Staphylococcus aureus Exoproteins on Nasal Epithelial Barrier in Chronic Rhinosinusitis

A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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DEDICATED to

"my Creator, who knit me together in my mother's womb"

ISAIAH 44:2A (NIV)

For everything, absolutely everything, above and below, visible and invisible... everything got started in him and finds its purpose in him (Jesus Christ).

COLOSSIANS 1:16 (MSG)

DECLARATION

I, Beula Subashini Panchatcharam certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Biofilm Exoproteins: Drivers of inflammation and host immune response in Chronic rhinosinusitis (CRS)

Beula Subashini Panchatcharam, Clare M Cooksley, Mahnaz Ramezanpour, Hua Hu, Ahmed Bassiouni, Peter J. Wormald, Alkis J. Psaltis, and Sarah Vreugde**.**

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Beula Subashini Panchatcharam, Clare M Cooksley, Mahnaz Ramezanpour, Shari Javadiyan, Rajan Sundaresan Vediappan, Ahmed Bassiouni, Peter J. Wormald, Alkis J. Psaltis and Sarah Vreugde.

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ABBREVIATIONS

ARS	Acute rhinosinusitis
AST	Antimicrobial susceptibility testing
Bap	Biofilm-associated protein
CAP	Community-acquired pneumonia
CFTR	Cystic fibrosis transmembrane conductance regulator
ClFA	Clumping factor, A
ClFB	Clumping factor B
CLSI	Clinical Laboratory Standards Institute
CLSM	Confocal laser scanning microscopy
CoNS	Coagulase-negative staphylococci
CRD	Carbohydrate domain
CRS	Chronic rhinosinusitis
DCs	Dendritic cells
DDG	1-O-dodecyl-rac-glycerol
E-test	Epsilometer test
Eap	Extracellular adherence protein
EGF	Epidermal growth factor
EPS	Extracellular polymeric substances
ESS	Endoscopic sinus surgey
ETs	Exfoliative toxins
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FESS	Functional endoscopic sinus surgery
MIC	Minimum inhibition concentration
mRNA	Messenger RNA
MRSA	Methicillin resistant S. aureus
MS	Mass spectrometry
MSA	Mannitol salt agar
MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
MUPP1	Multi-PDZ domain protein 1
NETs	Neutrophil extracellular traps
NO	Nitric oxide
Nuc	Nuclease
ORFs	Open reading frames
PAMPs	Pathogen associated molecular patterns
PBP	Penicillin binding proteins
PBPs	Penicillin-binding proteins
PCR	Polymerase chain reaction
PFTs	Pore-forming toxins
PIA	Polysaccharide intercellular adhesin
PIA	Polysaccharide intercellular adhesin
PMNs	Polymorphonuclear leukocytes
PNAG	Polymeric N-acetyl- glucosamine
PRR	Pattern recognition receptors
PSM	Phenol soluble modulins
PVL	Panton Valentine Leukotoxin
QRDR	Quinolone resistance–determining region
rCRS	Recalcitrant

FISH	Fuorescence in situ hybridization
FnBPA	Fibronectin-binding protein A
FnBPB	Fibronectin-binding protein B
GISA	Glycopeptide intermediate S. aureus
GML	Glycerol monolaurate
GRE	Glycopeptide resistant Enterococci
HAP	Hospital-acquired pneumonia
hBD	Human β defensins
HPA	Hypothalamus-pituitary-adrenal
Ig	Immunoglobulin
IL	Interleukin
ILCs	Innate lymphoid cells
INCS	Intranasal corticosteroids
JAMs	Junction adhesion molecules
LC	Liquid chromatography
LKS	Lund-Kennedy Score
LTAs	Lipoteichoic acids
MAbs	Monoclonal antibodies
MBC	Minimum Bactericidal Concentration
MBEC	Minimum biofilm eradication concentration
MBIC	Minimal biofilm inhibitory concentration
MGO	Methylglyoxal
MHC	Major histocompatibility complex class
RCT	Randomised controlled trial
RSV	Respiratory syncytial virus
SAgs	Superantigens
SaPIs	Staphylococcal pathogenicity islands
SCC	Staphylococcal cassette chromosome
Ses	Staphylococcal enterotoxins
SLPI	Secretory leukoprotease inhibitor
SNOT	Sinonasal outcome test
Spa	Staphylococcal protein A
SpA	Protein A
SSTI	Soft tissue infections
TEER	Transepithelial Electrical Resistance
TJ	Tight junctions
TLOs	Tertiary lymphoid organs
TLRs	Toll-like receptors
Treg	Regulatory T-cell
TSST	Toxic Shock Syndrome Toxin
VAS	Visual analogue scale
VISA	Vancomycin intermediate-resistant *S. aureus*
ZO	Zonula occludens
CBP	Collagen-binding protein (CBP)

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ABSTRACT

Chronic Rhinosinusitis (CRS) is characterised by a plethora of symptoms that patients suffer as a result of chronic inflammation of the sinus mucosa. These are associated with chronic relapsing infections caused by mucosal biofilms that are predominantly due to *Staphylococcus aureus*. CRS is a prevalent disease affecting around 10% of the general population in Western societies with significant impact on the socio-economical life. However, the pathophysiology of CRS and exact role of biofilms and *S. aureus* infections are poorly understood. Therefore, understanding the mechanism by which these micro-organisms and biofilms affect the mucosa and relate to chronic inflammation and relapsing infections is subject of intense ongoing research worldwide.

The first part of this thesis studied the pathogenicity of *S. aureus* clinical isolates isolated from patients who were suffering from CRS and that were grown in planktonic form and biofilm form. We collected the exoproteins secreted from those isolates and studied their effect on in-vitro models of the nasal epithelial barrier. As a result of this study we found *S. aureus* biofilm exoproteins disrupt the mucosal barrier structure in a time- and dose- dependent manner and are toxic to the barrier. This damage to the mucosal barrier is thought to play an important role in the etiopathogenesis of CRS.

In the second part of the thesis we explored the ability of *S. aureus* biofilms to induce a host immune response and we studied the relationship between *S. aureus* biofilm properties and host immune responses. *S. aureus* clinical isolates harvested from CRSwNP patients exhibited higher *S. aureus* biofilm exoprotein production and higher metabolic activity than *S. aureus* biofilms from CRSsNP patients. There was a significant strong positive correlation between the number of lymphocytes or eosinophils and the concentration of biofilm exoproteins secreted by corresponding clinical isolates and their metabolic activity. In contrast, exoprotein concentrations from planktonic cells did not correlate with inflammatory cell infiltration. The gradual increase in inflammation and Th2 polarisation in relation to *S. aureus* biofilm properties seen in that study imply that biofilm exoproteins might trigger inflammation in those patients.

The third part of this thesis involved studying the resistance pattern of *S. aureus* in planktonic and biofilm form, and to know if they were any different in their sensitivity to commonly used antibiotics used in routine clinical practice. By doing so it was our aim to show that persistence and severity of symptoms in recalcitrant (rCRS) disease is due to biofilms that resist treatment to conventional antibiotics, many times higher than their planktonic counterparts. We were able to show that *S. aureus* biofilm have unique properties of excessive exoprotein production which was directly proportionate to their biomass and metabolic activity. The minimum dosage for biofilm eradication (MBEC) was much higher than routinely used minimum inhibitory concentration (MIC) of planktonic forms of the organisms. MBEC values furthermore correlated with inflammatory cell counts, giving us valuable insights into the mechanisms by which the organism evade treatment and induce human suffering.

This study helps us to design future experiments in-vivo and study human response conditions thereby paving ways as a model for future therapies that could target the microorganism’s exoprotein production.

CHAPTER 1: LITERATURE REVIEW

1.1. Chronic rhinosinusitis (CRS)

1.1.1. Research definition

The term Rhinosinusitis (RS) refers to a group of disorders characterised by mucosal inflammation of the nasal cavity and paranasal sinuses. The Task Force on Rhinosinusitis, established in 1996, passed the first consensus to subclassify rhinosinusitis into acute and chronic based on the duration of symptoms:

1) < 4 weeks – Acute Rhinosinusitis

2) 4-12 weeks – Subacute Rhinosinusitis

3) > 12 weeks – Chronic Rhinosinusitis

The diagnosis of acute rhinosinusitis (ARS) is made based on the presence of either two major or one major and two minor symptoms[1] (Table 1.1). Chronic rhinosinusitis (CRS) holds a similar criterion used for acute sinusitis but for an extended period

(> 12 weeks). However, dependence on clinical diagnosis alone was considered as inaccurate [2], hence a committee of 30 physicians elected from 5 different specialties extended the guidelines[3] to incorporate objective evidence for mucosal inflammation. As per the guidelines, CRS is diagnosed as symptoms (major/minor) lasting for > 12 weeks along with objective evidence of inflammation by endoscopy and CT scan finding of the sinuses.

Table 1. 1 : Symptoms of sinusitis

Major Symptoms	Minor Symptoms
Nasal obstruction/blockage	Ear pain/pressure/fullness
Nasal discharge/purulence/ discoloured	Headache
Facial pain/pressure	Halitosis
Facial congestion/fullness	Fever (all nonacute)
Fever (acute rhinosinusitis only)	Fatigue
Nasal discharge / postnasal drainage	Dental pain
Hyposmia/anosmia	Cough

The European position (EPOS) paper[4] on rhinosinusitis and nasal polyps developed by European physicians proposed similar diagnostic criteria. CRS has also been classified based on the presence and absence of polyps as CRS with nasal polyps (CRSwNP) or CRS with absence of nasal polyps (CRSsNP).

1.1.2. Epidemiology

Epidemiological data reporting the prevalence of CRS varies according to geographical region and is influenced by the methods used for diagnosis. Prevalence of CRS in the USA[5] is reported to be between 12.5-16%, and is listed as the second most common chronic disease after orthopaedic impairments, being more common than ischaemic heart disease and hypertension. In Europe, based on the EP diagnostic criteria the prevalence ranges from 6.9%-27.9% [6]. A Korean study reported prevalence[7] of 6.95% from data collected using questionnaires and clinical examination by otolaryngologists. Sinusitis is one of the most common primary care presentations in Australia, with 1.4 in every 100 general practice outpatient visits estimated to be for rhinosinusitis [8]. In 2014-15, 7.1 million Australians were diagnosed to have a chronic respiratory condition by the National Health Survey (NHS) which includes 2.5 million people with asthma, 4.5 million people with allergic rhinitis and 1.9 million people with chronic sinusitis[9].

CRS contributes to a significant amount of healthcare expenditure due to direct costs arising from physician visits and medical treatment. Studies have reported that patients diagnosed with CRS undergo an additional 3.5 outpatient visits , requiring 5.5 prescriptions more than non-CRS patients, and also suffer excessive financial costs of $773 +/- 300 per year[10].The actual economic burden to society is significantly greater however due to the loss of productivity and absenteeism from work for health-related reasons.

Among the working age group, it has been estimated that those who suffer with CRS have an average loss of 4.8-5.7 days' work per year compared to those who do not

have the disease [11]. The impact of CRS on a patient's quality of life is also significant with comparative studies finding lower quality of life scores in CRS patients compared to those with congestive heart failure, angina, chronic obstructive pulmonary disease or back pain.

1.1.3. Factors associated with chronic rhinosinusitis (CRS)

CRS is a multifactorial disease, contributing factors have been broadly categorized into intrinsic (host related factors) and extrinsic (non-host related factors). Intrinsic factors that predispose to the development of CRS include anatomic / structural abnormalities, genetic diseases and immune mediated abnormalities. Anatomic variations thought to predispose to the development of CRS, are those that narrow the sinus drainage pathways such as the frontal recess and osteomeatal complex. Any inflammation in these areas can then lead to complete sinus obstruction, mucus stagnation and bacterial colonization and superinfection [12]. Alterations in the mucous layer due to inflammation or underlying genetic disorders such cystic fibrosis[13], primary ciliary dyskinesia can also reduce ciliary movement and mucocilary clearance, further contributing to mucus stasis and bacterial colonisation[14]. Non-infectious causes commonly seen associated with CRS are allergy and gastro-esophageal reflux disease[15] although their causative mechanism remains debated.

Defects in innate immune responses and/or the physical barrier can also contribute to the development of CRS when exposed to pathogens including microbes[16]. The innate immune system uses certain receptors for recognition of microbes that induce

cytokines and chemokines leading to activation of lymphocytes and the adaptive immune response. The immune system differentiates microbes from molecular structures on the host cells through Toll-like receptors and pathogen associated molecular patterns thereby inducing complement activation, inflammation and apoptosis in response to pathogens.

Though the majority of research on TLRs has focused on inflammation of lower-airway diseases, studies on human sinonasal tissue from patients with chronic rhinosinusitis and immortalized epithelial cell line have shown that TLRs 1 through 10 are identified in the sinonasal mucosa of CRS patients [17]. Real time PCR analysis has suggested that here might be differences in expression of TLRs in CRS patients compared to their controls. Compared to the innate immune response, the adaptive immunity, mediated through T and B lymphocytes, is characterized by specificity and memory. CRS is associated with CD4+ helper T cell activation. Functionally they are divided into T- helper (Th1) or T-helper 2 phenotype (Th2) based on the cytokines secreted[18]. CRS without nasal polyps (CRSsNP) is associated with Th 1 type responses and CRS with nasal polyps (CRSwNP) with the Th2 subtype.

The immune barrier hypothesis summarizes environmental and host factors that lead to inflammation in CRS. This hypothesis states that a weakened mechanical barrier can be due to primary genetic alterations in the host or due to epigenetic changes produced by the environment with increased sensitivity to exogenous agents [19].

Extrinsic factors such as microbial agents, cigarette smoke and environmental irritants can initiate inflammation in CRS. Use of tobacco is one of the common

extrinsic factors associated with CRS, leading to mucociliary dysfunction. Tobacco smoke on epithelial cells downregulates the cystic fibrosis transmembrane conductance regulator (CFTR) gene and thereby contributes to the formation of thick mucus, facilitating the growth of bacteria[20]. Studies have reported that tobacco smoke may enhance biofilm formation [21]. Animal studies have shown that environmental irritants such as air pollution can also cause structural damage to the ciliated epithelium and affect the mucociliary clearance [22]. A recent Canadian study also suggests that certain pollutants may predispose to polyp formation[23].

Microbes such as bacteria and fungi as well as bacterial superantigens and biofilms have all been associated with the development of CRS. Studies have reported that in 75 % of the human population bacteria isolated from sinuses are culturable[24]. Common organisms isolated from the adult population include aerobes such as coagulase negative Staphylococcus (35 %), *Corynebacteria spp* (23%) and *Staphylococcus aureus* (8%) gram-negative bacteria and anaerobic[25] organisms which include *Prevotella*, F*usobacterium, Peptostreptococcus* and *Propionibacterium.* In children *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* [26] are seen both in CRS and during episodes of acute exacerbations. Among the bacterial causes *Staphylococcus aureus* is the most common pathogen isolated by culture in both CRSwNP and CRSsNP [27]. Superantigens produced from bacteria such as *S. aureus* have been thought to play a pathogenic role in CRS. These antigens bypass antigen recognition and promote polyclonal T lymphocyte proliferation with massive release of cytokines which is very often seen in the case of polyp production[28].

Compared to free floating planktonic bacteria, those living as communities (biofilms)[29] contained within extracellular polymeric substances (EPS) have longer survival and propagation rates. Biofilm bacterial communities are complex and coexist with different strains and species. Cryer et al in 2004 was the first to provide evidence of biofilms on sinus mucosa[30]. Slow growing persister bacteria within biofilms resist conventional antimicrobial treatment leading to chronicity and disease recalcitrance [31]. Psaltis et al found that biofilms are associated with recalcitrance of disease especially in the post-operative period after endoscopic sinus surgery [32]. The presence of biofilms in CRS and absence in controls is a common finding in the literature[33], however the mechanism(s) by which they trigger and perpetuate inflammation are still unanswered and warrant further research.

The role of fungal infections in the pathogenesis of CRS has been debated for decades. They are broadly grouped into invasive and noninvasive fungal infections. The fungal hypothesis suggests that the presence of fungal elements in CRS activates T cells and eosinophils causing inflammation and damage to mucosa [34]. However, this theory was disproven when fungal elements were seen in control patients and antifungal treatments were ineffective to treat CRS patients [35]. In the absence of clinical improvement with antifungal therapy, the role of fungi in CRS is circumstantial, but studies have indicated that they may have a synergistic action with bacteria[36].

Viral infections of the upper respiratory tract play an important role in the etiopathogenesis of acute sinusitis rather than in CRS, however, the presence of virus in CRS patients has been shown to be associated with worse disease

severity[37], this may be in part due to an abnormal or exaggerated immune response reported in CRS patients with viral positivity [37,38].

1.1.4. Diagnosis and treatment of chronic rhinosinusitis

The diagnostic criteria of CRS as per EPOS guidelines requires documentation of the presence of sinus symptoms in combination with objective evidence of inflammation either on endoscopy or CT scan. To evaluate patient symptoms, formal, validated questionnaires have been developed. These questionnaires not only allow the severity of the disease to be assessed but also provides a tool for evaluating a patient's response to treatment. An example of a validated patient-reported outcome measures is the Sino-Nasal Outcome Test 22 (SNOT - 22)[4]. This is used to assess both rhinologic specific symptoms as well as general symptoms that can affect general quality of life.

The Adelaide Disease Severity Score is another scoring system that has been validated against the SNOT 22 and shown to have higher correlation with Lund–Mackay CT score and the Lund–Kennedy endoscopy score[39]. Unlike the SNOT22, this scoring system only assesses the severity of the 5 most common visual analogue scale (VAS)[40]. In this scale, patients graphically represent the severity of their symptoms on a continuous scale ranging from 1 to 10.

The tools used to document objective evidence of CRS are the Lund-Mackay and the Lund-Kennedy Score (LKS) scores. The Lund-Mackay CT scan staging system grades the opacification of the sinuses. Although commonly used it has never been

truly validated. It is however an easy and a reproducible method to assess treatment response in CRS [41]. The Lund-Kennedy Score (LKS) is an endoscopic scoring system that assesses the severity of disease based on the presence and severity of secretions, mucosal oedema, scarring and crusting. The Modified LKS system, proposed by Psaltis et al, is a refinement of the LKS, whereby scarring and crusting are removed from the scores. This allows the system to be used also for un-operated patients and removed the effect that poor surgical technique, often associated with crusting and scarring, may have on the score. Psaltis et al found higher intra and inter-rater reliability of the MLKS compared to the LKS and a higher correlation to symptom scores [42].

1.1.4.1. Medical treatment

CRS is a disease state of differing phenotypes and endotypes, there is sufficient evidence regarding it as an inflammatory disorder due to multiple factors and microbial infection being one of the main triggering factors. CRS is characterized by multiple symptoms which needs adequate medical therapy, this may include a combination of topical and systemic antibiotics along with the use of anti-inflammatory agents. Systematic reviews done of all the medical therapies in the last 5 decades could be classified into:

1.Local therapy– Saline Irrigation, Steroid Irrigation, Antibiotic irrigation & Antifungal application

2.Systemic therapy – Antibiotics, Steroids, Anti Leukotrine, Anti-Ig E & Immunotherapy

Local Therapy:

a. Saline: High-volume (>100ml) saline irrigations is a well-accepted and cost-effective treatment for all forms of CRS. The primary role of saline irrigation is removal of secretions/mucous with inflammatory substances in the sinuses. The use of saline along with antibiotics and/or adjunctive treatment is supported by A-1 evidence and is recommended by comprehensive international consensus statements[43,44]. Low-volume delivery either by spray topical or nebulization has been shown to be less efficacious and is generally not recommended[45]. Studies examining different formulations of saline irrigations have found similar effects with both Isotonic and hypertopnic saline, except with a higher rate of minor side effects while using hypertonic saolution[46].

b. Intranasal corticosteroids (INCS): Application of INCS are also considered first-line therapy for CRS [4]. This is supported by evidence published as Cochrane review randomised controlled trial (RCT's) which showed an improvement in symptom and endoscopic disease severity scores in both CRSsNP and CRSwNP patients by reducing the polyp size and associated improvement in olfaction [45]. Various types of INCS are in use such as beclomethasone propionate, mometasone furoate, fluticasone propionate and budesonide which have similar effects[45] . Early concerns of systemic absorption and its impact on the hypothalamus-pituitary-adrenal axis is now settled with very minimal side effects[47].

c. Saline Irrigation with Corticosteroids:

Topical delivery of glucocorticosteroids in combination with saline irrigation has gained increased popularity amongst clinicians despite its off-label use. RCTs have demonstrated the efficacy of controlling the disease with significant improvements in validated symptom and endoscopic scores of disease severity, particularly in the post-operative setting[48].Although safety studies suggest that steroid irrigations are generally well tolerated with little in the way of side effects on hypothalamus-pituitary-adrenal (HPA) axis suppression, a study by Soudry et al[49]did show mild but reversible asymptomatic HPA suppression in a small subset of patients, all of whom were concurrently using inhaled corticosteroids[50]. Early evidence suggests that high-volume corticosteroid irrigations (i.e., techniques that involve delivering >100 mL of solution into the nasal cavity[51]) (budesonide irrigations) are more effective than low-volume corticosteroid spray techniques (i.e., meter-dosed spray, atomized, or nebulized solutions[51] ,clinical trials are required before a recommendation on optimal delivery method can be provided.

d. Saline Irrigation with Antibiotics or Antifungal:

Systematic reviews[52-55]have evaluated topical antibiotics for chronic sinusitis without nasal polyps. Bardy et al demonstrated using a high-volume (240 mL divided between 2 nasal cavities) mupirocin irrigation compared with placebo in a specific cohort of patients with a sinus culture positive for *Staphylococcus aureus* [56]. There was improved short-term symptom score and no difference in sinus specific QOL.

Use of topical amphotericin B demonstrated no benefit compared with placebo for patients without nasal polyps in RCT's. Therefore, use of topical antifungals for chronic sinusitis without nasal polyps is not a routine practice.

Systemic Therapy:

a. Oral corticosteroids: Steroids as anti-inflammatory agents are now the accepted line of therapy that is widely accepted with numerous RCTs demonstrating significant improvements in symptom and quality of life metrics in CRSwNP patients when used for 2–3 weeks at a time[57] . A combination of oral and topical steroid therapy has also been shown to reduce polyp size more efficiently than topical treatment alone[58] Risk of side-effects due to high dose or frequent use suggest no more than 2–3 courses per year before considering endoscopic sinus surgey (ESS) [59]. Although high-level evidence exists supporting oral steroid use in CRSWNP patients, robust evidence supporting their use in CRSsNP is lacking[43] and therefore they are not routinely recommended in these patients.

b. Antibiotics: Despite CRS being one of the most common indications for which oral antibiotics are prescribed, evidence supporting their use in the treatment of CRS remains limited[60]. A Cochrane review assessing the value of antibiotics for CRS, revealed a limited overall benefit for their use in CRS, although transient improvement in QOL was seen in some CRS patients receiving 3 months of macrolide therapy[61].

A recent systematic review of published low-dose macrolide studies suggested that this antibiotics class may be more beneficial in CRSsNP than CRSwNP patients, with subgroup analysis showing more benefit when used for 24 weeks compared to shorter durations. No difference between 14-membered and 15-membered ring macrolides was noted[62] .The effect of low-dose macrolides is thought to be primarily mediated through their local immunomodulating/anti-inflammatory properties rather than their antibiotic action[63] .Although macrolides are generally well tolerated with a favorable side-effect profile, the FDA has released a recent warning of the possible increased risk of all-cause mortality over 10 years in patients with cardiac disease taking macrolides[64] suggesting caution in these patients. Given the lack of evidence supporting the use of non-macrolide oral antibiotics for CRS and the risk of serious adverse effects and emerging issues with antibiotic resistance, the use of these agents should be justified by endoscopic evidence of an infective exacerbation or complication. In such conditions, antibiotic therapy should ideally be culture directed. Although topical antibiotics may have a better systemic side effect profile than their oral counterparts, they are associated with an increase in local side effects such as burning, bleeding, and nasal dryness and to date, no high-level evidence supports their use for CRS.

c. Antifungal Therapy: The role of antifungal therapy for CRS remains uncertain. A Cochrane review of this topic was published in 2018 patients and included eight studies with 490 adults. This review demonstrated a lack of high-level evidence to support the use of oral or topical antifungal agents [65]. As a result there are no

generally recommended consensus guidelines for the routine use of these agents in treatment of CRS[66]

d. Monoclonal antibody:

Biological treatments for CRS are under trial using monoclonal antibodies, well-performed RCTs of five monoclonal antibody agents have all shown promise for their use in CRS. In two independent phase 3 study, CRS patients receiving subcutaneous injections of Omalizumab, an anti-IgE antibody approved for patients with refractory asthma, demonstrated improvements in polyp scores, nasal and asthma symptoms, radiological severity scores and QOL with 16 and 24 weeks of treatment, respectively [67,68]. These agents target the IL-5 pathway, which is thought to be central for Th2 eosinophilic inflammation associated with CRSwNP. Reslizumab and Mepolizumab, humanized monoclonal antibodies approved for the treatment of severe asthma, both showed a significant reduction in polyp size in patients treated for 4 weeks compared to placebo[69,70].

Dupilumab is a fully human monoclonal antibody that inhibits signaling of interleukin (IL)-4 and IL-13, key mediators of type 2 inflammation. Results from two multicenter, randomized, double-blind, placebo-controlled, parallel-group phase 3 trials involving 276 patients showed that in adult patients with severe CRSwNP,

dupilumab reduced polyp size, sinus opacification and severity of sinus symptoms. The medication was generally well tolerated although patients did experience a slightly high rate of side effects, namely headaches, epistaxis, injection site reactions nasopharyngitis and worsening of nasal polyps and asthma than placebo[71], and it is now FDA approved for treatment in CRS. Although biological agents promise a means of offering patients personalized endotype-driven therapy, their high cost and lack of clinical approval for use in CRS remain important prohibitive steps in their current use for this condition[72] .

Challenges in Medical Therapy: CRS remains the most common diagnosis treated with antibiotics[73]. Due to increasing levels of antibiotic resistance[74], treatment of CRS with antibiotics [75] are reserved only for those patients with evidence of bacterial infection manifesting as fever, purulent discharge and pain or pressure[76].

Rudmik et al[77] reviewed the use of topical therapies in CRS and recommended the use of irrigations with saline and nasal steroid treatment instead of the use of topical antimicrobials and antifungal treatments. The use of antifungals is being recognized

as ineffective in the treatment of CRS, as reported by a meta-analysis that included six studies (380 participants). The use of topical antifungals was investigated in five studies and one study investigated the effect of systemic antifungals. This pooled meta-analysis demonstrated that there was no significant benefit of topical or systemic antifungals over the use of placebo for any of the outcomes[78] .

Intranasal corticosteroids have been effective in both CRSsNP and CRSwNP patients as recommended by evidence-based review [79].The use of monoclonal antibodies [80](MAbs) as biologic therapies have been promising since they target inflammatory signaling molecules that serve as targets to treating inflammation in CRS. A recent study by Drilling et al[81] used bacteriophage (phage) for topical application in the treatment of CRS specifically in drug resistant clinical Isolates in sheep and found improved outcomes in comparison to saline.

The use of phage as a single dose proved to significantly decrease the formation of biofilms of *Pseudomonas aeruginosa* in vitro[82], and was shown to be safe and efficacious in an in vivo model when used as a cocktail (CT-PA) at a concentration of 10^8-10^{10} PFU/mL. Bacteriophage (phage) therapy as intranasal irrigation with the phage cocktail AB-SA01 of doses 3×10^9 plaque-forming units (PFU) for for 14 days was proven efficacious in the treatment of patients with recalcitrant CRS due to *S.aureus* [83].

The controversies of antibacterial therapy in CRS led investigators to look into

Cleland et al [84]studied the role of probiotic treatments to replace pathogenic bacteria with healthy commensal bacteria Staphylococcus epidermidis (SE) in a mouse sinusitis model supporting microbial rehabilitation as a promising avenue for the management of CRS.

1.1.4.2. Surgical treatment

Surgery is the last resort in patients who fail to respond to medical treatment. Definition of CRS has been put forward by multinational panel of rhinologists based on clinical symptoms and evidence of inflammation, however these definitions serve as better predictors of surgical intervention than indicators of progression of disease[85]. Optimizing patient selection in sinus surgery becomes crucial in terms of achieving better surgical outcomes. Patients with CRS have been frequently subdivided into two main groups, CRS with nasal polyps (CRSwNP) vs. CRS without nasal polyps (CRSsNP), this categorization has implications on surgical intervention [86] . Studies have reported that in CRSwNP, SNOT-22 (sinonasal outcome test) pre-operatively scores are worse compared to CRSsNP. However, CRSwNP patients show better improvements post-surgery in comparison to CRSsNP patients [87].

The primary goal of surgical management in CRSwNP are not only to remove the diseased tissue but open up drainage pathways, and allow for treatment with topical applications and rinses [43].

The effectiveness of ESS as reported by Chester et al[88] shows a greater improvement in symptoms such as nasal obstruction, compared to post-nasal drip and facial pain. ESS preserves the mucosa and opens up the drainage in the

ethmoid sinuses, by minimal invasive mucosa sparing endoscopic approach to enhance sinus ventilation and keep the ciliary action directed mucosal clearance functional, hence termed functional endoscopic sinus surgery (FESS). Recalcitrant sinus disease warrants surgical intervention in a large proportion of patients.

A systematic review evaluating a total of 33 studies reported symptomatic improvement from 78 to 88% for ESS compared with 43 to 84% for other techniques (including polypectomy, Caldwell-Luc and intranasal ethmoidectomy) showing that ESS is a safe and effective method of treatment [89]. The main drawbacks of ESS include complications due to proximity of anatomical structures such as orbit, skull base, dura, and brain[90]. Systematic reviews of treatment paradigms have shown surgical therapy performed for appropriate patients have experienced significant improvement in disease -specific quality of life score and objective endoscopic grading in the first 6 months and with stable scores between 6 and 12 months in comparison to medical therapy[91]. Studies also suggest early surgical intervention in refractory cases reduces the number of missed workdays and being cost effective[92,93].

For treatment of CRSsNP balloon dilation of sinus ostia is another surgical tool which has become popular, additionally, this has the advantage of being able to be performed in the outpatient clinics or in an operating room, avoiding the need for general anaesthesia[94,95]. Experts have agreed that this technique could be performed on a selected group of patients based on anatomy, phenotype and other co-morbidities. It is reported that CRSwNP patients benefit from surgical intervention when combined with post-operative antibiotics and perioperative steroids. Literature

also supports that CRSsNP benefit from correction of anatomic abnormalities along with post-operative antibiotics and topical steroids[96].

Though surgical instrumentation and techniques continue to evolve and have become less traumatic , surgery at least in the short term does not totally resolve inflammation in patients, hence an ongoing endoscopic surveillance, along with close communication between surgeon and patient in addition to monitoring patient adherence to their treatment regimens could enhance positive outcomes [97].

1.2. Staphylococcus aureus

1.2.1. Taxonomy and Microbiology

Genus *Staphylococcus* are gram-positive cocci (0.5 to1.5μm in diameter) arranged in grape like clusters as described by Ogston[98]. Staphylococci are catalase positive nonmotile, non–spore forming, and facultative anaerobes. Though they were classified along with micrococci, they differ from those in having a low guanidine-cytosine (G+C) content. The genus *staphylococcus* consists of 45 species and 24 subspecies [99] , the most virulent being *S. aureus* causing human infections. Other pathogenic species include *Staphylococcus lugdunensis* in humans and *Staphylococcus intermedius* in animals. Although *Staphylococcus epidermidis*, *Staphylococcus haemolyticus,* and *Staphylococcus saprophyticus* are associated with device- related and urinary tract infections they are less virulent compared to

S. aureus. Staphylococci colonize skin and mucosa of animals, including mammals and birds. *S. aureus* in humans colonises the anterior nares, axilla and perineum. *S. aureus* nasal carriage varies from 10% to 40% in both community and in hospitalised patients [100].

1.2.2. Biochemical characteristics

Although many phenotypic tests are available for the diagnosis of staphylococcal infections, coagulase remains the most valid confirmatory test. *S. aureus* forms beta haemolytic colonies on blood agar and golden yellow colonies on nutrient agar. Mannitol salt agar (MSA) is a good selective and differential media for isolation of staphylococci due to the presence of high salt content. MSA is used as a presumptive test for the identification of *S. aureus* and is also used for the differentiation of coagulase-positive staphylococci from coagulase-negative staphylococci (CoNS). *S. aureus* is positive for catalase, coagulase, mannitol fermentation and trehalose tests. Although diagnostic methods based on those characteristics provide a definitive identification, their time consuming nature becomes a disadvantage[101].

1.2.3. Virulence factors

To transform from a commensal into a pathogen *S. aureus* uses various virulence factors to alter the host defense system. These virulence factors [102] are divided mainly into two major types, the surface exposed proteins and the secretomes [103] .

Surface exposed proteins are the MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) which include the fibronectin-binding proteins (FnBPA, FnBPB), fibrinogen-binding proteins (Efb), clumping factors (ClfA, ClfB), staphylococcal protein A (Spa), collagen-binding protein (CBP) and extracellular matrix protein-binding protein (Emp) [104]. After colonisation *S. aureus* produces secretomes (table 1.2). These are proteins that enhance detachment and spread of infection and include enzymes and toxins. These toxins are the hemolysins (Hla, Hlb, Hlγ), leukocidins (LukD, LukE, LukM) and Panton-Valentine leukocidin (PVL), Phenol soluble modulins (PSMs), Exofoliative toxins (ETs — ETA, ETB, ETD) and Enterotoxins (SEs — SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI).

1.2.3.1. Cell surface determinants

1.2.3.1.1. Cell wall

Peptidoglycan constitutes 50 percent weight of staphylococcal cell wall; it comprises of alternating polysaccharide subunits of N-acetylglucosamine and N-acetylmuramic acid. Peptidoglycan exhibits endotoxin-like activity by activating macrophages, initiating complement activation and leading to aggregation of platelets. Staphylococcal strains with variations in peptidoglycan structure display alterations in their ability to produce disseminated intravascular coagulation[105]. Teichoic acids of the cell wall include the ribitol teichoic acids and Lipoteichoic acids (LTAs)[106] (Figure1.1). Teichoic acids serve as a site of attachment for cell wall, enzymes and other proteins thereby playing an important role in cell wall metabolism[106] . Teichoic

acids are involved in adherence to nasal epithelia, however their role in invasion and host inflammatory response remains unclear [107]. Lipoteichoic acids (LTAs)[108] are the plasma membrane-bound counterparts of teichoic acids which are composed of polyglycerol-phosphates. LTAs produce inflammation by triggering the release of cytokines and other activators of the innate immune system.

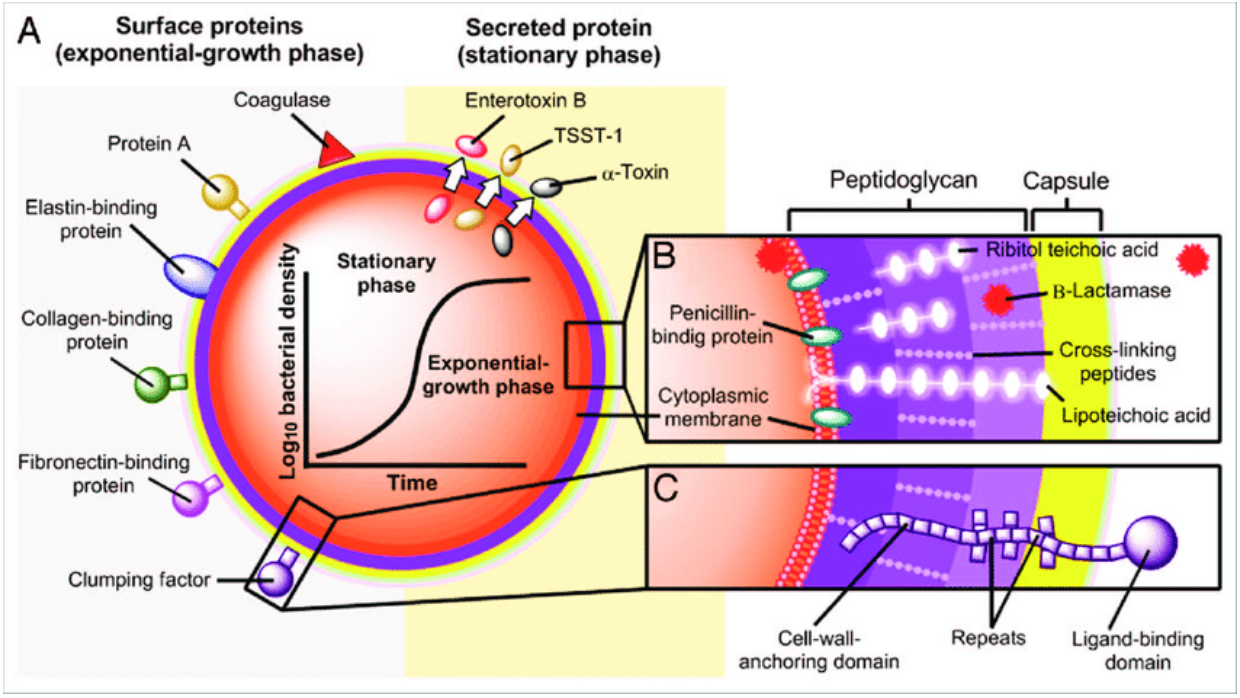

Figure 1. 1: Staphylococcus aureus virulence factors

(A) showing surface and secreted proteins, (B and C) are cross-sections of the cell envelope. Reprinted with permission[109]

1.2.3.1.2. Capsule

Surface-associated virulence factors aid *S. aureus* to adhere to the host and cause infection. Studies have reported that among the clinical isolates of *S. aureus* 90% of them produce a polysaccharidic capsule. Based on absorbed rabbit antiserum, Karakawa and Vann et al reported a capsular polysaccharide typing scheme for

S. aureus strains[110], describing eight capsular serotypes. Accordingly, heavily encapsulated strains called as M (type 1) and Smith (type 2) diffuse were denoted to serotypes 1 and 2 [111].Large quantities of polysaccharides produced by serotypes type 1 and 2 strains of *S. aureus* are often identified by their mucoid nature on culture plates. Clinical infections are seen among type 5 and type 8 accounting for 75% of infections[112]. The capsule enhances the persistence and colonization of bacteria by preventing phagocytosis and serves as a potential target for vaccination. The main function of the capsule in staphylococcal virulence is to interfere with phagocytosis [113].

