evidence [29]. Despite 20 years of progress in sino-nasal anatomy knowledge, imaging modalities, surgical techniques and instrumentation for ESS, it is accepted that it will not cure all CRS patients. As early as 1990 a study reporting results of ESS on 165 patients for a variety of different indications, showed that 50-87% of the patients in different subgroups described subjective improvement after a mean follow-up of 10 months, with CRSwNP patients describing the most symptomatic improvement [30]. However 37% of the operated sides showed poor objective result on post-operative endoscopy.   In 1992 Kennedy [31] analysed data from 120 patients undergoing ESS for improvements in subjective outcomes via symptom-based questionnaires and objective outcomes such as CT scans and endoscopy scores. Although 97% reported improvement in symptoms after an average follow-up of 18 months, the majority of patients showed persistent evidence of disease on endoscopy.

1.2 BIOFILMS

1.2.1 BIOFILM DISCOVERY

Biofilm like morphology has been identified in fossils from sedimentary rock and volcanogenic deposits indicating that this form of growth is a very old evolutionary adaptation present in the microbial world [32, 33]. The 'animaliculi' or 'tooth worm' that Anton Van Leeuwenhoek first described in his dental scrapings was possibly the first evidence of biofilm in human disease [34]. It was only in 1970s, when with the advent of the electron microscope, the slime layer found in various water treatment plants was found to be composed of microbial organisms, surrounded by a matrix [35, 36]. Based on further work with dental plaques and environmental water systems, the concept of biofilms was put forth aiming to explain the mechanisms and possible benefits of this microscopic ecologic system [37].

1.2.2 BIOFILM MORPHOLOGY AND PHYSIOLOGY

Biofilms have been defined as 'a microbially derived sessile community, characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of self produced extracellular polymeric substances, and exhibit an altered phenotype in terms of growth rate and genotype'[38]. With advances in scientific research and technology over the past 40 years, the definition of a biofilm has evolved from a structural description of assemblage of microbes enclosed in a polymeric matrix [39, 40], to encompass both structural and physiological features of microbes within this ecosystem.

The biofilm is basically composed of organised communities of microbes in a mosaic of extracellular polymeric substance, and is attached to a biotic or abiotic surface [41, 42].

The exopolysaccharide (EPS) matrix forms 85-90% of the biofilm [41-43] and is composed mainly of polysaccharides, protein, some nucleic acids and occasional dead microbial populations [42, 44-47]. The EPS expresses varying physiochemical features which protect the microbes contained in the biofilm. It also contains channels penetrating and separating the microbial communities that help in water, nutrients, and waste exchange. The microbes within the biofilms are clustered into functionally heterogeneous communities which display co-ordinated specialized phenotypes in different regions within the biofilm [48-50]. The localized specialization is seen as a varied expression of surface molecules, nutrient utilization, virulence factors, antibiotic resistance and division potential between different microbial communities within this micro-ecosystem of biofilms [51-55]. Intercellular communication via chemical secretions into surrounding EPS and channels ensures the synchronised existence of these microbes in the biofilm form that in turn enables them to survive under hostile conditions.

Biofilm formation is a staged process [50, 56, 57] with biofilm formation being initiated when few free floating planktonic microbes attach to a biotic or an abiotic surface. As a critical microbial density is reached, the process of 'quorum sensing' [58, 59] is initiated by which neighbouring microbes communicate by means of an altered genotype and a cascade of protein expression which begins defining the biofilm phenotype. The microbes then are seen to tightly adhere to the surface as small clusters of micro-colonies, which multiply and organise into layers or towers with intervening channels for exchange of water, nutrients and waste between the external environment and the biofilm microbes. It leads to the formation of a micro-ecosystem, with functionally heterogeneous microbial communities within the biofilm, bound by the exopolysaccharide matrix. Finally under the influence of altered environmental conditions such as shear forces, altered oxygen concentration, single to clusters of few cells detach from the biofilm mass and embolize to start the staged process of biofilm formation at a new site.

Biofilms do not have a singular common structure and formation of the heterogeneous biofilm ecosystem has been shown to be influenced by environmental factors like chemical composition of the substratum [60-62], available oxygen and nutrients [63-68], osmolarity [69], shear stresses [70, 71], and the host responses [48]. It has thus been suggested that biofilms forming on biotic surfaces like mucosa will differ phenotypically and genotypically from biofilm formed on inert surfaces like sewage/water pipes, indwelling medical devices or in-vitro conditions. The Centre for Genomic Sciences has coined the term 'mucosal biofilms' to describe biofilm structures identified on mucosal surfaces [72]. The host cellular and molecular components possibly contribute to the EPS and modulate the micro-environments of these mucosal biofilms via their immune systems [48].

1.2.3 BIOFILMS AND HUMAN DISEASES

Biofilms were first implicated in human diseases when they were identified in dental plaques by researchers at the Forsyth Dental Centre, Boston. [73]. Since then biofilms have been implicated in numerous infectious processes, such as cystic fibrosis pneumonia, endocarditis, chronic prostatitis, chronic cystitis, kidney stones, osteomyelitis, and periodontal disease [42]. Centre for Disease control and Prevention estimates that at least 65% of all human bacterial infectious processes involve biofilms [73]. Biofilms have been identified on medical devices like urinary catheters, venous and arterial catheters, contact lenses, artificial heart valves, pacemakers, orthopaedic prosthesis which once infected have to be removed or replaced as they are not amenable to resolution via conventional antimicrobials. The antibiotic treatments might kill the planktonic microbes shed into circulation from the biofilm, but the mother biofilms survive and form a nidus of infection to continue the systemic infection by sloughing off planktonic micro-organisms.

Bacteria in biofilm form are 1000 times less susceptible to antibiotic treatments when compared to their planktonic counterparts [74, 75] and multiple different mechanisms have

been put forward for the resistance of different bacteria towards various antibiotics. Biofilm bacteria have been shown to be metabolically slow and some of them have the ability to enter into a dormant (non-dividing) state that further reduces their susceptibility to certain antimicrobials. The antimicrobials acting on specific metabolic pathways or dividing bacteria will thus be ineffective on such 'persister cells' that are mostly present in the central core of the biofilm.[76-78]. It was earlier proposed that the extra-cellular matrix of the biofilm acted as a physical barrier impermeable to antibiotics. Now it is known to retard the rate of penetration of the antimicrobials, to give time for induction of gene expression in the biofilm that may mediate resistance to them [79]. The antibiotics may also be deactivated by binding with antibiotic-degrading enzymes [53] or as a consequence of the charge affinity with the EPS polymers [80]. There is also evidence suggesting that the transmembrane channels may be down regulated in the biofilms resulting in a phenotype less responsive to therapeutic concentrations of the antibiotics. Additional expressions of mutidrug efflux pumps within the biofilms further enhance their resistance to antimicrobials [81-84]. The biofilm ecosystem also favours adaptive mutations via plasmid transfers between microbes [38, 85-87]. They are also impermeable to antiseptics like hypochlorite [88] and sterilizing procedures like ultraviolet degradation [89] The above mechanisms help the bacteria form a persisting nidus of infection in host tissue protected from the host immune system and antibiotic regimes, leading to a chronic disease state with intermittent infections.

Formation of biofilms also appears to be a protective mechanism for the micro-organisms against the host immune system. Biofilm bacteria may not be vulnerable to phagocytosis by host immune cells as microscopy of clinical specimens has shown antibodies and inflammatory cells only surrounding biofilm communities and not penetrating them to reach the microbes [90, 91]. Some studies have shown the presence of leukocytes within the channels of the ECM, but with an un-stimulated morphology evidenced by lack of

motility, decreased oxidative potential or absent phagocytised cellular elements [92, 93]. Similar interaction has been seen between antibodies and biofilm aggregates with IgG observed bound to the periphery of the biofilms, and failing to penetrate the ECM and failing to eliminate the microbes via opsonisation [94, 95]. Though the immune system is ineffective against the biofilm, it causes collateral damage to surrounding host tissue leading to an inflammatory process which in turn possibly leads to disease causation. The presence of such an overt pathological inflammatory response differentiates colonization (microbial adherence or presence in asymptomatic hosts) from biofilm infections.

The bacteria within the biofilm form are difficult to culture and identify using the currently available clinical tests, and require specialised microscopy to detect them. Conventional microbiology techniques applied to suspect biofilm-specimens give negative results hinting at an absence of infectious pathologies, despite persistent signs and symptoms. This complexity in identifying biofilms in-vivo has led to the development of specific criteria for diagnosing biofilm infections in clinical specimens [96-98] which has been outlined in Table 2:

Table 2

Diagnostic criteria for biofilm infections
1. Pathogenic bacteria are associated with a surface.
2. Direct examination of infected tissue demonstrates aggregated cells in cell clusters encased in a matrix, which maybe of bacterial and host origin.
3. Infection is confined to a particular site in the host.
4. Recalcitrant to antibiotic treatment despite demonstrated susceptibility to planktonic bacteria.
5. Culture-negative results in spite of clinically documented high suspicion of infection.
6. Ineffective host clearance evidenced by the location of bacterial cell clusters in discrete areas in the host tissue associated with host inflammatory cells.

1.2.4 BIOFILMS IN OTOLARYNGOLOGY

Evidence of association of biofilms in various otolaryngology infections has existed for more than a decade [72, 99-103]. Using haemophilus influenzae biofilms in a chinchilla model of otitis media, Ehrlich and colleagues [56] described 5 stages in the formation of a mucosal biofilm, which is particularly representative of the biofilms seen on ciliated respiratory epithelium found lining the regions of the upper respiratory tract where biofilms have been identified.

Otitis Media with Effusion (OME) is being now increasingly recognised as a biofilm mediated disease with extensive evidence being present using both human and animal tissue [56, 104-110]. Presence of biofilms has also been seen in experimental and human cholesteatoma [111, 112], animal models of chronic suppurative otitis media (CSOM) [113], middle ear mucosal biopsies from patients of chronic otitis media undergoing tympanomastoid surgery [114, 115], and a patient with temporal bone osteoradionecrosis. Biofilms have now been described on a variety of indwelling otologic devices like cochlear implants in-vitro models [116, 117], cochlear implants removed as a result of device failure [118] or infection [119-124], ossicle reconstruction prosthesis removed at revision surgery [125], and tympanostomy tubes [126-136]. The chronicity and recurrent nature of tonsillitis is now being explained on the evidence of biofilms found on the surface and crypts of tonsils [137-140] and the pediatric nasopharynx [141]. Studies have found biofilms to completely cover adenoid tissue removed from children with CRS [142, 143] or OME [144, 145]. There are also reports of biofilms on voice prostheses [146-148] and tracheostomy tubes[149-152].

Over the past 7 years, mounting evidence has been presented to implicate biofilms in propogating the recalcitrant and chronic nature of Chronic Rhinosinusitis [62, 98, 153-160]. It has been suggested that CRS shows features similar to other biofilm mediated diseases i.e. a chronic course characterised by acute exacerbations, un-resolved by host defense mechanisms and resistant to antibiotic therapies.

1.3 BACTERIAL BIOFILMS IN CRS

1.3.1 EVIDENCE OF BACTERIAL BIOFILMS IN CRS

The first evidence of presence of biofilms on the sino-nasal mucosa was put forward by Cryer and colleagues in 2004 [161]. They used scanning electron microscopy (SEM) to evaluate biopsy specimens from 16 patients with continuing symptoms of chronic sinusitis, despite medical and surgical management. A near-total surface coverage of four mucosal specimens with a coating thicker than the normal mucociliary blanket made the authors conclude that this morphologically resembled ECM and hence possible biofilm presence in these patients. Structures resembling rod-shaped bacteria were seen in one of these four specimens and intra-operative cultures of sinus secretions yielded *P.aeruginosa*.

Later that year they described biofilm presence in all of the silicone frontal sinus stents removed from 6 patients 1 to 4 weeks after surgery, using SEM [162]. Five of these patients had sinus cultures positive for *Stapylooccus aureus* and one had *Pseudomonas aeruginosa* cultured. Further *in vitro* cultures of *Pseudomonas* on sialistic sheets, similar to those used in stents showed multiple structures with three-dimensional features, extra-cellular material and intercellular communications. The 3-dimensional structures on the in-vivo stents and in-vitro sheets appeared as pods of multiple smaller organisms. These findings were subsequently replicated in an animal model of sinusitis to demonstrate biofilms [163]. *Pseudomonas aeruginosa* biofilms were grown in the maxillary sinuses of 22 rabbits and the tissues were examined using SEM and light microscopy. Sheets and pods of multiple small organisms were detected with evidence of intercellular connections, extracellular ECM formation, water channels and destruction of surrounding cilia. The rabbit model was proposed to serve as a means to study various treatment options to eliminate biofilms.

Ramadan and colleagues examined ethmoid and maxillary mucosal biopsy specimens from 5 patients using SEM [164]. All showed evidence of biofilms with bacterial appearance similar to that of *Staphylococcal* species, along with denudation of ciliated epithelium and loss of goblet cells. Sanclament and colleagues took this pilot study a step further by searching for presence of biofilms in 30 patients with CRS undergoing ESS as compared to 4 control patients without CRS [165]. Using SEM morphologic criteria, and comparing them with 'hundreds of biofilm images', they concluded that 24 patients showed biofilm presence while none of the controls showed evidence of biofilm or inflammation. However their work also highlighted the limitations of SEM which was inadequate in identifying bacteria within the biofilm, and a further confirmation via transmission electron microscopy (TEM) was required.

Ferguson and colleagues reviewed 4 cases of CRS for the presence of biofilms using TEM [166]. In 2 of those 4 patients bacterial communities surrounded by a glycocalyx were seen above the mucosal surface and within layers of amorphous material overlying the mucosa. Cultured material from these 2 patients grew *P.aeruginosa*, and those agar colonies did not show any clumping of bacteria or glycocalyx haze surrounding them visible on TEM. This highlighted the fact that biofilm bacteria from tissues does not grow in the biofilm form on *in-vitro* agar cultures. Both patients symptoms were refractory to culture-directed antibiotics and it was thus concluded that biofilms were the possible cause of CRS refractory to antibiotics.

Ha and colleagues [167] have developed an animal model using sheep to study biofilms in rhinosinusitis. They standardised a technique for experimentally developing and identifying *Staphylococcus aureus* biofilms in the frontal sinuses of the sheep, which could be applied for further study of biofilms in the pathogenesis of CRS and novel treatment modalities. Psaltis and colleagues [168] applied the above standardised technique of

identifying bacterial biofilms via Confocal Scanning Laser Microscopy (CSLM) along with BacLight LIVE/DEAD kit for investigating biofilm presence in CRS patients. They prospectively analysed mucosal biopsy specimens from 38 CRS patients and 9 control patients undergoing ESS and detected biofilms in 17 (44%) of the 38 patients, but not in any of the controls. They thus put forward further evidence of existence of biofilms on the sino-nasal mucosa, and proposed that biofilms had a role to play in the pathogenesis of CRS. They also found that biofilms were detected in a higher number of patients who were undergoing revision endoscopic sinus procedures indicating that patients with biofilms possibly had a recalcitrant pattern of CRS disease.

Woodworth and colleagues [169] developed an *in-vitro* model of *Pseudomonas aeruginosa* biofilms on viable airway epithelial cell monolayer. Mouse nasal septal epithelium was cultured on air-liquid interfaces and incubated with biofilm forming strains of *Pseudomonas aeruginosa* under stationary horizontal and angulated positions, and in a tilting shaker. Confocal microscopy and scanning electron microscopy showed tower like biofilm formation in the inoculated wells supported by a viable airway epithelial cell surface monolayer. They have recommended the model as a reliable method for trialling novel treatment approaches for bacterial biofilm associated CRS. Dworniczek and colleagues [170] also demonstrated biofilms structures on 7 of 15 patients' mucosal specimens collected at time of sinus surgery and examining them using SEM and TEM.

1.3.2 DETECTION MODALITIES FOR MUCOSAL BIOFILMS

Mucosal biofilms have not only been identified on surgical biopsy specimens and in-dwelling devices, but have also been experimentally grown in-vivo in animal models, and in-vitro under controlled laboratory conditions. Since the microbial biofilm communities are not identifiable by the conventional microbiology culture-stain-microscopic methods, a

variety of specialized imaging techniques have been applied to visualize them including Scanning Electron Microscopy, Transmission Electron Microscopy, Confocal Scanning Laser Microscopy .

Ha and colleagues [167] analysed the three methods used to identify mucosal biofilms in the sheep model of biofilms in rhinosinusitis and concluded that CSLM was the most objective technique. CSLM permitted three-dimensional visualisation of biofilm structures on the sinonasal mucosa as compared to TEM which permits only a two-dimensional visualisation of the biofilms [165, 166]. They found that unlike SEM, CSLM it does not involve tissue preparing processes which dehydrate the ECM, distort the 3-dimensional configuration of the biofilms, reduce the biofilm size and possibly not detect very small biofilm communities. Tissue orientation was not a factor affecting imaging of the biofilms via CSLM, as unlike SEM it is not limited to imaging surface of the tissue and can identify biofilm structures which may be deeply embedded or growing between and within the tissues. False positive results from artefacts seen on SEM or TEM [165, 167] arising as a result of the dehydration, mucus and protein cross linking are also not seen in CSLM because the fluorescent stains used with CSLM are nucleic acid specific for cellular structures.

Confocal scanning laser microscopy can be used with nonspecific fluorescent stains like the Baclight stain [167, 168, 171] which bind to nucleic acids and thus stain both epithelial and microbial cells. However it does not provide for the identification of specific bacterial species forming the biofilms. For that purpose CSLM can also be used with specific Fluorescent in-situ hybridisation (FISH) probes for different bacterial species or fungal elements. Sanderson and colleagues [172] applied specific DNA probes for four common CRS bacterial pathogens - *Streptococcus pneumoniae, Staphylococcus aureus, Haemophilus influenzae,* and *Pseudomonas aeruginosa* to identify specific types of

bacteria forming biofilms on the sinus mucosa of 18 patients with CRS and 5 control patients using FISH (Fluorescent-In-Situ-Hybridisation) techniques. They identified biofilms in 14 of 18 CRS samples, with *H.influenzae* biofilm detected on all those samples, *S.pneumoniae* biofilm on 4 and *S.aureus* biofilms on 3 and *P.aeruginosa* biofilms on none of the samples. 2 of 5 controls also were positive for *H.influenzae* biofilms, and thus they suggested that biofilms may simply be colonizers and the exact role of biofilms in pathogenesis needed to be evaluated with larger sample sizes.

Foreman and colleagues [173] applied species specific probes for *Staphylococcus aureus, Haemophilus influenzae, Pseudomonas aeruginosa* and a universal fungal probe to sinonasal biopsy specimens obtained from 50 CRS patients and 10 control patients, and analysed them via CSLM. None of their control samples showed biofilms but 36 of the 50 CRS samples showed biofilms. More than half of the patients had polymicrobial biofilms with *S.aureus* biofilms being the most frequently detected in 25 of the 36 biofilm positive samples. These biofilms also were seen to coexist with the biofilm structures showing up with the universal fungal probe. Most of the techniques have one or the other limitation of sampling mucosa ex vivo, are expensive, and requiring skills limited to a few specialist centres.

With an aim to circumvent the specialised imaging techniques used to identify mucosal biofilms in research settings, Bendouah and colleagues presented an in-vitro technique to assay the biofilm forming capacity of bacteria isolated from CRS patients [174]. 31 clinical isolates of *P .aeruginosa, S .aureus*, and Coagulase negative *S .aureus* from CRS patients were cultured in 96-well plates and stained with crystal violet. Biofilm production was quantified by measuring optical density and comparing it with commercial positive and negative controls. They were able to show biofilm forming capacity in 22 of the 31 specimens, with biofilm forming capacity seen in all three of the bacterial species isolated

from the patients. They thus recommended the in-vitro assay as an inexpensive and easy method to assay biofilms formed by different species of bacteria in the clinical setting.

With an aim to determine the prevalence of biofilm forming bacteria in CRS patients, Prince and colleagues [175] also collected sinonasal culture from 157 patients and grew them under biofilm conditions. Approximately 30% of them formed biofilms of which 71% were polymicrobial cultures, *P .aeruginosa* and/or *S. aureus* cultures. They also found that a significantly more number of patients with prior ESS procedures showed biofilm formation. Oncel and colleagues [176] also cultured nasal swabs taken from areas of purulence at time of surgery and cultured them using conventional techniques. Samples which cultured bacteria were then evaluated for their ability to form slime by culturing them on congo red agar plates. 23 of their 30 samples grew *S .aureus* and *P .aeruginosa* on culturing, and 15 of those 23 showed biofilm formation. But these attempts to culture bacteria, in an environment distant from the original sampled milieu causes a change in the microbial phenotype, and thus the biofilm formation in-vitro cannot always be reflective of the biofilm formation in-vivo.

Non-planktonic bacteria which are not culturable can also be identified by applying molecular based techniques like polymerase chain reaction cloning, Denaturing gradient gel electrophoresis (DGGE), Temperature gradient gel electrophoresis (TGGE) [98]. Hochstim and colleagues [177] applied haematoxylin-eosin staining technique to samples from 24 CRS patients and 10 controls to identify biofilms. They identified biofilm structures in 15 of the 24 CRS patients and 1 control, seen as irregularly shaped groups of small basophilic bacteria with exopolymeric substance resting on mucosal epithelial surface. They recommended it as an easier and more cost-effective way of detecting biofilms in CRS patients as it gave results in concordance with FISH for biofilm detection. But unlike FISH it could not identify the type of bacterial species forming the biofilms.

Most of the above imaging modalities described above for studying biofilms provide a qualitative description of the biofilm presence on mucosal specimens. Attempts to quantify the imaged biofilms have used subjective or categorical measures which are not an optimal method for assaying biofilm structures. Hochstim and colleagues [177, 178] identified biofilms in patient samples using haematoxylin-eosin stains and subjectively graded the extent of biofilm as being 'extensive' if it covered more than 50% of the mucosal sample, and 'present' if it covered less than 50% of the sample surface. Le and Colleagues [179] assessed the percentage surface area covered by biofilm in sub-sections of tissue by visual inspection of individual CSLM image stacks which was insufficient and not an objective assay for quantifying the structural features of biofilms. In order to circumvent the specialised imaging techniques to assay biofilms, researchers have described *in-vitro* techniques to assay the biofilm forming capacity of bacteria isolated from CRS patients by culturing clinical isolates. The biofilm so produced *in-vitro* was quantified by either measuring the colony forming units (CFU) [180, 181] or optical density [174, 175, 182] and comparing it with commercial positive and negative controls. But as with all controlled experiments the biofilm formation *in-vitro* cannot be truly reflective of the biofilm formation *in-vivo*, which is significantly affected by the dynamic interactions with the host tissue. Thus an ideal technique for quantifying mucosal biofilms in CRS patients has been elusive to researchers so far.

1.3.3 IMPACT OF BACTERIAL BIOFILMS ON CRS

As the role biofilms play in the etiopathogenesis of recalcitrant CRS becomes better defined, the effect that these biofilms have on the treatments currently being used for managing CRS patients also becomes more and more critical to identify. With an aim to demonstrate the impact that biofilm-forming capacity of bacteria had on persistence of CRS following endoscopic sinus surgery, Bendouah and colleagues correlated in-vitro

biofilm forming capacity of 31 clinical isolates with a dichotomous outcome of favorable or unfavorable as assessed by questionnaire and endoscopy [174, 183]. They found that *Pseudomonas aeruginosa* and *Staphylococcus aureus* biofilms were associated with a poor evolution, but not coagulase-negative *staphylococcus*.

Psaltis and colleagues [184] retrospectively analysed symptom records and endoscopic evaluations of sinonasal mucosa of 40 patients who underwent ESS for CRS. Even the mildest of the symptoms reported by the patient at 8 months of follow-up was classified as 'ongoing symptoms' and ongoing post-operative mucosal inflammation (edema, pus, mucin or significant crusting) was classified as present or absent. 50% of the patients showed bacterial biofilms on confocal microscopy and these patients with biofilms had significantly more ongoing postoperative symptoms and mucosal inflammation. However, in the authors' own words this retrospective analysis had its limitations as it used non-validated symptom scoring methods, and only used nominal measures to report postoperative mucosal outcomes. Thus there had been a paucity of evidence of what influence the biofilms had on CRS patients and their management.

1.3.4 ROLE OF BACTERIAL BIOFILMS IN CRS

As described above ample evidence exists for the presence of biofilms in patients with CRS, but the precise mechanism by which the biofilms lead to the development and progression of a recalcitrant CRS has been more of a cause for hypotheses.

It is known that bioflm bacteria grow at a slow rate and are less susceptible to antibiotics and the host immune system. This helps it to form a nidus of infection in the host tissue which intermittently generates mobile planktonic bacteria. These planktonic bacteria are presumed to be reponsible for acute exacerbrations characteristically seen in patients with

the condition, and are more susceptible to antibiotics than their biofilm counterparts. Thus culture-directed antimicrobial therapies only eliminate the planktonic microbes and the source biofilm persists within the tissues. Though post-surgical sinus environment has been suggested as a possible stimulus for the biofilm form giving rise to the free-floating bacterium, it is still unclear as to how exactly this occurs and what maybe other possible stimuli for such a phenotypic change to occur.

Apart from the acute exacerbrations of the disease, it has been hypothesised that biofilms may be the common link in a web of causative factors leading to CRS as the endpoint, by potentially providing a continum of antigens like superantigens, exotoxins, cell surface markers and supporting fungal elements. In an attempt to characterize the inflammatory response elicited in biofilm mediated CRS, Hekiert and colleagues [185] collected sinonasal mucosal samples and peripheral blood from 60 CRS patients. 17 of those samples showed biofilms on SEM and the small subset of steroid-naïve biofilm positive patients among them had a local T_H1 inflammatory response with elevated levels of neutrophils and cytokines like INF-γ, granulocyte colony-stimulating factor in the sinonasal mucosa. This difference was not seen in the subset of patients on oral steroids. Recently Foreman and colleagues (in review/press) have found a coexistence of *S .aureus* biofilms and superantigens in CRS patients, and suggested the biofilms may be the source of superantigens thus providing a possible direct link between biofilms and CRS. They also demonstrated the existence of an eosinophilic, Th2 polarised immune response with increased levels of eosinophilic cationic protein and IL-5 in patients with S. *aureus* biofilms.

The above probable immune mechanisms still require further investigation to establish their cause and effect for the role biofilms may play in CRS. The biofilm mass is resistant to any type of host immune response mounted against it's presented antigens, possibly

protected by the surrounding EPS against phagocytosis, activated complement, antibody and immune cells. Instead there is collateral tissue damage caused through the cytotoxic, proteolytic and pro-inflammatory actions of the mileu of the activated complement-antibody-immune cells resulting in severe and sustained inflammation. Whatever be the type of immune response, the ineffective response against the biofilm thus possibly perpetuates disease in the surrounding host tissue.

1.4 FUNGAL BIOFILMS IN CRS

1.4.1 MYCOLOGY IN RHINOSINUSITIS

Ubiquitous fungal spores are continuously inhaled and deposited on the mucosal lining of the sino-nasal passages. Different studies have reported prevalence rates ranging from 0 to 100% [186-203] in the nose and paranasal sinuses of both CRS patients and controls, using different techniques for collection and identification of fungi. *Aspergillus, Penicillium, Cladosporium, Candida, Aureobasidium, and Alternaria* are the common species of fungi isolated from the sinonasal regions.

The spectrum of fungal rhinosinusitis (FRS) extends from benign colonisation of the nose and paranasal sinuses as fungal ball and Allergic FRS, to potentially life threatening invasive disease (Acute Invasive FRS, Chronic Invasive FRS, and Granulomatous invasive FRS) [204]. A fungal ball is a non-invasive disease in which a large mass of densely intertwined hyphae develop in the sino-nasal spaces without significant mucosal inflammation. In Allergic FRS, microscopic quantities of fungal hyphae in sinuses elicit an intense immune response, characterised by formation of allergic mucin containing numerous eosinophils and Charcot Leyden Crystals. Both these types of FRS are seen in immunocompetent individuals. Invasive fungal rhinosinusitis is seen in an immunocompromised individual, often as an acute fulminant disease that carries a high mortality rate. However, in patients whose immunologic deficiency is mild or unapparent, invasive FRS might run a more indolent chronic course. Management requires repeated surgical debridement, correction of any immunologic deficiency, and long-term systemic and topical antifungal therapy. Despite close medical attention, all invasive cases of fungal rhinosinusitis can progress to a fatal outcome or become a recurrent problem. Chronic invasive fungal rhinosinusitis has been divided into granulomatous and non-granulomatous subtypes on the basis of histopathology.

