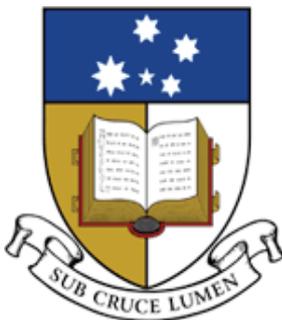# THE ROLE OF BACTERIAL BIOFILMS IN CHRONIC RHINOSINUSITIS

A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF ADELAIDE

Alkis James Psaltis

Department of Surgery, Faculty of Health Sciences

The Queen Elizabeth Hospital Adelaide, South Australia

October2008

Dedicated to my wonderful parents Jim and Lela
& to my beautiful wife Angela

"To climb steep hills requires slow pace at first."
William Shakespeare

iii

DECLARATION

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

I give consent to this copy of my thesis being made available in the University library. I acknowledge that copyright of published works contained within this thesis resides with the copyright holder/s of those works.

Alkis James Psaltis

1st June 2008

ABSTRACT

ALKIS J PSALTIS   PHD THESIS

THE ROLE OF BACTERIAL BIOFILMS IN CHRONIC RHINOSINUSITIS

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

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

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

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

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

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

In summary, this thesis provides further evidence that bacterial biofilms play a major role in the pathogenesis and disease persistence in a subset of CRS patients. Deficiencies in components of the innate immune system, such as lactoferrin, may play an important role in the predisposition of certain individuals to the initial development of bacterial biofilms.

ACKNOWLEDGEMENTS

ABBREVIATIONS USED IN THIS THESIS

CRS	- Chronic Rhinosinusitis		FISH	-Fluorescent In Situ Hybridization
EM	-Electron Microscopy		OSA	-Obstructive Sleep Apnoea
CSLM	-Confocal Scanning Laser Microscopy		TEM	-Transmission Electron Microscopy
			SEM	-Scanning Electron Microscopy
mRNA	-Messenger Ribosomal Nucleic Acid		PMN	-Polymorphonuclearcytes
GP	-General Practionner		HSV	-Herpes Simplex Virus
RCT	-Randomized Control Trial		CMV	-Cytomegalovirus
FESS	-Functional Endoscopic Sinus Surgery		HIV	-Human Immunodeficiency Virus
			HBV	-Hepatitis B Virus
ESS	-Endoscopic Sinus Surgery		HCV	-Hepatitis C Virus
NA	-Not available		RSV	-Respiratory Synctial Virus
CNS	-Coagulase Negative Staphylococci		LPS	-Lipopolysaccharide
SA	-*Staphylococcus aureus*		NO	-Nitric Oxide
SP	-*Streptococcus pneumoniae*		TNFa	-Tumour Necrosis Factor alpha
GNR	-Gram Negative Rods		IL8	-Interleukin 8
SV	-*Streptococcus viridans*		NK	-Natural Killer Cells
PA	-*Pseudomonas aeruginosa*		cDNA	-Complementary strand DNA
H inf	-*Haemophilus influenza.*		CF	-Cystic Fibrosis
DIC	-Differential Interference Contrast		ATCC	-American Type Culture Collection
PCR	-Polymerase Chain Reaction		MQ	-Milli-Q
MHC	-Major Histocompatability Complex		PBS	-Phosphate buffered solution
Th1	-T Helper Cell 1		RAST	-Radioallergosorbent testing
Th2	-T Helper Cell 2		CT	-Computerized tomography
SEA	-Staphylococcal Enterotoxin A		ICC	-Interobserver correlation coefficient
SEB	-Staphylococcal Enterotoxin B		AFS	-Allergic Fungal Sinusitis
IgG	-Immunoglobulin G		NAFES	-Non Allergic Fungal Eosinophilic sinusitis
CRS/NP	-Chronic Rhinosinusitis with Nasal Polyposis		NANFES	-Non allergic, Non Fungal Eosinophilic sinusitis
TSST-1	-Toxic Shock Syndrome Toxin 1		OR	-Odds Ratio
EPS	-Exopolysaccharide matrix		C.I.	-Confidence Interval
Bap	-Biofilm associated proteins		VAS	-Visual Analogue Scale
PIA	-Polysaccharide Intercellular Adhesin		CSS	-Chronic sinusitis survey
MDR	-Multidrug efflux pumps		qRT-PCR	-Quantitative real-time reverse-transcriptase polymerase chain reaction
CAM	-Cationic Antimicrobial Peptides			
OME	-Otitis Media with Effusion			
ELISA	-Enzyme linked immunosorbent assay		LF	-Lactoferrin
HPRT	-Hypoxanthine-guanine phosphoribosyltransferase			
Ct	-Cycle threshold			

ACCOMPLISHMENTS

AWARDS OBTAINED FOR RESEARCH ASSOCIATED WITH THIS THESIS

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

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

The Maurice H Cottle Award for the best paper presented at the Annual Meeting of the American Rhinologic Society, for paper titled " A sheep model for the study of biofilms in rhinosinusitis" Toronto September, 2006

The Sir Edwin Hughes Memorial Prize for Clinical Research in Surgery for the best presentation by an Australian or New Zealand Surgical Registrar, for the presentation titled "A new technique for the study of biofilms in sinusitis" Monash University, Victoria, October 2006

The Queen Elizabeth Hospital Research Day Presentation Award for Best Clinical Presentation in Higher Degree Category for the presentation titled " A sheep model to study the role of biofilms in rhinosinusitis" Adelaide, October 2006.

The R.P Jepson Medal awarded for the best presentation at the Annual Scientific Meeting at the Royal Australian College of Surgeons, SA and NT Sub-committee, for the presentation titled "The effect of biofilms on post-sinus surgical outcomes" Adelaide August 2007

The Best Poster Award at Inaugural Post-Graduate Research Expo for the Faculty of Health Sciences of the University of Adelaide for the poster titled "Development of a sheep model for the study of biofilms in rhinosinusitis" Adelaide, October 2007

The Best Presentation and Overall Winner at the Inaugural Research Expo for the Faculty of Health Sciences of the University of Adelaide, "Development of a sheep model for the study of biofilms in rhinosinusitis" Adelaide, October 2007

PUBLICATIONS ARISING FROM THE RESEARCH CONDUCTED FOR THIS THESIS

A sheep model for the study of biofilms in rhinosinusitis.

Psaltis AJ and Ha KR (Co-first authors), Tan L, Wormald PJ.
American Journal of Rhinology. 2007 May-Jun;21(3):339-45.
Erratum in: American Journal of Rhinology. 2007 Jul-Aug;21(4):519.

Confocal scanning laser microscopy evidence of biofilms in patients with chronic rhinosinusitis.

Psaltis AJ, Ha KR, Beule AG, Tan LW, Wormald PJ.
Laryngoscope. 2007 Jul;117(7):1302-6.

Nasal mucosa expression of lactoferrin in patients with chronic rhinosinusitis.

Psaltis AJ, Bruhn MA, Ooi EH, Tan LW, Wormald PJ.
Laryngoscope. 2007 Nov;117(11):2030-5.

Reduced Levels of Lactoferrin in Biofilm-Associated Chronic Rhinosinusitis.

Psaltis AJ, Wormald PJ, Ha KR, Tan LW.
Laryngoscope. 2008 Jan 21; [Epub ahead of print]

The effect of biofilms on post-sinus surgical outcomes.

Psaltis AJ, Weitzel E, Wormald P-J
American Journal of Rhinology. 2008 Jan-Feb;22(1):1-6.

In Vitro Activity of Mupirocin on Clinical Isolates of Staphylococcus aureus and its Potential Implications in Chronic Rhinosinusitis.

Ha KR, Psaltis AJ, Butcher AR, Wormald PJ, Tan LW.
Laryngoscope. 2007 Dec 3; [Epub ahead of print]

PRESENTATIONS

Lactoferrin And Biofilms
Biofilm Roundtable Discussion, Sydney Australia, October 2005.

The Heterogeneity of Lactoferrin Expression in Patients with Chronic Rhinosinusitis
Queen Elizabeth Hospital Research Day, Adelaide Australia, October 2005.

The Expression of Lactoferrin in Patients with Chronic Rhinosinusitis.
International Rhinological Society Meeting, Sydney Australia, October 2005.

Biofilms and the nose
Australasian Rhinological Society Annual Meeting, Barossa, South Australia, October 2006

A sheep model for the study of biofilms in Chronic Sinusitis
Annual Meeting of the American Rhinologic Society, Toronto, Canada September 2006

A new technique for the study of biofilms in sinusitis
The Cabrini Institute, Monash University, Melbourne Victoria October 2006

 An animal model to study biofilms in chronic rhinosinusitis
Queen Elizabeth Hospital Research Day, Adelaide, Australia, October 2006

CSLM study of biofilms in human CRS patients
ASOHNS Scientific Meeting, Adelaide, Australia, March 2007

Biofilms and Chronic Rhinosinusitis
Invited Speaker Divisions of Surgery Departmental Meeting, Adelaide, Australia June 2007

The effect of biofilms on post-sinus surgical outcomes.
Annual Scientific Meeting at the Royal Australian College of Surgeons, Adelaide, Aug 2007

 The effect of biofilms on post-sinus surgical outcomes.
American Rhinologic Society Meeting, Washington DC,USA September 2007

 The effect of biofilms on post-sinus surgical outcomes.
Queen Elizabeth Hospital Research Day, Adelaide, Australia October 2007

A sheep model for study of biofilm in rhinosinusitis.
Research Expo of the Faculty of Health Sciences, University of Adelaide, Australia Oct 2007

The role of biofilms in CRS.
Centre for Genomic Studies , Allegheny General Hospital Pittsburgh, PA. USA April 2008

A sheep model investigating several potential antibiofilm treatments. The American Triological Society of Otorhinolaryngology (COSM), Spring Meeting, Orlando Florida, USA May 2008

Biofilms and Chronic Rhinosinusitis –American Academy of Head and Neck Surgeons and Otolaryngologists, Annual Scientific Meeting , Chicago, Illinois, USA September 2008

TABLE OF CONTENTS

CHAPTER 1

LITERATURE REVIEW

1.1 CHRONIC RHINOSINUSITIS

1.1.1 RESEARCH DEFINITION

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

1.1.2 EPIDEMIOLOGY AND SOCIO-ECONOMIC IMPLICATIONS

CRS is a highly common condition affecting up to 16% of the US population. Its prevalence resembles that of hypertension and non-specific lower back pain and it remains the single most common self-reported chronic health condition affecting adults in the western world[3]. The financial burden of this condition is far reaching, with direct annual US health care expenditure in excess of $ 5.8 million US [4]. Estimates of restricted activity days in the USA exceed 73 million days/year making the true financial costs of CRS to society significantly

higher [5]. In 2004 the National Ambulatory Medical Care Survey estimated that 12.5 million

office-based doctor visits resulted in a diagnosis of CRS, with hospital out-patient visits

reaching more than 1.1 million [6]. Patients with CRS were shown to visit their GP 2 times

more often than non sufferers and had 5 times as many prescriptions filled [7]. Aside from

the enormous economic implications of CRS, numerous quality life and disability index

studies have repeatedly demonstrated the significant negative psycho-social impact that this

condition has on the sufferer. The disability and discomfort caused by CRS has been shown to

be comparable to that of other chronic diseases such as asthma, angina and lower back pain

[8].

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

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

1.1.3 AETIOLOGICAL/PATHOGENIC FACTORS

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

1.1.4 TREATMENTS FOR CHRONIC RHINOSINUSITIS

The treatment of CRS involves both medical and surgical interventions. Two recent surveys of US otolaryngologists found oral antibiotics and intranasal corticosteroids the most commonly employed first line agents in the management of CRS [17, 18]. Other agents also used, although less frequently include nasal douches and additional oral medications such as corticosteroids, decongestants, mucolytics and antihistamines. Although widely and liberally used, the level of evidence supporting the effectiveness of different medical therapies in the treatment of CRS is variable and often tenuous. Despite considerable disagreement

surrounding the role of bacteria in CRS, two prospective randomised control trials(RCT) have demonstrated the efficacy of long term antibiotic treatment (>12 weeks) in the management of CRS. Wallwork at al [19] showed a statistically significant improvement in symptom, endoscopic and rhinometric parameters in patients treated with long term macrolide antibiotics compared to those in the placebo arm. A similar improvement in objective and subjective outcome measures was also found by Ragab et al [20], in patients medically treated with long-term erythromycin, intra-nasal corticosteroids and alkaline douches compared to controls. As well as the numerous studies of Level III evidence, demonstrating clinical and radiological improvement in CRS patients after long term antibiotic therapy [21-25], multiple cohort studies have also shown clinical improvement in patients treated with shorter 4-6 week antibiotic courses with or without intra-nasal steroids [26-29]. The use of topical/aerosolized antibiotic treatments has also been investigated and although three cohort studies have demonstrated some benefit in CRS patients experiencing frequent acute exacerbations [30-32], the only prospective double blind RCT concerning topical antibiotic use, showed no additional benefit in the addition of tobramycin to nebulised saline in the treatment of CRS [33].

To date three RCTs investigating the use of topical steroids [34-36] and two RCTs evaluating the efficacy of intra-sinus instilled steroids [37, 38] in CRS have been published. Four of the five trials demonstrated a significant improvement in symptoms with no evidence of increased infection rates. Although the role of systemic steroids in the management of CRS has not been as extensively evaluated, a double-blind RCT exists demonstrating a clinically significant improvement in symptoms and pathology of nasal polyposis patients treated with a 14 day course of oral prednisolone [39] . Other lower evidence studies also suggest that that oral steroids may be particularly useful in the management of certain subtypes of CRS, particularly allergic fungal sinusitis [40],[41].

A systematic review of the literature by the Cochrane Collaboration, identified a number of randomised controlled trials evaluating the use of nasal saline irrigation for the symptoms of CRS [42]. Meta-analysis of three studies comparing the effect of saline vs no treatment [43-45], revealed a statistically significant improvement in symptoms and disease-specific quality of life scores in the saline arm. Although an improvement was also seen in both saline treatment arms in the RCT published by Heatley et al [46], this was not significantly better than the placebo arm. Two RCTs comparing hypertonic vs isotonic solutions have revealed conflicting results. Cordray et al [47], showed a significant improvement in the symptoms of CRS patients using hypertonic saline but not in those using isotonic saline, while Bachmann et al [48] showed both saline solutions improved symptom scores relative to baseline, with no significant difference between the two.

Functional endoscopic sinus surgery (FESS) has now become well established for the treatment of chronic rhinosinusitis refractory to medical management. A systematic review of the literature by the Cochrane collaboration in 2006 [49], revealed that the vast majority of studies examining the effectiveness of FESS for CRS were either cohort studies or case series of level III evidence at best. Although these studies generally demonstrated high efficacy of FESS [50-54], the 2 RCTs identified in the review [20, 55], did not demonstrate an overall significant difference in the clinical outcomes of FESS compared to medical management in the treatment of CRS. It must be kept in mind however that methodological flaws, ethical issues, differences in the patient populations, inherent difficulties in standardising and blinding surgical procedures as well as the fact that surgery is typically reserved for patients who have failed medical therapy, makes it not only difficult to statistically compare the results of the different studies but also to compare the efficacy of FESS versus medical treatment alone.

Numerous level III evidence studies have also attempted to determine possible factors affecting outcome following FESS. A history of previous polypectomy or sinus surgery [56-59], presence of allergy [57], smoking [10, 59] and more severe initial disease/inflammation on endoscopic or histopathological examination [58, 60] have all been shown to correlate with poorer post-operative outcomes and a more frequent need for revision surgery. Although some studies have also reported that polyposis and more severe radiological disease may also predispose to poorer outcomes [59, 61, 62], these findings have not been universal [20, 54, 63, 64].

1.2 BACTERIA AND CHRONIC RHINOSINUSITIS

1.2.1 CONTROVERSY REGARDING THE ROLE OF BACTERIA IN CRS

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

play a role [65] while others propose that gram negative bacteria may colonize or secondarily

infect because of underlying defects in host defences [84, 85].

Despite the frequent co-occurrence of bacteria with CRS, controversy still exists as to the role

that they play in the pathogenesis of this condition. It has been postulated that in many cases,

bacteria may simply be present as non-pathogenic bystanders invading already inflamed

tissue [86]. This opinion has largely stemmed from the following observations: (1) the finding

that the paranasal sinuses are not actually sterile as once thought, with more than half of all

healthy sinuses culturing bacteria [87-90]. (2) the relative absence of neutrophils and

predominance of eosinophils and mixed mononuclear cells in the inflammatory infiltrate [91,

92] (3) the poor correlation between clinical findings and microbiology [93] and (4) the often

short lived or poor response to seemingly appropriate culture-directed antibiotic therapy [29].

Although these observations have provided mounting evidence for the limited role of bacteria

in CRS, the recent discovery of bacterial superantigens in patients with CRS with nasal

polyposis; the new evidence of underlying bacterially driven osteitis in the bony walls of

inflamed sinuses and the re-discovery of an alternative form of bacterial existence, the

biofilm, has rekindled the debate of the importance of bacteria in the pathogenesis of CRS.

TABLE 2							Bacteriological Studies of CRS patients aged 17-79	
Author	**Year**	**Patient No.**	**Antibiotics <1wk before surgery**	**Aseptic technique**	**Sampling Method**	**Sample site**	**Micro-organisms**	
Doyle et al[9]	1991	59	Yes	Yes	Biopsy	Ethmoid	NA	CNS (73%), SA (34%) GNR
Hoyt et al[10]	1992	197	NA	Yes	Aspiration & Biopsy	Maxillary	NA	CNS,SA,GNR
Hsu et al[11]	1998	34	NA	Yes	Endoscopic	All sinuses	90%	CNS (28%) PA (17%) SA (13%)
Biel et al[12]	1998	174	Yes	Yes	Endoscopic	Maxillary	95%	CNS (36%) SA (25%), SV, Anaerobes
Brook et al[14]	2001	108	NA	Yes	Aspiration	Maxillary	NA	SA (16%) SV (13%) PA (11%), Anaerobes
Jiang et al[13]	2002	186	NA	Yes	Endoscopic	Middle Meatus Ethmoid	83%	CNS,GNR,SA
Finegold et al[15]	2002	150	NA	NA	Aspiration	Maxillary	76%	GNR,ACS, Anaerobes
Araujo et al[16]	2003	114	No	Yes	Endoscopic	Middle Meatus	92%	SA (36%) CNS (20%) SP (17%) Anaerobes (8%)
Kalcioglu et al[17]	2003	27	Yes	Yes	Aspirate	Maxillary	70%	SA (11%) SP (11%) H Inf (7%) Anaerobes (36%)
Merino Et al[18]	2003	510	No	Yes	Aspirates	Maxillary	98%	SV (28%) SP (12%) Coryn (12%) SA (9%) Anaerobes
Kingdom et al[19]	2004	101	NA	Yes	Endoscopic Swab and Biopsy	All sinuses	86%	CNS (45%) GNR (25%) SA (24%) PA (9%)
Yildrim et al[20]	2004	48	Yes	Yes	Endoscopic Swab	Middle Meatus Spheno-ethmoidal recess	Not applicable	CNS (46%) SP (17%) GNR (17%) SA (10%) PA (10%)
Busaba et al[21]	2004	179	No	Yes	Biopsy	Ethmoid	90%	CNS, SA, Anaerobes
Aneke et al[22]	2004	54	NA	NA	Aspirate	Maxillary	57%	SA,PA,GNR

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

1.2.2 BACTERIAL SUPER-ANTIGENS

The increasing evidence of superantigen mediated inflammation in chronic eosinophilic-lymphocytic inflammatory disorders such as atopic dermatitis [94, 95], allergic rhinitis [96] and asthma [97] has led researchers to believe that they may also play a role in the inflammation associated with CRS. Super-antigens are microbial derived toxins capable of triggering massive polyclonal T cell proliferation and activation. They do so by directly binding to and cross-linking the MHC class II molecule on antigen presenting cells to the variable region β chain of the T cell receptor. This bypasses the conventional MHC restrictions of the immune system, enabling them to activate up to 20-30% of the host T-cell population [98]. In the acute setting superantigens may lead to the sudden and massive release of Th1 and Th2 cytokines as seen in toxic shock syndrome, where as in chronic conditions they may act as allergens, promoting a systemic and local IgE response with histamine release on repeated exposure. In 2001 Bachert et al [99] published the first paper suggesting a possible role for bacterial superantigens in the pathogenesis of CRS with nasal polyposis. This study demonstrated the presence of specific IgE to staphylococcal enterotoxins A and B (SEA and SEB) in the polyp homogenates of CRS patients. The positive correlation they showed between the concentration of total and specific IgE to eosinophilic inflammation in the nasal polyp tissue, further strengthened their argument that the staphylococcal superantigens were inciting and maintaining the cellular inflammation. A similar study examining the serum of 23 CRS patients with nasal polyposis (CRS/NP), demonstrated the presence of IgE to staphylococcal superantigens (SEB and toxic shock syndrome toxin (TSST-1)) in a large proportion of the patients (60.9% and 39.1% respectively) and in none of the controls [100]. Further indirect evidence for the role of superantigens in CRS/NP is demonstrated in two studies which found significant clonal proliferation of specific variable β domains in lymphocytes located within the nasal polyp tissue of CRS patients [101, 102].The

11

recent direct detection of superantigens by enzyme-linked immunosorbent assay in the mucus and tissue of CRS/NP but not in healthy controls has now provided direct evidence for the role of these enterotoxins in CRS patients [103].

