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Strain-specific Intracellular *Staphylococcus aureus* in resistant chronic rhinosinusitis. Disease mechanisms and potential novel therapies.

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by

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Abstract

Faculty of Medicine
Clinical and Experimental Sciences

Doctor of Philosophy

Strain-specific Intracellular *Staphylococcus aureus* in resistant chronic rhinosinusitis. Disease mechanisms and potential novel therapies.

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Chronic rhinosinusitis (CRS) is a chronic inflammatory condition affecting the nose and paranasal sinuses. The disease affects up to 15% of the UK population and is recognised as the second most common chronic condition in Europe after arthritis. There are two well documented phenotypes of the condition; CRS without nasal polyps (CRSsNP) and CRS with polyps (CRSwNP). *S. aureus* colonises the nasal cavity in 60% of patients with CRSwNP, almost double that of the general population. Recently published research has demonstrated intracellular localisation of *S. aureus* within epithelial and mast cells in CRSwNP subjects. This appears to promote chronic inflammation, disease recalcitrance and treatment resistance.

A prospective study was undertaken to determine if *S. aureus* identified in CRS had an enhanced ability to internalise and survive within mast cells and to investigate the effectiveness of novel *S. aureus*-targeted therapies aimed at reducing the bacterial and pro-inflammatory cytokine burden. Nasal swabs of patients undergoing rhinological examination or surgical procedures were taken from non-CRS subjects, and from CRSsNP and CRSwNP patients and grown in *S. aureus* selective media. The cultured strains underwent short-read whole genome sequencing and were interrogated for the presence of virulence factors, antimicrobial resistance genes and stress proteins. Isolates obtained were used in mast and epithelial intracellular infection models to determine any differences in intracellular survival.

Our data demonstrate that there were differences between control, CRSsNP and CRSwNP-cultured *S. aureus*. There were very few virulence factors seen in CRSsNP patients and many associated with intracellular survival and infection in CRSwNP-cultured *S. aureus* compared to controls. Our HMC-1 and LAD2 mast cell models confirmed that a representative control strain was less able to internalise and replicate within the intracellular compartment compared to a CRSwNP *S. aureus* strain. Both strains exhibited similar toxicity levels.

The lipophilic statin, simvastatin was able to reduce both the extracellular and intracellular burden of a CRSwNP isolate of *S. aureus* in LAD2 mast cells in the prodrug, but not the activated form. Simvastatin was able to reduce the number of *S. aureus* colony-forming units internalised and lactate dehydrogenase release in mast cells in a dose-dependent fashion. Simvastatin appeared to have no effect on substance P signalling in mast cells. In air-liquid interface cultured epithelial cells, simvastatin appeared to reduce the production of pro-inflammatory cytokine expression (*TNF, TSLP and CXCL8* (IL-8)) although this failed to reach statistical significance.

In summary, our results demonstrate that CRSwNP-cultured *S. aureus* possesses a number of virulence factors which support intracellular localisation, infection and replication in mast cells. Simvastatin is an inexpensive, safe, and well tolerated oral cholesterol-lowering treatment, and our results indicate it may also reduce the burden of intracellular *S. aureus*. As such, it has the potential to be re-developed and re-branded for use as an anti-*S. aureus* adjunctive treatment in patients with *S. aureus*-related chronic inflammatory conditions such as CRS. Our findings therefore warrant further study of the antibacterial effects of simvastatin in primary cell culture and nasal explant models, and future clinical trials.

Papers
Hayes S, Biggs T, Goldie S, Harries P, Walls A, Allan R, Pender S, Salib R. *Staphylococcus aureus* internalization in mast cells in nasal polyps: Characterization of interactions and potential mechanisms. J Allergy Clin Immunol, 2020. 145(1): p. 147-159.