A recent review paper from a working group on fungal rhinosinusitis has supported the well established role of fungi in some of the above subtypes of FRS such as the invasive subtypes of FRS and fungal balls [205]. However the role of fungi in CRS per se or AFRS still remains unclear and very intensely debated in literature [206-211].

1.4.2 CONTROVERSIAL ROLE OF FUNGI IN CRS

AFRS was a term coined to describe a subtype of CRS associated with allergic eosinophilic mucus, and characterised by an IgE mediated inflammatory response to non-invasive fungi in the nose and sinuses [188, 204, 212]. Subsequent studies, applying novel sample collection and fungal detection methods have reported a prevalence of fungi in 90-100 % of CRS patients [187, 192, 196, 213], and it was suggested that fungi may play an important role in the pathogenesis of most of the CRS patients. However the role of fungi as significant pathogens in CRS has been questioned because different studies have shown similar fungal prevalence rates in CRS patients and healthy control groups [189, 190, 192-194, 196, 203, 213, 214], with similar types of species isolated [189, 190, 192, 196], and similar load of fungal allergens in the 2 groups[194].

Even research into the proposed IgE mediated allergy to fungi has shown similar sensitization rates in patients with or without fungi in the sinonasal regions [215], or between CRS patients and healthy controls [192, 216]. Some investigations into the type of cellular immune responses to fungi have further confounded the role of fungi in CRS. Shin and colleagues [216] demonstrated that CRS patients show exaggerated humoral and cellular responses with their PBMCs producing more IL-5 and IL-13 in response to common airborne fungi. These findings have supported the hypotheses that fungi on the sinus mucosal surface induce production of cytokines which in turn promote eosinophil migration, and ensuing EMCRS [217]. But Orlandi and colleagues were not able to

successfully replicate the result of the above study on a more heterogeneous sample and found only found minimal differences in cytokine levels [218]. Based on this theory that CRS is caused by an immune reaction to fungal presence in sinuses, antifungal treatments have been used in CRS patients with variable results [219-221], and some randomised placebo-controlled trials failing to show any improvement in subjective or objective outcome measures [195, 197, 222, 223].

Fungus may have a minor role in CRS as part of a more complex interplay among multiple factors. Alternatively, it may be the principal factor as some have claimed. Its relative importance and the exact pathophysiologic mechanism are crucial to understand, in order to determine whether it should be targeted in potential CRS treatments and, if so, how it should be addressed. The fungal analysis in all of the above studies has been based upon the conventional microbiology culture, microscopy and laboratory techniques that possibly identify only the ubiquitous non-biofilm forms of the fungi isolated from nasal swabs, secretions and lavages. To date evidence supporting the presence of fungal biofilms in CRS patients is lacking, as majority of the work has centred on bacterial biofilms.

1.4.3 FUNGAL BIOFILMS

Fungi are now being increasingly recognized as able to adopt a biofilm phenotype both on biotic and abiotic surfaces. Much of the work in fungal biofilm research has focused on *Candida* species [224-227], though other yeasts and filamentous fungi like *Cryptococcus, Blastoschizomyces, Malassezia, Trichosporon, Pneumocystis, Saccharomyces, Aspergillus, and Coccidioides* have also been implicated in fungal-biofilm mediated infections [228-238]. *Candida* biofilms have been isolated from numerous indwelling medical devices like cardiac pacemakers, prosthetic heart valves, CNS shunts, breast implants and joint replacements [239]. *Candida* biofilm has also been shown on cochlear implant implanted

in a child with CSOM who was given perioperative antibiotics[120]. *Cryptococcus neoformans* has been reported to form biofilms on ventricular shunts [240] , cardiac valves [241], peritoneal dialysis fistulae [242].

Numerous in-vitro models have been described which have shown that fungal biofilm formation occurs in stages similar to bacterial biofilm formation. The first step in biofilm formation is the attachment of spores to an inert or living surface, leading to colonization by hyphal proliferation. Extra-cellular matrix is then produced, followed by formation of pores and internal channels. Under the controlled conditions of the experimental models numerous factors like fungal strain / species, bacterial competition, substrate specificity have been found to influence fungal biofilm formation [239]. The models range from the earliest evidence of candida biofilms grown on catheter discs [243] or poly methyl-methacrylate strips [225] to the high throughput models like 96-well microtitre plate model [244], and flow models with the presence of flowing liquid over the biofilm surface [245, 246]. Dry weight measurements, tetrazolium salt (MTT) reduction assays [243], XTT reduction assays [225]are some of the complex assays applied to quantify fungal biofilms while analyzing antifungal susceptibility of the biofilms.

Villena & Gutierrez-Correa [247, 248] studied the morphology of filamentous fungi like *A.niger* biofilms grown adhering to a surface as compared to pellets grown in a suspension. They found that the biofilm form had orderly hyphal distribution forming a surface & inner channeled structure with evidence of ECM production. The pellet form showed no such organization and was composed of highly intertwined superficial hyphae & densely packed deep mycelium. Biofilm structures were also shown to have fivefold higher enzyme activities [249-251]. Beauvais and colleagues [252] demonstrated the hyphae on the surface of a colony of *A. fumigatus* were bound together by a hydrophobic extracellular matrix into a contiguous sheath, which was composed of significant antigens and

hydrophobins like galactomannan, α-1,3-glucans, monosaccharides, polyols, melanins, proteins. They also found that those *A.fumigatus* colonies were more resistant to polyene antifungals than a submerged shake mycelial growth.

Mowat and colleagues, standardized the first in-vitro model of *Aspergillus fumigatus* biofilms on microtitre plates [253]. Using LIVE/DEAD fluorescent stain and CSLM, they demonstrated *Aspergillus* to have formed coherent filamentous multicellular structures exhibiting acute angled dichotomous branching, with parallel-packed and cross linking of hyphae. They also found antifungal agents were at least 1000 times less effective at reducing the metabolic activity of cells in biofilms as compared to planktonic cells. While studying the biofilm formation they found that the conidia displayed a different antifungal susceptibility profile as compared to the complex hyphal structures formed. Further testing [254] with different antifungal agents showed that they are most effective during the early sporulation and hyphal proliferation stages of a developing *A .fumigatus* biofilm as compared to a fully developed mature multicellular-hyphal complex. Seidler and colleagues [255] applied some of Mowat's techniques and grew *Aspergillus fumigatus* biofilm on human bronchial epithelial cell lines. They demonstrated the formation of the ECM production by *A.fumigatus,* by assaying biofilm growth kinetics (dry weight measurements) and staining with safrannin. Using SEM and CSLM they identified filamentous multicellular structures exhibiting parallel-packed and cross-linking of hyphae, with EPS matrix in between. The biofilms adhered to the epithelial cells and showed lowered susceptibility to antifungal agents as compared to planktonic *A. fumigatus* strains.

1.4.4 EVIDENCE OF FUNGAL BIOFILMS IN CRS

The research implicating biofilms in the pathogenesis of CRS has mostly concentrated on bacterial biofilms, and not much evidence exists about fungal biofilms with respect to the

disease. Healy and colleagues [256] attempted to demonstrate the presence of fungal elements within bacterial biofilms on mucosal samples from sinuses of patients with CRS, EMCRS and AFRS. They applied FISH probes specific for *Haemophilus influenzae, Staphylococcus aureus, Pseudomonas aeruginosa, Streptococcus pneumophilia* and a general pan-fungal FISH probe to mucosal biopsies from 11 patients and 4 controls and analysed the samples using epifluorescent microscopy. They detected bacterial biofilms in 9 of the 11 patient samples and 2 of 3 control samples with *H. influenzae* biofilms being the commonest species forming biofilms. They stated that they demonstrated significant fungal elements in 7 out of 11samples, with more of them being present within AFRS and EMCRS samples as compared to CRS and controls. They did state that they however could not ascertain if they were viable fungal elements or if they were in a biofilm form.

Foreman and colleagues [173] used a pan-fungal FISH probe on tissue biopsies from CRS patients and demonstrated fungal biofilms like structures in 11/50 patients, and none from control samples. Interestingly they found a significant co-existence of fungal and bacterial biofilms in CRS patients. Out of the 11 patients in whom they identified robust fungal biofilms, 7 also demonstrated *S. aureus* biofilms, 2 had fungal–*H. influenza* biofilms present and the remaining 2 were solely fungal. They suggested that that the co-existence of fungal-bacterial biofilms could be an indication of possible symbiotic interactions known to augment biofilm survival by enhancing interspecies transfer of antimicrobial resistance traits, assisting surface adherence and improving the protection provided by the exo-polysaccharide matrix.

But once again, there has not been any direct evidence of the pathogenic filamentous fungi forming biofilms on the sinonasal mucosa of CRS patients.

1.5 AIMS UNDERPINNING STUDIES INCLUDED IN THESIS

At the time of the start of the work compiled in this thesis, evidence was present in peer-reviewed literature that biofilms existed on the mucosa of nose and sinuses obtained from CRS patients, and it had been hypothesised that they played a role in perpetuating the chronic, recurrent and recalcitrant course of the inflammatory disease.

- After extensively reviewing the available evidence, it was obvious that the research into the impact of biofilms on clinical outcomes of CRS patients was limited by the retrospective nature and methodology of the studies. To address these limitations and to find out more regarding the effect biofilms may have on the clinical outcomes and available treatments for CRS patients, a prospective, blinded study was conducted. The aim of the study was to analyse the post surgical outcomes of endoscopic sinus surgery, done in patients with chronic rhinosinusitis associated with biofilms, using internationally accepted and validated outcome measures.

- Different bacteria had been identified by different research groups as possible biofilm forming organisms on the sino-nasal mucosa, but again it was not known how the different bacterial species forming the biofilms affected the disease profile of the CRS patients. Thus a further evaluation of a cohort of CRS patients undergoing ESS was conducted in which the different organisms forming the biofilms were identified using species specific FISH probes and CSLM. With an aim of assessing the effect that different species forming biofilms had on the disease pattern and post-operative outcomes, their signs and symptoms of CRS were monitored using internationally accepted and validated outcome measures over a 12 month follow-up period.

- The research into mucosal biofilms in the nose and paranasal sinuses has so far applied only subjective parameters to quantify the biofilms assessed by different techniques.

Quantification of sino-nasal-mucosal biofilms can not only be of use in testing novel antibiofilm treatments, but may help in establishing a causal relationship of biofilms in the pathogenesis of CRS if a greater amount of biofilm is associated with a more severe disease. With an aim of finding an objective and reliable method of quantifying mucosal biofilms, we applied and standardised the COMSTAT computer program for quantifying biofilm structures seen on CSLM images of sinonasal mucosa.

• So far most of the published research in the field of biofilm in rhino-sinusitis has predominantly centered on bacterial biofilms. To characterize biofilms formed by filamentous fungi on sino-nasal mucosa and to build a tool that will further our understanding of the possible role that fungal biofilms may have in CRS, we aimed to develop an in-vitro model of *Aspergillus fumigatus* biofilm on Primary culture of Sino-Nasal Epithelial Cells.

CHAPTER 2

THE IMPACT OF BIOFILMS ON OUTCOMES AFTER ENDOSCOPIC SINUS SURGERY

- 32 -

Singhal, D., Psaltis, A.J., Foreman, A. & Wormald, P. (2010) The impact of biofilms on outcomes after endoscopic sinus surgery
American Journal of Rhinology & Allergy, v. 24 (3), pp. 169-174

- 53 -

CHAPTER 3

Staphylococcus aureus BIOFILMS: NEMESIS OF

ENDOSCOPIC SINUS SURGERY

- 54 -

NOTE:
This publication is included on page 54 in the print copy
of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

http://dx.doi.org/10.1002/lary.21805

Statement of Authorship

Staphylococcus Aureus Biofilms: Nemesis of Endoscopic Sinus Surgery

Deepti Singhal MS, Andrew Foreman BMBS, Josh-Jervis Bardy MBBS, Peter-John Wormald MD

From: The Department of Surgery- Otorhinolaryngology Head and Neck Surgery,
The Queen Elizabeth Hospital, and the University of Adelaide, Adelaide, South Australia
This study was supported by The Queen Elizabeth Hospital Research Foundation

Accepted for publication in Laryngoscope (2011) on 2ⁿᵈ March 2011

Singhal, D. (Candidate)

Project design, data collection, statistical data analysis, manuscript preparation

Foreman, A.

Sample processing and biofilm image analysis, manuscript editing

Bardy, J.J.

Data collection, manuscript editing

Wormald, P.J.

Project supervision, manuscript editing

By signing this document, I (the co-author) hereby acknowledge these to be accurate descriptions of the contribution I made to this paper and give permission for it to be included in the candidate's thesis

3.1 ABSTRACT

BACKGROUND:

Chronic Rhinosinusitis (CRS) patients with biofilms have persistent post-operative symptoms, ongoing mucosal inflammation & recurrent infections. Recent evidence, suggests that biofilms of differing species confer varying disease profiles in CRS patients.

AIMS:

To prospectively investigate effect of *Staphylococcus aureus, Pseudomonas aeruginosa, Haemophilus influenzae* and fungal biofilms on outcomes following ESS.

METHODS:

In this prospective blinded study, 39 patients undergoing ESS for CRS assessed their symptoms pre-operatively using internationally accepted standardised symptom scoring systems and QOL measures (10 point Visual Analogue Scale, Sino-Nasal-Outcome-Test-20, Global severity of CRS). Their sino-nasal mucosa was graded (Lund–Kennedy scale) & extent of radiological disease on CT scans scored (Lund-McKay scale). Random sino-nasal tissue samples were assessed for different bacterial species forming biofilms using Fluorescent In-Situ Hybridisation and Confocal Laser Microscopy. For 12 months after surgery, CRS symptoms, quality of life and objective evidence of persisting disease were assessed using the pre-operative tools.

RESULTS:

Different bacterial species combinations were found in 30/39 patients. 60% of these 30 biofilms were polymicrobial biofilms & 70% had S.aureus biofilms. Pre-operative nasendoscopy & radiological disease severity was significantly worse in patient with multiple biofilms (p=0.02 & p=0.01) and they had worse post-surgery mucosal outcomes on endoscopy (p=0.01) requiring significantly more number of post–operative visits (p=0.04). *S. aureus* biofilms progressed poorly with their symptom scores and quality of life outcomes, with significant differences in nasendoscopy scores (p=0.007).

CONCLUSION:

S .aureus biofilms play a dominant role in negatively affecting outcomes of ESS with persisting post-operative symptoms, ongoing mucosal inflammation & infections.

3.2 INTRODUCTION

Chronic Rhinosinusitis (CRS) is recurring, persistent inflammation of the sino-nasal tissues, and is known to cause significant physical symptoms, negatively affect quality of life and substantially impair daily functioning. While most patients do well after endoscopic sinus surgery (ESS), in some it continues to be a recalcitrant condition [263, 264]. Biofilms have been shown to negatively affect treatment outcomes in CRS patients [184, 265]. The presence of biofilms on the mucosa of CRS patients is associated with more severe disease preoperatively, persistent post-operative symptoms, ongoing mucosal inflammation & infections following ESS [265]. However the above studies used Baclight for diagnosis and this technique precludes species identification. Bendouah et al. detected the biofilm-forming capacity of bacteria and correlated that with dichotomous post-surgical outcome [174, 183] and found that *Pseudomonas aeruginosa* and *Staphylococcus aureus* biofilms were associated with a more unfavourable surgical outcome.

Fluorescence in situ hybridization (FISH) techniques have now been applied with species specific probes to identify some of the bacterial species forming biofilms in CRS patients[173]. A retrospective analysis of CRS patients in whom biofilm forming organisms were known demonstrated that different biofilm species display different disease characteristics[266]. *Haemophilus influenzae* biofilms were found in patients with mild disease, whereas *Staphylococcus aureus* biofilms were associated with a more severe surgically recalcitrant disease profile. But the results of the study were limited as it was a retrospective review of a relatively small number of patients and used non-validated symptom scoring methods and nominal reporting of post-operative endoscopic outcomes. Thus a prospective blinded study of CRS patients undergoing ESS was conducted using internationally accepted, standardised symptom, radiologic and endoscopic scoring systems to more conclusively report on the different disease and treatment outcomes seen with different biofilm species.

3.3 METHODS

STUDY DESIGN

A prospective, blinded study of patients undergoing Endoscopic Sinus Surgery (ESS) for Chronic Rhinosinusitis (CRS) in a tertiary rhinology clinic was conducted. Approval was obtained from the Ethics Committee and informed consent was obtained. . The 39 recruited patients fulfilled the criteria for CRS diagnosis as per the Rhinosinusitis task force definition for the disease [2] and were considered for ESS after a poor response to maximal medical therapy. Patients under 18 years of age and those with ciliary dysmotility or immunocompromised conditions as well as any patients taking steroids or antibiotics in the three weeks prior to their surgery were excluded from the study.

PRE-OPERATIVE DATA COLLECTION

Demographic and clinical data (age, sex, past medical and surgical history, allergies, and previous ESS procedures) were recorded. Before undergoing ESS patients completed questionnaires documenting the severity of their rhinosinusitis. They graded their individual symptoms (nasal congestion / obstruction, nasal discharge, alteration in sense of smell, headache, facial pain / pressure, sneezing), and their combined symptoms' score using the widely accepted and validated 10-point Visual Analogue Scoring system (VAS) [267]. They assessed the effect of sinusitis on their quality of life using the Sino-Nasal-Outcome-Test-20 (SNOT 20) symptom inventory and the 'Global Assessment of Rhinosinusitis Symptom severity' 7 point Visual Analogue Scale (GARS) [2]. The treating surgeon graded the patient's sino-nasal mucosa using the Kennedy-Lund scoring system [259]. All patients underwent pre-operative computerized tomography (CT) scanning, which was scored using Lund-Mackay scoring system [260].

PERI-OPERATIVE DATA COLLECTION

Presence/absence of pus, polyps and eosinophilic mucous at the time of the sinus procedure were documented, swabs were sent from the sinonasal areas of all patients for microscopy and culture for isolation of possible bacteria or fungi. To ensure that all patients received the same standard of surgical care, all the endoscopic sinus procedures were performed by the senior surgeon only. Sinonasal tissue biopsies were sent for histopathological evaluation and two random mucosal samples were taken from either the osteomeatal region or the sinuses of each patient at the time of their surgery and transported on ice to our laboratory in Dulbecco's Modified Eagle medium (Gibco, Invitrogen Corp., Grand Island, NY), where it was cryo-preserved for delayed processing.

TISSUE ANALYSIS

Biofilm characterization was performed by an independent investigator (AF) who was blinded to the patient questionnaire responses and the operative findings of the surgeon. The specimens were processed and analysed using a FISH protocol described previously [173], using species-specific probes for *S. aureus, H. influenzae*, and *P. aeruginosa*, as well as a universal fungal probe. The hybridized slides were analysed using a Leica TCS SP5 confocal scanning laser microscope (Leica Microsystems, Wetzlar, Germany), and biofilms identified as per the previously established biofilm definitions of FISH-CSLM protocol [173].

POST-OPERATIVE DATA COLLECTION

The patients were all followed-up after surgery by the operating surgeon, who was blinded to the biofilm status of the patients so that a standardised post-operative care was ensured for each patient. As per the standard post-operative care, the patients were assessed at 2 week, 6 week, 6 months, and 12 months. The patients also remained blinded to their

Chapter 3

Staphylococcus aureus Biofilms: Nemesis of Endoscopic Sinus Surgery
Laryngoscope, 2011 July; **121** *(7): p.1578-1583*

biofilm results throughout the follow-up period and on each visit, the patients graded their symptoms and quality of life on the same scales as they had done pre-operatively (i.e. VAS, SNOT 20, and GARS). Their sino-nasal mucosa was graded endoscopically at each visit by the same surgeon. Any deviation from the standardised post-operative surgical care was recorded.

STATISTICAL ANALYSIS

Statistical analysis was performed using Graph Pad Prism 5.0 software (San Diego, CA). All data was considered non-parametric, and hence median and interquartile ranges (IQR) are being reported in the results. All statistical tests were considered to be significant at a p value = 0.05. Differences were analysed using the Fisher's exact test for dichotomous data, the Mann-Whitney-U-Test for 2-way independent samples. For analysis of the data, the patients were divided first into Biofilm Positive or Biofilm Negative groups depending upon the presence / absence of any biofilm on FISH-Confocal analysis. Patients with biofilms were further grouped based on the number of species contained within their biofilm, into 'Unimicrobial biofilms' or 'Polymicrobial biofilms'. Patients with unimicrobial biofilms had evidence of only one bacterial species forming biofilms on FISH i.e. *S. aureus* or *P. aeruginosa* or *H. influenzae* or Fungal biofilms. Patients with polymicrobial biofilms had different combinations of *S. aureus, P. aeruginosa, H. influenzae* or Fungal biofilms. These polymicrobial were further divided into two groups – i). Staphylococcal-polymicrobial biofilms ii) Non-staphylococcal-polymicrobial biofilm. The staphylococcal biofilms had *S. aureus* biofilm detected with *P.aeruginosa* and/or *H. influenzae* and/or Fungal biofilms. Non-staphylococcal polymicrobial biofilms had only combinations of *P. aeruginosa* and/or *H. influenzae* and/or Fungal biofilms with no *S. aureus* biofilms detected in analysed tissue specimens.

Staphylococcus aureus Biofilms: Nemesis of Endoscopic Sinus Surgery

3.4 RESULTS

DEMOGRAPHIC FACTORS

Amongst the 39 patients who met the inclusion criteria and participated in the study, 19 were males (48.7%) and 20 were females (51.3%). The median age of the population under study was 51.5 years (IQR: 37.3 to 57.7 years). More than three-fourths of the study population had suffered with sinusitis for more than 6 years and 70% of them reported that they had symptoms 'all the time'. 21 patients had co-existing asthma, and 6 had a history of aspirin sensitivity. Approximately 2/3 of the study population was non-smokers and only 2 subjects gave a current history of smoking.

BIOFILM STATUS

With CSLM 30 of the 39 (76.9%) patients in our study showed evidence of biofilms on confocal microscopy. A total of 50 biofilms were identified by FISH probes in these 30 patients in one of the various combinations. 18 (60%) of the 30 patients with biofilms had polymicrobial biofilms and 15 of those 18 were polymicrobial biofilms with *S. aureus* and the remaining 3 were other combinations of microbes forming biofilms without *S. aureus.* In the patients with staphylococcal-polymicrobial biofilms 5 samples had *P. aeruginosa* biofilms with *S. aureus* biofilms, 4 had *S. aureus & H. influenzae* biofilms, 3 had *S.aureus* biofilms & fungal elements, 3 had *S.aureus* with both *P. aeruginosa* biofilms & fungal elements, 2 had *S. aureus, P. aeruginosa & H. influenzae* biofilms and 1 had *S .aureus & H. influenzae* biofilms with *Fungal* elements. Out of the 3 samples with nonstaphylococcal-polymicrobial biofilms 2 had *P. aeruginosa & H. influenzae* biofilms, and 1 had *H. influenzae* biofilm with co-existing fungal elements. Unimicrobial biofilms were seen in 12 (40%) of the 30 biofilm positive patients with *S. aureus* biofilms being the most common uni-microbial biofilm. 6 of the 12 unimicrobial biofilm patients had *S. aureus* biofilms, 4 patients showed *H. influenzae* biofilms, and 2 had fungal biofilms only.

PRE-OPERATIVE DATA ANALYSIS

Pre-operative Severity of CRS

The pre-operative subjective and objective measures of severity of sinusitis for the different patient groups in the study are compared in Table I. The preoperative subjective quality of life measures (GARS and SNOT-20) were comparably similar in the polymicrobial and unimicrobial biofilms, whereas the nasendoscopy and radiology evidence of disease was significantly worse in patient with multiple biofilms (p=0.02 and p=0.01 respectively), and these patient also had higher symptom scores when compared with those patients with single-species biofilms (p=0.053).

	GARS (1-7) Median, IQR	VAS symptoms (0 – 70) Median, IQR	SNOT 20 (0-60) Median, IQR	Nasendoscopy scores (0-22) Median, IQR	CT scores (0-24) Median, IQR
Biofilm Negative (n=9)	6.0 (5.7-6.5)	42.0 (31.5-50.0)	29 (22.0-38.5)	6.0 (5.0-7.0)	13 (9.5-15.0)
Poly-microbial Biofilms (n=18)	6.0 (5.0-7.0)	41 (33.0-51.7)	32.5 (20-41.2)	8.0 (6.7-12.0)	14.0 (12.5-20.2)
a) **With S.aureus (n=15)**	6.0 (5.0-6.0)	41 (33.0-50.0)	31 (23.0-41.0)	8.0 (2.0-12.0)	14 (13.0-20.0)
b) **Without S.aureus (n=3)**	7.0 (6.0-7.0)	41 (22.0-68.0)	34 (11.0-43.0)	8.0 (7.0-13.0)	13 (11.0-21.0)
Mono-microbial biofilms (n=12)	5.5 (5.0-6.0)	33 (22.7-42.0)	32.5 (24.7-38.7)	6.0 (3.2-7.7)	9.5 (8.0-14.7)
a) **S.aureus biofilm (n=6)**	5.75 (5.0-6.6)	37.0 (30.0-45.0)	35.5 (29.2-43.7)	6.5 (4.2-10.2)	12.5 (10.2-19.6))
b) **H.influenzae biofilm (n=4)**	5.0 (4.3-5.8)	23.5 (12.2-38.5)	26 (19.5-37.0)	5 (3.2-7.5)	6.0 (2.5-8.0)
c) **Fungal biofilm (n=2)**	5.0 (5.0-5.0)	37.5 (30.0-45.0)	32.5 (27.0-38.0)	4.0 (2.0-6.0)	11.5 (8.0-15.0)

Table 1: Pre-operative Objective and Subjective measures of CRS severity in the different species specific patient subgroups.

n: number of patients; IQR: Interquartile Range;
GARS: Global Assessment of Rhinosinusitis Severity; VAS: Visual Analog Scale;
SNOT: Sino-nasal Outcome Test; CT: Computerized Tomography

Previous surgery

25 of the 39 patients had undergone at least one previous ESS procedure. The average number of prior procedures was 2.2 (range 0-9) for patients with polymicrobial biofilms and 1.2 (range 0-6) for patients having unimicrobial biofilms. Patients who had S.aureus biofilm (alone or in combination) had undergone an average of 2 (range 0-9) prior procedures in comparison to 1.3 (0-5) in patients with no S.aureus biofilms. However the above differences in number of procedures weren't statistically significant.

PERI-OPERATIVE DATA ANALYSIS

Intra-operative swabs cultured 31 bacterial isolates from 28 of the 39 patients. Non-pathogenic respiratory flora was identified from 8 of those and the remaining 23 of the 31 cultures demonstrated pathogenic species. S. aureus was the most common isolate, being cultured in 11 samples. Other pathogens cultured included 5 cultures of *S. pneuomoniae*, 2 of *E. coli*, and one each of *P. mirabilis, P. aeruginosa, M. catarrhalis, Acinetobacter* & coagulase negative *Staphylococcus*. There was no correlation between the bacteria isolated via culture and the species specific biofilm identified via FISH, in keeping with the biofilm hypothesis of biofilm bacteria not being culturable via the conventional techniques. Eosinophilic mucin was identified on histology in 15 of the 39 patients and nasal polyps in 23 of the 39 patients with no specific correlation with any of the specific biofilm-forming species.

Chapter 3

Staphylococcus aureus Biofilms: Nemesis of Endoscopic Sinus Surgery
*Laryngoscope, 2011 July; **121** (7): p.1578-1583*

POST-OPERATIVE RESULTS

Follow-up Visits

The patients were reviewed at 2 weeks, 6 weeks, 6 months, and 12 months as a part of their post-surgical care. But based on patient needs/symptoms and/or the surgeon's assessment, 25 of the 39 patients required extra visits apart from those specified above. The median number of total visits and extra visits required by each of the patient subgroup is described in table II. The patients with multiple bacterial biofilms required significantly more number of extra visits to the rhinology clinic when compared with the patients with single species biofilms (p=0.04). As seen from the data in table 2, patients with *S. aureus* biofilms by itself or in combination with other biofilms had come for more follow-up visits when compared with other subgroup patients within their group.

	Median no. of follow-up visits in first year after ESS	Average no. of extra visits required
Biofilm Negative (n=9)	4.5	1
Poly-microbial Biofilms (n=18)	6.0	2.1
i.　With S.aureus　　　(n=15)	6.5	2.1
ii.　Without S.aureus　　(n=3)	6.0	1.7
Mono-microbial Biofilms (n=12)	4.5	0.9
i.　S.aureus biofilm　　(n=6)	5.0	1.5
ii.　H.influenzae biofilm (n=4)	4.0	1.0
iii.　Fungal biofilm　　　(n=2)	4.0	0

Table II: Total and extra follow-up visits for the different species specific groups.