1.2.3 BACTERIAL MEDIATED OSTEITIS

Histological changes of the bone underlying the sinus mucosa were first described in animal studies of bacterial induced CRS [104, 105]. The later findings of inflammatory- mediated bony changes in adjacent and distant non-infected animal sinuses [106, 107], led some authors to compare this inflammatory process with the histological diagnosis of osteomyelitis [2]. The fact that sinus bones are flat bones lacking marrow spaces resulted in the term "osteitis" being used rather than osteomyelitis to describe the pathological process occurring in the bony walls of sinuses.

Only a limited number of human clinical studies have been performed in CRS patients to examine the possible role of osteitis in the pathogenesis of this condition. Both Kennedy et al [108] and Giacchi et al [109] have found histomorphometric and histopathological evidence of bone remodelling in human ethmoid sinuses. Using radionucleotides Jang et al [110] also demonstrated a higher uptake of radioisotope in the paranasal sinus bones of patients with poorer outcomes post endoscopic sinus surgery, suggesting that ongoing osteitis may perpetuate mucosal inflammation post operatively. Whether the mechanism of bone remodelling is as consequence of direct infection of the bone or as a result of bacterial-induced release of inflammatory mediators, that in turn stimulate osteoblastic activity, remains unknown [107]. The fact that bacterial organisms have yet to be identified in the bone of either human patients or animal models of CRS had led some researchers to believe the latter is a more likely explanation. It should be noted however that even in the well established bacterial entity of osteomyelitis, the recovery of bacterial organisms is often very

difficult. Nevertheless, it is certainly possible that underlying bony changes may explain the often recalcitrant and difficult to treat nature of CRS. Further human studies are most certainly required.

1.3 BACTERIAL BIOFILMS

1.3.1 HISTORICAL PERSPECTIVES

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

1.3.2 DEFINITION

The definition of a biofilm is a constantly evolving one, reflecting advances in scientific research and technology. The early definitions focussed entirely on the structural composition of the biofilm, namely the bacterial clusters and their encasing matrix [111],[113]. Soon after, it became evident that biofilms were not static, homogenous structures but rather exhibited spatial and temporal heterogeneity as well as many differences to their planktonic counterparts in terms of growth, metabolic rate and genetic expression [114],[115, 116]. As a consequence the most recent definition put forward by Donlan and Costerton encompasses both the readily observable structural features of a biofilm as well as the specific

physiological features of the organisms existing within these structures. They now define a biofilm as a *microbially derived sessile community, characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of self produced extracellular polymeric substances, and exhibit an altered phenotype in terms of growth rate and genotype* [117]. This definition has important implications with respect to research, as previous bacterial populations classified as biofilms according to early structural definitions, have now been shown to merely be micro-colonies of planktonic bacteria lacking the inherently resistant phenotype of true biofilms.

1.3.3 Biofilm Ultrastructure

The conceptual understanding of biofilm ultrastructure has evolved with the advent of new imaging modalities. From early light and transmission electron microscopy studies, biofilms were viewed as homogenous, unstructured, planar accretions of bacterial cells embedded within the cells' exopolysaccharide matrices [118]. This misperception of biofilm ultrastructure arose from the inherent flaws associated with these imaging modalities. Light microscopy suffers from out-of-focus effects that are thought to cause distortion of the structure being viewed, while the complete dehydration of specimens required for electron microscopy could theoretically dehydrate and collapse the typically well hydrated biofilm matrix. New imaging technologies allowing more detailed and less invasive biofilm imaging have led to new conceptual models, casting serious doubt on biofilms existing as homogeneous flat structures. Using the differential interference contrast (DIC) microscope to study water-system biofilms, Keevil et al [119] formulated their 'heterogeneous mosaic model' of biofilm growth, following their observation of biofilms growing as numerous microcolony stacks within an exopolysaccharide matrix. The application of Confocal Scanning

Laser Microscopy (CSLM) to biofilm research probably represents the most significant advancement in our understanding of biofilms. CSLM circumvents many of the problems of other imaging modalities, by allowing the fresh processing of specimens and by eliminating out-of-focus distortion through the use of optical sectioning. Using CSLM, the current model of biofilms resembling dense, confluent mushroom-type structures penetrated by interstitial voids, has emerged. Although the initial CSLM studies of biofilms were entirely descriptive [120], the use of CSLM has since been expanded to examine the species and chemical composition of biofilms, their physiological properties and their relationship with the substratum. Numerous factors influencing the formation of biofilms at different times have been identified and are summarised in table 3 adapted from Wimmpeny et al's review of biofilms' heterogeneous structure [121].

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

1.3.4 BIOFILM COMPOSITION

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

Another important structure adding both organization and fortification to the biofilm is the extracellular matrix, produced by the constituent microbial cells. The composition of the matrix is complex and variable among different bacterial species and even within the same species under different environmental conditions [125]. Despite their heterogeneous composition, exopolysaccharides and proteins are common essential features of most biofilm matrices, providing a scaffold around which the microbial communities arrange themselves. The recent finding of commonality amongst different bacterial biofilms in the presence of certain exopolysaccharides (cellulose and B-1,6-linked N-acetylglucosamine) as well as some secondary signal pathway associated proteins (GGDEF-domain-containing proteins) and surface proteins (Bap-related proteins) has led researchers to believe that common essential elements may exist in the biofilm formation process [126]. Other important

components identified within the matrix albeit to lesser extents include lipids, extracellular DNA, metal ions, divalent cations and other biopolymers [127]. Although the role of these minor compounds remains largely unknown, research has suggested that they may be of critical importance in the establishment of the overall biofilm structure. Extracellular DNA, initially thought to be simply a by-product of cell lysis has now been shown to be actively released through an exocytotic mechanism involving the outer bacterial membrane [128, 129]. A study by Whitchurch et al of P. *aeruginosa* biofilms demonstrated that in the absence of extracellular DNA, biofilm formation was inhibited [130], and that enzymatic degradation of extracellular DNA could dissolve immature biofilms, implying the importance of DNA in early biofilm establishment.

1.3.5 BIOFILM FORMATION

The evidence of biofilm formation in the early fossil record (more than 3.25 billion years ago) and their commonality throughout a diverse range of organisms has led researchers to hypothesise biofilm formation to be an ancient and integral component of the prokaryotic life cycle[131]. It is now thought that 99.9 % of bacteria adopt this form for the survival benefits it confers. Recently, there is a growing concept that biofilms may in actual fact represent an earlier evolutionary form than the planktonic state, and may indeed be the default form of growth for some microbial species [124, 132]. Although the evolutionary order of bacterial growth forms remains debated, there is a general consensus that bacteria freely interchange between the planktonic and biofilm form depending on the environmental conditions. This inter-form transition is rapid, owing to the enormous plasticity of bacterial genomes and is mediated by either plasmid exchange, chemotactic signalling or through diffusible signals arising from a process called quorum signalling [133-135].

Biofilm formation has been shown to occur by at least three mechanisms: (1) the redistribution of attached cells by surface motility [136, 137], (2) the binary division of attached cells [138] and (3) the recruitment of cells from the bulk fluid to the development of the biofilm [139]. The relative contribution of each mechanism will depend on the interplay between the organism and surface involved as well as environmental physical and chemical properties.

1.3.6 BIOFILM LIFECYCLE

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

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

(2) Irreversible attachment employs molecularly mediated binding between specific microbial adhesins and the surface. Bacteria have been shown to produce multiple different adhesins, many of which are regulated at the transcriptional level. This permits the rapid transition between planktonic and sessile forms depending on environmental factors [142]. One such example is the polysaccharide intercellular adhesin (PIA) that mediates the cell-cell interactions in some staphylococcal biofilms [143, 144]. Further consolidation of adhesion in this stage occurs through the microbial production of exopolysaccharides that complex with

surface molecules and/or receptor-specific ligands located on pili, fimbriae, and fibrillae [145]. At the conclusion of this stage, the biofilm's attachment is considered irreversible making these structures extremely difficult to remove without chemical intervention or considerable mechanical force.

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

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

There is recent evidence to suggest that all these stages of biofilm formation and development growth and development may be under the regulation of population density-dependent gene expression mediated by cell-to-cell signalling molecules such as acylated homoserine lactones.[134, 147, 148]
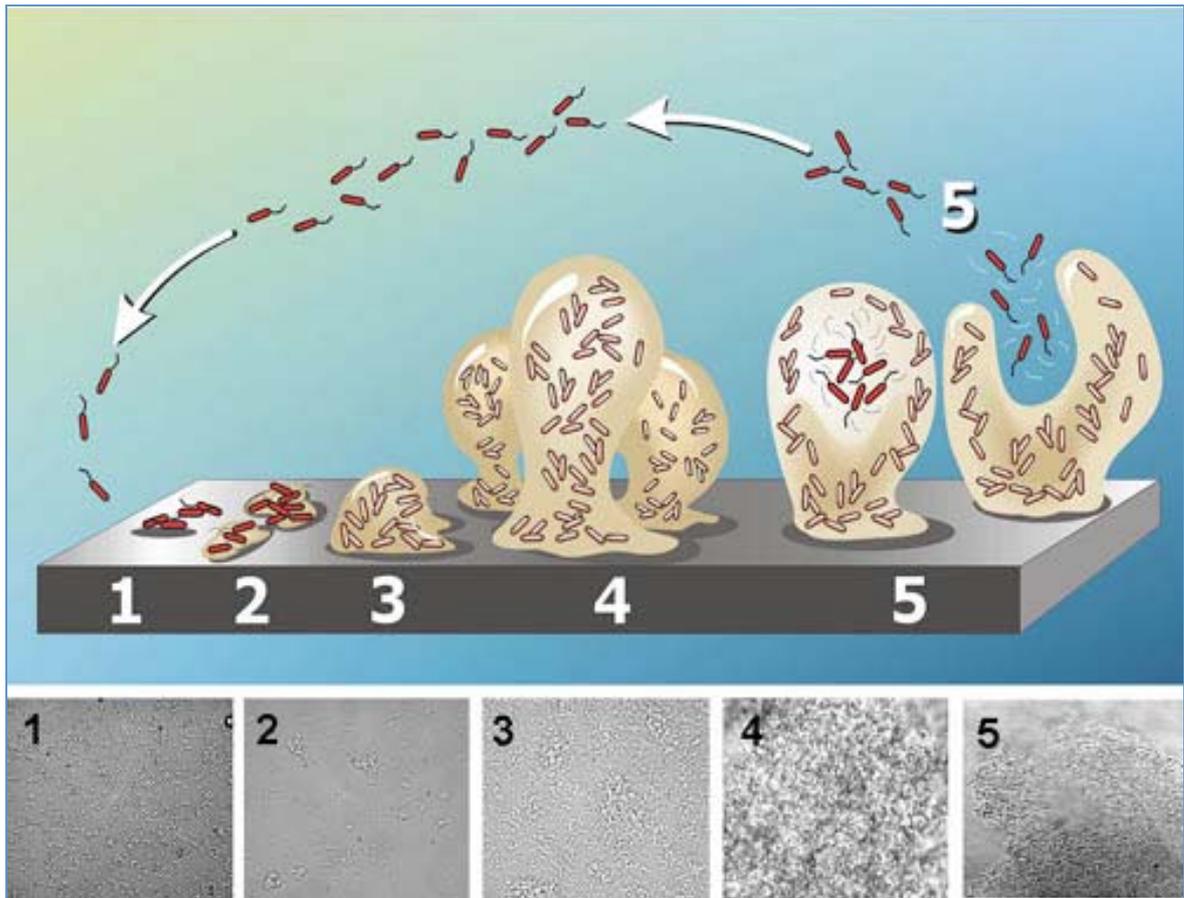
Figure 1: "Reprinted, with permission, from the Annual Review of Microbiology, Volume 56 ©2002 by Annual Reviews www.annualreviews.org

1.3.7 BIOFILMS AND ANTIBIOTIC RESISTANCE

When attached , bacteria show a profound resistance, rendering biofilm cells 10-1000 fold less susceptible to various antimicrobial agents, disinfectants and biocides than the same bacterium grown in planktonic cultures [149-151]. Although this resistance was initially postulated to be mediated by a single generalizable mechanism, recent studies suggest that it more likely to be a multi-factorial process and that the mechanism may vary among different organisms. The main hypotheses have been summarised below.

 (a) **Delayed antibiotic penetration of biofilms.** The presence of the exopolysaccharide matrix of biofilms has long been held to have a role in limiting the penetration of

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

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

effective in killing rapidly growing cells, this slow growth undoubtedly also contributes to biofilm resistance to antimicrobial killing [175].

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

(c) Persisters. It has been proposed that within the heterogeneous population of biofilm microbial cells, a small sub-population of cells, referred to as persisters, may exist. It is thought that these cells adopt a unique and highly protected, phenotypic state, akin to spore formation and are thought to serve as a nidus for biofilm regeneration following antimicrobial treatment[179-182]. Data in support of this persister hypothesis includes measurements of biphasic biofilm killing in which the majority of the cells are killed but a fraction remain unaffected despite prolonged antibiotic treatment [176, 183] Multiple specific genes that contribute to the persister state have now been isolated, an example of which is the high level persistence gene (hip) described in E.coli [181, 184, 185]

1.3.8 BIOFILMS AND THE IMMUNE SYSTEM

Although biofilms are known to be less susceptible to antimicrobial drugs, less is known about their susceptibility to the innate immune system. The innate immune system represents the first line of defence against bacterial colonization and infection. It provides humans with antigen-independent mechanisms of coping with infectious challenges in the absence of pre-existing adaptive immunity and has been shown to be of critical importance in the early stages of infection [186]. This system is multi-tiered and encompasses factors that prevent bacterial adherence to mucous membranes (e.g. mucus and ciliary movement), limit bacterial growth and replication (e.g. antimicrobial peptides) and direct host immune cell responses through specific recognition (Toll-Like receptor activation). Through these mechanisms it is thought that the innate immune system may prevent pathogens gaining a foothold and may also provide the host with the time needed to mobilize the more slowly developing mechanisms of adaptive immunity. It is becoming increasingly evident that pathogenic bacteria use very efficient strategies to circumvent and misguide these host defences in order to colonise and invade human tissues [187]. One such strategy is through the formation of thick biofilms that may prevent the recognition and/or inactivation by antimicrobial molecules and phagocytes [188, 189]. Research examining pseudomonal biofilms have shown that the exopolysaccharide matrix may afford protection against human neutrophils and interferon-γ mediated macrophage killing [190],[191]. Although not well characterised, several mechanisms have been proposed for this increased biofilm resistance to human leukocyte killing; (1) the inactivation or suppression of leukocyte-specific proteases by the biofilm matrix or bacterial components; (2) the decreased ability of leukocytes to phagocytise biofilm bacteria (3) the presence of global response regulators and quorum sensing that increase resistance to leukocytes in biofilms and/or (4) specific genetic switches that lead to increased resistance to components of the human innate system. Although it was initially thought that

24

limited biofilm penetration of leukocytes and their antimicrobial products may play a role, Leid et al [192] have recently shown that in some biofilms leukocytes may penetrate the matrix. Interestingly, Walker et al [193] have also shown that when the host fails to eradicate an infection, neutrophils can undergo necrosis and serve as a biological matrix that may further facilitate microbial biofilm formation.

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

1.3.9 BIOFILMS AND CHRONIC DISEASES

Progress in microbiology and the development of antibiotics and vaccines has led to the successful treatment and in some cases almost complete eradication of many acute epidemic bacterial diseases. With these advances also have emerged the less aggressive and more persistent chronic diseases. Until the rediscovery of biofilms, many chronic diseases were thought to be sterile inflammatory conditions that persisted after the eradication of all micro-organisms. This belief stemmed from the following: (1) it was often difficult to successfully

apply Koch's postulates, the long standing paradigm of bacterial causation of disease, to many chronic conditions, (2) in many chronic diseases, bacteria recovery using conventional culturing methods was not possible, (3) on the occasions when bacteria were isolated and found to be sensitive to antibiotics in laboratory cultures, use of these antibiotics yielded little or no benefit in the treatment of the chronic disease and (4) unlike acute infections which generally involved either host immune-suppression or highly virulent pathogenic organisms, many of the pathogens isolated from chronic diseases were common environmental organisms with seemingly low virulence and poorly defined pathogenic mechanisms. The unequivocal demonstration, using molecular diagnostics, of the presence and metabolic activity of bacteria within many of these "sterile" conditions, accompanied by the increasing environmental and industrial evidence of bacteria existing in an alternate biofilm form, led researchers to revisit the role that bacteria may play in chronic disease. Based on direct examinations of material from device related and other chronic infections, and on patterns of inherent resistance to antibiotics, and to host clearance mechanism, the US Centre for disease control and prevention (CDC) estimated in 1999 that biofilms may be responsible for in excess of 65% of all infections in the developed world [199]. A partial list of common biofilm mediated diseases is shown in table 4 taken from reference [199].