Oral presentations
Goldie S *et al.* Can we repurpose Simvastatin to treat recalcitrant *S. aureus*-mediated chronic rhinosinusitis (2023) European Rhinological Society meeting, Sofia, Bulgaria

Goldie S *et al.* Is there a role for Staphylococcus *pseudintermedius* in resistant chronic rhinosinusitis (2023) European Rhinological Society meeting, Sofia, Bulgaria

Goldie, S *et al.* Enhanced pathogenicity of CRS-related *S. aureus* strains may mediate early disease recurrence (2022) American Rhinological Society annual meeting, Philadelphia, Pennsylvania, United States of America

Goldie, S *et al.* Repurposing Simvastatin to target intracellular *Staphylococcus aureus* in resistant chronic rhinosinusitis (2022) British Rhinological Society meeting, Sheffield, United Kingdom

Goldie, S *et al. Staphylococcus aureus* within nasal mast cells as a driver of chronic rhinosinusitis: A role for simvastatin in disease management? (2021) European mast cell and basophil research network meeting.

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Research Thesis: Declaration of Authorship

Print name: Simon Patrick Goldie

Title of thesis: Strain-specific Intracellular Staphylococcus aureus in resistant chronic rhinosinusitis. Disease mechanisms and potential novel therapies.

I declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

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3. Where I have consulted the published work of others, this is always clearly attributed;
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Abbreviations

AD	**Atopic dermatitis**
AERD	**Aspirin exacerbated respiratory disease**
AFRS	**Allergic fungal rhinosinusitis**
AGR	**Accessory gene regulator protein**
agr	**Accessory gene regulator gene**
AIP	**Autoinducing peptide**
ALI	**Air-liquid interface**
APC	**Antigen-presenting cell**
APES	**3-aminopropropyltriethoxysilane**
BSA	**Bovine serum albumin**
C 48/80	**Compound 48/80**
CBA	**Columbia blood agar**
CFU	**Colony forming unit**
cDNA	**Complementary deoxyribonucleic acid**
CI	**Confidence interval**
CCL	**C-C chemokine ligand**
CCR	**C-C chemokine receptor**
CRES	**Chronic Rhinosinusitis Epidemiology Study**
CRS	**Chronic rhinosinusitis**
CRSsNP	**Chronic rhinosinusitis without nasal polyps**
CRSwNP	**Chronic rhinosinusitis with nasal polyps**
CST	**Community state type**
CVID	**Common variable immunodeficiency**
CT	**Computed tomography**
Ct	**Cycle threshold**
DAPI	**4',6-diamidino-2-phenylindole**
DNA	**Deoxyribonucleic acid**
EMT	**Epithelial-to-mesenchymal transition**
ERK	**Extracellular signal-regulated kinase**
ESKAPE	***Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*** **and** ***Enterobacter*** **species**
EPOS	**European Position Paper on Rhinosinusitis and Nasal Polyps**
FAM	**Fluorescein amidite**
FBS	**Foetal bovine serum**
FcεRI	**High-affinity IgE receptor**
FESS	**Functional endoscopic sinus surgery**
FISH	**Fluorescence in situ hybridisation**
FnBP	**Fibronectin binding repeat**
GA^{2}LEN	**Global Allergy and Asthma European Network**
GM-CSF	**Granulocyte-macrophage colony-stimulating factor**
HBSS	**Hank's balanced salt solution**
HIV	**Human immunodeficiency virus**
HMC-1	**Human mast cell line 1**
HMG-COA	**3-hydroxy-3-methylglutaryl-coenzyme A**
HSP	**Heat shock protein**