Symptom Outcomes

The VAS symptom scores did not show any dependence on the number of different bacterial biofilms found in the patients' mucosa. In the unimicrobial biofilm patients, the median total sinus symptom score of 34 pre-operatively showed some initial improvement to 24.5 (IQR 16-36) at 2 wks, 18 (IQR 8 to 35) at 6 wks. But after that it stabilized at that value at 6 months, and showed marginal improvement to 13 (8 to 18) at 12 months. The Polymicrobial group had median total sinus symptom scores of 26.5 (IQR 18.2-30.2) at 2 wks, improving to 11 (7 to 16.5) at 6 wks, again stabilising at 12 (5 to 22.75)) at 6 months and 13 (5 to 22.5) at 12 months. However, the symptom scores of the further biofilm subgroups progressed differently over the follow-up period as shown in Figure 1. The patients with *S. aureus* biofilms (either by itself or in combination) progressed poorly with their symptom scores and the VAS symptom scores were significantly different between unimicrobial *S. aureus* and *H. influenzae* biofilm (p=0.01 on ANOVA test).
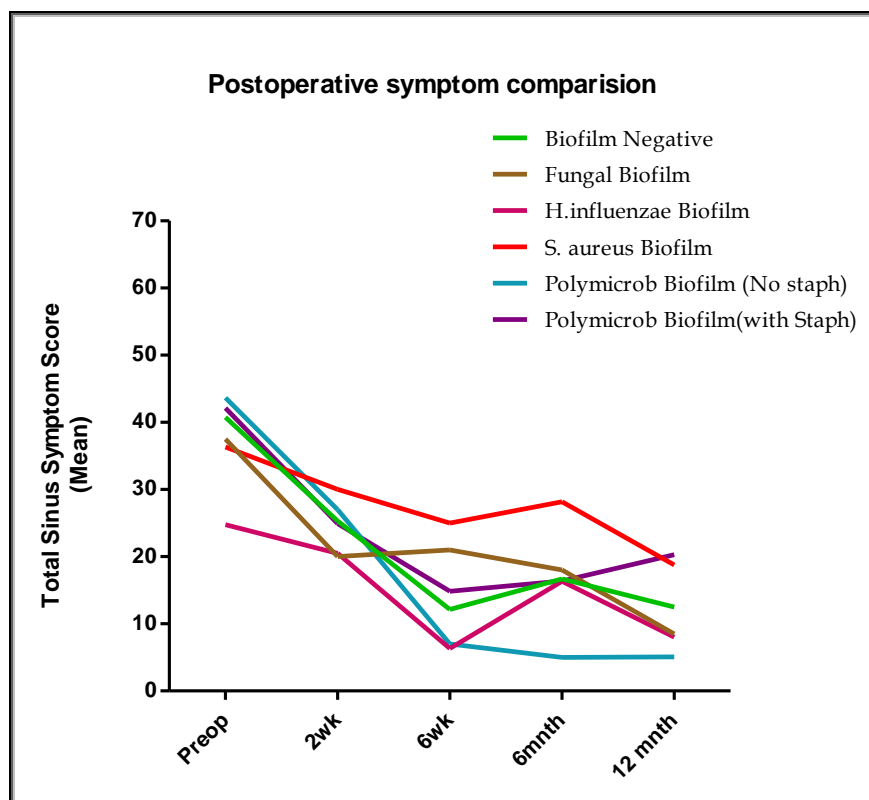
Figure 1: Comparison of Post-operative symptoms (10-point visual analog scale)

Nasendoscopy Outcomes

The Polymicrobial biofilm patients had median (with IQR) Kennedy Lund scores of 6 (3.7 to 9.2) at 2 wks, 4.5 (2 to 11.5) at 6 wks, 3 (1 to 8) at 6 months, 5.5 (2 to 8.2) at 12 months. At the same follow up period unimicrobial biofilm patients had median nasendoscopy scores of 4 (2 to 8.7), 1 (0 to 7.5), 3 (0 to 8), 2 (0 to 5). And these different scores were statistically significant between the 2 groups (p=0.01).The difference in nasendoscopy scores between the different species biofilm subgroups as shown in Figure 2 was also statistically very significant (p=0.001 on ANOVA test)
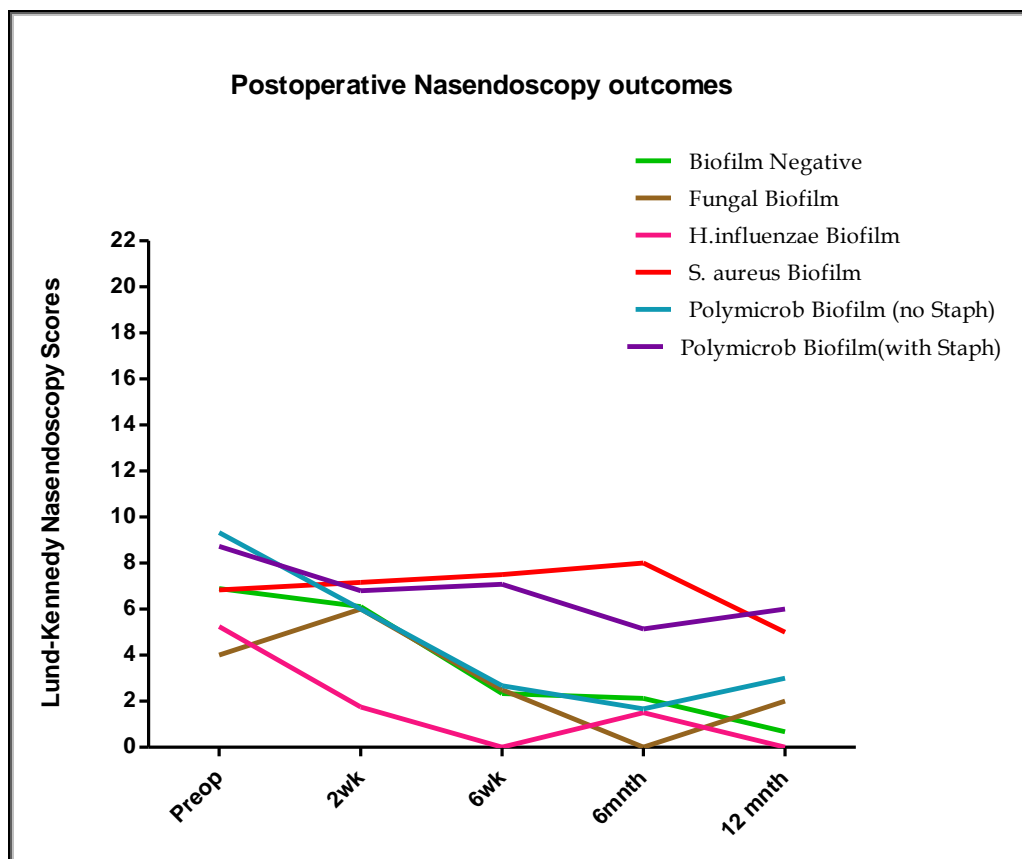
Figure 2: Comparison of Post-operative Nasendoscopy scores
(Lund-Kennedy Scale)

Quality of Life (QOL) Outcomes

The median Global assessment of severity of CRS score when compared between the different subgroups (Figure 3) was significantly worse for the *S. aureus* biofilms (p=0.007) with the scores slowly creeping towards the pre-operative baseline in the follow-up period.
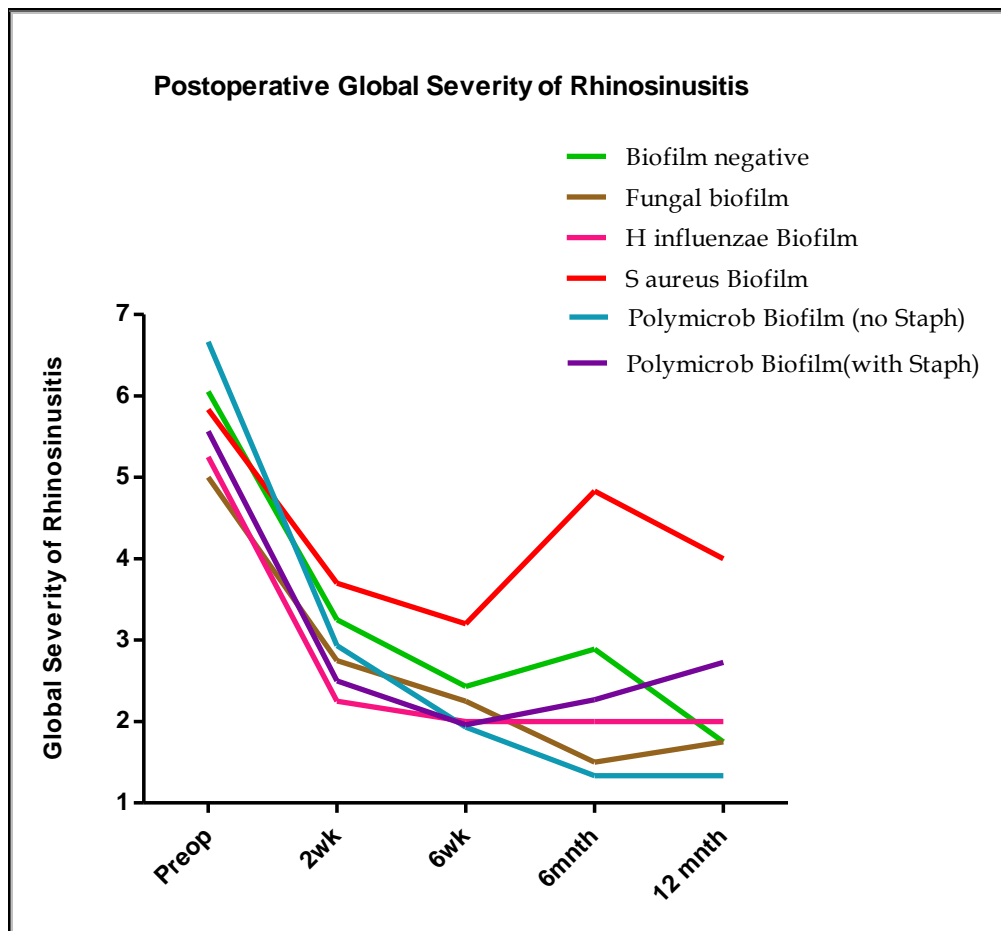
Figure 3: Global Assessment of Rhinosinusitis Severity (GARS) Comparison

The median total SNOT 20 scores for the different subgroups are described in table III are not showing statistical significance, but do show that *S. aureus* biofilms (alone or combination) have worse SNOT-20 scores when compared with other subgroup patients within their group.

Median SNOT-20 scores	Pre Surgery	2 wk	6 wk	6 mnth	12 mnth
Biofilm Negative	29.0	17.0	10.0	12.5	8.5
Poly-microbial Biofilms	32.5	21.0	11.0	12.0	9.0
i) With S.aureus	31.0	21.0	12.0	13.0	14.5
ii) Without S.aureus	34.0	14.0	9.0	7.0	1.0
Mono-microbial Biofilms	32.5	12.0	11.0	13.5	4.5
i) S.aureus biofilm	35.5	17.0	11.0	25.0	9.0
ii)H.influenzae biofilm	26.0	11.5	8.5	11.0	3.0
iii) Fungal biofilm	32.5	7.5	15.0	16.0	6.0

Table III: SNOT-20 Scores for the different species specific subgroups

3.5 DISCUSSION

This study is a prospective analysis of the impact of different biofilm species on the outcomes of endoscopic sinus surgery done for medically recalcitrant CRS and it has shown that *S. aureus* biofilms have a negative impact on the post-surgical outcomes, both as a single species biofilm or in combination with other species biofilms.

Rhinologists are increasingly being faced with a subgroup of CRS patients whose disease process fails to improve despite maximal medical and surgical management. Factors like nasal polyposis, aspirin sensitivity, smoking, acid regurgitation have been reported to affect post-Endoscopic Sinus Surgery (ESS) outcomes [268-271]. This study confirms the deleterious role of *S. aureus* in influencing the outcome after ESS [173, 183, 184, 265]. *S. aureus, H. influenzae, P. aeruginosa*, fungal biofilms have been characterized as the commonly occurring species specific biofilms in different study populations [172-174, 183, 256]. In our study we used FISH probes specific for the species described above along with validated outcome measures, to identify which species specific biofilms are associated with poor post-treatment outcomes. However, as there is a limit to the number of species specific FISH probes that can be used for biofilm detection, we did not able identify some of the other clinically relevant species like *S. pneumoniae* and *M. catarrhalis* and define their disease outcomes.

CRS is typically a polymicrobial disease on the basis of standard culture techniques. Additionally, biofilms in CRS are polymicrobial and it appears that the number of species within a biofilm is also a significant predictor of disease progression in our study. Multiple microbes forming biofilms on the sinonasal mucosa was associated with more severe disease pre-operatively as seen by higher symptom VAS, nasendoscopy and radiology scores as compared with patients with single species biofilms. Post-operatively these patients had worse post-surgery mucosal outcomes on endoscopy requiring significantly more number of post–operative visits to the rhinology clinic.

Chapter 3

Staphylococcus aureus Biofilms: Nemesis of Endoscopic Sinus Surgery
*Laryngoscope, 2011 July; **121** (7): p.1578-1583*

S. aureus biofilms (either by itself or in combination with other species) were identified in 21 (70%) of the 30 biofilm positive patients and this group progressed poorly with their subjective and objective post-ESS outcomes. *S. aureus* has been identified in different study populations as the most common bacteria isolated from patients with surgically recalcitrant disease [272-276], and also the most common biofilm forming organism found on intraoperative mucosal specimens [173]. Our prospective analysis of patients displaying *S. aureus* biofilms on their sinonasal mucosa has provided direct evidence that these patients have worse VAS symptom scores, worse nasendoscopy scores and worse quality of life outcomes after endoscopic sinus surgery in comparison to the patients with other microbial biofilms. These patients also required more follow-up visits when compared to the remaining biofilm patients indicating that repeated infections and persistent disease prevail in this subgroup. The patients also had a tendency to have had more prior sinus surgeries reflecting the more severe and recalcitrant nature of the disease when associated with *S. aureus* biofilm. This negative impact on post-treatment outcomes is more pronounced when the *S. aureus* biofilms occur as single species biofilms and is still seen in a somewhat attenuated manner when they occur along with other microbial biofilms.

Further work done in our department (in press) has found a coexistence of *S.aureus* biofilms and superantigens in CRS patients, and suggested the biofilms may be the source of superantigens. An eosinophilic, Th2 polarised immune response with increased levels of eosinophilic cationic protein and IL-5 in patients with S.*aureus* biofilms has also been observed possibly providing a link in the pathogenesis of CRS. The biofilm phenotype exhibits resistance to this immmune response, possibly protected by the surrounding EPS against phagocytosis, antibody and immune cells, which instead lead to collateral tissue damage caused through their cytotoxic, proteolytic and pro-inflammatory actions, and

result in severe and sustained inflammation. Further investigation is required regarding the precise immune pathway so that possible treatments targeting it can be devised to control the resulting inflammation.

The role of *H. influenzae* in CRS has recently been reinvigorated, with studies identifying *H. influenzae* biofilms commonly on the sinonasal mucosal specimens of CRS patients [172, 173, 256]. The disease profile of patients with *H. influenzae* biofilms has been described as mild with rapid resolution of signs and symptoms following surgery[266]. In our study 12 patients had *H. influenzae* biofilms, of which 4 were unimicrobial biofilms and 8 were found in various combinations with other microbial biofilms. Patients with unimicrobial *H. influenzae* biofilms had the least severity of disease pre-operatively with the lowest symptom and nasendoscopy scores. Over the first 6-8 weeks they had a rapid resolution of symptoms and mucosal signs on endoscopy, with continued marked improvement of quality of life outcome measures similar to patients without biofilms. This significant improvement was not evident when *H. influenzae* and *S. aureus* biofilms were found together; indicating that it's pathogenic effect may have been either weak from the start, or overpowered by the refractory and recalcitrant pathogenic mechanisms surrounding *S. aureus* biofilm. When *H. influenzae* biofilms combined with *P. aeruginosa* or fungal biofilms, as a polymicrobial biofilm without *S. aureus* the disease pattern was once again milder and reflective of the possibly mild combined pathogenic effects of these bacterial biofilms. These two pieces of information further support the theory that *S. aureus* plays a dominant role in CRS biofilms.

No unimicrobial *P. aeruginosa* biofilms were seen in our study population, and hence the disease profile associated with them could not be defined. Of the 10 *P. aeruginosa* polymicrobial biofilms 8 were along with *S. aureus* and thus disease outcomes for *P. aeruginosa* are difficult to differentiate from the staphylococcal biofilm. There were only

2 patients with single species fungal biofilms and the disease pattern in this very small population was mild, though the recovery of signs and symptoms was more protracted. The remaining 4 fungal biofilms co-existed with *S. aureus* biofilms and the patterns were again reflective of a staphylococcal biofilm. Thus in this small study population, *S. aureus* biofilms are spread through the biofilm subgroups, causing a loss of possible differences in the outcome measures and also indicating that *S. aureus* biofilms play a dominant role in determining disease severity and guiding postoperative course.

The study thus shows that different bacterial biofilms are associated with different disease progression after ESS. Thus a clinically relevant species directed biofilm analysis will help in determining the possible at risk group of patients and guide a more directional research towards the elimination of those biofilms. This specific knowledge may help in developing novel and species specific anti-biofilm treatments which may enable us to aggressively treat these patients and help in improving the post-surgical outcomes of ESS.

3.6 CONCLUSION

S. aureus biofilms play a dominant role in negatively affecting the outcomes of ESS with persistence of post-operative symptoms, ongoing mucosal inflammation & infections. Patients with unimicrobial *H. influenzae* biofilms have a milder disease pattern with a rapid resolution of symptoms, and mucosal signs following ESS. Future studies evaluating therapeutic intervention specifically targeting S.aureus biofilms are suggested.

3.7 REFERENCES

1. Senior BA, K.D., Tanabodee J, Kroger H, Hassab and L.D. M, *Long-term results of functional endoscopic sinus surgery.* Laryngoscope, 1998. **108**: p. 151-157.

2. Khalil, H., Nunez, D. A., *Functional endoscopic sinus surgery for chronic rhinosinusitis.* Cochrane database of Systematic Reviews, 2009(4).

3. Psaltis, A.J., Weitzel, E. K., Ha, K. R., Wormald, P. J., *The effect of bacterial biofilms on post-sinus surgical outcomes.* American Journal of Rhinology, 2008. **22**(1): p. 1-6.

4. Singhal, D., Psaltis, A.J., Foreman, A., Wormald, P.J., *The impact of biofilms on outcomes after endoscopic sinus surgery.* Am J Rhinol Allergy, 2010 May. **24.**(3): p. 169-74.

5. Bendouah, Z., Barbeau, J., Hamad, W. A., Desrosiers, M., *Use of an in vitro assay for determination of biofilm-forming capacity of bacteria in chronic rhinosinusitis.* American Journal of Rhinology, 2006. **20**(5): p. 434-438.

6. Bendouah, Z., Barbeau, J., Hamad, W. A., Desrosiers, M., *Biofilm formation by Staphylococcus aureus and Pseudomonas aeruginosa is associated with an unfavorable evolution after surgery for chronic sinusitis and nasal polyposis.* Otolaryngology - Head and Neck Surgery, 2006. **134**(6): p. 991-996.

7. Foreman, A., Psaltis, A. J., Tan, L. W., Wormald, P. J., *Characterization of bacterial and fungal biofilms in chronic rhinosinusitis.* American Journal of Rhinology and Allergy, 2009. **23**(6): p. 556-561.

8. Foreman, A., Wormald, P. J., *Different biofilms, different disease? A clinical outcomes study.* Laryngoscope. **120**(8): p. 1701-1706.

9. Meltzer, E.O., Hamilos, D. L., Hadley, J. A., and et al., *Rhinosinusitis: Establishing definitions for clinical research and patient care.* Otolaryngol Head Neck Surg,, 2004. **131**(**6 Suppl**): p. S1-62.

10. Kennedy, D.W., Draf, W., Friedman, W. H., et al., *Quantification for staging sinusitis.* Annals of Otology, Rhinology and Laryngology, 1995. **104**(10 I): p. 17-21.

11. Lund, V.J., Kennedy, D.W., , *Staging for rhinosinusitis.* Otolaryngol Head Neck Surg, , 1997. **117**: p. s35-s40.

12. Lund, V.J., Mackay, I.,, *Staging in rhinosinusitis.* Rhinology, 1993. **31**: p. 183-184.

13. Briggs, R.D., Wright, S.T., Cordes, S., Calhoun, K.H., *Smoking in chronic rhinosinusitis: a predictor of poor long-term outcome after endoscopic sinus surgery.* Laryngoscope, 2004. **114**: p. 126-128.

14. Chambers, D.W., Davis, W.E., Cook, , N. P.R., G.J., , and D.T. Rudman, *Long-term outcome analysis of functional endoscopic sinus surgery: correlation of symptoms with endoscopic examination findings and potential prognostic variables.* Laryngoscope, 1997. **107**: p. 504-510.

15. Smith, T.L., Mendolia-Loffredo, S., Loehrl, T. A., et al., *Predictive factors and outcomes in endoscopic sinus surgery for chronic rhinosinusitis.* Laryngoscope, 2005. **115**(12): p. 2199-2205.

16. Deal, R.T., Kountakis, S.E.,, *Significance of nasal polyps in chronic rhinosinusitis: symptoms and surgical outcomes.* Laryngoscope 2004. **114**: p. 1932-1935.

17. Healy, D.Y., Leid, J. G., Sanderson, A. R., Hunsaker, D. H., *Biofilms with fungi in chronic rhinosinusitis.* Otolaryngology - Head and Neck Surgery, 2008. **138**(5): p. 641-647.

18. Sanderson, A.R., Leid, J. G., Hunsaker, D., *Bacterial biofilms on the sinus mucosa of human subjects with chronic rhinosinusitis.* Laryngoscope, 2006. **116**(7): p. 1121-1126.

19. Bhattacharyya, N., *The microbiology and pathophysiology of infection in chronic rhinosinusitis.* Otorinolaringologia, 2003. **53**(3): p. 79-87.

20. Lin, A., Busaba, N. Y., *Staphylococcus aureus and endoscopic sinus surgery.* Current Opinion in Otolaryngology and Head and Neck Surgery, 2006. **14**(1): p. 19-22.

21. Bhattacharyya, N., Gopal, H. V., Lee, K. H., *Bacterial Infection after Endoscopic Sinus Surgery: A Controlled Prospective Study.* Laryngoscope, 2004. **114**(4): p. 765-767.

22. Jervis-Bardy, J., Foreman, A., Field, J., Wormald, P. J., *Impaired mucosal healing and infection associated with Staphylococcus aureus after endoscopic sinus surgery.* American Journal of Rhinology and Allergy, 2009. **23**(5): p. 549-552.

23. Ramadan, H.H., *What is the bacteriology of chronic sinusitis in adults?.* Am J Otolaryngol, 1995. **16**: p. 303-306.

CHAPTER 4

Aspergillus fumigatus BIOFILM

ON

PRIMARY HUMAN SINONASAL EPITHELIAL

CULTURE

NOTE:

This publication is included on pages 76-99 in the print copy
of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

http://dx.doi.org/10.2500/ajra.2011.25.3622

CHAPTER 5

QUANTITATIVE ANALYSIS OF IN-VIVO MUCOSAL

BIOFILMS

NOTE:
This publication is included on page 101 in the print copy
of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

http://dx.doi.org/10.1002/alr.20082

<u>Statement of Authorship</u>

Quantitative analysis of mucosal bacterial biofilms

Deepti Singhal MS, Sam Boase BMBS, John Field PhD,
Camille Jardeleza, M.D., Andrew Foreman BMBS , Peter-John Wormald, MD

From: The Department of Surgery- Otorhinolaryngology Head and Neck Surgery,
The Queen Elizabeth Hospital, and the University of Adelaide, Adelaide, South Australia

Submitted for review to publish in American Journal of Allergy & Rhinology

Singhal, D. (candidate)
Project design, Sheep surgical work, Tissue processing and image analysis, statistics and manuscript preparation

Boase, S.
Sheep surgical work, manuscript editing

Field, J.,
Data analysis

Jardeleza, C.
Biofilm image analysis, manuscript editing

Foreman, A.
Biofilm image analysis, manuscript editing

Wormald, P.J.
Project supervision, manuscript editing

By signing this document, I (the co-author) hereby acknowledge these to be accurate descriptions of the contribution I made to this paper and give permission for it to be included in the candidate's thesis

5.1 ABSTRACT

BACKGROUND:

Quantitative assays of mucosal biofilms on ex-situ samples are challenging with the currently applied specialised microscopic techniques to identify them. COMSTAT2 computer program has been applied to in-vitro biofilm models for quantifying biofilm structures seen on confocal scanning laser microscopy (CSLM).

AIM:

Quantify *S.aureus* biofilms seen via CSLM on ex-situ samples of sinonasal mucosa, using COMSTAT2 program.

METHODS:

S.aureus biofilms were grown in frontal sinuses of 4 merino sheep as per previously standardised sheep sinusitis model for biofilms. 2 sinonasal mucosal samples, 10mm x 10mm size from each of the 2 sinuses of the 4 sheep were analysed for biofilm presence with Baclight stain & CSLM. 2 random image-stacks of mucosa with *S.aureus* biofilm were recorded from each sample, and analysed using COMSTAT2 software which translates image stacks into simplified 3-dimensional matrix of biofilm mass by eliminating surrounding host tissue. 3 independent observers analysed images using COMSTAT2 and 3 repeated rounds of analyses were done to calculate biofilm biomass.

RESULTS:

COMSTAT2 application uses observer dependent threshold setting to translate CSLM biofilm images into a simplified 3-dimensional output for quantitative analysis. Intraclass correlation coefficient (ICC) between thresholds set by the 3 observers for each image stacks was 0.59 (P=0.0003). Threshold values set at different points of time by single observer also showed significant correlation as seen by ICC of 0.80 (P<0.001)

CONCLUSION:

COMSTAT2 can be applied to quantify and study the complex 3-dimensional biofilm structures that are recorded via CSLM on mucosal tissue like the sinonasal mucosa.

5.2 INTRODUCTION

The Centres for Disease control and Prevention estimate that at least 65% of all human bacterial infectious processes involve biofilms [73], and over the past 7 years mounting evidence has been presented to implicate biofilms in propogating the recalcitrant and chronic nature of chronic rhinosinusitis (CRS) [62, 98, 153, 154]. Biofilms are organised communities of bacteria, attached to a biotic or abiotic surface, embedded in a mosaic of self-produced extracellular polymeric substance. Within this micro-ecosystem, synchronised existence is seen between the various bacterial communities exhibiting specialised phenotypes, such as differing expression of surface molecules, nutrient utilization, antibiotic resistance and division potential which enables the bacteria to survive in hostile conditions [49]. However, bacteria within the biofilm are difficult to culture and thus, not easy to assay using the currently available microbiology and clinical tests.

A variety of specialised microscopic techniques including Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM), Confocal Scanning Laser Microscopy (CSLM) have been applied to show the presence of *in-vivo* bacterial biofilms on sino-nasal mucosa in animal models and humans. The literature reporting biofilms *in-vivo* has mostly been descriptive, and lacking in quantitative detail. Quantification of biofilms can be useful in studying the temporal nature of biofilm development, as well as assessing the influence of varying environmental conditions on biofilm phenotype, and analyzing the effects of novel treatments for biofilm related infections. In this study we describe the application of the COMSTAT2 computer program [278] for quantifying biofilm structures seen on CSLM images of sinonasal mucosa, in a sheep model of rhinosinusitis.

5.3 METHODS

At The Queen Elizabeth Hospital surgical workshop facility in Adelaide, South Australia, with the approval of Animal Ethics Committees of the University of Adelaide and the Institute for Medical and Veterinary Science, four adult male merino sheep were included in the study to form bacterial biofilms in the frontal sinuses as per the previously established sheep biofilm sinusitis models [167].

BACTERIAL INOCULUM

A pure strain of *Staphylococcus aureus* clinical isolate with proven *in-vitro* biofilm forming capacity was procured from the department of Microbiology at The Queen Elizabeth Hospital in Adelaide. The frozen glycerol stocks were first subcultured overnight at 37°C in 3ml CSF broth (Oxoid) on a shaker and again on Columbia Horse Blood Agar (Oxoid) for 12-18 hrs at 37°C. To prepare the inoculum, single colony units from the plate cultures were added to 0.45% sterile saline and adjusted to a 0.5 McFarland units, and transported on ice for instillation into sheep sinuses.