1.3.10 BIOFILMS AND OTOLARYNGOLOGY

Research in the field of otorhinolaryngology has demonstrated bacterial biofilms in a variety of common ENT conditions previously thought to have non bacterial aetiologies. For many years the belief that Otitis media with effusion (OME) was an inflammatory condition, largely stemmed from the often sterile cultures obtained from middle ear aspirates of patients suffering from this condition. With the advent of newer technologies such as the polymerase-

chain reaction (PCR) based assay system, bacterial DNA has been found in a significant percentage of middle ear effusions, sterile by culture [200, 201]. This coupled with the large number of genomic equivalents present in surgically treated cases of OME and the presence of bacterially produce endotoxins within the effusion fluid has led researchers to believe that viable metabolically active bacteria may be contributing to the pathogenesis of OME [200, 202, 203]. The biofilm paradigm of live yet difficult to culture and antibiotic resistant bacteria seemed to offer a plausible explanation for the clinical and microbiological findings of OME. Further evidence supporting the role of biofilms in this condition was provided by the demonstration of mucosal biofilms on the middle ear mucosa in an experimentally infected chinchilla model for OME [204]. Since then CSLM, FISH and immuno-staining examination have provided direct evidence of biofilm presence on the middle ear mucosa of children with OME, further strengthening the biofilm hypothesis of disease causation/persistence [205]. Biofilms have also been demonstrated on tonsillar and adenoidal tissue. Galli et al [206], used scanning electron microscopy to show structures resembling biofilms in 21/25 patients with adenotonsilitis. Furthermore they isolated *Haemophilus influenza*, with a high in vitro biofilm forming capacity, in large proportion of the adenoidal and tonsillar specimens taken from these patients. Also using SEM Zuliani et al [207], demonstrated almost universal mucosal coverage (mean biofilm coverage 94.9%) of adenoidal tissue in all 7 CRS patients included in their study while only 4 of 9 children with OSA and no CRS showed biofilm structures (mean biofilm coverage of 1.9%). These findings coupled with the recent CSLM evidence of biofilms on the adenoidal tissue of 54% of children with chronic or recurrent otitis media, suggests that biofilms in the nasopharynx and in particular the adenoidal pad, may serve as a reservoir for recurrent and persistent infection of neighbouring anatomical structures [208]. This may also explain why mechanical removal of these biofilms via adenoidectomy is often accompanied by marked clinical improvement in the chronic sinus and ear related conditions suffered by children.

Aside from the above conditions, biofilms have also been demonstrated in the keratin accumulations of patients with cholesteatoma [209], in the tonsillar crypts of patients with chronic tonsillitis [210, 211], and on otolarnygologicial-related prosthetic devices such as tracheostomy tubes [212], endotracheal tubes [213, 214], cochlear implants [215-217] voice prostheses [218-220] and tympanostomy tubes [221, 222].

Table 4 Chronic diseases thought to be mediated by biofilms

Infection or disease	Common biofilm bacterial species
Nosocomial infections:	
ICU pneumonia	Gram-negative rods
Sutures	*Staphylococcus epidermidis* and *Staphylococcus aureus*
Exit sites	*S. epidermidis* and *S. aureus*
Arteriovenous shunts	*S. epidermidis* and *S. aureus*
Schleral buckles	Gram-positive cocci
Contact lens	*Pseudomonas aeruginosa* and Gram-positive cocci
Urinary catheter cystitis	*Escherichia coli* and other Gram-negative rods
Peritoneal dialysis (CAPD) peritonitis	A variety of bacteria and fungi
IUDs	*Actinomyces israelii* and many others
Endotracheal tubes	A variety of bacteria and fungi
Hickman catheters	*S. epidermidis* and *Candida albicans*
Central venous catheters	*S. epidermidis* and others
Mechanical heart valves	*S. aureus* and *S. epidermidis*
Vascular grafts	Gram-positive cocci
Biliary stent blockage	A variety of enteric bacteria and fungi
Orthopedic devices	*S. aureus* and *S. epidermidis*
Penile prostheses	*S. aureus* and *S. epidermidis*
Dental caries	Acidogenic Gram-positive cocci (e.g. *Streptococcus*)
Periodontitis	Gram-negative anaerobic oral bacteria
Otitis media	Non-typable strains of *Haemophilus influenzae*
Musculoskeletal infections	Gram-positive cocci (e.g. staphylococci)
Necrotizing fasciitis	Group A streptococci
Biliary tract infection	Enteric bacteria (e.g. *E. coli*)
Osteomyelitis	Various bacteria and fungal species – often mixed
Bacterial prostatitis	*E. coli* and other Gram-negative bacteria
Native valve endocarditis	Viridans group streptococci
Cystic fibrosis pneumonia	*P. aeruginosa* and *Burkholderia cepacia*
[a]Abbreviations: CAPD, continuous ambulatory peritoneal dialysis; ICU, intensive care unit; IUD, intrauterine device.	

1.3.11 BIOFILMS AND RHINOLOGY

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

The first study suggesting biofilm presence on sinus mucosa of CRS patients was a descriptive paper published by Cryer et al in 2004 [223]. This small study of 16 CRS patients, who had failed maximal medical and surgical treatment of their condition, utilised SEM to analyse sinus mucosal specimens. Their findings of near-total surface coverage of four specimens by a coating thicker than the normal mucocilliary blanket, led the authors to speculate biofilm presence in these patients. It is important to note however, that structures resembling actual bacterial elements were only seen in one of these four specimens. Despite the lack of conclusive evidence, the similarity between the SEM appearances of this single specimen to that of previously published images of biofilms, led the authors to conclude that there was "compelling" evidence of bacterial biofilms in patients with recalcitrant CRS. They further hypothesized that these structures may explain the treatment resistant nature of this disease.

Similar pilot studies using electron microscopy were published the following year in 2005. Ramadam et al [224] reported staphylococcal-type biofilm in all 5 CRS patients they examined using SEM. According to the authors, biofilm presence was determined by using strict SEM morphologic criteria as described in the referenced literature, as well as by comparing the images they obtained with "hundreds of biofilm photographs". Unfortunately, of the ten papers referenced by the authors, no evidence of specific SEM morphologic criteria for biofilm detection could be found in any of them. Furthermore two of the included references made no mention of the structure of biofilms at all [225, 226], and only two used CSLM or epifluorescent microscopy as part of their methodology [127, 226]. Additionally the sources of the comparative photographs used, were not provided by the authors. Importantly, in their discussion, the authors do address some of the possible limitations of using SEM to visualise biofilms. They acknowledge that artefacts arising from the dehydration and protein cross-linking that accompany standard SEM preparation, may be confused with biofilms and raise concerns of inadvertent biofilm removal by the harsh SEM preparation process.

Other early electron microscopy research also revealed that biofilms were not only limited to the sinus mucosa. Perloff et al [227] visualised multicellular syncytia coated with extracellular matrix on all frontal stents removed post-operatively from 6 CRS patients. The similarity of these structures to known images of biofilms as well as to structures visible on sterile stents cultured in vitro with known biofilm forming organisms, suggested, that they may represent biofilms. The absence of these structures on stents that did not undergo in vitro culture with bacteria, supported the author's hypothesis that frontal sinus stents may serve as a reservoir for biofilms. Ferguson and Stolz [228]visualized biofilms within the amorphous material overlying the mucosa in 2 of the 4 CRS patients they analysed with TEM. In the two patients without biofilms, a non-bacterial aetiology was discovered. Interestingly both patients with bacterial biofilms had failed medical management with culture-directed antibiotics,

topical steroids, and nasal lavage, further supporting Cryer et al's [223] initial hypothesis that biofilms may not only be involved in the pathogenesis of CRS but more importantly may explain its recalcitrant and often resistant nature.

Although these early studies represented significant advances in biofilm research in the field of rhinology, their small sample size and lack of healthy control specimens, made it difficult to draw definitive conclusion about the role of biofilms in the pathogenesis of CRS. To address this, Perloff and Palmer created a biofilm model in NZ white rabbits [229]. In this study they consistently created sinusitis in each of the 22 maxillary sinuses inoculated with *Pseudomonas aeruginosa*. Mucosa from the middle turbinate and maxillary sinus of the contra-lateral uninoculated side was used as the control. The absolute correlation they observed between the macroscopic features of sinusitis and the presence of biofilms under SEM, and the absence of any biofilms in the controls further strengthened the possible causal relationship of bacterial biofilms and CRS. Furthermore they noted that mature biofilms at day 5 underwent little morphological change at day 10 or 20, suggesting a plateau stage of biofilm development. Although their rabbit model provided further evidence of biofilm involvement in CRS, methodological flaws exist in this model. Firstly, although rabbit models have been used extensively in sinusitis research since Hilding first introduced their use in 1941 [230-238], the universal colonization of rabbits with *Pasturella multilocoda*, a known biofilm forming organism, may render their use limited in biofilm research. Furthermore , a study by Juan et al in 1995, showed that despite the macroscopic normal clinical appearance of healthy New Zealand white rabbit sinuses, 70% demonstrated histological evidence of sinusitis and positive bacterial cultures [239]. A second problem identified with Perloff's model pertains to the external surgical technique used to achieve ostial obstruction. This technique which relies upon surgical violation of the sinus cavity, may not only introduce external bacterial contamination but is also to thought to alter the normal physiology of the

sinus mucosa by interrupting normal sinus capillary flow and pre-existing mucocilliary flow patterns [240, 241]. Extrapolation of the findings of this non-physiologic "sinogenic" model of sinusitis to that seen in humans with CRS may be erroneous. Other additional specific problems with their proposed model involve: (1) the lack of consistent control tissue, with middle turbinate tissue often used instead of sinus tissue (2) the lack of a study arm to examined the contribution of the surgical procedure itself to the formation of sinusitis and (3) the use of cyanomethacrylate glue to ensure maintenance of the ostial plug. Research has shown that shorter-chain derivatives (methyl- and ethyl) of cyanoacrylate are histotoxic to sinus mucosa causing acute inflammation, tissue necrosis, and a chronic foreign body giant cell reaction [242]. Finally the over-riding flaw of this study was the reliance on SEM alone to document biofilm presence, despite previously raised concerns of its subjectivity, and potential problems associated with dehydration, distortion, and artefact introduction.

In an attempt to address many of the theoretical concerns of using SEM in biofilm research, Sanclement et al conducted a human study of 30 CRS patients [243]. In this study, 24 CRS specimens and all 4 controls were processed using standard SEM techniques while 6 CRS specimens were prepared with advanced cryofixation methods for SEM and TEM analysis. According to the authors, the purpose of this smaller experimental group was to examine (a) whether standard SEM preparation caused dehydration or artefactual error and (b) whether structures resembling bacterial biofilms on surface analysis, demonstrated bacterial structures on cross-sectional analysis with TEM. This study reported an absence of biofilms on control specimens and 80% prevalence in CRS patients. The authors also concluded that standard SEM preparation did not introduce significant artefacts from protein cross linking and that although it caused some reduction in biofilm size from dehydration, biofilms were still easily recognizable. They also reported an absolute correlation between structures deemed biofilms on SEM and TEM. When critically reviewing this study however, it is important to note the

following; (1) the very small number of controls (n=4) included in the study, (2) the fact that the majority of specimens (24 of 30) were only analysed with SEM , (3) specimens from the same patient were not used in the comparison of standard SEM preparation and cryofixation and (4) that no control or non-biofilm patients were included in the group analysed with both SEM and TEM. Such methodological flaws, not only throw into question the high biofilm prevalence rate observed in this study, but also make it difficult to substantiate many of the authors' conclusions.

1.4 LACTOFERRIN

1.4.1 INTRODUCTION

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

1.4.2 STRUCTURE

The complete amino acid sequence of human serum lactoferrin was first determined in 1984 by Metz-Boutigue [252]. He not only demonstrated its 60% sequence identity with serum transferrin, another iron binding protein, but also showed 40% internal homology of its two domains, the N- and C-terminals. In addition approximately 70% amino acid sequence identity has since been demonstrated between different species (human, bovine, buffalo, camel, goat, horse, mouse, pig), suggesting early evolutionary importance of this antimicrobial peptide [253]. In 1990 , the complete cDNA sequence was isolated and found to encode a signal peptide of 19 amino acids followed by a mature protein, 692 amino acids in length [254],[255].

Using crystallographic analysis with a resolution of 2.8 Ã, the tertiary structure of lactoferrin, has been well characterized [256]. The polypeptide chain of human lactoferrin has been shown to be folded into two almost homologous globular lobes each with the same fold, reflecting their previously eluded to sequence identity. These lobes consist of an N terminal half of amino acid residues 1-333 and a C terminal half of amino acid residues 345-691 linked together by a 10 residue peptide chain (residues 334-344) that forms an extended 3-turn alpha-helix [257]. Within each lobe 2 alpha/beta domains exist that enclose a deep cleft surrounding the iron binding site. The iron binding sites are chemically and geometrically ideal for high affinity but reversible iron binding, and any mutagenesis of these ligands substantially weakens their iron binding ability [258],[259],[260]. See figure 2 for the diagrammatic representation of lactoferrin's structure. At least four isoforms of lactoferrin, α, β,γ,δ are now known to exist. Although they share very similar chemical compositions, they have been shown to differ in their iron binding activity (only possessed by the α isoform), and enzymatic activity (with β and γ having RNase activity but not α). It is thought that these differences may explain the many diverse functions that have been attributed to lactoferrin.

Figure 2   Lactoferrin's three dimensional structure

Iron Bound   Iron Free
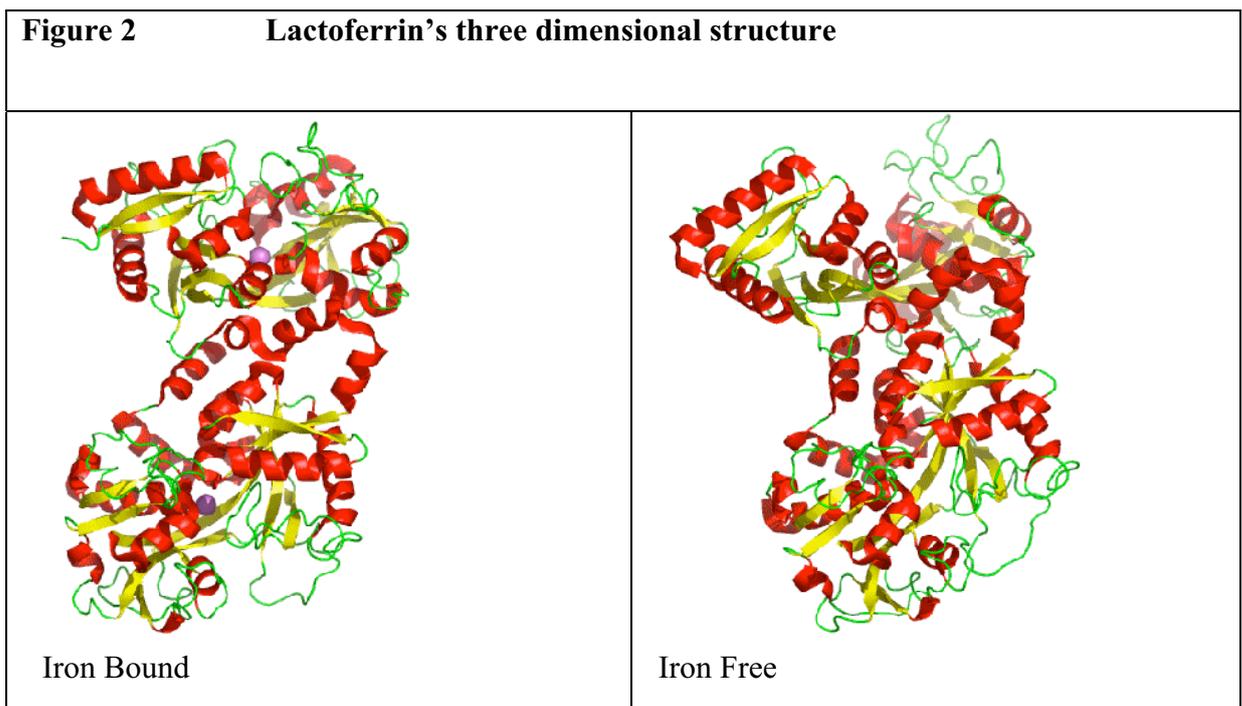
1.4.3 LACTOFERRIN'S ANTIMICROBIAL ACTION

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

Lactoferrin and lactoferricin have also been shown to have antiviral activity against both enveloped viruses (HSV 1and 2 CMV, HIV, HBV, HCV, RSV) as well as naked viruses (rotavirus, poliovirus, adenovirus and enterovirus) [273]. This action is again thought to be iron independent and is postulated to either be mediated by lactoferrin directly binding to viral

particles before they infect host cells, or by it binding to and altering host cell molecules which viruses ordinarily use as receptors to enter cells [274]. This direct viral binding capacity of lactoferrin has led some researchers to explore the use of this anti-microbial peptide as a selective delivery system for antiviral medications.

1.4.4 IMMUNE REGULATION BY LACTOFERRIN

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

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

1.4.5 ANTI-BIOFILM ACTION

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

Deficiencies in the level or activity of lactoferrin are thought to partly explain the propensity of biofilm formation in chronic diseases such as cystic fibrosis. In 1993 Britigan et al [286] showed that lactoferrin was proteolytically degraded in the lungs of cystic fibrosis patients infected with Pseudomonas aeruginosa by proteases such as neutrophil elastase and Pseudomonas elastase. Later in 2004, Rogan et al [287] established that this failure to achieve the expected increase in levels of lactoferrin despite large amounts of neutrophil degradation

was due to the action of another elastolytic protease called cathepsin. They showed that cathepsins, which cleave and inactivate lactoferrin, reducing its antimicrobial and anti-biofilm actions, had higher activity in CF patients with pseudomonas positive sputum. Although the trigger for the increase in cathepsin activity is unknown, studies suggest that bacterial LPS and cytokines (IFN gamma) may be involved [288, 289].

1.4.6 LACTOFERRIN AND CRS

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

1.5 CONCLUSIONS FROM REVIEW OF THE LITERATURE.

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

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

1.6 STUDIES TO BE CONDUCTED

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

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

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

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

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

CHAPTER 2

A SHEEP MODEL FOR THE STUDY OF BIOFILM IN SINUSITIS

CHAPTER 3

CONFOCAL SCANNING LASER MICROSCOPY EVIDENCE OF BACTERIAL

BIOFILMS IN PATIENTS WITH CHRONIC RHINOSINUSITIS

CHAPTER 4

THE EFFECT OF BACTERIAL BIOFILMS ON POST SINUS SURGICAL OUTCOMES

CHAPTER 5

NASAL MUCOSA EXPRESSION OF LACTOFERRIN IN PATIENTS

WITH CHRONIC RHINOSINUSITIS

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

CHAPTER 6

REDUCED LEVELS OF LACTOFERRIN IN

BIOFILM-ASSOCIATED CHRONIC RHINOSINUSITIS

CHAPTER 7

DISCUSSION AND CONCLUSIONS

7.1. OVERVIEW

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

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

7.1.1 THE OVINE MODEL IS A SUITABLE MODEL FOR THE INVESTIGATION BACTERIAL BIOFILMS IN RHINOSINUSITIS.

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

Our department has had significant experience with the sheep rhinologic model [301, 318, 357-366]. In Australia sheep are relatively inexpensive and easily obtainable. Their large size and blood volume allow them to withstand multiple long surgical procedures requiring general anaesthesia. Studies have also demonstrated sheep to possess an almost identical sinus mucosa histology and analogous sinus anatomy to humans [308, 309]. In addition, the sheep's large nasal cavity makes it superior to smaller animals in surgical models trying to replicate endoscopic procedures conducted in humans. Ostial obstruction can be achieved entirely endoscopically, avoiding the need for large externally created surgical windows that could potentially disrupt the normal mucocilliary and capillary blood flow patterns within the sinuses. In the model described in this thesis, the frontal sinus was selected to obstruct and inoculate with bacteria. Preceding cadaveric studies of sheep heads showed that as in humans, the frontal sinus is superficially located with an anterior table thickness of 5–12mm. The thin anterior table allows for mini-trephination that not only assists in the confirmation of the frontal sinus' drainage tract but also provides a minimally traumatic portal for intra-sinus bacterial instillation. The site for safe and effective trephination was determined to be located at the bisection of a perpendicular line drawn from the midpoint of the bony orbit and a para-

sagittal line 1cm lateral to the midline. Using these landmarks, mini-trephination was successfully performed in all 48 sheep frontal sinuses without adverse event. The additional advantage of the mini-trephine is that it can be left in post-operatively and potentially used in treatment extensions of the model we have developed.