1.2.3.1.3. Surface Adhesins

The MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) enable attachment to the host matrix proteins [114]by altering their organization and averting the organism recognition by the host immune system[100] . The most common MSCRAMMs involved in pathogenesis include clumping factor A and B (ClFA/B) which comprises of the fibronectin-binding protein A and B (FnBPA/B) and fibrinogen binding proteins. FnBPA has been associated with

S. aureus mediated endocarditis[115] and ClFA causes platelet aggregation at areas of tissue injury[116] . ClFB is seen in colonization of *S. aureus* in the anterior nares due to its ability to bind strongly with cytokeratin 10, which is an important component of keratinizing squamous epithelium in the anterior nares. Various other factors that contribute to bacterial cell adhesion include collagen binding adhesin and coagulase [117] .

1.2.3.1.4. Protein A (SpA)

Protein A plays a crucial role in the pathogenesis of *Staphylococcus aureus and* is present in almost all clinical stains[118]. *Staphylococcus aureus* surface is coated with protein A, which is covalently linked to peptidoglycan layer and has affinity to bind to Fc receptor of immunoglobulin (Ig)G1, IgG2, and IgG4 thereby preventing immune clearance by hindering the phagocytosis of opsonized bacteria. This binding of protein A to Fc receptor causes alteration in the recognition of Fc receptors on polymorphonuclear leukocytes (PMNs) thereby making them unable to appropriately distinguish and phagocytose these pathogens [119]. Studies have explained that protein A deficient strains of *S. aureus* are phagocytosed efficiently by neutrophils[100]. SpA like superantigens, induces B cell clonal expansion[120], B cell proliferation in the presence of TLR 2 ligands, suggesting its participation in innate immune mechanisms[121]. SpA also plays an important role in biofilm formation[122] by stimulating bacterial cell aggregation, thereby enhancing formation of biofilms on host tissue and surfaces of medical devices.

1.2.3.1.5. Biofilm

Biofilms are defined as microbially derived sessile bacterial communities, characterized by cells that are irreversibly attached to a substratum or interface. Biofilms are embedded in a self-produced extracellular polymeric substances (EPS) matrix and have a manifest altered phenotype compared to their planktonic counterparts [123]. Biofilms are made of microbial cells constituting up to 15% of the biofilm volume and the EPS matrix forming the remaining part. The *S. aureus* biofilm matrix encases all of the cells in the mature structure and it is composed of secreted and lysis derived proteins, host factors, polysaccharides and eDNA[124].

The polysaccharide intercellular adhesin (PIA) also called polymeric N-acetyl-glucosamine (PNAG) forms the main constituent of the biofilm matrix. PIA is made of β-1,6-linked *N*-acetyl glucosamine polymer, and is produced by enzymes that are encoded by the *ica ADBC* locus[125]. The PIA plays an important role in maintaining the structural integrity of the biofilms; however, these matrix components were later depicted to be proteins and eDNA that function as intercellular adhesins[126] .

Proteins are major contributors of attachment and biofilm matrix development, such as protein A (surface-associated proteins), biofilm-associated protein (Bap), fibrinogen- binding proteins (FnBPA and FnBPB), *S. aureus* surface protein (SasG) and clumping factor B (ClFB). In addition, other secreted proteins such as beta toxin (Hlb) and extracellular adherence protein (Eap) play a major role in the maturation of biofilm[127]. Intracellular proteins identified within the biofilm matrix are released during

cell lysis and incorporated into the matrix, but their importance is yet to be understood.

The eDNA component of the biofilm matrix was identified recently. It functions as an electrostatic polymer anchoring cells to the host factors and to the surface[128]. eDNA production occurs during the autolysis of a subpopulation of the biofilm cells[129], mediated by murein hydrolases that are encoded by the *atl* and *lytM* genes. These murein hydrolases play a role in cell division and cell wall rearrangements by degrading the peptidoglycan layer[129].

During the formation of biofilms, the free-floating bacteria become adherent and undergo a process of reversible attachment forming aggregates. In the next stage they undergo irreversible attachment that enable the binding between specific microbial adhesins and the surface. At the end of this stage the biofilm's attachment is considered irreversible making it difficult to remove without chemical intervention or mechanical forces. Aggregation and maturation occur when the surface bound organisms begin to actively replicate, increasing the overall density and complexity of the biofilm. Proteomic studies show that during this stage, biofilm bacteria have radically different levels of genetic and protein expression in comparison to their planktonic counterparts[130]. Detachment or dispersal occurs during the final stage of the biofilm lifecycle, where cells separate as in groups or as single organisms from the major biomass after attaining a critical mass. Exo-enzymes and surfactants produced by *S. aureus* degrade the PMS matrices. Proteases produced by *S. aureus* include two cysteine proteases (SspB and ScpA), seven serine proteases (SspA and SplA-F) and one metalloprotease (Aur). Elevated levels of these proteases lead to

degradation of matrix proteins and the destabilization of biofilms[131]. The expression of proteases and phenol soluble modulins (PSM) is regulated by the *agr* quorum sensing system [132].

Two extracellular nucleases produced by *S. aureus* include the nuclease (Nuc), nuclease 2 (Nuc 2)[133] and the major staphylococcal nuclease (micrococcal nuclease). These nucleases lead to the disruption of neutrophil extracellular traps (NETs), thereby modulating biofilm development. Studies have demonstrated that the expression of nuclease results in reduced levels of biofilm formation[128]. Other enzymes such as hyaluronidases and lipases are also involved in the dispersal of biofilm, but their exact mechanisms need to be investigated[134]. The importance of each enzyme that degrades biofilm matrix depends on the strain specific composition of the biofilm matrix. Both in vivo and in vitro studies have demonstrated that biofilms resist treatment to antibiotics, disinfectants and various surfactants [135]. Hence, the ability of *S. aureus* to form biofilms in an important determinant for chronicity of infections.

1.2.3.2. Secreted virulence factors

The secreted factors are broadly classified as enzymes and toxins (Table 1.2)

1.2.3.2.1. Staphylococcal enzymes

1.2.3.2.1.1. Catalase

The enzyme catalase produced by *S. aureus* converts hydrogen peroxide into water and oxygen and neutralizes the bactericidal effects of hydrogen peroxide to protect from their effects. The catalase test differentiates streptococci (negative test) from staphylococci [136](positive test).

1.2.3.2.1.2. Coagulase

Coagulase production is important for the identification of *S. aureus* in the laboratory. All strains of *S. aureus* do not produce coagulase but do possess a coagulase gene (coa) [137]. *S. aureus* strains produce two forms of coagulases; free and bound (clumping factor) coagulase. Bound coagulase converts fibrinogen to insoluble fibrin bound to the staphylococcal cell wall causing the staphylococci to clump. The cell-free coagulase causes clotting of plasma in a test tube and produces coagulase-reacting factor which combines with globulin to form staphylothrombin. This enhances fibrinogen to form insoluble fibrin. Fibrin formed causes a mesh around staphylococcal abscesses thus localizing the infection and inhibiting phagocytosis. Production of coagulase is one of the markers of *S. aureus* virulence.

1.2.3.2.1.3. Proteases

S. aureus extracellular proteases include cysteine (staphopain A and B), serine (V8 protease) and serine protease-like (Spl proteases) and metalloproteases[138] . Staphylococcal serine protease [V8 protease] (SspA) modulates the stability of

S. aureus by cleaving surface protein A [139] and thereby enhancing the potential of these invasive phenotypes. Massimi et al have demonstrated that Staphopain B degenerates fibronectin and aids in the spread of infection caused by *S. aureus* [140]. By cleaving antimicrobial peptides, proteases produced by *S. aureus* aid in acquiring nutrients and protein richness.

1.2.3.2.1.4. Lipases

S. aureus produces lipases that enhance its ability to invade host tissue and disseminate to other sites. Lipases hydrolyze lipids[141] which is necessary for the survival of staphylococci in the sebaceous parts of the body, thus enhancing the invasive capacity of *S. aureus* [142]. The Glycerol Ester Hydrolase lipase of *S. aureus* (Geh) is secreted as a pro-Geh which is a precursor enzyme (72 kDa) and is processed into the mature form (Geh, 42 kDa). The direct association of lipase in the pathogenesis of *S. aureus* especially in CRS is not yet determined. Lipase produced by *S. aureus* is known to interfere with the host granulocyte function and increase the survival of the bacteria[143].

S. aureus as a common colonizer of humans must cope with other innate defense mechanisms of the skin that include the secretion of sebum by the sebaceous glands[144]. Sebum, a liquid concoction of antimicrobial lipids, is composed of free fatty acids, triglycerides and wax esters. *S. aureus* is exposed to linoleic acid from nasal secretions while colonizing the anterior nares. It has been suggested that lipase supports the persistence of *S. aureus* in the fatty secretions in human skin and therefore has an indirect influence on its pathogenic potential. Studies have reported that lipase is associated with formation of biofilms, when lipase encoding genes were found to be among the up-regulated genes in *S. aureus* biofilms [145] . The use of farnesol[145], a lipase inhibitor, has been shown to reduce biofilm production of *S. aureus*. Literature shows that lipase plays an important role in *S. aureus* pathogenesis, demonstrating anti-lipase IgG antibodies in patients suffering from *S. aureus* infections, emphasizing the virulent potential of lipase[146]. It is perplexing as to how *S. aureus* can persist on human skin while still producing lipase that would liberate toxic fatty acids from the sebum triglyceride fraction [147] .

1.2.4. Staphylococcal toxins

S .aureus secretes a repertoire of extracellular toxins to enhance its ability to disseminate infection[148] . Toxins constitute proteins that are secreted during the post exponential and early stationary phases of *S. aureus* growth. The cytolytic property of these toxins enables bacterial growth by acquiring nutrients like iron from lysed

cells. Toxins secreted by *S. aureus* are mainly composed of three groups namely exfoliative toxins (ETs) , superantigens (SAgs) and pore-forming toxins (PFTs) [149].

1.2.4.1. Hemolysins

Pore-forming toxins (α- haemolysin) or α- toxin of *S. aureus* is one among the important cytolysins produced by *S. aureus* during invasion causing pores in the cell membrane leading to lysis. α -toxin affects different cell types such as the endothelial, erythrocytes, macrophages and T-cells. The α-hemolysin binds to the metalloprotease protein receptor ADAM10 leading to formation of pores. Further Ca^{2+} influx and K efflux leads to osmotic lysis and necrotic cell death[150]. α -toxins have been associated with in vivo biofilm formation on mucosal surfaces [151], by disorganizing the epithelium which in turn enhances bacterial adhesion and growth. α -toxins cause impairment of the mucocilliary structure leading to persistence of biofilms in Cystic Fibrosis as reported by Hoiby et al[152]. β-hemolysin, a sphingomyelinase, hydrolyses sphingomyelin from membranes [143], is a non-pore-forming toxin, cytotoxic preferentially to monocytes and not to lymphocytes and platelets[153].

1.2.4.2. Leukotoxins

Leukotoxins are bi-component toxins causing lysis of leucocytes, produced by most strains of *S. aureus* [154]. Panton Valentine Leukotoxin (PVL) is produced by ≤ 2%

S. aureus strains and seen in community-acquired CA-MRSA[155]. Panton-Valentine toxin causes skin and soft tissue infections (SSTI), necrotizing pneumonia and severe hemorrhagic pneumonia in children [156].

1.2.4.3. Phenol-soluble modulins

Phenol-soluble modulins (delta toxin, δ-toxin) are α-helical peptides (Figure: 1.2) in virulent strains of *S. aureus*. They were initially described in *S. epidermidis*, but later also shown in virulent strains of *S. aureus* which have the ability to lyse neutrophils[157]. Phenol-soluble modulins are principal factors in biofilm formation and also enable dissemination of infection. Based on the length of amino acids they are classified as either longer (~44 aa) β-type PSM and a shorter α-type PSM (~20-25 amino acids)[158]. δ- toxin has been reported to act as an immune modulator and also induces mast cell degranulation in patients with allergic dermatitis, suggesting a mechanistic role of *S. aureus* in allergy[159] .

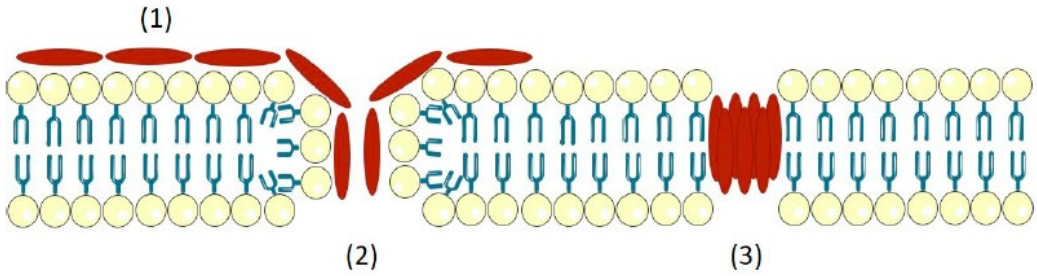

Figure 1. 2: Pore formation mechanism of S. aureus

Phenol-Soluble Modulins (1) Attachment, (2) Membrane Disintegration (3) Aggregate into Oligomers. Reprinted with Permission[160].

1.2.4.4. Staphylococcal exfoliative toxins

Exfoliative toxins (ETs) are serine proteases that destroy protein desmoglein[161] by colonizing and disrupting the epidermal layers. Staphylococcal exfoliative toxins (ETs) cause staphylococcal scalded skin syndrome (SSSS) and staphylococcal bullous impetigo described in newborns and infants[162].

1.2.4.5. Staphylococcal enterotoxins

Staphylococcal enterotoxins (SEs) cause food-borne diseases presenting with diarrhea and vomiting. These toxins are heat stable and belong to five groups: enterotoxin A (SEA), SEB, SEC, SED and SEE[163]. Enterotoxins belong to pyrogenic exotoxins that behave like superantigens (SAgs), were first elucidated by Bernhard Fleischer and Hubert Schrezenmeier in 1988. T lymphocytes are activated by superantigens (SAgs) by direct cross-linking of T cell receptor Vβ domains with the major histocompatibility complex class II (MHC II) molecules (Figure 1.3). This produces a cytokine storm as large amounts of pro-inflammatory cytokines (IL-2, IFN-γ and TNF) are released which leads to clinical symptoms such as high fever, hypotension, vomiting and diarrhea and results in multiorgan failure [164]. Studies have reported that SAgs aggravate formation of polyps, asthma, and Chronic Obstructive Pulmonary Disease (COPD) by accentuating the TH-2 immune response. The Toxic Shock Syndrome Toxin (TSST) is not a true enterotoxin as it lacks the emetic form[165]. TSST is produced at the site of infection (vaginal tampons) and then

spreads to the blood stream releasing massive amounts of cytokines leading to shock syndrome[166]. Superantigens bind to MHC class II molecules of antigen presenting cells and Vβ region of T-cell receptor in a non-antigen-specific manner leading to massive release of cytokines and chemokines.

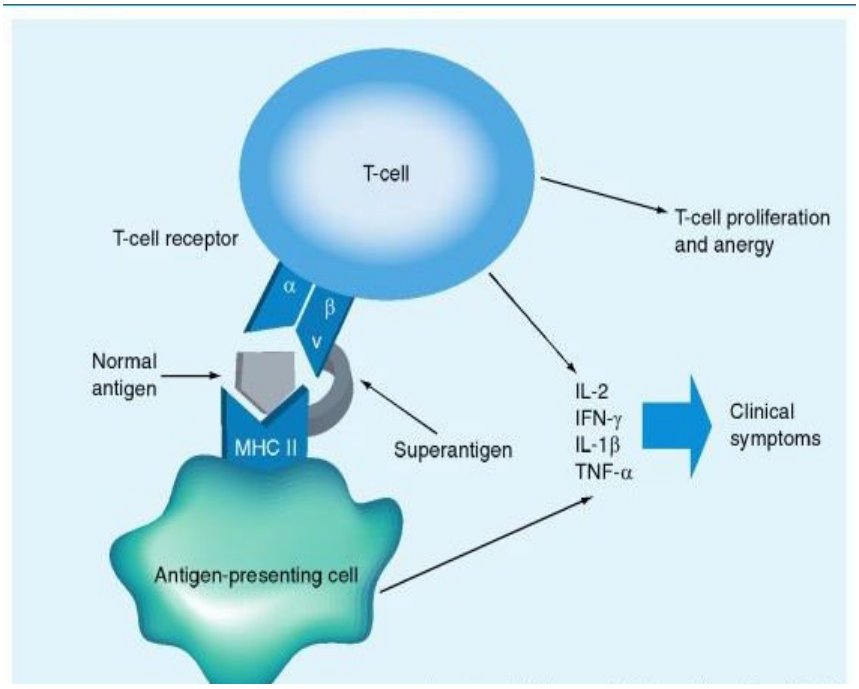

Figure 1. 3 : Mechanism of action of Superantigens. Reprinted with permission[164]

Table 1. 2: Staphylococcal virulence factors involved in bacterial pathogenesis

Virulence factor	Functions
Exfoliative toxins	Act as serine proteases; activate T cells
Staphylokinase	Plasminogen activation; inactivate antimicrobial peptides
Lipase	Inactivate fatty acids
Hyaluronidase	Degradation of hyaluronic acid
Toxic shock syndrome toxin 1 Staphylococcal enterotoxins	Activate T cells and macrophages
Serine proteases; cysteine proteases; aureolysin	Inactivate neutrophil proteolytic activity; inactivate antimicrobial peptides
Cytolysins (α-, β-, γ-, δ-toxins); phenol- soluble modulin-like peptides; leukocidins (PVL, LukD/E)	Induce apoptosis (at low concentration) and lysis of various cell types, including erythrocytes, lymphocytes, monocytes, epithelial cells; target specificity varies

1.2.4.6. Regulation of virulence

The *S. aureus* genome is divided mainly into two, the core and the accessory genomes. The core genome consists of the stable and the variable regions[167]. The latter contains genes that encode for factors such as the superantigens, toxins and the exoenzymes. Staphylococcal pathogenicity islands[167] (SaPIs) that carry genes encoding virulence factors and toxins are located at constant positions in the chromosome. Two major regulons described are the two-component signal transduction pathways (agrA/C, saeR/S, srrA/B, arlR/S, rap/traP) and the second SarA (staphylococcal accessory regulator) family. The effector molecule RNAIII in *agr* (Figure 1.4) is produced during the mid-exponential to post-exponential phase of growth resulting in global changes in gene expression. This molecule down regulates Protein A, collagen binding protein and fibrinogen binding protein expressed at early stages of bacterial growth, while it upregulates the toxins α, β and extracellular enzymes secreted during late phase of growth. The surface expressed proteins needed by *S. aureus* for adhesion to the host is synthesized early, followed later by the secretion of enzymes to attack the host[168]. The agr locus[169] controls both the sensor and response regulator components that drive the secretion of surface proteins, enzymes and toxins[170]. The agr operon consists of two transcriptional units RNAII and RNAIII, driven by the promoters P2 and P3. Agr A activates RNAIII expression increasing the secretion of *S. aureus* toxins and enzymes.

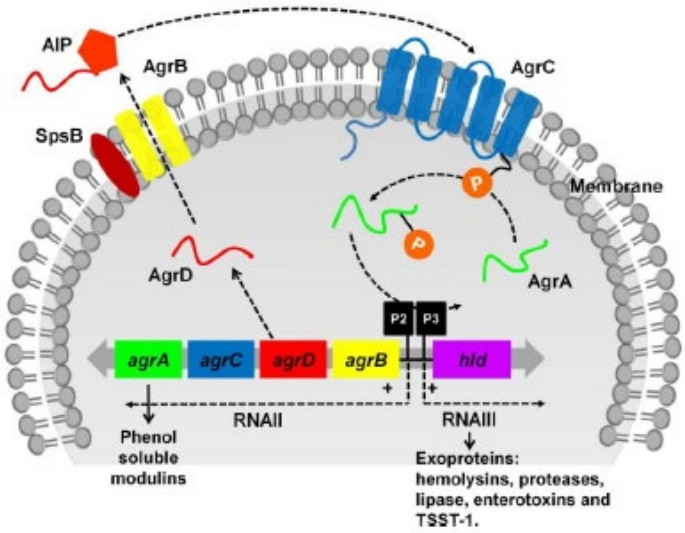

Figure 1. 4 : S. aureus agr regulatory system [170]

1.2.4.7. Detection of toxins

The gold standard to confirm whether *S. aureus* is the cause of a disease is isolation of the organism by culture from a suspected sample that could be the source of contamination or patient specimen. Detection of virulence factors in diseases includes identification of the toxin by detecting the toxin gene and confirming the toxic activity by identifying the gene or the activity of protein.

1.2.4.7.1. Molecular Methods

Polymerase chain reaction (PCR) targeting *S. aureus* genes (toxins) using specific nucleotide primers and probes is a sensitive and specific method and still remains the molecular method of choice. Discovery-based toxin analysis detection is possible by whole genome sequencing[171]. PCR is quick to perform and can be used to detect more than one toxin, however identification needs expertise and knowledge of genomic sequences as detection of a specific gene alone by PCR does not provide evidence about the expression of protein[172].

1.2.4.7.2. Biological Assays

Animal models have served as a common method for studying the biological activity of *S. aureus* toxins and their disease progression. The advantage of using animal models is that it can be easily compared with human disease processes, however they can be expensive for studies that include a large sample size and for high-throughput toxin testing. Various animals that are used include rats, mice, rabbits, guinea pigs and monkeys. Toxins are fed to these animals and information of biological activity is monitored [173]. The limitations of using this technique includes low sensitivity / specificity and variability of results[172].

1.2.4.7.3. Serological assays

Antibodies in serum are detected using methods based on antibody–antigen binding [173].Classical examples of antigen–antibody reactions include the agglutination and gel immunodiffusion assays. Double gel immunodiffusion depends on the reaction between the antibody and the toxin forming a band of precipitation. Though this method has the advantage of using small sample volumes, it is very subjective and at times produces semiquantitative results that require large amounts of purified antibodies[174]. Latex agglutination depends mainly on the agglutination of toxin with antibody-coated cells. It can be time consuming and difficult to interpret[175]. Immunoassays are highly sensitive and specific[171] as they can detect toxins from complex samples and are rapid to yield results, however, limitations include multiple washes and long incubation periods. Radioimmunoassay use radioactivity and highly sensitive methods to detect <1 ng/g[174], require facilities to handle and dispose hazardous materials and pitfalls such as nonspecific reactions could occur similar to other precipitation and diffusion assays. Newer technologies such as liquid chromatography (LC) with mass spectrometry (MS) have been used for toxin detection. The proteins are degraded into smaller peptides[176] and liquid chromatography (LC) is used to separate them based on their molecular weight followed by visualisation using the mass spectrometry (MS)[177].

These can detect lower levels of toxins, however, require experts and expensive equipment for analysis[178]. Newer techniques that use similar principles required for antigen–antibody reactions such as Aptamer-based bioassays use chemically

engineered antibody portions that interact with specific antigens [179]. Aptamers are ssDNA or RNA molecules that bind to target sites similar to antibodies. They are cost effective, have a long shelf life for toxin detection and exhibit low or no levels of immunogenicity in therapeutic models[179]. The Vβ-TCR Bio-Plex assay method for detecting toxins uses synthetically engineered enterotoxin-specific Vβ domain of the TCRs (Vβ-TCRs)[180]. By using magnetic microspheres, the fluorescence-based bead array enables the detection of toxins. These methods are highly specific and spare the need to use animals for raising antibodies[181].

1.2.4.8. Targeting toxins

1.2.4.8.1. Direct approach

With the emergence of antibiotic resistance, the need for novel therapeutic drugs as alternative treatment for *S. aureus* infections becomes necessary[182]. These anti-virulence therapies affect virulence determinants such as enzymes, toxins and surface proteins that alter regulatory systems are a promising option. Compared to conventional antibiotics, these methods reduce the selective pressure imposed on resistance development[183]. Allen et al has suggested the use of combination of anti-virulence drugs along with a proper treatment environment is an appropriate solution [184]. As hemolysins are the major toxins produced by most *S. aureus* strains, Hemolysin (α hemolysin) (Hla) has been the focus as a target for anti-toxin drug development (anti α hemolysin). Targeting α hemolysin using neutralizing antibodies to treat staphylococcal pneumonia in animal models[185] have additive effects when

used in combination with antibiotics (vancomycin or linezolid)[186]. A β -hemolysin neutralizing antibody to inhibit the Hlb hemolytic activity *in vitro* has been demonstrated using antibody phage display[187].

A number of molecules or compounds that block the activity of hemolysins secreted by *S. aureus* have been discovered, including β cyclodextrin derivatives and compounds that inhibit the activity of α hemolysis[188]. Toxins that have a specific host receptor allow therapies to be designed to antagonize these receptors. ADAM10, the zinc-dependent metalloprotease receptor for Hla[189] is one such target for the inhibitor to block the toxin-receptor, decreasing the severity of skin and soft tissue infections. In connection with the leukotoxins produced by *S. aureus*, PVL toxin remains one of the main targets in CA-MRSA. Human intravenous polyclonal immunoglobulin preparations (IVIg) containing PVL antibodies inhibit PVL-neutrophil binding and demonstrate anti-PVL leucotoxic effects[190]. Studies have reported that appropriate antibiotic treatment in combination with IVIg anti-toxin therapy improved symptoms of patients suffering with severe necrotizing pneumonia due to PVL-positive *S. aureus* strains, further supporting the use of intravenous polyclonal immunoglobulin[191]. Targeted therapies against Staphylococcal Enterotoxins (SEB) include monoclonal antibodies that bind and neutralize their effects on cell receptors and decreasing the amount of pro-inflammatory cytokine production thereby protecting from its toxic effects[192]. The use of Intravenous immunoglobulin from pooled antibodies (IVIG) to treat patients with TSS has been effective in treating patients with sepsis [193].

Glycerol monolaurate (GML), a preservative and emulsifier used in food and cosmetic industry exhibits inhibitory and immunomodulatory effects against

S. aureus and blocks the effect of β-lactamases and exoproteins mainly the TSST-1 and α-toxin[194]. GML decreases the production of proinflammatory cytokines and prevents lysis of human and rabbit erythrocytes from hemolysins. The compound 1-O-dodecyl-rac-glycerol (DDG) is noted to have similar effects as GML, hence, it is used as topical agent to treat TSS due to *S. aureus* infections.

The direct approach has been successful against many pathogens such as *Clostridia spp, Bacillus spp* and *Bordetella pertussis. S. aureus* specific anti-toxins that inhibit hemolysins, leukotoxins and enterotoxins have been promising in animal models, but none of them are successful in clinical trials[169].

1.2.4.8.2. Indirect approach

In addition to direct neutralization of toxins as described previously, targeting

S. aureus toxins can also be done indirectly by disrupting the regulatory mechanisms that control expression. The main regulators of virulence in *S. aureus* are the two-component signal transduction systems (Agr and Sae) and SarA transcriptional regulator[195]. The Agr system plays a key role as a virulence regulator and also serves as a target for the development of anti-virulence therapy. The agr locus contains two transcription units RNAII and RNAIII. The RNAII unit contains four open reading frames (ORFs)[196], its transcription unit regulates the transcription of genes that encode secreted enzymes and toxins at the same time repressing the

transcription of genes that encode the cell surface proteins[197]. In addition the RNAIII effector molecule controls the expression of many virulence factors, thereby targeting RNAIII is a novel approach to decrease the production of toxins and their expression[198]. Studies have reported that various bioactive components isolated from natural products, including isorhamnetin, chrysin, capsaicin, and puerarin decrease RNAIII expression and subsequently hla expression and have been seen to successfully protect the host against pneumonia due to MRSA and MSSA strains [199]. A highly potent anti-virulence compound, savarin, identified by Sully et al[200] is a compound that inactivates the Agr system by downregulation of agr-regulated genes as well as genes encoding PVL, PSM, protease, lipase and hemolysins.

Mansson et al [201] isolated Solonamide A and Solonamide B from photobacteria which antagonize the α - and β -globins that target the SrrA–SrrB two-component system that regulates the activity of α -hemolysin. Targeting *S. aureus* toxins and virulence factors are key approaches for future drug therapies, however one of the main constraints is probing virulence factors and infection within the context of a human infection. Hence, the use of the worm C. elegans as an early animal model to test such therapies has been adopted in laboratories as an *in vivo* model for the discovery of anti-infectives[202]. To identify new therapeutic molecules towards *S. aureus* with a mode of action that is different from conventional antibiotics, laboratories have developed this *in vivo* Caenorhabditis elegans—*S. aureus* liquid-based screen model to identify compounds that do not kill the pathogen but disrupt *S. aureus* virulence[203].

The Caenorhabditis elegans model serves a dual purpose by being able to detect anti-virulence compounds and to study the infection processes in an intact host-pathogen interaction[204]. The advantages of using a worm model are that it is simple, inexpensive to maintain and easy to manipulate. There are currently no anti-virulence agents approved for clinical use in bacterial infections in humans, however, there is growing evidence of the usefulness of these agents in infections. Although efforts to use novel anti-virulence methods have been met with varying degrees of success, there is still lack of pharmacology and toxicology data for this approach[169].

1.2.5. Staphylococcus aureus infections

S. aureus is an important human pathogen causing infections both in healthy immuno-competent people in the community and hospitalised patients with decreased immunity. *S. aureus* is found on the skin and in the nasopharynx of the human body. A minor breech due to trauma or surgery leads *S. aureus* to enter deeper tissue leading to local abscess formation [205] (Table 1.3). On reaching the lymphatic channels or blood it can cause septicaemia [148]. *S. aureus* produces various extracellular toxins, such as toxic shock syndrome toxin-1 (TSST-1) , enterotoxin A-E and exfoliative toxins A and B[206].

Contaminated food containing enterotoxin produced by *S. aureus* can cause food poisoning. Those strains carrying the TSST-1 gene[207] lead to toxic shock syndrome (TSS). TSS infections are mostly seen in menstruating women, those using

tampons. Staphylococcal scalded skin syndrome (SSSS) is due to the exfoliative toxins, that constitute toxic epidermal necrolysis and scarlatiniform erythema.

S. aureus is proficient in infecting foreign devices such as prosthetic joints and prosthetic cardiac valves due to its ability to form biofilms. The formation of biofilms on these surfaces of foreign devices makes it resistant to host immune responses and antimicrobial treatment[208].

Risk factors for developing *S. aureus* bacteraemia include intravenous drug use, intravascular catheter infection and prosthetic heart valves. *S. aureus* is the most common organism causing infective endocarditis contributing to 10-15 % of bacteraemia. Patients on pacemakers are at higher risk of developing *S. aureus* bacteraemia. Chamis et al has reported that in 33 patients with a cardiac device, the rate of device infection was identified to be 45 percent [209]. Progression of joint pain or back pain increases the suspicion of an occult site of infection in patients who had recent episode of *S. aureus* bacteraemia. Diagnosis of vertebral osteomyelitis is usually delayed by a month from the time of onset of symptoms leading to chronic disability and poor long-term outcomes. Septic arthritis generally presents as an acute episode with tenderness and swelling of the affected joint in > 80 % of patients at the time of presentation with fever present in only 30-50 % of them[210].

S. aureus is an important cause of community-acquired pneumonia (CAP), hospital-acquired pneumonia (HAP), ventilator-associated pneumonia (VAP), and health care-associated pneumonia (HCAP). HCA-MRSA is commonly seen to occur in the

elderly with comorbid conditions taking a course similar to gram-negative organisms. The development of bacteraemia indicates poor prognosis leading to a mortality of 50 % in both MSSA and MRSA cases. *S. aureus* is the most common organism isolated from culture in patients with chronic rhinosinusitis[211]. Boase et al[27] have reported on 513 patients with CRS showing 83 % of them had positive cultures; *S. aureus* was detected in 35 % of cultures and 9 % of them were positive for *P. aeruginosa.* Other reports that used molecular methods reported *S. aureus* in 71.4% of patients with CRSwNP[211]. The presence of *S. aureus* intra-operatively during sinus surgery predicts that there is a high likelihood of the organism being isolated in the post-operative period[212]. *S. aureus* is a strong biofilm producer and it is thought that the conditions within the diseased sinus, such as low PH, low oxygen tension and poor vascularity, enable *S. aureus* to transform into the biofilm phenotype[213].

S. aureus adapts itself to evade the host immune attack enabling it to reside and persist in the mucosa. *S. aureus* becomes facultative intracellular in neutrophils, endothelial cells and fibroblasts. By assuming intracellular residence, *S. aureus* remains as a reservoir for recurrent episodes of CRS [214]. Once inside the mammalian cell, *S. aureus* can switch to become small colony variants (SCV), transforming into slow growers with impaired cell separation activity[215]. Biofilm formation mechanisms are also reported to be involved in SCV infections.

1.2.6. Treatment of Staphylococcus aureus infections

The emergence of multiple drug resistant *S. aureus* strains have risen due to excessive use of antibiotics[148]. Penicillin was the first drug used for treating *S. aureus* infections in the 1940s and was effective in decreasing morbidity and mortality until the emergence of resistance due to the bacteria developing penicillinase[216]. Resistance to antibiotics can be coded by genes present on plasmids, accounting for their rapid spread amongst different bacterial strains[217]. Later, methicillin was introduced for treatment, however, in 1961, Jevons[218] discovered the emergence of methicillin resistant *S. aureus* (MRSA) which has since then spread worldwide as threatening nosocomial pathogen. MRSA strains produce altered penicillin binding proteins (PBP) designated as PBP2/, which have a low affinity for ß-lactam antibiotics creating resistance to treatment[219]. Altered penicillin binding proteins (PBP2/) are encoded for the mec A gene, located in mec region found only in the methicillin resistant strains. A glycopeptide, Vancomycin has been the first option against MRSA infections[220]. Studies have suggested that the use of combination of drugs along with aminoglycosides increase the bactericidal activity while decreasing resistance levels[221]. Rifampicin is an active drug against *S. aureus,* especially when an intracellular infection is suspected. In life threatening infections such as endocarditis and osteomyelitis, antibiotics are administered for four weeks or longer.

Prevention of infections due to *S. aureus* involves adherence to antimicrobial stewardship guidelines and infection control practices[222] . The treatment of carriers is

crucial as this decreases the risk of infections and the transmission rates. Intranasal mupirocin along with skin disinfectants are effective for treating carriers. The use of vaccines to prevent *S. aureus* have been a failure due to the presence of numerous virulence and immune evasion mechanisms in *S. aureus*[223]. Studies have reported that targeting only one protein or antigen is not sufficient. Understanding the molecular regulation of the host and the interaction with bacteria, provides hope that a multi-antigen vaccine could be efficient to prevent *S. aureus* infections.

Table 1. 3 : S. aureus infections in humans

Disease due to direct organ invasion:
Skin and soft tissue infections
Folliculitis
Furuncle, carbuncle
Cellulitis
Impetigo
Mastitis
Surgical wound infections
Musculoskeletal infections
Septic arthritis
Osteomyelitis
Pyomyositis
Psoas abscess
Respiratory tract infections
Community-acquired pneumonia
Nosocomial pneumonia
Empyema
Disease caused by exotoxin release:
Gastroenteritis
Toxic shock syndrome
Scalded skin syndrome
Disease due to bacteremia:
Sepsis and septic shock
Metastatic foci of infection
Infective endocarditis (native/prosthetic valves and injection drug use)
Device related infections:
Intravascular catheters
Orthopedic devices
Ventilator-associated pneumonia
Prosthetic valves
Urological instrumentation

1.3. Nasal epithelial Barrier

Epithelial cells form a physical barrier to prevent penetration of foreign allergens and pathogens. The upper respiratory epithelium consists of pseudostratified ciliated columnar epithelial cells and dendritic cells that present antigens to CD4+ T cells. Tight junctions (TJ) are adhesion complexes between adjacent epithelial cells. They are necessary to maintain the function of the epithelial barrier. Disruption of the epithelial TJ has been documented in inflammatory diseases such as celiac disease, inflammatory bowel disease and in allergic diseases such as nasal allergy and asthma[224]. Genetic causes of disruption to TJ barrier are seen in patients suffering from atopic dermatitis. Mucociliary clearance, intercellular apical junctional complexes that regulate paracellular permeability and the production of antimicrobial peptides are the three primary components of the barrier function of the airway tract[20]. These three components coordinate together to avoid pathogens, allergens and particulate matter to penetrate and helps to maintain tissue homeostasis. Furthermore, epithelial cells are also involved in the pathogenesis of inflammatory respiratory diseases, that are partly mediated by an impaired permeability of the mucosa[225]. The nasal mucosa produces cytokines and chemokines that play a crucial role in controlling innate and acquired immune responses.

In addition, studies have reported that disorders of innate immune receptors contribute to exaggerated innate immune activation leading to chronic inflammation [226]. Understanding the structural integrity and functional ability of the barrier is essential in chronic diseases such as CRS.

1.3.1. Mucociliary Function

The mucus and cilia play a major role in the mucociliary function of the airway epithelium. Inhaled pathogens are trapped by mucus, further swept away from the airways by coordinated beating cilia [227]. Mucus secreted by both goblet cells and submucosal glands contains more than 200 proteins; mucins form the main component that constitute its structural framework [228]. Inflammatory mediators, such as tumor necrosis factor α, IL-17, IL-13, IL-1β, neutrophil elastase and environmental factors such as cigarette smoke and infectious agents stimulate increased secretion of mucus[229]. Defective ciliary function leads to impaired mucociliary clearance as seen in patients with ciliary dyskinesia[230]. In patients with COPD, excessive mucus production increased the viscosity of mucus and reduces ciliary beating leading to impaired mucociliary function as reported by Yaghi et al[231]. In patients with cystic fibrosis and ciliary dyskinesia, impaired mucociliary function, in addition to causing obstruction, also leads to persistent and recurrent infections[232]. Mucin glycoproteins interact and bind to respiratory pathogens such as *Pseudomonas aeruginosa, Staphylococcus aureus, Heamophilus influenzae, Streptococcus pneumoniae, Burkholderia cenocepacia*, influenza virus, adenovirus, and coronavirus[233]. During impaired mucociliary clearance these pathogens that are cleared during normal conditions persist in the airway lumen and lead to initiation of an inflammatory response which can further cause damage to the airway epithelium[234].

1.3.2. Airway Epithelial Permeability

Tight and adherens junctions of airway epithelial cells contribute significantly to the barrier function of airway epithelium. The paracellular permeability of airway epithelium is maintained by structural components: tight junctions and adherens junctions present on the apicolateral membranes[235]. Tight junctions (TJs)[236] (Figure 1.5) regulate the paracellular permeability of ions and fluids across the apical and basolateral compartments [237]. Tight junctions are made up of transmembrane proteins such as claudins, junctional adhesion molecule (JAM) and scaffolding proteins (zonula occludens (ZO)-1, ZO-2, ZO-3), cingulin, and multi-PDZ domain protein 1 (MUPP1)[238]. Transmembrane proteins seen at the junction help to connect the membranes of adjacent cells to make a seal, while scaffolding proteins anchor transmembrane proteins. The claudin family of proteins comprises more than 25 members. They regulate the paracellular permeability in airway epithelium and their expression differs among tissues. Some claudins such as Claudin 1, 4, 13 and 14 are expressed in the nasal mucosa of humans [239]. Occludin is described to be universally expressed by epithelial cells and regulates assembly of tight junctions [240]. These intercellular junctions also serve as signaling platforms regulating gene expression, differentiation and cell proliferation [241].