ENDOSCOPIC SINUS SURGERY

The first part of the procedure for biofilm formation in the frontal sinuses is endonasal endoscopic access to the frontal sinus under a general anaesthetic. Intra-venous induction with sodium thiopentone via the internal jugular vein, followed by endotracheal intubation and maintenance anaesthesia with 1.5-2% isoflurane inhalation was given. The sheep were placed supine on the operating table and the nasal mucosa topically decongested with co-phenylcaine forte nasal spray (ENT technologies, Victoria, Australia). An endoscopic middle turbinectomy with removal of the anterior ethmoid complex was performed to reveal the frontal sinus ostium in each nasal cavity of all the sheep.

The sinonasal mucosa was give time to heal over a 3-4 wk period and the sheep then underwent a second general anaesthetic for the second part of the procedure which commenced with frontal sinus trephination, and identification of the frontal ostium intra-nasally with the help of 1% fluorescein flush through the frontal trephine. Vaseline-coated gauze was used to occlude the frontal recess endoscopically to ensure a water tight seal of the frontal sinus. 1 ml of the prepared dilution of *S. aureus* inoculum was instilled into both the frontal sinuses via the mini-trephines, which were then capped and left *in-situ.* The sheep were then held in approved pens for 7 days to allow for bacterial biofilms to form. After the 7-day incubation period, for optimum biofilm formation to have occurred, the occluding packs were removed from the frontal recesses of the sheep to allow ventilation of the sinuses again.

TISSUE COLLECTION

The sheep were euthanized 24 hrs later and the mucosa lining the inoculated frontal sinus was exposed by removing the forehead skin and anterior table of the sinus. The sinus mucosa was carefully dissected off the bony walls taking care to maintain its structural integrity as far as possible. It was transported in Dulbecco's Modified Eagle's Medium (with no antibiotic additive) (Gibco, Invitrogen, Grand Island, NY) to the laboratory for further analysis. Two random samples, 10mm x 10mm in size were cut off from the sinus mucosa of both sinuses of each of the 4 sheep; thus a total of 16 specimen samples were obtained for staining and imaging from the 4 sheep.

Chapter 5
Quantitative analysis of in-vivo mucosal biofilms
Int Forum Allergy Rhinol, 2011; 00:xx-xx

SAMPLE PREPARATION

The specimens were processed and analysed within 2 hours of collection using the Live-Dead Baclight stain-CSLM protocol described elsewhere [168]. Briefly, each tissue sample was washed thoroughly in three separate beakers of sterile MQ water to remove any planktonic bacteria. The sample was then immersed in 1 mL of sterile MQ water, to which 1.5uL aliquots of component A (syto9) and component B (Propidium Iodide) of BacLight LIVE/DEAD kit (Invitrogen, Molecular Probes) are added. With this combination of stains, cells & bacteria with intact cell membranes stain fluorescent green, whereas those with damaged membranes stain fluorescent red. After incubation in darkness at room temperature for 15 minutes, sample was rinsed in sterile MQ water to remove excess stain.

IMAGE ACQUISITION

The specimens were mounted on cover slips for analysis with a Leica TCS SP5 confocal scanning laser microscope (Leica Microsystems, Wetzlar, Germany). The entire area of each specimen was scanned for evidence of biofilm structures using a 488nm Argon laser and water immersion lens at 20x magnification. Biofilms were identified as clusters & towers of immobile, irreversibly attached, intensely fluorescing, live green cocci shaped bacteria, approximately 0.5-2 μm diameter. Two different sets of image stacks were recorded from the samples - i) 2 random z-stacks, with a total thickness of each z-stack at 85 μm, at a distance of 0.5μm per slice, were recorded from each of the 16 samples, making a total of 32 image-stacks imaged for biofilm evidence. ii) In 4 of the 16 samples, an additional 10 image stacks were recorded to map out representative areas of the sampled tissue, and compared with the 2 image stacks recorded previously. These 10 image stacks were taken randomly from different positions on the specimen. The physical dimensions of each stack as recorded by the Leica Confocal microscope at 20x magnification were 775.0μm x 775.0μm x 85 μm, with a volume of 5.1×10^7 μm^3 of tissue being imaged per image stack.

IMAGE ANALYSIS USING COMSTAT2

Using the COMSTAT2 software (http://www.COMSTAT2.dk, Lyngby, Denmark)[277, 278], a threshold was set for each z-stack by which the fluorescence emitted by epithelial debris in the image background was minimised, while maintaining the biofilm fluorescence as much as possible . After applying the individually set thresholds to each stack, biofilm biomass (volume/area or $\mu m^3/\mu m^2$) was then calculated using the same software. Bio-volume or Biomass as calculated by COMSTAT 2 is defined as 'the number of biomass pixels in all images of a stack multiplied by the voxel size [(pixel size)$_x$ X (pixel size)$_y$ X (pixel size)$_z$] divided by the substratum area of the image stack'. It is described as volume by surface area of the image ($\mu m^3/\mu m^2$) and represents the volume of the biofilm in the image stack.

Threshold values for the first set of 32 z-stacks were set by 3 independent investigators (DS, CJ, and AF). All 3 observers manually set the threshold for each image stack at a setting that they thought, maintained maximum biofilm visibility in the sample tissue, but minimised the background fluorescence emitted by mucosal epithelial cells which is not required in the assessment. The threshold values and biomass calculated for each of the 32 stacks by each of the 3 observers were compared, to identify and assess the degree of inter-observer variability of threshold settings, which greatly dictate the biofilm biomass values. These 32 image stacks were then analysed by a single investigator (DS) three times, at monthly intervals to assess the intra-observer variability present in the thresholds set by the same observer on the same images at different points in time. The second set of images was analysed to compare the biofilm biomass derived from sampling almost the entire area of a tissue sample by recording 10 image stacks vs. the biomass calculated from only 2 random image stacks taken from the same sample.

5.4 RESULTS

S. aureus biofilms were identified using the Baclight-CSLM protocol on all 32 mucosal samples as clusters & towers of immobile, irreversibly attached, intensely fluorescing, live green cocci shaped bacteria, approximately 0.5-2 μm diameter, seen interspersed between the viable (green fluorescence) epithelium and dead necrotic (red fluorescence) tissue (Fig 1). As seen in Fig 1a,b,c the images obtained from confocal microscopy of 3 different samples clearly show different biofilm formation on the mucosa but it is not possible to give a detailed and accurate estimate of what is the precise difference in the three biofilms seen on visual assessment only.
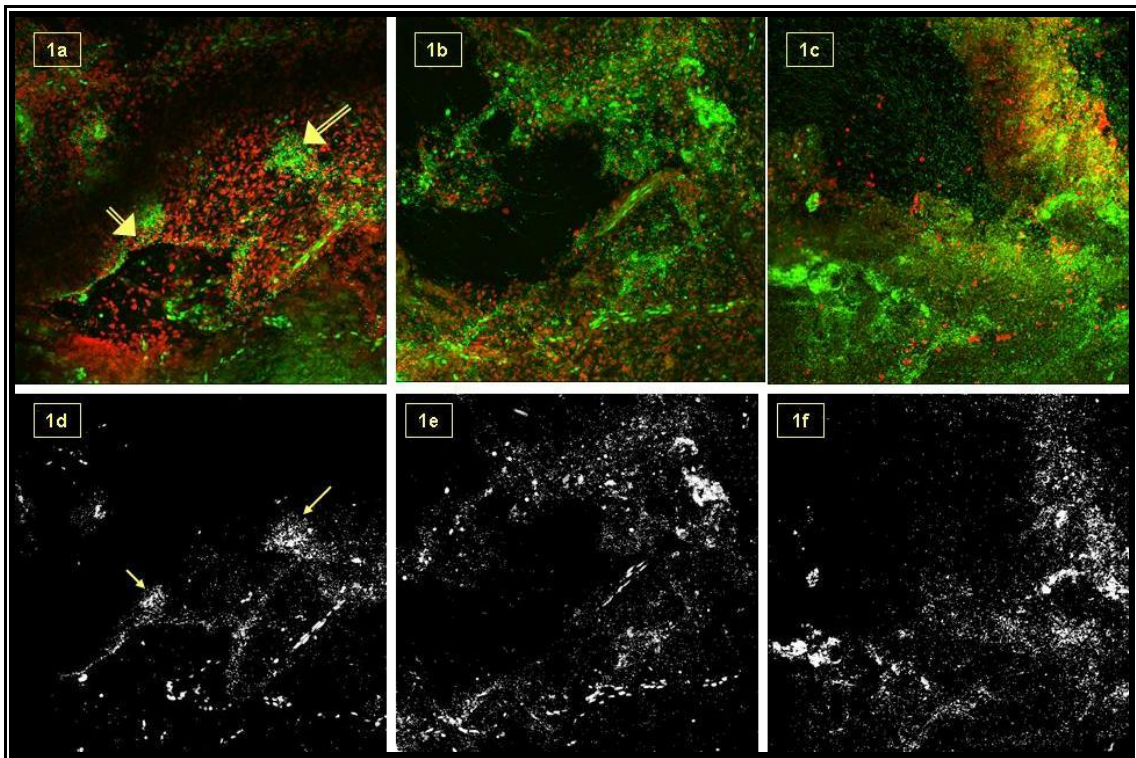
Figure 1a, 1b, 1c: 20x image stacks recorded from sample by CSLM after staining with Baclight. Small arrows show *S. aureus* biofilms as clusters of intensely fluorescing, live green cocci shaped bacteria, approximately 0.5-2 μm diameter, seen interspersed between the green fluorescing viable epithelium and red fluorescing dead tissue

Figure 1d, 1e, 1f: COMSTAT2 translation of the above CSLM images into a simplified 3-dimensional output of the biofilm mass in the tissue by eliminating the fluorescence from the background noise, epithelial tissue, and debris.

When using the COMSTAT2 application, the image recorded by confocal microscopy is translated into a simplified 3-dimensional output of the biofilm mass in the tissue (Fig d,e,f). This is done by setting 'threshold' levels for each imaged stack, based on the software definition of the three-dimensional matrix which has a absolute value of 'one' in the positions where the pixel values in the original image fluoresces above or equal to the threshold value and 'zero' where the pixel values are below the threshold value. Thus, intensely fluorescing bacteria in the biofilms were retained in the output image whereas the subtle fluorescence from the background noise, epithelial tissue, and debris were eliminated from quantitative analysis.

INTER-OBSERVER RESULTS

As the threshold setting is an observer dependent step in the analysis, 3 independent observers (I, II, III) set the threshold for all 32 stacks separately (Fig 2), and an Intraclass correlation coefficient (ICC) was calculated between the thresholds set by the 3 observers for each image stack The ICC was 0.59 (P=0.0003, 95% confidence interval 0.25-0.79).
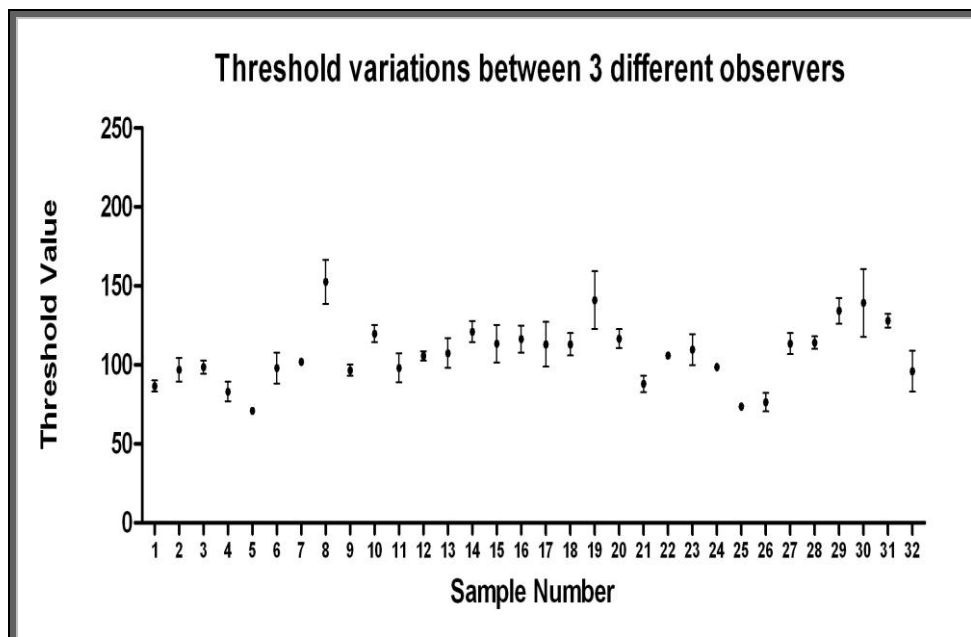
Figure 2: Threshold set by 3 independent observers (I, II, III) on 32 image stacks and Intraclass correlation coefficient (ICC) was 0.59 (P=0.0003, 95% CI 0.25-0.79)

For further evaluating the inter-observer variability using the 32 stacks, an analysis of variance was applied to the set of threshold values recorded by the 3 investigators. The means for each observer over the 32 stacks are given in Table 1. Means followed by the same letter are not significantly different at P=0.05. On average observer II chose thresholds which were significantly lower than either observer I or observer III (P<0.001). The residual standard deviation was 11.2.

Observer	I	II	III
Average threshold	115.6 b	95.5 a	110.3 b

Table 1: Means for each observer

Means of threshold values recorded by each observer over the 32 stacks for analysis of variance. Means followed by the same letter are not significantly different at P=0.05.

INTRA-OBSERVER RESULTS

Threshold values set at different points of time by a single observer were also found to have significant correlation as seen by the ICC: 0.80 (P<0.001, 95% CI 0.68-0.89). The means of the 32 stacks for the single observer's readings on the three occasions for the analysis of variance in assessing the intra-observer variability are given in Table 2. Means followed by the same letter are not significantly different at P=0.05. On average the observer assigned lower threshold values on the second occasion than on the first or third occasion (P=0.02). The residual standard deviation was 8.6, which is about 77% of the rsd for inter-observer variability. Thus there is less variability for the one observer over different occasions than there is between different observers. This is confirmed by the ICCs.

Observer	T1	T2	T3
Average threshold	110.3b	105.0 a	110.6 b

Table 2: Means for each occasion

Means of 32 stacks for single observer's readings on three occasions for analysis of variance. Means followed by the same letter are not significantly different at P=0.05.

As seen in the figure 3 the average biomass calculated from 2 random z-stacks was not significantly different from the average biomass values obtained from 10 z-stacks mapping the sample, (p > 0.81in all 4 samples, Mann-Whitney t-test).
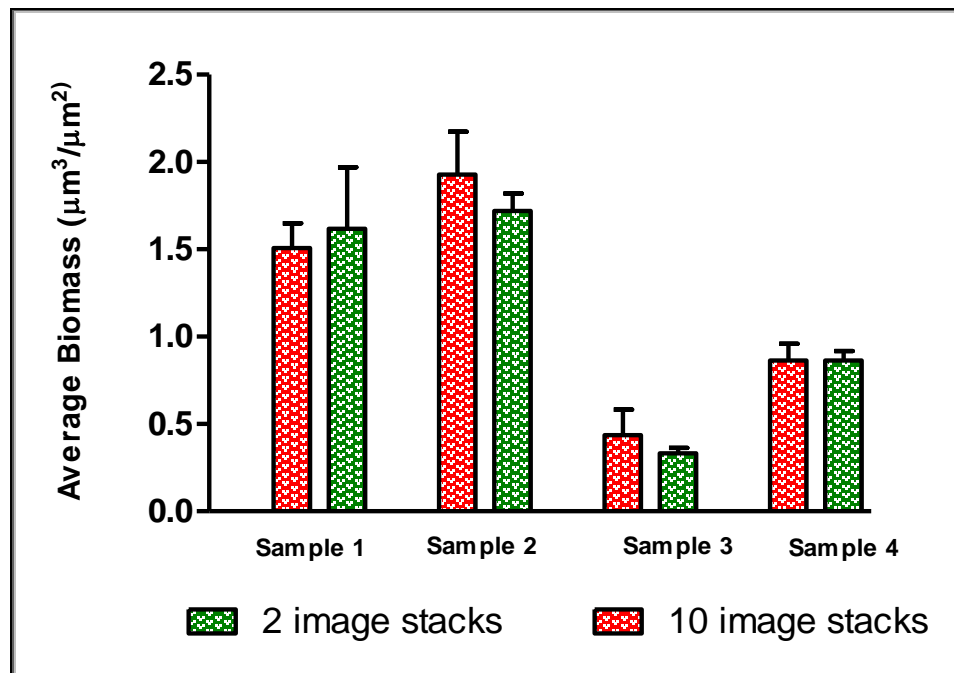
Figure 3: Comparison of biomass from 10 stacks and 2 stacks within samples

To further estimate the precision of biomass estimates with respect to sample size, the 12 stacks from one of the above samples were further analysed, assuming that the variation in the 12 biomass values used is 'typical' of the variation which will be observed during future experimentation. They had a mean biomass of 0.819 ± 0.344. These 12 values were used to estimate the standard error and 95% confidence limits for the mean biomass for sample size values of 2 to 50. This was done using 10,000 bootstrap replications at each sample size 2, 3, 4, … , 20, 22, 24, … , 50. The results are shown in Figure 4 which plots the mean biomass estimate (0.819) together with 95% confidence limits. We can see that the width of the confidence interval and standard error decreases as the sample size increases. The question of how many image stacks to record per sample thus depends on the precision that is required for the estimate of biomass. If the object is to simply estimate biomass of a biofilm then two stacks will provide the necessary information but estimate of the biomass will have wider confidence limits. If two treatments are to be compared then the minimum clinical difference between the two treatments will thus dictate the higher number of image-stacks thus to be recorded based on the information above.
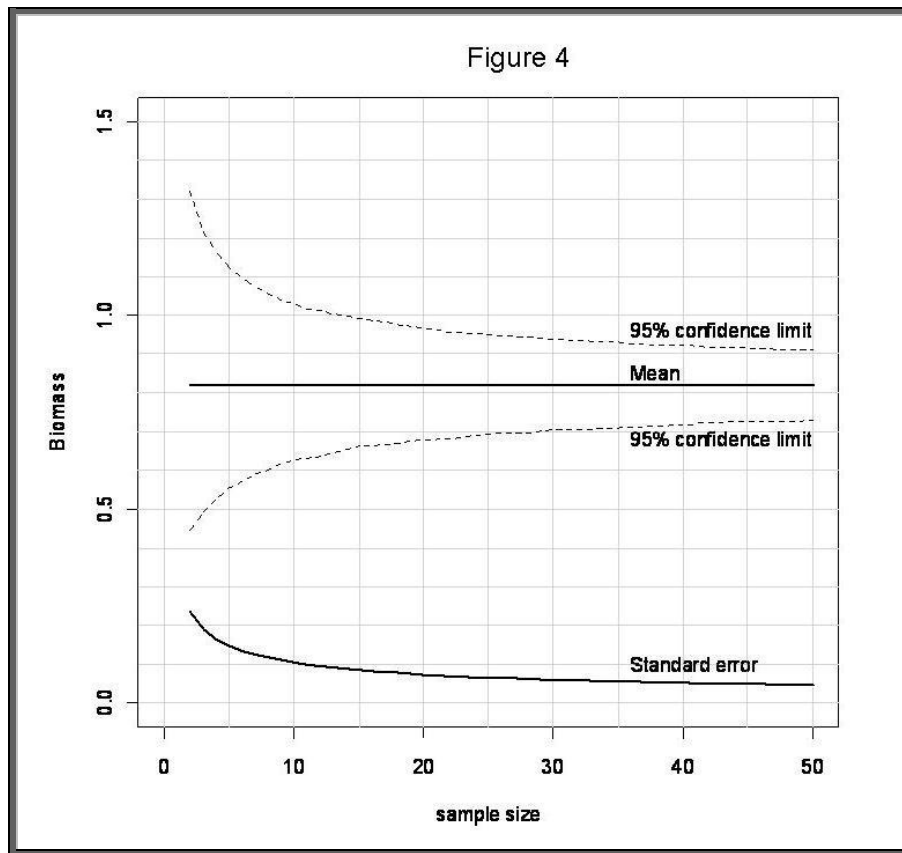
Figure 4: The plot shows the results of bootstrap replications carried out to estimate the standard error and 95% confidence limits for the mean biomass for sample size values of 2 to 50 on the basis of a mean biomass estimate from 12 z-stacks of 0.819 with a standard deviation of 0.344. It plots the mean biomass estimate (0.819) together with 95% confidence limits. The separate line at the bottom of the plot is the standard error of the mean.

5.5 DISCUSSION

In this paper we have described a method to quantify the biofilms identified on sino-nasal mucosa with Confocal Scanning Electron Microscopy using the COMSTAT2 2 software, which is a quick and simple way for analysing large amounts of three-dimensional biofilm image data.

Biofilms formed in natural conditions *in-vivo* have been difficult to investigate and a number of laboratory based experimental biofilm model systems have been developed to study biofilms under controlled conditions [289]. Analyses of various parameters such as thickness variability [290]; mean thickness and roughness of cultures [291]; fractal dimensions [292]; density, porosity, surface area and mean pore radius [293]; textural entropy, area porosity, fractal dimension and maximum diffusion distance [294]; bio-volumes and areas of microbial colonization [295] have all been described in various *in-vitro* models to quantitatively analyse biofilms. The application of the above techniques to assay mucosal biofilms is not possible as all of the above parameters have been assessed on pure monomicrobial biofilms under controlled *in-vitro* conditions, on inert surfaces where confounding variables linked to the substratum are not involved.

While describing mucosal biofilms in the nose and sinuses some researchers have attempted to provide a subjective and categorical assessment of extent of biofilm seen in the samples. Hochstim and colleagues [177, 178] identified biofilms in patient samples using haematoxylin-eosin stains and subjectively graded the extent of biofilm as being 'extensive' if it covered more than 50% of the mucosal sample, and 'present' if it covered less than 50% of the sample surface. Le and Colleagues [179] assessed the percentage surface area covered by biofilm in sub-sections of tissue analysed by confocal microscopy which was not representative the entire quantity of biofilm present in the samples.

Thus, visual inspection of individual CSLM image stacks is insufficient and not an objective assay for quantifying the structural features of biofilms. With an aim to circumvent the specialised imaging techniques to assay biofilms, researchers [174, 175, 182] have described *in-vitro* techniques to assay the biofilm forming capacity of bacteria isolated from CRS patients by culturing clinical isolates in 96-well plates. Biofilm production from these clinical isolates *in-vitro* was quantified by measuring optical density and comparing it with commercial positive and negative controls. But as with all controlled experiments the biofilm formation *in-vitro* cannot be truly reflective of the biofilm formation *in-vivo*, which is significantly affected by the dynamic interactions with the host tissue.

COMSTAT2 2 can be applied to assay a three dimensional image recorded by Confocal Scanning Laser Microscopy which is being accepted as the imaging modality of choice for identifying biofilms. It calculates different features[278, 296] relating to biofilms like biovolume, area occupied by bacteria in each layer, thickness distribution and mean thickness, area distribution of micro-colonies at the substratum, volumes of micro-colonies identified at the substratum, fractal dimension, roughness coefficient, distribution of diffusion distances, average and maximum diffusion distance, surface to volume ratio. In the present study, only the biomass was assayed as it is the simplest to interpret in biological and physical dimensions. Area of at least 1×10^5 μm^2 has been recommended as the minimum area to be investigated in order to obtain representative data for *Pseudomonal* biofilms [297, 298]. Each image stack recorded in our study assayed an area of 6×10^5 μm^2. With 2 random image stacks being recorded from each sinus we assayed a total of 12×10^5 μm^2 per sinus which was obviously more than 10 times the surface area recommended above.

At the same time we showed in the study that biomass calculated from 2 image stacks was comparable to the biomass calculated from 10 z-stacks which were thought to sample out greater amount of the specimen. Considering the time and logistics of recording and analysing the images, biomass assessed from 2 randomly recorded image-stacks gave reliable biomass quantification.

While applying COMSTAT2 2 to the images, setting the threshold for each image stack is the critical step which is observer dependent and defines the amount of biomass in the image that will be assessed. If only one investigator acquires and records all image stacks, then the gain voltage and offset of the confocal microscope will also be adjusted more or less at the same grey level each time for each sample. This in turn takes out the variability related to recording of the images at different intensities which can affect the thresholds set for COMSTAT2. In our experiment, a single investigator recorded all the images and thus the adjusted threshold value varied only between different observers assessing the recorded images via COMSTAT2. The threshold set by the 3 different observers in the above analysis showed significant correlation suggesting that COMSTAT2 could be reliably applied by different observers to obtain comparable results.

There are some points of caution while recording and assessing the images via CSLM and COMSTAT2 2. Firstly, quantification from small micro volumes of tissue may lead to some misinterpretation of the spread and extent of biofilm in the more extensive mucosal lining of the sinonasal tissue, and hence multiple samples from different regions should be sampled to minimise this sampling error. Secondly, over-setting the threshold value may at times hide some of the small biofilm elements from the assessment which may be significant in the very small biofilm communities being assessed. At times very intensely fluorescing cellular components or under-valuing the threshold, can lead to some of the background epithelial nuclei being included in the quantification as biomass components.

Quantitative analysis of biofilms can be used for a temporal analysis of biofilm development, comparison of different types of biofilms formed under controlled conditions, analysis of influence of varying environmental factors on biofilms and effect of different antibiofilm treatments. Quantification of mucosal biofilms by the above software application may help us to understand the degree of biofilm involvement in sinusitis, and possibly analyse if greater sinonasal mucosal coverage of mucosal biofilms correlates with more significant disease, hence establishing another link to the causal relationship of bacterial biofilms in the pathogenesis of sinusitis.

5.6 CONCLUSION

COMSTAT2 software can be reliably used to assay and quantitatively describe the three dimensional biofilm structures that are recorded via confocal scanning microscopy in sino-nasal mucosal samples. This has multiple potential future applications both in understanding the aetiologic role of biofilms in CRS as well as objectively assessing novel anti-biofilm therapies.

5.7 REFERENCES

1.   Potera, C., *Forging a link between biofilms and disease.* Science, 1999. **283**(5409): p. 1837, 1839.

2.   Cohen, M.K., J. Nayak, J. V. Palmer, J. N. Chiu, A. G. Leid, J. G. Cohen, N. A., *Biofilms in chronic rhinosinusitis: A review.* American Journal of Rhinology and Allergy, 2009. **23**(3): p. 255-260.

3.   Harvey, R.J., Lund, V. J., *Biofilms and chronic rhinosinusitis: Systematic review of evidence, current concepts and directions for research.* Rhinology, 2007. **45**(1): p. 3-13.

4.   Palmer, J.N., *Bacterial biofilms: Do they play a role in chronic sinusitis?* Otolaryngologic Clinics of North America, 2005. **38**(6): p. 1193-1201.

5.   Suh, J.D., V. Ramakrishnan, and J.N. Palmer, *Biofilms.* Otolaryngologic Clinics of North America. **43**(3): p. 521-530.

6.   Hall-Stoodley, L. and P. Stoodley, *Evolving concepts in biofilm infections.* Cellular Microbiology, 2009. **11**(7): p. 1034-1043.

7.   Heydorn, A., et al., *Quantification of biofilm structures by the novel computer program COMSTAT.* Microbiology, 2000. **146**(10): p. 2395-2407.

8.   Ha, K.R., Psaltis, A. J., Tan, L., Wormald, P. J., *A sheep model for the study of biofilms in rhinosinusitis.* American Journal of Rhinology, 2007. **21**(3): p. 339-345.

9.   Psaltis, A.J., et al., *Confocal scanning laser microscopy evidence of biofilms in patients with chronic rhinosinusitis.* Laryngoscope, 2007. **117**(7): p. 1302-6.

10.   Heydorn, A., et al., *Experimental reproducibility in flow-chamber biofilms.* Microbiology, 2000. **146**(10): p. 2409-2415.

11.   Palmer Jr, R.J., *Microscopy Flowcells: Perfusion chambers for real time study of biofilms*, in *Methods in Enzymology*. 1999. p. 160-166.

12.   Stewart, P.S., et al., *Quantitative observations of heterogeneities in Pseudomonas aeruginosa biofilms.* Applied and Environmental Microbiology, 1993. **59**(1): p. 327-329.

13.   Murga, R., P.S. Stewart, and D. Daly, *Quantitative analysis of biofilm thickness variability.* Biotechnology and Bioengineering, 1995. **45**(6): p. 503-510.

14.   Hermanowicz, S.W., U. Schindler, and P. Wilderer, *Fractal structure of biofilms: New tools for investigation of morphology*, in *Water Science and Technology*. 1995. p. 99-105.

15.   Zhang, T.C. and P.L. Bishop, *Density, porosity, and pore structure of biofilms.* Water Research, 1994. **28**(11): p. 2267-2277.

16.   Lewandowski, Z., et al., *Quantifying biofilm structure*, in *Water Science and Technology*. 1999. p. 71-76.

17.   Kuehn, M., et al., *Automated confocal laser scanning microscopy and semiautomated image processing for analysis of biofilms.* Applied and Environmental Microbiology, 1998. **64**(11): p. 4115-4127.