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

Using this model we were able to successfully and consistently create an inflammatory reaction consistent with sinusitis in the frontal sinuses of sheep. Through the use of different study groups, the relative and independent contribution of both anatomical ostial obstruction and bacterial load was assessed. As demonstrated by our findings; to create consistent sinusitis involving the entire sinus cavity, both obstruction and bacterial instillation were required. Without both of these interventions, the degree of mucosal inflammation was significantly reduced, less generalised and in some cases absent. Also of significance was the finding of largely localised inflammation around the cotton wool pledget in the sinus group randomised to ostial obstruction only. Such a localised inflammatory process would suggest a foreign body reaction rather than sinusitis and so this unrepresentative area of mucosa was excluded from microscopic examination for biofilms.

In addition to its demonstrated usefulness in the study of rhinosinusitis, the sheep model was also shown to be of use in the investigation of the role that biofilms play in this condition. The findings of an absence of biofilms in the control sinuses and an almost absolute correlation of their presence with macroscopic evidence rhinosinusitis in the remaining sinus groups, added support to earlier research suggesting their possible importance in the pathogenesis of CRS.

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

CSLM is generally accepted by the scientific community to be the gold standard imaging modality for biofilms. Despite this, biofilm research in the field of rhinology has relied solely upon electron microscopy until recently. To address this, the sheep model we developed was also used to evaluate and compare CSLM to traditional electron microscopy in the imaging of biofilms on sinus mucosa. In this study, CSLM was found to be the most reliable modality for the detection of biofilms, demonstrating the highest concordance between macroscopic and microscopic findings. SEM, although a seemingly very sensitive modality, tended to over-estimate biofilm presence, while the converse was found with TEM. In our study we experienced first-hand many of the previously postulated theoretical problems associated with EM. Dehydration and distortion of specimens during their preparation, was evident both macroscopically and microscopically. Several specimens confirmed as containing biofilms on both CSLM and TEM, lacked the typical three dimensional structures usually seen on SEM imaging. Rather biofilms appeared as collapsed and unorganised amorphous masses. Correct orientation of EM specimens after critical point drying was extremely difficult and inconsistent, making the incorrect mounting of samples for sputter coating highly likely. We

believe that this poses a real problem for new researchers who may make erroneous conclusions regarding biofilm presence, following inadvertent imaging of the non-mucosal surface of the specimen. From this study, and later from the findings of the study in chapter 6, it became evident that under SEM, mucus and the biofilm matrix, both composed of long chain polysaccharides, appear almost identical. Previous SEM sheep studies from our department have shown that the mucus layer which typically obstructs visualisation of the underlying ciliated mucosa is very difficult to remove. These studies required the use of ultra-sound energy to dislodge the mucus [359, 365]. Unfortunately ultra-sound could not be used in our study as it has also been shown to be an effective method of removing biofilms [305]. From close examination of sheep and later human SEM specimens, we observed that the only distinguishing feature between the biofilm matrix and mucus under SEM was the presence of clusters of bacterial elements. Although not mentioned as an important criterion for biofilm detection by previous SEM studies, we found the visualisation of such structures critical for the definitive documentation of biofilms. This is further highlighted by the findings of the study in chapter 6, which showed that when this criterion was adhered to, the SEM biofilm prevalence rate decreased significantly from 73% to 34% and more closely resembled that seen with CSLM. Unfortunately it must be noted however, that even though such strict criteria may improve the specificity of SEM, its sensitivity may suffer. This is because SEM only allows visualisation of surface structures and bacteria residing deep with the biofilm matrix will not be seen. Although our sheep study showed that TEM may improve the visualisation of these internal bacteria, its requirement for ultra-thin sectioning of specimens, also decreases the sensitivity of this modality. From these findings we concluded that when investigating biofilm prevalence, TEM and SEM should not be used alone.

CSLM seemed to circumvent many of the problems associated with EM. The larger specimen size accommodated by the CSLM's stage meant that specimen orientation was much easier. Furthermore, it allowed a much larger surface area of mucosa to be imaged quickly, thereby reducing the chance of sampling error created by a biofilm's typical patchy mucosal distribution. Unlike SEM and TEM, specimens were processed fresh avoiding aldehyde or ethanol-related dehydration. In addition the avoidance of formalin fixatives removed any possibility of artefactual error related to protein cross-linking. We found that through the use of nucleic acid probes and optical sectioning, CSLM allowed the entire three dimensional biofilm structure to be visualised and mucus could be easily distinguished from the biofilm matrix. In addition when combined with FISH, it has also been shown to be useful in the precise identification of the specific bacterial species within the biofilm[314] . It is for these reasons that we believe CSLM has emerged as the imaging modality of choice for biofilm research in the wider scientific community and should be used in future CRS studies.

7.1.3 OBJECTIVE EVIDENCE OF THE PRESENCE OF BIOFILMS IN CRS PATIENTS.

Although biofilms have been previously documented on the mucosa of CRS patients, it was not until 2006 that the first CSLM study was published. In this study, biofilms were demonstrated using fluorescent in situ hybridization (FISH) in 14 of 18 CRS patients and in 2 of 5 healthy control specimens[314]. Using specific radio-fluorescent labelled probes, bacterial identification was possible with *H.influenza* being found to be most common organism isolated within the biofilms (identified in 14/18 CRS specimens). Although *S. pneumoniae* and *S.aureus* were also detected their presence was far less common. Importantly this study was also the first to demonstrate bacterial biofilms on control specimens leading the

authors to postulate a possible commensal or non-pathogenic role of biofilms in CRS. Although this conclusion may prove to hold true, planktonic contamination as a consequence of methodological deficiencies such as delayed processing time, absence of specimen washing and lengthy specimen preparation times cannot be excluded and may explain this finding. Such contamination may also explain the high biofilm prevalence rate observed in this study. Nevertheless, the use of FISH with CSLM in this study represented a significant advance in rhinological related biofilm research.

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

7.1.4 CLINICAL FEATURES OF BIOFILM PATIENTS

In the study performed in chapter 4, an attempt was made to elicit any differences in the clinical characteristics of biofilm patients compared to those in whom biofilms were not detected. Demographically no difference was found with respects to age or gender distribution. Interestingly, although no statistical difference was seen in the symptom scores reported by the two groups prior to surgery (median biofilm symptom score 17 with inter-quartile range 13.5-18.5 compared to median non-biofilm symptom score 16 (13.5-18.0), patients with biofilms had significantly higher Lund-Mackay scores on pre-op CT imaging (median score biofilm 18.5 with inter-quartile range 17.0-22.0 compared to median score non-biofilm14.5 with inter-quartile range14.0-18.0). This perhaps reflects a more severe and diffuse disease process in this sub-group of patients. It is important to note that the lack of correlation between CRS symptom scores and the overall radiological score observed in this study, is in concordance with the majority of previously published studies concerning this matter [322].

Due to the tertiary referral nature of the practice from which the patients were recruited, there were a high percentage of revision FESS cases. Worthy of note however, was the particularly high proportion of patients with biofilms requiring revision surgery compared to those without (75% vs 45%) and the trend for them having undergone a higher number of previous surgical procedures (average number 2.7 vs 1.8 previous FESS operations). This findings provides further support to the speculation by earlier researchers that biofilms may predispose to a persistent and recalcitrant sinus disease process, often refractory to surgical intervention [223, 228, 311].

Another important finding of our CSLM study was the lack of statistical relationship of positive planktonic bacterial culture rates and the detection of biofilms. This finding is consistent with that of Sanderson et al [314] and is in accordance with the proposed biofilm paradigm; that bacteria existing within biofilms exhibit much slower growth and metabolic rates than their planktonic counterparts, making them extremely difficult to culture using standard techniques.

7.1.5 THE EFFECT OF BIOFILMS ON POST ENDOSCOPIC SINUS SURGICAL OUTCOMES

The success rate of FESS is extremely variable. Although some studies claim resolution or improvement of symptoms in up to 83-92% of patients [57, 319], the majority of this research is of low level evidence [53]. A recent systematic review of the literature by the Cochrane Collaboration Group concluded that based on the evidence available FESS does not confer any additional benefits to that obtained by medical treatments of CRS [49]. To date multiple studies have been performed addressing possible outcome predictors following FESS, These include demographic differences, smoking, presence of nasal polyposis and disease severity [54, 57, 61, 320, 321]. The first paper to address the possible effect of biofilms on post-operative outcomes was published by Bendouah et al in 2006 [311]. This study examined the relationship between the in vitro biofilm forming capacity of bacteria recovered from CRS patients and the evolution of their disease post-operatively. A significant correlation was reported between the biofilm-producing capacity of clinically isolated *Pseudomonas aeruginosa* and *Staphylococcus aureus* and an unfavourable evolution after FESS. The authors concluded that this provided further evidence for the role of biofilms in CRS.

In the study outlined in chapter 4, we retrospectively analysed prospectively collected data from 40 CRS patients who underwent ESS for symptoms of CRS. Of all the clinical and histological features evaluated, only the presence of biofilms and/or fungus were statistically related to a worse post-operative outcome in terms of ongoing symptoms and evidence of mucosal inflammation. The documented extreme difficulty in completely removing both biofilms and fungus from mucosal surfaces may partly explain this, with residual colonies of micro-organisms potentially serving as a nidus for re-infection. Interestingly, the presence of pus, polyps or eosinophilic mucus, all factors also believed to be associated with poorer post-operative outcomes, were not shown to have an adverse influence on ESS outcomes in this study. The reason for this is unclear but may be in part due to the fact that the scoring of post-operative outcomes in this study was nominal rather than ordinal, with any evidence of mucosal inflammation recorded as unfavourable. To address this, a blinded prospective study with endoscopic grading of post-operative mucosal outcomes is currently being undertaken in our department.

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

Despite lactoferrin's abundance in airway secretions and its presumed importance as a first line of defence against a broad spectrum of invading pathogens, there is little known about the role it plays in CRS. The recent identification of lactoferrin's anti-biofilm activity provided the motivation for the studies outlined in chapter 5 and 6. To our knowledge, these are the first two published studies to objectively quantify the expression of this antimicrobial peptide in the sino-nasal mucosa of CRS patients All previous studies had relied upon the subjective and at best semi-quantitative methodology of Immunohistochemistry (IHC) [292-294] .

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

Our study also demonstrated that although all sub-groups of patients with chronic rhinosinusitis seem to have a down regulation of lactoferrin in their nasal mucosa compared to

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

7.1.7 REDUCED LEVELS OF LACTOFERRIN EXPRESSION IN BIOFILM ASSOCIATED RHINOSINUSITIS

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

It has already been documented in cystic fibrosis patients that a reduction in the antimicrobial action or gene expression of innate peptides such as lactoferrin may predispose individuals to

recurrent infections and inflammation [287, 339]. Given its diverse anti-pathogenic activity, it also seems plausible that a decrease in the synthesis and production of lactoferrin in certain people may also predispose them to chronic infections like CRS. It must be noted however, that although an association between reduced lactoferrin levels and biofilm presence was evident in this study, a cause and effect relationship could not be deduced. As a consequence the possibility of impaired lactoferrin production by the biofilm itself or through a secondary mediator produced by the biofilm cannot be excluded and further investigation is warranted.

7.2 TREATMENT OF BACTERIAL BIOFILMS

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

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

b) Antibiotic based treatments – Although biofilm bacteria have known resistance against antimicrobials at standard concentrations, in vitro evidence now exists that when used at very high concentrations, antibiotics may be effective in reducing biofilm load. Moxifloxacin and Mupirocin have both shown in vitro efficacy against biofilms when used at concentrations easily attainable in topical solution [370, 371]. Such findings have rekindled interest in the use of topical therapies in the treatment of CRS. In

addition to these studies, novel therapies have also shown improved antibiotic susceptibility of biofilm bacteria in both electric fields and from ultrasonic stimulation [372, 373].

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

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

7.3 CONCLUSION

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

BIBLIOGRAPHY

1. Jackson, L.L. and S.E. Kountakis. *Classification and management of rhinosinusitis and its complications.* Otolaryngol Clin North Am 2005; **38** (6): p. 1143-1153.
2. Meltzer, E.O., D.L. Hamilos, J.A. Hadley, D.C. Lanza, B.F. Marple, R.A. Nicklas, C. Bachert, J. Baraniuk, F.M. Baroody, M.S. Benninger, I. Brook, B.A. Chowdhury, H.M. Druce, S. Durham, B. Ferguson, J.M. Gwaltney, M. Kaliner, D.W. Kennedy, V. Lund, R. Naclerio, R. Pawankar, J.F. Piccirillo, P. Rohane, R. Simon, R.G. Slavin, A. Togias, E.R. Wald, and S.J. Zinreich. *Rhinosinusitis: establishing definitions for clinical research and patient care.* J Allergy Clin Immunol. 2004; **114** (6 Suppl): p. 155-212.
3. Anand, V.K. *Epidemiology and economic impact of rhinosinusitis.* Ann Otol Rhinol Laryngol Suppl 2004; **193**: p. 3-5.
4. Ray, N.F., J.N. Baraniuk, M. Thamer, C.S. Rinehart, P.J. Gergen, M. Kaliner, S. Josephs, and Y.H. Pung. *Healthcare expenditures for sinusitis in 1996: contributions of asthma, rhinitis, and other airway disorders.* J Allergy Clin Immunol 1999; **103** (3 Pt 1): p. 408-414.
5. Collins, J.G. *Prevalence of selected chronic conditions: United States, 1990-1992.* Vital Health Stat 10 1997; (194): p. 1-89.
6. Hing, E. and C.W. Burt. *Characteristics of office-based physicians and their practices: United States, 2003-04.* Vital Health Stat 13 2007; (164): p. 1-34.
7. Raofi, S. and S.M. Schappert. *Medication therapy in ambulatory medical care: United States, 2003-04.* Vital Health Stat 13 2006; (163): p. 1-40.
8. Gliklich, R.E. and R. Metson. *The health impact of chronic sinusitis in patients seeking otolaryngologic care.* Otolaryngol Head Neck Surg 1995; **113** (1): p. 104-109.
9. Wolf, C. *Urban air pollution and health: an ecological study of chronic rhinosinusitis in Cologne, Germany.* Health Place 2002; **8** (2): p. 129-139.
10. Briggs, R.D., S.T. Wright, S. Cordes, and K.H. Calhoun. *Smoking in chronic rhinosinusitis: a predictor of poor long-term outcome after endoscopic sinus surgery.* Laryngoscope 2004; **114** (1): p. 126-128.
11. Chen, Y., R. Dales, and M. Lin. *The epidemiology of chronic rhinosinusitis in Canadians.* Laryngoscope 2003; **113** (7): p. 1199-1205.
12. Asero, R. and G. Bottazzi. *Nasal polyposis: a study of its association with airborne allergen hypersensitivity.* Ann Allergy Asthma Immunol 2001; **86** (3): p. 283-285.
13. Gutman, M., A. Torres, K.J. Keen, and S.M. Houser. *Prevalence of allergy in patients with chronic rhinosinusitis.* Otolaryngol Head Neck Surg 2004; **130** (5): p. 545-552.
14. Wang, X., J. Kim, R. McWilliams, and G.R. Cutting. *Increased prevalence of chronic rhinosinusitis in carriers of a cystic fibrosis mutation.* Arch Otolaryngol Head Neck Surg 2005; **131** (3): p. 237-240.
15. Armengot, M., G. Juan, C. Carda, J. Montalt, and J. Basterra. *Young's syndrome: a further cause of chronic rhinosinusitis.* Rhinology 1996; **34** (1): p. 35-37.
16. Baroody, F.M. *Mucociliary transport in chronic rhinosinusitis.* Clin Allergy Immunol 2007; **20**: p. 103-119.
17. Sharp, H.J., D. Denman, S. Puumala, and D.A. Leopold. *Treatment of acute and chronic rhinosinusitis in the United States, 1999-2002.* Arch Otolaryngol Head Neck Surg 2007; **133** (3): p. 260-265.
18. Kaszuba, S.M. and M.G. Stewart. *Medical management and diagnosis of chronic rhinosinusitis: A survey of treatment patterns by United States otolaryngologists.* Am J Rhinol 2006; **20** (2): p. 186-190.

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

20. Ragab, S.M., V.J. Lund, and G. Scadding. *Evaluation of the medical and surgical treatment of chronic rhinosinusitis: a prospective, randomised, controlled trial.* Laryngoscope 2004; **114** (5): p. 923-930.

21. Nishi, K., M. Mizuguchi, H. Tachibana, T. Ooka, T. Amemiya, S. Myou, M. Fujimura, and T. Matsuda. *[Effect of clarithromycin on symptoms and mucociliary transport in patients with sino-bronchial syndrome].* Nihon Kyobu Shikkan Gakkai Zasshi 1995; **33** (12): p. 1392-1400.

22. Scadding, G.K., V.J. Lund, and Y.C. Darby. *The effect of long-term antibiotic therapy upon ciliary beat frequency in chronic rhinosinusitis.* J Laryngol Otol 1995; **109** (1): p. 24-26.

23. Hashiba, M. and S. Baba. *Efficacy of long-term administration of clarithromycin in the treatment of intractable chronic sinusitis.* Acta Otolaryngol Suppl 1996; **525**: p. 73-78.

24. Ichimura, K., Y. Shimazaki, T. Ishibashi, and R. Higo. *Effect of new macrolide roxithromycin upon nasal polyps associated with chronic sinusitis.* Auris Nasus Larynx 1996; **23**: p. 48-56.

25. Suzuki, H., A. Shimomura, K. Ikeda, T. Oshima, and T. Takasaka. *Effects of long-term low-dose macrolide administration on neutrophil recruitment and IL-8 in the nasal discharge of chronic sinusitis patients.* Tohoku J Exp Med 1997; **182** (2): p. 115-124.

26. Namyslowski, G., M. Misiolek, E. Czecior, E. Malafiej, B. Orecka, P. Namyslowski, and H. Misiolek. *Comparison of the efficacy and tolerability of amoxycillin/clavulanic acid 875 mg b.i.d. with cefuroxime 500 mg b.i.d. in the treatment of chronic and acute exacerbation of chronic sinusitis in adults.* J Chemother 2002; **14** (5): p. 508-517.

27. Legent, F., P. Bordure, C. Beauvillain, and P. Berche. *A double-blind comparison of ciprofloxacin and amoxycillin/clavulanic acid in the treatment of chronic sinusitis.* Chemotherapy 1994; **40 Suppl 1**: p. 8-15.

28. McNally, P.A., M.V. White, and M.A. Kaliner. *Sinusitis in an allergist's office: analysis of 200 consecutive cases.* Allergy Asthma Proc 1997; **18** (3): p. 169-175.

29. Subramanian, H.N., K.B. Schechtman, and D.L. Hamilos. *A retrospective analysis of treatment outcomes and time to relapse after intensive medical treatment for chronic sinusitis.* Am J Rhinol 2002; **16** (6): p. 303-312.

30. Scheinberg, P.A. and A. Otsuji. *Nebulized antibiotics for the treatment of acute exacerbations of chronic rhinosinusitis.* Ear Nose Throat J 2002; **81** (9): p. 648-652.

31. Vaughan, W.C. and G. Carvalho. *Use of nebulized antibiotics for acute infections in chronic sinusitis.* Otolaryngol Head Neck Surg 2002; **127** (6): p. 558-568.

32. Solares, C.A., P.S. Batra, G.S. Hall, and M.J. Citardi. *Treatment of chronic rhinosinusitis exacerbations due to methicillin-resistant Staphylococcus aureus with mupirocin irrigations.* Am J Otolaryngol 2006; **27** (3): p. 161-165.

33. Desrosiers, M.Y. and M. Salas-Prato. *Treatment of chronic rhinosinusitis refractory to other treatments with topical antibiotic therapy delivered by means of a large-particle nebulizer: results of a controlled trial.* Otolaryngol Head Neck Surg 2001; **125** (3): p. 265-269.