Junction adhesion molecules (JAMs) play an important role in barrier function. Dimerization of this protein across cell membranes is needed for the regulation of barrier function. JAM proteins are also expressed from surfaces of blood cells and endothelial cells. They are necessary for the assembly of TJs, platelet activation and

migration. Cytoplasmic scaffolding proteins including Zonula Occludens proteins are expressed in a tissue specific manner and play an important role in stabilization of TJs in the apical membrane [242]. Studies have shown that changes in the expression of ZO proteins, for example upon stimulation of bronchial epithelial cells with poly (I:C) and respiratory syncytial virus (RSV) can alter barrier function[243]. Sustained injury and insult that affects these junctional complexes will disrupt the barrier function and interferes with normal repair and differentiation of airway epithelium.

Abnormally differentiated, leaky and hyperproliferative airway epithelium has been described in smokers, patients with asthma and COPD[244]. Microbial invasion by viruses or bacteria [245], allergens such as pollens, house dust mite, air pollution and cigarette smoke cause transient disruption of TJ and adherens junctions[243]. Increase in permeability occurs due to cell death or detachment of the cell membrane leading to entry of allergens and microbial pathogens into the subepithelial space thereby activating the adaptive and innate immune responses and promoting mucosal inflammation. Barrier disruption can occur due to direct invasion by inhalation or indirectly by host defence factors such as interferons and tumor necrosis factor-α that prolong the disruption of these TJs even after the infection is cleared allowing the passage of inhaled pollutants and allergens [246].

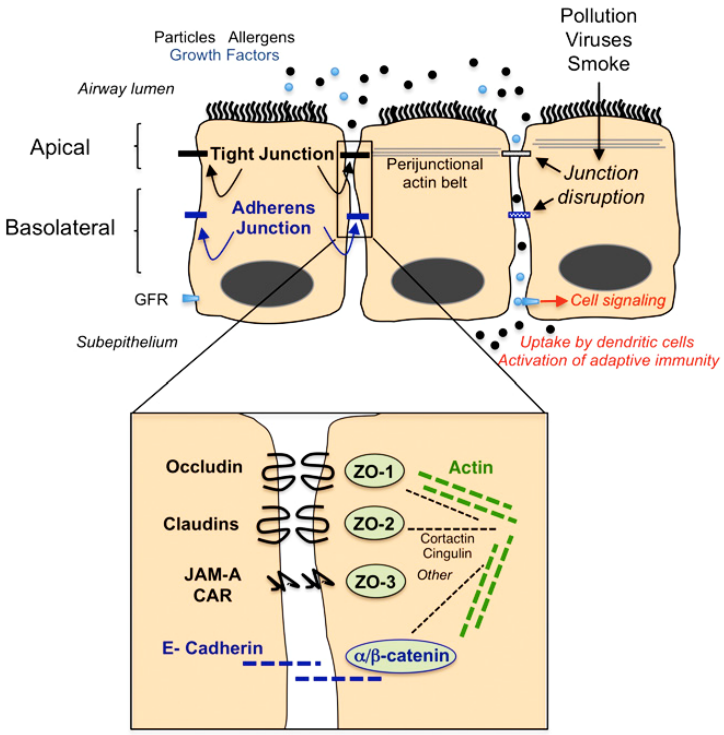

Figure 1. 5 : Organization of junctional structures in the airway epithelium

tight junctions (black) and adherens junctions (blue) [236]

1.3.3. Antimicrobial Products of Airway Epithelium

Apart from being a physical barrier, the airway epithelium also functions as a biochemical barrier against invading pathogens. Antimicrobial substances such as antimicrobial peptides, enzymes and protease inhibitors secreted by airway epithelial cells help in killing inhaled pathogens. The enzyme lysozyme found in airway epithelial secretions has antimicrobial effects on gram-positive bacteria, by degrading their peptidoglycan layer [247]. Lysozyme is also effective against gram-

negative bacteria in the presence of lactoferrin, which disrupts the outer membrane, allowing lysozyme to gain access to the peptidoglycan layer [248]. Lactoferrin, an iron-chelator, sequesters iron from growing micro-organisms and depletes the iron needed for their respiration, thereby inhibiting the growth of these organisms [249]. Lactoferrin also exhibits antiviral activity against both RNA and DNA viruses by inhibiting the viral binding to the virus or its binding to the host cells. Studies have reported that in COPD patients, low levels of salivary lysozyme correlated with increased risk of exacerbations, due to degradation of this enzyme by proteases produced by bacteria or neutrophils[250].

Protease inhibitors, such as the elastase inhibitor α1-antiprotease, and antichymotrypsin and secretory leukoprotease inhibitor (SLPI) are produced by epithelial cells. These protease inhibitors avert the effects of proteases produced by pathogens. Mc Elvaney et al has described that administration of SLPI led to decrease in the levels of IL-8 and elastase activity in patients with cystic fibrosis [251]. Hence, maintaining a balance between proteases and antiproteases in the airway lumen during infection is vital in preventing lung inflammation.

Human β defensins (hBD), being the most abundant antimicrobial peptides secreted on the surface of airway epithelium, are effective against a range of bacteria and viruses[252]. Smoking and exposure to air pollutants have been reported to decrease defensin levels in the airways[253]. Oxidants such as nitric oxide (NO) and hydrogen peroxide are also generated by airway epithelial cells. The three main NO synthases that contribute to NO production are the nitric oxide synthase (NOS)1 and NOS3 and inducible NOS2. Airway epithelial cells in COPD demonstrated a decrease in

expression of NOS2 which led to impaired clearance of rhinovirus[254]. The airway epithelial barrier coordinates mucociliary clearance, expression of tight junction proteins and the secretion of antimicrobial peptides effectively to clear inhaled pathogens and allergens.

1.3.4. Restoring Barrier dysfunction

Literature has reported various mechanisms to restore barrier function. Those include improving the innate immune function [255], use of growth factors (epidermal growth factor (EGF), keratinocyte growth factor) and by using corticosteroids. Toll-like receptors (TLRs) are crucial receptors involved in inducing an innate immune response against invading pathogens. Studies report that TLR2 activation of Calu-3 bronchial epithelial cells showed an increase in the TEER. This increase in the barrier function was linked to the upregulation of levels of the TJ proteins ZO-1 and claudin-1[256]. Micro-RNAs, endogenous noncoding RNAs, regulate the formation of TJs, epithelial barriers and inflammatory responses in the nasal mucosa[257]. The use of microRNA inhibitors increased the barrier function of HNECs by increasing the expression of TJ proteins (occludin, claudin-1, JAM)[258]. Treatment with Apoliprotein A1, a lipoprotein with anti-inflammatory properties, demonstrates enhanced barrier function. Studies conducted in primary bronchial epithelial cells from asthmatics treated *in vitro* with apolipoprotein A1 showed restoration of occludin and ZO-1 similar to controls which is promising as a potential strategy to repair impaired barrier by enhancing the expression of TJs.

Epidermal growth factor (EGF) and keratinocyte growth factor have been found to increase the epithelial barrier integrity by healing damaged mucosa. Transforming Growth Factor-b produced by most cell types in the lung prevents barrier damage produced by cigarette smoke by restoring ZO-1 and ZO-2[259]. Apart from promoting cell growth, EGF also enhances the homeostasis of the epithelial barrier. Xiao et al have reported that treatment of bronchial epithelial cells from asthmatic patients with EGF increased barrier function, by increasing the TEER and the expression of occludin and ZO-1[260]. Corticosteroids, due to their anti-inflammatory properties, have been used for allergic diseases. A study by Sekiyama et al[261] shows that treatment of bronchial epithelial cell lines with fluticasone propionate and dexamethasone increased the expression of occludin and ZO-1 [262].

1.4. Innate Immunity in the paranasal sinuses

The innate immune system acts as a physical and chemical barrier, recruits immune competent cells to the site of infection, activates the adaptive immune system and the complement system. The innate immune system is non-specific and does not depend on prior exposures for optimal function. The sinonasal tract is the first point of contact with inhaled pathogens, lined by the respiratory epithelium it plays a crucial role in innate immunity[263].

The respiratory epithelium is actively involved in mucociliary clearance, recognizing pathogens by receptors, secreting anti-microbial peptides, inflammatory mediators and activating the adaptive immune system[264]. Apart from the lymphocytes, tissue

bound epithelial cells, fibroblasts, dendritic cells and innate lymphoid cells (ILCs) also participate in innate immunity leading to perpetuating inflammation.

These cell types lack the ability to generate antigen-specific receptors by gene recombination and employ pattern recognition receptors (PRR) to recognise antigens. The innate immune system recognizes conserved molecular structures such as pathogen associated molecular patterns (PAMPs) present in microbes (bacteria, viruses, parasites or fungi). These PAMPs include lipopolysaccharide (LPS), lipoteichoic acid (LTA), peptidoglycan, mannans, bacterial DNA, and double stranded RNA[265]. Some of the important features of PAMPs are that they are produced by microbial pathogens but not by the host cells, and represent specific conserved regions required for survival of the pathogen.

Receptors for the innate immune system are present on effector cells such as dendritic cells (DCs), macrophages, B cells and respiratory epithelial cells. The dendritic cells bind to PAMPs leading to the activation of T cells. The Endocytic pattern recognition receptors engulf pathogens and present to lymphocytes. The Toll-like receptors (TLRs) play an important role in the activation of adaptive immunity [266]. Nasal epithelial cells also play a role as antigen-presenting cells and modulate the inflammatory response at the local tissue level. Cells that are involved in the functioning of the innate immune system include polymorphonuclear neutrophils, macrophages, eosinophils and basophils. Neutrophils secrete bactericidal lysozyme, lactoferrin, proteases and antimicrobial peptides while basophils and mast cells produce mediators such as leukotrienes and histamines.

Complement system in plasma comprises of more than 30 proteins that are involved in opsonization, leukocyte activation linking the innate and adaptive immunity [267]. Complement C5b, C6, C7, C8, and C9 are involved in the formation of membrane attack complexes which lead to cell lysis. The antimicrobial peptides that play a key role at the mucosal surface include the cathelicidins and defensins along with lactoferrin and lysozyme that are found in the sinus mucosa and nasal secretions. The iron-binding protein lactoferrin is abundant in the specific granules of human neutrophils. It inhibits microbial growth by depletion of iron needed for respiration[268]. Inflammation due to infection is characterized by increased levels of neutrophils and cytokines such as the IL-1β and IL-6. Interleukins IL-1β, IL-6 and IL-8 play an important role in innate immunity [266] .

The four major surfactant proteins SP-A, SP-B, SP-C, and SP-D called collectins are involved in the innate immunity against pathogens such as viruses, fungi and bacteria. These collectins recognize the carbohydrate domain (CRD) [269] and bind to carbohydrate moieties on the surface of pathogens such as bacteria and fungi. Surfactant protein D binds to both Gram-positive and Gram-negative bacteria and viruses. Collectins also recognize the mannan like high-mannose structures on the surface of fungi[270] such as *A. fumigatus, C. albicans and Pneumocystis* and LPS present on gram negative bacteria.

1.4.1. Dysregulated innate immunity and chronic sinus infection

Increased mucociliary flow is an important nonspecific defense mechanism used by the sinonasal epithelium as a response to airborne irritants. Impaired mucociliary function leads to stasis of mucus, giving an opportunity for inhaled organisms to grow within the sinonasal cavities, thus enhancing infectious inflammation [271]. Loss of mucosal homeostasis can occur due to either underactivity or overactivity of innate immune processes. Any imbalance in the interaction between the host and environment will stimulate an inflammatory response. The ability of sinonasal epithelial cells to alter and adapt their mucociliary function to environmental stimuli may be impaired in CRS[271]. It is difficult to determine whether the role of bacteria in CRS pathogenesis is one of cause or effect. Bacterial biofilms are complex structures providing bacteria with resistance to systemic and topical antibiotics [272]. The presence of bacterial biofilms provokes a chronic inflammatory reaction. Studies have highlighted that the level of lactoferrin in nasal secretions was observed to be decreased in CRS patients with paranasal sinus biofilms[273]. As TLRs play an important role in innate immunity and activation of adaptive immunity, any alteration in expression of TLRs are associated with refractory CRS[274]. Studies have reported that TLR2, TLR4, and TLR7 messenger RNA (mRNA) and protein levels are lower in CRSsNP than controls[275]. A different class of pathogen recognition receptors (PRR), the taste receptors, has been described to be expressed by nasal solitary chemosensory cells and to modulate epithelial cell innate immune activity[276]. Dysregulation of these taste receptors in CRS has been suggested but not demonstrated yet. Downregulation of PRRs or their upregulation implies either

dysfunction or dysregulation of innate immunity that could contribute to the pathogenesis of CRS[277]. Decreased production and expression of the peptides of the innate immune system causes recurrent infections while hyperactivity of the same leads to persistent damaging inflammation. Elevated levels of antimicrobial peptides serve as the driving force of inflammation in CRS trying to eliminate infection [278].

1.5. Adaptive Immunity in Chronic Rhinosinusitis

In contrast to innate immunity, adaptive immunity reflects a specific and long-lasting reaction to the presence of antigens. Innate immunity plays an important role in priming and modulating the adaptive immune response by stimulating cytokine production. These cytokines in turn drive inflammatory responses and promote T-cell differentiation. The innate and adaptive immune system highly depend on each other and are linked intricately. The major cell type of the adaptive immune system, the lymphocyte, gets activated upon binding of the antigen to specific receptors and the release of chemokines and cytokines. These chemokines in turn recruit leukocytes and modulate inflammatory processes[279] (Figure 1.6). Based on the pattern of cytokine secretion, T cells are divided as “Th1” or “Th2” phenotype. Th1 cells secrete interferon-gamma (IFN-γ) and tumor necrosis factor α (TNF-α), which in turn activate macrophages and cytotoxic T lymphocytes [280]. IL-4, IL-5, IL-9, and IL-13 are the cytokines secreted by Th2 cells. Th1, Th2, and Th17 T cell types produce the dominantly expressed cytokines of the adaptive immune response, however, in CRS

the inflammatory process demonstrates Th2 skewing with eosinophilia as a feature of CRSwNP. A study by Jyonouchi et al has reported increased levels of IFN-γ , IL-5, IL-8, IL-10, and IL-18 from sinonasal lavage in treatment- resistant CRS patients[281]. Evidence from the sinusitis literature supports that interleukin-13 is a central mediator in eosinophilic inflammation[282]. Elevated levels of IL-13 are seen in sinus lavages of patients with sinusitis as compared to controls, while greater amounts of IL-13 mRNA-positive cells were found in the sinus epithelium of CRS patients[283]. Regulatory T-cell (Treg) dysfunction has been reported in CRS; however, its role in CRS remains unclear[284]. Increased levels of B-cells, chemotactic factors and cytokines are seen in nasal polyps compared to controls and CRSsNP[285]. An understanding of the different inflammatory patterns within the tissue and sinuses of CRS patients may help to pave the way to specifically target inflammatory pathways that contribute to disease pathogenesis in CRS.

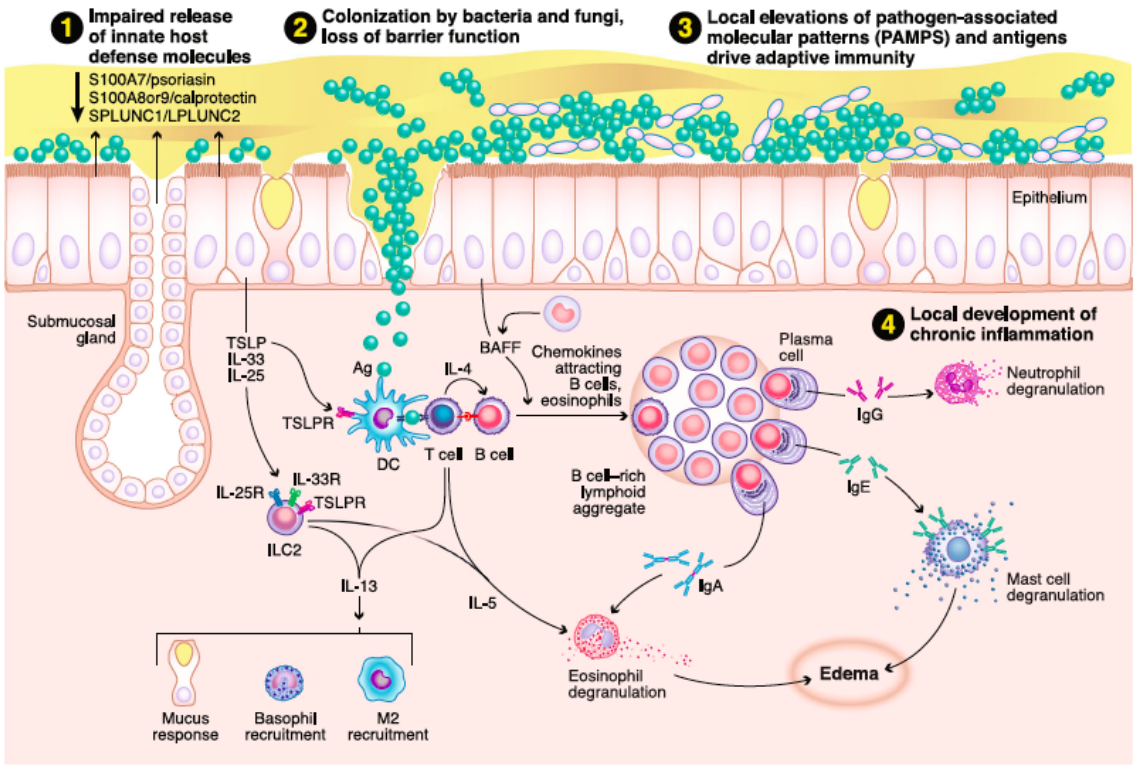

Figure 1.6: Mechanisms of inflammation associated with CRS. Reprinted with permission[279]

1.6. Antibiotic Resistance in Staphylococcus aureus

Antibiotic resistance is a rapidly evolving health issue that has emerged as a prominent global health concern in the 21st century. The evolution of antimicrobial resistance among bacterial species stems from various factors that include inappropriate use of antimicrobial agents. Inadequate dosages and extensive use of these agents to feed animals to enhance their growth has led to the relative ease of spread of antimicrobial-resistant bacteria [286-288].

Infections due to *Staphylococcus aureus* are of major clinical concern in patients and are often associated with increasing burden of anti-microbial resistance to multiple antibiotics (Figure 1.7). Hospital acquired and community acquired infections due to *Staphylococcus aureus* are a substantial burden in terms of healthcare costs, morbidity and mortality [289,290]. Penicillin, the first antibiotic introduced to treat staphylococcal infection was promising till 1942, when resistance to penicillin was identified in hospitals and later in the community[291]. Inactivation of penicillin by strains of *S. aureus* was identified by Kirby[292] , and the role of penicillinase seen in more than 90% of staphylococcal isolates was reported by Bondi and Dietz[293]. Penicillin resistance is mainly spread by resistant strains that process the gene for β -lactamase which forms a part of a transposable element present on a plasmid, often accompanied with additional antimicrobial resistance genes for antibiotics such as erythromycin and gentamicin. The gene blaZ encoding β-lactamase plays an important role in resistance of *S. aureus* to penicillin. The enzyme β-lactamase is synthesized on exposure to β-lactam antibiotics, it hydrolyses their β-lactam ring thereby making them inactive.

The regulatory genes antirepressor blaR1 and the repressor blaI control the expression of blaZ [294]. Studies have demonstrated that cleavage of these proteins BlaR1 and BlaI is essential for the synthesis of β-lactamase[295,296]. BlaR1, a transmembrane sensor-transducer cleaves by itself on exposure to β-lactams. These cleaved proteins then act as a protease and cleave the repressor BlaI, in turn allowing blaZ to proceed with the synthesis of the enzyme [297]. Methicillin was the first

of the semisynthetic penicillinase resistant penicillins that was introduced in 1961. The methicillin-resistant *S. aureus* (MRSA) strain was identified in the UK after two years of introduction of methicillin[298,299]. Clinically, the spread of these methicillin-resistant strains has been a critical one because outcome of infections due to MRSA was considered to be worse than those from methicillin-sensitive strains[300]. MRSA was detected in hospital settings [301], but now is increasingly seen in the community[302], reported in patients from both urban and rural settings[303-305]. CA-MRSA strains are generally more susceptible to antimicrobial agents compared to HA-MRSA, but remain more virulent due to the presence of virulence genes such as the Panton-Valentine leucocidin or enterotoxins, and have higher mortality rates [306-308].

The mecA gene (causing methicillin resistance) reported by Katayama et al[309] forms part of a staphylococcal cassette chromosome mec (SCCmec). Four different SCCmec elements varying in size from 21 to 67 kb have been characterized. The upsurge of community-acquired MRSA infections reported in patients from different countries was associated with the detection of a novel and unique SCCmec type IV [310]. This element is also identified in a range of MSSA genetic backgrounds, which suggests that it is more often transferred from other staphylococcal species[311]. Resistance to methicillin needs the presence of the chromosomally localized mecA gene[312] which is essential for the synthesis of penicillin-binding protein 2a (PBP2a; also called PBP2′) . Penicillin-binding proteins (PBPs) are enzymes that enable transpeptidation, a reaction needed for cross-linkage of peptidoglycan chains [313].

PBP2a due to low affinity for all β-lactam antibiotics allow the survival of staphylococci to high doses of β-lactam agents. Resistance to methicillin includes resistance to all β-lactam agents, including cephalosporins.

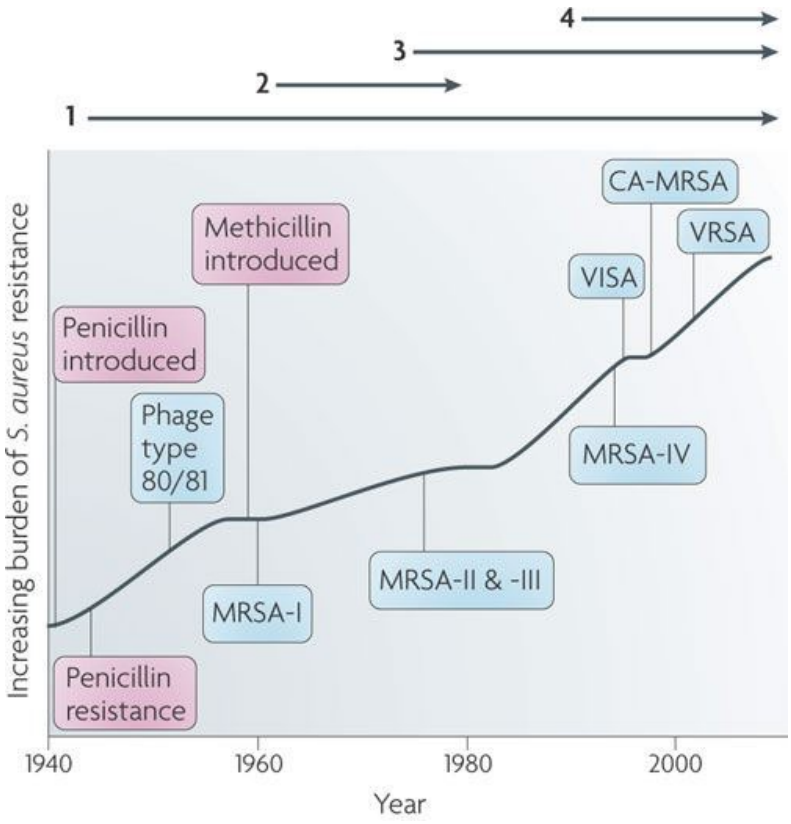

Figure 1.7: Antibiotic resistance in Staphylococcus aureus. Reprinted with permission[314]

Waves of clonal dissemination of methicillin resistant *S. aureus* (MRSA) strains spread rapidly across the world, accounting for different proportions of nosocomial *S. aureus* infections reported from different countries[315].

MRSA prevalence varies from > 40% in Greece, the UK and Italy, while < 1% in northern Europe (Sweden, Denmark and The Netherlands). This indicates that there is 100-fold difference in the prevalence of MRSA proportions[316] reported across the countries. The prevalence rates of CA-MRSA reported by a meta-analysis shows 0.2% MRSA colonisation rates in community members with no contact to healthcare workers, suggesting that these reservoirs of CA-MRSA could continue to expand which might lead to endemicity[317]. Outbreaks of MRSA within the hospitals and their emergence in various regions are due to dissemination of clonal strains typically due to clonal expansion[315]. The spread of MRSA strains can be prevented by adhering to good infection control protocols such as hand hygiene, isolation practices and rational antimicrobial prescription policies[318].

Fluoroquinolones were introduced to treat Gram-negative bacterial infections in the 1980s. Due to their Gram-positive bacterial coverage they are appropriate to treat infections caused by pneumococci and staphylococci also. The fluoroquinolones inhibit DNA synthesis, by binding to the enzyme-DNA complex they stabilize DNA strand breaks created by DNA gyrase and topoisomerase IV. The quinolone resistance–determining region (QRDR) is a critical region of the enzyme- DNA complex, any changes in the amino acids of this region leads to decrease in the affinity of its targets[319,320]. Accumulation of resistance mutations in the QRDR sites

can lead to development of resistance mutations. In addition, induction of the NorA efflux pump is another mechanism of resistance in *S. aureus* [321]. Bisognano et al have reported that fibronectin-binding protein expression is increased when a quinolone-resistant isolate is exposed to a quinolone, suggesting the linkage of antimicrobial resistance to virulence of the organism[322]. The newer fluoroquinolones (moxifloxacin) remain active *in vitro* against ciprofloxacin-resistant staphylococci [323], however Entenza et al [324] has reported the differences in their *in vitro* susceptibility and *in vivo* therapeutic efficiency. Increase in use of vancomycin to treat infections caused by methicillin resistant staphylococci has led to the emergence of vancomycin-resistant staphylococci[325]. Resistance to vancomycin by staphylococci was first identified in a clinical strain of *Staphylococcus haemolyticus*, while vancomycin intermediate-resistant *S. aureus* (VISA) was first reported in Japan in 1997[326]. VISA strains are reported to have minimum inhibition concentration (MIC) for vancomycin ranging from of 8–16 μ g/ml, but pre-VISA resistance (heteroresistant) strains even though they remain susceptible, do contain resistant subpopulations within them [327]. Resistance in VISA strains occurs due to alterations in peptidoglycan synthesis, while VRSA acquire resistance by plasmids containing the vanA operon acquired from Enterococcus faecalis (vancomycin-resistant)[328] leading to alteration of cell wall terminal peptide D-alanyl-D-alanine to D-alanyl-D-lactate [329,330]. Quinupristin-dalfopristin and linezolid belong to the newer antimicrobial agents against VISA and VRSA strains in vitro; both cover Gram-positive spectrum by inhibiting protein synthesis[331].

Prevention of spread of antimicrobial resistance among S. *aureus* isolates is by application of strict infection control practices[332]. Isolation of patients colonised with

S. aureus allows the containment of these infections and enables to limit their spread. Nasal carriage of *S. aureus* increases the risk of subsequent infection hence the use of topical antimicrobials eliminates carriage, thereby decreasing infection rates[333-335]. Novel agents, such as endopeptidases, lysostaphin, or phage lytic enzymes have been considered effective to prevent nasal carriage[336-338]. The multidrug resistant nature of *S. aureus* is only one example for diminishing efficacy of antimicrobial agents required for treatment of infections due to these organisms. The rise in antimicrobial resistance is alarming for *S. aureus* due to diversity of infections caused by this pathogen. It is hoped that new classes of antibiotics will help to overcome the threat posed by the spread of *S. aureus* strains that are resistant to older agents.

1.6.1. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing (AST) is used clinically to detect antibiotic sensitivity profiles of bacterial isolates. This guides antibiotic treatment decisions and predicts treatment outcomes[339]. The role of a clinical microbiology laboratory is to perform antimicrobial susceptibility testing of bacterial isolates to detect resistance patterns and provide susceptibility patterns for treatment of infections. Most commonly used methods include microbroth dilution, disk diffusion, gradient diffusion

method and automated methods. As the test results obtained from all the above described methods vary widely under different test conditions, the procedures used for testing should be standardized for intra- and inter-laboratory reproducibility. Two established organizations and committees, the Clinical Laboratory Standards Institute (CLSI) [340]and the European Committee on Antimicrobial Susceptibility Testing (EUCAST)[341] have laid guidelines for AST testing procedures. The MIC interpretation of susceptible and resistant was based on the breakpoints and definitions as per CLSI [340]and EUCAST[341]. The CLSI is an interdisciplinary, international organization which promotes accuracy and reproducibility of antimicrobial susceptibility testing (AST), appropriate reporting by developing reference methods, criteria for interpretation and establishing quality control for reporting. The CLSI is approved by FDA-USA and recommended by WHO. According to their protocols to produce accurate and reproducible results, factors that control the results should be kept in mind and followed accurately. Only media formulations such as Mueller-Hinton medium tested according to and those that meet the acceptance limits should be used. The Mueller-Hinton medium shows acceptable batch-to-batch reproducibility, it is low in sulphanamide, trimethoprim, and tetracycline inhibitors, it gives satisfactory growth of most non fastidious pathogens hence it is ideal for performing AST testing.

Other factors that are crucial while performing susceptibility testing include pH, moisture, effects of thymidine or thymine and effects of variation in divalent cations. The pH of each batch of Mueller-Hinton agar needs to be checked; agar medium

should have a pH between 7.2 and 7.4 at room temperature. If the pH is too low, certain drugs will appear to lose potency (e.g., aminoglycosides, macrolides and quinolones,), while other agents like tetracyclines may appear to have excessive activity; if the pH is too high, the opposite effects can be expected. Media containing excessive amounts of thymidine or thymine can reverse the inhibitory effect of sulphonamides and trimethoprim[342], thus yielding smaller and less distinct zones resulting in false-resistance reports. Variation in divalent cations, such as magnesium and calcium affect the interpretation of aminoglycoside and tetracycline tests with *P. aeruginosa* strains[343]. Excessive cation content will reduce zone sizes, low cation content may result in unacceptably large zones of inhibition, while excess zinc ions may reduce zone sizes of carbapenems. If protocols are implemented accurately according to standard procedures described, AST test results can be comparable between different laboratories.

1.6.1.1. Broth dilution tests

The tube dilution and microbroth dilution methods were the earliest methods used for the detection of anti-microbial susceptibilities of microorganisms in doubling dilutions. Tube dilution is a simple method for testing single or few isolates; the advantage of using this method is the generation of quantitative results, and these tubes can be further used for minimum bactericidal tests as well. It also has better reproducibility and uses smaller amounts of reagents due to miniaturization of the tests. The use of micro trays with a final volume of 0.1 ml has made broth dilution methods more popular and practical. The disadvantages of the microdilution method include its

tedious nature, preparation of antibiotics for each test manually and errors that occur during preparation[344]. The minimum inhibitory concentration (MIC) test can be performed by broth microdilution method using disposable microdilution trays which contain 96 wells to allow minimal volume (0.1 mL) and results may be determined visually or by automated instruments[345].

The minimum inhibitory concentration (MIC) is defined as the lowest concentration of antibiotic required to inhibit the growth of bacteria. This determines the susceptibility of the organism to the antimicrobial agent used[344]. Absence of growth in MIC does not imply death of bacteria alone but lack of growth, thus, Minimum Bactericidal Concentration (MBC) becomes useful when the tube with no growth is sub-cultured to detect the actual presence of viable bacteria. Interpretation of AST to categorize[346] isolates as susceptible, intermediate, or resistant are based on breakpoints set and updated by national organizations such as the Clinical Laboratory Standards Institute [340](CLSI) in the USA and the European Committee[341] on Antimicrobial Susceptibility Testing. MIC values are important parameters to determine phenotypic resistance and also to monitor global resistance patterns. MIC values are considered to be gold standard to assess and evaluate other AST techniques.

Antimicrobial gradient method uses the principle of dilution and diffusion of antimicrobial agent in a concentration gradient in an agar medium to detect susceptibility. This quantitative method applies both the diffusion and dilution of the antibiotic into the medium. The E-test (bioMe´rieux AB BIODISK) is commercially available, it consists of a predefined antimicrobial gradient on an insert carrier strip.

A minimum of 5 strips can be placed in a 150 mm agar plate in a radial arrangement. Epsilometer test (E-test) can be used to determine MIC for fastidious organisms such as *N. gonorrhoea, Haemophilus spp, S. pneumoniae* and β- hemolytic Streptococci. E-test detects antimicrobial susceptibility of Non- fermenting Gram Negative bacilli (NFGNB) such as *Pseudomonas spp and Burkholderia pseudomallei* as well as glycopeptide resistant Enterococci (GRE) and glycopeptide intermediate *S. aureus* (GISA). The drug impregnated on the E-strip is released when it comes in contact with the agar following which a symmetrical inhibition ellipse is produced. The intersection of the zone of inhibition and the calibrated carrier strip indicates MIC value (> 10 dilutions). In general E-test results correlate well with MICs from agar or broth dilution methods. However, for some organisms, the MIC by broth dilution methods does not correlate with the MICs from E-test which represents a potential short coming [347].

1.6.1.2. Disk diffusion test

The Stokes and Kirby-Bauer methods are used for antimicrobial susceptibility testing recommended by CLSI. This is a simple and practical method that uses bacterial inoculum of 1-2 x 10^8 CFU/ml on a plate of (150 mm diameter) Mueller-Hinton agar plate [348]. A minimum of 12 discs commercially prepared antibiotics of fixed concentration on paper discs are placed on agar inoculated with bacterial culture and incubated for 16-18 h at 37°C. Growth inhibition as a zone around the disc is measured and interpreted using the CLSI guidelines. Disc diffusion method is simple

and easy to use but lacks mechanization or automation. This method has been adopted for testing fastidious organisms such as *Streptococci, Hemophilus influenzae* by using specialized media and specific zone sizes.

1.6.1.3. Automated instrument systems

FDA has approved the use of three automated instruments that generate rapid antimicrobial susceptibility results (3.5-16 h) and one that generates overnight results. The Micro Scan Walk Away (Siemens Healthcare Diagnostics) is a device with an incubator for analysis of 40-96 microdilution trays. The Walk Away incubates manually inoculated trays for required time and examines periodically. It has separate gram positive and negative panels that read within 3.5-7 h and 4.5-18 h respectively.

The BD Phoenix Automated Microbiology System (BD Diagnostics) uses both turbidimetric and colorimetric growth reduction. It tests for both gram-negative and gram-positive organisms generating MIC in 6-16 hrs. The Vitek 2 System (bioMe´rieux) is compact, contains reagent cards that have antibiotics and test media in 64 wells, employs turbidimetric monitoring for rapidly growing gram positive and gram-negative aerobic bacteria and *S. pneumoniae* within 4-10 h. The Phoenix, Sensititre ARIS 2X, Vitek 1 and 2, and Walk Away instruments use enhanced computer software for determination of susceptibility patterns[349].

The use of rapid methods to detect susceptibility of organisms enhances rapid identification and appropriate antimicrobial treatment and shortens hospital stay,

however limitations include decreased ability to detect inducible β-lactamases and vancomycin resistance. Emerging mechanisms of resistance require constant vigilance with regard to the ability of these methods to detect resistance.

1.6.2. Antimicrobial resistance in bacterial biofilms

Resistance mechanisms in bacteria forming biofilms are mainly due to mutations, modifying enzymes and efflux pumps [350]. Decreased and delayed penetration of the antibiotic into the biofilm is the most common hypothesis that explains the resistance mechanism involved among biofilm forms. Deactivation of antibiotic in biofilm leads to retardation in the rate of diffusion as seen with ampicillin that penetrates through biofilm of β-lactamase-negative strain of *K. pneumoniae* not seen with β-lactamase-positive wild type strain[351]. The second hypothesis includes alteration in the microenvironment within the biofilm. Studies [352] have shown that oxygen depletion in the superficial layers can lead to anaerobic pockets in the deep layers leading to concentration gradients in metabolic substrates. Reduction in pH[353] and accumulation of toxic waste metabolites antagonize the effect of the antibiotic and avoid killing of bacteria present in a non- growing state and thereby leading to osmotic stress responses. The third mechanism speculates that altered phenotypic changes within a subpopulation of microorganisms within the biofilms forming a spore like state that resist treatment with antibiotics and disinfectants [354].

The multicellular nature of biofilms remains as the most common cause for biofilm resistance[355] by collectively resisting antibiotic treatments that would kill a single cell.

Strategies to disrupt the multicellular nature of biofilms serve as new approaches [356]for treatment of biofilms. Specific treatments include enzymes that solubilize the matrix polymers of biofilm, those that block matrix synthesis and those that stop cell to cell communication [357].

1.6.3. Antimicrobial susceptibility testing in S. aureus biofilm

Biofilms are recognized as most relevant drivers of persistent infection leading to inflammation. In vitro antimicrobial susceptibility tests (ASTs) performed in clinical laboratories provide the breakpoints for antimicrobial therapy, however these are not appropriate for the treatment of biofilm forming bacteria [358,359]. Studies have suggested that a minimum biofilm eradication concentration (MBEC) could be better indicators of the antibiotic concentration needed to eliminate bacteria in biofilm forms[360].

In addition to determining the MBEC other tests to study the biomass can also be performed[361]. Several in vitro models to evaluate antimicrobial activity on biofilms

have been implemented over the past few years. Based on the nutrient delivery system used they are classified as closed (batch culture) and open systems (continuous culture) [362]. Closed systems include the microtitre plate method and the Calgary biofilm device model, while the open systems include flow cell models and the CDC biofilm reactor model. Closed models are simple and applicable in high-

throughput analysis, whereas open models demonstrate better control of growth parameters and dynamics.

The microtitre plate (96-well plate) is a simple quantitative assay; wells are inoculated with sterile media and bacteria, different concentrations of test antibiotic agents are added at various concentrations over the biofilms to determine their ability to prevent or eliminate biofilms [363]. Quantification of biofilm production could be obtained by performing crystal violet staining and biofilm viability can be determined by the activity of resazurin dye[364,365]. The Calgary biofilm device method is another example of a closed system that grows biofilms on pegs suspended from the lid of a microtitre plate and incubated at 37°C[366]; this system allows incubation along with antibiotics [367]. The biofilm structure can be studied by using the scanning electron microscopy or the confocal laser scanning microscopy (CLSM) after staining the biofilms with appropriate fluorophores[368].