18.   Hochstim, C.J., et al., *Biofilm detection with hematoxylin-eosin staining.* Archives of Otolaryngology - Head and Neck Surgery, 2010. **136**(5): p. 453-456.

19.   Hochstim, C.J., R. Masood, and D.H. Rice, *Biofilm and persistent inflammation in endoscopic sinus surgery.* Otolaryngology - Head and Neck Surgery. **143**(5): p. 697-698.

20.   Le, T., et al., *The efficacy of topical antibiofilm agents in a sheep model of rhinosinusitis.* American Journal of Rhinology, 2008. **22**(6): p. 560-567.

21.   Bendouah, Z., Barbeau, J., Hamad, W. A., Desrosiers, M., *Use of an in vitro assay for determination of biofilm-forming capacity of bacteria in chronic rhinosinusitis.* American Journal of Rhinology, 2006. **20**(5): p. 434-438.

22.   Prince, A.A., et al., *Prevalence of biofilm-forming bacteria in chronic rhinosinusitis.* American Journal of Rhinology, 2008. **22**(3): p. 239-245.

23.   Hai, P.V.T., C. Lidstone, and B. Wallwork, *The effect of endoscopic sinus surgery on bacterial biofilms in chronic rhinosinusitis.* Otolaryngology - Head and Neck Surgery, 2010. **142**(3 SUPPL. 1): p. S27-S32.

24.   Korber, D.R., et al., *Programs for determining statistically representative areas of microbial biofilms.* Binary, 1992. **4**: p. 204-210.

25.   Korber, D.R., et al., *Analysis of spatial variability within mot+ and mot- Pseudomonas fluorescens biofilms using representative elements.* Biofouling, 1993. **7**(4): p. 339-358.

CHAPTER 6

DISCUSSION

6.1 OVERVIEW OF BIOFILMS IN CRS

Rhinologists are increasingly being faced with a subgroup of CRS patients whose disease signs and symptoms fails to improve despite maximal medical and surgical management. Factors like nasal polyposis, aspirin sensitivity, smoking, acid regurgitation have been reported to affect post-Endoscopic Sinus Surgery (ESS) outcomes [268-271]. Along with this multifactorial pathophysiology, the presence of bioflims may also contribute to the ongoing inflammation seen in the recalcitrant sub-group of CRS patients, who continue to have persistent sinonasal inflammation and recurrent acute exacerbrations, despite long-term culture-directed antibiotic therapy and well performed sinus surgery.

Biofilms are organised communities of bacteria, attached to a biotic or abiotic surface, embedded in a mosaic of self-produced extracellular polymeric substance. Within the biofilm ecosystem, synchronised existence is seen between the various microbial communities exhibiting specialised phenotypes, which enable the micro-organisms to survive in the host by resisting the onslaught of the immune system and antibiotic treatments. Almost like a highly self sufficient fortress, they persist within tissues, leading to chronic inflammation in their surrounding tissues with intermittent acute exacerbations, which are the hallmark of biofilm mediated diseases.

With the detection of biofilms on the sino-nasal mucosa, it has been extensively speculated that these structures may be responsible for propogating recalcitrant and chronic pathophysiological processes seen in CRS patients [62, 98, 153-157]. CRS does fulfill the diagnostic criteria laid out for identifying biofilm mediated infections [97, 98] i.e 1) Micro-organisms (bacteria and fungi) are found attached, embedded or within the mucosa lining the sino-nasal region, 2) Examination of sinonasal biopsies via various imaging techniques has shown evidence of microbial clusters/microcolonies surrounded by the ECM, 3) The disease is localised to the nose and paranasal sinuses, 4) The infective process is difficult to

control despite culture-directed antibiotic therapies, 5) The samples often are difficult to culture despite obvious signs of mucopus and associated inflammation.

At the same time some studies have described biofilm structures in control specimens [172], suggesting that the much hyped role of biofilms in CRS be evaluated further. Though it is unlikely that biofilm is passively present in such a high percentage of patients who fail medical or surgical management, a recent study [299, 300] based on SEM evidence of biofilm presence in non-CRS patients, has stated that sino-nasal biofilms are 'just a dummy' and 'another name for a regular mucosal blanket'. It is noteworthy that the results of that study are based on SEM which is known to give false positive results if no methods are applied to remove the mucous layer covering a normal sinus mucosa or artefacts arising out of tissue prepration techniques. Also no species specific evaluation of the biofilm was conducted in the study to establish if it was composed of pathogenic or commensal microbes on a normal mucosa as the normal microbial flora in the body is also known to exist in biofilm form.

Thus mucosal biofilm mediated CRS still needs to be carefully differentiated from the in-vivo adherence or colonization of the mucosa by mere bystander microbial organisms. The associated overt pathological inflammatory response seen in CRS patients to the presence of mucosal biofilm structures is a distinguishing feature. Future research should be directed at understanding the naturally occurring commensal biofilm systems in the sino-nasal regions and their balanced existence with the host tissue which possibly may prevent the pathogenic biofilm formation.

6.2 NEGATIVE IMPACT ON POST-SURGICAL OUTCOMES

Ever since the first evidence of mucosal biofilms in CRS patients was published in literature, they have been touted as possible factors in propagating the recalcitrant nature of CRS. But none of the subsequent publications had conclusively documented the negative impact that biofilms may have had on outcomes of ESS for CRS. Bendouah and colleagues [183] had found *Pseudomonas aeruginosa* and *Staphylococcus aureus* biofilms were associated with unfavourable surgical outcomes one year post ESS procedure and a retrospective analysis of 40 CRS patients had also shown that patients with biofilms were more likely to have symptoms and show evidence of ongoing mucosal inflammation following sinus surgery [184]. It is important to note however that both the studies had been limited by their in vitro and retrospective design and they used non-validated symptom scoring methods and dichotomous or nominal reporting of post-operative outcomes.

As described in Chapter 2, our prospective study of the impact of biofilms on the post-surgical outcomes, used internationally accepted, standardised symptom, radiologic and endoscopic scoring systems [2, 259, 260] and has shown that biofilm positive patients had worse objective disease and poorer post-operative outcomes than biofilm negative patients. Biofilms were detected in 71% of our study population using the Baclight-CSLM protocol standardised previously [168]. Pre-operatively, the biofilm positive group had significantly worse Lund-Mackay radiology scores [260] and significantly poor Lund-Kennedy nasendoscopy scores [259] as compared to the biofilm negative group, indicative of a more severe pre-operative disease in those patients. The biofilm positive patients' symptoms and quality of life outcome measures started worsening significantly starting six months after surgery, indicating that this subgroup of CRS patients continues to have an ongoing

relapsing and recalcitrant course. The patients with biofilms had ongoing infective episodes and ensuing inflammation resulting in mucosal oedema, discharge and crusts.

Some more recently reported studies have shown similar poor outcomes in patients with biofilms after endoscopic sinus surgery. Hochstim and colleagues [178] provided evidence for association of biofilms with persistent inflammation after endoscopic sinus surgery. They identified biofilms in 15 of the 24 patients using FISH and Confocal scanning microscopy, and found that patients with biofilms had significantly persistent and prolonged inflammation as indicated by mucosal edema or erythema as compared to patients without biofilms. They however did not find any association between the presence of biofilms and prior ESS, and once again used nominal outcome measures. Zhang and colleagues [301] identified biofilms intra-operatively in 20 out of 27 patients in their study population, and looked at identifying bacterial biofilms on postoperative sinonasal mucosal biopsies taken from areas of post-surgical edema or scar identified on nasendoscopy from these patients. They demonstrated biofilms in 4/6 scar tissue samples and 5/9 edema samples using SEM, indicating a persistence or regrowth of biofilms after treatment. In their study too, the patients with intra-operative and post-operative biofilms had more severe preoperative radiology scores and worse Lund-Kennedy endoscopy scores after 6 months of follow-up.

Hai and colleagues [302] aimed to determine if endoscopic sinus surgery had any impact on bacterial biofilms. They assessed the in-vitro biofilm forming capacity of bacterial isolates cultured before and 3 months after the surgery from endoscopic nasal swabs, and found that ESS brought about a reduction in bacterial biofilms, but did not eliminate them totally. The decrease in biofilms however did not correlate with improvement in nasal endoscopic score after surgery. Thus the presence of bioflims may well contribute to the ongoing inflammation even after well-performed surgery.

6.3 BURDENING MEDICAL COSTS

Patients who have biofilms on their sino-nasal mucosa tend to have more severe disease which persists with ongoing mucosal inflammation and recurrent infections, even after well performed surgery by experienced surgeons. Postoperatively, three fourths of the biofilm positive patients in our study described in chapters 2 and 3, required extra visits to a tertiary rhinologic clinic, apart from the standard planned care [265, 303]. These additional visits resulted from the patients developing sinus symptoms requiring medical opinion or due to ongoing inflammation in the sino-nasal region prompting the surgeon to review them at more frequent intervals than expected. These extra consults reflected the on-going disease in these patients, possibly as a result of the inflammation set up by the persistence of biofilms within mucosa preserved at surgery. The extra-consults in turn result in an increased burden on the healthcare system, extra financial costs to the patient and work days lost.

Also as seen in our study [265] as well as described in other publications [168, 184], patients with biofilms underwent more number of previous sinus surgeries. Almost 75% of the patients who were undergoing revision sinus surgeries in our studies showed evidence of biofilms. This supports the hypothesis that biofilms may play a role in perpetuating the recalcitrant nature of chronic sinusitis, with the patients requiring repeated surgical interventions despite maximal medical management. Additionally, more than 90% of the patients with biofilms in our study showed a tendency to undergo more extensive procedures like Full-FESS and another third of them required a frontal drillout procedure. Whereas only 67% of the biofilm negative patients underwent a Full-FESS and only a tenth of them required a drillout procedure. This was once again reflecting the more severe disease seen in patients with biofilms, necessitating a more extensive surgical intervention.

Residual biofilms after surgery may reseed the regenerating epithelium and serve as a nidus for further biofilm formation. The biofilm may act as a stimulus for ongoing inflammatory response, as well as releasing planktonic forms of bacteria periodically as a part of its life cycle leading to acute exacerbations on top of the chronic process. The high rates of intra-operative and post-operative bacteria cultured from the biofilm positive group in our studies reflects this periodic planktonic transformation of biofilm bacteria. In 50% of the patients in the study described in Chapter 2 mucopurulent discharge was detected on endoscopy during the post-surgery follow-up. Swabs taken for microbiology evaluation from them cultured bacteria in 70% of biofilm positive cases post-operatively. 40% biofilm positive cases cultured bacteria on more than one follow-up visit; and 60% of the biofilm positive patients grew the same bacteria on multiple different occasions. All of those patients underwent repeated antibiotic treatments based on conventional microbiology principles of identifying antimicrobial sensitivities, which targeted only the planktonic microbial forms and not the biofilm forms. Thus, the misdirected antimicrobial treatments only increased the health care costs associated with each acute exacerbation of the chronic disease, while a therapy directed against the biofilm forms might have been more fruitful in improving the outcomes.

6.4 SPECIES SPECIFIC BIOFILMS: PROGNOSTIC INDICATORS?

As described above, we had clearly shown that in general the presence of biofilms on the mucosa of CRS patients is associated with more severe disease preoperatively, persistent post-operative symptoms, ongoing mucosal inflammation & infections following ESS [265]. However, that study had used Baclight stain to identify the mere presence and absence of biofilms on the sinus mucosa and that technique precludes species identification. Thus we could not describe which bacterial species formed biofilms in the clinically symptomatic patients or which species specific biofilms were associated with poorer outcomes after surgery. Fluorescence in situ hybridization (FISH) techniques using species specific probes have been described to identify the various bacterial species forming biofilms in CRS patients [172, 173, 256]. And a retrospective analysis of CRS patients in whom biofilm forming organisms were known demonstrated that different biofilm species display different disease characteristics [266]. *Haemophilus influenzae* biofilms were found in patients with mild disease, whereas *Staphylococcus aureus* biofilms were associated with a more severe surgically recalcitrant disease profile. But the results of the study were limited as it was a retrospective review and applied non-validated symptom scoring methods and nominal reporting of post-operative endoscopic outcomes.

Thus, we conducted a prospective study and used FISH probes for the commonly identified bacterial species forming biofilms identified in different study populations [98, 172, 173, 256] i.e. *S. aureus, H. influenzae, P. aeruginosa* and fungal probes, to identify which species specific biofilms are associated with poor post-treatment outcomes. It is obvious that we may have possibly missed out identifying some clinically relevant biofilm forming species like *S. pneumoniae* and *M. catarrhalis* and thus were unable to define their disease outcomes. But we were limited by the number of species specific probes that could

be applied to tissue samples as a result of limitations imposed on by the logistics related to the size of the biopsy specimen, cost of the probes and time for processing the samples.

We used standardised internationally accepted symptom, radiologic and endoscopic scoring systems [2, 259, 260] as the validated outcome measures, and thus more convincingly found that different bacterial biofilms are associated with different disease progression after ESS. As described in chapter 3, *S. aureus* biofilms were identified in 70% of the biofilm positive patients in our study and this group progressed poorly with their subjective and objective post-ESS outcomes. They were found to play a dominant role in negatively affecting the outcomes of ESS with persistence of post-operative symptoms, ongoing mucosal inflammation & infections. The patients with *S. aureus* biofilms thus required more follow-up visits when compared to the remaining biofilm patients indicating that repeated infections and persistent disease prevail in this subgroup. *S. aureus* has been identified in different study populations as the most common bacteria isolated from patients with surgically recalcitrant disease [272, 274, 276, 304], and we have also identified it as the most common biofilm forming organism found on intraoperative mucosal specimens and post-operative sinonasal cultures from infected sinuses [265].

On the other hand, in our study, patients with unimicrobial *H.influenzae* biofilms had the least severity of disease pre-operatively and post-operatively had a rapid resolution of signs, symptoms and quality of life outcome measures similar to patients without biofilms. The interest in the role *H. influenzae* may play in pathogenesis of CRS has recently been rekindled, with studies identifying *H. influenzae* biofilms commonly on sinonasal biopsies [172, 173, 256]. Surprisingly, no unimicrobial *P. aeruginosa* biofilms were seen in our study population, though it has been commonly reported to occur as a predominant bacterial species forming biofilms in some other studies. In our research it was found to coexist with other microbial species and hence the disease profile associated with a *P.*

aeruginosa biofilm could not be defined. The very small population of patients with single species fungal biofilms in our study had mild disease, though the recovery of signs and symptoms was more protracted.

Thus a clinically relevant species directed biofilm analysis will help in determining the possible at risk group of patients and guide a more directional research towards the elimination of those biofilms. This specific knowledge may help in developing novel and species specific anti-biofilm treatments against biofilms like those formed by *S.aureus* which may enable us to aggressively treat those patients and help in improving the post-surgical outcomes of ESS.

6.5 POLYMICROBIAL BIOFILMS

CRS is typically a polymicrobial disease described on the basis of standard culture techniques. Biofilms in CRS are also formed by different species of bacteria and *Staphylococcus aureus, Streptococcus pneumoniae, Haemophilus influenzae, Pseudomonas aeruginosa* are some of the bacterial species that have been frequently identified growing in the biofilm form on the sino-nasal mucosa of CRS patients [172, 173]. Additionally, 50-70% of the biofilms in CRS specimens have been shown to be polymicrobial based on FISH-species specific probes and Confocal microscopy [173, 175]. As described in chapter 2, we found that the number of species within a biofilm is also a significant predictor of disease progression as they are associated with more severe disease pre-operatively, when compared with patients with single species biofilms. Post-operatively these patients had worse post-surgery mucosal outcomes on endoscopy requiring significantly more number of post–operative visits to the rhinology clinic.

In our small study population, the patients in whom *H .influenzae, P. aeruginosa*, fungal biofilms co-existed with *S. aureus* biofilms disease patterns were again reflective of the refractory and recalcitrant pathogenic mechanisms of staphylococcal biofilms, indicating that *S. aureus* biofilms play a dominant role in determining disease severity and guiding postoperative course. When *H. influenzae*, *P. aeruginosa* or fungal biofilms combined together as a polymicrobial biofilm without *S. aureus* the disease pattern was once again milder and reflective possibly of their mild pathogenic effects. These two pieces of information further support the theory that *S. aureus* plays a dominant role in CRS biofilms.

The polymicrobial complexity of biofilms has been intensely studied in conditions like periodontitis and dental plaques, and many significant interspecies interactions have been

highlighted since [305]. Co-culture models have shown that in a polymicrobial biofilm, *Streptococcus pneumoniae* is protected against β-lactam antibiotics when it co-exists with β-lacatmase-producing strain of *Moraxella catarrhalis* [306].   In our study, the negative impact on post-treatment outcomes was observed to be more pronounced when the *S. aureus* biofilms occurred as single species biofilm and was somewhat attenuated when they occurred along with other microbial biofilms.

6.6 FUNGAL BIOFILMS IN CRS

Direct evidence for the existence of fungal biofilms in CRS has been conspicuously absent despite the extensive knowledge in the scientific world regarding the role that fungal biofilms play in various environmental and medical scenarios. There have been a few studies which have described fungal elements using FISH probes and CSLM in rhinology. Whilst studying bacterial biofilms on sinonasal tissue, Healy and colleagues [256] incidentally detected fungal elements within bacterial biofilms but could not determine if these fungi produced biofilms. Foreman and colleagues [173] using a pan-fungal Fluorescent In Situ Hybridisation (FISH) probe to screen tissue biopsies from CRS patients found non-specific fungal elements showing biofilm like structures, which may have been non-specific attached fungal elements rather than embedded biofilms containing the possible pathogenic filamentous fungi. As described in chapter 5 we have been able to demonstrate that pathogenic filamentous fungi, often identified in CRS patients, can form biofilms in-vitro on primary sinonasal epithelial cell cultures. The techniques applied to develop the in-vitro fungal biofilm model have further helped in developing an animal sheep model of fungal sinusitis in which fungal biofilms have been grown in the frontal sinuses of sheep [280].

A. fumigatus biofilm grown under controlled in-vitro conditions on the primary culture of sino-nasal epithelial cells was seen to form structurally and developmentally similar to those described in other *Aspergillus* biofilm models [248, 253, 255]. We also found that the appearance of *A. fumigatus* attached to the epithelial cell monolayer differed markedly from the smoky gray-green woolly to cottony textured colonies described when grown on potato dextrose agar. On microscopy the 'cottony' foci of growth from agar slopes displayed a dense clump of branching hyphae radiating from a central clump of spores with no matrix produced around them. In contrast the biofilm form of growth described in chapter 5 was seen to start when the spores of *A. fumigatus* first attached to the surface of

the cells and this was then followed by the formation of a coordinated network of hyphae spreading in all directions, in between the host cells, cross-linking with each other to form a parallel-packed matrix. This led to the formation of a structure of high stability and protection with an intricate network of channels and passages. The mature multi-layered *A. fumigatus biofilm* formed a thick mat of hyphae and cells, which appeared to be bound by a thick, impermeable gel like substance which in vivo may act as a physical barrier between the fungal cells and clinically used anti-fungal agents thus leading to the ongoing colonisation of fungi in the sinuses despite maximal treatment. Loussert et al [281] have recently described galactosaminogalactan and galactomannan as the major polysaccharides forming the ECM in *A. fumigatus* biofilms, which may be a possible target for anti-fungal treatments directed at *Aspergillus* biofilms.

It is probable that this biofilm form of filamentous fungi may provide links in addressing the controversies regarding the role of fungi in pathogenesis of CRS or other forms like AFRS. It has been speculated that some *Aspergillus* allergens (Asp f proteins) secreted after conidia have germinated into hyphae participate in the development of the differential protective / allergic immune immune processes [286], but the exact mechanism by how this happens has been elusive. The biofilm form of *A. fumigatus* may prove to be the yet unidentified source of the differential antigen expression favoring the initiation of a protective (TH1–TH17) or a destructive allergic (TH2) immune response as studies on antigenic and protein expression of fungi like *Candida* [287, 288] have shown differential protein expression in fungal biofilm forms as compared to planktonic forms. Filamentous fungi in biofilm form are also known to have more productive and metabolically efficient enzymatic activities [247]. The biofilm form of *A. fumigatus* can be a possible efficient source of factors like gliotoxin and other uncharacterized high-molecular weight factors, which if produced in a sufficient quantity in-vivo, can contribute to airway pathology by

damaging epithelial cells and reducing ciliary beat frequency [285], and thus facilitate the

pathogenesis of chronic rhinosinusitis.

6.7 HETEROTYPIC BIOFILMS IN CRS

Fungal-bacterial biofilms (FBB) [284] are mixed biofilms in which fungi or bacteria act as the biotic surface to which another bacteria or fungus adhere. In such 'heterotypic biofilms', symbiotic interactions between bacteria and fungi augment survival by assisting surface adherence and improving the protection provided by the complex ECM. These mixed species biofilms are known to have better growth and colonization abilities.

Co-existence of fungal and bacterial biofilms as heterotypic biofilms has been reported in varied environments [283, 284]. One such study focusing on bacterial–fungal interactions has shown that 78% of the 50 patients with stomatitis had *C. albicans* and *S. aureus* existing as a mixed species biofilm [307]. This study demonstrated that the bacterial biofilm possibly makes the host environment more conducive for fungal biofilm formation by lowering the pH in the surrounding tissues. Implant related biofilm infections are also frequently reported as mixed species biofilm infections especially in case of indwelling catheters [308-310], and they display a more complex ECM which makes them much more resistant to antibiotic and antifungal therapy. Another study examining interactions between *C. albicans* biofilms and other bacterial biofilms [311] showed the ECM had an important role to play in the co-colonization of bacterial-fungal biofilms. Specifically, bacteria which produce glycocalyx, such as *S. aureus*, were better able to adhere to *Candida* biofilms.

Co-existence of fungal elements and bacterial biofilms has been described in CRS patients. Foreman and colleagues [173] found that out of the 11 patients in whom they identified fungal biofilm like structures, 7 also demonstrated *S. aureus* biofilms, 2 had *H. influenza* biofilms present. Healy and colleagues [256] also demonstrated the presence of fungal elements within bacterial biofilms on mucosal samples from sinuses of 7 of the 11 patients in their study. However they could not ascertain if they were viable fungal elements or if they were in a biofilm form. In our study described in chapter 2, we found fungal detection

correlating with bacterial biofilm presence in CRS patients [265]. Fungus was isolated from 6 of the 36 biofilm positive subjects by either staining or culture, but no fungus was isolated from any of the biofilm negative patients. 80% of patients who had eosinophilic mucin, suggestive of hypersensitivity to undetected fungi showed bacterial biofilms. All of these findings indicated towards some form of interaction between fungi and bacteria possibly in a biofilm form that had so far not been identified and could possibly hold an important key to understanding the etiopathogenesis of bacterial/fungal CRS.

Further work done to develop an in-vivo animal model of fungal sinusitis [280] has applied the techniques described in Chapter 5 to identify the possible proliferation of fungal spores into biofilm form in the sinuses of sheep. That study has shown that fungi alone do not readily form biofilm structures in otherwise non-inflamed sinuses and *S. aureus* biofilms were identified as an important co-factor for fungal persistence and proliferation in the sinuses in a biofilm form. Following inoculation of sheep sinuses with *Aspergillus fumigatus* and *Alternaria alternata* spores only, no fungal growth was detected. However when the fungal spores were co-inoculated with *S. aureus,* 80 % of the sinuses showed evidence of fungal biofilm as well as *S. aureus* biofilm formation. In this model, it is possible that the mucosal reaction to *S. aureus*, with the associated inflammatory milieu, resulted in an environment where fungi could 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 signalling.

Complex symbiotic interactions between bacteria and fungi can have profound effects on the virulence and survival of these organisms. Thus the possible existence of bacteria and fungi in biofilm form in CRS patients would also indicate a symbiotic co-existence, better protected from the host defenses and antimicrobial therapies, thus playing a key role in CRS pathogenesis.

6.8 IN-VITRO MUCOSAL BIOFILM MODELS

As dictated by Koch's postulates, chronic infectious processes have so far been targeted at a molecular, cellular, organism level instead of a community level as seen in biofilms. The standard microbiological techniques developed to identify pathogenic microbes are based upon the simplistic principle of single organisms multiplying in culture media and thus fail to identify biofilm communities which form and develop via more complex interactions. The antibiotic susceptibility tests are also limited by the use of above conventional techniques as they test the efficacy against suspensions or cultures of planktonic microbes. Conventional antimicrobials target individual micro-organisms via cellular or molecular mechanisms and are not aimed against microbial communities.

Biofilms can be studied *in-situ* within their natural environment or *ex-situ* by removing intact biofilm materials and analysing them in a laboratory. In addition biofilms can also be investigated via various *in-vivo* and *in-vitro* models. Although clinical specimens or in-vivo animal models have provided an ideal method to investigate biofilm related pathological conditions, in-vitro models are equally important as they allow investigation of specific biofilm phenotypes and mechanisms of resistance under controlled conditions. *In-vitro* models described range from simple and basic closed system biofilm models like those grown on agar plates or multi-well plates; to open system biofilm models like flow cells, biofilm fermenters, and reactors [312]. But the lack of an ideal model system has led to selection of different models based on user preferences, with the need to simulate *in-situ* conditions with ease, which can be reliably replicated.

Apart from *ex-situ* clinical specimens to study biofilms on sinonasal mucosa, and some animal models to replicate biofilm environments within sinuses [163, 167], laboratory models are also being adopted and refined to study biofilms in the pathogenesis of CRS

[174, 175]. Woodworth and colleagues [169] grew *Pseudomonas aeruginosa* biofilms supported on mouse nasal septal epithelium in air-liquid interfaces, to develop an in-vitro model for trialling novel anti-biofilm treatments for CRS. In the study described in Chapter 5, we used primary cultures of sino-nasal epithelial tissue taken from patients undergoing ESS, so that the substratum on which the biofilms grew was as similar as possible to those in-vivo conditions in CRS patients. Although the influence of the innate immune system could not be simulated with just the epithelial monolayer of cells, the continuous-flow co-cultures were set up to mimic mucociliary clearance and to evaluate the formation of the biofilm in conditions similar to the sino-nasal physiology.

6.9 QUANTIFYING MUCOSAL BIOFILMS

Microbes within the biofilm are difficult to culture and thus, not easy to assay using the conventional microbiology and clinical tests. A variety of specialised microscopic techniques have been described in literature to identify, image and describe mucosal biofilms in-vivo and in-vitro conditions. Techniques assaying biofilm growth kinetics by dry-weight measurements have been applied to demonstrate the increased formation of ECM in biofilms[255]. But the dry weight measurement is applicable only to a pure biofilm developed in-vitro setting where confounding contribution from an underlying substratum like host cells in mucosal samples is not present. Colny forming units from a disrupted biofilm [180] or comparing the optical density of crystal violet or safrannin stained biofilms [174, 175, 255, 313] with commercially available standards has also been used for quantitative assays of biofilms formed in-vitro conditions. Again, these assays are not applicable to the biofilms detected in-vivo conditions or on ex-situ tissue samples.

Confocal Scanning Laser Microscopy is widely being accepted as the imaging modality of choice for identifying and studying the 3-dimensional structural formation of biofilms. With CSLM, a more accurate imaging of viable biofilms is possible with minimal disruption of their 3 dimensional structures. But even with the use of CSLM, the literature reporting mucosal biofilms in rhinology has been mostly descriptive, and lacking in quantitative detail. As described in Chapter 4, biofilms identified on sino-nasal mucosa with Confocal Scanning Electron Microscopy can be quantified using the Comstat 2 software, which is a quick and simple way for analysing large amounts of three-dimensional biofilm image data.

Comstat 2 calculates different features [278, 296] relating to biofilms like biovolume, area occupied by bacteria in each layer, thickness distribution and mean thickness, area

distribution of micro-colonies at the substratum, volumes of micro-colonies identified at the substratum, fractal dimension, roughness coefficient, distribution of diffusion distances, average and maximum diffusion distance, surface to volume ratio. There are some points of caution while recording and assessing the images via CSLM and Comstat 2. All samples need to be imaged and recorded by the same investigator to ensure that the gain voltage and offset of the confocal microscope is set at the same level, which in turn takes out the variability relating to the images recorded at different fluorescing intensities, which in turn can affect the thresholds set for Comstat. Over-setting the observer dependent threshold value may at times hide some of the small biofilm elements from the assessment which may be significant in the very small biofilm communities being assessed. At times, very intensely fluorescing cellular components or under-valuing the threshold can lead to some of the background epithelial nuclei being included in the quantification as biomass components.