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

35. Parikh, A., G.K. Scadding, Y. Darby, and R.C. Baker. *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.

36. Sykes, D.A., R. Wilson, K.L. Chan, I.S. Mackay, and P.J. Cole. *Relative importance of antibiotic and improved clearance in topical treatment of chronic mucopurulent rhinosinusitis. A controlled study.* Lancet 1986; **2** (8503): p. 359-360.

37. Lavigne, F., L. Cameron, P.M. Renzi, J.F. Planet, P. Christodoulopoulos, B. Lamkioued, and Q. Hamid. *Intrasinus administration of topical budesonide to allergic patients with chronic rhinosinusitis following surgery.* Laryngoscope 2002; **112** (5): p. 858-864.

38. Cuenant, G., J.P. Stipon, G. Plante-Longchamp, C. Baudoin, and Y. Guerrier. *Efficacy of endonasal neomycin-tixocortol pivalate irrigation in the treatment of chronic allergic and bacterial sinusitis.* ORL J Otorhinolaryngol Relat Spec 1986; **48** (4): p. 226-232.

39. Hissaria, P., W. Smith, P.J. Wormald, J. Taylor, M. Vadas, D. Gillis, and F. Kette. *Short course of systemic corticosteroids in sinonasal polyposis: a double-blind, randomized, placebo-controlled trial with evaluation of outcome measures.* J Allergy Clin Immunol 2006; **118** (1): p. 128-133.

40. Kupferberg, S.B., J.P. Bent, 3rd, and F.A. Kuhn. *Prognosis for allergic fungal sinusitis.* Otolaryngol Head Neck Surg 1997; **117** (1): p. 35-41.

41. Landsberg, R., Y. Segev, A. DeRowe, T. Landau, A. Khafif, and D.M. Fliss. *Systemic corticosteroids for allergic fungal rhinosinusitis and chronic rhinosinusitis with nasal polyposis: a comparative study.* Otolaryngol Head Neck Surg 2007; **136** (2): p. 252-257.

42. Harvey, R., S.A. Hannan, L. Badia, and G. Scadding. *Nasal saline irrigations for the symptoms of chronic rhinosinusitis.* Cochrane Database Syst Rev 2007; (3): p. CD006394.

43. Garavello, W., M. Romagnoli, L. Sordo, R.M. Gaini, C. Di Berardino, and A. Angrisano. *Hypersaline nasal irrigation in children with symptomatic seasonal allergic rhinitis: a randomized study.* Pediatr Allergy Immunol 2003; **14** (2): p. 140-143.

44. Garavello, W., F. Di Berardino, M. Romagnoli, G. Sambataro, and R.M. Gaini. *Nasal rinsing with hypertonic solution: an adjunctive treatment for pediatric seasonal allergic rhinoconjunctivitis.* Int Arch Allergy Immunol 2005; **137** (4): p. 310-314.

45. Rabago, D., A. Zgierska, M. Mundt, B. Barrett, J. Bobula, and R. Maberry. *Efficacy of daily hypertonic saline nasal irrigation among patients with sinusitis: a randomized controlled trial.* J Fam Pract 2002; **51** (12): p. 1049-1055.

46. Heatley, D.G., K.E. McConnell, T.L. Kille, and G.E. Leverson. *Nasal irrigation for the alleviation of sinonasal symptoms.* Otolaryngol Head Neck Surg 2001; **125** (1): p. 44-48.

47. Cordray, S., J.B. Harjo, and L. Miner. *Comparison of intranasal hypertonic dead sea saline spray and intranasal aqueous triamcinolone spray in seasonal allergic rhinitis.* Ear Nose Throat J 2005; **84** (7): p. 426-430.

48. Bachmann, G., G. Hommel, and O. Michel. *Effect of irrigation of the nose with isotonic salt solution on adult patients with chronic paranasal sinus disease.* Eur Arch Otorhinolaryngol 2000; **257** (10): p. 537-541.

49. Khalil, H.S. and D.A. Nunez. *Functional endoscopic sinus surgery for chronic rhinosinusitis.* Cochrane Database Syst Rev 2006; **3**: p. CD004458.

50. Metson, R.B. and R.E. Gliklich. *Clinical outcomes in patients with chronic sinusitis.* Laryngoscope 2000; **110** (3 Pt 3): p. 24-28.

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

52. Damm, M., G. Quante, M. Jungehuelsing, and E. Stennert. *Impact of functional endoscopic sinus surgery on symptoms and quality of life in chronic rhinosinusitis.* Laryngoscope 2002; **112** (2): p. 310-315.

53. Smith, T.L., P.S. Batra, A.M. Seiden, and M. Hannley. *Evidence supporting endoscopic sinus surgery in the management of adult chronic rhinosinusitis: a systematic review.* Am J Rhinol 2005; **19** (6): p. 537-543.

54. Poetker, D.M., S. Mendolia-Loffredo, and T.L. Smith. *Outcomes of endoscopic sinus surgery for chronic rhinosinusitis associated with sinonasal polyposis.* Am J Rhinol 2007; **21** (1): p. 84-88.

55. Hartog, B., P.P. van Benthem, L.C. Prins, and G.J. Hordijk. *Efficacy of sinus irrigation versus sinus irrigation followed by functional endoscopic sinus surgery.* Ann Otol Rhinol Laryngol 1997; **106** (9): p. 759-766.

56. Bhattacharyya, N. *Clinical outcomes after revision endoscopic sinus surgery.* Arch Otolaryngol Head Neck Surg 2004; **130** (8): p. 975-978.

57. Dursun, E., H. Korkmaz, A. Eryilmaz, U. Bayiz, D. Sertkaya, and E. Samim. *Clinical predictors of long-term success after endoscopic sinus surgery.* Otolaryngol Head Neck Surg 2003; **129** (5): p. 526-531.

58. Watelet, J.B., B. Annicq, P. van Cauwenberge, and C. Bachert. *Objective outcome after functional endoscopic sinus surgery: prediction factors.* Laryngoscope 2004; **114** (6): p. 1092-1097.

59. Sobol, S.E., E.D. Wright, and S. Frenkiel. *One-year outcome analysis of functional endoscopic sinus surgery for chronic sinusitis.* J Otolaryngol 1998; **27** (5): p. 252-257.

60. Giger, R., B.N. Landis, C. Zheng, D.D. Malis, A. Ricchetti, A.M. Kurt, D.R. Morel, and J.S. Lacroix. *Objective and subjective evaluation of endoscopic nasal surgery outcomes.* Am J Rhinol 2003; **17** (6): p. 327-333.

61. Deal, R.T. and S.E. Kountakis. *Significance of nasal polyps in chronic rhinosinusitis: symptoms and surgical outcomes.* Laryngoscope 2004; **114** (11): p. 1932-1935.

62. Sil, A., I. Mackay, and J. Rowe-Jones. *Assessment of predictive prognostic factors for functional endoscopic sinus surgery in a 5-year prospective outcome study.* Am J Rhinol 2007; **21** (3): p. 289-296.

63. Bhattacharyya, N. *Radiographic stage fails to predict symptom outcomes after endoscopic sinus surgery for chronic rhinosinusitis.* Laryngoscope 2006; **116** (1): p. 18-22.

64. Bhattacharyya, N. *Influence of polyps on outcomes after endoscopic sinus surgery.* Laryngoscope 2007; **117** (10): p. 1834-1838.

65. Brook, I. *Acute and chronic bacterial sinusitis.* Infect Dis Clin North Am 2007; **21** (2): p. 427-448, vii.

66. Brook, I. and E.H. Frazier. *Microbiology of recurrent acute rhinosinusitis.* Laryngoscope 2004; **114** (1): p. 129-131.

67. Doyle, P.W. and J.D. Woodham. *Evaluation of the microbiology of chronic ethmoid sinusitis.* J Clin Microbiol 1991; **29** (11): p. 2396-2400.

68. Hoyt, W.H., 3rd. *Bacterial patterns found in surgery patients with chronic sinusitis.* J Am Osteopath Assoc 1992; **92** (2): p. 205, 209-212.

69. Hsu, J., D.C. Lanza, and D.W. Kennedy. *Antimicrobial resistance in bacterial chronic sinusitis.* Am J Rhinol 1998; **12** (4): p. 243-248.

70. Biel, M.A., C.A. Brown, R.M. Levinson, G.E. Garvis, H.M. Paisner, M.E. Sigel, and T.M. Tedford. *Evaluation of the microbiology of chronic maxillary sinusitis.* Ann Otol Rhinol Laryngol 1998; **107** (11 Pt 1): p. 942-945.

71. Jiang, R.S., J.F. Lin, and C.Y. Hsu. *Correlation between bacteriology of the middle meatus and ethmoid sinus in chronic sinusitis.* J Laryngol Otol 2002; **116** (6): p. 443-446.

72. Brook, I. and E.H. Frazier. *Correlation between microbiology and previous sinus surgery in patients with chronic maxillary sinusitis.* Ann Otol Rhinol Laryngol 2001; **110** (2): p. 148-151.

73. Finegold, S.M., M.J. Flynn, F.V. Rose, H. Jousimies-Somer, C. Jakielaszek, M. McTeague, H.M. Wexler, E. Berkowitz, and B. Wynne. *Bacteriologic findings associated with chronic bacterial maxillary sinusitis in adults.* Clin Infect Dis 2002; **35** (4): p. 428-433.

74. Araujo, E., B.C. Palombini, V. Cantarelli, A. Pereira, and A. Mariante. *Microbiology of middle meatus in chronic rhinosinusitis.* Am J Rhinol 2003; **17** (1): p. 9-15.

75. Kalcioglu, M.T., B. Durmaz, E. Aktas, O. Ozturan, and R. Durmaz. *Bacteriology of chronic maxillary sinusitis and normal maxillary sinuses: using culture and multiplex polymerase chain reaction.* Am J Rhinol 2003; **17** (3): p. 143-147.

76. Merino, L.A., M.C. Ronconi, G.E. Hrenuk, and M.G. de Pepe. *Bacteriologic findings in patients with chronic sinusitis.* Ear Nose Throat J 2003; **82** (10): p. 798-800, 803-794, 806.

77. Yildirim, A., C. Oh, H. Erdem, and T. Kunt. *Bacteriology in patients with chronic sinusitis who have been medically and surgically treated.* Ear Nose Throat J 2004; **83** (12): p. 836-838.

78. Busaba, N.Y., N.S. Siegel, and S.D. Salman. *Microbiology of chronic ethmoid sinusitis: is this a bacterial disease?* Am J Otolaryngol 2004; **25** (6): p. 379-384.

79. Aneke, E.C. and B.C. Ezeanolue. *Profile of aerobic bacteria isolated in chronic maxillary sinusitis patients.* Niger Postgrad Med J 2004; **11** (2): p. 116-120.

80. Jiang, R.S., C.Y. Hsu, and J.W. Jang. *Bacteriology of the maxillary and ethmoid sinuses in chronic sinusitis.* J Laryngol Otol 1998; **112** (9): p. 845-848.

81. Gordts, F., S. Halewyck, D. Pierard, L. Kaufman, and P.A. Clement. *Microbiology of the middle meatus: a comparison between normal adults and children.* J Laryngol Otol 2000; **114** (3): p. 184-188.

82. Nadel, D.M., D.C. Lanza, and D.W. Kennedy. *Endoscopically guided cultures in chronic sinusitis.* Am J Rhinol 1998; **12** (4): p. 233-241.

83. Bhattacharyya, N. and L.J. Kepnes. *The microbiology of recurrent rhinosinusitis after endoscopic sinus surgery.* Arch Otolaryngol Head Neck Surg 1999; **125** (10): p. 1117-1120.

84. Meltzer, E.O., D.L. Hamilos, J.A. Hadley, D.C. Lanza, B.F. Marple, R.A. Nicklas, C. Bachert, J. Baraniuk, F.M. Baroody, M.S. Benninger, I. Brook, B.A. Chowdhury, H.M. Druce, S. Durham, B. Ferguson, J.M. Gwaltney, Jr., M. Kaliner, D.W. Kennedy, V. Lund, R. Naclerio, R. Pawankar, J.F. Piccirillo, P. Rohane, R. Simon, R.G. Slavin, A. Togias, E.R. Wald, and S.J. Zinreich. *Rhinosinusitis: Establishing definitions for clinical research and patient care.* Otolaryngol Head Neck Surg 2004; **131** (6 Suppl): p. S1-62.

85.   Bhattacharyya, N., H.V. Gopal, and K.H. Lee. *Bacterial infection after endoscopic sinus surgery: a controlled prospective study.* Laryngoscope 2004; **114** (4): p. 765-767.

86.   Rontal, M., J.M. Bernstein, E. Rontal, and J. Anon. *Bacteriologic findings from the nose, ethmoid, and bloodstream during endoscopic surgery for chronic rhinosinusitis: implications for antibiotic therapy.* Am J Rhinol 1999; **13** (2): p. 91-96.

87.   Brook, I. *Aerobic and anaerobic bacterial flora of normal maxillary sinuses.* Laryngoscope 1981; **91** (3): p. 372-376.

88.   Su, W.Y., C. Liu, S.Y. Hung, and W.F. Tsai. *Bacteriological study in chronic maxillary sinusitis.* Laryngoscope 1983; **93** (7): p. 931-934.

89.   Jiang, R.S., K.L. Liang, J.W. Jang, and C.Y. Hsu. *Bacteriology of endoscopically normal maxillary sinuses.* J Laryngol Otol 1999; **113** (9): p. 825-828.

90.   Cook, H.E. and J. Haber. *Bacteriology of the maxillary sinus.* J Oral Maxillofac Surg 1987; **45** (12): p. 1011-1014.

91.   Nguyen, L.H., S. Fakhri, S. Frenkiel, and Q.A. Hamid. *Molecular immunology and immunotherapy for chronic sinusitis.* Curr Allergy Asthma Rep 2003; **3** (6): p. 505-512.

92.   Kennedy, D.W. *Pathogenesis of chronic rhinosinusitis.* Ann Otol Rhinol Laryngol Suppl 2004; **193**: p. 6-9.

93.   Damm, M., G. Quante, T. Jurk, and J.A. Sauer. *Nasal colonization with Staphylococcus aureus is not associated with the severity of symptoms or the extent of the disease in chronic rhinosinusitis.* Otolaryngol Head Neck Surg 2004; **131** (3): p. 200-206.

94.   Bunikowski, R., M.E. Mielke, H. Skarabis, M. Worm, I. Anagnostopoulos, G. Kolde, U. Wahn, and H. Renz. *Evidence for a disease-promoting effect of Staphylococcus aureus-derived exotoxins in atopic dermatitis.* J Allergy Clin Immunol 2000; **105** (4): p. 814-819.

95.   Taskapan, M.O. and P. Kumar. *Role of staphylococcal superantigens in atopic dermatitis: from colonization to inflammation.* Ann Allergy Asthma Immunol 2000; **84** (1): p. 3-10; quiz 11-12.

96.   Shiomori, T., S. Yoshida, H. Miyamoto, and K. Makishima. *Relationship of nasal carriage of Staphylococcus aureus to pathogenesis of perennial allergic rhinitis.* J Allergy Clin Immunol 2000; **105** (3): p. 449-454.

97.   Lee, J.Y., H.M. Kim, Y.M. Ye, J.W. Bahn, C.H. Suh, D. Nahm, H.R. Lee, and H.S. Park. *Role of staphylococcal superantigen-specific IgE antibodies in aspirin-intolerant asthma.* Allergy Asthma Proc 2006; **27** (5): p. 341-346.

98.   Herman, A., J.W. Kappler, P. Marrack, and A.M. Pullen. *Superantigens: mechanism of T-cell stimulation and role in immune responses.* Annu Rev Immunol 1991; **9**: p. 745-772.

99.   Bachert, C., P. Gevaert, G. Holtappels, S.G. Johansson, and P. van Cauwenberge. *Total and specific IgE in nasal polyps is related to local eosinophilic inflammation.* J Allergy Clin Immunol 2001; **107** (4): p. 607-614.

100.   Tripathi, A., D.B. Conley, L.C. Grammer, A.M. Ditto, M.M. Lowery, K.A. Seiberling, P.A. Yarnold, B. Zeifer, and R.C. Kern. *Immunoglobulin E to staphylococcal and streptococcal toxins in patients with chronic sinusitis/nasal polyposis.* Laryngoscope 2004; **114** (10): p. 1822-1826.

101.   Bernstein, J.M., M. Ballow, P.M. Schlievert, G. Rich, C. Allen, and D. Dryja. *A superantigen hypothesis for the pathogenesis of chronic hyperplastic sinusitis with massive nasal polyposis.* Am J Rhinol 2003; **17** (6): p. 321-326.

102. Conley, D.B., A. Tripathi, K.A. Seiberling, L.A. Suh, K.E. Harris, M.C. Paniagua, L.C. Grammer, and R.C. Kern. *Superantigens and chronic rhinosinusitis II: analysis of T-cell receptor V beta domains in nasal polyps.* Am J Rhinol 2006; **20** (4): p. 451-455.

103. Seiberling, K.A., D.B. Conley, A. Tripathi, L.C. Grammer, L. Shuh, G.K. Haines, 3rd, R. Schleimer, and R.C. Kern. *Superantigens and chronic rhinosinusitis: detection of staphylococcal exotoxins in nasal polyps.* Laryngoscope 2005; **115** (9): p. 1580-1585.

104. Norlander, T., K.M. Westrin, and P. Stierna. *The inflammatory response of the sinus and nasal mucosa during sinusitis: implications for research and therapy.* Acta Otolaryngol Suppl 1994; **515**: p. 38-44.

105. Bolger, W.E., D. Leonard, E.J. Dick, Jr., and P. Stierna. *Gram negative sinusitis: a bacteriologic and histologic study in rabbits.* Am J Rhinol 1997; **11** (1): p. 15-25.

106. Perloff, J.R., F.H. Gannon, W.E. Bolger, K.T. Montone, R. Orlandi, and D.W. Kennedy. *Bone involvement in sinusitis: an apparent pathway for the spread of disease.* Laryngoscope 2000; **110** (12): p. 2095-2099.

107. Khalid, A.N., J. Hunt, J.R. Perloff, and D.W. Kennedy. *The role of bone in chronic rhinosinusitis.* Laryngoscope 2002; **112** (11): p. 1951-1957.

108. Kennedy, D.W., B.A. Senior, F.H. Gannon, K.T. Montone, P. Hwang, and D.C. Lanza. *Histology and histomorphometry of ethmoid bone in chronic rhinosinusitis.* Laryngoscope 1998; **108** (4 Pt 1): p. 502-507.

109. Giacchi, R.J., R.A. Lebowitz, H.T. Yee, J.P. Light, and J.B. Jacobs. *Histopathologic evaluation of the ethmoid bone in chronic sinusitis.* Am J Rhinol 2001; **15** (3): p. 193-197.

110. Jang, Y.J., T.W. Koo, S.Y. Chung, and S.G. Park. *Bone involvement in chronic rhinosinusitis assessed by 99mTc-MDP bone SPECT.* Clin Otolaryngol Allied Sci 2002; **27** (3): p. 156-161.

111. Costerton, J.W., G.G. Geesey, and K.J. Cheng. *How bacteria stick.* Sci Am 1978; **238** (1): p. 86-95.

112. Morris, D.P. and A. Hagr. *Biofilm: why the sudden interest?* J Otolaryngol 2005; **34 Suppl 2**: p. S56-59.

113. Costerton, J.W., K.J. Cheng, G.G. Geesey, T.I. Ladd, J.C. Nickel, M. Dasgupta, and T.J. Marrie. *Bacterial biofilms in nature and disease.* Annu Rev Microbiol 1987; **41**: p. 435-464.

114. Characklis, W.G. and K.C. Marshall, *Biofilms: a basis for an interdisciplinary approach.* Biofilms, ed. W.G. Characklis and K.C. Marshall. 1990, New York, N.Y.: John Wiley and Sons. 3-15.