Abbreviations

IFN	**Interferon**
IgE	**Immunoglobulin E**
IL	**Interleukin**
IMDM	**Iscove's Modified Dulbecco's Medium**
IMS	**Industrial methylated spirits**
LAD2	**Laboratory of Allergic Diseases cell line 2**
LAMP	**Lysosomal associated membrane protein**
LDH	**Lactate dehydrogenase**
MLMS	**Modified Lund-Mackay score**
MLST	**Multilocus sequence typing**
LTC_4	**Leukotriene C4**
MCP	**Monocyte chemoattractant protein**
MHC	**Major histocompatibility complex**
MIC	**Minimal inhibitory concentration**
MOI	**Multiplicity of infection**
MPO	**Myeloperoxidase**
MRGPRX2	**Mas-related G protein-coupled receptor X2**
MRI	**Magnetic resonance imaging**
MRSA	**Methicillin-resistant *Staphylococcus aureus***
NIHR	**National Institute for Health and Care Research**
NHS	**National Health Service**
NF-κB	**Nuclear factor kappa-light-chain-enhancer of activated B-cells**
OR	**Odds ratio**
PAMP	**Pathogen associated molecular pattern**
PBS	**Phosphate-buffered saline**
PCR	**Polymerase chain reaction**
PGD2	**Prostaglandin D2**
PKB	**Protein kinase B**
PVL	**Panton-Valentine leukocidin**
qRT-PCR	**Quantitative real-time polymerase chain reaction**
RANTES	**Regulated on activation, normal T cell expressed and secreted**
RBL-2H3	**Rat basophilic leukaemia 2H3 cell line**
RNA	**Ribonucleic acid**
ROT	**Repressor of toxins**
RPMI	**Roswell Park Memorial Institute**
RRR	**Relative risk ratio**
SEB	***Staphylococcus aureus* enterotoxin B**
SCC*mec*	**Staphylococcal chromosome cassette *mec***
SCF	**Stem cell factor**
SCIN	**Staphylococcal complement inhibitor**
SCV	**Small colony variant**
SNOT-22	**Sino-Nasal Outcome Test-22**
TEER	**Trans-epithelial electrical resistance**
Th	**T helper**
TLR	**Toll-like receptor**
TNF	**Tumour necrosis factor**

Abbreviations

TSLP	**Thymic stromal lymphopoietin**
VCAM	**Vascular cell adhesion molecule**
VLA	**Very late antigen**
VRSA	**Vancomycin-resistant *Staphylococcus aureus***

Chapter 1 **Introduction**

1.1 Overview of study

Chronic rhinosinusitis (CRS) is an inflammatory condition affecting the nasal cavity and sinuses. The disease affects 15% of the European population and costs the National Health Service (NHS) £16.8 billion annually in repeat prescriptions (steroids and antibiotics) and multiple surgeries (1). It represents the leading reason for an antibiotic prescription from primary care (2).

Recent work has identified intracellular *Staphylococcus aureus (S. aureus)* within mast and epithelial cells in CRS nasal tissue which leads to early disease relapse post-surgery and a more resistant disease phenotype (3). Patients with the condition demonstrate higher burdens of intranasal *S. aureus* and higher levels of immunoglobulin E (IgE) towards *S. aureus* enterotoxins (3, 4). Given *S. aureus* are commensal bacteria in the nasal cavities of subjects without the condition, we hypothesise that these bacteria have an increased ability to survive intracellularly in patients with CRS compared to subjects without the disease. This may be explained by the presence of extra genes known as virulence factors which increase the pathogenicity of *S. aureus*. Interestingly, lipophilic statins have been shown to reduce the incidence of CRS as well as acting as potent antistaphylococcal compounds by suppressing virulence factor production and killing *S. aureus* (5-7).

This study set out to identify if there were differences in *S. aureus* virulence factors between strains cultured from control subjects and those with CRS without polyps (CRSsNP) and those with polyps (CRSwNP). It also aimed to explore the intracellular survival capabilities of these strains between the conditions. Furthermore, we investigated simvastatin's ability to treat the intracellular burden of CRS-related *S.*

aureus and the effect this has on pro-inflammatory cytokine production. Our objective was to improve our understanding of the pathogenic role of *S. aureus* in CRS, and highlight potential novel therapies which can target these bacteria with the ultimate aim of improving the quality of life of sufferers whilst reducing our dependence on antibiotics. In turn, this would also reduce the risk of antimicrobial resistance which has reached near epidemic proportions.