A 10mm x 10mm sample taken for analysis at the time of surgery is a very small representation of the entire sino-nasal mucosa covering the multiple sinuses in patients. The biofilms possibly have a patchy distribution on the sino-nasal mucosa and quantification from such small micro volumes of tissue may lead to some misinterpretation of the spread and extent of biofilm in the more extensive mucosal lining of the sinonasal tissue. In order to minimise this sampling error, multiple samples from different regions and a standardised technique for sampling the sinus mucosa may be required, so that Comstat can be applied convincingly to better understand the community structure of mucosal biofilms visualised by Confocal Microscopy.

Sanderson and colleagues [172] had found *H. influenzae* biofilms on sinus mucosal samples of 2/5 control patients and described them as 'not as floridly' positive as the biofilms seen in CRS patient samples. They also found that *H. influenzae* and *S. aureus*

biofilms observed on CRS patient samples were more extensively distributed as compared to *S. pneumoniae* samples. This had led to the speculation that biofilms may simply be colonizers of the sinonasal cavity or there may be a critical biofilm mass at which an inflammatory response may be elicited by the host leading to CRS manifestation. Quantification of mucosal biofilms by the above software application may help us to understand the degree of biofilm involvement in sinusitis, and possibly analyse if greater sinonasal mucosal coverage of mucosal biofilms correlates with more significant disease, hence establishing another link to the causal relationship of bacterial biofilms in the pathogenesis of sinusitis. It will be a useful tool for assessing the *in-vivo* effects of novel anti-biofilm treatments, and possibly give an answer to the question of how much biofilm is pathologically and clinically significant.

6.10 TREATMENT OPTION FOR BIOFILMS:

Studies trialling possible medical therapies against biofilms in CRS have shown that the Minimal Biofilm Eradication Concentration (MBEC) was 100 to 1000 fold greater than the MIC for some of the currently used antimicrobials [181, 314]. This high concentration required to eliminate a biofilm focus in the body is accompanied by adverse effects from the antimicrobial agent if administered systemically. Thus the experimental approach has mostly focused on topical administration of antimicrobials which can possibly be administered in larger doses to achieve higher concentrations in the sinonasal tissues, while minimizing systemic absorption and avoiding ensuing adverse effects. Mupirocin is one such agent that has shown 90% reduction in *S. aureus* biofilms in-vitro and animal models [179, 315], with significant improvement in signs and symptoms in CRS patients [316] when used as an adjunct to nasal lavages.

Lavages with chemical surfactants like baby shampoo have also shown to be of benefit in post surgical CRS patients [317]. Other surfactants like combination of soap like surfactant and a calcium ion sequestrating agent, aiming to break the bonds in the EPS have also been reported to effectively eradicate *S.aureus* and *P. aeruginosa* biofilms under controlled in-vitro settings. Mechanical disruption of the biofilm by applying hydrodynamic sheer force has been shown to further increase the effectiveness of the above surfactant agents in-vitro settings [180]. However, hydro dynamically applied surfactant to in-vivo mucosal biofilms in the sheep model showed only an immediate reduction in the biofilm, with the effect not lasting over a longer period of time [179].

While the ideal method or agent for controlling and eradicating biofilms is being researched, ESS still remains the option which is offered repeatedly to patients with recalcitrant CRS. Removal of diseased tissues, unobstructed well aerated sinus cavities and

restoration of mucociliary clearance mechanism of the sino-nasal region have been the basic fundamentals of treating CRS via ESS. But in attempting to preserve the healthy appearing mucosa for a rapid return of the mucociliary clearance mechanism, the biofilm foci may also be inadvertently retained which in turn rekindles and perpetuates the inflammatory process. In such patients undergoing frequent exacerbations of the disease requiring repeated medical treatments despite prior maximal medical management or revision surgical interventions despite well-performed prior surgeries, the principle of preservation of the healthy appearing sino-nasal mucosa needs to be reconsidered. This does make for a difficult choice of either heading back from advanced minimally invasive endoscopic sinus surgeries to the more radical surgical approaches in managing patients with recalcitrant CRS or of keeping patients on palliative care till an optimum antibiofilm-treatment is discovered.

APPENDICES

APPENDIX A:

ABBREVIATIONS IN THESIS

AFRS	… Allergic Fungal Rhinosinusitis	FISH	… Fluorescent In-Situ Hybridisation
ANOVA	… Analysis of Variance Analysis	FRS	… Fungal Rhinosinusitis
ATCC	… American Type Culture Collection	GARS	… Global Assessment of Rhinosinusitis Severity
CFC	… Continuous Flow Culture	ICC	… Intra Class Coefficient
CFU	… Colony Forming Unit	INF-γ	… Interferon- γ
CI	… Confidence Interval	IL-5	… Interleukin-5
CIVS	… Cylindrical Intra-Vacuolar Structures	IL-13	… Interleukin-13
		IgE	… Immunoglobulin E
Con A	… Concanavalin A	IQR	… Inter Quartile Range
CO_2	… Carbon Dioxide	MBEC	… Minimal Biofilm Eradication Concentration
COPD	… Chronic Obstructive Pulmonary Disease	MIC	… Minimal Inhibitory Concentration
CNS	… Central Nervous System	MELP	… Modified Endoscopic Lothrop's Procedure
CRS	… Chronic RhinoSinusitis	MQ	… Milli-Q
CRSwNP	… Chronic RhinoSinusitis with Nasal Polyps	mm	… Millimetre
CRSsNP	… Chronic RhinoSinusitis without Nasal Polyps	mL	… Millilitre
		µL	… Micro litre
CSF	… Cerebrospinal Fluid	OME	… Otitis Media with Effusion
CSOM	… Chronic Suppurative Otitis Media	PBMC	… Peripheral Blood Mononuclear Cells
CSLM	… Confocal Scanning Laser Microscopy	PBS	… Phosphate Buffer Saline
		QOL	… Quality of Life
CT	… Computerised Tomography	RCT	… Randomized Controlled Trials
DGGE	… Denaturing Gradient Gel Electrophoresis	SEM	… Scanning Electron Microscopy
DMEM	… Dulbeco's Modified Eagle Medium	SNOT-20	… Sino Nasal Outcome Test-20
		StC	… Static Co-culture
DNA	… Deoxyribo Nucleic Acid	TEM	… Transmission Electron Microscopy
ESS	… Endoscopic sinus Surgery		
ECM	… Extra Cellular Matrix	TGGE	… Temperature gradient gel electrophoresis
EMCRS	… Eosinophilic Mucin Rhinosinusitis	T_H1	… T Helper Cell 1
EPS	… Exopolysaccharide Matrix	T_H2	… T Helper Cell 2
FBB	… Fungal Bacterial Biofilm	VAS	… Visual Analogue Scale
FCS	… Foetal Calf Serum		
FESS	… Functional Endoscopic Sinus Surgery		

APPENDIX B:

PRESENTATIONS 0F WORK DONE IN THE THESIS

✦ **Staphylococcus Aureus Biofilms: Nemesis of Endoscopic Sinus Surgery**

The Surgical Research Society 47th Annual Scientific Meeting,
Basil Hetzel Institute, Adelaide, Australia.
19th November 2010

✦ **Impact of Biofilms on Outcomes of Endoscopic Sinus Surgery**

Sir Edward Hughes Memorial Award
The Cabrini-Monash University, Melbourne, Australia
17th October 2009

✦ **Impact of Biofilms on Outcomes of Endoscopic Sinus Surgery**

Annual Meeting of The American Rhinology Society
San Diego, California, USA
3rd October 2009

✦ **Post Surgical Outcomes of Endoscopic Sinus Surgery, done for chronic Rhinosinusitis associated with Biofilms**

Annual Meeting of the Australasian Society of Head Neck Surgery
Gold Coast, Queensland, Australia
23rd May 2009

PUBLICATIONS FROM WORK DONE FOR THE THESIS

✦ **The impact of biofilms on outcomes after endoscopic sinus surgery**

Singhal D, Psaltis A.J, Foreman A, Wormald P.J.
American Journal of Rhinology & Allergy, 2010 May; **24** (3): p. 169-74.

✦ *Staphylococcus Aureus* **Biofilms: Nemesis of Endoscopic Sinus Surgery**

Singhal D, Bardy J.J, Foreman A, Wormald PJ,
Laryngoscope, 2011 July 2011; 121 (7): p. 1578-1583

✦ *Aspergillus fumigatus* **biofilm on primary sinonasal epithelial culture**

Singhal D, Baker L, Wormald PJ, Tan LW
American Journal of Rhinology & Allergy, 2011 July/August; **25** (4): p. 219-225

✦ **Quantitative analysis of in-vivo mucosal biofilms**

Singhal D, Boase S, Field J, Jardeleza C, Foreman A, Wormald PJ
International Forum of Allergy & Rhinology, 2011; 00:xx-xx

- 148 -

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Fokkens, W., Lund, V., Mullol, J., *European position paper on rhinosinusitis and nasal polyps 2007.* Rhinology. Supplement, 2007(20): p. 1-136.

2. Meltzer, E.O., Hamilos, D. L., Hadley, J. A., et al., *Rhinosinusitis: Establishing definitions for clinical research and patient care.* Otolaryngol Head Neck Surg,, 2004. **131(6 Suppl)**: p. S1-62.

3. Stewart M, F.B., Fromer L, *Epidemiology and burden of nasal congestion.* International Journal of General Medicine, 2010. **3**: p. 37-45.

4. Ahsan, S.F., Jumans, S., Nunez , D.A., *Chronic rhinosinusitis: A comparitive study of disease occurence in North of Scotland and Southern Carribean otolaryngology outpatient clinics over a two month period.* Scott Med J, 2004 Nov. **49**(4): p. 130-3.

5. Pleis, J.R., Coles, R., *Summary health statistics for US adults: National health Interview Survey, 1998. National Centre for Health Statistics.* Vital Health Stat, 2002. **10**: p. 1-113.

6. Collins, J., *Prevalence of selected chronic conditions: United states, 1990-1992.* Vital Health Stat, 1997. **10**(194): p. 1-89.

7. Chen, Y., Dales, R., Lin, M., *The epidemiology of chronic rhinosinusitis in Canadians.* Laryngoscope, 2003. **113**(7): p. 1199-205.

8. Ray, N.F., Baraniuk, J.N., Thamer, M. et al, , *Healthcare expenditure for sinusitis in 1996: contributions of asthma, rhinitis and other airway disorders.* Journal of Allergy and Clinical Immunology, 1999. **103**: p. 408-414.

9. Anand, V., *Epidemiology and economic impact of rhinosinusitis.* Ann Otol Rhinol Laryngol Suppl, 2004. **193**: p. 3-5.

10. Gliklich, R.E., Metson, R., *The health impact of chronic sinusitis in patients seeking otolaryngologic care.* Otolaryngology - Head and Neck Surgery, 1995. **113**(1): p. 104-109.

11. Hadfield, P.J., Rowe-Jones, J.M., Mackay, I.S., *The prevalence of nasal polyps in adults with cystic fibrosis.* Clinical Otolaryngol, 2000. **25**(1): p. 19-22.

12. Krause, H.F., *Allergy and chronic rhinosinusitis.* Otolaryngol Head Neck Surg,, 2003. **128**(1): p. 14-6.

13. Bosquet, J., Van Cauwenberge, P., Khaltaev, N., *Allergic rhinitis and its impact on asthma.* Journal of Allergy and Clinical Immunology, 2001 Nov. **108**(5(suppl)): p. s147-334.

14. Ogino, S., Harada, T., Okawachi, I., Irifune, M., Matsunaga, T. , *Aspirin induced asthma and nasal polyps.* Acta Otolaryngol Suppl, 1986. **430**: p. 21-7.

15. Chee, L., Graham, S.M., Carothers, D.G., Ballas, Z.K.,, *Immune dysfunction in refractory sinusitis in a tertiary care setting.* Laryngoscope, 2001. **111**(2): p. 233-5.

16.   Ozdek, A., Cirak, M.Y., Samim, E., Bayiz, U., Safak, M.A., Turet, S., *A possible role of Helicobacter pylori in chronic rhinosinusitis: a preliminary report.* Laryngoscope, 2003. **113**(4): p. 679-82.

17.   Chiu, A.G., *Osteitis in chronic rhinosinusitis.* Otolaryngologic Clinics of North America, 2005. **38**(6): p. 1237-1242.

18.   Lal, D., Scianna, J. M., Stankiewicz, J. A., *Efficacy of targeted medical therapy in chronic rhinosinusitis, and predictors of failure.* American Journal of Rhinology and Allergy, 2009. **23**(4): p. 396-400.

19.   Fokkens, W.J., Lund, V., Mullol, J., *European position paper on rhinosinusitis and nasal polyps 2007.* Rhinology, 2007. **45**(SUPPL. 20): p. 1-136.

20.   Wood, A.J., Douglas, R. G., *Pathogenesis and treatment of chronic rhinosinusitis.* Postgraduate Medical Journal, 2010. **86**(1016): p. 359-364.

21.   Wallwork, B., Coman, W.,Mackay-Sim, A.,Cervin, A., *Effect of Clarithromycin on Nuclear Factor-ÎºB and Transforming Growth Factor-Î² in Chronic Rhinosinusitis.* Laryngoscope, 2004. **114**(2): p. 286-290.

22.   Wallwork, B., Coman, W., Feron, F., Mackay-Sim, A., Cervin, A., *Clarithromycin and prednisolone inhibit cytokine production in chronic rhinosinusitis.* Laryngoscope, 2002. **112**(10): p. 1827-1830.

23.   Wallwork, B., Coman, W., Feron, F., Mackay-Sim, A., Greiff, L., Cervin, A., *A double-blind, randomized, placebo-controlled trial of macrolide in the treatment of chronic rhinosinusitis.* Laryngoscope, 2006. **116**(2): p. 189-193.

24.   Harvey, R., Hannan, S. A., Badia, L., Scadding, G., *Nasal saline irrigations for the symptoms of chronic rhinosinusitis.* Cochrane database of Systematic Reviews, 2007(3).

25.   Hissaria, P., Smith, W., Wormald, PJ. et al, *Short course of systemic corticosteroids in sinonasal polyposis: a double-blind, randomized placebo-controlled trial with evaluation of outcome measures.* Journal of Allergy and Clinical Immunology, 2006. **116**: p. 1257-81.

26.   Parikh, A., Scadding, G. K., Darby, Y., Baker, R. C., *Topical corticosteroids in chronic rhinosinusitis: A randomized, double-blind, placebo-controlled trial using fluticasone propionate aqueous nasal spray.* Rhinology, 2001. **39**(2): p. 75-79.

27.   Lund, V.J., Black, J. H., SzabÃ³, L. Z., Schrewelius, C., Ãkerlund, A., *Efficacy and tolerability of budesonide aqueous nasal spray in chronic rhinosinusitis patients.* Rhinology, 2004. **42**(2): p. 57-62.

28.   Poetker, D.M., Smith, T. L., *Adult chronic rhinosinusitis: Surgical outcomes and the role of endoscopic sinus surgery.* Current Opinion in Otolaryngology and Head and Neck Surgery, 2007. **15**(1): p. 6-9.

29.   Smith, T.L., Batra, P. S., Seiden, A. M., Hanley, M., *Evidence supporting endoscopic sinus surgery in the management of adult chronic rhinosinusitis: A systematic review.* American Journal of Rhinology, 2005. **19**(6): p. 537-543.

30. Vleming, M., De Vries, N., *Endoscopic paranasal sinus surgery: Results.* Am J Rhinol, 1990. **4**(1): p. 13-17.

31. Kennedy, D.W., *Prognostic factors, outcomes and staging in ethmoid sinus surgery.* Laryngoscope, 1992. **102**(12 II): p. 1-18.

32. Westall, F., de Wit, M.J., Dann, J., van der Gaast, S., et al, *Early archean fossil bacteria and biofilms in hydrothermally influenced sediments from the Barberton greenstone belt, South Africa.* Precambrian Res, 2001. **106**(93-116).

33. Rasmussen, T.B., *Filamentous microfossils in a 3,235-million-year-old volcanogenic massive sulphide deposit.* Nature, 2000. **405**(676-679).

34. Ring, M.E., *Antoni van Leeuwenhoek and the tooth worm.* Journal of American Dent Assoc, 1971. **83**(999-1001).

35. Jones, H.C., RothI, I.L., Sanders, W.M. 3rd, , *Electron microscopic study of a slime layer.* J Bacteriol,, 1969. **99**(1): p. 316-25.

36. Characklis, W., *Attached Microbial Growths-II. Fritional resistance to microbial slimes.* Water Research, 1973. **7**: p. 1249-58.

37. Costerton, J.W., Geesey, G. G., Cheng, K. J., *How bacteria stick.* Scientific American, 1978. **238**(1): p. 86-95.

38. Donlan, R.M., Costerton, J. W., *Biofilms: Survival mechanisms of clinically relevant microorganisms.* Clinical Microbiology Reviews, 2002. **15**(2): p. 167-193.

39. Donlan, R.M., *Biofilms: Microbial life on surfaces.* Emerging Infectious Diseases, 2002. **8**(9): p. 881-890.

40. Donlan, R.M., *Biofilms and Device-Associated Infections.* Emerging Infectious Diseases, Mar-Apr 2001. **7**(2): p. 277-281.

41. Lawrence, J.R., Korber, D. R., Hoyle, B. D., Costerton, J. W., Caldwell, D. E., *Optical sectioning of microbial biofilms.* Journal of Bacteriology, 1991. **173**(20): p. 6558-6567.

42. Costerton, W., Veeh, R., Shirtliff, M., Pasmore, M., Post, C., Ehrlich, G., *The application of biofilm science to the study and control of chronic bacterial infections.* Journal of Clinical Investigation, 2003. **112**(10): p. 1466-1477.

43. Mah, T.F.C., O'Toole, G.A., *Mechanisms of biofilm resistance to antimicrobial agents* Trends in Microbiology, 2001. **9**(1): p. 6558-6567.

44. Webb, J.S., Thompson, L. S., James, S., Charlton, T., Tolker-Nielsen, T., Koch, B., Givskov, M., Kjelleberg, S., *Cell death in Pseudomonas aeruginosa biofilm development.* Journal of Bacteriology, 2003. **185**(15): p. 4585-4592.

45. Sutherland, I.W., *The biofilm matrix - An immobilized but dynamic microbial environment.* Trends in Microbiology, 2001. **9**(5): p. 222-227.

46. Sutherland, I.W., *Exopolysaccharides in biofilms, flocs and related structures*, in *Water Science and Technology*. 2001. p. 77-86.

47.   Whitchurch, C.B., Tolker-Nielsen, T., Ragas, P. C., Mattick, J. S., *Extracellular DNA required for bacterial biofilm formation.* Science, 2002. **295**(5559): p. 1487.

48.   Hall-Stoodley, L., Costerton, J. W., Stoodley, P., *Bacterial biofilms: From the natural environment to infectious diseases.* Nature Reviews Microbiology, 2004. **2**(2): p. 95-108.

49.   Hall-Stoodley, L., Stoodley, P., *Evolving concepts in biofilm infections.* Cellular Microbiology, 2009. **11**(7): p. 1034-1043.

50.   Stoodley, P., Sauer, K., Davies, D. G., Costerton, J. W., *Biofilms as complex differentiated communities*, in *Annual Review of Microbiology*. 2002. p. 187-209.

51.   Zhang, L., Mah, T. F., *Involvement of a novel efflux system in biofilm-specific resistance to antibiotics.* Journal of Bacteriology, 2008. **190**(13): p. 4447-4452.

52.   Lenz, A.P., Williamson, K. S., Pitts, B., Stewart, P. S., Franklin, M. J., *Localized gene expression in Pseudomonas aeruginosa biofilms.* Applied and Environmental Microbiology, 2008. **74**(14): p. 4463-4471.

53.   Bagge, N., Hentzer, M., Andersen, J. B., Ciofu, O., Givskov, M., HÃ¸iby, N., *Dynamics and Spatial Distribution of Î²-Lactamase Expression in Pseudomonas aeruginosa Biofilms.* Antimicrobial Agents and Chemotherapy, 2004. **48**(4): p. 1168-1174.

54.   Jurcisek, J.A., Bakaletz, L. O., *Biofilms formed by nontypeable Haemophilus influenzae in vivo contain both double-stranded DNA and type IV pilin protein.* Journal of Bacteriology, 2007. **189**(10): p. 3868-3875.

55.   Pearson, M.M., Laurence, C. A., Guinn, S. E., Hansen, E. J., *Biofilm formation by Moraxella catarrhalis in vitro: Roles of the UspA1 adhesin and the Hag hemagglutinin.* Infection and Immunity, 2006. **74**(3): p. 1588-1596.

56.   Ehrlich, G.D., Veeh, R., Wang, X., Costerton, W.J., Hayes, J. D., Hu, F. Z., Daigle, B. J., Ehrlich, M. D., Christopher Post, J., *Mucosal biofilm formation on middle-ear mucosa in the chinchilla model of otitis media.* Journal of the American Medical Association, 2002. **287**(13): p. 1710-1715.

57.   Sauer, K., Camper, A. K., Ehrlich, G. D., Costerton, J. W., Davies, D. G., *Pseudomonas aeruginosa displays multiple phenotypes during development as a biofilm.* Journal of Bacteriology, 2002. **184**(4): p. 1140-1154.

58.   Davies, D.G., Parsek, M. R., Pearson, J. P., Iglewski, B. H., Costerton, J. W., Greenberg, E. P., *The involvement of cell-to-cell signals in the development of a bacterial biofilm.* Science, 1998. **280**(5361): p. 295-298.

59.   Parsek, M.R., Greenberg, E. P., *Quorum sensing signals in development of Pseudomonas aeruginosa biofilms*, in *Methods in Enzymology*. 1999. p. 43-55.

60.   Cunliffe, D., Smart, C. A., Alexander, C., Vulfson, E. N., *Bacterial adhesion at synthetic surfaces.* Applied and Environmental Microbiology, 1999. **65**(11): p. 4995-5002.

61.   Dalton, H.M., Poulsen, L. K., Halasz, P., Angles, M. L., Goodman, A. E., Marshall, K. C., *Substratum-induced morphological changes in a marine bacterium and their relevance to biofilm structure.* Journal of Bacteriology, 1994. **176**(22): p. 6900-6906.

62.   Suh, J.D., Ramakrishnan, V., Palmer, J. N., *Biofilms.* Otolaryngologic Clinics of North America. **43**(3): p. 521-530.

63.   MÃ¸ller, S., Korber, D. R., Wolfaardt, G. M., Molin, S., Caldwell, D. E., *Impact of nutrient composition on a degradative biofilm community.* Applied and Environmental Microbiology, 1997. **63**(6): p. 2432-2438.

64.   Grotenhuis, J.T.C., Smit, M., Plugge, C. M., Yuansheng, X., Van Lammeren, A. A. M., Stams, A. J. M., Zehnder, A. J. B., *Bacteriological composition and structure of granular sludge adapted to different substrates.* Applied and Environmental Microbiology, 1991. **57**(7): p. 1942-1949.

65.   Nielsen, A.T., Tolker-Nielsen, T., Barken, K. B., Molin, S., *Role of commensal relationships on the spatial structure of a surface-attached microbial consortium.* Environmental Microbiology, 2000. **2**(1): p. 59-68.

66.   Picioreanu, C., Van Loosdrecht, M. C. M., Heijnen, J. J., *Mathematical modeling of biofilm structure with a hybrid differential- discrete cellular automaton approach.* Biotechnology and Bioengineering, 1998. **58**(1): p. 101-116.

67.   Wimpenny, J.W.T., Colasanti, R., *A unifying hypothesis for the structure of microbial biofilms based on cellular automaton models.* FEMS Microbiology Ecology, 1997. **22**(1): p. 1-16.

68.   Wolfaardt, G.M., Lawrence, J. R., Robarts, R. D., Caldwell, S. J., Caldwell, D. E., *Multicellular organization in a degradative biofilm community.* Applied and Environmental Microbiology, 1994. **60**(2): p. 434-446.

69.   O'Toole, G.A., Kolter, R., *Initiation of biofilm formation in Pseudomonas fluorescens WCS365 proceeds via multiple, convergent signalling pathways: A genetic analysis.* Molecular Microbiology, 1998. **28**(3): p. 449-461.

70.   Stoodley, P., Lewandowski, Z., Boyle, J. D., Lappin-Scott, H. M., *Structural deformation of bacterial biofilms caused by short-term fluctuations in fluid shear: An in situ investigation of biofilm rheology.* Biotechnology and Bioengineering, 1999. **65**(1): p. 83-92.

71.   Van Loosdrecht, M.C.M., Eikelboom, D., Gjaltema, A., Mulder, A., Tijhuis, L., Heijnen, J. J., *Biofilm structures*, in *Water Science and Technology*. 1995. p. 35-43.

72.   Post, J.C., Stoodley, P., Hall-Stoodley, L., Ehrlich, G. D., *The role of biofilms in otolaryngologic infections.* Current Opinion in Otolaryngology and Head and Neck Surgery, 2004. **12**(3): p. 185-190.

73.   Potera, C., *Forging a link between biofilms and disease.* Science, March 1999. **283**(5409): p. 1837-1839.

74.   Ceri, H., Olson, M. E., Stremick, C., Read, R. R., Morck, D., Buret, A., *The Calgary Biofilm Device: New technology for rapid determination of antibiotic*

susceptibilities of bacterial biofilms. Journal of Clinical Microbiology, 1999. **37**(6): p. 1771-1776.

75. Hoyle, B.D., Costerton, J. W., *Bacterial resistance to antibiotics: The role of biofilms.* Progress in Drug Research, 1991. **37**: p. 91-105.

76. Lewis, K., *Persister cells and the riddle of biofilm survival.* Biochemistry (Moscow), 2005. **70**(2): p. 267-274.

77. Lewis, K., *Persister cells, dormancy and infectious disease.* Nature Reviews Microbiology, 2007. **5**(1): p. 48-56.

78. Lewis, K., *Multidrug tolerance of biofilms and persister cells*, in *Current Topics in Microbiology and Immunology.* 2008. p. 107-131.

79. Jefferson, K.K., Goldmann, D. A., Pier, G. B., *Use of confocal microscopy to analyze the rate of vancomycin penetration through Staphylococcus aureus biofilms.* Antimicrobial Agents and Chemotherapy, 2005. **49**(6): p. 2467-2473.

80. Walters, M.C., Roe, F.,Bugnicourt, A., Franklin, M. J., Stewart, P. S., *Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of Pseudomonas aeruginosa biofilms to ciprofloxacin and tobramycin.* Antimicrobial Agents and Chemotherapy, 2003. **47**(1): p. 317-323.

81. Li, X.Z., Nikaido, H., *Efflux-mediated drug resistance in bacteria: An update.* Drugs, 2009. **69**(12): p. 1555-1623.

82. De Kievit, T.R., Parkins, M. D., Gillis, R. J., Srikumar, R., Ceri, H., Poole, K., Iglewski, B. H., Storey, D. G., *Multidrug efflux pumps: Expression patterns and contribution to antibiotic resistance in Pseudomonas aeruginosa biofilms.* Antimicrobial Agents and Chemotherapy, 2001. **45**(6): p. 1761-1770.

83. Gilbert, P., Allison, D. G., McBain, A. J., *Biofilms in vitro and in vivo: do singular mechanisms imply cross-resistance?* Symposium series (Society for Applied Microbiology), 2002(31): p. 98S-110S.

84. Gillis, R.J., White, K. G., Choi, K. H., Wagner, V. E., Schweizer, H. P., Iglewski, B. H., *Molecular basis of azithromycin-resistant Pseudomonas aeruginosa biofilms.* Antimicrobial Agents and Chemotherapy, 2005. **49**(9): p. 3858-3867.

85. Hausner, M., Wuertz, S., *High rates of conjugation in bacterial biofilms as determined by quantitative in situ analysis.* Applied and Environmental Microbiology, 1999. **65**(8): p. 3710-3713.

86. Patel, R., *Biofilms and antimicrobial resistance.* Clinical Orthopaedics and Related Research, 2005(437): p. 41-47.

87. Wuertz, S., Hendrickx, L., Kuehn, M., Rodenacker, K., Hausner, M., *In situ quantification of gene transfer in biofilms*, in *Methods in Enzymology.* 2001. p. 129-143.

88. De Beer, D., Srinivasan, R., Stewart, P. S., *Direct measurement of chlorine penetration into biofilms during disinfection.* Applied and Environmental Microbiology, 1994. **60**(12): p. 4339-4344.

89.   Elasri, M.O., Miller, R. V., *Study of the response of a biofilm bacterial community to UV radiation.* Applied and Environmental Microbiology, 1999. **65**(5): p. 2025-2031.

90.   Lam, J., Chan, R., Lam, K., Costerton, J. W., *Production of mucoid microcolonies by Pseudomonas aeruginosa within infected lungs in cystic fibrosis.* Infection and Immunity, 1980. **28**(2): p. 546-556.