115. Costerton, J.W., Z. Lewandowski, D.E. Caldwell, D.R. Korber, and H.M. Lappin-Scott. *Microbial biofilms.* Annu Rev Microbiol 1995; **49**: p. 711-745.

116. Davies, D.G. and G.G. Geesey. *Regulation of the alginate biosynthesis gene algC in Pseudomonas aeruginosa during biofilm development in continuous culture.* Appl Environ Microbiol 1995; **61** (3): p. 860-867.

117. Donlan, R.M. and J.W. Costerton. *Biofilms: survival mechanisms of clinically relevant microorganisms.* Clin Microbiol Rev 2002; **15** (2): p. 167-193.

118. Nyvad, B. and O. Fejerskov. *Assessing the stage of caries lesion activity on the basis of clinical and microbiological examination.* Community Dent Oral Epidemiol 1997; **25** (1): p. 69-75.

119. Keevil, C.W. and J.T. Walker. *Normarski DIC microscopy and image analysis of biofilms.* BINARY 4 1992: p. 93-95.

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

121. Wimpenny, J., W. Manz, and U. Szewzyk. *Heterogeneity in biofilms.* FEMS Microbiol Rev 2000; **24** (5): p. 661-671.

122. Shapiro, J.A. *Thinking about bacterial populations as multicellular organisms.* Annu Rev Microbiol 1998; **52**: p. 81-104.

123. Christensen, B.B., J.A. Haagensen, A. Heydorn, and S. Molin. *Metabolic commensalism and competition in a two-species microbial consortium.* Appl Environ Microbiol 2002; **68** (5): p. 2495-2502.

124. Jefferson, K.K. *What drives bacteria to produce a biofilm?* FEMS Microbiol Lett 2004; **236** (2): p. 163-173.

125. Branda, S.S., S. Vik, L. Friedman, and R. Kolter. *Biofilms: the matrix revisited.* Trends Microbiol 2005; **13** (1): p. 20-26.

126. Lasa, I. *Towards the identification of the common features of bacterial biofilm development.* Int Microbiol 2006; **9** (1): p. 21-28.

127. Sutherland, I.W. *The biofilm matrix--an immobilized but dynamic microbial environment.* Trends Microbiol 2001; **9** (5): p. 222-227.

128. Muto, Y. and S. Goto. *Transformation by extracellular DNA produced by Pseudomonas aeruginosa.* Microbiol Immunol 1986; **30** (7): p. 621-628.

129. Kadurugamuwa, J.L. and T.J. Beveridge. *Virulence factors are released from Pseudomonas aeruginosa in association with membrane vesicles during normal growth and exposure to gentamicin: a novel mechanism of enzyme secretion.* J Bacteriol 1995; **177** (14): p. 3998-4008.

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

131. Hall-Stoodley, L., J.W. Costerton, and P. Stoodley. *Bacterial biofilms: from the natural environment to infectious diseases.* Nat Rev Microbiol 2004; **2** (2): p. 95-108.

132. Bhinu, V.S. *Insight into biofilm-associated microbial life.* J Mol Microbiol Biotechnol 2005; **10** (1): p. 15-21.

133. Dow, J.M., L. Crossman, K. Findlay, Y.Q. He, J.X. Feng, and J.L. Tang. *Biofilm dispersal in Xanthomonas campestris is controlled by cell-cell signaling and is required for full virulence to plants.* Proc Natl Acad Sci U S A 2003; **100** (19): p. 10995-11000.

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

135. Ben-Jacob, E. and H. Levine. *Self-engineering capabilities of bacteria.* J R Soc Interface 2006; **3** (6): p. 197-214.

136. Korber, D.R., J.R. Lawrence, H.M. Lappin-Scott, and J.W. Costerton, *Growth of microorganisms on surfaces.* In Microbial Biofilms, ed. H.M. Lappin-Scott and J.W. Costerton. 1995, Cambridge, UK:: Cambridge Univ. Press. 15-45.

137. O'Toole, G.A. and R. Kolter. *Flagellar and twitching motility are necessary for Pseudomonas aeruginosa biofilm development.* Mol Microbiol 1998; **30** (2): p. 295-304.

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

139. Tolker-Nielsen, T., U.C. Brinch, P.C. Ragas, J.B. Andersen, C.S. Jacobsen, and S. Molin. *Development and dynamics of Pseudomonas sp. biofilms.* J Bacteriol 2000; **182** (22): p. 6482-6489.

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

141. Stoodley, P., K. Sauer, D.G. Davies, and J.W. Costerton. *Biofilms as complex differentiated communities.* Annu Rev Microbiol 2002; **56**: p. 187-209.

142. An, Y.H., R.B. Dickinson, R.J. Doyle, and *Mechanisms of bacterial adhesion and pathogenesis of implant and tissue infections.* Handbook of bacterial adhesion: principles, methods, and applications., ed. Y.H.A.a.R.J. Friedman. 2000, Totowa, N.J. : Humana Press. 1-27.

143. Ziebuhr, W., V. Krimmer, S. Rachid, I. Lossner, F. Gotz, and J. Hacker. *A novel mechanism of phase variation of virulence in Staphylococcus epidermidis: evidence for control of the polysaccharide intercellular adhesin synthesis by alternating insertion and excision of the insertion sequence element IS256.* Mol Microbiol 1999; **32** (2): p. 345-356.

144. Wang, I.W., J.M. Anderson, M.R. Jacobs, and R.E. Marchant. *Adhesion of Staphylococcus epidermidis to biomedical polymers: contributions of surface thermodynamics and hemodynamic shear conditions.* J Biomed Mater Res 1995; **29** (4): p. 485-493.

145. Dunne, W.M., Jr. *Bacterial adhesion: seen any good biofilms lately?* Clin Microbiol Rev 2002; **15** (2): p. 155-166.

146. Whiteley, M., M.G. Bangera, R.E. Bumgarner, M.R. Parsek, G.M. Teitzel, S. Lory, and E.P. Greenberg. *Gene expression in Pseudomonas aeruginosa biofilms.* Nature 2001; **413** (6858): p. 860-864.

147. McLean, R.J., M. Whiteley, D.J. Stickler, and W.C. Fuqua. *Evidence of autoinducer activity in naturally occurring biofilms.* FEMS Microbiol Lett 1997; **154** (2): p. 259-263.

148. Allison, D.G., B. Ruiz, C. SanJose, A. Jaspe, and P. Gilbert. *Extracellular products as mediators of the formation and detachment of Pseudomonas fluorescens biofilms.* FEMS Microbiol Lett 1998; **167** (2): p. 179-184.

149. Luppens, S.B., M.W. Reij, R.W. van der Heijden, F.M. Rombouts, and T. Abee. *Development of a standard test to assess the resistance of Staphylococcus aureus biofilm cells to disinfectants.* Appl Environ Microbiol 2002; **68** (9): p. 4194-4200.

150. Ceri, H., M.E. Olson, C. Stremick, R.R. Read, D. Morck, and A. Buret. *The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms.* J Clin Microbiol 1999; **37** (6): p. 1771-1776.

151. Hoyle, B.D. and J.W. Costerton. *Bacterial resistance to antibiotics: the role of biofilms.* Prog Drug Res 1991; **37**: p. 91-105.

152. Anderl, J.N., M.J. Franklin, and P.S. Stewart. *Role of antibiotic penetration limitation in Klebsiella pneumoniae biofilm resistance to ampicillin and ciprofloxacin.* Antimicrob Agents Chemother 2000; **44** (7): p. 1818-1824.

153. Jouenne, T., O. Tresse, and G.A. Junter. *Agar-entrapped bacteria as an in vitro model of biofilms and their susceptibility to antibiotics.* FEMS Microbiol Lett 1994; **119** (1-2): p. 237-242.

154. Dunne, W.M., Jr., E.O. Mason, Jr., and S.L. Kaplan. *Diffusion of rifampin and vancomycin through a Staphylococcus epidermidis biofilm.* Antimicrob Agents Chemother 1993; **37** (12): p. 2522-2526.

155. Yasuda, H., Y. Ajiki, T. Koga, and T. Yokota. *Interaction between clarithromycin and biofilms formed by Staphylococcus epidermidis.* Antimicrob Agents Chemother 1994; **38** (1): p. 138-141.

156. Suci, P.A., M.W. Mittelman, F.P. Yu, and G.G. Geesey. *Investigation of ciprofloxacin penetration into Pseudomonas aeruginosa biofilms.* Antimicrob Agents Chemother 1994; **38** (9): p. 2125-2133.

157. Yasuda, H., Y. Ajiki, T. Koga, H. Kawada, and T. Yokota. *Interaction between biofilms formed by Pseudomonas aeruginosa and clarithromycin.* Antimicrob Agents Chemother 1993; **37** (9): p. 1749-1755.

158. Shigeta, M., G. Tanaka, H. Komatsuzawa, M. Sugai, H. Suginaka, and T. Usui. *Permeation of antimicrobial agents through Pseudomonas aeruginosa biofilms: a simple method.* Chemotherapy 1997; **43** (5): p. 340-345.

159. Kumon, H., K. Tomochika, T. Matunaga, M. Ogawa, and H. Ohmori. *A sandwich cup method for the penetration assay of antimicrobial agents through Pseudomonas exopolysaccharides.* Microbiol Immunol 1994; **38** (8): p. 615-619.

160. Nichols, W.W., M.J. Evans, M.P. Slack, and H.L. Walmsley. *The penetration of antibiotics into aggregates of mucoid and non-mucoid Pseudomonas aeruginosa.* J Gen Microbiol 1989; **135** (5): p. 1291-1303.

161. Walters, M.C., 3rd, F. Roe, A. Bugnicourt, M.J. Franklin, and P.S. Stewart. *Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of Pseudomonas aeruginosa biofilms to ciprofloxacin and tobramycin.* Antimicrob Agents Chemother 2003; **47** (1): p. 317-323.

162. Gordon, C.A., N.A. Hodges, and C. Marriott. *Antibiotic interaction and diffusion through alginate and exopolysaccharide of cystic fibrosis-derived Pseudomonas aeruginosa.* J Antimicrob Chemother 1988; **22** (5): p. 667-674.

163. Giwercman, B., E.T. Jensen, N. Hoiby, A. Kharazmi, and J.W. Costerton. *Induction of beta-lactamase production in Pseudomonas aeruginosa biofilm.* Antimicrob Agents Chemother 1991; **35** (5): p. 1008-1010.

164. Anderl, J.N., J. Zahller, F. Roe, and P.S. Stewart. *Role of nutrient limitation and stationary-phase existence in Klebsiella pneumoniae biofilm resistance to ampicillin and ciprofloxacin.* Antimicrob Agents Chemother 2003; **47** (4): p. 1251-1256.

165. Farber, B.F., M.H. Kaplan, and A.G. Clogston. *Staphylococcus epidermidis extracted slime inhibits the antimicrobial action of glycopeptide antibiotics.* J Infect Dis 1990; **161** (1): p. 37-40.

166. Konig, C., S. Schwank, and J. Blaser. *Factors compromising antibiotic activity against biofilms of Staphylococcus epidermidis.* Eur J Clin Microbiol Infect Dis 2001; **20** (1): p. 20-26.

167. Souli, M. and H. Giamarellou. *Effects of slime produced by clinical isolates of coagulase-negative staphylococci on activities of various antimicrobial agents.* Antimicrob Agents Chemother 1998; **42** (4): p. 939-941.

168. Wimmpenny, J.W.T. and S.L. Kinniment, *Biochenical reactions and the establishment of gradients within biofilms.* Microbial iofilms, ed. H.M. Lappin-Scott and J.W. Costerton. 1995, Cambridge: Cambridge University Press. 99-117.

169. Tack, K.J. and L.D. Sabath. *Increased minimum inhibitory concentrations with anaerobiasis for tobramycin, gentamicin, and amikacin, compared to latamoxef, piperacillin, chloramphenicol, and clindamycin.* Chemotherapy 1985; **31** (3): p. 204-210.

170. Retsema, J.A., L.A. Brennan, and A.E. Girard. *Effects of environmental factors on the in vitro potency of azithromycin.* Eur J Clin Microbiol Infect Dis 1991; **10** (10): p. 834-842.

171. Venglarcik, J.S., 3rd, L.L. Blair, and L.M. Dunkle. *pH-dependent oxacillin tolerance of Staphylococcus aureus.* Antimicrob Agents Chemother 1983; **23** (2): p. 232-235.

172. Wentland, E.J., P.S. Stewart, C.T. Huang, and G.A. McFeters. *Spatial variations in growth rate within Klebsiella pneumoniae colonies and biofilm.* Biotechnol Prog 1996; **12** (3): p. 316-321.

173. Sternberg, C., B.B. Christensen, T. Johansen, A. Toftgaard Nielsen, J.B. Andersen, M. Givskov, and S. Molin. *Distribution of bacterial growth activity in flow-chamber biofilms.* Appl Environ Microbiol 1999; **65** (9): p. 4108-4117.

174. Xu, K.D., P.S. Stewart, F. Xia, C.T. Huang, and G.A. McFeters. *Spatial physiological heterogeneity in Pseudomonas aeruginosa biofilm is determined by oxygen availability.* Appl Environ Microbiol 1998; **64** (10): p. 4035-4039.

175. Costerton, J.W., P.S. Stewart, and E.P. Greenberg. *Bacterial biofilms: a common cause of persistent infections.* Science 1999; **284** (5418): p. 1318-1322.

176. Brooun, A., S. Liu, and K. Lewis. *A dose-response study of antibiotic resistance in Pseudomonas aeruginosa biofilms.* Antimicrob Agents Chemother 2000; **44** (3): p. 640-646.

177. Mah, T.F., B. Pitts, B. Pellock, G.C. Walker, P.S. Stewart, and G.A. O'Toole. *A genetic basis for Pseudomonas aeruginosa biofilm antibiotic resistance.* Nature 2003; **426** (6964): p. 306-310.

178. Espinosa-Urgel, M., A. Salido, and J.L. Ramos. *Genetic analysis of functions involved in adhesion of Pseudomonas putida to seeds.* J Bacteriol 2000; **182** (9): p. 2363-2369.

179. Keren, I., N. Kaldalu, A. Spoering, Y. Wang, and K. Lewis. *Persister cells and tolerance to antimicrobials.* FEMS Microbiol Lett 2004; **230** (1): p. 13-18.

180. Keren, I., D. Shah, A. Spoering, N. Kaldalu, and K. Lewis. *Specialized persister cells and the mechanism of multidrug tolerance in Escherichia coli.* J Bacteriol 2004; **186** (24): p. 8172-8180.

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

182. Stewart, P.S. *Mechanisms of antibiotic resistance in bacterial biofilms.* Int J Med Microbiol 2002; **292** (2): p. 107-113.

183. Goto, T., Y. Nakame, M. Nishida, and Y. Ohi. *In vitro bactericidal activities of beta-lactamases, amikacin, and fluoroquinolones against Pseudomonas aeruginosa biofilm in artificial urine.* Urology 1999; **53** (5): p. 1058-1062.

184. Pedersen, K., S.K. Christensen, and K. Gerdes. *Rapid induction and reversal of a bacteriostatic condition by controlled expression of toxins and antitoxins.* Mol Microbiol 2002; **45** (2): p. 501-510.

185. Wada, A. *Growth phase coupled modulation of Escherichia coli ribosomes.* Genes Cells 1998; **3** (4): p. 203-208.

186. Medzhitov, R. and C. Janeway, Jr. *Innate immunity.* N Engl J Med 2000; **343** (5): p. 338-344.

187. Hornef, M.W., M.J. Wick, M. Rhen, and S. Normark. *Bacterial strategies for overcoming host innate and adaptive immune responses.* Nat Immunol 2002; **3** (11): p. 1033-1040.

188. Gotz, F. *Staphylococcus and biofilms.* Mol Microbiol 2002; **43** (6): p. 1367-1378.

189. Vuong, C., J.M. Voyich, E.R. Fischer, K.R. Braughton, A.R. Whitney, F.R. DeLeo, and M. Otto. *Polysaccharide intercellular adhesin (PIA) protects Staphylococcus*

epidermidis against major components of the human innate immune system. Cell Microbiol 2004; **6** (3): p. 269-275.

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

191. Leid, J.G., C.J. Willson, M.E. Shirtliff, D.J. Hassett, M.R. Parsek, and A.K. Jeffers. *The exopolysaccharide alginate protects Pseudomonas aeruginosa biofilm bacteria from IFN-gamma-mediated macrophage killing.* J Immunol 2005; **175** (11): p. 7512-7518.

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

193. Walker, T.S., K.L. Tomlin, G.S. Worthen, K.R. Poch, J.G. Lieber, M.T. Saavedra, M.B. Fessler, K.C. Malcolm, M.L. Vasil, and J.A. Nick. *Enhanced Pseudomonas aeruginosa biofilm development mediated by human neutrophils.* Infect Immun 2005; **73** (6): p. 3693-3701.

194. Vuong, C., S. Kocianova, J.M. Voyich, Y. Yao, E.R. Fischer, F.R. DeLeo, and M. Otto. *A crucial role for exopolysaccharide modification in bacterial biofilm formation, immune evasion, and virulence.* J Biol Chem 2004; **279** (52): p. 54881-54886.

195. Begun, J., J.M. Gaiani, H. Rohde, D. Mack, S.B. Calderwood, F.M. Ausubel, and C.D. Sifri. *Staphylococcal biofilm exopolysaccharide protects against Caenorhabditis elegans immune defenses.* PLoS Pathog 2007; **3** (4): p. e57.

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

197. Doering, T.L., J.D. Nosanchuk, W.K. Roberts, and A. Casadevall. *Melanin as a potential cryptococcal defence against microbicidal proteins.* Med Mycol 1999; **37** (3): p. 175-181.

198. Singh, P.K., M.R. Parsek, E.P. Greenberg, and M.J. Welsh. *A component of innate immunity prevents bacterial biofilm development.* Nature 2002; **417** (6888): p. 552-555.

199. Costerton, J.W. *Cystic fibrosis pathogenesis and the role of biofilms in persistent infection.* Trends Microbiol 2001; **9** (2): p. 50-52.

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

201. Hendolin, P.H., A. Markkanen, J. Ylikoski, and J.J. Wahlfors. *Use of multiplex PCR for simultaneous detection of four bacterial species in middle ear effusions.* J Clin Microbiol 1997; **35** (11): p. 2854-2858.

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

203. Dingman, J.R., M.G. Rayner, S. Mishra, Y. Zhang, M.D. Ehrlich, J.C. Post, and G.D. Ehrlich. *Correlation between presence of viable bacteria and presence of endotoxin in middle-ear effusions.* J Clin Microbiol 1998; **36** (11): p. 3417-3419.

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

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

206. Galli, J., L. Calo, F. Ardito, M. Imperiali, E. Bassotti, G. Fadda, and G. Paludetti. *Biofilm formation by Haemophilus influenzae isolated from adeno-tonsil tissue samples, and its role in recurrent adenotonsillitis.* Acta Otorhinolaryngol Ital 2007; **27** (3): p. 134-138.

207. Zuliani, G., M. Carron, J. Gurrola, C. Coleman, M. Haupert, R. Berk, and J. Coticchia. *Identification of adenoid biofilms in chronic rhinosinusitis.* Int J Pediatr Otorhinolaryngol 2006; **70** (9): p. 1613-1617.

208. Kania, R.E., G.E. Lamers, M.J. Vonk, E. Dorpmans, J. Struik, P. Tran Ba Huy, P. Hiemstra, G.V. Bloemberg, and J.J. Grote. *Characterization of mucosal biofilms on human adenoid tissues.* Laryngoscope 2008; **118** (1): p. 128-134.

209. Chole, R.A. and B.T. Faddis. *Evidence for microbial biofilms in cholesteatomas.* Arch Otolaryngol Head Neck Surg 2002; **128** (10): p. 1129-1133.