The Open systems models used for biofilm culture replicate the *in vivo* conditions through the controlled delivery of nutrients, flow, and temperature. The advantage of using this method is that the biofilms effects can be studied without the hindrance of planktonic cells as they are eliminated by the flow. Moreover, these systems make possible the implementation of pharmacokinetic/pharmacodynamic (PK/PD) models, as well as allowing observation by microscopy. The flow cell system has been indicated to be the best approach for modelling biofilm formation as real-time imagining can be performed using CLSM[369]. Biofilms formed in this model are thicker, however, its time-consuming nature remains a constraint [370]. Compared to

the flow cell system the CDC biofilm reactor (open system) model is a robust culture method with high reproducibility in which biofilms can be grown for various analyses. Biofilms are allowed to develop on coupons suspended from the lid that is immersed in growth medium and at the same time different antimicrobial agents can be tested by adding them into the fluid phase and exposing the coupons. The biofilm structure can be observed with CLSM by staining the coupons[371,372].

Similar to the conventional MIC performed for planktonic bacteria, various pharmacodynamic parameters are being used to quantify the biofilm-growing bacteria. Of these the minimal biofilm inhibitory concentration (MBIC), was first defined by Moskowitz et al [373] using the Calgary device, as the lowest concentration of drug that resulted in an OD650 nm difference of ≤10% of the mean of two positive control well readings. Another parameter commonly used is the minimal biofilm-eradication concentration (MBEC), defined as the lowest concentration of antimicrobial agent that prevents visible growth[374]. All these pharmacodynamic parameters quantify the antimicrobial activity in biofilm-growing bacteria, however, these are not standardised guidelines to treat clinical infections. Hence standardized guidelines to use of biofilm susceptibility endpoint parameters is the need of the hour to improve the clinical validity of future anti-biofilm assays.

1.6.4. S. aureus biofilm-associated infections, detection and treatment

S. aureus biofilms play an important role in infections among the immunocompromised causing diseases such as skin and soft tissues infections, osteomyelitis, endocarditis, cystic fibrosis and implant infections[375]. Colonization of staphylococcal biofilms on prosthetic and implanted devices plays a key role in leading to chronicity of infections. Staphylococcal biofilm can develop on various structures such as catheters, prosthetic joints, cerebrospinal fluid shunts and pacemakers. These implanted devices coated with host proteins enhance and facilitate the attachment of *S. aureus* biofilms.

S. aureus biofilm-associated infections remain resistant to antibiotics. Various studies [376] have stated that decreased penetration, accumulation of antibiotic-degrading enzymes, slow growers and persister cells[377] contribute to this resistance. The mechanisms involved are mainly altered gene expression, nutrient starvation and horizontal gene transfer or mutations that favor antimicrobial resistance[378].

Treatment of *S. aureus* biofilm infections remains a therapeutic challenge in cases of formed and mature biofilms as described by Ceri et al. The MBEC for *S. aureus* biofilms remains to be 10–1000 times higher compared to planktonic bacteria[374]. This finding has been observed in *in vitro* studies of *S. aureus* biofilm models [379].

The use of antibiotics combinations seems to be promising for enhancing effective treatment. Parra-Ruiz et al[380] have indicated that combination therapy including daptomycin or moxifloxacin with clarithromycin is more efficient in treating methicillin susceptible *S. aureus* (MSSA) strains compared to individual treatments.

Biofilms can be removed by the use of saline or surfactants such as baby shampoo. A study by Chiu et al showed that the use of 1 % baby shampoo[381] post-ESS caused 60% reduction in the symptoms of post nasal discharge. In a sheep study the use of hydrodebrider[382] system that is used along with saline was efficacious in the treatment of biofilms due to *S. aureus.* Colloidal silver suspended in water shows the ability to decrease *S. aureus* biofilms in vitro[383]. The use of enzymes such as deoxyribonuclease, alginate lyase and dispersin B act on the biofilm matrix and cause their dispersion[384]. Dispersin B targets the biofilm matrix polysaccharide poly-N-acetylglucosamine (PNAG) and degrades it preventing interaction with bacterial proteins[385].

Prevention is a better option compared to treatment strategies (Figure 1.8), hence, detection methods such as quantitative polymerase chain reaction (qPCR) techniques to detect the ica genes encoding Polysaccharide intercellular adhesin (PIA) enables early detection of biofilms. However, the ica operon is not present in all strains of *S. aureus*, hence cannot be used as a universal biomarker [386]. The use of non-invasive diagnostic tests of PNAG detection for *S. aureus* biofilm were comparable with results obtained from both culture methods and fluorescence in situ hybridization (FISH) of CRS patients[387]. The Quantitative analysis of bacterial

biofilms by BacLight™ Live/Dead® staining and image analysis could enhance early detection and confirmation of biofilm-positive disease[388].

The main therapeutic strategies to eliminate biofilms include disruption of quorum sensing, mechanical disruption and the use of biofilm matrix degrading enzymes[389]. These methods target biofilms and unseal the planktonic forms to increase susceptibility to antibiotics. Disruption of signaling molecules acyl-homoserine lactone in *P aeruginosa* increases the susceptibility to sodium dodecylsulphate[390]. Neutralizing antibodies[391] could be used to reduce opportunities for initial attachments as adhesins. The use of topical antibiotics to deliver higher concentrations of antibiotics has been studied by Ha et al[392]. *S. aureus* biofilms treated with high concentration of topical antimicrobials showed that topical mupirocin reduced *S. aureus* biofilm mass by 90% in comparison to ciprofloxacin and vancomycin that were ineffective. Singhal et al reports, that the use of novel non-antibiotic antimicrobial agents such as N, N-dichloro-2,2-dimethyltaurine (NVC-422) decreased *S. aureus* biofilms compared to control patients [393]. Sheep models of CRS showed decrease in biofilm biomass when manuka honey containing methylglyoxal [394] (MGO) was used in comparison to saline irrigations.

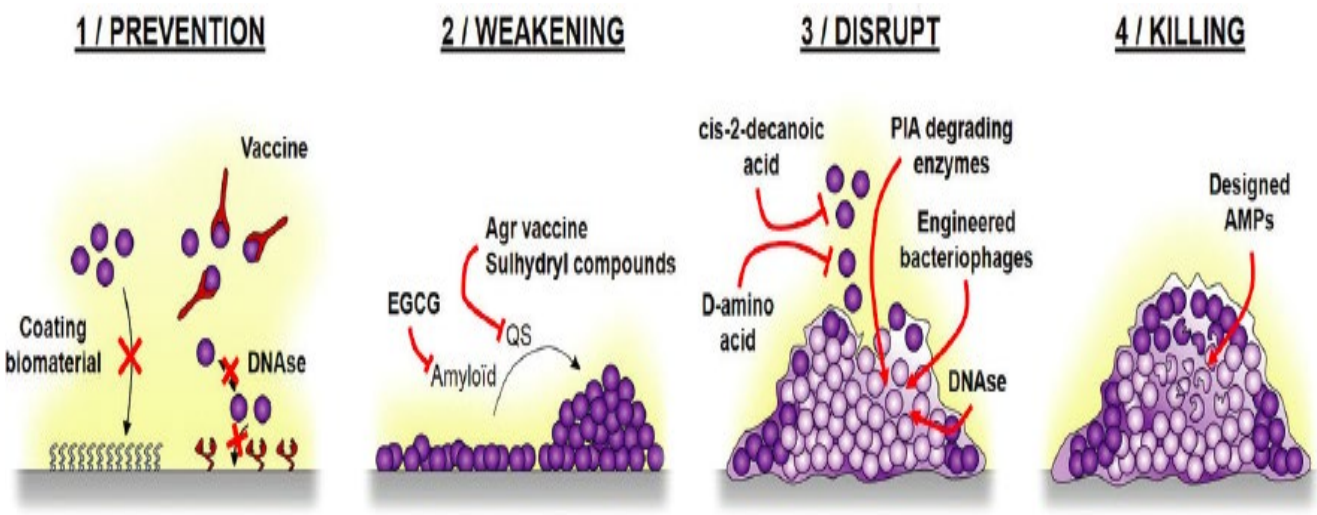

Figure 1. 8 :Strategies "anti-S. aureus to eliminate biofilms" [395].

Adjunctive or parallel approaches are necessary to circumvent the potential problem of antibiotic-resistance due to biofilms. Further research is necessary however, to see the success of these treatments and validation of these approaches to different types of clinical infections caused by *S. aureus* biofilms.

1.7. Summary of Literature Review

Chronic rhinosinusitis (CRS) is a heterogeneous inflammatory disease of multifactorial aetiology and affects the nasal and the paranasal sinuses leading to mucociliary dysfunction. A spectrum of factors such as defects in innate immunity, aberrant host responses, allergy, barrier dysfunction, biofilm formation and microbial invasion have been attributed in the etiopathogenesis of this disease, yet the direct evidence of how the role of bacteria, in particular biofilms breaks through the nasal epithelial barrier and cause CRS needs to be unravelled.

Infectious aetiology caused by bacteria is believed to be the most plausible influence on the inflammatory process in different ways. These include adherence and invasion into host cells enabling colonisation and infection, directly influencing and driving the inflammatory process or affecting the mucosal barrier structure and function. In CRS patients, however, the way this happens on the cellular level is a subject to be investigated. It is now well established that *S. aureus* is the commonest of the colonisers in sino-nasal cavities that cause CRS. *S. aureus* in their way towards gaining supremacy in the microbiome of the sino-nasal cavity undergoes phenotype switching into small-colony variants and biofilms and are directly linked to disease recalcitrance.

Bacterial products such as toxins and exoproteins secreted from different pathogens including *S. aureus* have been shown to negatively affect mucosal barrier structure and function. Hyperpermeability of the epithelial barrier is thought to allow

submucosal penetration of antigens leading to an exacerbated immune response. This being the premise of the immune barrier hypothesis as a cause of CRS. Despite the evidence that *S. aureus* is the most common organism in CRS, that *S. aureus* biofilms are associated with CRS disease recalcitrance, the effect of biofilm exoproteins on the barrier structure and function and how that compares to exoproteins from planktonic forms is unclear and yet to be explored.

Hence, we address these questions, in our first study aiming to determine the effects of *S. aureus* exoproteins from matched biofilm and planktonic clinical isolates from CRS patients on the nasal epithelial barrier.

S. aureus is at the core of the sinonasal tract microbiome, along with *Corynebacterium* spp, *Moraxella* spp, *Streptococcus* spp and *Haemophilus* spp. Interestingly, *S. aureus* and *P. aeruginosa* are the most commonly cultured pathogenic organisms in patients with CRS. This suggests that *S. aureus* in the sinuses use various mechanisms to remain stealthy in the biofilms, and intracellularly in the form of Small Colony Variants (SCV). They also switch to a slow growth pathogenic mode by reduced expression of “exoproteins” such as α-haemolysin (α-toxin) and elevated levels of adhesins such as IgG-binding protein Protein-A and Fibronectin binding protein-A (FnbA), this is alluded to lead to recurrent and persistent inflammation in CRS.

Exoproteins are not only harmful to the nasal epithelium but also trigger a host of immune response activity along the barrier which further accentuates the inflammatory process. *S. aureus* has traditionally been thought to affect its host by its

secretions / exoproteins and the interaction of its surface proteins with the host barriers. The exoproteins are in the form of enzymes and toxins, but the exact nature of this interaction in CRS has not been investigated. The inflammatory response in a host to the presence of biofilms, especially in a clinical scenario, is an evolving concept. Studies have shown that biofilms associated with defective epithelium have raised counts of immune cells, however direct causation is yet to be determined. The relationship between the bacterial biofilm and the host inflammatory response remains to be elucidated.

To date, no primary defects in epithelial tight junction proteins have been described in patients with CRS or in nasal polyps driven by infection and how much by inflammation still remains subject of investigation. Although ample studies have demonstrated that nasal mucosal immunity is altered due to the presence of S. *aureus* biofilms, the subsequent cytokine response needs to be elucidated, the relationship between biofilms, tertiary lymphoid organs (TLOs) and inflammation in CRS needs investigation.

Hence, we attempt to answer these questions by investigating the relation between exoproteins secreted in planktonic form and biofilms and their association with inflammation in CRS in our second study.

Biofilms exoproteins are implicated to induce ongoing inflammation and negatively affect treatment outcomes in patients with CRS. Planktonic forms differ from biofilm forms within the same bacterial species due to differences in their phenotypic and genotypic expression. Biofilm grown organisms resist susceptibility to antibiotics

when their planktonic cultures of the same remain susceptible. Biofilm producing bacteria cause recurrent nosocomial infections leading to prolonged hospital stays, thereby increasing morbidity and mortality. Hence elimination of biofilms is crucial for eradicating disease in recalcitrant CRS (rCRS). Minimum inhibitory concentration (MIC) a standard method for antibiotic susceptibility testing for planktonic organisms remains an important reference for treatment of infections, however application of MIC to treat chronic infections due to biofilms forms is ineffective. The concentrations of antibiotics used in standard anti-microbial susceptibility testing for planktonic forms do not predict the concentrations needed to eradicate biofilms. Minimum biofilm eradication concentration (MBEC) are better predictors of antibiotic concentrations required for eradication of biofilms. Hence, in our last study we aimed to determine the susceptibility profile of *S. aureus* clinical isolates from patients with rCRS to commonly used antibacterial agents grown in planktonic and in biofilm forms.

1.8. Studies to be Conducted

Project 1: Hypothesis; *Staphylococcus aureus* produces exoproteins that affect the mucosal epithelial barrier structure and function in chronic sinusitis which is related to disease severity.

Aim: To determine the relationship between the effects of *Staphylococcus aureus* exoproteins from planktonic form and as biofilms on the nasal epithelial barrier in relation to severity of disease.

Objective; To Correlate the effects of Staphylococcus aureus exoproteins on the nasal epithelial barrier with symptom scores (SNOT 22), Lund-Mackay CT scores and inflammatory cell counts.

Project 2: Hypothesis; *Staphylococcus aureus* exoproteins affecting mucosal epithelial barrier are associated with inflammation in CRS.

Aim; To determine the relationship between *Staphylococcus aureus* exoproteins and inflammation in CRS.

Objective 1; To correlate *S. aureus* exoprotein-induced nasal barrier inflammation with symptom scores (SNOT 22), Lund-Mackay CT scores.

Objective 2; To investigate the association between *Staphylococcus aureus* exo-proteins and the host immune response.

Project 3: Hypothesis; *Staphylococcus aureus* biofilm resist treatment to antimicrobial agents compared to their planktonic counterparts in CRS and is related to disease severity.

Aim; To determine the relationship between antimicrobial resistance patterns among biofilm and planktonic forms in CRS in relation to severity of disease and exoprotein production.

Objective; To Correlate the resistance of *Staphylococcus aureus* in different phenotypes in CRS with disease severity by symptom scores (SNOT 22), Lund-Mackay CT scores and tissue Inflammation.

CHAPTER 2: Staphylococcus aureus biofilm exoproteins are cytotoxic to human nasal epithelial barrier in Chronic Rhinosinusitis

Conducted in the Department of Otolaryngology – Head and Neck Surgery

The University of Adelaide, Adelaide, Australia

Financial assistance provided by The University of Adelaide

Staphylococcus aureus biofilm exoproteins are cytotoxic to human nasal epithelial barrier in chronic rhinosinusitis

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Background: **Chronic rhinosinusitis patients (CRS) suffer from chronic inflammation of the sinus mucosa associated with chronic relapsing infections. Mucosal biofilms, associated with *Staphylococcus aureus*, have been implicated as a cause. We compared the effect of exoproteins secreted from clinical isolates of *S aureus* from CRS patients in planktonic and biofilm form on the nasal epithelial barrier.**

Methods: **Clinical *S aureus* isolates from 39 CRS patients were grown in planktonic and biofilm forms and their exoproteins concentrated. These were applied to primary human nasal epithelial cells grown at the air-liquid interface. Transepithelial electrical resistance, permeability of flourescein isothiocyanate-dextrans, and cytotoxicity were measured. Structure and expression of tight junctions zona occludens-1, and claudin-1 proteins were assessed by electron microscopy and immunofluorescence. The Wilcoxon signed rank test was used for statistical analyses.**

Results: ***S aureus*** **biofilm exoproteins showed dose- and time-dependent reduction of transepithelial electrical resistance, increased cell toxicity, and increased permeability ($p < 0.001$) compared with equal concentrations of planktonic cultures. Discontinuity in zona occludens-1 and claudin-1 immunofluorescence was confirmed as disrupted tight junctions on electron microscopy.**

Conclusion: ***S aureus*** **biofilm exoproteins disrupt the mucosal barrier structure in a time- and dose-dependent manner and are toxic. Damage to the mucosal barrier by *S aureus* biofilm exoproteins may play a major role in CRS etiopathogenesis.** © 2020 ARS-AAOA, LLC.

Key Words:
biofilm; chronic rhinosinusitis; claudin-1; mucosal barrier; *Staphylococcus aureus* exoproteins; tight junction; transepithelial electrical resistance; zona occludens-1

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Chronic rhinosinusitis (CRS) is defined as an inflammation of the mucosal lining of the nose and paranasal sinuses for >12 weeks.[1] The financial burden due to CRS, both from direct health expenditure and loss of productivity, is significant, as is its negative impact on quality of life.[2] *Staphylococcus aureus* is the most commonly isolated pathogenic bacteria in CRS patients[3–5]; however, *S aureus* sinonasal colonization can also occur in patients without CRS.[6,7] Phenotype switching in the small-colony variant form and the ability to form *S aureus*–dominant biofilms have nevertheless been linked to CRS disease recalcitrance.[8–10] It is thought that strain-specific differences in toxin and enzyme production could account for the variation in *S aureus* pathogenicity and type of inflammation.[11] Such virulence factors can influence the inflammatory process in different ways. These include allowing adherence and invasion into host cells enabling colonization and infection,[12,13] directly influencing and driving the inflammatory process,[11] or affecting the mucosal barrier structure and function.[14–16]

Biofilms enable the bacteria to survive within a self-produced matrix consisting of extracellular polymeric substances (EPS), consisting of proteins, polysaccharides, and extracellular DNA. These viscoelastic biopolymers

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***Staphylococcus aureus* biofilm exoproteins are cytotoxic to human nasal epithelial barrier in Chronic Rhinosinusitis**

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2.1 ABSTRACT

Background:

Chronic Rhinosinusitis (CRS) patients suffer from chronic inflammation of the sinus mucosa associated with chronic relapsing infections. Mucosal biofilms, associated with *Staphylococcus aureus,* have been implicated as a cause. We compared the effect of exoproteins secreted from clinical isolates of *S. aureus* from CRS patients in planktonic and biofilm form on the nasal epithelial barrier.

Methods:

Clinical *S. aureus* isolates from 39 CRS patients were grown in planktonic and biofilm forms and their exoproteins concentrated. These were applied to primary human nasal epithelial cells (HNECs) grown at Air Liquid Interface (ALI). Transepithelial Electrical Resistance (TEER), permeability of FITC-dextrans and cytotoxicity were measured. Structure and expression of tight junctions (TJs) Zona Occludens-1 (ZO-1) and claudin-1 proteins were assessed by electron microscopy (EM) and immunofluorescence (IF). Wilcoxon signed rank test was used for analysis.

Results:

S. aureus biofilm exoproteins showed dose- and time-dependent reduction of TEER, increased cell toxicity, and increased permeability ($P < 0.001$) compared to equal concentrations of planktonic cultures. Discontinuity in ZO-1 and claudin-1 immunofluorescence was confirmed as disrupted TJs on EM.

Conclusion:

S. aureus biofilm exoproteins disrupt the mucosal barrier structure in a time- and dose- dependent manner and are toxic. Damage to the mucosal barrier by *S. aureus* biofilm exoproteins might play an important role in CRS etiopathogenesis.

2.2 INTRODUCTION

Chronic rhinosinusitis (CRS) is defined as an inflammation of the mucosal lining of the nose and paranasal sinuses for more than 12 weeks[396]. The financial burden due to CRS, both from direct health expenditure and loss of productivity, is significant, as is the negative impact on quality of life[397]. *Staphylococcus aureus* is the most commonly isolated pathogenic bacteria in CRS patients [398-400], however, *S. aureus* sinonasal colonisation can also occur in patients that do not suffer from CRS [401,402]. Phenotype switching in the small-colony variant form and the ability to form *S. aureus* dominant biofilms have nevertheless been linked to CRS disease recalcitrance [31,215,403]. It is thought that strain-specific differences in toxin and enzyme production could account for variation in *S. aureus* pathogenicity and type of inflammation [404]. Such virulence factors can influence the inflammatory process in different ways. These include allowing adherence and invasion into host cells enabling colonisation and infection [405,160], directly influencing and driving the inflammatory process [404] or affecting the mucosal barrier structure and function [406-408].

Biofilms enable the bacteria to survive within a self-produced matrix consisting of extracellular polymeric substances (EPS), consisting of proteins, polysaccharides and extracellular DNA. These viscoelastic biopolymers facilitate attachment to inert or mucosal surfaces, enable intercellular communication and exchange of genetic information and shield the bacteria from a hostile environment [409]. This makes the bacteria within biofilms less susceptible to antibiotics than their planktonic forms. Bacteria within the biofilm differ from their planktonic counterparts phenotypically and genotypically[410,411] with enrichment of many proteins involved in the pathogenesis of inflammation in biofilm EPS [412].

Bacterial products from different pathogens including *S. aureus* have been shown to negatively affect mucosal barrier structure and function [406-408,413]. Tight junctions (TJ), cell adhesion complexes between epithelial cells composed of occludin, junction adhesion molecules (JAMs) and zonula occludens (ZO) proteins, are necessary to maintain normal epithelial barrier function [414] . Hyperpermeability of the epithelial barrier is thought to allow submucosal penetration of antigens leading to an exacerbated immune response [415,416]. This is the premise of the immune barrier hypothesis as a cause of CRS, and is also seen in other inflammatory conditions such as asthma, atopic dermatitis, inflammatory bowel disease and allergic rhinitis [417].

Despite the evidence that *S. aureus* is the most common organism in CRS [27,418], that *S. aureus* biofilms are associated with CRS disease recalcitrance [31,403,419] and that *S. aureus* secreted products negatively affect the mucosal barrier [406-408], the effect of biofilm exoproteins on the barrier structure and function and how that compares to

exoproteins from planktonic forms is unclear and yet to be explored. To address these questions, our study aims to determine the effects of *S. aureus* exoproteins from matched biofilm and planktonic clinical isolates from CRS patients on the nasal epithelial barrier.

2.3 METHODS

2.3.1. *S. aureus* clinical isolate collection and clinical parameters

The study was performed at The Queen Elizabeth Hospital (TQEH) in Adelaide, South Australia with ethics approval and written consent obtained prior to the study for the use of *S. aureus* clinical isolates (HREC/18/CALHN/69) and for the collection of primary human nasal epithelial cells (HNECs). *S. aureus* clinical isolates were obtained from patients with CRS at the time of endoscopic sinus surgery and isolated by an independent pathology laboratory (Adelaide Pathology Partners, Adelaide South Australia) and stored at - 80°C. CRS patients fulfilled the diagnostic criteria for CRS according to the recent position papers by the American Academy of Otolaryngology and Head and Neck Surgery and the European Position Statement (EPOS) [4] on CRS. Patients with CRS were further sub-classified according the absence (CRSsNP) or presence (CRSwNP) of nasal polyps as defined by EPOS guidelines[66]. Clinical data from the patients was collected prospectively including age, gender and history of asthma. Disease severity was measured based on preoperative patient reported Sino-Nasal Outcome Test (SNOT -22) questionnaires[420], the disease specific 5 questions-based Adelaide Severity score (ADS)[421] and the objective CT-scan scoring of Lund Mackay (LMS)[422] .

2.3.2. *Staphylococcus aureus* culture and collection of exoproteins

Clinical isolates of *Staphylococcus aureus* were streaked onto nutrient agar (Oxoid, UK) from glycerol stocks, cultured in nutrient broth (NB) and incubated on a shaking platform (180 rpm) at 37° C for 14-16 hours. The following day, fresh NB was inoculated to a starting optical density of 0.01 at 600 nm. Growth was followed by measuring optical density on an hourly basis. At 6 hours[423,424] (late exponential phase of growth) liquid cultures were centrifuged at 4 °C for 10 minutes at 1500 x g. Supernatants were removed and passed through a 0.22 µm syringe filter followed by passage through a 3 KDa filter to collect exoproteins (Pierce Protein Concentrator, Thermofisher, IL, USA) as described [425]. Exoproteins were stored at -80 ºC for further analysis. Biofilm cultures for the same clinical isolates were set up with NB in six well plates after adjusting the opacity to 1.0 ± 0.1 Mac Farland units and incubating at 37°C for 48 hours[426,427] on a rotating platform (3D Gyratory Mixer; Ratek Instruments, Boronia, Australia) at 70 rpm. Supernatants collected from the biofilm forms were centrifuged following the same protocol used for the planktonic forms.

2.3.3. Nano Orange Protein Assay

Nano Orange Protein Quantitation Kit (Molecular Probes, Eugene, Oregon, USA) was used for the protein quantification for both planktonic and biofilm forms in black-walled 96-well plates as per the manufacturer's instructions. Fluorescence was

measured using excitation at 485 nm and emission at 590 nm using the FLUOstar OPTIMA plate reader (BMG Labtech, Ortenberg, Germany).

2.3.4. Human Nasal Epithelial Cell Harvesting and Culture

Nasal brushings were collected during surgery from patients with CRS from the inferior turbinate using cytology brushes (McFarlane Medical Equipment, Pty Ltd, Surrey Hills, VIC, Australia) as described[428]. The nasal brushings were transported using PneumaCult™ Ex Plus Basal Medium (STEMCELL Technologies, Cambridge, UK). Processing of the cells from the brushes was performed after centrifuging the brushes at 300 g for 5 minutes at 4 ºC and suspending in 2 ml Pneuma Cult™ Ex Plus Basal Medium. The pellet was resuspended using a 10 ml syringe to create a single cell suspension into a petri dish containing anti-CD68 antibody (Dako, Carpinteria, CA, USA) to deplete the cell suspension from monocytes. HNECs were expanded on type 1 collagen -coated T25 flasks (Thermo Scientific, Walthman, MA, USA) and incubated at 37 ºC with 5 % CO_2 [429].

2.3.5. Air -Liquid Interface Culture

HNECs reaching 80- 90 % confluence were maintained at Air Liquid Interface (ALI) using the PneumaCult™ Ex Plus ALI culture method (STEMCELL Technologies, Cambridge, UK) as described [430,431]. Transwells, 6.5 mm permeable (Costar, California, USA) were seeded with 70,000 cells in 100 µL 100 µl Pneuma Cult-EX medium (in the apical chamber) and 500 µl of Pneuma Cult-EX growth medium were

added into the basal chambers. The Transwells were incubated at 37 ºC with 5 % CO_2. The medium in the apical chambers was removed after 72 hours and 500 µL Pneuma Cult-ALI differentiation medium (STEMCELL Technologies, Cambridge, UK) was added to the basal chamber. Pneuma Cult-ALI medium differentiation medium was changed from the basal chamber every second day. HNECs at ALI (HNEC-ALI) were maintained for 4 to 6 weeks for development of tight junctions.

2.3.6. Transepithelial Electrical Resistance

Transepithelial electrical resistance (TEER) was measured from the apical chambers using the EVOM2 epithelial volt-ohmmeter (World Precision Instruments, Sarasota, Florida, USA). Pneuma Cult-ALI medium (100 µL) was added into the apical chamber to produce an electrical circuit transmitted from the apical cells to the basal chambers. Those cells which were mature with resistance measuring > 1000 Ω/cm^2 were considered appropriate for the experiment. ALI cultures were rinsed with phosphate buffered saline (PBS) to remove all accumulated soluble mucus and baseline TEER (time point=0) was recorded following which the exoproteins were added and the TEER noted at different time intervals. First, optimization experiments were performed in triplicates using *S. aureus* ATCC 13565 (ATCC, Manassas, VA, USA) known to disrupt the mucosal barrier. Filtered exoproteins at various concentrations (200 µg/ml, 100 µg/ml, 50 µg/ml, 20 µg/ml, 10 µg/ml and 5µg/ml) were applied to the HNEC-ALI cultures to determine the optimal concentration that produced a consistent decrease in TEER. For the remainder of the experiments,

exoproteins were applied onto the HNEC-ALI cultures at a concentration of 20 µg/ml of planktonic or matched biofilm protein, followed by measuring TEER, paracellular permeability and cell viability (Lactate dehydrogenase assay). A positive control of 2% Triton-X 100, and a negative control of undiluted NB were used. TEER was normalized against the negative control and expressed as Ohms per square centimeter (Ω/cm^2). TEER measurements were taken after 5 minutes, 1 hour, 2 hours and 3 hours, each time placing the HNEC-ALI plate on a heating platform maintained at 37ºC.

2.3.7. Proteinase K and heat treatment of exoproteins

To confirm that the effects of the exoproteins on ALI cultures were indeed due to proteins, inactivation experiments were performed. Exoproteins of clinical isolates, both planktonic and biofilm forms, were subjected to heat treatment at 100°C for 30 minutes, after which they were allowed to cool to room temperature for 15 minutes and added to the ALI-culture to document the effect on the TEER. To a separate aliquot of exoproteins, proteinase K 1 mg/mL (Sigma-Aldrich) was added and incubated overnight at 37°C followed by addition of 5 mM phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich) to inactivate the activity of the enzyme before addition to ALI cultures. A separate control, replacing proteinase K with PBS was included along with a positive control (2% Triton-X 100) and a negative control (undiluted NB).

2.3.8. Permeability Assay

The paracellular permeability across the HNEC-ALI cultures was measured by adding a 4 -kDa fluorescein isothiocyanate (FITC) labeled dextran (Sigma- Aldrich, St. Louis, MO, USA). After exoprotein treatment of the cells for 3 hours, the upper chambers were filled with 0.3 mg/mL FITC-dextran in 100 µL PneumaCult-ALI medium and incubated for 2 hours at 37 °C. The amount of dextran present in the basolateral compartment was measured using the FLUO star Optima 96-well fluorescence microplate reader (BMG Lab tech, Ortenberg, Germany) with wavelengths 485 nm and 520 nm excitation and emission respectively.

2.3.9. Lactate Dehydrogenase (LDH) Assay

Cell viability of the HNEC-ALI cells was determined using the Cytotoxicity Detection Kit (Promega CytoTox 96, Promega, Fitchburg, Wisconsin, USA) after 20 µg/ml exoprotein treatment of the cells for 3 hours. 50 µL Lactate Dehydrogenase **(**LDH) reagent was added to 50 µL of the ALI supernatants in a 96-well clear bottom plate and incubated for 30 minutes at 37 ºC after adding 50 µl of stop solution to each well. The absorbance was read using the FLUO star Optima plate reader (BMG Labtec, Ortenberg, Germany) at 490 nm. The results were calculated as a percentage viability relative to the negative control.

2.3.10. Immunofluorescence of Claudin-1 and ZO-1

After 20 µg/ml exoprotein treatment of the cells for 3 hours HNEC-ALI cultures were fixed using 2.5 % formalin in phosphate-buffered saline (PBS) for 15 minutes, permeated using 0.1% Triton X-100 in PBS and blocked using serum free blocker (SFB; Dako, Glostrup, Denmark) at room temperature (RT) for 60 minutes. Culture support membranes were incubated overnight with 10 µg/ml Claudin-1 rabbit polyclonal antibody and 5µg/mL ZO-1 mouse monoclonal antibodies (both Invitrogen, Carlsbad, CA, USA) diluted in tris-buffered saline-0.5 % Tween (TBST)-10 % SFB (both Invitrogen, Carlsbad, CA, USA). Cells were washed 2x10 minutes with TBST followed by addition of 2 µg/mL anti-mouse Cy3 and anti-rabbit Alexa-488 conjugated secondary antibody (Jackson Immunoresearch Labs Inc., West Grove, PA, USA) for 60 minutes at RT. After the third wash 200 ng/ml of 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) was added and incubated for 10 minutes at RT in the humid chamber, protected from light, to visualize the nucleus. Membranes were mounted onto a glass slide using anti-fade medium (Dako, Glostrup, Denmark) and imaging was performed using the LSM700 confocal laser scanning microscope (Carl Zeiss, Germany).

2.3.11. Transmission Electron Microscopy (TEM)

2.3.11.1. Fixation and embedding of HNEC-ALI cells

HNEC-ALI cells on culture support membranes were fixed by adding 1.25 % glutaraldehyde, 4% paraformaldehyde in PBS containing 4% sucrose to both apical

(0.5 ml) and basal (1.5 ml) chambers and incubated overnight at 4ºC. Cells were washed in PBS containing 4% sucrose and post fixed in 2% Osmium tetroxide (OsO4) for 1 hour (ProSciTech, QLD, Australia). The wash step was repeated, and cells were dehydrated through a graded ethanol series (70%, 90%, 100%, 3 x 10 minutes each and final 20 minutes in 100% ethanol). Following dehydration, the cell monolayers were infiltrated with 1:1 ethanol (100%): Resin (100%, epon-araldite) overnight and 100% resin (3 x 3 hr). The transmembrane was embedded in fresh resin and polymerized at 70 °C for 24 hours.

2.3.11.2. Sectioning and Imaging

Areas of interest were noted, and 1 µm sections were made followed by ultrathin (70nm) sections. These were placed on copper grids (Proscitech 200 mesh). Sections were stained with uranyl acetate (4%) followed by Reynolds lead citrate (ProSciTech, QLD, Australia) for 10 minutes each. Transmission electron microscopy (TEM, FEI Tecnai G2 Spirit, Oregon, USA) was used to image tight junctions between cells in control and experimental (planktonic and biofilm) treatments.

2.3.11.3. Statistical Analysis

Mean results of TEER, FITC-dextran permeability assays and LDH measurements were compared between the planktonic and biofilm *S. aureus* treatments using a

paired Wilcoxon signed rank test. Comparison of (non-paired) means of standard negative control, positive control and standard ATCC planktonic samples were compared against the rest of the groups using a pairwise non-parametric Mann-Whitney test with the Benjamini-Hochberg False Discovery Rate (FDR) correction. All statistics performed using GraphPad Prism version 8.0.0 (Graph Pad Software, San Diego, California USA). Statistical significance was taken at the traditional alpha=0.05 level.

2.4 RESULTS

2.4.1. Clinical Characteristics

A total of 39 *S. aureus* clinical isolates (CIs) from CRS patients were included in the study, of which 16 were female and 23 males, with mean age 58 years

(range =29-89). The cohort of patients included 23 CRSwNP and 16 CRSsNP. The symptoms scores (SNOT22 and ADS) and the Lund Mackay Scores for each group are summarized in table 2.1.

Table 2.1. Demographics of S. aureus clinical isolates

I.D	Age	Sex	Polyp Status	Asthma	SNOT 22	Lund-Mackay Score	Adelaide Disease Severity Score
CL1	52	M	CRSsNP	Positive	38	21	NA
CL2	29	F	CRSsNP	Negative	NA	4	NA
CL3	73	F	CRSwNP	Negative	25	NA	13
CL4	59	M	CRSwNP	Positive	34	19	12
CL5	50	F	CRSwNP	Negative	63	14	16
CL6	50	M	CRSwNP	Positive	36	18	14
CL7	58	M	CRSwNP	Positive	78	NA	18
CL8	64	F	CRSwNP	Positive	34	19	25
CL9	48	F	CRSsNP	Negative	76	10	NA
CL10	58	M	CRSwNP	Positive	NA	NA	NA
CL11	37	F	CRSwNP	Positive	NA	20	NA
CL12	56	M	CRSwNP	Negative	NA	12	NA
CL13	75	F	CRSwNP	Positive	49	18	19
CL14	54	M	CRSsNP	Negative	46	3	18
CL15	60	M	CRSwNP	Positive	NA	19	NA
CL16	76	M	CRSsNP	Negative	NA	7	NA
CL17	74	M	CRSsNP	Negative	67	NA	19
CL18	40	F	CRSwNP	Positive	44	23	19
CL19	47	M	CRSwNP	Positive	36	NA	7
CL20	77	F	CRSsNP	Positive	NA	NA	NA
CL21	55	F	CRSwNP	Positive	79	18	19
CL22	76	M	CRSwNP	Negative	15	19	13
CL23	77	M	CRSsNP	Negative	NA	5	NA
CL24	29	F	CRSwNP	Positive	NA	11	NA
CL25	54	M	CRSsNP	Negative	65	14	17
CL26	49	M	CRSsNP	Positive	46	13	12
CL27	45	F	CRSsNP	Negative	NA	15	NA
CL28	70	M	CRSwNP	Positive	NA	14	NA
CL29	35	F	CRSwNP	Negative	33	17	8
CL30	73	M	CRSsNP	Negative	NA	13	NA
CL31	39	M	CRSsNP	Negative	32	NA	8
CL32	79	M	CRSsNP	Negative	NA	13	NA
CL33	61	F	CRSwNP	Negative	NA	18	NA
CL34	57	M	CRSwNP	Negative	36	NA	8
CL35	89	F	CRSwNP	Negative	38	15	18
CL36	77	M	CRSwNP	Positive	6	NA	4
CL37	77	M	CRSwNP	Positive	43	22	17
CL38	74	M	CRSsNP	Negative	NA	1	NA
CL39	37	F	CRSwNP	Negative	46	NA	14

CL= clinical Isolate
CRSsNP= chronic rhinosinusitis without nasal polyps
CRSwNP= chronic rhinosinusitis with nasal polyps
NA= Not available

2.4.2. *Staphylococcus aureus* exoproteins reduce the Transepithelial Electrical Resistance of HNEC-ALI cultures

For uniformity, the HNEC-ALI cells used in the experiments were obtained from CRSsNP patients (n=7, age range 35-75 years) without a history of smoking, asthma or allergy. The Transepithelial Electrical Resistance (TEER) was measured at time = 0 and 5 minutes, 1 hour, 2 hours and 3 hours after adding 20 µg/ml *S. aureus* exoproteins of matched planktonic and biofilm forms to HNEC-ALI cultures. Both biofilm and planktonic derived *S. aureus* exoproteins showed an immediate reduction in TEER values within 5 minutes of application ($p < 0.001$, one-sample Wilcoxon signed rank test). There was a further time dependant decrease in TEER over 3 hours (Friedman with post hoc wilcoxon, multiple comparisons $p < 0.001$, original FDR method of Benjamini and Hochberg) (Figures 2.1A & 2.1B).