91.   Kirketerp-MÃ¸ller, K., Jensen, P. Ã., Fazli, M., Madsen, K. G., Pedersen, J., Moser, C., Tolker-Nielsen, T., HÃ¸iby, N., Givskov, M., Bjarnsholt, T., *Distribution, organization, and ecology of bacteria in chronic wounds.* Journal of Clinical Microbiology, 2008. **46**(8): p. 2717-2722.

92.   Leid, J.G., Shirtliff, M. E., Costerton, J. W., Stoodley, P., *Human leukocytes adhere to, penetrate, and respond to Staphylococcus aureus biofilms.* Infection and Immunity, 2002. **70**(11): p. 6339-6345.

93.   Jesaitis, A.J., Franklin, M. J., Berglund, D., Sasaki, M., Lord, C. I., Bleazard, J. B., Duffy, J. E., Beyenal, H., Lewandowski, Z., *Compromised host defense on Pseudomonas aeruginosa biofilms: Characterization of neutrophil and biofilm interactions.* Journal of Immunology, 2003. **171**(8): p. 4329-4339.

94.   Cerca, N., Jefferson, K. K., Oliveira, R., Pier, G. B., Azeredo, J., *Comparative antibody-mediated phagocytosis of Staphylococcus epidermidis cells grown in a biofilm or in the planktonic state.* Infection and Immunity, 2006. **74**(8): p. 4849-4855.

95.   De Beer, D., Stoodley, P., Lewandowski, Z., *Measurement of local diffusion coefficients in biofilms by microinjection and confocal microscopy.* Biotechnology and Bioengineering, 1997. **53**(2): p. 151-158.

96.   Fux, C.A., Stoodley, P., Hall-Stoodley, L., Costerton, J. W., *Bacterial biofilms: A diagnostic and therapeutic challenge.* Expert Review of Anti-Infective Therapy, 2003. **1**(4): p. 667-683.

97.   Parsek, M.R., Singh, P. K., *Bacterial Biofilms: An Emerging Link to Disease Pathogenesis*, in *Annual Review of Microbiology.* 2003. p. 677-701.

98.   Harvey, R.J., Lund, V. J., *Biofilms and chronic rhinosinusitis: Systematic review of evidence, current concepts and directions for research.* Rhinology, 2007. **45**(1): p. 3-13.

99.   Macassey, E., Dawes, P., *Biofilms and their role in otorhinolaryngological disease.* Journal of Laryngology and Otology, 2008. **122**(12): p. 1273-1278.

100.   Vlastarakos, P.V., Nikolopoulos, T. P., Maragoudakis, P., Tzagaroulakis, A., Ferekidis, E., *Biofilms in ear, nose, and throat infections: How important are they?* Laryngoscope, 2007. **117**(4): p. 668-673.

101.   Manning, S., *Basics of biofilm and clinical otolaryngology.* Ear Nose Throat Journal, 2003. **82**: p. 18-20.

102.   Morris, D.P., *Bacterial biofilm in upper respiratory tract infections.* Current Infectious Disease Reports, 2007. **9**(3): p. 186-192.

103. Post, J.C., Hiller, N. L., Nistico, L., Stoodley, P., Ehrlich, G. D., *The role of biofilms in otolaryngologic infections: Update 2007.* Current Opinion in Otolaryngology and Head and Neck Surgery, 2007. **15**(5): p. 347-351.

104. Gok, U., Bulut, Y., Keles, E., Yalcin, S., Doymaz, M.Z., *Bacteriological and PCR analysis of clinical material aspirated from otitis media with effusions.* Int J Pediatr Otorhinolaryngol., 2001. **60**(1): p. 49-54.

105. Tonnaer, E.L.G.M., Graamans, K., Sanders, E.A.M., Curfs, J.H.A.J, *Advances in inderstanding the pathogenesis of pneumococcal otitis media.* Pediatric infectious Disease Journal, 2006. **25**(6): p. 546-552.

106. Post, J.C., *Direct evidence of bacterial biofilms in otitis media.* Laryngoscope, 2001. **111**(12): p. 2083-2094.

107. Kerschner, J.E., *Bench and bedside advances in otitis media.* Current Opinion in Otolaryngology and Head and Neck Surgery, 2008. **16**(6): p. 543-547.

108. Hall-Stoodley, L., Hu, F. Z., Gieseke, A., Nistico, L., Nguyen, D., Hayes, J., Forbes, M., Greenberg, D. P., Dice, B., Burrows, A., Wackym, P. A., Stoodley, P., Post, J. C., Ehrlich, G. D., Kerschner, J. E., *Direct detection of bacterial biofilms on the middle-ear mucosa of children with chronic otitis media.* Journal of the American Medical Association, 2006. **296**(2): p. 202-211.

109. Dohar, J.E., Hebda, P. A., Veeh, R., Awad, M., Costerton, J. W., Hayes, J., Ehrlich, G. D., *Mucosal biofilm formation on middle-ear mucosa in a nonhuman primate model of chronic suppurative otitis media.* Laryngoscope, 2005. **115**(8): p. 1469-1472.

110. Post, J.C., Preston, R. A., Aul, J. J., Larkins-Pettigrew, M., Rydquist-White, J., Anderson, K. W., Wadowsky, R. M., Reagan, D. R., Walker, E. S., Kingsley, L. A., Magit, A. E., Ehrlich, G. D., *Molecular analysis of bacterial pathogens in otitis media with effusion.* Journal of the American Medical Association, 1995. **273**(20): p. 1598-1604.

111. Chole, R.A., Faddis, B. T., *Evidence for microbial biofilms in cholesteatomas.* Archives of Otolaryngology - Head and Neck Surgery, 2002. **128**(10): p. 1129-1133.

112. Wang, E.W., Jung, J.Y., Pashia, M.E., Nason, R., Scholnick, S., Chole, R.A. and *Otopathogenic Pseudomonas aeruginosa Strains as Competent Biofilm Formers.* Arch Otolaryngol Head Neck Surg., 2005. **131**: p. 983-98.

113. Dohar JE, H.P., Veeh R, Awad M, Costerton JW, Hayes J, Ehrlich GD., *Mucosal biofilm formation on middle-ear mucosa in a nonhuman primate model of chronic suppurative otitis media.* Laryngoscope, 2005Aug. **115**(8): p. 1469-72.

114. Pinar, E., Ã–ncel, S., KaragÃ¶z, Ãœ, Åžener, G., Ã‡alli, Ã‡, Tatar, B., *Demonstration of bacterial biofilms in chronic otitis media.* Mediterranean Journal of Otology, 2008. **4**(2): p. 64-68.

115. Lee, M.R., Pawlowski, K. S., Luong, A., Furze, A. D., Roland, P. S., *Biofilm presence in humans with chronic suppurative otitis media.* Otolaryngology - Head and Neck Surgery, 2009. **141**(5): p. 567-571.

116. Loeffler, K.A., Johnson, T. A., Burne, R. A., Antonelli, P. J., *Biofilm formation in an in vitro model of cochlear implants with removable magnets.* Otolaryngology - Head and Neck Surgery, 2007. **136**(4): p. 583-588.

117. Johnson, T.A., Loeffler, K. A., Burne, R. A., Jolly, C. N., Antonelli, P. J., *Biofilm formation in cochlear implants with cochlear drug delivery channels in an in vitro model.* Otolaryngology - Head and Neck Surgery, 2007. **136**(4): p. 577-582.

118. Ruellan, K., Frijns, J. H. M., Bloemberg, G. V., Hautefort, C., Van Den Abbeele, T., Lamers, G. E. M., Herman, P., Ba Huy, P. T., Kania, R. E., *Detection of bacterial biofilm on cochlear implants removed because of device failure, without evidence of infection.* Otology and Neurotology, 2010. **31**(8): p. 1320-1324.

119. Pawlowski, K.S., Wawro, D., Roland, P. S., *Bacterial biofilm formation on a human cochlear implant.* Otology and Neurotology, 2005. **26**(5): p. 972-975.

120. Cristobal, R., Edmiston Jr, C. E., Runge-Samuelson, C. L., Owen, H. A., Firszt, J. B., Wackym, P. A., *Fungal biofilm formation on cochlear implant hardware after antibiotic-induced fungal overgrowth within the middle ear.* Pediatric infectious Disease Journal, 2004. **23**(8): p. 774-778.

121. Antonelli, P.J., Lee, J. C., Burne, R. A., *Bacterial biofilms may contribute to persistent cochlear implant infection.* Otology and Neurotology, 2004. **25**(6): p. 953-957.

122. Makarem, A.O., Schaudinn, C., Webster, P., Linthicum, F. H., *Possible role of biofilm in fulminant meningitis related to cochlear implantation of dysplastic inner ear.* Revue de Laryngologie Otologie Rhinologie, 2009. **129**(4-5): p. 245-248.

123. Brady, A.J., Farnan, T. B., Toner, J. G., Gilpin, D. F., Tunney, M. M., *Treatment of a cochlear implant biofilm infection: A potential role for alternative antimicrobial agents.* Journal of Laryngology and Otology. **124**(7): p. 729-738.

124. Kos, M.I., Stenz, L., François, P., Guyot, J. P., Schrenzel, J., *Immuno-detection of Staphylococcus aureus biofilm on a cochlear implant.* Infection, 2009. **37**(5): p. 450-454.

125. Jaryszak, E.M., Sampson, E. M., Antonelli, P. J., *Effect of ossicular prosthesis biofilms on middle ear scarring and hearing outcomes.* Otology and Neurotology, 2009. **30**(8): p. 1191-1195.

126. Barakate, M., Beckenham, E., Curotta, J.,Da Cruz, M. D., *Bacterial biofilm adherence to middle-ear ventilation tubes: Scanning electron micrograph images and literature review.* Journal of Laryngology and Otology, 2007. **121**(10): p. 993-997.

127. Berry, J.A., Biedlingmaier, J. F., Whelan, P. J., *In vitro resistance to bacterial biofilm formation on coated fluoroplastic tympanostomy tubes.* Otolaryngology - Head and Neck Surgery, 2000. **123**(3): p. 246-251.

128. Biedlingmaier, J.F., Samaranayake, R., Whelan, P., *Resistance to biofilm formation on otologic implant materials.* Otolaryngology - Head and Neck Surgery, 1998. **118**(4): p. 444-451.

129. Jang, C.H., Park, H., Cho, Y. B., Choi, C. H., *Effect of vancomycin-coated tympanostomy tubes on methicillin-resistant Staphylococcus aureus biofilm formation: In vitro study.* Journal of Laryngology and Otology. **124**(6): p. 594-598.

130. Jang, C.H., Cho, Y. B., Choi, C. H., *Structural features of tympanostomy tube biofilm formation in ciprofloxacin-resistant Pseudomonas otorrhea.* International Journal of Pediatric Otorhinolaryngology, 2007. **71**(4): p. 591-595.

131. Jang, C.H., Park, H., Cho, Y. B., Choi, C. H.,Park, I. Y., *The use of piperacillin-tazobactam coated tympanostomy tubes against ciprofloxacin-resistant Pseudomonas biofilm formation: An in vitro study.* International Journal of Pediatric Otorhinolaryngology, 2009. **73**(2): p. 295-299.

132. Malaty, J., Antonelli, P. J., *Effect of blood and mucus on tympanostomy tube biofilm formation.* Laryngoscope, 2008. **118**(5): p. 867-870.

133. Mehta, A.J., Lee, J. C., Stevens, G. R., Antonelli, P. J., *Opening plugged tympanostomy tubes: Effect of biofilm formation.* Otolaryngology - Head and Neck Surgery, 2006. **134**(1): p. 121-125.

134. Oxley, K.S., Thomas, J. G., Ramadan, H. H., *Effect of ototopical medications on tympanostomy tube biofilms.* Laryngoscope, 2007. **117**(10): p. 1819-1824.

135. Saidi, I.S., Biedlingmaier, J. F., Whelan, P., *In vivo resistance to bacterial biofilm formation on tympanostomy tubes as a function of tube material.* Otolaryngology - Head and Neck Surgery, 1999. **120**(5): p. 621-627.

136. Tatar, E.C., Ãœnal, F. Ã–, Tatar, I., Celik, H. H., Gursel, B., *Investigation of surface changes in different types of ventilation tubes using scanning electron microscopy and correlation of findings with clinical follow-up.* International Journal of Pediatric Otorhinolaryngology, 2006. **70**(3): p. 411-417.

137. Chole, R.A., Faddis, B. T., *Anatomical evidence of microbial biofilms in tonsillar tissues: A possible mechanism to explain chronicity.* Archives of Otolaryngology - Head and Neck Surgery, 2003. **129**(6): p. 634-636.

138. Galli, J., Ardito, F., CalÃ², L., Mancinelli, L., Imperiali, M., Parrilla, C., Picciotti, P. M., Fadda, G., *Recurrent upper airway infections and bacterial biofilms.* Journal of Laryngology and Otology, 2007. **121**(4): p. 341-344.

139. Kania, R.E., Lamers, G. E. M., Vonk, M. J., Huy, P. T. B., Hiemstra, P. S., Bloemberg, G. V., Grote, J. J., *Demonstration of bacterial cells and glycocalyx in biofilms on human tonsils.* Archives of Otolaryngology - Head and Neck Surgery, 2007. **133**(2): p. 115-121.

140. Stoodley, P., deBeer, D., Longwell, M., Nistico, L., Hall-Stoodley, L., Wenig, B., Krespi, Y. P., *Tonsillolith: Not just a stone but a living biofilm.* Otolaryngology - Head and Neck Surgery, 2009. **141**(3): p. 316-321.

141. Coticchia, J., Zuliani, G., Coleman, C., Carron, M., Gurrola Ii, J., Haupert, M., Berk, R., *Biofilm surface area in the pediatric nasopharynx: Chronic rhinosinusitis vs obstructive sleep apnea.* Archives of Otolaryngology - Head and Neck Surgery, 2007. **133**(2): p. 110-114.

142. Zuliani, G., Carron, M., Gurrola, J., Coleman, C., Haupert, M., Berk, R., Coticchia, J., *Identification of adenoid biofilms in chronic rhinosinusitis.* International Journal of Pediatric Otorhinolaryngology, 2006. **70**(9): p. 1613-1617.

143. Ungkanont, K., Damrongsak, S., *Effect of adenoidectomy in children with complex problems of hinosinusitis and associated diseases.* International Journal of Pediatric Otorhinolaryngology, 2004. **68**(447-51).

144. Abdul-Baqi, K.J., Shakhatreh, F.M., Khader, Q.A., *Use of adenoidectomy and adenotonsillectomy in children with otitis media with effusion.* Ear Nose Throat Journal, 2001 Sep. **80**(9): p. 647-50.

145. Saylam, G., Tatar, E. Ã‡, Tatar, I., Ãzdek, A., Korkmaz, H., *Association of adenoid surface biofilm formation and chronic otitis media with effusion.* Archives of Otolaryngology - Head and Neck Surgery. **136**(6): p. 550-555.

146. Buijssen, K.J.D.A., Harmsen, H. J. M., van der Mei, H. C., Busscher, H. J., van der Laan, B. F. A. M., *Lactobacilli: Important in biofilm formation on voice prostheses.* Otolaryngology - Head and Neck Surgery, 2007. **137**(3): p. 505-507.

147. Everaert, E.P.J.M., Mahieu, H. F., Van De Belt-Gritter, B., Peeters, A. J. G. E., Verkerke, G. J., Van Der Mei, H. C., Busscher, H. J., *Biofilm formation in vivo on perfluoro-alkylsiloxane-modified voice prostheses.* Archives of Otolaryngology - Head and Neck Surgery, 1999. **125**(12): p. 1329-1332.

148. Elving, G.J., Van der Mei, H., Busscher, H., Van Weissenbruch, R., Albers, F., *Influence of different combinations of bacteria and yeasts in voice prosthesis biofilms on air flow resistance.* ANTONIE VAN LEEUWENHOEK, 2003. **83**(1): p. 45-55.

149. Jarrett, W.A., Ribes, J., Manaligod, J. M., *Biofilm formation on tracheostomy tubes.* Ear, Nose and Throat Journal, 2002. **81**(9): p. 659-661.

150. Meslemani, D., Yaremchuk, K., Rontal, M., *Presence of biofilm on adult tracheostomy tubes.* Ear, Nose and Throat Journal. **89**(10): p. 496-504.

151. Perkins, J., Mouzakes, J., Pereira, R., Manning, S., *Bacterial Biofilm Presence in Pediatric Tracheotomy Tubes.* Archives of Otolaryngology - Head and Neck Surgery, 2004. **130**(3): p. 339-343.

152. Solomon, D.H., Wobb, J., Buttaro, B. A., Truant, A., Soliman, A. M. S., *Characterization of bacterial biofilms on tracheostomy tubes.* Laryngoscope, 2009. **119**(8): p. 1633-1638.

153. Cohen, M., Kofonow, J., Nayak, J.V., Palmer, J.N., Chiu, A.G.,Leid, J.G., Cohen, N.A.,, *Biofilms in chronic rhinosinusitis: A review.* American Journal of Rhinology and Allergy, 2009. **23**(3): p. 255-260.

154. Palmer, J.N., *Bacterial biofilms: Do they play a role in chronic sinusitis?* Otolaryngologic Clinics of North America, 2005. **38**(6): p. 1193-1201.

155. Kilty, S.J., Desrosiers, M. Y., *The role of bacterial biofilms and the pathophysiology of chronic rhinosinusitis.* Current Allergy and Asthma Reports, 2008. **8**(3): p. 227-233.

156. Kilty, S.J., Desrosiers, M. Y., *Are Biofilms the Answer in the Pathophysiology and Treatment of Chronic Rhinosinusitis?* Immunology and Allergy Clinics of North America, 2009. **29**(4): p. 645-656.

157. Hunsaker, D.H., Leid, J. G., *The relationship of biofilms to chronic rhinosinusitis.* Current Opinion in Otolaryngology and Head and Neck Surgery, 2008. **16**(3): p. 237-241.

158. Suh, J.D., Cohen, N. A., Palmer, J. N., *Biofilms in chronic rhinosinusitis.* Current Opinion in Otolaryngology and Head and Neck Surgery. **18**(1): p. 27-31.

159. Palmer, J., *Bacterial biofilms in chronic rhinosinusitis.* The Annals of otology, rhinology & laryngology. Supplement., 2006. **196**: p. 35-39.

160. Ramadan, H.H., *Chronic rhinosinusitis and bacterial biofilms.* Current Opinion in Otolaryngology and Head and Neck Surgery, 2006. **14**(3): p. 183-186.

161. Cryer, J., Schipor, I., Perloff, J. R., Palmer, J. N., *Evidence of bacterial biofilms in human chronic sinusitis.* ORL, 2004. **66**(3): p. 155-158.

162. Perloff, J.R., Palmer, J. N., *Evidence of bacterial biofilms on frontal recess stents in patients with chronic rhinosinusitis.* American Journal of Rhinology, 2004. **18**(6): p. 377-380.

163. Perloff, J.R., Palmer, J. N., *Evidence of bacterial biofilms in a rabbit model of sinusitis.* American Journal of Rhinology, 2005. **19**(1): p. 1-6.

164. Ramadan, H.H., Sanclement, J. A., Thomas, J. G., *Chronic rhinosinusitis and biofilms.* Otolaryngology - Head and Neck Surgery, 2005. **132**(3): p. 414-417.

165. Sanclement, J.A., Webster, P., Thomas, J., Ramadan, H. H., *Bacterial biofilms in surgical specimens of patients with chronic rhinosinusitis.* Laryngoscope, 2005. **115**(4): p. 578-582.

166. Ferguson, B.J., Stolz, D. B., *Demonstration of biofilm in human bacterial chronic rhinosinusitis.* American Journal of Rhinology, 2005. **19**(5): p. 452-457.

167. Ha, K.R., Psaltis, A. J., Tan, L., Wormald, P. J., *A sheep model for the study of biofilms in rhinosinusitis.* American Journal of Rhinology, 2007. **21**(3): p. 339-345.

168. Psaltis, A.J., Ha, K. R., Beule, A. G., Tan, L. W., Wormald, P. J., *Confocal scanning laser microscopy evidence of biofilms in patients with chronic rhinosinusitis.* Laryngoscope, 2007. **117**(7): p. 1302-1306.

169. Woodworth, B.A., Tamashiro, E., Bhargave, G., Cohen, N. A., Palmer, J. N., *An in vitro model of Pseudomonas aeruginosa biofilms on viable airway epithelial cell monolayers.* American Journal of Rhinology, 2008. **22**(3): p. 235-238.

170. Dworniczek, E., Fraczek, M., Seniuk, A., Kassner, J., SobieszczaÅ„ska, B., Adamski, J., Ciesielska, U., *Bacterial biofilms in patients with chronic rhinosinusitis.* Folia Microbiologica, 2009. **54**(6): p. 559-562.

171. Foreman, A., Singhal, D., Psaltis, A. J., Wormald, P. J., *Targeted imaging modality selection for bacterial biofilms in chronic rhinosinusitis.* Laryngoscope, 2010. **120**(2): p. 427-431.

172. Sanderson, A.R., Leid, J. G., Hunsaker, D., *Bacterial biofilms on the sinus mucosa of human subjects with chronic rhinosinusitis.* Laryngoscope, 2006. **116**(7): p. 1121-1126.

173. Foreman, A., Psaltis, A. J., Tan, L. W., Wormald, P. J., *Characterization of bacterial and fungal biofilms in chronic rhinosinusitis.* American Journal of Rhinology and Allergy, 2009. **23**(6): p. 556-561.

174. Bendouah, Z., Barbeau, J., Hamad, W. A., Desrosiers, M., *Use of an in vitro assay for determination of biofilm-forming capacity of bacteria in chronic rhinosinusitis.* American Journal of Rhinology, 2006. **20**(5): p. 434-438.

175. Prince, A.A., Steiger, J. D., Khalid, A. N., Dogrhamji, L., Reger, C., Claire, S. E., Chiu, A. G., Kennedy, D. W., Palmer, J. N., Cohen, N. A., *Prevalence of biofilm-forming bacteria in chronic rhinosinusitis.* American Journal of Rhinology, 2008. **22**(3): p. 239-245.

176. Oncel, S., Pinar, E., Sener, G., Calli, C., Karagoz, U., *Evaluation of bacterial biofilms in chronic rhinosinusitis.* Journal of Otolaryngology - Head and Neck Surgery, 2010. **39**(1): p. 52-55.

177. Hochstim, C.J., Choi, J. Y., Lowe, D., Masood, R., Rice, D. H., *Biofilm detection with hematoxylin-eosin staining.* Archives of Otolaryngology - Head and Neck Surgery, 2010. **136**(5): p. 453-456.

178. Hochstim, C.J., Masood, R., Rice, D.H., *Biofilm and persistent inflammation in endoscopic sinus surgery.* Otolaryngology-head and neck surgery, 2010. **143**: p. 697-698.

179. Le, T., Psaltis, A., Tan, L. W., Wormald, P. J., *The efficacy of topical antibiofilm agents in a sheep model of rhinosinusitis.* American Journal of Rhinology, 2008. **22**(6): p. 560-567.

180. Desrosiers, M., Myntti, M., James, G., *Methods for removing bacterial biofilms: In vitro study using clinical chronic rhinosinusitis specimens.* American Journal of Rhinology, 2007. **21**(5): p. 527-532.

181. Desrosiers, M., Bendouah, Z., Barbeau, J., *Effectiveness of topical antibiotics on Staphylococcus aureus biofilm in vitro.* American Journal of Rhinology, 2007. **21**(2): p. 149-153.

182. Hai, P.V.T., Lidstone, C., Wallwork, B., *The effect of endoscopic sinus surgery on bacterial biofilms in chronic rhinosinusitis.* Otolaryngology - Head and Neck Surgery, 2010. **142**(3 SUPPL. 1): p. S27-S32.

183. Bendouah, Z., Barbeau, J., Hamad, W. A., Desrosiers, M., *Biofilm formation by Staphylococcus aureus and Pseudomonas aeruginosa is associated with an unfavorable evolution after surgery for chronic sinusitis and nasal polyposis.* Otolaryngology - Head and Neck Surgery, 2006. **134**(6): p. 991-996.

184. Psaltis, A.J., Weitzel, E. K., Ha, K. R., Wormald, P. J., *The effect of bacterial biofilms on post-sinus surgical outcomes.* American Journal of Rhinology, 2008. **22**(1): p. 1-6.

185. Hekiert, A.M., Kofonow, J. M., Doghramji, L., Kennedy, D. W., Chiu, A. G., Palmer, J. N., Leid, J. G., Cohen, N. A., *Biofilms correlate with TH1 inflammation in the sinonasal tissue of patients with chronic rhinosinusitis.* Otolaryngology - Head and Neck Surgery, 2009. **141**(4): p. 448-453.

186. Braun, H., Stammberger, H., Buzina, W., Freudenschuss, K., Lackner, A., Beham, A., *Incidence and detection of fungi and eosinophilic granulocytes in chronic rhinosinusitis.* Häufigkeit und nachweis von pilzen und eosinophilen granulozyten bei chronischer rhinosinusitis, 2003. **82**(5): p. 330-340.

187. Taylor, M.J., Ponikau, J. U., Sherris, D. A., Kern, E. B., Gaffey, T. A., Kephart, G., Kita, H., *Detection of fungal organisms in eosinophilic mucin using a fluorescein-labeled chitin-specific binding protein.* Otolaryngology - Head and Neck Surgery, 2002. **127**(5): p. 377-383.

188. Katzenstein, A.L.A., Sale, S. R., Greenberger, P. A., *Allergic Aspergillus sinusitis: A newly recognized form of sinusitis.* Journal of Allergy and Clinical Immunology, 1983. **72**(1): p. 89-93.

189. Kim, S.T., Choi, J. H., Jeon, H. G., *Comparison between polymerase chain reaction and fungal culture for the detection of fungi in patients with chronic sinusitis and normal controls.* Acta Otolaryngol, 2005. **125**: p. 72-75.

190. Murr, A.H., Goldberg, A. N., Vesper, S., *Fungal speciation using quantitative polymerase chain reaction (QPCR) in patients with and without chronic rhinosinusitis.* Laryngoscope, 2006. **116**(8): p. 1342-1348.

191. Polzehl, D., Weschta, M., Podbielski, A., Riechelmann, H., Rimek, D., *Fungus culture and PCR in nasal lavage samples of patients with chronic rhinosinusitis.* Journal of Medical Microbiology, 2005. **54**(1): p. 31-37.

192. Ponikau, J.U., *The diagnosis and incidence of allergic fungal sinusitis.* Mayo Clinic Proceedings, 1999. **74**(9): p. 877-884.

193. Rao, A.K., Mathers, P. H., Ramadan, H. H., *Detection of fungi in the sinus mucosa using polymerase chain reaction.* Otolaryngology - Head and Neck Surgery, 2006. **134**(4): p. 581-585.

194. Scheuller, M.C., Murr, A. H., Goldberg, A. N., Mhatre, A. N., Lalwani, A. K., *Quantitative Analysis of Fungal DNA in Chronic Rhinosinusitis.* Laryngoscope, 2004. **114**(3): p. 467-471.

195. Weschta, M., Rimek, D., Formanek, M., Polzehl, D., Podbielski, A., Riechelmann, H., *Topical antifungal treatment of chronic rhinosinusitis with nasal polyps: A randomized, double-blind clinical trial.* Journal of Allergy and Clinical Immunology, 2004. **113**(6): p. 1122-1128.

196. Buzina, W., Braun, H., Freudenschuss, K., Lackner, A., Habermann, W., Stammberger, H., *Fungal biodiversity - As found in nasal mucus.* Medical Mycology, 2003. **41**(2): p. 149-161.

197. Kennedy, D.W., Kuhn, F. A., Hamilos, D. L., Zinreich, S. J., Butler, D., Warsi, G., Poster, P. J., Tavakkol, A., *Treatment of chronic rhinosinusitis with high-dose oral terbinafine: A double blind, placebo-controlled study.* Laryngoscope, 2005. **115**(10 I): p. 1793-1799.

198. Corradini, C., Del Ninno, M., Buonomo, A., Nucera, E., Paludetti, G., Alonzi, C., Sabato, V., Schiavino, D., Patriarca, G., *Amphotericin B and lysine acetylsalicylate in the combined treatment of nasal polyposis associated with mycotic infection.* Journal of Investigational Allergology and Clinical Immunology, 2006. **16**(3): p. 188-193.

199. Granville, L., Chirala, M., Cernoch, P., Ostrowski, M., Truong, L. D., *Fungal Sinusitis: Histologic Spectrum and Correlation with Culture.* Human Pathology, 2004. **35**(4): p. 474-481.

200. Jiang, R.S., Su, M. C., Lin, J. F., *Nasal mycology of chronic rhinosinusitis.* American Journal of Rhinology, 2005. **19**(2): p. 131-133.