210. Chole, R.A. and B.T. Faddis. *Anatomical evidence of microbial biofilms in tonsillar tissues: a possible mechanism to explain chronicity.* Arch Otolaryngol Head Neck Surg 2003; **129** (6): p. 634-636.

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

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

213. Inglis, T.J., T.M. Lim, M.L. Ng, E.K. Tang, and K.P. Hui. *Structural features of tracheal tube biofilm formed during prolonged mechanical ventilation.* Chest 1995; **108** (4): p. 1049-1052.

214. Inglis, T.J. *Evidence for dynamic phenomena in residual tracheal tube biofilm.* Br J Anaesth 1993; **70** (1): p. 22-24.

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

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

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

218. Free, R.H., H.J. Busscher, G.J. Elving, H.C. van der Mei, R. van Weissenbruch, and F.W. Albers. *Biofilm formation on voice prostheses: in vitro influence of probiotics.* Ann Otol Rhinol Laryngol 2001; **110** (10): p. 946-951.

219. Leunisse, C., R. van Weissenbruch, H.J. Busscher, H.C. van der Mei, F. Dijk, and F.W. Albers. *Biofilm formation and design features of indwelling silicone rubber tracheoesophageal voice prostheses--an electron microscopical study.* J Biomed Mater Res 2001; **58** (5): p. 556-563.

220. Everaert, E.P., H.F. Mahieu, B. van de Belt-Gritter, A.J. Peeters, G.J. Verkerke, H.C. van der Mei, and H.J. Busscher. *Biofilm formation in vivo on perfluoro-alkylsiloxane-modified voice prostheses.* Arch Otolaryngol Head Neck Surg 1999; **125** (12): p. 1329-1332.

221. Barakate, M., E. Beckenham, J. Curotta, and M. da Cruz. *Bacterial biofilm adherence to middle-ear ventilation tubes: scanning electron micrograph images and literature review.* J Laryngol Otol 2007; **121** (10): p. 993-997.

222. Mehta, A.J., J.C. Lee, G.R. Stevens, and P.J. Antonelli. *Opening plugged tympanostomy tubes: effect of biofilm formation.* Otolaryngol Head Neck Surg 2006; **134** (1): p. 121-125.

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

224. Ramadan, H.H., J.A. Sanclement, and J.G. Thomas. *Chronic rhinosinusitis and biofilms.* Otolaryngol Head Neck Surg 2005; **132** (3): p. 414-417.

225. Becker, P., W. Hufnagle, G. Peters, and M. Herrmann. *Detection of differential gene expression in biofilm-forming versus planktonic populations of Staphylococcus aureus using micro-representational-difference analysis.* Appl Environ Microbiol 2001; **67** (7): p. 2958-2965.

226. Xu, K.D., G.A. McFeters, and P.S. Stewart. *Biofilm resistance to antimicrobial agents.* Microbiology 2000; **146 (Pt 3)**: p. 547-549.

227. Perloff, J.R. and J.N. Palmer. *Evidence of bacterial biofilms on frontal recess stents in patients with chronic rhinosinusitis.* Am J Rhinol 2004; **18** (6): p. 377-380.

228. Ferguson, B.J. and D.B. Stolz. *Demonstration of biofilm in human bacterial chronic rhinosinusitis.* Am J Rhinol 2005; **19** (5): p. 452-457.

229. Perloff, J.R. and J.N. Palmer. *Evidence of bacterial biofilms in a rabbit model of sinusitis.* Am J Rhinol 2005; **19** (1): p. 1-6.

230. Kelemen, G. *The nasal and paranasal cavities of the rabbit in experimental work.* AMA Arch Otolaryngol 1955; **61** (5): p. 497-512.

231. Maeyama, T. *A study of experimental sinusitis in rabbits.* Auris Nasus Larynx 1981; **8** (2): p. 87-97.

232. Kumlien, J. and H. Schiratzki. *Blood flow in the rabbit sinus mucosa during experimentally induced chronic sinusitis. Measurement with a diffusible and with a non-diffusible tracer.* Acta Otolaryngol 1985; **99** (5-6): p. 630-636.

233. Drettner, B., P. Johansson, and J. Kumlien. *Experimental acute sinusitis in rabbit. A study of mucosal blood flow.* Acta Otolaryngol 1987; **103** (5-6): p. 432-434.

234. Johansson, P., J. Kumlien, B. Carlsoo, B. Drettner, and C.E. Nord. *Experimental acute sinusitis in rabbits. A bacteriological and histological study.* Acta Otolaryngol 1988; **105** (3-4): p. 357-366.

235. Westrin, K.M., P. Stierna, B. Carlsoo, and C.E. Nord. *Mucosubstance histochemistry of the maxillary sinus mucosa in experimental sinusitis: a model study on rabbits.* ORL J Otorhinolaryngol Relat Spec 1991; **53** (5): p. 299-304.

236. Min, Y.G., Y.M. Lee, B.J. Lee, H.W. Jung, and S.O. Chang. *The effect of ostial opening on experimental maxillary sinusitis in rabbits.* Rhinology 1993; **31** (3): p. 101-105.

237. Benninger, M.S., J. Kaczor, and C. Stone. *Natural ostiotomy vs. inferior antrostomy in the management of sinusitis: an animal model.* Otolaryngol Head Neck Surg 1993; **109** (6): p. 1034-1042.

238. Hilding, A. *Experimental sinus surgery: effects of operative windows on normal sinuses.* Ann Otol Rhinol Laryngol 1941; **50**: p. 379-392.

239. Juan, K.H., C.F. Peng, and H.J. Lin. *Bacteriology of maxillary sinuses in rabbits.* Gaoxiong Yi Xue Ke Xue Za Zhi 1995; **11** (10): p. 552-556.

240. Hinni, M.L., T.V. McCaffrey, and J.L. Kasperbauer. *Early mucosal changes in experimental sinusitis.* Otolaryngol Head Neck Surg 1992; **107** (4): p. 537-548.

241. Marks, S.C. *Acute sinusitis in the rabbit: a new rhinogenic model.* Laryngoscope 1997; **107** (12 Pt 1): p. 1579-1585.

242. Toriumi, D.M., W.F. Raslan, M. Friedman, and M.E. Tardy. *Histotoxicity of cyanoacrylate tissue adhesives. A comparative study.* Arch Otolaryngol Head Neck Surg 1990; **116** (5): p. 546-550.

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

244. Johansson, B. *Isolation of an iron conatining red protein from human milk.* Acta Chem Scand 1960; **14**: p. 510-512.

245. Thompson, A.B., T. Bohling, F. Payvandi, and S.I. Rennard. *Lower respiratory tract lactoferrin and lysozyme arise primarily in the airways and are elevated in association with chronic bronchitis.* J Lab Clin Med 1990; **115** (2): p. 148-158.

246. van de Graaf, E.A., T.A. Out, A. Kobesen, and H.M. Jansen. *Lactoferrin and secretory IgA in the bronchoalveolar lavage fluid from patients with a stable asthma.* Lung 1991; **169** (5): p. 275-283.

247. Uchida, K., R. Matsuse, S. Tomita, K. Sugi, O. Saitoh, and S. Ohshiba. *Immunochemical detection of human lactoferrin in feces as a new marker for inflammatory gastrointestinal disorders and colon cancer.* Clin Biochem 1994; **27** (4): p. 259-264.

248. Baggiolini, M., C. De Duve, P.L. Masson, and J.F. Heremans. *Association of lactoferrin with specific granules in rabbit heterophil leukocytes.* J Exp Med 1970; **131** (3): p. 559-570.

249. Green, I., C.H. Kirkpatrick, and D.C. Dale. *Lactoferrin--specific localization in the nuclei of human polymorphonuclear neutrophilic leukocytes.* Proc Soc Exp Biol Med 1971; **137** (4): p. 1311-1317.

250. Lash, J.A., T.D. Coates, J. Lafuze, R.L. Baehner, and L.A. Boxer. *Plasma lactoferrin reflects granulocyte activation in vivo.* Blood 1983; **61** (5): p. 885-888.

251. Bennett, R.M. and J.L. Skosey. *Lactoferrin and lysozyme levels in synovial fluid: differential indices of articular inflammation and degradation.* Arthritis Rheum 1977; **20** (1): p. 84-90.

252. Metz-Boutigue, M.H., J. Jolles, J. Mazurier, F. Schoentgen, D. Legrand, G. Spik, J. Montreuil, and P. Jolles. *Human lactotransferrin: amino acid sequence and structural comparisons with other transferrins.* Eur J Biochem 1984; **145** (3): p. 659-676.

253. Baker, H.M. and E.N. Baker. *Lactoferrin and iron: structural and dynamic aspects of binding and release.* Biometals 2004; **17** (3): p. 209-216.

254. Powell, M. and J. Ogden. *Nucleotide sequence of human lactoferrin cDNA.* Nucleic Acids Res 1990; **18**: p. 4013.

255. Rey, M., S. Woloshuk, M. deBoer, and F. Pieper. *Complete nucleotide sequence of human mammary gland lactoferrin.* Nucleic Acids Res 1990; **18**: p. 5288.

256. Anderson, B., H. Baker, G. Norris, D. Rice, and E. Baker. *Structure of human lactoferrin: crystallographic structure analysis and refinement at 2.8 A resolution.* J Mol Biol 1989; **209**: p. 711-734.

257. Anderson, B., H. Baker, and E. Dodson. *Structure of human Lactoferrin at 3.2-A resolution.* Proc Natl Acad Sci USA 1987; **84**: p. 1769-1773.

258. Faber, H.R., C.J. Baker, C.L. Day, J.W. Tweedie, and E.N. Baker. *Mutation of arginine 121 in lactoferrin destabilizes iron binding by disruption of anion binding: crystal structures of R121S and R121E mutants.* Biochemistry 1996; **35** (46): p. 14473-14479.

259. Nicholson, H., B.F. Anderson, T. Bland, S.C. Shewry, J.W. Tweedie, and E.N. Baker. *Mutagenesis of the histidine ligand in human lactoferrin: iron binding properties and crystal structure of the histidine-253-->methionine mutant.* Biochemistry 1997; **36** (2): p. 341-346.

260. He, Q.Y., A.B. Mason, R. Pakdaman, N.D. Chasteen, B.K. Dixon, B.M. Tam, V. Nguyen, R.T. MacGillivray, and R.C. Woodworth. *Mutations at the histidine 249 ligand profoundly alter the spectral and iron-binding properties of human serum transferrin N-lobe.* Biochemistry 2000; **39** (6): p. 1205-1210.

261. Bellamy, W., M. Takase, K. Yamauchi, H. Wakabayashi, K. Kawase, and M. Tomita. *Identification of the bactericidal domain of lactoferrin.* Biochim Biophys Acta 1992; **1121** (1-2): p. 130-136.

262. Tomita, M., W. Bellamy, M. Takase, K. Yamauchi, H. Wakabayashi, and K. Kawase. *Potent antibacterial peptides generated by pepsin digestion of bovine lactoferrin.* J Dairy Sci 1991; **74** (12): p. 4137-4142.

263. Bellamy, W., M. Takase, H. Wakabayashi, K. Kawase, and M. Tomita. *Antibacterial spectrum of lactoferricin B, a potent bactericidal peptide derived from the N-terminal region of bovine lactoferrin.* J Appl Bacteriol 1992; **73** (6): p. 472-479.

264. Yamauchi, K., M. Tomita, T.J. Giehl, and R.T. Ellison, 3rd. *Antibacterial activity of lactoferrin and a pepsin-derived lactoferrin peptide fragment.* Infect Immun 1993; **61** (2): p. 719-728.

265. Appelmelk, B.J., Y.Q. An, M. Geerts, B.G. Thijs, H.A. de Boer, D.M. MacLaren, J. de Graaff, and J.H. Nuijens. *Lactoferrin is a lipid A-binding protein.* Infect Immun 1994; **62** (6): p. 2628-2632.

266. Hwang, P.M. and H.J. Vogel. *Structure-function relationships of antimicrobial peptides.* Biochem Cell Biol 1998; **76** (2-3): p. 235-246.

267. Hwang, P.M., N. Zhou, X. Shan, C.H. Arrowsmith, and H.J. Vogel. *Three-dimensional solution structure of lactoferricin B, an antimicrobial peptide derived from bovine lactoferrin.* Biochemistry 1998; **37** (12): p. 4288-4298.

268. Bellamy, W., H. Wakabayashi, M. Takase, K. Kawase, S. Shimamura, and M. Tomita. *Killing of Candida albicans by lactoferricin B, a potent antimicrobial peptide derived from the N-terminal region of bovine lactoferrin.* Med Microbiol Immunol (Berl) 1993; **182** (2): p. 97-105.

269. Dial, E.J. and L.M. Lichtenberger. *Effect of lactoferrin on Helicobacter felis induced gastritis.* Biochem Cell Biol 2002; **80** (1): p. 113-117.

270. Qiu, J., D.R. Hendrixson, E.N. Baker, T.F. Murphy, J.W. St Geme, 3rd, and A.G. Plaut. *Human milk lactoferrin inactivates two putative colonization factors expressed by Haemophilus influenzae.* Proc Natl Acad Sci U S A 1998; **95** (21): p. 12641-12646.

271. Valenti, P., R. Greco, G. Pitari, P. Rossi, M. Ajello, G. Melino, and G. Antonini. *Apoptosis of Caco-2 intestinal cells invaded by Listeria monocytogenes: protective effect of lactoferrin.* Exp Cell Res 1999; **250** (1): p. 197-202.

272. Gahr, M., C.P. Speer, B. Damerau, and G. Sawatzki. *Influence of lactoferrin on the function of human polymorphonuclear leukocytes and monocytes.* J Leukoc Biol 1991; **49** (5): p. 427-433.

273. Valenti, P., F. Berlutti, M.P. Conte, C. Longhi, and L. Seganti. *Lactoferrin functions: current status and perspectives.* J Clin Gastroenterol 2004; **38** (6 Suppl): p. S127-129.

274. van der Strate, B.W., L. Beljaars, G. Molema, M.C. Harmsen, and D.K. Meijer. *Antiviral activities of lactoferrin.* Antiviral Res 2001; **52** (3): p. 225-239.

275. Ishikado, A., H. Imanaka, M. Kotani, A. Fujita, Y. Mitsuishi, T. Kanemitsu, Y. Tamura, and T. Makino. *Liposomal lactoferrin induced significant increase of the interferon-alpha (IFN-alpha) producibility in healthy volunteers.* Biofactors 2004; **21** (1-4): p. 69-72.

276. Kruzel, M.L., Y. Harari, D. Mailman, J.K. Actor, and M. Zimecki. *Differential effects of prophylactic, concurrent and therapeutic lactoferrin treatment on LPS-induced inflammatory responses in mice.* Clin Exp Immunol 2002; **130** (1): p. 25-31.

277. Machnicki, M., M. Zimecki, and T. Zagulski. *Lactoferrin regulates the release of tumour necrosis factor alpha and interleukin 6 in vivo.* Int J Exp Pathol 1993; **74** (5): p. 433-439.

278. Baveye, S., E. Elass, D.G. Fernig, C. Blanquart, J. Mazurier, and D. Legrand. *Human lactoferrin interacts with soluble CD14 and inhibits expression of endothelial adhesion molecules, E-selectin and ICAM-1, induced by the CD14-lipopolysaccharide complex.* Infect Immun 2000; **68** (12): p. 6519-6525.

279. Mazurier, J., D. Legrand, B. Leveugle, E. Rochard, J. Montreuil, and G. Spik. *Study on the binding of lactotransferrin (lactoferrin) to human PHA-activated lymphocytes and non-activated platelets. Localisation and description of the receptor-binding site.* Adv Exp Med Biol 1994; **357**: p. 111-119.

280. Shinoda, I., M. Takase, Y. Fukuwatari, S. Shimamura, M. Koller, and W. Konig. *Effects of lactoferrin and lactoferricin on the release of interleukin 8 from human polymorphonuclear leukocytes.* Biosci Biotechnol Biochem 1996; **60** (3): p. 521-523.

281. Shau, H., A. Kim, and S.H. Golub. *Modulation of natural killer and lymphokine-activated killer cell cytotoxicity by lactoferrin.* J Leukoc Biol 1992; **51** (4): p. 343-349.

282. Damiens, E., J. Mazurier, I. el Yazidi, M. Masson, I. Duthille, G. Spik, and Y. Boilly-Marer. *Effects of human lactoferrin on NK cell cytotoxicity against haematopoietic and epithelial tumour cells.* Biochim Biophys Acta 1998; **1402** (3): p. 277-287.

283. Longhi, C., M.P. Conte, L. Seganti, M. Polidoro, A. Alfsen, and P. Valenti. *Influence of lactoferrin on the entry process of Escherichia coli HB101 (pRI203) in HeLa cells.* Med Microbiol Immunol (Berl) 1993; **182** (1): p. 25-35.

284. Longhi, C., M.P. Conte, W. Bellamy, L. Seganti, and P. Valenti. *Effect of lactoferricin B, a pepsin-generated peptide of bovine lactoferrin, on Escherichia coli HB101 (pRI203) entry into HeLa cells.* Med Microbiol Immunol (Berl) 1994; **183** (2): p. 77-85.

285. Singh, P.K. *Iron sequestration by human lactoferrin stimulates P. aeruginosa surface motility and blocks biofilm formation.* Biometals 2004; **17** (3): p. 267-270.

286. Britigan, B.E., M.B. Hayek, B.N. Doebbeling, and R.B. Fick, Jr. *Transferrin and lactoferrin undergo proteolytic cleavage in the Pseudomonas aeruginosa-infected lungs of patients with cystic fibrosis.* Infect Immun 1993; **61** (12): p. 5049-5055.

287. Rogan, M.P., C.C. Taggart, C.M. Greene, P.G. Murphy, S.J. O'Neill, and N.G. McElvaney. *Loss of microbicidal activity and increased formation of biofilm due to decreased lactoferrin activity in patients with cystic fibrosis.* J Infect Dis 2004; **190** (7): p. 1245-1253.

288. Petanceska, S., P. Canoll, and L.A. Devi. *Expression of rat cathepsin S in phagocytic cells.* J Biol Chem 1996; **271** (8): p. 4403-4409.

289. Maubach, G., M.C. Lim, S. Kumar, and L. Zhuo. *Expression and upregulation of cathepsin S and other early molecules required for antigen presentation in activated*

hepatic stellate cells upon IFN-gamma treatment. Biochim Biophys Acta 2007; **1773** (2): p. 219-231.

290. Casado, B., L.K. Pannell, S. Viglio, P. Iadarola, and J.N. Baraniuk. *Analysis of the sinusitis nasal lavage fluid proteome using capillary liquid chromatography interfaced to electrospray ionization-quadrupole time of flight- tandem mass spectrometry.* Electrophoresis 2004; **25** (9): p. 1386-1393.

291. Niehaus, M.D., J.M. Gwaltney, Jr., J.O. Hendley, M.J. Newman, P.W. Heymann, G.P. Rakes, T.A. Platts-Mills, and R.L. Guerrant. *Lactoferrin and eosinophilic cationic protein in nasal secretions of patients with experimental rhinovirus colds, natural colds, and presumed acute community-acquired bacterial sinusitis.* J Clin Microbiol 2000; **38** (8): p. 3100-3102.

292. Zhang, L., D. Han, and Z. Wang. *[Lysozyme and lactoferrin in human uncinate process mucosa during chronic sinusitis].* Zhonghua Er Bi Yan Hou Ke Za Zhi 1998; **33** (4): p. 219-221.

293. Liu, Z., J. Kim, J.P. Sypek, I.M. Wang, H. Horton, F.G. Oppenheim, and B.S. Bochner. *Gene expression profiles in human nasal polyp tissues studied by means of DNA microarray.* J Allergy Clin Immunol. 2004; **114** (4): p. 783-790.