1.2 Nasal cavity anatomy

1.2.1 Sinonasal anatomy

The nasal cavity and sinuses form part of the upper respiratory tract along with the pharynx and the larynx. The nasal cavity is a paired structure beginning at the nostrils and ending at the choanae posteriorly. The medial wall dividing both nasal cavities, formed anteriorly by the nasal septum also known as the quadrangular cartilage, is a large hyaline cartilaginous structure. It attaches inferiorly to the maxillary crest and vomer of the maxillary bone and superiorly to the perpendicular plate of the ethmoid bone and the inferior aspect of the nasal bones known as the rhinion. Immediately inferior to the rhinion is the keystone area, which is the major structural support of the middle one-third of the nose, constituting 10-15 mm of bony cartilaginous septal junction. The septal cartilage is covered by a thin perichondral layer with keratinised squamous epithelium anteriorly between the nares and the anterior nasal valve. The remainder of the septum and nasal cavity is covered in columnar respiratory epithelium. The floor of each nasal cavity is formed by the palatine process of the maxilla. The lateral wall of the nasal cavity is made up of the lower and upper lateral cartilages, frontal process of the maxilla, lacrimal bone, turbinate bones and the medial pterygoid plate of the sphenoid bone (8). There are 3-4 turbinates on each lateral nasal wall, the inferior, middle and superior turbinates with 60% of individuals having a supreme turbinate (9). The turbinates project medially and inferiorly into the nasal cavity to create meatuses, which are spaces between the lateral nasal cavity and the lateral aspects of the turbinates (Figure 1-1) (8).

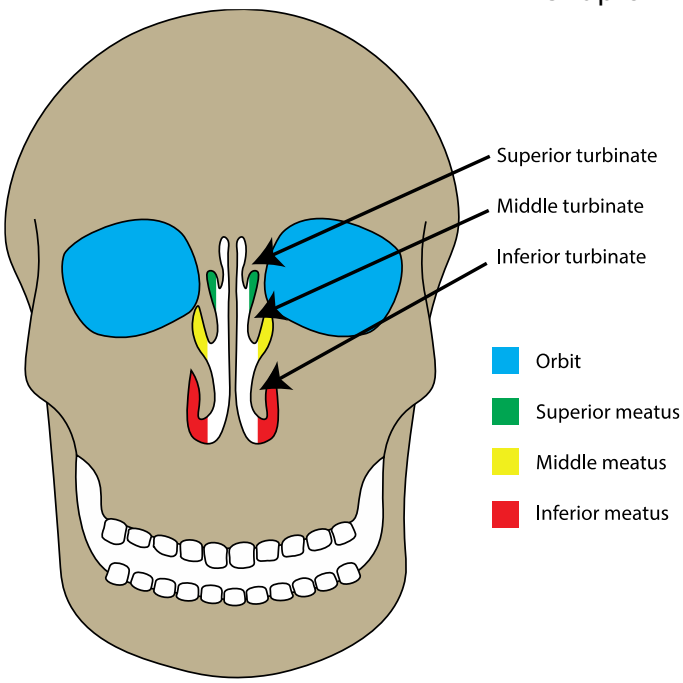


Figure 1-1 Nasal cavity anatomy

Anatomy of the nasal cavity demonstrating the superior, middle, and inferior turbinates and the associated meatuses.

The meatuses contain openings for sinuses and ducts. The inferior meatus provides an opening for the lacrimal duct via Hasner's valve. The middle meatus contains the infundibulum shielded by the uncinate process, and provides a common drainage pathway for the maxillary, anterior ethmoid and frontal sinuses. The superior meatus allows drainage of the posterior ethmoid sinuses. Behind the turbinates, the sphenoethmoidal recess is a space anterior to the sphenoid but behind the posterior ethmoid sinuses.

The roof of the nasal cavity is formed by the nasal bones, nasal spine of the frontal bone, cribriform plate and the anterior surface of the sphenoid bone. The area inferior to the cribriform plate known as the olfactory recess, is covered in a thin non-ciliated epithelium known as Schneiderian membrane, allowing the passage of olfactory nerve fibres from the olfactory bulb (8).