201. Kostamo, K., Richardson, M., Virolainen-Julkunen, A., Leivo, I., Malmberg, H., Ylikoski, J., Toskala, E., *Microbiology of chronic hyperplastic sinusitis.* Rhinology, 2004. **42**(4): p. 213-218.

202. Collins, M.M., Nair, S.B., Wormald, P.J.. *Prevalence of noninvasive fungal sinusitis in South Australia.* American Journal of Rhinology, 2003. **17**(3): p. 127-132.

203. Catten, M.D., Murr, A. H., Goldstein, J. A., Mhatre, A. N., Lalwani, A. K., *Detection of fungi in the nasal mucosa using polymerase chain reaction.* Laryngoscope, 2001. **111**(3): p. 399-403.

204. Deshazo, R.D., et al.,   . (): p. . *A new classification and diagnostic criteria for invasive fungal sinusitis.* Archives of Otolaryngology - Head and Neck Surgery,, 1997. **123**(11): p. 1181-1188.

205. Chakrabarti, A., Denning, D.W., Ferguson, B.J., et al. , *Fungal rhinosinusitis: a categorization and definitional schema addressing current controversies.* Laryngoscope, 2009. **119**: p. 1809-18.

206. Orlandi, R.R., Marple, B.F.,, *The Role of Fungus in Chronic Rhinosinusitis.* Otolaryngol Clin N Am 2010. **43**: p. 531-537.

207. Ebbens, F.A., Georgalas, C., Rinia, A.B., van Drunen, C.M., Lund, V.J., Fokkens, W.J., *The fungal debate: Where do we stand today?* Rhinology, 2007. **45**(3): p. 178-189.

208. Ebbens, F.A., Fokkens, W. J., *The mold conundrum in chronic rhinosinusitis: Where do we stand today?* Current Allergy and Asthma Reports, 2008. **8**(2): p. 93-101.

209. Ebbens, F.A., Georgalas, C., Fokkens, W. J., *The mold conundrum in chronic hyperplastic sinusitis.* Current Allergy and Asthma Reports, 2009. **9**(2): p. 114-120.

210. Ebbens, F.A., Georgalas, C., Fokkens, W. J., *Fungus as the cause of chronic rhinosinusitis: The case remains unproven.* Current Opinion in Otolaryngology and Head and Neck Surgery, 2009. **17**(1): p. 43-49.

211. Fokkens, W.J., Ebbens, F., van Drunen, C. M., *Fungus: A Role in Pathophysiology of Chronic Rhinosinusitis, Disease Modifier, Â Treatment Target, orÂ No Role at All?* Immunology and Allergy Clinics of North America, 2009. **29**(4): p. 677-688.

212. Bent Iii, J.P., Kuhn, F. A., *Diagnosis of allergic fungal sinusitis.* Otolaryngology - Head and Neck Surgery, 1994. **111**(5): p. 580-588.

213. Braun, H., Buzina, W., Freudenschuss, K., Beham, A., Stammberger, H., *'Eosinophilic fungal rhinosinusitis': A common disorder in Europe?* Laryngoscope, 2003. **113**(2): p. 264-269.

214. Ponikau, J.U., Sherris, D. A., Kephart, G. M., Adolphson, C., Kita, H., *The role of ubiquitous airborne fungi in chronic rhinosinusitis.* Current Allergy and Asthma Reports, 2005. **5**(6): p. 472-476.

215. Tosun, F., Hidir, Y., Saracli, M. A., Caliskaner, Z., Sengul, A., *Intranasal fungi and chronic rhinosinusitis: What is the relationship?* Annals of Otology, Rhinology and Laryngology, 2007. **116**(6): p. 425-429.

216. Shin, S.H., Ponikau, J. U., Sherris, D. A., Congdon, D., Frigas, E., Homburger, H. A., Swanson, M. C., Gleich, G. J., Kita, H., *Chronic rhinosinusitis: An enhanced immune response to ubiquitous airborne fungi.* Journal of Allergy and Clinical Immunology, 2004. **114**(6): p. 1369-1375.

217. Sasama, J., Sherris, D. A., Shin, S. H., Kephart, G. M., Kern, E. B., Ponikau, J. U., *New paradigm for the roles of fungi and eosinophils in chronic rhinosinusitis.* Current Opinion in Otolaryngology and Head and Neck Surgery, 2005. **13**(1): p. 2-8.

218. Orlandi, R.R., Marple, B. F., Georgelas, A., Durtschi, D., Barr, L., *Immunologic response to fungus is not universally associated with rhinosinusitis.* Otolaryngology - Head and Neck Surgery, 2009. **141**(6): p. 750-756.e2.

219. Ponikau, J.U., Sherris, D. A.,Kita, H.,Kern, E. B., *Intranasal antifungal treatment in 51 patients with chronic rhinosinusitis.* Journal of Allergy and Clinical Immunology, 2002. **110**(6): p. 862-866.

220. Ponikau, J.U., Sherris, D. A.,Weaver, A.,Kita, H., *Treatment of chronic rhinosinusitis with intranasal amphotericin B: A randomized, placebo-controlled, double-blind pilot trial.* Journal of Allergy and Clinical Immunology, 2005. **115**(1): p. 125-131.

221. Ricchetti, A., Landis, B. N., Giger, R., Zheng, C., Lacroix, J. S., *Effect of local antifungal treatment on nasal polyposis.* Oto-Rhino-Laryngologia Nova, 2002. **12**(2): p. 48-51.

222. Ebbens, F.A., Georgalas, C., Luiten, S., Van Dru Nen, C. M., Badia, L., Scadding, G. K., Hellings, P. W., Jorissen, M., Mullol, J., Cardesin, A., Bachert, C., Van Zele, T. P. J., Lund, V. J., Fokkens, W. J., *The effect of topical amphotericin B on*

inflammatory markers in patients with chronic rhinosinusitis: A multicenter randomized controlled study. Laryngoscope, 2009. **119**(2): p. 401-408.

223.   Liang, K.L., Su, M. C., Shiao, J. Y., Tseng, H. C., Hsin, C. H., Lin, J. F., Jiang, R. S., *Amphotericin B irrigation for the treatment of chronic rhinosinusitis without nasal polyps: A randomized, placebo-controlled, double-blind study.* American Journal of Rhinology, 2008. **22**(1): p. 52-58.

224.   Ten Cate, J.M., Klis, F.M., Pereira-Cenci, T., Crielaard, W., de Groot, P.W.J.,, *Molecular and Cellular Mechanisms That Lead to Candida Biofilm Formation.* Journal of Dental Research, 2009. **88**(2): p. 105-115.

225.   Chandra, J., Kuhn, D. M., Mukherjee, P. K., Hoyer, L. L., McCormick, T., Ghannoum, M. A., *Biofilm formation by the fungal pathogen Candida albicans: Development, architecture, and drug resistance.* Journal of Bacteriology, 2001. **183**(18): p. 5385-5394.

226.   Mukherjee, P.K., Zhou, G., Munyon, R., Ghannoum, M. A., *Candida biofilm: A well-designed protected environment.* Medical Mycology, 2005. **43**(3): p. 191-208.

227.   Seneviratne, C.J., Jin, L., Samaranayake, L.P.,, *Biofilm Lifestyle of candida: a mini review.* Oral Diseases, Dec 2007. **14**(7): p. 582-590.

228.   Martinez, L.R., Casadevall, A., *Susceptibility of Cryptococcus neoformans biofilms to antifungal agents in vitro.* Antimicrobial Agents and Chemotherapy, 2006. **50**(3): p. 1021-1033.

229.   Martinez, L.R., Casadevall, A., *Cryptococcus neoformans cells in biofilms are less susceptible than planktonic cells to antimicrobial molecules produced by the innate immune system.* Infection and Immunity, 2006. **74**(11): p. 6118-6123.

230.   Martinez, L.R., Christaki, E., Casadevall, A., *Specific antibody to Cryptococcus neoformans glucurunoxylomannan antagonizes antifungal drug action against cryptococcal biofilms in vitro.* Journal of Infectious Diseases, 2006. **194**(2): p. 261-266.

231.   Martinez, L.R., Casadevall, A., *Cryptococcus neoformans biofilm formation depends on surface support and carbon source and reduces fungal cell susceptibility to heat, cold, and UV light.* Applied and Environmental Microbiology, 2007. **73**(14): p. 4592-4601.

232.   Cannizzo, F.T., Eraso, E., Ezkurra, P. A., Villar-Vidal, M., Bollo, E., CastellaÌ•, G., CabanÌ ƒes, F. J., Vidotto, V., QuindoÌ•s, G., *Biofilm development by clinical isolates of Malassezia pachydermatis.* Medical Mycology, 2007. **45**(4): p. 357-361.

233.   Cushion, M.T., Collins, M. S., Linke, M. J., *Biofilm formation by Pneumocystis spp.* Eukaryotic Cell, 2009. **8**(2): p. 197-206.

234.   D'Antonio, D., Parruti, G., Pontieri, E., Di Bonaventura, G., Manzoli, L., Sferra, R., Vetuschi, A., Piccolomini, R., Romano, F., Staniscia, T., *Slime production by clinical isolates of Blastoschizomyces capitatus from patients with hematological malignancies and catheter-related fungemia.* European Journal of Clinical Microbiology and Infectious Diseases, 2004. **23**(10): p. 787-789.

235. Davis, L.E., Cook, G., William Costerton, J., *Biofilm on ventriculoperitoneal shunt tubing as a cause of treatment failure in coccidioidal meningitis.* Emerging Infectious Diseases, 2002. **8**(4): p. 376-379.

236. Di Bonaventura, G., Pompilio, A., Picciani, C., Iezzi, M., D'Antonio, D., Piccolomini, R., *Biofilm formation by the emerging fungal pathogen Trichosporon asahii: Development, architecture, and antifungal resistance.* Antimicrobial Agents and Chemotherapy, 2006. **50**(10): p. 3269-3276.

237. Walsh, T.J., Schlegel, R., Moody, M. M., *Ventriculoatrial shunt infection due to Cryptococcus neoformans: An ultrastructural and quantitative microbiological study.* Neurosurgery, 1986. **18**(3): p. 373-375.

238. Mowat, E., Williams, C., Jones, B., McChlery, S., Ramage, G., *The characteristics of Aspergillus fumigatus mycetoma development: is this a biofilm?* Med Mycol, 2008. **47**(SUPPL. 1): p. S1-S7.

239. Ramage, G., Mowat, E., Jones, B., Williams, C., Lopez-Ribot, J., *Our Current Understanding of Fungal Biofilms* Critical Reviews in Microbiology, 2009. **35**(4): p. 340-355.

240. Bach, M.C., Tally, P. W., Godofsky, E. W., *Use of cerebrospinal fluid shunts in patients having acquired immunodeficiency syndrome with cryptococcal meningitis and uncontrollable intracranial hypertension.* Neurosurgery, 1997. **41**(6): p. 1280-1283.

241. Banerjee, U., Gupta, K., Venugopal, P., *A case of prosthetic valve endocarditis caused by Cryptococcus neoformans var. neoformans.* Journal of Medical and Veterinary Mycology, 1997. **35**(2): p. 139-141.

242. Braun, D.K., Janssen, D. A., Marcus, J. R., Kauffman, C. A., *Cryptococcal infection of a prosthetic dialysis fistula.* American Journal of Kidney Diseases, 1994. **24**(5): p. 864-867.

243. Hawser, S.P., Douglas, L. J., *Biofilm formation by Candida species on the surface of catheter materials in vitro.* Infection and Immunity, 1994. **62**(3): p. 915-921.

244. Pierce, C.G., Uppuluri, P., Tristan, A. R., Wormley Jr, F. L., Mowat, E., Ramage, G., Lopez-Ribot, J. L., , *A simple and reproducible 96-well plate-based method for the formation of fungal biofilms and its application to antifungal susceptibility testing.* Nature Protocols, 2008. **3**(9): p. 1494-1500.

245. Hawser, S.P., Baillie, G. S.,Douglas, L. J., *Production of extracellular matrix by Candida albicans biofilms.* Journal of Medical Microbiology, 1998. **47**(3): p. 253-256.

246. Elving, G.J., van der Mei, H., Busscher, H., van Weissenbruch, R., Albers, F., *Influence of different combinations of bacteria and yeasts in voice prosthesis biofilms on air flow resistance.* Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology, 2003. **83**(1): p. 45-55.

247. Villena, G.K., Gutiérrez-Correa, M., *Morphological patterns of Aspergillus niger biofilms and pellets related to lignocellulolytic enzyme productivities.* Letters in Applied Microbiology, 2007. **45**(3): p. 231-237.

248. Villena, G.K., Fujikawa, T., Tsuyumu, S., Gutiérrez-Correa, M., *Structural analysis of biofilms and pellets of Aspergillus niger by confocal laser scanning microscopy and cryo scanning electron microscopy.* Bioresource Technology, 2009.

249. Villena, G., Moreno, P., Correa, M. G., *Cellulase production by fungal biofilms on polyester cloth.* Agro Food Industry Hi-Tech, 2001. **12**(1): p. 32-35.

250. Villena, G.K., Gutiérrez-Correa, M., *Production of cellulase by Aspergillus niger biofilms developed on polyester cloth.* Letters in Applied Microbiology, 2006. **43**(3): p. 262-268.

251. Villena, G.K., GutieÌ•rrez-Correa, M., *Production of lignocellulolytic enzymes by Aspergillus niger biofilms at variable water activities.* Electronic Journal of Biotechnology, 2007. **10**(1).

252. Beauvais, A., Schmidt, C., Guadagnini, S., Roux, P., Perret, E., Henry, C., Paris, S., Mallet, A., Prévost, M. C., Latgé, J. P., *An extracellular matrix glues together the aerial-grown hyphae of Aspergillus fumigatus.* Cellular Microbiology, 2007. **9**(6): p. 1588-1600.

253. Mowat, E., Butcher, J., Lang, S., Williams, C., Ramage, G., *Development of a simple model for studying the effects of antifungal agents on multicellular communities of Aspergillus fumigatus.* Journal of Medical Microbiology, 2007. **56**(9): p. 1205-1212.

254. Mowat, E., Lang, S., Williams, C., McCulloch, E., Jones, B., Ramage, G., *Phase-dependent antifungal activity against Aspergillus fumigatus developing multicellular filamentous biofilms.* Journal of Antimicrobial Chemotherapy, 2008. **62**(6): p. 1281-1284.

255. Seidler, M.J., Salvenmoser, S., MÃller, F. M. C., *Aspergillus fumigatus forms biofilms with reduced antifungal drug susceptibility on bronchial epithelial cells.* Antimicrobial Agents and Chemotherapy, 2008. **52**(11): p. 4130-4136.

256. Healy, D.Y., Leid, J. G., Sanderson, A. R., Hunsaker, D. H., *Biofilms with fungi in chronic rhinosinusitis.* Otolaryngology - Head and Neck Surgery, 2008. **138**(5): p. 641-647.

257. Psaltis, A.J., Wormald, P. J., Ha, K. R., Tan, L. W., *Reduced levels of lactoferrin in biofilm-associated chronic rhinosinusitis.* Laryngoscope, 2008. **118**(5): p. 895-901.

258. Piccirillo, J.F., Merritt, M.G. Jr, Richards, M.L., *Psychometric and clinimetric validity of the 20-Item Sino-Nasal Outcome Test (SNOT-20).* Otolaryngol Head Neck Surg,, 2002. **126**: p. 41-47.

259. Lund, V.J., Kennedy, D. W., *Staging for rhinosinusitis.* Otolaryngology - Head and Neck Surgery, 1997. **117**(3 II SUPPL.): p. S35-S40.

260. Lund, V.J., Mackay, I. S., *Staging in rhinosinusitus.* Rhinology, 1993. **31**(4): p. 183-184.

261. Landis, J.R., Koch, G.G., *The measurement of observer agreement for categorical data.* Biometrics, 1997. **33**: p. 159-174.

262. Bester, E., Wolfaardt, G., Joubert, L., et al. , *Planktonic-cell yield of a pseudomonad biofilm.* Appl Environ Microbiol 2005. **71**: p. 7792-7798.

263. Senior, B.A., Kennedy, D. W., Tanabodee, J., Kroger, H., Hassab, M., Lanza, D., *Long-term results of functional endoscopic sinus surgery.* Laryngoscope, 1998. **108**(2): p. 151-157.

264. Khalil, H., Nunez, D. A., *Functional endoscopic sinus surgery for chronic rhinosinusitis.* Cochrane database of Systematic Reviews, 2009(4).

265. Singhal, D., Psaltis, A.J.,, Foreman, A., Wormald, P.J., *The impact of biofilms on outcomes after endoscopic sinus surgery.* Am J Rhinol Allergy, 2010 May. **24.**(3): p. 169-74.

266. Foreman, A., Wormald, P. J., *Different biofilms, different disease? A clinical outcomes study.* Laryngoscope. **120**(8): p. 1701-1706.

267. Kennedy, D.W., Draf, W., Friedman, W. H., et al., *Quantification for staging sinusitis.* Annals of Otology, Rhinology and Laryngology, 1995. **104**(10 I): p. 17-21.

268. Chambers, D.W., Davis, W.E., Cook, P.R., Nishioka, G.J., Rudman, D.T. , *Long-term outcome analysis of functional endoscopic sinus surgery: correlation of symptoms with endoscopic examination findings and potential prognostic variables.* Laryngoscope, 1997. **107**: p. 504-510.

269. Briggs, R.D., Wright, S.T., Cordes, S., Calhoun, K.H. , *Smoking in chronic rhinosinusitis: a predictor of poor long-term outcome after endoscopic sinus surgery.* Laryngoscope, 2004. **114**: p. 126-128.

270. Smith, T.L., Mendolia-Loffredo, S., Loehrl, T. A., Sparapani, R., Laud, P. W., Nattinger, A. B., *Predictive factors and outcomes in endoscopic sinus surgery for chronic rhinosinusitis.* Laryngoscope, 2005. **115**(12): p. 2199-2205.

271. Deal, R.T., Kountakis, S.E.,, *Significance of nasal polyps in chronic rhinosinusitis: symptoms and surgical outcomes.* Laryngoscope 2004. **114**: p. 1932-1935.

272. Bardy, J.J., Foreman, A., Field, J., Wormald, P. J., *Impaired mucosal healing and infection associated with Staphylococcus aureus after endoscopic sinus surgery.* American Journal of Rhinology and Allergy, 2009. **23**(5): p. 549-552.

273. Bhattacharyya, N., *The microbiology and pathophysiology of infection in chronic rhinosinusitis.* Otorinolaringologia, 2003. **53**(3): p. 79-87.

274. Lin, A., Busaba, N. Y., *Staphylococcus aureus and endoscopic sinus surgery.* Current Opinion in Otolaryngology and Head and Neck Surgery, 2006. **14**(1): p. 19-22.

275. Bhattacharyya, N., Gopal, H. V., Lee, K. H., *Bacterial Infection after Endoscopic Sinus Surgery: A Controlled Prospective Study.* Laryngoscope, 2004. **114**(4): p. 765-767.

276. Ramadan, H.H., *What is the bacteriology of chronic sinusitis in adults? .* Am J Otolaryngol, 1995. **16**: p. 303-306.

277. Heydorn, A., Ersbøll, B.K., Hentzer, M., Parsek, M.R., Givskov, M., and Molin, S. , *Experimental Reproducibility in Flow-Chamber Biofilms.* Microbiology 2000. **146**(10): p. 2409-15.

278. Heydorn, A., Nielsen, A. T., Hentzer, M., Sternberg, C., Givskov, M.,Ersboll, B. K., Molin, S., *Quantification of biofilm structures by the novel computer program COMSTAT.* Microbiology, 2000. **146**(10): p. 2395-2407.

279. Vorregaard M, Ersbøll BK, Yang L, Haagensen JAJ, Heydorn A, Molin S, Sternberg C, Personal communication. .

280. Boase, S., Valentine, R., Singhal, D., Tan, L.W., Wormald, P.J., *A sheep model to investigate the role of fungal biofilms in sinusitis: fungal & bacterial synergy.* In Press.

281. Loussert, C., Schmitt, C., Prevost, M. C., Balloy, V., Fadel, E., Philippe, B., Kauffmann-Lacroix, C., Beauvais, A., *In vivo biofilm composition of Aspergillus fumigatus.* Cellular Microbiology, 2010. **12**(3): p. 405-410.

282. Uppuluri, P., Chaturvedi, A. K., Lopez-Ribot, J. L., *Design of a simple model of Candida albicans biofilms formed under conditions of flow: Development, architecture, and drug resistance.* Mycopathologia, 2009. **168**(3): p. 101-109.

283. Wargo, M.J., Hogan, D. A., *Fungal-bacterial interactions: a mixed bag of mingling microbes.* Current Opinion in Microbiology, 2006. **9**(4): p. 359-364.

284. Seneviratne, G., Zavahir, J.S., Bandara, W.M.M., et al, *Fungal-bacterial biofilms: their development for novel biotechnological applications.* World J Microbiotechnol, 2008. **24**: p. 739-743.

285. Amitani, R., Murayama, T., Nawada, R., Lee, W. J., Niimi, A., Suzuki, K., Tanaka, E., Kuze, F., *Aspergillus culture filtrates and sputum sols from patients with pulmonary aspergillosis cause damage to human respiratory ciliated epithelium in vitro.* European Respiratory Journal, 1995. **8**(10): p. 1681-1687.

286. Chaudhary, N., Staab, J. F., Marr, K. A., *Healthy human T-cell responses to Aspergillus fumigatus antigens.* PLoS ONE. **5**(2).

287. Jain, N., Fries, B. C., *Antigenic and phenotypic variations in fungi.* Cellular Microbiology, 2009. **11**(12): p. 1716-1723.

288. Thomas, D.P., Bachmann, S. P., Lopez-Ribot, J. L., *Proteomics for the analysis of the Candida albicans biofilm lifestyle.* Proteomics, 2006. **6**(21): p. 5795-5804.

289. Palmer Jr, R.J., *Microscopy Flowcells: Perfusion chambers for real time study of biofilms*, in *Methods in Enzymology*. 1999. p. 160-166.

290. Stewart, P.S., Peyton, B. M., Drury, W. J., Murga, R., *Quantitative observations of heterogeneities in Pseudomonas aeruginosa biofilms.* Applied and Environmental Microbiology, 1993. **59**(1): p. 327-329.

291. Murga, R., Stewart, P. S., Daly, D., *Quantitative analysis of biofilm thickness variability.* Biotechnology and Bioengineering, 1995. **45**(6): p. 503-510.

292. Hermanowicz, S.W., Schindler, U., Wilderer, P., *Fractal structure of biofilms: New tools for investigation of morphology*, in *Water Science and Technology*. 1995. p. 99-105.

293. Zhang, T.C., Bishop, P. L., *Density, porosity, and pore structure of biofilms.* Water Research, 1994. **28**(11): p. 2267-2277.

294. Lewandowski, Z., Webb, D., Hamilton, M., Harkin, G., *Quantifying biofilm structure*, in *Water Science and Technology*. 1999. p. 71-76.

295. Kuehn, M., Hausner, M., Bungartz, H. J., Wagner, M., Wilderer, P. A., Wuertz, S., *Automated confocal laser scanning microscopy and semiautomated image processing for analysis of biofilms.* Applied and Environmental Microbiology, 1998. **64**(11): p. 4115-4127.

296. Heydorn, A., et al., *Experimental reproducibility in flow-chamber biofilms.* Microbiology, 2000. **146**(10): p. 2409-2415.

297. Korber, D.R., Lawrence, J. R., Hendry, M. J., Caldwell, D. E., *Programs for determining statistically representative areas of microbial biofilms.* Binary, 1992. **4**: p. 204-210.

298. Korber, D.R., Lawrence, J. R., Hendry, M. J., Caldwell, D. E., *Analysis of spatial variability within mot+ and mot- Pseudomonas fluorescens biofilms using representative elements.* Biofouling, 1993. **7**(4): p. 339-358.

299. Mladina, R., SkitareliÄ‡, N., *Biofilm - The other name for the regular mucosal blanket.* Medical Hypotheses, 2010. **75**(4): p. 391-392.

300. Mladina, R., SkitareliÄ, N., MusiÄ‡, S., RistiÄ‡, M., *A biofilm exists on healthy mucosa of the paranasal sinuses: A prospectively performed, blinded, scanning electron microscope study.* Clinical Otolaryngology, 2010. **35**(2): p. 104-110.

301. Zi, Z., Demin, H., Shengzhong, Z., Yehua, H., Wei, D., Erzhong, F., Ying, L., Yunchuan, L., Deyun, W., *Biofilms and mucosal healing in postsurgical patients with chronic rhinosinusitis.* American Journal of Rhinology and Allergy, 2009. **23**(5): p. 506-511.

302. Hai, P.V.T., C. Lidstone, and B. Wallwork, *The effect of endoscopic sinus surgery on bacterial biofilms in chronic rhinosinusitis.* Otolaryngology - Head and Neck Surgery, 2010. **142**(3 SUPPL. 1): p. S27-S32.

303. Singhal, D., Bardy, J.J., Foreman, A., Wormald, P.J., *Staphylococcus Aureus Biofilms: Nemesis of Endoscopic Sinus Surgery.* Laryngoscope, 2011.

304. Bhattacharyya, N., Kepnes, L. J., *The microbiology of recurrent rhinosinusitis after endoscopic sinus surgery.* Archives of Otolaryngology - Head and Neck Surgery, 1999. **125**(10): p. 1117-1120.

305. Kuramitsu, H.K., He, X., Lux, R., Anderson, M. H., Shi, W., *Interspecies interactions within oral microbial communities.* Microbiology and Molecular Biology Reviews, 2007. **71**(4): p. 653-670.

306.   Budhani, R.K., Struthers, J. K., *Interaction of Streptococcus pneumoniae and Moraxella catarrhalis: Investigation of the indirect pathogenic role of Î²-lactamase-producing moraxellae by use of a continuous-culture biofilm system.* Antimicrobial Agents and Chemotherapy, 1998. **42**(10): p. 2521-2526.

307.   Baena-Monroy, T., Moreno-Maldonado, V., Franco-Martínez, F., Aldape-Barrios, B., Quindós, G., Sánchez-Vargas, L.O., *Candida albicans, Staphylococcus aureus and Streptococcus mutans colonization in patients wearing dental prosthesis.* Med Oral Pathol Oral Cir Bucal, 2005. **1**(10 Suppl 1): p. E 27-39.

308.   Marrie, T.J., Costerton, J.W., *Scanning and Transmission Electron Microscopy of In Situ Bacterial Colonization of Intravenous and Intraarterial Catheters.* Journal of Clinical Microbiology, 1984. **19**(5): p. 687-693.

309.   Crump, J.A., Collignon, P. J., *Intravascular catheter-associated infections.* European Journal of Clinical Microbiology and Infectious Diseases, 2000. **19**(1): p. 1-8.

310.   Ramage, G., Saville, S. P., Thomas, D. P., Loì • pez-Ribot, J. L., *Candida biofilms: An update.* Eukaryotic Cell, 2005. **4**(4): p. 633-638.

311.   El-Azizi, M.A., Starks, S. E., Khardori, N., *Interactions of Candida albicans with other Candida spp. and bacteria in the biofilms.* Journal of Applied Microbiology, 2004. **96**(5): p. 1067-1073.

312.   McBain, A.J., *Chapter 4 In Vitro Biofilm Models. An Overview*, in *Advances in Applied Microbiology*. 2009. p. 99-132.

313.   Fessia, S.L., Griffin, M.J.,, *A method for assaying biofilm capacity on polyurethane-coated slides.* Perit. Dial. Int, 1991. **11**: p. 144-146.

314.   Chiu, A.G., Antunes, M. B., Palmer, J. N., Cohen, N. A., *Evaluation of the in vivo efficacy of topical tobramycin against Pseudomonas sinonasal biofilms.* Journal of Antimicrobial Chemotherapy, 2007. **59**(6): p. 1130-1134.

315.   Ha, K.R., Psaltis, A. J., Butcher, A. R., Wormald, P. J., Tan, L. W., *In vitro activity of mupirocin on clinical isolates of Staphylococcus aureus and its potential implications in chronic rhinosinusitis.* Laryngoscope, 2008. **118**(3): p. 535-540.

316.   Uren, B., Psaltis, A., Wormald, P. J., *Nasal lavage with mupirocin for the treatment of surgically recalcitrant chronic rhinosinusitis.* Laryngoscope, 2008. **118**(9): p. 1677-1680.

317.   Chiu, A.G., Palmer, J. N., Woodworth, B. A., Doghramji, L., Cohen, M. B., Prince, A., Cohen, N. A., *Baby shampoo nasal irrigations for the symptomatic post-functional endoscopic sinus surgery patient.* American Journal of Rhinology, 2008. **22**(1): p. 34-37.