294. Fukami, M., P. Stierna, B. Veress, and B. Carlsoo. *Lysozyme and lactoferrin in human maxillary sinus mucosa during chronic sinusitis. An immunohistochemical study.* Eur Arch Otorhinolaryngol 1993; **250** (3): p. 133-139.

295. Seiberling, K.A., L. Grammer, and R.C. Kern. *Chronic rhinosinusitis and superantigens.* Otolaryngol Clin North Am 2005; **38** (6): p. 1215-1236, ix.

296. Perez-Novo, C.A., J.B. Watelet, C. Claeys, P. Van Cauwenberge, and C. Bachert. *Prostaglandin, leukotriene, and lipoxin balance in chronic rhinosinusitis with and without nasal polyposis.* J Allergy Clin Immunol 2005; **115** (6): p. 1189-1196.

297. Chiu, A.G. *Osteitis in chronic rhinosinusitis.* Otolaryngol Clin North Am 2005; **38** (6): p. 1237-1242.

298. Costerton, J.W., L. Montanaro, and C.R. Arciola. *Biofilm in implant infections: its production and regulation.* Int J Artif Organs 2005; **28** (11): p. 1062-1068.

299. Brook, I. *Microbiology and antimicrobial management of sinusitis.* J Laryngol Otol 2005; **119** (4): p. 251-258.

300. Domenico, P., L. Baldassarri, P.E. Schoch, K. Kaehler, M. Sasatsu, and B.A. Cunha. *Activities of bismuth thiols against staphylococci and staphylococcal biofilms.* Antimicrob Agents Chemother 2001; **45** (5): p. 1417-1421.

301. Shaw CL., C.A., Wormald P.J. *Standardization of the sheep as a suitable model for studying endoscopic sinus surgery.* . 2001; **4** (No 1): p. 23-26.

302. Palmer, J.N. *Bacterial biofilms: do they play a role in chronic sinusitis?* Otolaryngol Clin North Am 2005; **38** (6): p. 1193-1201, viii.

303. Stoodley, P., S. Kathju, F.Z. Hu, G. Erdos, J.E. Levenson, N. Mehta, B. Dice, S. Johnson, L. Hall-Stoodley, L. Nistico, N. Sotereanos, J. Sewecke, J.C. Post, and G.D. Ehrlich. *Molecular and imaging techniques for bacterial biofilms in joint arthroplasty infections.* Clin Orthop Relat Res 2005; (437): p. 31-40.

304. MacGillivray, R.T., S.A. Moore, J. Chen, B.F. Anderson, H. Baker, Y. Luo, M. Bewley, C.A. Smith, M.E. Murphy, Y. Wang, A.B. Mason, R.C. Woodworth, G.D. Brayer, and E.N. Baker. *Two high-resolution crystal structures of the recombinant N-lobe of human transferrin reveal a structural change implicated in iron release.* Biochemistry 1998; **37** (22): p. 7919-7928.

305. Trampuz, A., D.R. Osmon, A.D. Hanssen, J.M. Steckelberg, and R. Patel. *Molecular and antibiofilm approaches to prosthetic joint infection.* Clin Orthop Relat Res 2003; (414): p. 69-88.

306. Paul, S.R. and T.D. Bunch. *Chronic frontal sinusitis and osteolysis in desert bighorn sheep.* J Am Vet Med Assoc 1978; **173** (9): p. 1178-1180.

307. Rings, D.M. and J. Rojko. *Naturally occurring nasal obstructions in 11 sheep.* Cornell Vet 1985; **75** (2): p. 269-276.

308. Gardiner, Q., M. Oluwole, L. Tan, and P.S. White. *An animal model for training in endoscopic nasal and sinus surgery.* J Laryngol Otol 1996; **110** (5): p. 425-428.

309. Illum, L. *Nasal delivery. The use of animal models to predict performance in man.* J Drug Target 1996; **3** (6): p. 427-442.

310. Lanza, D.C., H.J. Dhong, P. Tantilipikorn, J. Tanabodee, D.M. Nadel, and D.W. Kennedy. *Fungus and chronic rhinosinusitis: from bench to clinical understanding.* Ann Otol Rhinol Laryngol Suppl 2006; **196**: p. 27-34.

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

312. Benninger, M.S., B.J. Ferguson, J.A. Hadley, D.L. Hamilos, M. Jacobs, D.W. Kennedy, D.C. Lanza, B.F. Marple, J.D. Osguthorpe, J.A. Stankiewicz, J. Anon, J. Denneny, I. Emanuel, and H. Levine. *Adult chronic rhinosinusitis: definitions, diagnosis, epidemiology, and pathophysiology.* Otolaryngol Head Neck Surg 2003; **129** (3 Suppl): p. S1-32.

313. Ha, K., A.J. Psaltis, L.W. Tan, and P.J. Wormald. *A Sheep Model for the Study of Biofilms in Chronic Rhinosinusitis.* In Press 2006.

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

315. Landis, J.R. and G.G. Koch. *The measurement of observer agreement for categorical data.* Biometrics 1977; **33** (1): p. 159-174.

316. Fleiss, J.L. *Measuring nominal scale agreement among many raters.* Psychological Bulletin 1971; **76** (5): p. 378-382.

317. Pant, H., F.E. Kette, W.B. Smith, P.J. Macardle, and P.J. Wormald. *Eosinophilic mucus chronic rhinosinusitis: clinical subgroups or a homogeneous pathogenic entity?* Laryngoscope 2006; **116** (7): p. 1241-1247.

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

319. Iro, H., S. Mayr, C. Wallisch, B. Schick, and M.E. Wigand. *Endoscopic sinus surgery: its subjective medium-term outcome in chronic rhinosinusitis.* Rhinology 2004; **42** (4): p. 200-206.

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

321. Batra, P.S., R.C. Kern, A. Tripathi, D.B. Conley, A.M. Ditto, G.K. Haines, 3rd, P.R. Yarnold, and L. Grammar. *Outcome analysis of endoscopic sinus surgery in patients with nasal polyps and asthma.* Laryngoscope 2003; **113** (10): p. 1703-1706.

322. Stewart, M.G. and R.F. Johnson. *Chronic sinusitis: symptoms versus CT scan findings.* Curr Opin Otolaryngol Head Neck Surg 2004; **12** (1): p. 27-29.

323.	Stewart, M.G., M.W. Sicard, J.F. Piccirillo, and P.J. Diaz-Marchan. *Severity staging in chronic sinusitis: are CT scan findings related to patient symptoms?* Am J Rhinol 1999; **13** (3): p. 161-167.

324.	Robinson, S., V. Der-Haroutian, D. Grove, G. Rees, and P.J. Wormald. *Prevalence of pus in radiologically diseased sinuses in patients undergoing surgery for chronic rhinosinusitis.* Otolaryngol Head Neck Surg 2005; **133** (2): p. 181-184.

325.	Bester, E., G. Wolfaardt, L. Joubert, K. Garny, and S. Saftic. *Planktonic-cell yield of a pseudomonad biofilm.* Appl Environ Microbiol 2005; **71** (12): p. 7792-7798.

326.	Ponikau, J.U., D.A. Sherris, E.B. Kern, H.A. Homburger, E. Frigas, T.A. Gaffey, and G.D. Roberts. *The diagnosis and incidence of allergic fungal sinusitis.* Mayo Clin Proc 1999; **74** (9): p. 877-884.

327.	Collins, M.M., S.B. Nair, and P.J. Wormald. *Prevalence of noninvasive fungal sinusitis in South Australia.* Am J Rhinol 2003; **17** (3): p. 127-132.

328.	Schubert, M.S. *Allergic fungal sinusitis.* Otolaryngol Clin North Am 2004; **37** (2): p. 301-326.

329.	Klotz, S.A., N.K. Gaur, R. De Armond, D. Sheppard, N. Khardori, J.E. Edwards, Jr., P.N. Lipke, and M. El-Azizi. *Candida albicans Als proteins mediate aggregation with bacteria and yeasts.* Med Mycol 2007; **45** (4): p. 363-370.

330.	Toros, S.Z., S. Bolukbasi, B. Naiboglu, B. Er, C. Akkaynak, H. Noshari, and E. Egeli. *Comparative outcomes of endoscopic sinus surgery in patients with chronic sinusitis and nasal polyps.* Eur Arch Otorhinolaryngol 2007.

331.	Harbitz, O., A.O. Jenssen, and O. Smidsrod. *Lysozyme and lactoferrin in sputum from patients with chronic obstructive lung disease.* Eur J Respir Dis 1984; **65** (7): p. 512-520.

332.	Kotlar, H.K., O. Harbitz, A.O. Jenssen, and O. Smidsrod. *Quantitation of proteins in sputum from patients with chronic obstructive lung disease. II. Determination of albumin, transferrin, alpha1-acid glycoprotein, IgG, IgM, lysozyme and C3-complement factor.* Eur J Respir Dis 1980; **61** (4): p. 233-239.

333.	Meltzer, E.O., D.L. Hamilos, J.A. Hadley, D.C. Lanza, B.F. Marple, R.A. Nicklas, A.D. Adinoff, C. Bachert, L. Borish, V.M. Chinchilli, M.R. Danzig, B.J. Ferguson, W.J. Fokkens, S.G. Jenkins, V.J. Lund, M.F. Mafee, R.M. Naclerio, R. Pawankar, J.U. Ponikau, M.S. Schubert, R.G. Slavin, M.G. Stewart, A. Togias, E.R. Wald, and B. Winther. *Rhinosinusitis: Developing guidance for clinical trials.* Otolaryngol Head Neck Surg 2006; **135** (5 Suppl): p. S31-80.

334.	Livak, K.J. and T.D. Schmittgen. *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods 2001; **25** (4): p. 402-408.

335.	Jeney, E.V., G.D. Raphael, S.D. Meredith, and M.A. Kaliner. *Abnormal nasal glandular secretion in recurrent sinusitis.* J Allergy Clin Immunol 1990; **86** (1): p. 10-18.

336.	Raphael, G.D., E.V. Jeney, J.N. Baraniuk, I. Kim, S.D. Meredith, and M.A. Kaliner. *Pathophysiology of rhinitis. Lactoferrin and lysozyme in nasal secretions.* J Clin Invest 1989; **84** (5): p. 1528-1535.

337.	Ramos-Vara, J.A. *Technical aspects of immunohistochemistry.* Vet Pathol. 2005; **42** (4): p. 405-426.

338.	Kalfa, V.C., S.L. Spector, T. Ganz, and A.M. Cole. *Lysozyme levels in the nasal secretions of patients with perennial allergic rhinitis and recurrent sinusitis.* Ann Allergy Asthma Immunol 2004; **93** (3): p. 288-292.

339. Laube, D.M., S. Yim, L.K. Ryan, K.O. Kisich, and G. Diamond. *Antimicrobial peptides in the airway.* Curr Top Microbiol Immunol 2006; **306**: p. 153-182.

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

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

342. Ganz, T. *Antimicrobial polypeptides in host defense of the respiratory tract.* J Clin Invest 2002; **109** (6): p. 693-697.

343. Cavestro, G.M., A.V. Ingegnoli, G. Aragona, V. Iori, N. Mantovani, N. Altavilla, N. Dal Bo, A. Pilotto, A. Bertele, A. Franze, F. Di Mario, and L. Borghi. *Lactoferrin: mechanism of action, clinical significance and therapeutic relevance.* Acta Biomed 2002; **73** (5-6): p. 71-73.

344. Psaltis, A.J., M.A. Bruhn, E.H. Ooi, L.W. Tan, and P.J. Wormald. *Nasal Mucosa Expression of Lactoferrin in Patients With Chronic Rhinosinusitis.* Laryngoscope 2007.

345. Orsi, N. *The antimicrobial activity of lactoferrin: current status and perspectives.* Biometals 2004; **17** (3): p. 189-196.

346. Ardehali, R., L. Shi, J. Janatova, S.F. Mohammad, and G.L. Burns. *The effect of apo-transferrin on bacterial adhesion to biomaterials.* Artif Organs 2002; **26** (6): p. 512-520.

347. Berlutti, F., M. Ajello, P. Bosso, C. Morea, A. Petrucca, G. Antonini, and P. Valenti. *Both lactoferrin and iron influence aggregation and biofilm formation in Streptococcus mutans.* Biometals 2004; **17** (3): p. 271-278.

348. Rhodes, E.R., S. Menke, C. Shoemaker, A.P. Tomaras, G. McGillivary, and L.A. Actis. *Iron acquisition in the dental pathogen Actinobacillus actinomycetemcomitans: what does it use as a source and how does it get this essential metal?* Biometals 2007; **20** (3-4): p. 365-377.

349. Longhi, C., M.P. Conte, W. Bellamy, L. Seganti, and P. Valenti. *Effect of lactoferricin B, a pepsin-generated peptide of bovine lactoferrin, on Escherichia coli HB101 (pRI203) entry into HeLa cells.* Med Microbiol Immunol 1994; **183** (2): p. 77-85.

350. Oho, T., M. Mitoma, and T. Koga. *Functional domain of bovine milk lactoferrin which inhibits the adherence of Streptococcus mutans cells to a salivary film.* Infect Immun 2002; **70** (9): p. 5279-5282.

351. Kobayashi, H. *Airway biofilms: implications for pathogenesis and therapy of respiratory tract infections.* Treat Respir Med 2005; **4** (4): p. 241-253.

352. Caraher, E.M., K. Gumulapurapu, C.C. Taggart, P. Murphy, S. McClean, and M. Callaghan. *The effect of recombinant human lactoferrin on growth and the antibiotic susceptibility of the cystic fibrosis pathogen Burkholderia cepacia complex when cultured planktonically or as biofilms.* J Antimicrob Chemother 2007; **60** (3): p. 546-554.

353. Leitch, E.C. and M.D. Willcox. *Lactoferrin increases the susceptibility of S. epidermidis biofilms to lysozyme and vancomycin.* Curr Eye Res 1999; **19** (1): p. 12-19.

354. Kara, C.O., C.B. Cetin, N. Colakoglu, M. Sengul, and E. Pakdemirli. *Experimentally induced rhinosinusitis in rabbits.* J Otolaryngol 2002; **31** (5): p. 294-298.

355. Grouzmann, E., M. Monod, B. Landis, S. Wilk, N. Brakch, K. Nicoucar, R. Giger, D. Malis, I. Szalay-Quinodoz, C. Cavadas, D.R. Morel, and J.S. Lacroix. *Loss of dipeptidylpeptidase IV activity in chronic rhinosinusitis contributes to the neurogenic*

inflammation induced by substance P in the nasal mucosa. Faseb J 2002; **16** (9): p. 1132-1134.

356. Jacob, A., B.T. Faddis, and R.A. Chole. *Chronic bacterial rhinosinusitis: description of a mouse model.* Arch Otolaryngol Head Neck Surg 2001; **127** (6): p. 657-664.

357. Rajapaksa, S., D. McIntosh, A. Cowin, D. Adams, and P.J. Wormald. *The effect of insulin-like growth factor 1 incorporated into a hyaluronic acid-based nasal pack on nasal mucosal healing in a healthy sheep model and a sheep model of chronic sinusitis.* Am J Rhinol 2005; **19** (3): p. 251-256.

358. Thomas, D.C. and P.J. Wormald. *Standardization of diagnostic criteria for eosinophilic chronic rhinosinusitis in the Oestrus ovis infected sheep model.* Am J Rhinol 2007; **21** (5): p. 551-555.

359. Rajapaksa, S.P., A. Cowin, D. Adams, and P.J. Wormald. *The effect of a hyaluronic acid-based nasal pack on mucosal healing in a sheep model of sinusitis.* Am J Rhinol 2005; **19** (6): p. 572-576.

360. Robinson, S., D. Adams, and P.J. Wormald. *The effect of nasal packing and prednisolone on mucosal healing and reciliation in a sheep model.* Rhinology 2004; **42** (2): p. 68-72.

361. Ha, K.R., A.J. Psaltis, L. Tan, and P.J. Wormald. *A sheep model for the study of biofilms in rhinosinusitis.* Am J Rhinol 2007; **21** (3): p. 339-345.

362. McIntosh, D., A. Cowin, D. Adams, and P.J. Wormald. *The effect of an expandable polyvinyl acetate (Merocel) pack on the healing of the nasal mucosa of sheep.* Am J Rhinol 2005; **19** (6): p. 577-581.

363. Rajapaksa, S.P., A. Ananda, T. Cain, L. Oates, and P.J. Wormald. *The effect of the modified endoscopic Lothrop procedure on the mucociliary clearance of the frontal sinus in an animal model.* Am J Rhinol 2004; **18** (3): p. 183-187.

364. Rajapaksa, S.P., A. Ananda, T.M. Cain, L. Oates, and P.J. Wormald. *Frontal ostium neo-osteogenesis and restenosis after modified endoscopic Lothrop procedure in an animal model.* Clin Otolaryngol Allied Sci 2004; **29** (4): p. 386-388.

365. McIntosh, D., A. Cowin, D. Adams, T. Rayner, and P.J. Wormald. *The effect of a dissolvable hyaluronic acid-based pack on the healing of the nasal mucosa of sheep.* Am J Rhinol 2002; **16** (2): p. 85-90.

366. Shaw, C.K., A. Cowin, and P.J. Wormald. *A study of the normal temporal healing pattern and the mucociliary transport after endoscopic partial and full-thickness removal of nasal mucosa in sheep.* Immunol Cell Biol 2001; **79** (2): p. 145-148.

367. Ha, K.R., A.J. Psaltis, A.R. Butcher, L.W. Tan, and P.-J. Wormald. *In vitro activity of mupirocin on clinical isolates of Staphylococcus aureus and its potential implications in chronic rhinosinusitis.* Laryngoscope 2008.

368. Chiu, A.G., J.N. Palmer, B.A. Woodworth, L. Doghramji, M.B. Cohen, A. Prince, and N.A. Cohen. *Baby shampoo nasal irrigations for the symptomatic post-functional endoscopic sinus surgery patient.* Am J Rhinol 2008; **22** (1): p. 34-37.

369. Desrosiers, M., M. Myntti, and G. James. *Methods for removing bacterial biofilms: in vitro study using clinical chronic rhinosinusitis specimens.* Am J Rhinol 2007; **21** (5): p. 527-532.

370. Desrosiers, M., Z. Bendouah, and J. Barbeau. *Effectiveness of topical antibiotics on Staphylococcus aureus biofilm in vitro.* Am J Rhinol 2007; **21** (2): p. 149-153.

371. Ha, K.R., A.J. Psaltis, A.R. Butcher, P.J. Wormald, and L.W. Tan. *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.

372.   Costerton, J.W., B. Ellis, K. Lam, F. Johnson, and A.E. Khoury. *Mechanism of electrical enhancement of efficacy of antibiotics in killing biofilm bacteria.* Antimicrob Agents Chemother 1994; **38** (12): p. 2803-2809.

373.   Pitt, W.G., M.O. McBride, J.K. Lunceford, R.J. Roper, and R.D. Sagers. *Ultrasonic enhancement of antibiotic action on gram-negative bacteria.* Antimicrob Agents Chemother 1994; **38** (11): p. 2577-2582.

374.   Ensing, G.T., D. Neut, J.R. van Horn, H.C. van der Mei, and H.J. Busscher. *The combination of ultrasound with antibiotics released from bone cement decreases the viability of planktonic and biofilm bacteria: an in vitro study with clinical strains.* J Antimicrob Chemother 2006; **58** (6): p. 1287-1290.

375.   Qian, Z., R.D. Sagers, and W.G. Pitt. *Investigation of the mechanism of the bioacoustic effect.* J Biomed Mater Res 1999; **44** (2): p. 198-205.

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