Compared to the negative control both the planktonic and biofilm exoproteins caused a significant decrease in the normalized (to time = 0) TEER values after 3 hours from 0.97 Ω/cm^2 in the negative control to 0.37Ω/cm^2 and 0.29 Ω/cm^2 in planktonic and biofilm forms respectively ($p < 0.001$, Mann-Whitney). 2% Triton-X 100 along with 20 µg/ml planktonic ATCC 13565 exoproteins, known to disrupt the mucosal barrier, were used as positive control and significantly reduced TEER levels compared to negative control as expected[425,432] . Compared to exoproteins from

planktonic cultures, equal concentrations of exoproteins from matched biofilm cultures significantly reduced TEER readings ($p < 0.001$) (Figure 2.1C).

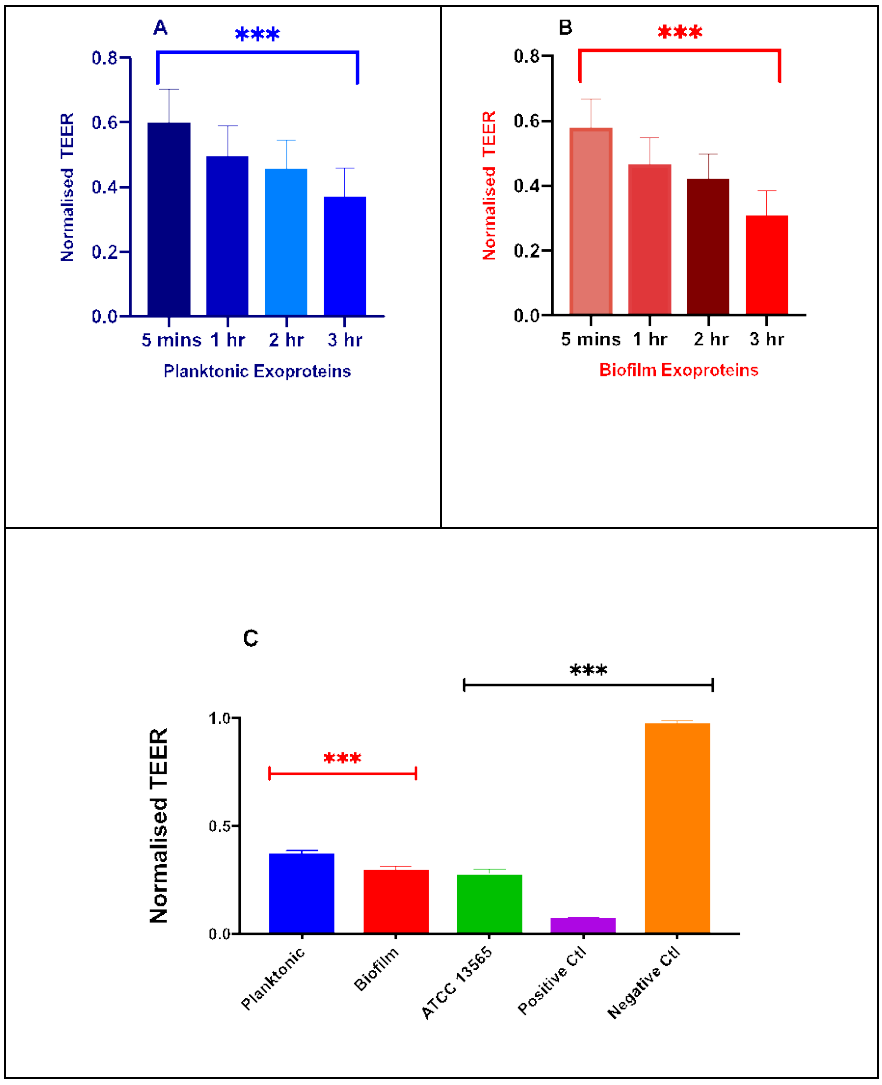


Figure 2.1: Time dependant decrease in TEER on addition of S. aureus exoproteins over 3 hours.

*Exoproteins from 39 clinical isolates planktonic forms (A) and biofilm forms (B) were added to the HNEC-ALI cultures at a concentration of 20µg/ml. TEER was measured at baseline, 5 minutes, 1 hr, 2 hrs and 3 hrs. TEER values were normalised to values at baseline with significant reductions at 5 minutes and a further reduction at 3 hours for both planktonic and biofilm exoproteins. Mean normalised TEER measurements at 3 hours were lower for biofilm exoproteins than for planktonic exoproteins (C). Planktonic ATCC 13565 exoproteins and positive control (2% Triton-X 100) reduced TEER compared to negative control (undiluted nutrient broth). TEER measurements expressed as mean ± SEM; *** p < 0.001in 2.1A and 2.1B, Wilcoxon signed rank test & ***p < 0.001, Mann-Whitney in 2.1C. HNEC-ALI = human nasal epithelial cells grown at air liquid interface; TEER =trans epithelial electrical resistance.*

2.4.3. *Staphylococcus aureus* exoproteins increased the paracellular permeability of FITC-dextrans

HNEC-ALI cultures were then treated with *S. aureus* exoproteins of planktonic and biofilm forms for 3 hours followed by measuring the paracellular permeability of FITC-dextrans. Compared to the negative control, the results showed a significant increase in permeability induced by exoproteins from planktonic (29 fold) and biofilm (250 fold) forms ($p < 0.001$; Wilcoxon Rank Sum) as well as positive control (360 fold) and planktonic ATCC 13565 (200 fold) ($p < 0.001$; Mann-Whitney). Biofilm exoproteins significantly increased the relative permeability of FITC-dextrans compared to their planktonic counterparts ($p < 0.001$) (Figure 2.2).

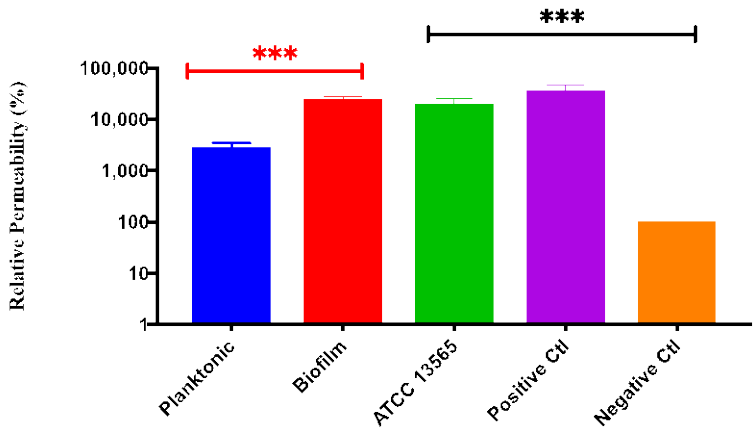


Figure 2.2: Paracellular permeability of HNEC-ALI cultures exposed to S. aureus exoproteins. *Equal concentrations of planktonic and biofilm exoproteins from S. aureus CIs (n=39) and planktonic ATCC 13565 as well as positive control (2% Triton-X 100) caused a significant increase in permeability of FITC-dextrans relative to the negative control (undiluted nutrient broth). Alterations in paracellular permeability to FITC-dextrans reported as mean ± standard error of mean (SEM),*

**** p < 0.001, Wilcoxon signed rank test ***p < 0.001, Mann-Whitney. FITC= fluorescein isothiocyanate.*

2.4.4. Dose dependent reduction in TEER

To assess the dose-dependent effect of the exoproteins on TEER, exoproteins from three *S. aureus* CIs in planktonic and biofilm form were applied at 5 µg/ml, 20 µg/ml and 200 µg/ml for up to 6 hours. Results showed that the lowest concentration of 5µg/ml did not cause a reduction in TEER. Concentrations of 20 µg/ml and 200 µg/ml however caused a significant dose- and time-dependant reduction in TEER values (Fig 2.3 A & 2.3B for planktonic and biofilm form respectively)

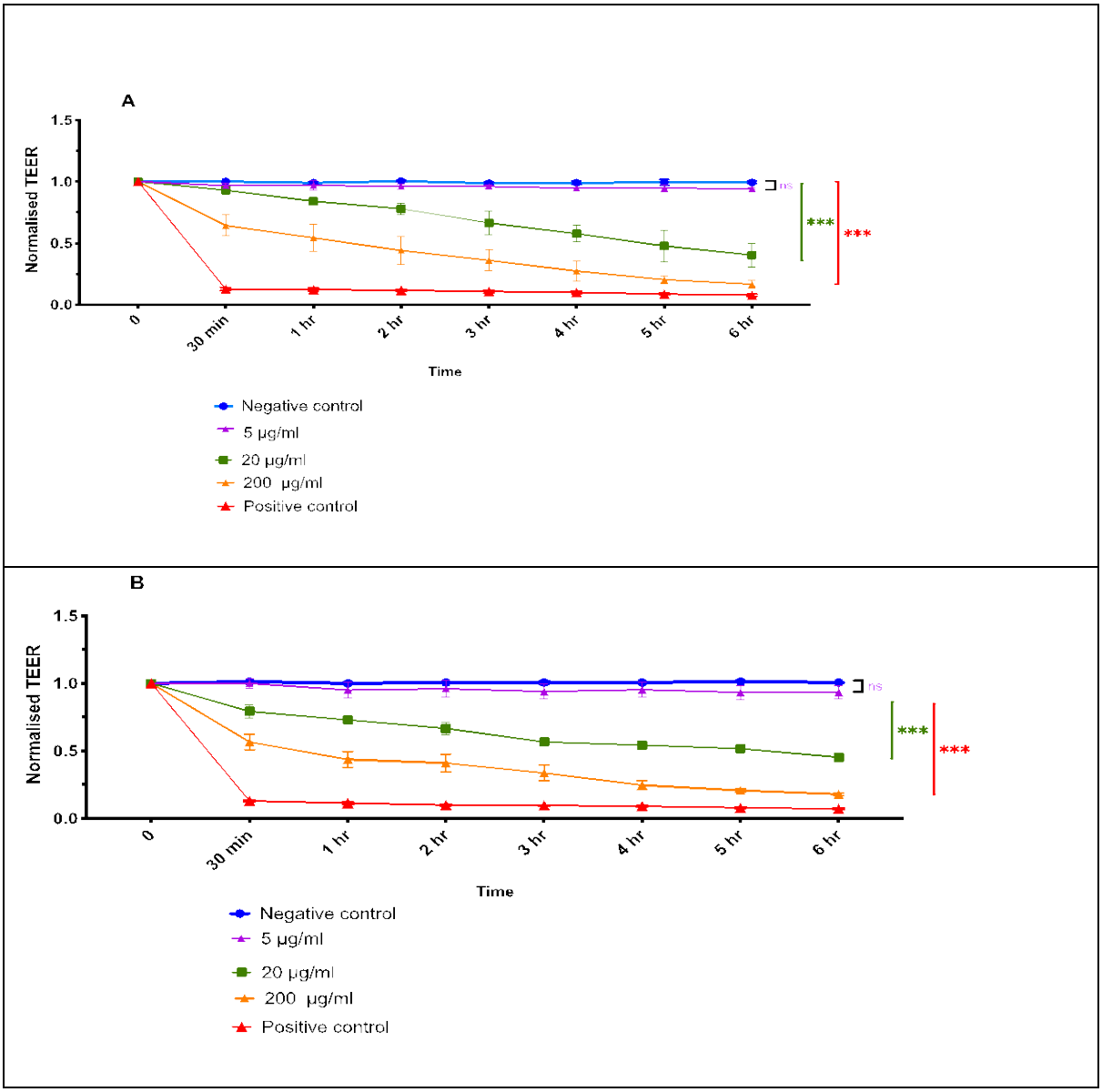


Figure 2.3: Dose dependent reduction in TEER on addition of S. aureus exoproteins

*Positive control was 2% Triton-X 100 and negative control was undiluted nutrient broth. TEER measurements expressed as mean ± SEM for three biologically independent clinical isolates in 3 replicates each A (Planktonic) & B (Biofilm). Significance denoted as ns (not significant), ***p<0.001 (Mann -Whitney test). TEER =trans epithelial electrical resistance.*

2.4.5. Prolonged exposure leads to increased loss in TEER of HNEC-ALI cultures To assess the reversibility of the effect of exoproteins on TEER measurements, exoproteins from 3 CIs in planktonic and biofilm form were added to HNEC-ALI cultures at a concentration of 20 µg/ml for different exposure times (5 minutes and 2 hours) followed by measuring TEER for up to 6 hours. TEER measurements at the 6-hour time point normalized to t = 0 showed a significant reduction ($p < 0.001$)(Mann -Whitney test) in HNEC-ALI cultures exposed to exoproteins for 2 hrs compared to those exposed for 5 minutes (Figure 2.4 A & 2.4B for planktonic and biofilm). After a short 5-minute exposure time to exoproteins from planktonic cells and biofilms, TEER values decreased further with a maximal reduction after 15 minutes and 2 hours for the planktonic and biofilm exoproteins respectively. TEER values reversed back to normal at the 6-hour time point for both planktonic and biofilm exoproteins with no significant differences between TEER values of short 5-minute *S. aureus* exoproteins challenged cells and control cells. In contrast, there was no evidence of recovery of TEER values at the 6-hour time point after prolonged exposure times of 2 hours.

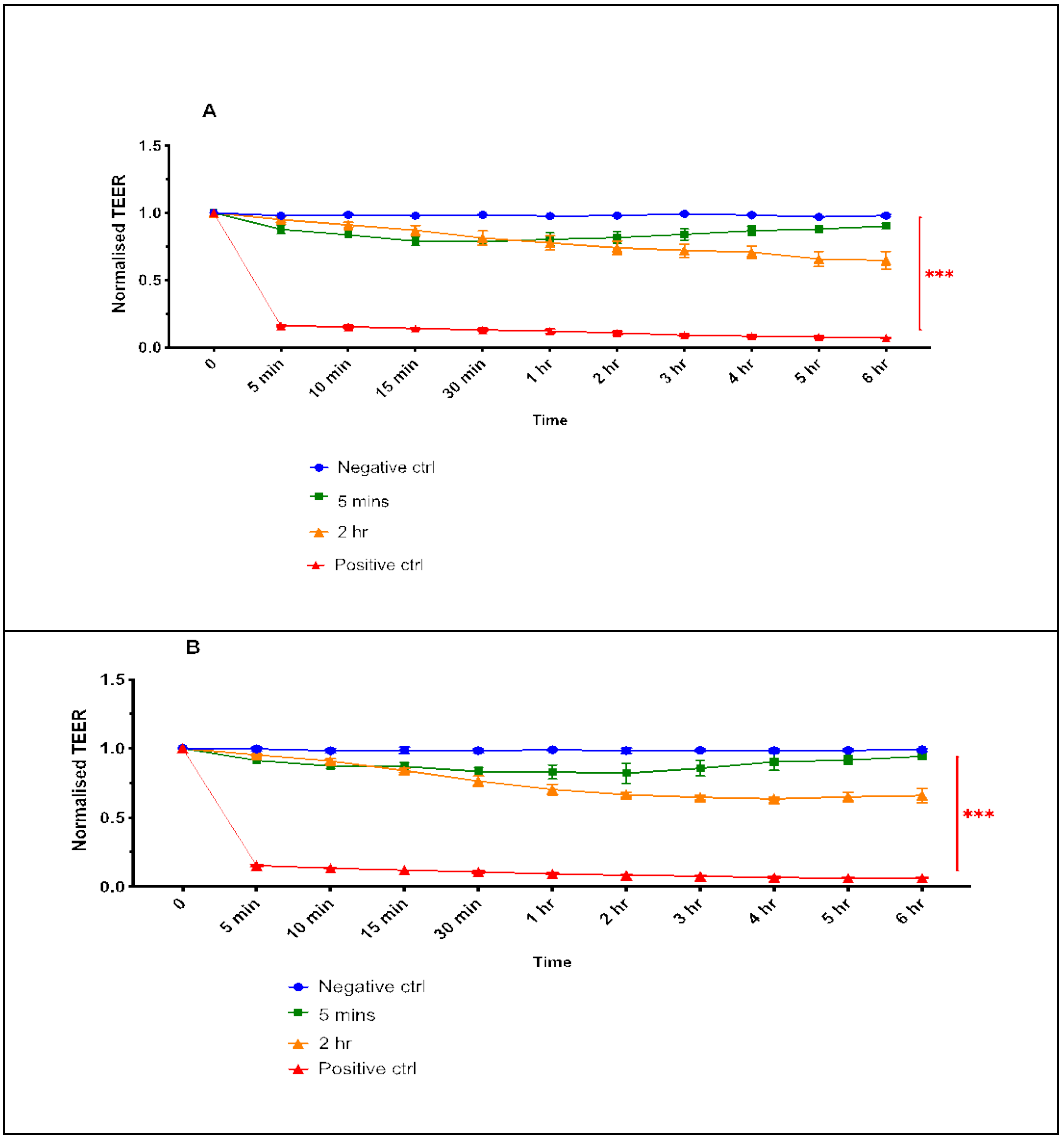


Figure 2.4: Recovery in TEER after short but not prolonged exposure to S. aureus exoproteins.

*S. aureus exoproteins (20 µg/ml) from planktonic (A) and biofilm (B) were added at time = 0 and removed after 5 minutes (green line) or 2 hours (red line) followed by measuring TEER to 6 hrs; positive control is 2% Triton-X 100 and negative control is undiluted nutrient broth. TEER expressed as mean ± SEM for three biologically independent clinical isolates in 3 replicates, normalised to time = 0. Significance denoted as ***p<0.001 (Mann -Whitney test). TEER =trans epithelial electrical resistance.*

2.4.6. Proteinase K and Heat treatment abort the TEER reducing effects

To confirm that the detrimental effect of the exoproteins on the mucosal barrier was in fact due to proteinaceous particles, exoproteins were inactivated by heat or proteinase K treatment. The TEER-reducing effect on the ALI-cultures was lost when exoproteins from planktonic forms and biofilms were heat treated to 100°C for 30 minutes or incubated overnight at 37 °C with proteinase K (Figures 2.5A & 2.5B). Exoproteins incubated overnight in PBS retained their ability to reduce the TEER on the HNEC-ALI culture similar to the effect of the untreated exoproteins.

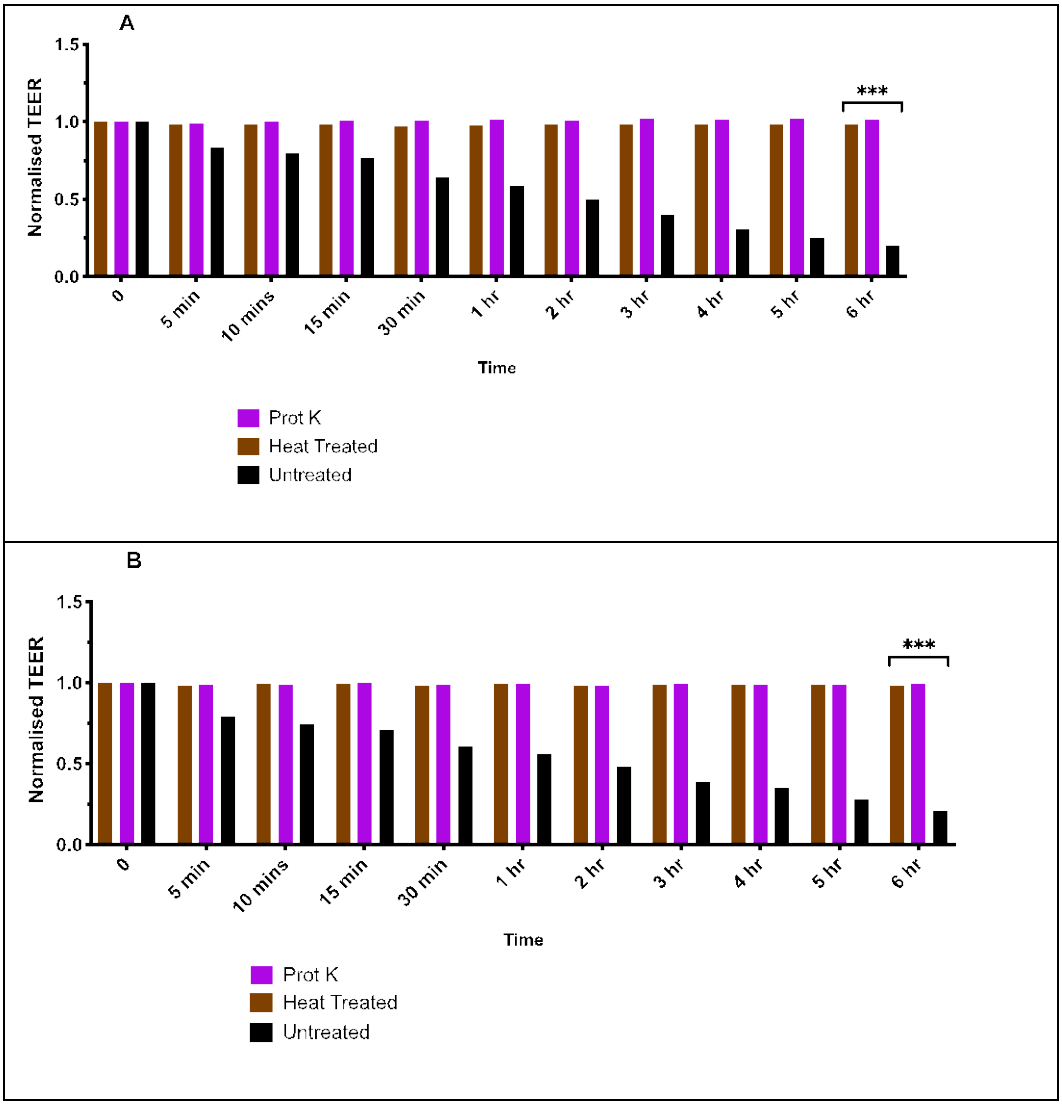


Figure 2.5. Effect of Proteinase K and heat inactivation of S. aureus planktonic and biofilm exoproteins on their barrier disrupting ability. *HNEC-ALI cultures were treated with exoproteins (20 µg/ml) from planktonic (A) and biofilm (B) forms of S. aureus left untreated or exposed to 100°C for 30 minutes, to proteinase K, Positive control was 2% Triton-X 100 and negative control was undiluted nutrient broth. The TEER was measured at 5, 10, 15 and 30 minutes, 1, 2, 3, 4, 5 and 6 h. Values are shown as mean ± SEM for three biologically independent clinical isolates. Significance denoted as ***p<0.001(Mann -Whitney test). TEER =trans epithelial electrical resistance.*

2.4.7. *S. aureus* biofilm exoproteins decrease cell viability:

The cytotoxic effect of S. *aureus* exoproteins (n=39) on HNECs was assessed by measuring the concentration of LDH in the medium 3 hours after application of the exoproteins. Planktonic exoproteins did not significantly affect the cell viability of HNEC-ALI cultures compared to negative control. In contrast, biofilm exoproteins significantly reduced the viability of HNEC-ALI cultures compared to their planktonic counterparts and compared to the negative control (Figure 2.6).

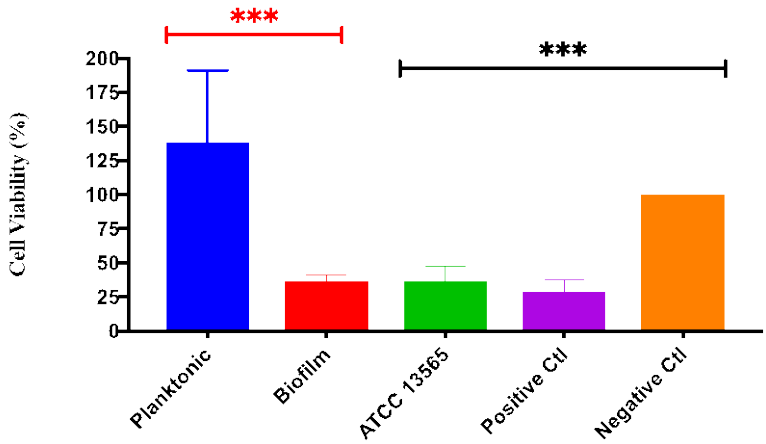


Figure 2 .6: Cytotoxic effects of S. aureus exoproteins on HNEC-ALI cultures.

Exoproteins from S. aureus planktonic and biofilm forms (n=39), ATCC 13565 planktonic exoproteins, positive control (2% Triton-X 100) and negative control (undiluted nutrient broth) were applied for 3 hours to HNEC-ALI cultures, followed by measuring cell viability using Lactate Dehydrogenase (LDH) assay. Cell viability is

expressed as relative to untreated cells (negative control set at 100 %). *** *$p < 0.001$ Wilcoxon signed rank test, ***$p < 0.001$, Mann-Whitney.*

2.4.8. Exoproteins from *S. aureus* cause disruption of the Tight Junction Proteins ZO-1 and claudin-1

Immunofluorescence staining and confocal laser scanning microscopy were used to assess the localization of Zona Occludens-1 (ZO-1) and claudin-1 after exposing the HNEC-ALI cultures to the exoproteins from biofilm and planktonic forms for 3 hours. Whilst in control cells, ZO-1 and Claudin-1 showed clear co-localization, application of planktonic exoproteins reduced the co-localization of both proteins. Application of biofilm exoproteins induced a complete degradation of these TJ proteins compared to negative controls (nutrient broth) as illustrated in Figure 2.7.

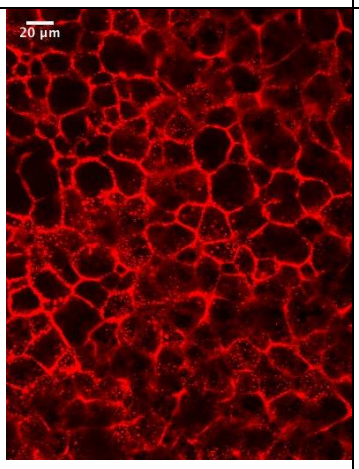
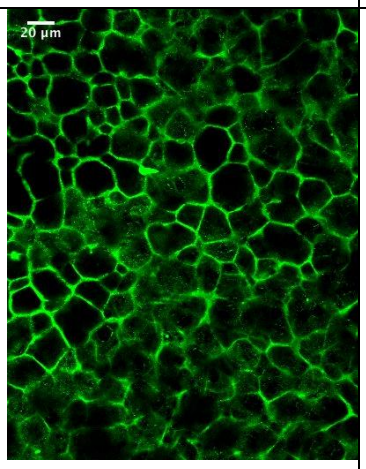
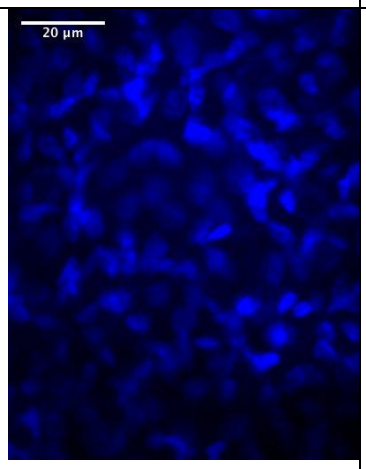
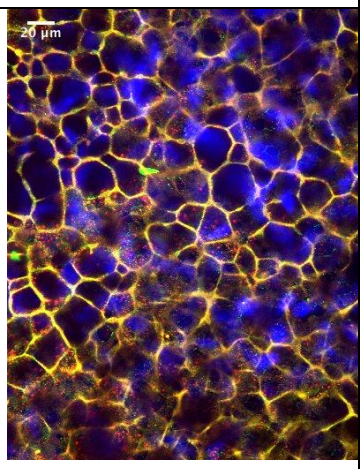
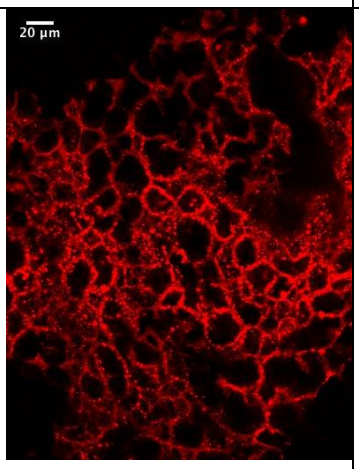
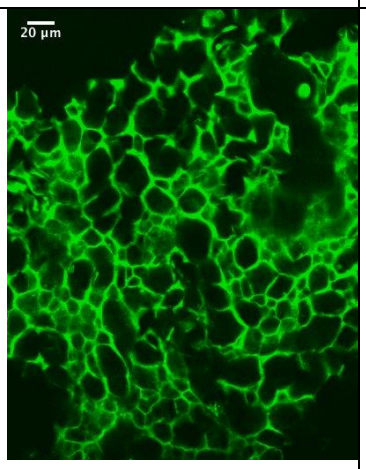
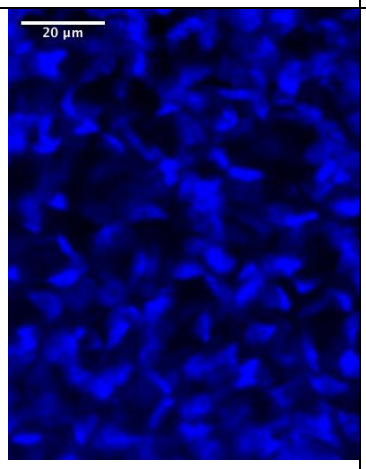
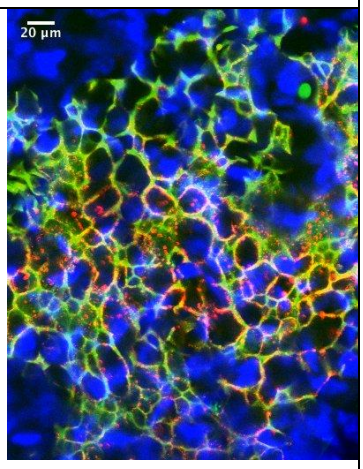
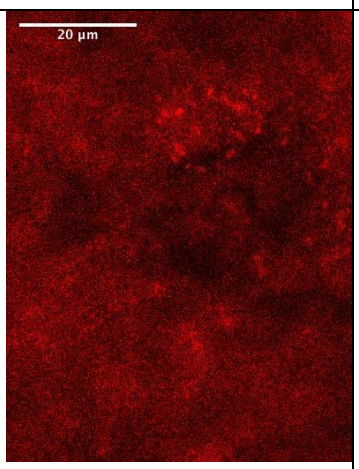
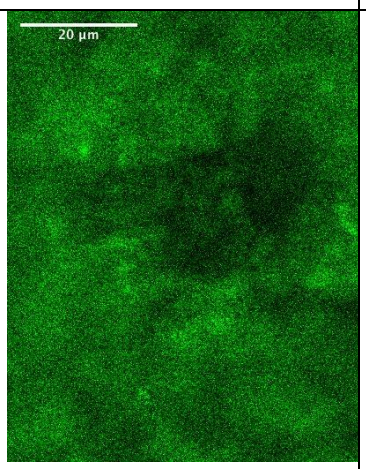
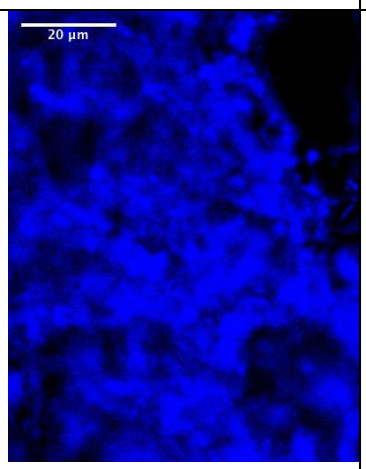
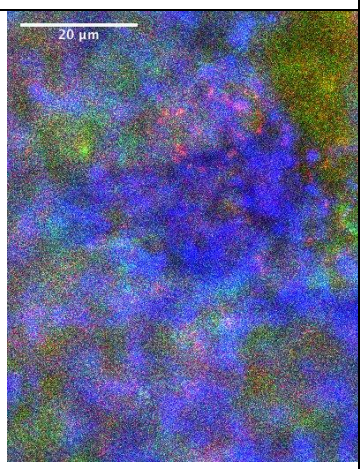
	Claudin-1	ZO-1	DAPI	Overlay
A				
B				
C				

Figure 2 .7: Representative confocal scanning laser microscopy images

Control cells (A) showed a consistent co-localization of Claudin-1 (red) and ZO-1 (green) proteins evidenced by orange colour in the overlay. In contrast, cells exposed to S. aureus planktonic exoproteins (B) showed reduced co-localization of both proteins (mixed green, orange and red colours in overlay). Cells exposed to S. aureus biofilm exoproteins (C) showed a complete degradation of ZO-1 and Claudin-1 with absence of distinct immunostaining patterns. DAPI stains nuclei blue. All images were viewed with 20x objective power (Scale bar: 20 µm)

2.4.9. Transmission electron microscopy shows disruption of TJs

The apical junctional complexes of the negative controls were seen as ‘kissing” cells, illustrating the close contact of the apical region of adjacent cells with normal and intact tight junctions (Figure 2.8A). In contrast, HNECs exposed to planktonic *S. aureus* exoproteins exhibit a gap between adjacent cells, characteristic of tight junction disruption (Figure 2.8B). HNECs exposed to the biofilm exoproteins exhibit severe destruction of the cell membrane with no trace of tight junctions or scattered cilia and loss of normal cell structures, with presence of cell death seen as reduced nuclear chromatin density (Figure 2.8C).

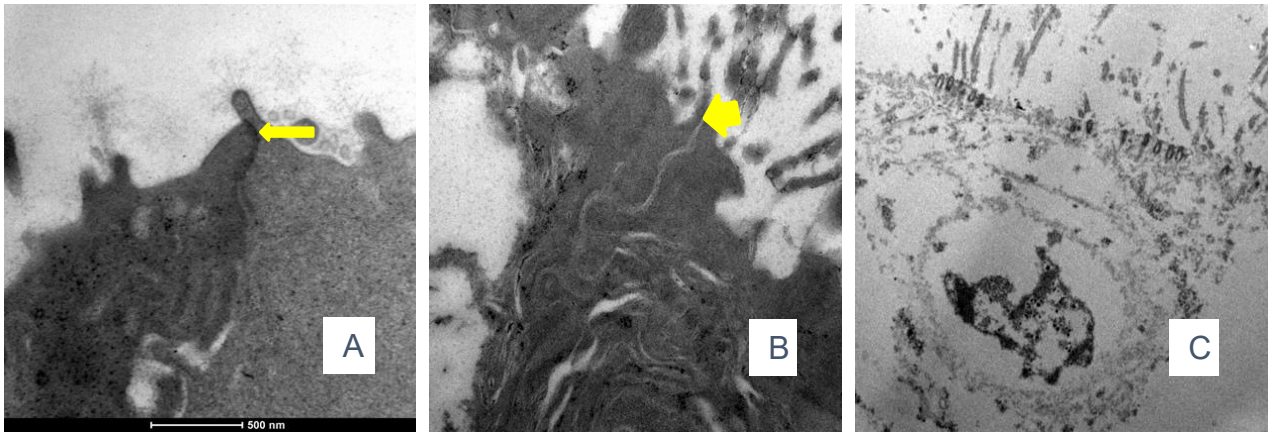


Figure 2.8: Representative Transmission Electron Microscopic images of HNEC-ALI cultures exposed to control solution (A), planktonic exoproteins (B) and biofilm exoproteins (C)

Tight Junctions in cells exposed to control solution (kissing cells) (yellow arrow in A, scale bar: 500 nm). In cells exposed to planktonic exoproteins, disrupted tight junctions are seen as increased space between cells, noted as a white line extending between adjacent cells (yellow arrow in B, scale bar: 500 nm). HNEC-ALI cultured cells treated with biofilm exoproteins show destruction of cell morphology along with nuclear chromatin condensation (C, scale bar: 20 µm).

2.5 DISCUSSION

This study is the first to use *S. aureus* clinical isolates from CRS patients to demonstrate that *S. aureus* exoproteins have dose-and time-dependent detrimental effects on the mucosal barrier structure of HNEC-ALI cultures *in vitro.* Our results indicate that compared to *S. aureus* planktonic exoproteins, equal concentrations of exoproteins derived from their biofilm counterparts have more profound detrimental effects on the mucosal barrier structure and function and are cytotoxic.

Chronic relapsing bacterial infections are a hallmark of CRS. Oral antibiotics, often combined with topical rinses, form the cornerstone of CRS management and in fact, adult rhinosinusitis is the main diagnosis for which antibiotics are prescribed [433]. *S. aureus* remains the most common pathogen isolated in CRS patients, particularly those with nasal polyps [27,418]. Recently, CRSwNP patients have been shown to have a defective mucosal barrier with reduced expression of tight junction proteins [434,435]. Interestingly an association has also been demonstrated between *S. aureus* infection in CRSwNP patients and decreased levels of tight junction proteins[434]. The respiratory epithelium protects the airway against allergens[436] and pathogens by creating a barrier held together by intercellular tight junctions. Destruction of these TJ proteins allow direct exposure of the submucosal tissue to antigens, thereby contributing to the development of inflammation. Hence, TJ dysfunction and loss of barrier integrity is thought to play a crucial role in the pathogenesis of CRS by forming a leaky junction between opposing cells [437].

In line with our current findings, previous studies have shown exoproteins from *S. aureus* planktonic cells and specific proteases disrupt the mucosal barrier of HNEC-ALI cultures [406-408]. However, those studies used laboratory strains and the relevance of those findings in strains that are isolated from the sinonasal cavities is unclear. Interestingly, whilst 5 µg/ml *S. aureus* exoproteins did not affect the mucosal barrier, 20 µg/ml exoproteins harvested from all *S. aureus* clinical isolates tested had acute detrimental effects on the mucosal barrier, regardless of whether those exoproteins were harvested from planktonic cells or biofilms. These effects were long lasting, dose-dependent and were abrogated with heat- or proteinase K-inactivation. These results indicate that *S. aureus* clinical isolates from the sinonasal cavities of CRS patients universally secrete proteins that have strong immediate barrier disruptive effects. The identity of those proteins and their exact mechanism of action are not known and warrant further investigation.

Exoproteins express staphylococcal virulence[438] and induction of inflammation, however, this is not restricted to the effect of a single toxin. Rather, a repertoire of proteins such as enterotoxins, hemolysins and proteases[439] are thought to contribute to the inflammation observed, but only some of those are likely contributing to the actual barrier disruption. This was also shown in our previous studies where from a number of toxins and virulence factors tested, only the V8 protease was shown to disrupt the integrity of HNEC-ALI cultures [408]. Interestingly, our results indicate planktonic exoproteins, at a concentration of 20 µg/ml, could disrupt the barrier without causing significant cytotoxicity. Those exoproteins induced a 29-fold increase in paracellular permeability of 4 kDa dextrans, indicating that antigens and proteins

of similar size might traverse the mucosal layer in the presence of similar planktonic cell exoprotein concentrations. Exoproteins such as lipoteichoic acid (LTA) are released in substantial amounts during *S. aureus* growth and infection with levels as high as 10 µg/ml present in wash fluid samples of atopic dermatitis (AD) patients with levels correlating with *S. aureus* colony-forming unit (cfu) counts and AD disease severity [440]. Hence, taking into consideration that LTA would be expected to be only one of many proteins present in *S. aureus* exoproteins, the exoprotein concentrations found to affect the barrier in this study might be relevant also *in vivo.* This could occur for example, during the course of acute *S. aureus* infections, where relatively large quantities of exoproteins can be produced [440].

S. aureus, however, often resides within mucosal biofilms, protecting the bacteria from a hostile environment and rendering them less susceptible to antibiotics. *S. aureus* biofilms have therefore been associated with recurrence of disease and poor post-operative outcomes [441,442]. Although many studies have demonstrated an association between mucosal biofilms and CRS disease recalcitrance [443], a causative link, their toxicity and how they interact with the host mucosa is largely unknown.

Our study indicates *S. aureus* biofilm exoproteins had more profound effects on the mucosal barrier and were more toxic than equal concentrations of matched planktonic exoproteins, increasing the paracellular permeability >250 fold.

The increased permeability could at least in part be due to the cell toxicity induced by biofilm exoproteins. Together, these results indicate that qualitative differences exist

between exoproteins secreted by planktonic cells and biofilms. Indeed, studies have shown that bacteria living in a biofilm form express a different set of genes and more virulence factors compared to their planktonic counterparts [412,444].Further research is needed to determine the specific *S. aureus* biofilm proteins that cause toxicity and disrupt the mucosal barrier and to evaluate how that relates to the extent and type of inflammation in the context of CRS. Anti-toxin treatment and targeted barrier protection could be avenues of novel therapies in the treatment of *S. aureus* associated CRS.

2.6 CONCLUSION

S. aureus exoproteins produce a dose- and time- dependent detrimental effect on HNEC-ALI barrier structure and function. In comparison to planktonic exoproteins, biofilm exoproteins produced irreversible damage to the HNEC-ALI cells with increased paracellular permeability and decreased cell viability. Unraveling the identity and role of exoproteins produced by *S. aureus* biofilms in the inflammatory process is crucial towards understanding the pathophysiology of CRS.

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CHAPTER 3: Biofilm Exoproteins: Drivers of inflammation and host immune response in Chronic rhinosinusitis (CRS)

Conducted in the Department of Otolaryngology – Head and Neck Surgery

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Statement of Authorship

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Publication Details	Beula Subashini Panchatcharam, Clare M Cooksley, Mahnaz Ramezanpour, Hua Hu, Ahmed Bassiouni , Peter J. Wormald , Alkis J. Psaltis, and Sarah Vreugde *

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Name of Principal Author (Candidate)	Beula Subashini Panchatcharam		
Contribution to the Paper	Conduct of study, Collection of data, interpretation of results drafting and editing the manuscript		
Overall percentage (%)	72%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	10/4/2020

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Signature		Date	3/7/2020

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Overall percentage Contribution to the Paper	4% Designing the study, supervision, interpretation of data and results, drafting, editing of manuscript and final approval for submission		
Signature		Date	3/7/2020

Biofilm Exoproteins: Drivers of inflammation and host immune response in Chronic rhinosinusitis (CRS)

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3.1 ABSTRACT

Introduction:

Biofilms in CRS are often polymicrobial, but when dominated by *S. aureus* are associated with CRS disease recalcitrance. However, biofilms are also found in healthy patients and thus the role of biofilms in CRS is unclear. This study used *S. aureus* clinical isolates (CIs) and tissue sections from corresponding CRS patients to evaluate the relationship between *in vitro S. aureus* biofilm properties and inflammation.

Methods:

Tissue and *S. aureus* CIs from CRS patients were collected, and isolates cultured in both planktonic and biofilm forms. Exoproteins were quantified and applied to primary human nasal epithelial cells (HNECs) grown at Air Liquid Interface (ALI) followed by IL-6 protein quantification using ELISA. Biofilm metabolic activity and biomass were determined via Alamar Blue and crystal violet assays. Tissue sections were evaluated for inflammatory cell infiltration and presence of tertiary lymphoid organs (TLOs) using histopathology.

Results :

Fifty *S. aureus* CIs and 50 tissue samples from corresponding CRS patients were used in this study. There was a strong positive correlation between biofilm exoprotein concentration and biofilm metabolic activity ($r = 0.715$; $p < 0.0001$) or biofilm biomass ($r=0.68$, $p<0.0001$) and between the biofilm biomass and biofilm metabolic activity ($r=0.61$, $p<0.0001$). There was a significant strong positive correlation between the number of lymphocytes or eosinophils and the concentration of biofilm exoproteins secreted by corresponding CIs ($r =0.66$ and $r = 0.69$ for lymphocyte and eosinophil numbers respectively, $p < 0.0001$) and their metabolic activity ($r =0.50$ and $r = 0.52$ for lymphocyte and eosinophil numbers respectively, $p<0.001$).

Conclusion:

S. aureus biofilm exoproteins and metabolic activity are increased in CRSwNP patients compared to CRSsNP patients in correlation with eosinophil and lymphocyte cell numbers. Our results indicate biofilm properties might differ between patients and account for differences in severity of inflammation.

3.2 INTRODUCTION

The aetiopathogenesis of CRS remains elusive with both environmental factors and host factors postulated as having a role. The immune response in CRS is heterogeneous, ranging from a predominantly Th1 response to a Th2 polarisation in patients without (CRSsNP) and with nasal polyps (CRSwNP), respectively [445]. *Staphylococcus aureus* is at the core of the sinonasal tract microbiome along with *Corynebacterium spp, Moraxella spp, Streptococcus spp* and *Haemophilus spp* [446]. *S. aureus* is also the most commonly cultured organism in patients with chronic rhinosinusitis (CRS)[418]. Mucosal biofilms in the context of CRS are often polymicrobial but can be dominated by a single species. *S. aureus* dominant mucosal biofilms are associated with CRS disease recalcitrance [403,447.] Recalcitrant CRS patients often have nasal polyps, tissue eosinophilia and comorbid asthma [448-450] and frequently exhibit organised infiltrations of immune cells named tertiary lymphoid organs (TLOs) [451,452]. However, *S. aureus* and mixed species biofilms are also found in healthy sinuses, therefore the exact role of *S. aureus* and mucosal biofilms in the pathophysiology of CRS and how that relates to TLO formation is unknown and highly controversial [401,453.]

Bacterial biofilms are organised clusters of bacterial cells encompassed by a self-produced Extracellular Polymeric Substance (EPS) matrix consisting of viscoelastic biopolymers, which facilitate strong mucosal attachment and account for 50-90% [131] of the total organic matter in the biofilm.[409,454]

The EPS matrix consists mainly of polysaccharides, proteins, glycoproteins and extracellular DNA. Bacteria living in a biofilm express a different set of genes and proteins than their planktonic counterparts with an enrichment of many proteins involved in the pathogenesis of inflammation, such as toxins (e.g. leukocidin and beta-hemolysin), immunomodulatory proteins (e.g. lipoprotein and protein A) and a large number of proteins involved in carbohydrate metabolism [412].

These secreted proteins (exoproteins) are not only harmful to the nasal epithelium but also trigger a host immune response and can disrupt the barrier function which further exacerbates the inflammatory process[455] . However, also exoproteins released by planktonic *S. aureus* affect the mucosal barrier structure and function in different ways[425,432,456] and it is unclear whether differences exist between planktonic and biofilm secreted proteins on how they affect the immune response. Studies using sinonasal explants have shown that *S. aureus* biofilms induce apoptosis and a host of pro-inflammatory cytokines[457]. Furthermore, evidence for biofilms contributing to chronic sinus inflammation and their role as external disease triggers has been well established in previous studies[419,458]. A study by Wood et al showed that in CRS patients, biofilms that were directly opposed to a disrupted epithelial layer were associated with inflammatory cell infiltration whereas areas of mucosa without bacteria present were not [459]. Together, these studies indicate an association of *S. aureus* biofilms and inflammation in the context of CRS. However, it is not known whether differences in biofilm properties might relate to differences between patients in their levels and type of inflammation.

This study used *S. aureus* clinical isolates and tissue sections from corresponding CRS patients to evaluate the relationship between *in vitro S. aureus* biofilm properties and measures of inflammation.

3.3 METHODS

3.3.1 Ethics approval, bacterial isolates and tissue collection

Ethics clearance was obtained from The Queen Elizabeth Hospital (TQEH) Human Research Ethics Committee in Adelaide, South Australia (HREC/18/CALHN/69).

S. aureus ATCC 13565 was from American Type Culture Collection (Manassas, Virginia USA). *S. aureus* clinical isolates from the sinus cavities of patients with Chronic rhinosinusitis (CRS) were stored at - 80°C in glycerol stocks before use. Matching tissue samples (nasal mucosa from CRS patients without nasal polyps (CRSsNP) and polyp tissue from CRS with nasal polyps (CRSwNP)) were fixed in 10% formalin before embedding in paraffin for histological analysis. CRS patients were divided into CRSsNP and CRSwNP patients according to the guidelines of the European Position Paper of Rhinosinusitis [460]. Clinical data from the patients was collected retrospectively including age, gender, history of asthma and past surgeries. Disease severity was measured based on preoperative patient reported quality of life Sino-Nasal Outcome Test (SNOT-22) questionnaires[420], CT-scan scoring of Lund Mackay (LMS) [461] and Adelaide Severity score (ADS)[421].

3.3.2 Bacterial culture and concentration of supernatants

ATCC 13565, ATCC 25923 and *S. aureus* clinical isolates were streaked onto nutrient agar from glycerol stocks and cultured in nutrient broth overnight at 37 ºC. The following day, fresh nutrient broth was inoculated to a starting optical density (OD) of 0.01 at 600 nm. Growth was followed by measuring Optical Density (OD) on an hourly basis. At 6 hours (stationary phase), liquid cultures were centrifuged using Pierce Protein Concentrator (Thermofisher, IL, USA) at 4 ºC for 10 minutes at 1500 x g. Supernatants were removed and passed through a 0.22 µm syringe filter. They were then concentrated using a 3 KDa, Pierce Protein Concentrator (Thermofisher, IL, USA) and sterility of the supernatants was checked by streaking onto nutrient agar plates to rule out the presence of viable bacteria. Concentrated supernatants were stored at -80 ºC for further analysis. Biofilm cultures for the same clinical isolates and ATCC 25923 were set up in six well plates after adjusting the opacity to 1.0 ± 0.1 Mac Farland units and incubating at 37°C for 48 hours on a rotating platform (3D Gyratory Mixer; Ratek Instruments, Boronia, Australia) at 70 rpm. Supernatants collected from the biofilm forms were centrifuged and concentrated following the same protocol used for the planktonic forms.

3.3.3. Alamar Blue activity assay

S. aureus isolates were streaked onto nutrient agar and incubated overnight at 37°C. Using a cotton swab a single colony of *S. aureus* was suspended in 0.9 % saline to turbidity reading 1.0 ± 0.1 McFarland Units. A dilution of 1 in 15 in nutrient broth was prepared and 150 µl bacterial mix was added into each well of a black 96 well plate for biofilm formation. 150 µl nutrient broth was added to control wells. The plate was incubated for 48 h at 37°C on a rotating plate set at 70 rpm (3D Gyratory Mixer, Ratek Instruments, Australia). Following incubation, a 1:10 dilution of Alamar Blue reagent (Life Technologies, Scoresby, Australia) was prepared in nutrient broth and 200 ul added to all wells after washing the wells twice with phosphate buffered saline (PBS). The plate was returned to the gyratory mixer at 37 ºC and OD read after half an hour followed by hourly readings using a FLUO star OPTIMA plate reader (BMG Lab tech, Ortenberg, Germany) and fluorescence read at λ excitation = 530 nm/λ emission= 590 nm, with maximum intensities reaching within 6 hours incubation period for all isolates. The maximum metabolic activity of the individual isolates was recorded; all experiments were performed as 3 biological replicates and 6 technical replicates with a sterility control (nutrient broth) and ATCC 25923 positive control in each batch.

3.3.4. Nano Orange Protein Assay

Protein quantification of all isolates, both planktonic and biofilm forms, was performed using the Invitrogen Nano Orange Protein Quantitation Kit (Molecular Probes, Eugene, Oreg, USA) in black-walled 96-well plates as per the manufacturer's instructions. Fluorescence was measured using excitation at 485 nm and emission at 590 nm using the FLUOstar OPTIMA plate reader (BMG Labtech, Ortenberg, Germany).

3.3.5. Measurement of biofilm formation using crystal violet staining

S. aureus biofilms were grown as described with the exception that clear 96 well plates (Nunc, Thermo Fisher Scientific, Waltham, MA, USA) were used. After incubation, biofilms were washed to remove free planktonic bacteria with phosphate buffered saline (PBS). Biofilms were stained using 0.1% crystal violet solution for 15 min at room temperature. The plate was washed 3 times with sterile MilliQ water following which adherent dye was removed by adding 30% glacial acetic acid to all wells and incubating on a plate shaker for 1 hour. Absorbance was measured at 595 nm with a plate reader (BIORAD laboratories, CA, USA). Readings from negative control wells (media only) were subtracted from all other readings. All experiments were performed as 3 biological replicates and 6 technical replicates, and all measurements normalised to absorbance of *S. aureus* ATCC 25923 in each replicate.

3.3.6. Enumeration of biofilm colony forming units (CFU)

Following the growth of biofilms for 48 hrs, the medium was removed, and wells were washed with saline. The 6 well plates were then wrapped with parafilm and sonicated for 15 mins in a sonicating water bath. After sonication serial dilutions were performed in 0.9% saline up to 10^6 dilution and plated onto nutrient agar. The nutrient agar plates were incubated for 24 hrs at 37°C and the CFU/ml were calculated.

3.3.7. Hematoxylin & Eosin (H &E) staining of tissue sections

Paraffin embedded tissue was cut as 4 µm thick sections with a microtome (Thermo Scientific HM 325 Rotary Microtome). Slides were stained with Hematoxylin & Eosin (H &E) (Thermo Fisher Scientific, Waltham, MA, USA). All slides were nano-zoomed using the digital whole-slide imaging technology (WSI) on Nano Zoomer Digital Pathology system (Hamamatsu Photonics, Hamamatsu City, Japan) and visualized at 40 X objective magnification. For each specimen, at least 5 areas of the lamina propria measuring 0.035 mm^2 each were randomly selected and lymphocytes and eosinophils were counted as described [462]. This surface area of 0.035 mm^2 was selected as it equals to high-power field using the light microscope (Nikon Eclipse 90i Microscope; Nikon Corporation, Tokyo, Japan). The presence of tertiary lymphoid organs (TLO) was identified by histology as reported [463,464].

3.3.8. Primary human nasal epithelial Cells

Ethics clearance for collection of primary human nasal epithelial cells (HNEC) was approved by the Human Research Ethics Committee of the Queen Elizabeth Hospital and the University of Adelaide (HREC/15/TQEH/132) and patients were included after written informed consent. Inclusion criteria included non-smokers of more than 18 years of age. Nasal brushings were collected from the inferior turbinate of seven patients with CRSsNP and transported in PneumaCult™ Ex Plus Basal Medium (STEMCELL Technologies, Cambridge, UK). Processing of the cells from the brushes was performed after centrifuging the brushes at 300 g for 5 mins at 4 ºC and suspending in 2 ml PneumaCult™ Ex Plus Basal Medium. The pellet was resuspended using a 10 ml syringe to create a single cell suspension into a petri dish containing anti-CD 68 antibody (Dako, Carpinteria, CA) to deplete the cell suspension from monocytes. HNECs were expanded on type 1 collagen coated T25 flask (Corning, Corning, New York) and incubated at 37 ºC with 5 % CO_2.

3.3.9. Air -Liquid Interface Culture and treatment with bacterial supernatants

Air Liquid Interface (ALI) cultures were established using the PneumaCult™ Ex Plus, ALI culture method (STEMCELL Technologies, Cambridge, UK) as reported [431]. Transwells, 6.5 mm permeable (Costar, California, USA), were seeded with 70,000 HNECs in 100 µL B-ALI growth medium in the apical chamber of the transwell plate and 500 µL B-ALI growth medium was added into the basal chambers. The

Transwells were incubated at 37 ºC with 5 % CO_2. The media in the apical chambers was removed after 72 hrs and 500 µL PneumaCult-ALI differentiation medium (STEMCELL Technologies, Cambridge, UK) was added to the basal chamber. B-ALI differentiation medium was changed from the basal chamber every second day. HNECs at ALI (HNEC-ALI) were maintained for 4 to 6 weeks for development of tight junctions[430,431].

3.3.10. IL-6 Enzyme-Linked Immunosorbent Assay (ELISA)

Interleukin-6 (IL-6) protein levels were estimated with an ELISA kit using rat anti-human IL-6 antibodies (BD Biosciences, New Jersey, USA), according to the manufacturer's instructions. IL-6 protein levels were detected in supernatants collected following 24-hour exposure to both planktonic and biofilm exoproteins using a sandwich ELISA as previously described [429]. All measurements were performed in duplicate using a FLUOstar OPTIMA plate reader (BMG Labtech, Ortenberg, Germany).

3.3.11. Statistical Analysis

Spearman correlation was used to correlate the amount of planktonic and biofilm protein with inflammatory cell counts and various clinical scores including the Snot 22, Adelaide disease severity and Lund Mackay scores. Mean IL-6 measurements were compared between the planktonic and biofilm *S. aureus* treatments using a paired Wilcoxon signed rank test. Comparison of (non-paired) means of standard negative control, standard ATCC planktonic samples, mean inflammatory cell counts

between CRSwNP and CRSsNP patients and between TLO-positive and TLO-negative patients were compared using a pairwise non-parametric Mann-Whitney test. All statistical analysis was performed using the scipy Scientific Python library. Statistical significance was taken at the traditional alpha=0.05 level.

3.4 RESULTS

3.4.1. Clinical isolates and patient characteristics

A total of 50 *S. aureus* clinical isolates (CIs) from CRS patients were selected based on the availability of matched *S. aureus* clinical isolates, tissue sections and clinical disease severity scores. These included twenty-eight patients with nasal polyps (CRSwNP) and twenty-two patients without nasal polyps (CRSsNP), aged 23-89 years, including 29 males and 21 females. Histological analysis identified sixteen patients as TLO-positive and thirty-four patients as TLO-negative. Demographic data is detailed in Table 3.1.

I.D	Age	Sex	Polyp Status	Asthma	Planktonic protein (μg/ml)	Biofilm protein (μg/ml)	L	E	TLO	Biomass	Alamar Blue	eDNA
CL1	52	M	CRSsNP	Positive	25.60	352.07	48	8	Positive	1.429	21250	
CL2	29	F	CRSsNP	Negative	91.09	189.79	2	1	Negative	0.921	19905	
CL3	73	F	CRSwNP	Negative	25.20	496.60	32	8	Positive	1.226	21308	2.15
CL4	59	M	CRSwNP	Positive	480.59	632.95	53	6	Positive	1.94	21674	3.6
CL5	50	F	CRSwNP	Negative	120.16	481.25	42	9	Positive	0.934	20652	2.97
CL6	50	M	CRSwNP	Positive	128.40	477.32	33	10	Positive	1.029	20817	
CL7	58	M	CRSwNP	Positive	23.09	323.54	14	4	Positive	0.836	20017	
CL8	64	F	CRSwNP	Positive	82.00	354.27	53	4	Positive	1.187	20833	
CL9	48	F	CRSsNP	Negative	13.49	201.31	8	0	Negative	0.93	19000	
CL10	58	M	CRSwNP	Positive	43.73	271.18	28	2	Positive	0.848	19641	
CL11	37	F	CRSwNP	Positive	100.22	243.89	5	1	Negative	1.295	20638	
CL12	56	M	CRSwNP	Negative	23.66	172.30	36	6	Positive	0.63	20174	
CL13	75	F	CRSwNP	Positive	41.22	185.88	2	1	Negative	0.65	19365	
CL14	54	M	CRSsNP	Negative	150.00	166.00	12	2	Negative	0.63	19562	
CL15	60	M	CRSwNP	Positive	131.04	152.93	5	0	Negative	0.427	20325	
CL16	76	M	CRSsNP	Negative	68.04	195.23	2	0	Negative	0.988	18438	
CL17	74	M	CRSsNP	Negative	106.01	208.27	5	1	Negative	0.94	20195	
CL18	40	F	CRSwNP	Positive	152.58	220.87	14	2	Negative	0.987	21038	
CL19	47	M	CRSwNP	Positive	63.58	384.29	38	6	Positive	1.029	12552	
CL20	77	F	CRSsNP	Positive	58.97	116.03	3	1	Negative	0.564	18427	
CL21	55	F	CRSwNP	Positive	112.18	237.57	5	1	Negative	1.137	19703	2.96
CL22	76	M	CRSwNP	Negative	152.58	220.87	3	2	Negative	1.276	19129	
CL23	77	M	CRSsNP	Negative	193.13	233.94	5	2	Negative	1.291	19203	
CL24	29	F	CRSwNP	Positive	95.00	183.00	8	1	Negative	0.92	18510	
CL25	54	M	CRSsNP	Negative	133.06	199.71	2	0	Negative	0.978	19017	
CL26	49	M	CRSsNP	Positive	90.74	283.00	22	1	Positive	1.328	21124	
CL27	45	F	CRSsNP	Negative	51.86	76.53	2	0	Negative	0.52	18707	2.88
CL28	70	M	CRSwNP	Positive	74.00	129.00	3	1	Negative	0.733	17005	
CL29	35	F	CRSwNP	Negative	85.00	168.00	4	1	Negative	0.653	17560	3.95
CL30	73	M	CRSsNP	Negative	16.38	97.29	5	0	Negative	0.397	16206	2.68
CL31	39	M	CRSsNP	Negative	22.00	48.00	3	1	Negative	0.33	16515	2.79
CL32	79	M	CRSsNP	Negative	30.86	82.99	3	0	Negative	0.411	16628	2.38
CL33	61	F	CRSwNP	Negative	109.00	554.27	34	2	Positive	1.32	20928	2.35
CL34	57	M	CRSwNP	Negative	35.00	351.00	38	3	Positive	0.945	20619	
CL35	89	F	CRSwNP	Negative	39.00	340.00	28	2	Positive	0.624	21532	2.52
CL36	77	M	CRSwNP	Positive	89.00	463.00	30	1	Positive	1.61	20786	
CL37	77	M	CRSwNP	Positive	73.594	482.94	19	1	Positive	1.795	20909	
CL38	74	M	CRSsNP	Negative	214.464	320.9	3	1	Negative	1.237	20227	
CL39	37	F	CRSwNP	Negative	122.002	285.79	3	2	Negative	1.382	21351	
CL40	45	M	CRSsNP	Negative	137.525	219.78	3	1	Negative	1.219	19950	
CL41	70	F	CRSwNP	Positive	139.236	210.80	3	1	Negative	2.841	21453	
CL42	69	F	CRSwNP	Negative	81.7625	157.00	4	1	Negative	0.729	20278	
CL43	81	M	CRSwNP	Negative	33.0425	52.71	4	0	Negative	0.35	15819	
CL44	80	M	CRSsNP	Negative	94.686	120.86	3	0	Negative	2.238	19686	
CL45	65	M	CRSsNP	Negative	52.9915	105.78	2	0	Negative	0.618	18909	
CL46	57	F	CRSsNP	Negative	81.0485	100.60	4	0	Negative	0.537	18475	
CL47	23	M	CRSsNP	Negative	123.696	198	3	1	Negative	0.846	20575	
CL48	68	F	CRSsNP	Negative	109.864	184.85	8	1	Negative	2.276	20517	
CL49	51	F	CRSsNP	Negative	71.452	118.60	3	1	Negative	0.942	19273	
CL50	28	M	CRSwNP	Negative	126.5905	133.75	4	2	Negative	0.916	19685	

Table 3.1.Clinical data

CL= clinical Isolate
CRSsNP= chronic rhinosinusitis without nasal polyps
CRSwNP= chronic rhinosinusitis with nasal polyps
L= Lymphocytes
E= Eosinophils

3.4.2. *S. aureus* clinical isolates from CRSwNP and TLO-positive CRS patients demonstrate higher *S. aureus* biofilm exoprotein production and higher metabolic activity than clinical isolates from CRSsNP and TLO-negative CRS patients

We first measured the exoprotein concentration, metabolic activity, biomass and Colony Forming Units (CFUs) of 48-hour biofilms and protein production by planktonic cells for all 50 clinical isolates. eDNA concentration was also determined for representative clinical isolates only. Results are detailed in Table 3.1.

Protein concentrations of exoproteins from planktonic and biofilm forms varied widely between isolates ranging from 20 micrograms/ml to 480 micrograms/ml in the planktonic form and from 48 micrograms/ml to 633 micrograms/ml in the biofilm form. Alamar Blue metabolic activity and biomass of the biofilms also differed amongst isolates ranging from 12552 to 21674 OD 590 nm for the metabolic activity and 0.33 to 2.841 OD 595 nm for the biomass.

Values were then compared between CRSwNP and CRSsNP patients and between TLO-positive and TLO-negative patients. Compared to clinical isolates from CRSsNP patients, those from CRSwNP patients produced more biofilm exoproteins (mean

concentration of 309 μg/ml +/- 146.0 vs 166 μg/ml +/- 74.5 in CRSwNP and CRSsNP respectively, p<0.001) and showed higher metabolic activity (mean 19983 OD +/- 1953 vs 18980 OD +/-1355, p<0.001). However, the biofilm biomass and planktonic protein production were not different when comparing clinical isolates from CRSwNP and CRSsNP patients (p>0.05) (Figure 3.1, A-D).

Compared to clinical isolates from TLO-negative CRS patients, clinical isolates from TLO-positive CRS patients demonstrated higher biofilm exoprotein concentration (mean 401.2 μg/ml +/- 118.2 vs 169.6 μg/ml +/- 64.2 in CRSwNP and CRSsNP respectively, p<0.0001), higher metabolic activity (mean 20301 OD +/-2135 vs 19155 OD +/-1451, p<0.001) and higher biofilm biomass (mean 1.17 OD +/- 0.38 vs 0.97 OD +/- 0.55, P=0.02). In contrast, the planktonic protein production was not different when comparing clinical isolates from TLO-positive and TLO-negative CRS patients, (p>0.05) (Figure 3.1 E-H).

Spearman correlation demonstrated a strong positive correlation between biofilm exoprotein concentration and biofilm metabolic activity ($r = 0.715$; $p < 0.0001$) or biofilm biomass ($r=0.68$, $p<0.0001$) and between the biofilm biomass and biofilm metabolic activity ($r=0.61$, $p<0.0001$) (Figure 3.2). There were no significant correlations between exoprotein concentrations of clinical isolates in biofilm form and planktonic form and with colony forming unit (CFU) counts or eDNA content of 48-hour biofilms (data not shown).

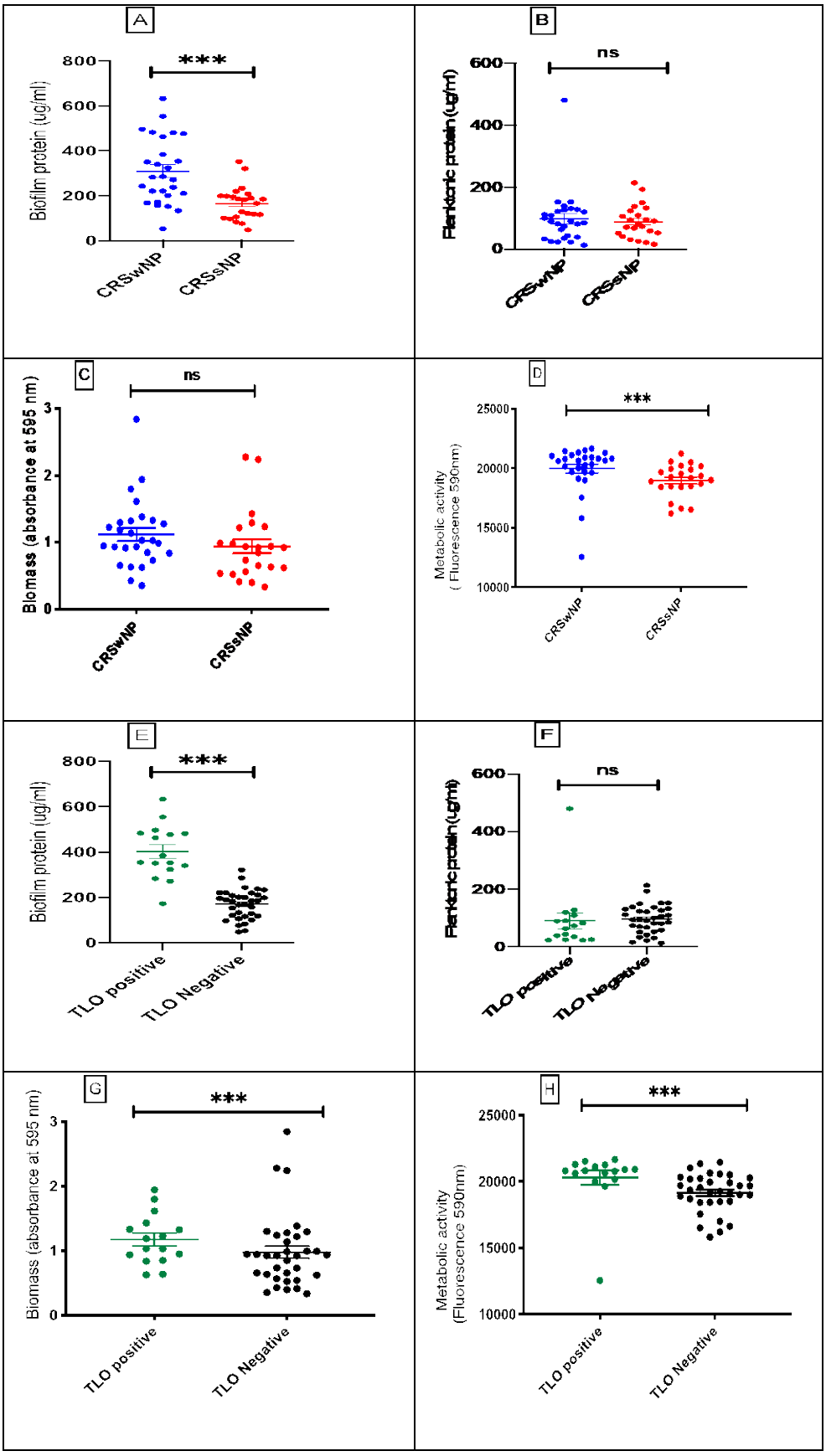

A
***
Biofilm protein (ug/ml)
800
600
400
200
0
CRSwNP
CRSsNP
B
ns
Planktonic protein (ug/ml)
600
400
200
0
CRSwNP
CRSsNP
C
ns
Biomass (absorbance at 595 nm)
3
2
1
0
CRSwNP
CRSsNP
D
***
Metabolic activity
(Fluorescence 590nm)
25000
20000
15000
10000
CRSwNP
CRSsNP
E
***
Biofilm protein (ug/ml)
800
600
400
200
0
TLO positive
TLO Negative
F
ns
Planktonic protein (ug/ml)
600
400
200
0
TLO positive
TLO Negative
G
***
Biomass (absorbance at 595 nm)
3
2
1
0
TLO positive
TLO Negative
H
***
Metabolic activity
(Fluorescence 590nm)
25000
20000
15000
10000
TLO positive
TLO Negative

Figure 3.1: Exoprotein production, biofilm biomass and metabolic activity in S. aureus clinical isolates from CRS patients

*(A-H) Biofilm exoproteins (*μg/ml) (A, E), planktonic exoproteins *(*μg/ml) (B, F), biofilm biomass (OD at 595 nM) (C, G) and biofilm metabolic activity (OD at 590 nM) (D, H) in *S. aureus* clinical isolates harvested from CRSwNP and CRSsNP patients (A-D) or TLO-positive and TLO-negative CRS patients (E-H). n=50 clinical isolates

*Measurements reported as mean ± standard error of mean (SEM). Mann-Whitney: ***p <0.001.*

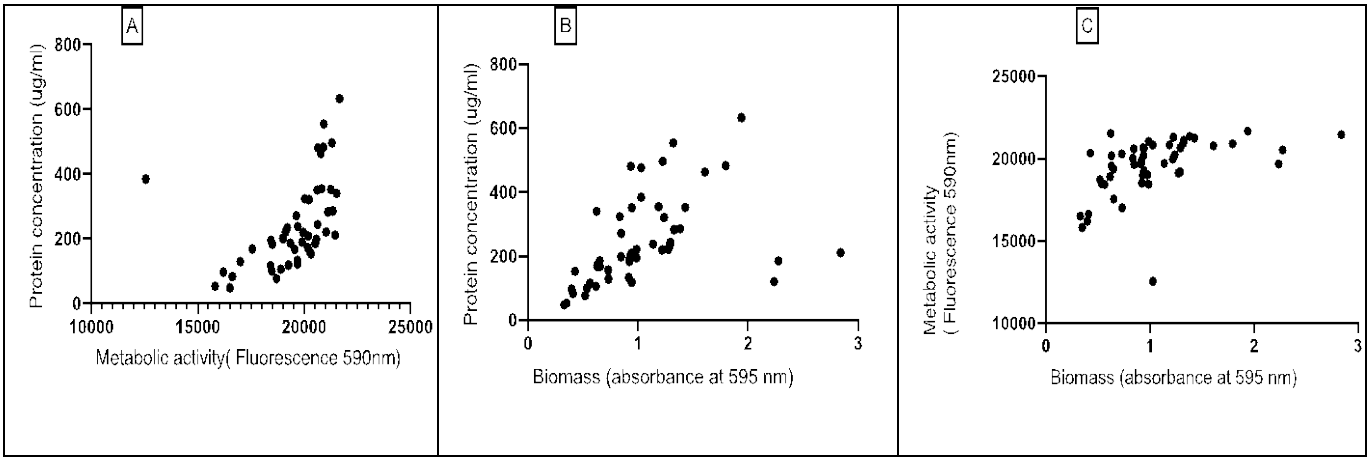


Figure 3.2: Correlation between biofilm exoprotein, biofilm metabolic activity and biofilm biomass as a scatter plot.

*Spearman correlation between biofilm exoprotein (*μg/ml) and metabolic activity (OD 590 nm) (A), *biofilm exoprotein (*μg/ml) and biofilm biomass (OD 595 nm) (B),

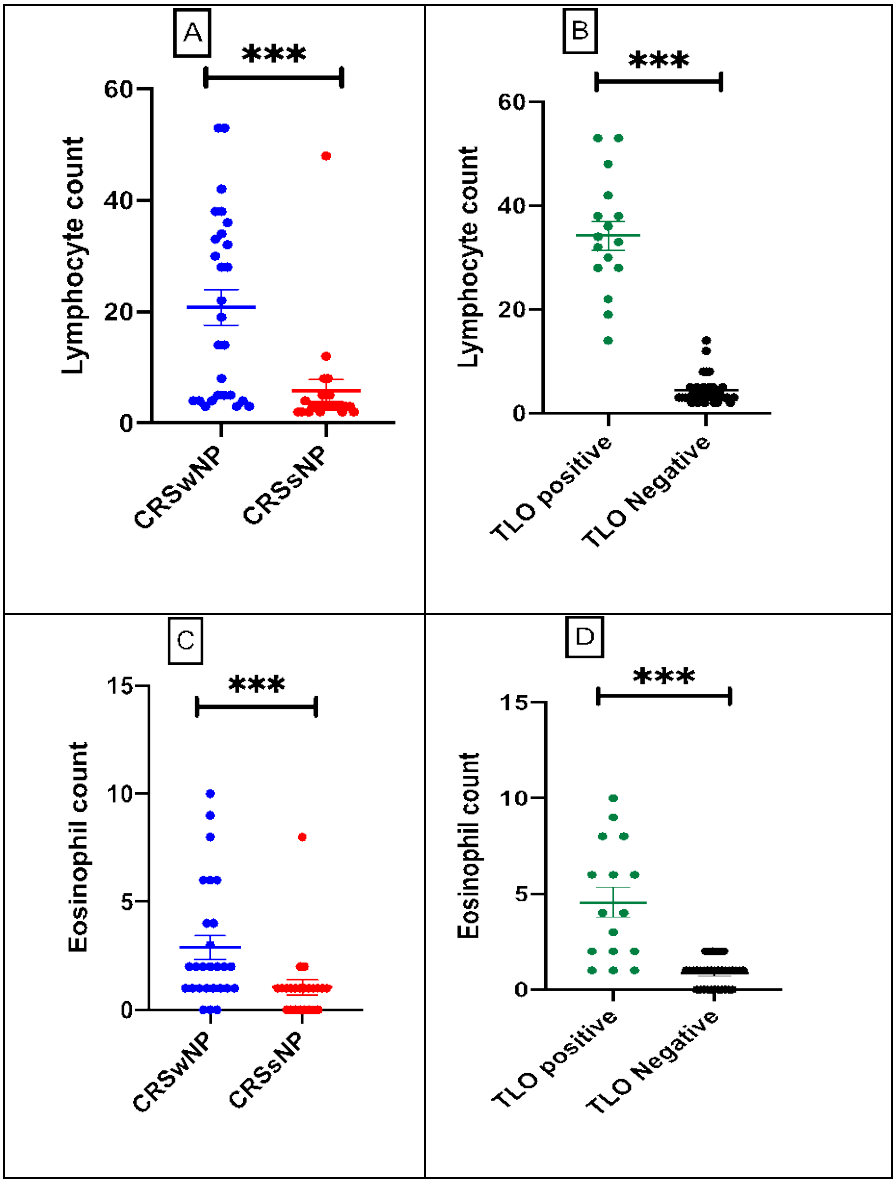
metabolic activity (OD 590 nm) and biofilm biomass (normalised to biomass *of ATCC 25923)* (OD 595 nm) (C). *n=50 S. aureus clinical isolates with averaged values of 3 biological replicates and 6 technical replicates.*

3.4.3. Inflammatory cell counts correlate with biofilm protein levels

Sino nasal tissue samples from CRS patients corresponding to all 50 clinical isolates were stained with H&E and immune cells counted. Compared to CRSsNP patients and TLO-negative CRS patients, CRSwNP and TLO-positive CRS patients had significantly increased mean total inflammatory cell counts (mean 20.8 cells/HPF +/- 16.69 and 34.2 cells/HPF +/- 11.19 vs 5.8 cells/HPF +/- 9.5 and 4.3 cells/HPF +/- 2.75 in CRSwNP and TLO-positive vs CRSsNP and TLO-negative respectively, $p<0.001$) and increased number of eosinophils (mean 2.8 eosinophils/HPF +/- 2.8 and 4.6 eosinophils/HPF +/- 3.05 vs 1.0 eosinophils/HPF +/- 1.63 and 0.8 eosinophils/HPF +/- 0.70 in CRSwNP and TLO-positive vs CRSsNP and TLO-negative respectively, $p<0.001$) (Supplementary Figure 3.1).

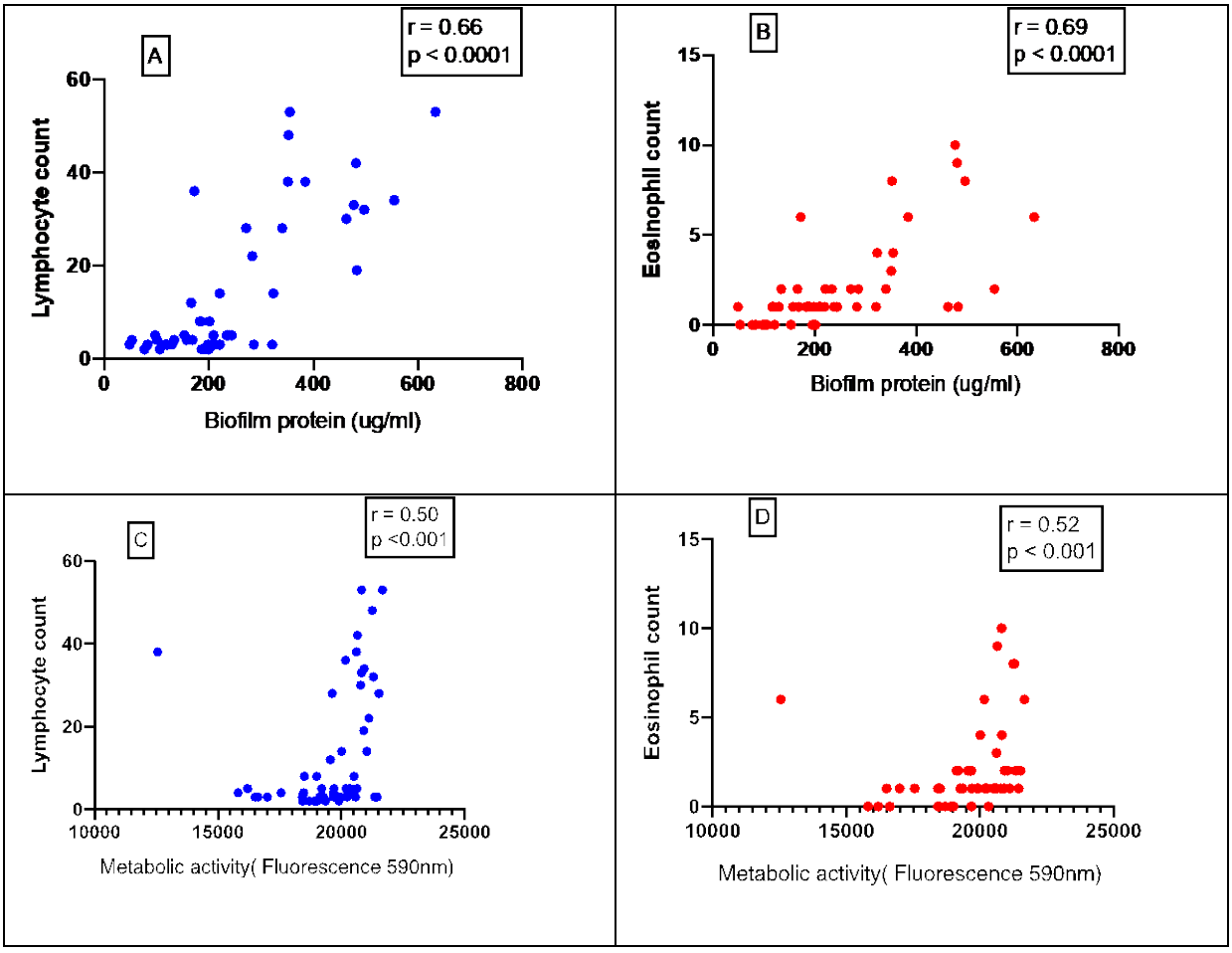
There was a significant strong positive correlation between the number of lymphocytes and eosinophils and the concentration of biofilm exoproteins secreted by corresponding clinical isolates ($r =0.66$ and $r = 0.69$ for lymphocyte and eosinophil numbers respectively, $p < 0.0001$) and their metabolic activity ($r =0.50$ and $r = 0.52$ for lymphocyte and eosinophil numbers respectively, $p<0.001$). There was also a

correlation between eosinophil cell number and biofilm biomass ($r=0.33$, $p<0.05$) but not between the number of lymphocytes or eosinophils and planktonic protein production ($p>0.05$) (Figure 3.3 and results not shown).



Supplementary figure 3.1: CRSwNP and TLO-positive CRS patients produce higher inflammation

CRSwNP had significantly high inflammatory cell counts lymphocytes (p=0.00012, Fig A) and eosinophils (p =0.00084, Fig C) and TLO-positive patients had increased inflammatory cell counts lymphocytes (p= 3.839190e-08, Fig B) and eosinophils (p=2.036803e-06, Fig D) respectively. Measurements reported as mean ± standard error of mean (SEM), (p<0.0001, Mann-Whitney).



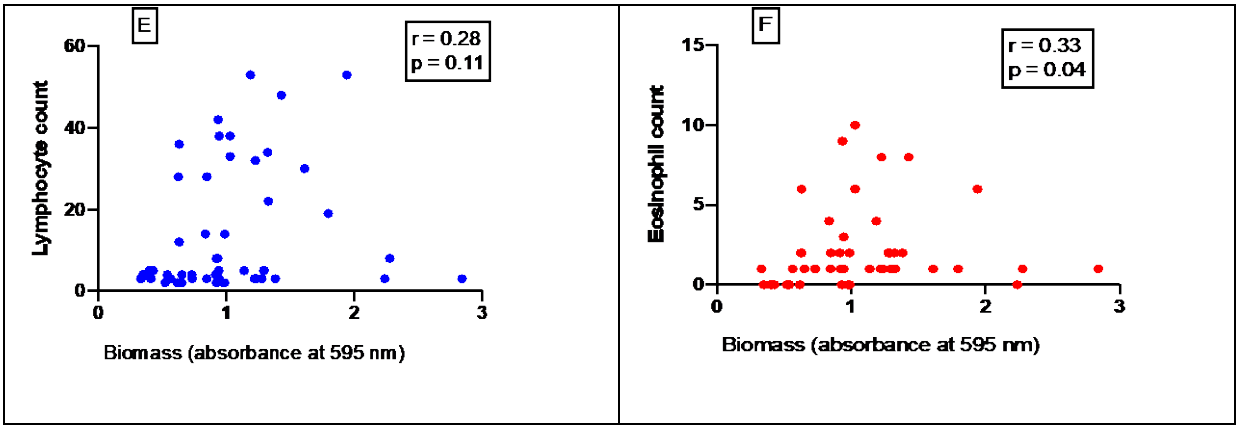


Figure 3.3: Correlation between biofilm exoproteins, biofilm metabolic activity, biofilm biomass and inflammatory cell counts.

*Spearman correlation between lymphocyte (A, C, E) and eosinophil counts (B, D, F) and biofilm exoprotein (*μg/ml) (A, B), biofilm metabolic activity (OD 590 nm) (C, D), biofilm biomass (OD 595 nm) (E, F). *n=50 S. aureus clinical isolates and corresponding tissue sections with averaged cell counts of 6 High Power Fields/tissue section and 3 biological and 6 technical replicates for each of the 50 clinical isolates.*

3.4.4. Elevated Interleukin-6 induction by biofilm exoproteins

Thirty-nine clinical isolates (from 24 CRSwNP and 15 CRSsNP patients) were used where proteins from planktonic cultures and biofilms were purified and applied to air liquid interface cultures (ALI) of primary human nasal epithelial cells (HNECs). HNEC-ALI cells were obtained from seven CRSsNP patients with ages ranging from 35-75 yrs. These patients were nonsmokers with no history of asthma or allergy.

Interleukin-6 (IL-6) ELISA was performed on supernatants from the basal compartment of the HNEC-ALI cultures after 24 hours exposure to biofilm or planktonic exoproteins. A significant increase in IL-6 was seen in cells treated with biofilm exoproteins compared to those treated with planktonic exoproteins ($p < 0.001$; Wilcoxon). All isolates were tested in comparison with exoproteins from planktonic ATCC13565 and negative control (nutrient broth).

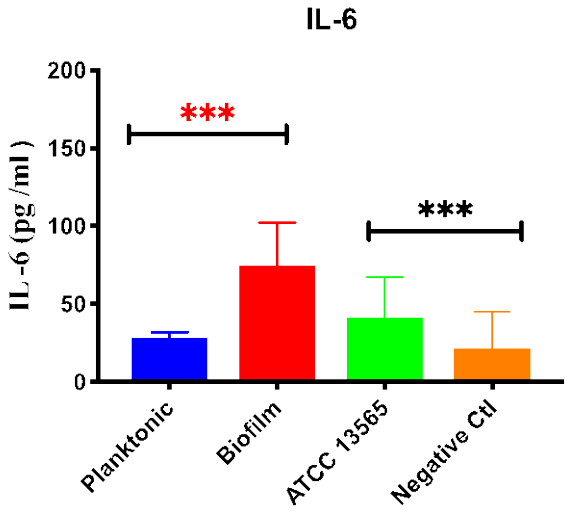


Figure 3.4: IL-6 protein levels in supernatants of primary human nasal epithelial cells challenged with S. aureus biofilm and planktonic exoproteins.

Exoproteins (100 μg/ml) of *S. aureus* planktonic and biofilm cultures (n=39, 2 technical replicates each) and of planktonic *ATCC 13565 or negative control (nutrient broth) were applied to* primary human nasal epithelial cells at air-liquid interface (HNEC-ALI, n=7) for 24 hours, followed by measuring IL-6 concentration (pg/ml). *** *p < 0.001, Wilcoxon signed rank test* ****p < 0.001, Mann-Whitney.*

3.5 DISCUSSION

This study is the first to link *in vitro S. aureus* biofilm properties with the severity and type of inflammation in CRS, indicating that the *S. aureus* biofilm matrix is of critical importance in the pathophysiology of CRS. The study shows that *S. aureus* clinical isolates harvested from CRSwNP patients and TLO-positive CRS patients exhibit higher *S. aureus* biofilm exoprotein production and higher metabolic activity than *S. aureus* biofilms from CRSsNP and TLO-negative patients. There was a significant strong positive correlation between the number of lymphocytes or eosinophils and the concentration of biofilm exoproteins secreted by corresponding Clinical isolates and their metabolic activity. In contrast, exoprotein concentrations from planktonic cells did not correlate with inflammatory cell infiltration.

Around 10% of CRS patients suffer from recalcitrant CRS. Those patients often have *S. aureus* mucosal biofilms, tertiary lymphoid organs, tissue eosinophilia and comorbid asthma [30,32,410,451,465,466]. However, given that *S. aureus* and mixed species mucosal biofilms are also found in healthy sinuses, the exact role of *S. aureus* mucosal biofilms in the pathophysiology of CRS is unknown [401,453]. A number of studies have implicated *S. aureus* toxins, mainly enterotoxins, in the pathophysiology of CRSwNP[467-469]. A more recent study furthermore showed that *S. aureus* superantigens expand $CD4^+$ T cells exhibiting Th2 phenotypes and that such influx in seen in CRSwNP patients in direct relationship with CRS disease recalcitrance[470] .

This study adds to those findings in that a higher grade of eosinophilic inflammation and thus Th2 skewing was seen in patients that harbour *S. aureus* isolates that appear metabolically more active, have a higher biomass and produce more exoproteins. Our previous studies have already shown that proteases and exoproteins from planktonic *S. aureus* disrupt the mucosal barrier of HNEC-ALI cultures [406-408]. A more recent study has furthermore shown that *S. aureus* biofilm exoproteins have a more pronounced detrimental effect on the mucosal barrier structure and function than equal concentrations of exoproteins from planktonic counterparts [455]. The study furthermore showed a dose-response relationship between biofilm exoprotein challenge and mucosal barrier disruption. Also, CRSwNP patients have been shown to have a defective mucosal barrier along with reduced expression of tight junction proteins [434,435,471]. A study by Wood et al has furthermore demonstrated that biofilms that were present on a disrupted epithelial layer were associated with more T lymphocytes and more macrophages than areas of mucosa without bacteria present [472]. Together with the results of the present study, it is therefore postulated that thicker, metabolically more active *S. aureus* biofilms are characteristic of recalcitrant CRSwNP patients also in vivo. The increased production of exoproteins is thought to result in a dose-dependent mucosal barrier disruption and immune activation with Th2 polarisation in those patients. Interestingly, CRS patients with corresponding *S. aureus* biofilms producing more or less than 320 µg/ml exoproteins were TLO positive in 13/13 (100%) and 3/26 (11%) cases respectively and none of the biofilms that produced less than 170 µg/ml exoproteins were found in TLO positive patients. Also, eosinophil counts showed a significant

increase in TLO positive CRS patients, which is in line with our previous findings that recalcitrant CRS patients demonstrate TLOs in correlation with tissue eosinophilia and that TLO positive CRS patients demonstrate Th2- gene expression signatures[451,452]. TLOs are a pathological hallmark of severe chronic inflammation and have been found in chronic obstructive pulmonary disease (COPD), inflammatory bowel disease (IBD), cancer, transplant rejection and auto-immune diseases[473] . They have recently also been found in the context of eosinophilic recalcitrant CRS[451,452] implying persistent antigenic stimulation in those patients. In the context of infections, only a few microbial species (including the *Influenza* virus*, Borrelia burgdorferi, Helicobacter* sp.) have been associated with TLOs[473] and the triggers and pathways of TLO induction and maintenance and their role in the context of CRS are unknown. Our data showing that TLO formation in CRS patient tissues is linked to *S. aureus* biofilm properties and exoprotein production provides a platform for understanding potential TLO induction and maintenance pathways. The gradual increase in inflammation, Th2 polarisation and TLO positivity in relation to *S. aureus* biofilm properties seen in the present study indeed imply that biofilm exoproteins might trigger inflammation in those patients. More research is needed in relevant animal models to evaluate a potential causative relationship between *S. aureus* biofilm produced exoproteins and inflammation with Th2 polarisation and TLO-formation.

Interestingly, the strong relationship between *in vitro* biofilm properties and in situ levels of inflammation indicate lasting phenotypic changes in *S. aureus* clinical isolates, even when grown in optimal conditions *in vitro*. The molecular basis of this

finding warrants further investigation, in particular to evaluate potential underlying genotypic and/or epigenetic changes in those isolates.

Foreman et al has shown that the identity of bacteria within the biofilm determines the virulence nature of the biofilm and influences clinical outcomes. [403] Specifically, they showed that *S. aureus* dominant biofilms were associated with more severe disease and this is in line with our current findings. However, studies universally show that the sinonasal microbiome harbours many genera and that mucosal biofilms are often polymicrobial [27,403,474]. In view of the polymicrobial nature of the sinonasal microbiome, it is unclear how *S. aureus* biofilm properties affect other genera present within the niche. In particular whether *S. aureus* biofilm properties such as exoprotein production have a direct role in eliciting inflammation or merely mark a more virulent microbiome with also other potential genera present in that biofilm having an altered phenotype.

Future animal models to study the in vivo immune response to *S. aureus* exoproteins would enable us to better understand the inflammatory heterogeneity of CRS disease and create avenues to determine new treatment modalities based on these findings.

3.6 CONCLUSION

This study indicates strain-specific differences in *S. aureus* biofilm matrix quantity and metabolic activity that are directly linked to the severity and type of inflammation in CRS. Our study shows that *S. aureus* clinical isolates from CRSwNP patients exhibit higher *S. aureus* biofilm exoprotein production and metabolic activity than clinical isolates harvested from CRSsNP patients in correlation with eosinophil and lymphocyte cell numbers. The findings provide a platform and rationale for the development of new and improved treatment strategies for recalcitrant CRS patients.

CHAPTER 4:

Reduced antimicrobial susceptibility of Staphylococcus aureus biofilms correlates with chronic rhinosinusitis eosinophilic inflammation

Conducted in the Department of Otolaryngology – Head and Neck Surgery

The University of Adelaide, Adelaide, Australia

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Contribution to the Paper	Conduct of study,Collection of data, interpretation of results drafting and editing the manuscript		
Overall percentage (%)	70%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Signature		Date	3/7/2020

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Contribution to the Paper Overall percentage (%)	Designing the study, supervision, interpretation of data, drafting, editing of manuscript and final approval for submission 4%		
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Signature		Date	3/7/2020

Reduced antimicrobial susceptibility of *Staphylococcus aureus* biofilms correlates with chronic rhinosinusitis eosinophilic inflammation

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4.1 ABSTRACT

Abstract

Introduction:

Bacterial biofilms play a crucial role in chronic rhinosinusitis (CRS), leading to persistence of symptoms. Bacteria within biofilms resist treatment with antibiotics, however, the relationship between biofilm properties, antibiotic susceptibility and measures of inflammation has not been defined. This study aimed to determine the susceptibility profile of *S. aureus* clinical isolates from patients diagnosed with CRS to four commonly used antibacterial drugs in planktonic and biofilm forms in relation to biofilm properties and severity of disease.

Methods:

Clinical *S. aureus* isolates and tissue were collected from CRS patients at the time of endoscopic sinus surgery. Antimicrobial susceptibility of isolates to Augmentin (amoxicillin and clavulanate, Aug), Clindamycin (Clin), Azithromycin (Azi) and Mupirocin (Mupi) was determined by minimum inhibitory concentration (MIC) and minimum biofilm eradication concentration (MBEC) by the broth microdilution technique. 48-hour biofilms were evaluated for quantity of exoprotein production, biomass, determined by crystal violet staining and metabolic activity measured via Alamar blue assay. Disease severity was measured via patient reported disease severity scores and histopathology. Spearman correlation was used to correlate the

MIC/ MBEC with various parameters and Mann Whitney test was used for comparison of subgroups using the scipy Scientific Python library.

Results: 10 *S. aureus* clinical isolates and ATCC 25923 were evaluated. The MBEC was significantly higher than the MIC for all four antibiotics ($p < 0.001$). These clinical isolates had variable biofilm biomass, metabolic activity and exoprotein production. The biofilm exoprotein production, biomass and metabolic activity each significantly correlated with the MBEC values for Clindamycin and Mupirocin. There was a significant correlation between lymphocyte cell counts and MBEC for Clindamycin and eosinophil cell counts correlated with MBEC for Amoxicillin- clavulanate and Mupirocin. Disease severity scores such as Lund-Mackay scores, Adelaide scores and SNOT 22, did not show any relationship with the MBEC or MIC values for all four antibiotics.

Conclusion: *S. aureus* biofilm properties including exoprotein production, biomass and metabolic activity positively correlated with MBEC values for different antibiotics. MBEC values correlated with inflammatory cell counts. Antibacterial therapy to treat CRS should be aimed at eliminating biofilms, for which MBEC is a better guide to determine the antibiotic susceptibility than the MIC.

4.2 INTRODUCTION

Staphylococcus aureus, the most frequent pathogen associated with chronic rhinosinusitis (CRS) produces biofilms leading to recalcitrant disease [418]. Studies indicate that the presence of biofilms predicts the persistence of symptoms of CRS and evidence of inflammation post-surgery [32,465]. Biofilms are bacterial communities within a self-produced extracellular polymeric matrix made of polysaccharides, proteins, lipids and extracellular DNA[419]. Planktonic forms differ from biofilm forms within the same bacterial species due to differences in their phenotypic and genotypic expression[475]. Studies have shown that bacteria within biofilms exhibit tolerance to conventional antibiotics 10 to 1000 times greater than planktonic forms[135,476-478]. Biofilm producing bacteria are associated with recurrent nosocomial infections and chronic infectious diseases leading to prolonged hospital stays, thereby increasing morbidity and mortality[479,480]. Hence elimination of biofilms is crucial for eradicating chronic relapsing infections in the context of diseases such as recalcitrant CRS (rCRS). Minimum inhibitory concentration (MIC) is usually determined in diagnostic labs as a standard method for antibiotic susceptibility testing for planktonic organisms and remains an important reference for the treatment of acute infections. However application of MIC to guide therapeutic protocols and dosages to treat chronic infections due to biofilm forms is ineffective[481]. The concentrations of antibiotics used in standard anti-microbial susceptibility testing for planktonic forms do not predict the concentrations needed to eradicate biofilms[482]. Studies have demonstrated that sub-inhibitory doses of antibiotics either impede or stimulate biofilm formation, hence adequate dosing is

imperative when treating biofilm-related infections [483]. Minimum biofilm eradication concentration (MBEC) is known to be a better predictor of antibiotic concentrations required for eradication of biofilms[484]. Determining the MBEC would prove clinically useful to assess and characterize the relationship between antibiotic dosages needed for the treatment of biofilm-associated infections and the nature and degree of biofilm and its biomass[485]. Using MBEC rather than MIC values to support clinical decision making and defining the dosage of antibiotics that can eradicate biofilms may improve clinical outcomes and decrease the burden of antimicrobial resistance formation[486]. Biofilm properties such as biomass, protein production and metabolic activity differ between isolates, but it is unclear how those properties affect antibiotic susceptibility. Efforts have been taken to develop laboratory biofilm models that depict *in vivo* conditions in cystic fibrosis [487] and chronic wound infections[488], but the validity of these models is lacking. Hence, we aimed to determine the susceptibility profile of *S. aureus* clinical isolates from patients with rCRS to commonly used antibacterial agents grown in planktonic and in biofilm forms in relation to severity of disease and biofilm properties.

4.3 METHODS

4.3.1. Clinical Strains and Culture collection

All bacterial isolates and tissue samples were obtained from the sino nasal cavities of patients undergoing endoscopic sinus surgery for a problem of chronic rhinosinusitis (CRS) at The Queen Elizabeth Hospital (TQEH) in Adelaide, South Australia. Ethics approval was obtained from The Queen Elizabeth Hospital Human Research Ethics Committee, Woodville, SA, Australia (HREC/18/CALHN/69) and all patients signed an informed consent form. CRS patients were classified as CRS with nasal polyps (CRSwNP) or CRS without nasal polyps (CRSsNP) according to the international CRS (EPOS) diagnostic criteria [4,396]. Clinical isolates were cultured and identified by an independent pathology laboratory (Adelaide Pathology Partners, Adelaide South Australia) and were stored in glycerol stocks at - 80°C. Pregnant patients, patients with systemic immunosuppressive diseases, age less than 18 years and active smokers were excluded. Clinical isolates (CIs 1-10) of *Staphylococcus aureus* from patients with CRS were included in the study. *Staphylococcus aureus* ATCC 25923 was obtained from the American Type Culture Collection (ATCC, Manassas, USA).

Severity of disease was assessed by preoperative patient reported Sino-Nasal Outcome Test (SNOT -22) questionnaires[420], objective CT-scan scoring of Lund Mackay (LMS) [461] and disease specific 5 questions-based Adelaide Severity score (ADS)[421] (refer to Table 4.1).

Table 4.1. Demographics and disease severity scores of S. aureus clinical isolates

I.D	Age	Sex	Polyp Status	Asthma	Planktonic protein (μg/ml)	Biofilm protein (μg/ml)	L	E	Biomass	Alamar Blue	LMS	SNOT 22	ADS
CL1	52	M	CRSsNP	Positive	25.60	352.07	48	8	1.429	21250	21	38	NA
CL2	29	F	CRSsNP	Negative	91.09	189.79	2	1	0.921	19905	NA	78	18
CL3	73	F	CRSwNP	Negative	25.20	496.60	32	8	1.226	21308	NA	No score	NA
CL4	59	M	CRSwNP	Positive	480.59	632.95	53	6	1.94	21674	10	76	NA
CL5	50	F	CRSwNP	Negative	120.16	481.25	42	9	0.934	20652	4	No score	NA
CL6	50	M	CRSwNP	Positive	128.40	477.32	33	10	1.029	20817	NA	25	13
CL7	58	M	CRSwNP	Positive	23.09	323.54	14	4	0.836	20017	18	36	14
CL8	64	F	CRSwNP	Positive	82.00	354.27	53	4	1.187	20833	NA	34	12
CL9	48	F	CRSsNP	Negative	13.49	201.31	8	0	0.93	19000	14	63	16
CL10	58	M	CRSwNP	Positive	43.73	271.18	28	2	0.848	19641	19	34	25

CL= clinical Isolate
L= Lymphocytes
E= Eosinophils
NA= Not available
ADS = Adelaide Disease Severity Score
CRSsNP= chronic rhinosinusitis without nasal polyps
CRSwNP= chronic rhinosinusitis with nasal polyps

4.3.2 Antibacterial Agents

The following antibiotics (all from Sigma) were used in this study based on their relevance in treatment of CRS: Augmentin (amoxicillin and clavulanate, Aug) Clindamycin (Clin), Azithromycin (Azi), Mupirocin (Mupi). They were dissolved at a concentration of 10 mM in sterile Milli-Q Water (Milli-Q Plus 185; Merck Millipore, Darmstadt, Germany) except for Mupirocin which was dissolved in dimethyl sulphoxide (DMSO, Sigma-Aldrich, St. Louis, USA). All were stored at −20 °C for further use. For minimum inhibitory concentration (MIC) antibiotic solutions were made in sterile microtiter plates containing Mueller–Hinton broth (MHB) (Oxoid, UK) recommended by the Clinical and Laboratory Standards Institute (CLSI). Mupirocin was first dissolved in DMSO and was subsequently diluted in MHB. The antibiotic solutions were prepared fresh on the day of experiments after filter sterilised using a 0.22 μm filter.

4.3.3 Antimicrobial Susceptibility and MIC determination

Microdilution method was used to determine the MIC as described by Wiegand et al[344]. Briefly, bacterial clinical isolates grown overnight (18-24 hrs) on 1.5 % nutrient agar plates were suspended in 0.9% normal saline at 0.5 MacFarland Units (MFU). The bacterial suspension was diluted 1:100 in MH broth in a 96 well plate containing serially diluted antibiotics (concentration range 0.125–64 μg/ml). The micro titre plates were incubated at 37 °C for 18–24 h. The MIC is the lowest concentration of

antimicrobial agent that completely inhibits growth of the organism in the microdilution wells by measuring the optical density at 595 nm using a plate reader from BIO-RAD laboratories, CA, USA. All MIC assays were performed as 2 technical and 3 biological replicates. A positive growth control (GC) containing MH broth and bacterial suspension along with a negative sterility control (SC) containing only MH broth were included in all tests performed. The MIC interpretation of susceptible and resistant was based on the breakpoints and definitions as per CLSI guidelines[340].

4.3.4. Micro-titre Biofilm Eradication Assay

Susceptibility assays in microtitre plates were performed as described [489]. Briefly, bacterial clinical isolates were subcultured on nutrient agar plates and isolated single colonies in 0.9% saline were adjusted to 1.0 ± 0.1 McFarland Units. 150 ul of bacterial suspension was added to black 96 well plates along with controls and incubated for 48 hrs at 37°C on a rotator set at 70 rpm (3D Gyratory Mixer, Ratek Instruments, Australia) for the formation of biofilms. Antibiotics Augmentin (Aug), Clindamycin (Clin), Azithromycin (Azi) and Mupirocin (Mupi) were added in serially diluted manner at a concentration range 0.25 –128 µg/ml and plates incubated for a further 24 h at 37°C on the rotator to determine the MBEC (minimum biofilm eradication concentration) of antibiotics. After the incubation period wells were washed twice with phosphate buffered saline (PBS) to remove loosely adherent planktonic cells following which, 200 ul of 1:10 diluted Alamar Blue reagent in nutrient broth (Life Technologies, Scoresby, Australia) was added to all wells. To

determine the biofilm eradication ability of the antibiotics the plates were read at hourly intervals using the FLUO star OPTIMA plate reader (BMG Lab tech, Ortenberg, Germany) using fluorescence at λ excitation = 530 nm/λ and emission= 590 nm, with maximum intensities reached at 6 hours incubation period. The percentage eradication of biofilm after treatment with antibiotics was calculated as

% BE= [(FC – FT) / FC] x 100% Antimicrobial activity of the antibiotics was expressed as the percentage of biofilm eradication (%BE), where FC is the fluorescence of the untreated biofilms used as controls (100% bacterial growth) and FT is the fluorescence of treated biofilms, the FC and FT were corrected for background fluorescence from sterile media (SC). All eradication experiments were performed as 2 technical and 3 biological replicates.

4.3.5. Biofilm biomass quantification by crystal violet staining

Determination of biofilm biomass was performed as previously described [490]. Briefly, biofilm plates were set up using *S. aureus* as described in section 2.4, except that clear walled plates were used instead of black. Plates were incubated at 37˚C for 48 h on a rotating plate set at 70 rpm (3D Gyratory Mixer, Ratek Instruments, Australia). Following the incubation all the liquid from each well was aspirated and rinsed twice with 1x PBS to remove the planktonic cells. Crystal violet (0.1% in water, 180µL/well) was added and incubated for 15 min at room temperature to stain the biofilms. The wells were washed with 200 ul sterile MilliQ water and after drying the plates, 180 µL/well of 30% acetic acid was added and incubated on a plate shaker until crystal

violet was solubilized and the solution in the wells appeared homogeneous (≅1 hour). A BIORAD plate reader was used to measure absorbance at 595 nm. Biomass assays were performed as 6 technical replicates, 3 biological replicates including *S. aureus* ATCC 25923 in each experiment as a reference control. The biomass of each isolate (OD sample, ODs) was calculated by subtracting the OD of sterile control (nutrient broth) and normalised with the OD of the ATCC 25923 reference strain.

4.3.6. Planktonic and Biofilm Exoprotein concentration and quantification

Biofilm cultures for clinical isolates (CL1-10) were set up with opacity adjusted to 1.0 ± 0.1 Mac Farland units following which it was diluted to 1:15 in NB as per the 96 well plates and incubated at 37°C for 48 hours on a rotating platform (3D Gyratory Mixer; Ratek Instruments, Boronia, Australia) at 70 rpm. Following incubation, the medium was collected and centrifiged at 4 ºC for 10 minutes at 1500 x g and passed through a 0.22 µm syringe filter. Proteins were concentrated using 3 KDa Pierce Protein Concentrators (Thermofisher, IL, USA) according to the manufacturer's instructions.

Planktonic cultures were set up for the same clinical isolates of *S. aureus* by inoculating nutrient broth with culture starting at optical density (OD) of 0.01 at 600 nm. The growth curve was followed, and supernatants were collected at 6 hours (stationary phase), followed by centrifugation and filtering using the same protocol as for biofilm supernatants. Supernatants of both planktonic and biofilm forms were

stored at -80 ºC until required. Protein quantification of biofilm and planktonic concentrates was performed using the Invitrogen Nano Orange Protein Quantitation Kit (Molecular Probes, Eugene, Oreg, USA) according to manufacturer's instructions. Fluorescence was measured using a FLUOstar OPTIMA plate reader (BMG Labtech, Ortenberg, Germany) with excitation at 485 nm and emission at 590 nm.

4.3.7. *S. aureus* biofilm formation and Alamar blue assay

Isolated single bacterial colonies of *S. aureus* from nutrient agar plates were suspended in 0.9% saline and adjusted to 1.0 ± 0.1 McFarland units. The Alamar blue assay was performed in black 96-well microtiter plate (Costar; Corning, Inc, Corning, NY, USA) by inoculating the bacterial suspension diluted 1:15 in nutrient broth (Oxoid, UK). A total of 150 µL was added into each well and incubated for 48 hours onto a rotating platform (3D Gyratory Mixer; Ratek Instruments, Boronia, Australia) at 70 rpm. The biofilm was washed with PBS twice, following which the metabolic activity of biofilm was determined by adding the Alamar blue reagent (Life Technologies, Scoresby, Australia) which consisted of 200 µL of a freshly prepared 10% dilution in NB. The plates were incubated on the rotating platform at 37° C for 7 hours, hourly fluorescence was recorded using the FLUOstar Optima plate reader at λ excitation = 530 nm/λ emission = 590 nm.

4.3.8. Staining of tissue sections with Hematoxylin & Eosin (H &E)

Hematoxylin & Eosin (H & E) (Thermo Fisher Scientific, Waltham, MA, USA) staining technique was used to stain the slides made from paraffin embedded tissue; 4 µm

thick sections were made with microtome (Thermo Scientific HM 325 Rotary Microtome). Nano zoomed slides (whole-slide imaging technology (WSI) on Nano Zoomer Digital Pathology system (Hamamatsu Photonics, Hamamatsu City, Japan) were magnified to 40 X. Five areas were selected measuring 0.035 mm^2 (equals to high-power field using the light microscope (Nikon Eclipse 90i Microscope; Nikon Corporation,Tokyo, Japan) and lymphocytes and eosinophils within this area were counted as described [462].

4.3.9. Statistical Analysis

Spearman correlation was used to correlate the MIC/ MBEC of planktonic and biofilm isolates with protein concentration, biomass, inflammatory cell counts and various clinical scores such as the SNOT 22, Adelaide disease severity and Lund Mackay scores. Mann Whitney test was used for comparison of subgroups CRSwNP and CRSsNP. All statistical analysis was performed using the scipy Scientific Python library. Statistical significance was taken at the traditional alpha=0.05 level.

4.4 RESULTS

4.4.1. Clinical Isolates

Ten patients with rCRS undergoing surgery at TQEH were selected based on their clinical symptoms and clinical course to represent various levels of disease severity. This included seven CRSwNP and three CRSsNP (age 29-73 years, 5 females and 5 males); six patients among them were asthmatics. Details of disease severity scores are depicted in Table 4.1. *S. aureus* clinical isolates and *S. aureus* ATCC 25923 were cultured as planktonic form and biofilm forms.

4.4.2. Determination of the MIC for *S. aureus* Planktonic form

MIC was determined using the broth microdilution method for four antibiotics Amoxicillin-clavulanate (AUG), Clindamycin (CLI), Azithromycin (AZI) and Mupirocin (MUP). Their susceptibility profile is shown in Table 4.2 and had a similar pattern as compared with their clinically reported susceptibility pattern. Accordingly all strains were reported susceptible to Augmentin and Mupirocin and CL6 and CL10 were resistant to Clindamycin while Azithromycin was not tested for *S. aureus* clinical isolates by the independent pathology laboratory (Adelaide Pathology Partners, Adelaide South Australia) as per the guidelines[340].

Table 4.2: Minimum Inhibitory Concentration 90 (MIC 90)

	Antibiotic (μg/mL)			
Isolate	**Amoxicillin-clavulanate (AUG)**	**Clindamycin (CLI)**	**Azithromycin (AZI)**	**Mupirocin (MUP)**
CI1	2	0.25	4	0.25
CI 2	2	0.25	4	0.25
CI 3	0.5	0.25	4	0.25
CI 4	0.5	0.25	8	0.25
CI 5	0.5	0.25	4	0.25
CI 6	1	4	64	0.5
CI 7	1	0.25	4	0.5
CI 8	2	0.25	4	0.25
CI 9	2	1	8	0.25
CI 10	1	8	8	0.25
ATCC 25923	0.125	0.125	0.125	0.25

MIC90 = Minimum Inhibitory Concentration required to inhibit the growth of 90% of organisms in planktonic form. ATCC= American type culture collection

4.4.3. Determination of the MBEC for *S. aureus* Biofilm forms

The Minimum Biofilm Eradication Concentration (MBEC) of *S. aureus* biofilm was tested with four antimicrobial agents by the Broth Microdilution method: Amoxicillin-clavulanate (AUG), Clindamycin (CLI), Azithromycin (AZI) and Mupirocin (MUP). Ten clinical isolates were tested, along with the ATCC 25923 reference strain. The MBEC was determined after 24 h and is summarised in Table 4.3.

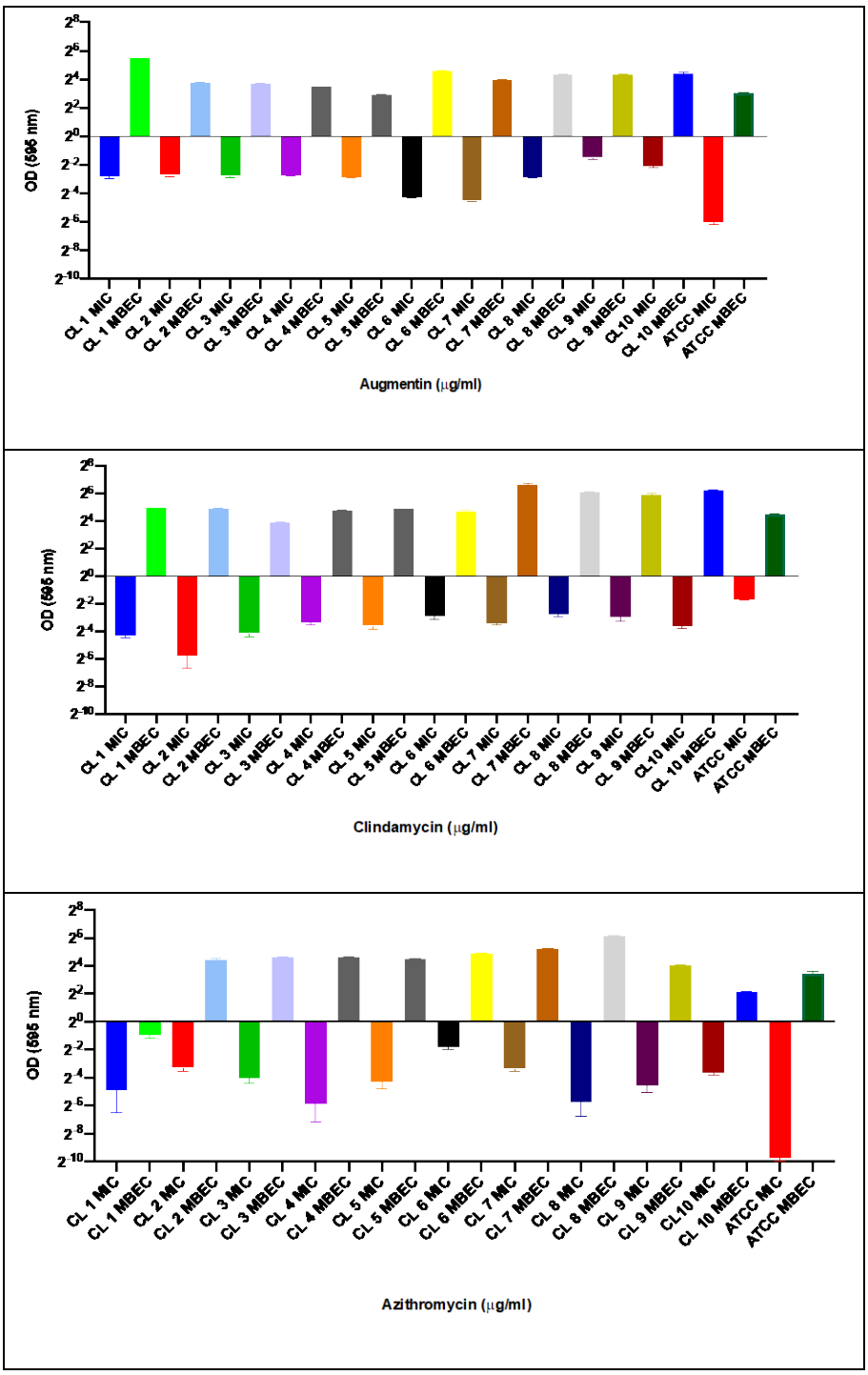
Table 4 .3: Minimum Biofilm Elimination Concentration 90 (MBEC 90)

	Antibiotic (μg/mL)			
Isolate	**Amoxicillin-clavulanate (AUG)**	**Clindamycin (CLI)**	**Azithromycin (AZI)**	**Mupirocin (MUP)**
CI1	8	8	16	4
CI 2	8	4	16	2
CI 3	4	8	8	2
CI 4	4	4	64	2
CI 5	2	4	8	2
CI 6	16	32	128	8
CI 7	16	16	128	8
CI 8	32	64	256	16
CI 9	16	8	64	8
CI 10	8	16	16	4
ATCC 25923	1	0.5	1	1

MBEC 90 = Concentration required to kill 90% of organisms of established biofilms. ATCC= American type culture collection

4.4.4. Comparison of the antibiotic susceptibility profile among *S. aureus* planktonic and biofilm forms of clinical isolates

The susceptibility of *S. aureus* planktonic and biofilm forms of the ten clinical isolates (CL 1-10) and ATCC 25923 to four antimicrobial agents differed significantly between the two groups. The average MBEC was significantly higher for amoxicillin-clavulanate (AUG) ($p<0.00027$), Clindamycin (CLI) ($p=0.00047$), Azithromycin (AZI) ($p= 0.00085$), Mupirocin (MUP) ($p< 0.0002$) compared to the corresponding MIC values respectively (Figure not shown). The fold increase between MBEC and MIC value varied between isolates and for the different antibiotics (fold increase ranged from 4X-16X for AUG, 16X-32X for CLI, 16X-32X for AZI and 2X-8X for MUP). Comparisons are shown in Supplementary Figure 4.1.


OD (595 nm)
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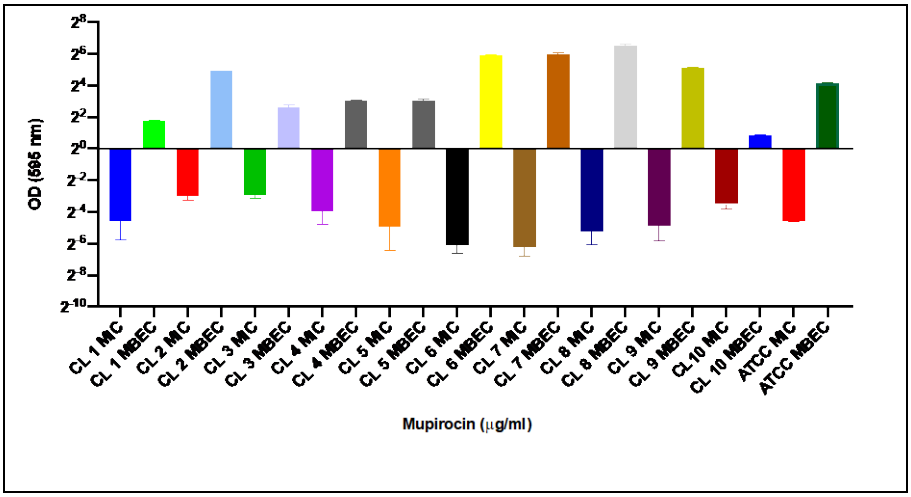
ATCC MIC
ATCC MBEC
Augmentin (µg/ml)
Clindamycin (µg/ml)
Azithromycin (µg/ml)



Supplementary figure 4.1: Comparison of MIC/ MBEC of Amoxicillin- clavulanate (AUG), Clindamycin (CLI), Azithromycin (AZI) and Mupirocin (MUP) in S. aureus.

The susceptibility to four antibiotics was tested on ten clinical isolates of S. aureus and ATCC 25923 in planktonic and biofilm forms. All tests were performed as 3 biological replicates and 6 technical replicates with a growth control (GC) and a sterility control (SC) in each assay. Measurements expressed as mean ± SEM.

4.4.5. Correlation of MIC/ MBEC with levels of exoprotein production

We measured the exoprotein production of all isolates in planktonic (6-hour cultures) and biofilm form (48-hour biofilms). Exoprotein production ranged between 13.49 µg/ml and 480.59 µg/ml in planktonic form and between 201.31 µg/ml and 632.95 µg/ml in biofilm form. Results are detailed in Table 4.1. We then correlated

susceptibility patterns of all clinical isolates with the exoprotein production by both planktonic and biofilm forms of *S. aureus*. The biofilm exoprotein production significantly correlated with the MBEC 90 values among all four antibiotics Clindamycin (CLI) ($r=0.87$, $p=0.021$), Amoxicillin- clavulanate (AUG) ($r= 0.97$, $p < 0.0001$), Azithromycin (AZI), ($r=0.798$, $p=0.041$), Mupirocin ($r=0.953$, $p<0.00062$) as illustrated in Figure 4.1. The planktonic exoprotein production did not show any correlation with MIC values.

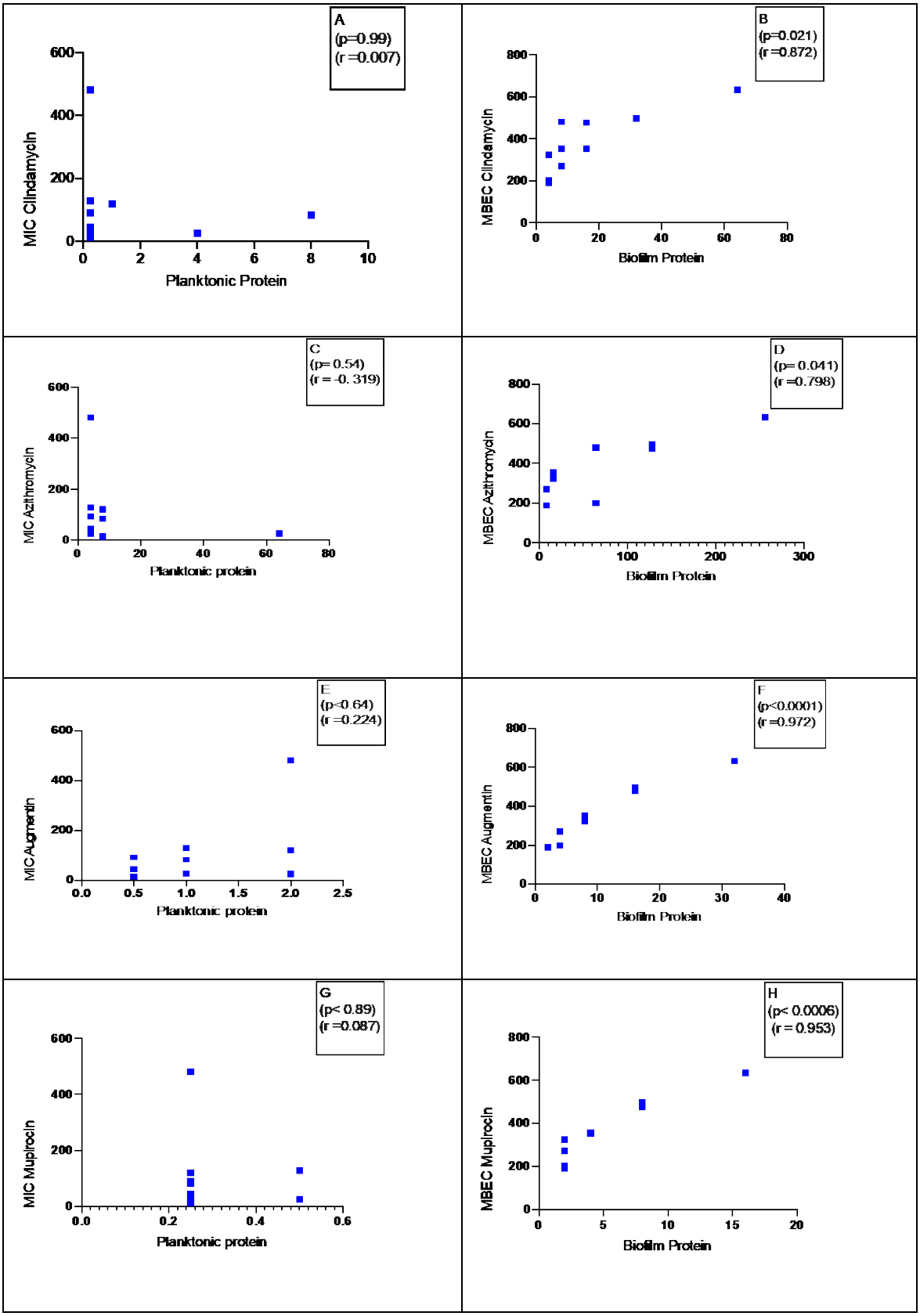


Figure 4.1: Minimum Biofilm Eradication Concentration (MBEC) correlates with biofilm exoprotein production.

MIC values (A, C, E, G) and MBEC values (B, D, F, H) for 10 *S. aureus* clinical isolates for Clindamycin (A, B), Azithromycin (C, D), Amoxicillin- clavulanate (E, F), Mupirocin (G, H) in relation to planktonic and biofilm protein production (in µg/ml). Each dot represents 1 clinical isolate. All tests performed as 3 biological replicates and 6 technical replicates with a growth control (GC) and a sterility control (SC) in each assay.

4.4.6. Biomass of biofilm forms of *S. aureus* correlates with biofilm eradication concentration

Biomass was determined for all of the ten clinical isolates using crystal violet. Each assay was performed with a positive growth control (GC) and a negative sterility control (SC) containing sterile nutrient broth. Absorbance of all isolates was read at OD 595 nm and normalised with the OD values of ATCC 25923. The biomass of these isolates was significantly different between isolates ($p = 0.03$).

The biomass production significantly correlated with the Minimum Biofilm Eradication Concentration (MBEC) for Clindamycin (CLI) ($r= 0.78$, $p= 0.04$) and Mupirocin (MUP) ($r= 0.79$, $p= 0.04$) but not for Amoxicillin- clavulanate (AUG) ($r=0.65$, $p=0.16$) and Azithromycin (AZI) ($r =0.62$, $p= 0.16$) (Figure 4.2)

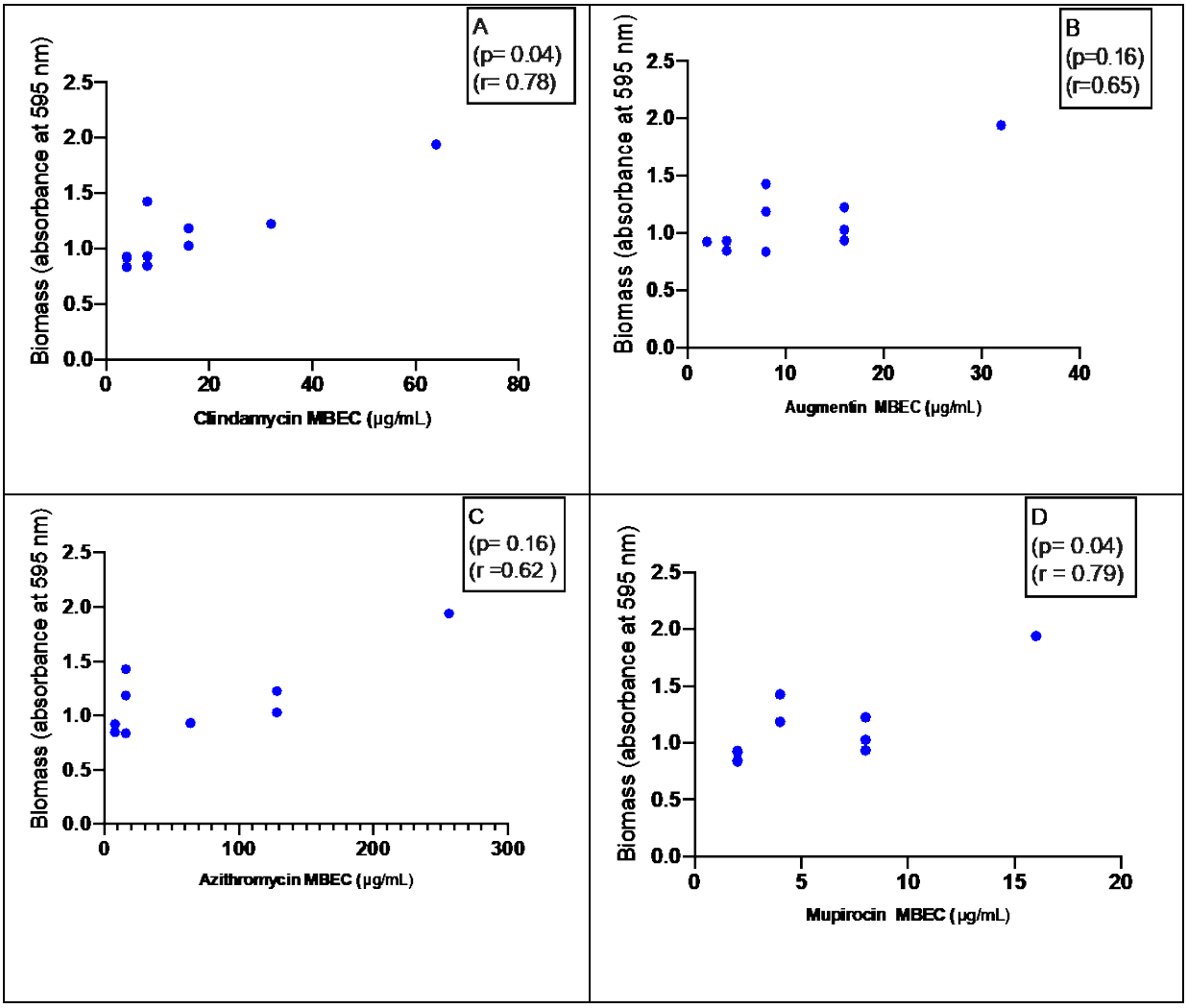
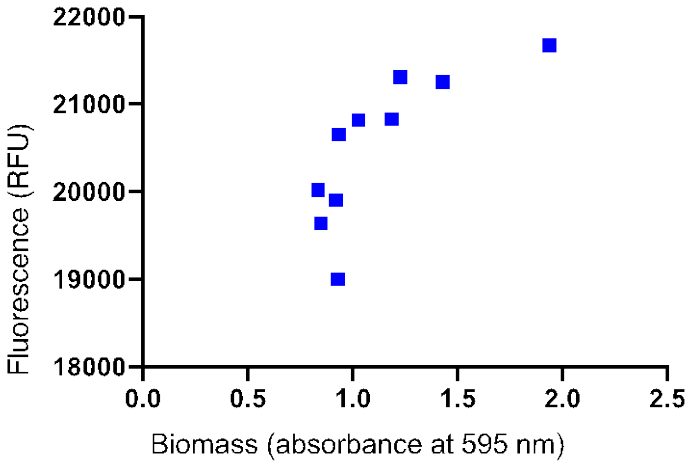


Figure 4.2: Biofilm biomass correlation with MBEC for clindamycin and mupirocin

Biofilm biomass (OD 595 nm) among 10 S. aureus clinical isolates significantly correlated with MBEC (in µg/mL) of Clindamycin (A) and Mupirocin (D) but not Amoxicillin- clavulanate (AUG) (B) and Azithromycin (AZI) (C). All tests were performed as 3 biological replicates and 6 technical replicates with a growth control (GC) and a sterility control (SC) in each assay.

4.4.7. Biofilm metabolic activity correlates with biofilm biomass and minimum biofilm eradication concentration (MBEC)

The metabolic activity among isolates varied and biomass production showed a positive correlation with metabolic activity of these isolates (r = 0.8788; p = 0.0016) (Supplementary Figure 4.2). The metabolic activity significantly correlated with the MBEC 90 among Clindamycin (CLI) (r =0.8352, p= 0.004), Amoxicillin- clavulanate (AUG) (r =0.7854, p= 0.009), Mupirocin (MUP) (r = 0.826, p = 0.005) but not Azithromycin (AZI) (r =0.5884, p= 0.07) (Figure 4.3)



Supplementary Figure 4. 2: Biofilm biomass correlates with metabolic activity

The biofilm biomass (expressed as absorbance at 595 nm) was positively correlated with biofilm metabolic activity (expressed as fluorescence (RFU)). Experiments performed as 3 biological replicates and 6 technical replicates with a growth control (GC) and a sterility control (SC) in each assay, Spearman correlation (r = 0.8788; p = 0.0016.)

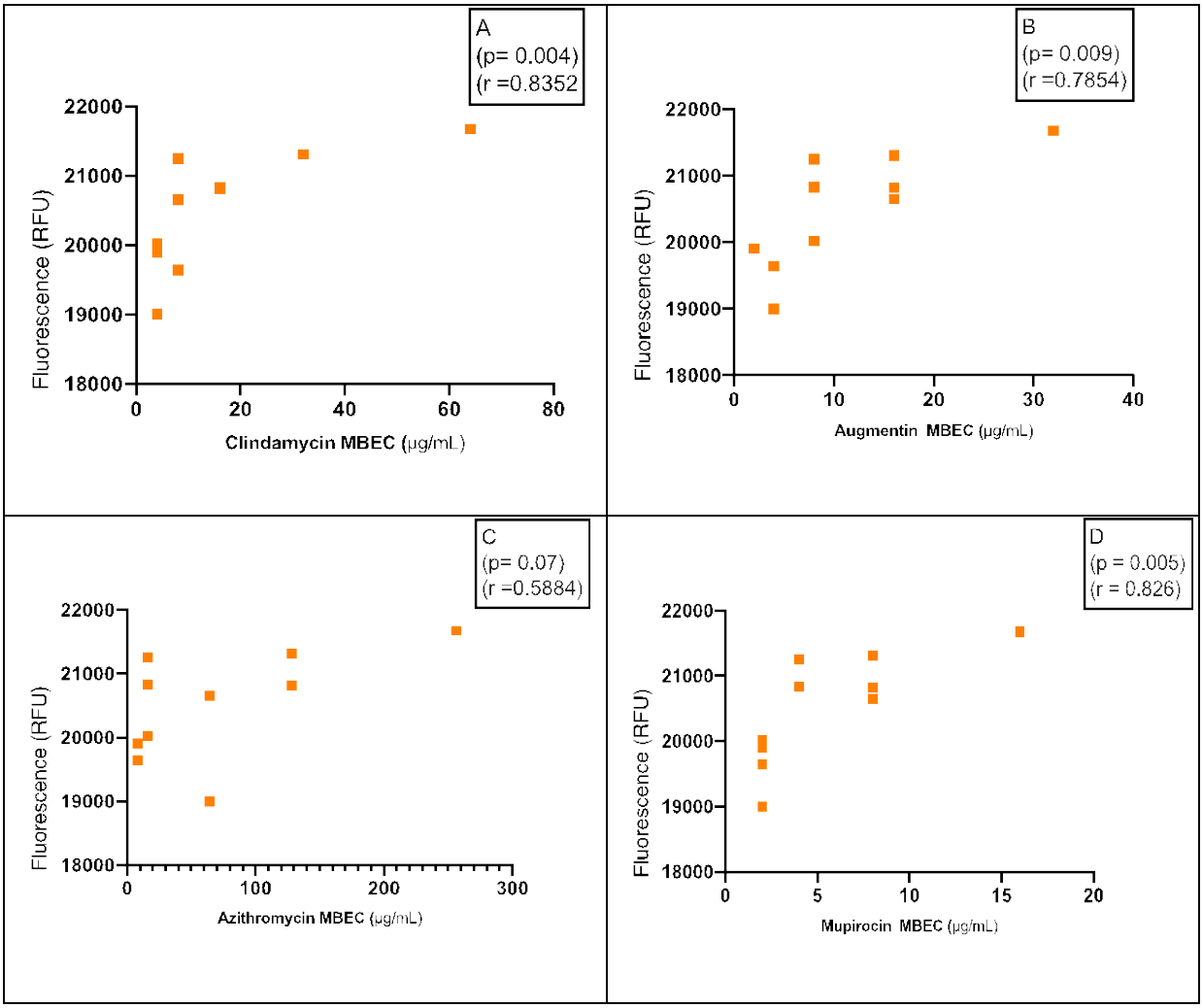


Figure 4.3: Biofilm metabolic activity correlates with MBEC values

Metabolic activity of S. aureus biofilm (expressed as fluorescence (RFU)) in relation to MBEC (in µg/mL) *of Clindamycin (A), Amoxicillin- clavulanate (B), Azithromycin (C) Mupirocin (D) (Spearman correlation). All assays performed as 3 biological replicates and 6 technical replicates with a growth control (GC) and a sterility control (SC) in each test.*

4.4.8. Minimum biofilm eradication concentration (MBEC) correlates with Inflammatory cell counts

Tissue samples from ten clinical isolates, including three CRSsNP samples and seven CRSwNP samples were stained with H&E and inflammatory cells per high-power field were counted for lymphocytes and eosinophils. Results are detailed in Table 4.1.

MIC values did not show any correlations with inflammatory cell numbers (data not shown). Correlation was seen among lymphocyte cell counts and MBEC for Clindamycin (CLI) (r =0.75, p=0.05) but not for Azithromycin (AZI) (r =0.42, p=0.421), Amoxicillin- clavulanate (AUG) (r =0.68, p=0.12) and Mupirocin (MUP) (r = 0.73,p= 0.07). Eosinophil cell counts correlated with MBEC for Amoxicillin- clavulanate (AUG) (r =0.80, p=0.04) and Mupirocin (r = 0.78, p=0.04) but not for Azithromycin (AZI) (r =0.55, p= 0.24) or Clindamycin (CLI) (r =0.58, p= 0.21) (Figure 4.4)

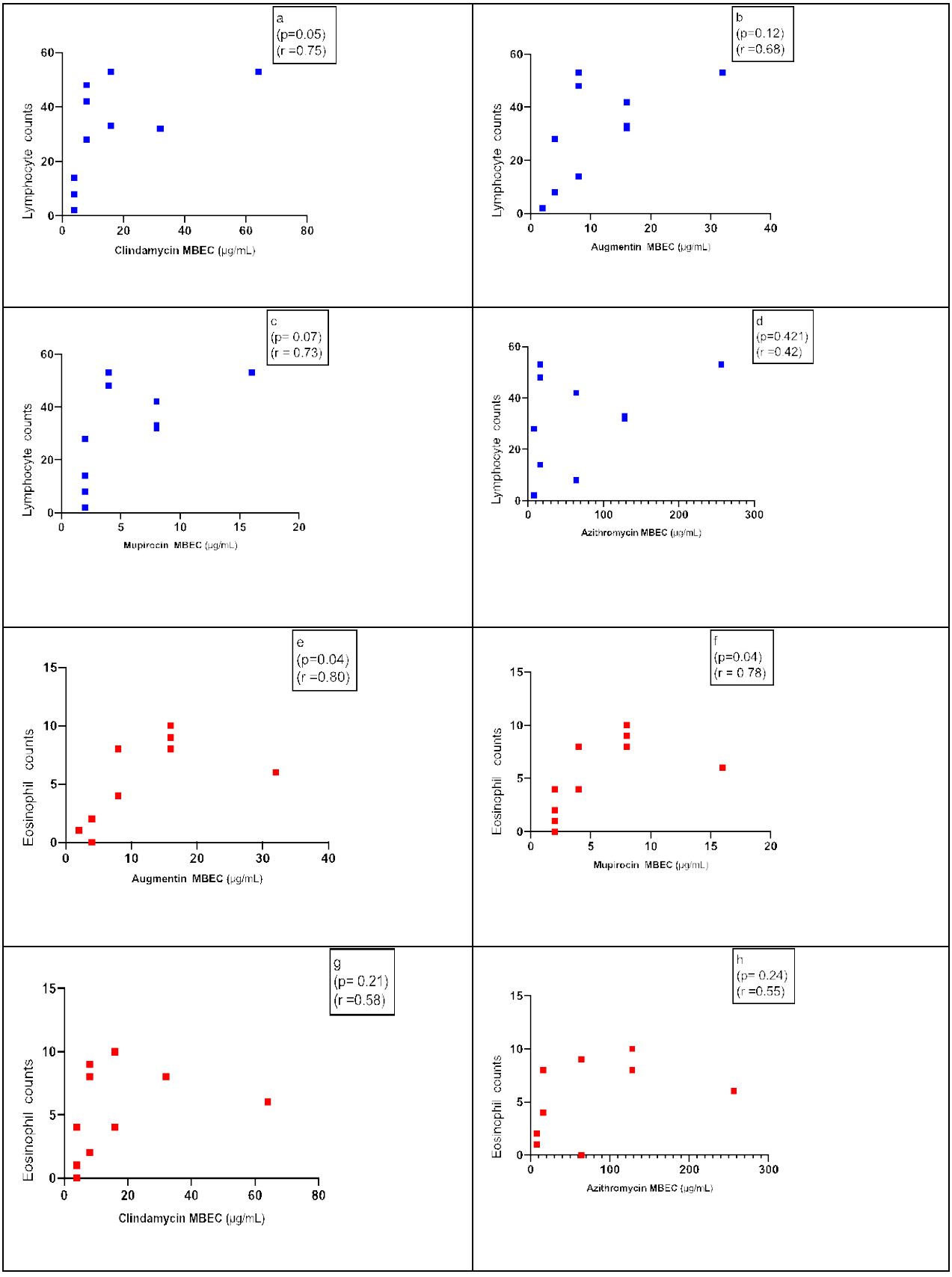

a
(p=0.05)
(r =0.75)
Lymphocyte counts
Clindamycin MBEC (µg/mL)
b
(p=0.12)
(r =0.68)
Lymphocyte counts
Augmentin MBEC (µg/mL)
c
(p= 0.07)
(r = 0.73)
Lymphocyte counts
Mupirocin MBEC (µg/mL)
d
(p=0.421)
(r =0.42)
Lymphocyte counts
Azithromycin MBEC (µg/mL)
e
(p=0.04)
(r =0.80)
Eosinophil counts
Augmentin MBEC (µg/mL)
f
(p=0.04)
(r = 0.78)
Eosinophil counts
Mupirocin MBEC (µg/mL)
g
(p= 0.21)
(r =0.58)
Eosinophil counts
Clindamycin MBEC (µg/mL)
h
(p= 0.24)
(r =0.55)
Eosinophil counts
Azithromycin MBEC (µg/mL)

Figure 4.4: Inflammatory counts correlates with MBEC

Lymphocyte (a-d) and eosinophil (e-h) cell counts per high power field in relation to MBEC values (in µg/mL) for Clindamycin (a, g) Amoxicillin-clavulanate (Augmentin, b, e), Mupirocin (c, f) and Azithromycin (d, h) (Spearman correlation).

4.4.9. Relationship of MBEC with disease severity scores

The spearman correlation between disease severity scores including Lund-Mackay scores, Adelaide scores and SNOT 22 did not show any relationship with the MBEC or MIC values for all four antibiotics (data not shown).

4.5 DISCUSSION

This study demonstrates that clinical isolates of *S. aureus* show a reduced susceptibility pattern to antibiotics in biofilm form in comparison to their planktonic forms. The biofilm biomass of established 48-hour biofilms was linked to the metabolic activity and exoprotein production of these biofilms and each of these values significantly correlated with the MBEC values for Clindamycin and Mupirocin. There was a significant correlation between lymphocyte cell counts and MBEC for Clindamycin and eosinophil cell counts correlated with MBEC for Amoxicillin-clavulanate and Mupirocin. In contrast, MIC values did not relate to biofilm properties or inflammatory cell counts.

Studies have increasingly shown that biofilms play a significant role in chronic infections such as in the context of CRS, leading to disease recalcitrance and poor clinical outcomes after surgery[491]. Biofilms exhibit multiple mechanisms of resistance and tolerance that act together to provide an increased overall level of resistance to antibiotics[492,493]. This was evident also in our study where all isolates exhibited an increased tolerance to all 4 antibiotics when grown in biofilm form than their planktonic counterparts. Interestingly, the fold increase between MBEC and MIC varied for the different antibiotics and was lowest for mupirocin and highest for clindamycin. These results indicate that mupirocin might have better penetration through biofilms and/or affect bacteria within biofilms more easily than clindamycin. Also, whereas most isolates had similar MIC values for the different antibiotics, there was a high variability between isolates in their MBEC values. These findings underscore the importance of revisiting the diagnostic work-up needed to design therapies to effectively treat biofilm-related chronic infections. Sinusitis is the most common diagnosis for which antibiotics are prescribed[494] yet antibiotics are ineffective to eradicate the infection and associated inflammation. This might at least in part be due to the inability of antibiotics to eradicate biofilms, in particular those biofilms that are found in patients with a higher disease load. Decision making of which antibiotic therapies to use and at which dosages is indeed based on the susceptibility profile of planktonic cells. Given similar MIC values for planktonic cells between isolates are seen as shown in this study, prescribed dosages will also be similar. With some variability depending on the antibiotic used, those dosages will inevitably be insufficient to kill the biofilm nidus. In fact, it is likely that at least some

bacterial cells in biofilm are exposed to subinhibitory levels of antibiotics during antimicrobial treatment due to diffusion gradients for antibiotics in biofilm[493]. This is likely to be more pronounced in those isolates that have higher MBEC/MIC ratio's as higher antibiotics concentrations would be needed to kill those bacteria within those biofilms. Exposure to subinhibitory concentrations of antibiotics is known to promote biofilm formation and can also induce mutagenesis resulting in a higher probability of antibiotic resistance development[495]. Also, biofilm-producing isolates have a higher tendency to exhibit antimicrobial resistance and methicillin resistance compared to sensitive isolates [496]. Piechota et al reported similar findings in MRSA strains that had significantly higher ability of biofilm formation than MSSA strains from respiratory specimens [497]. It is well known that the biofilm lifestyle is stressful for the bacteria due to the scarcity of nutrients, excess of waste products, hypoxia, and antimicrobials. Reduced antimicrobial biofilm susceptibility in relation to increased biomass and metabolic activity as seen in the present study might simply reflect biofilm cells in those patients chronically responding to stress. Biofilms present in recalcitrant CRS patients would indeed have a frequent and often long-term exposure to antibiotics since antibiotics are the mainstay of CRS medical therapy. It might be that the increased biofilm biomass seen in isolates harvested from those patients contributes to the reduced antibiotic susceptibility. Increased biomass would in turn lead to phenotypic changes secondary to increased exposure to waste products and hypoxia, further exacerbating the biofilm biomass production. Further in vitro and in vivo research is required to evaluate the effect of chronic antibiotic

exposure on *S. aureus* biofilm biomass and how that relates to changes in the composition and metabolism of the biofilm and signalling pathways involved.

Interestingly clinical isolates that had high MBEC values also secreted high amounts of exoproteins and showed higher numbers of inflammatory cells within their tissue compared to isolates with lower MBEC values. *S. aureus* exoproteins induce barrier disruption of HNEC-ALI cultures[406,408,425], thereby playing a critical role in the pathogenesis of CRS. Cytolysins, proteases and phenol-soluble modulins (PSMs) secreted by *S. aureus* lead to dispersion of biofilm forms in turn enabling them to become planktonic [498,499]. Bacteria living in a biofilm furthermore express a different set of genes and proteins than their planktonic counterparts with an enrichment of many proteins involved in the pathogenesis of inflammation, such as toxins (e.g. leukocidin and beta-hemolysin) and immunomodulatory proteins (e.g. lipoprotein and protein A) [412]. These exoproteins are not only harmful to the nasal epithelium but might also exacerbate a host immune response. Further research is needed to validate these findings in more antibiotics classes and using more clinical isolates and to evaluate whether indeed chronic exposure to antibiotics might enhance inflammation by contributing to the formation of a more virulent *S. aureus* biofilm.

Our results support the need of pursuing MBEC as better predictor of antibiotic concentrations required for eradication of biofilms especially in recalcitrant CRS patients and underscore the differences in biofilm properties that may contribute to the treatment failure seen in those patients.

4.6 CONCLUSION

This study determined that *S. aureus* clinical isolates exhibited a reduced susceptibility to antibiotics in biofilm form than in planktonic form. Moreover, MBEC values were significantly correlated with *S. aureus* biofilm properties such as biomass, metabolic activity and exoprotein production. Increased MBEC values were furthermore related to increased inflammatory cell infiltration in sinonasal tissue sections from corresponding patients. Since most therapeutic guidelines are applicable only to planktonic forms of bacteria and not sufficient to treat infections due to biofilm forms, there is an urgent need for new therapeutic guidelines that target *S. aureus* in biofilms. Antibacterial therapy to treat recalcitrant CRS should be aimed at eliminating biofilms, for which MBEC values are a better guide to determine the antibiotic susceptibility in comparison to MIC.

CHAPTER 7: THESIS SYNOPSIS

7.1 THESIS SUMMARY

The work presented in this PhD thesis has explored the relationship of *S. aureus* exoproteins and their effect on the sinonasal mucosa following an extensive review of literature (Chapter 1). The literature review summarizes CRS as a heterogeneous inflammatory disease of multifactorial aetiology and emphasises the role of *S. aureus* in CRS. Bacterial products such as toxins and exoproteins secreted by *S. aureus* in planktonic form had already been shown to negatively affect mucosal barrier structure and function. However, the effect of biofilm exoproteins on the barrier structure and function was not clearly understood.

The first part of this project (Chapter 2) investigated the effect of *S. aureus* exoproteins on the mucosal barrier structure and function. We observed that *S. aureus* exoproteins have dose-and time-dependent detrimental effects on the mucosal barrier structure of HNEC-ALI cultures *in vitro.* In comparison to *S. aureus* planktonic exoproteins, equal concentrations of exoproteins from their biofilm counterparts showed more profound detrimental effects on the mucosal barrier structure and function and were cytotoxic. These results indicated that qualitative differences existed between exoproteins secreted by planktonic cells and biofilms. Our study is the first to use *S. aureus* clinical isolates from CRS patients to address those important questions and provides insights into the possible role of *S. aureus* exoproteins in the pathophysiology of CRS. In this study we were able to demonstrate that the effects on mucosal barrier structure and function were long lasting, dose-dependent and were abrogated with heat- or proteinase K-inactivation.

The second part of the project (Chapter 3) evaluated the relationship between *in vitro S. aureus* biofilm properties and inflammation. This study showed that *S. aureus* clinical isolates harvested from CRSwNP patients and TLO-positive CRS patients exhibit higher *S. aureus* biofilm exoprotein production and higher metabolic activity than *S. aureus* biofilms from CRSsNP and TLO-negative patients. There was a significant positive correlation between the number of lymphocytes or eosinophils and the concentration of biofilm exoproteins secreted by corresponding clinical isolates and their metabolic activity. In contrast, exoprotein concentrations from planktonic cells did not correlate with inflammatory cell infiltration. This study also showed that TLO formation in CRS patient tissues is linked to *S. aureus* biofilm., These findings provide a platform and rationale for the development of new and improved treatment strategies for recalcitrant CRS patients. Interestingly, we also observed a strong relationship between *in vitro* biofilm properties and *in situ* levels of inflammation. These findings indicate lasting phenotypic changes in *S. aureus* clinical isolates, even when grown in optimal conditions *in vitro*.

The molecular basis of this finding warrants further investigation, in particular to evaluate potential underlying genotypic and/or epigenetic changes in those isolates. Along with studying the various types of proteins that are found in these exoproteins at different disease states / phenotypes to cause varying degree of inflammation.

Future animal models are required to study the *in vivo* immune response to *S. aureus* exoproteins to enable us to better understand the inflammatory heterogeneity of CRS disease and create avenues to determine new treatment modalities based on these findings.

Finally, in Chapter 4 we were interested to determine the relationship between antimicrobial resistance patterns among *S. aureus* biofilm and planktonic forms in CRS in relation to severity of disease and exoprotein production. This would enable us to understand at a clinical practice level, the way the isolates behaved and to determine if there was need for change in addressing the antibiotic strategy. We observed that clinical isolates of *S. aureus* show a reduced susceptibility pattern to antibiotics in biofilm form in comparison to their planktonic forms. The biofilm biomass of established 48-hour biofilms was linked to the metabolic activity and exoprotein production of these biofilms and each of these values significantly correlated with the MBEC values. Interestingly, there was a significant correlation between eosinophil cell counts of tissue sections of corresponding patient biopsies and *in vitro* MBEC values. However, this study demonstrates these findings in a limited population of ten patients with rCRS, hence further research is needed to validate these findings in more antibiotics classes and using more clinical isolates. This could further clarify whether chronic exposure to antibiotics might enhance inflammation by contributing to the formation of a more virulent *S. aureus* biofilm. The importance of findings from this last project will become clear when taking this research to clinical setting, in particular the need to pursue MBEC as better predictor of antibiotic concentrations for eradication of biofilms especially in recalcitrant CRS patients.

The findings from this research enhance our understanding of the role of exoproteins of *S. aureus* in planktonic and biofilm forms on the nasal epithelial barrier, its relation to inflammation and resistance patterns in CRS. The outcomes of this PhD research

set ground for the development of new approaches for recalcitrant CRS patients and for new therapeutic guidelines such as MBEC aimed at eliminating biofilms in comparison to conventional MIC thereby translating research from “bench-to-bedside”.

7.2: CONCLUSION

In conclusion, this PhD thesis has provided insights to the role of exoproteins of

S. aureus in CRS. We have identified that *S. aureus* exoproteins disrupt the mucosal barrier structure, are toxic and damage the mucosal barrier which might play an important role in CRS etiopathogenesis.The concepts of strain-specific differences in *S. aureus* biofilm matrix quantity and metabolic activity have been raised as potential mechanisms linking to the severity, type of inflammation and resistance patterns in CRS. These findings have laid a foundation to guide further investigation into

S. aureus exoproteins in CRS, with a hope that it may provide a rationale for the development of better treatment strategies and guidelines for recalcitrant CRS patients.

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