There are four sets of air-filled paranasal sinuses surrounding the nasal cavity named from the bones in which they form. The maxillary and ethmoid sinuses grow during embryological development, while the sphenoid typically develops between the sixth month and fourth year of life. The frontal sinus develops around age seven. All sinuses are lined with mucus-secreting columnar ciliated respiratory epithelium (10).

The frontal sinus is the most superior and anterior sinus located between the anterior and posterior tables of the frontal bone. The maxillary sinus is located lateral to the nasal cavity within the maxillary bone and is related to the floor of the orbit (roof), molars and premolars (floor), the infratemporal fossa (posteriorly) and the skin of the face (laterally and anteriorly). The ethmoid sinuses create a honeycomb structure and are divided into anterior and posterior by the posterior insertion of the middle turbinate known as the basal (ground) lamella (Figure 1-2). The borders of these sinuses include the medial wall of the orbit (lamina papyracea) and the caudal edge of the middle turbinate (8).

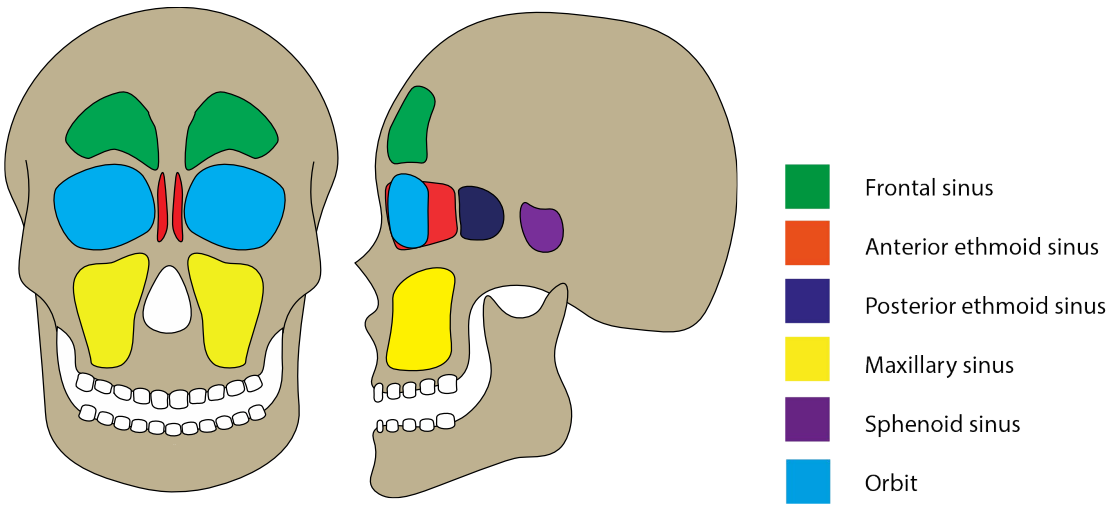


Figure 1-2 Paranasal sinus anatomy

Anatomical location of the paranasal sinuses within the human skull.

The nasal cavity receives a rich blood supply from both the internal and external carotid arteries. The sphenopalatine artery, a terminal branch of the maxillary artery, supplies up to 60% of blood flow to the nasal cavity and arises from the sphenopalatine foramen, where it proceeds to create posterior lateral nasal branches and posterior septal branches, which supply most of the lateral wall and the posterior aspect of the nasal septum. The anterior and posterior ethmoidal arteries mainly supply the roof and the nasal septum. The posterior ethmoid artery originates from the cribriform plate. The anterior ethmoidal artery passes through the lamina papyracea, typically over a groove in the cribriform plate, although the artery can be dehiscent. The greater palatine artery and the superior labial artery supply the inferior aspect of the nasal cavity, arising through the incisive canal and soft tissue of the lip respectively. All arteries anastomose at a point on the nasal septum known as Little's area or Kiesselbach's plexus. The venous supply closely resembles the distribution of the arterial supply and shares similar nomenclature (10).

1.2.2 Physiology of the nasal cavity

The nasal cavity and sinuses have several functions. At birth, neonates are obligate nasal breathers for the first few months of life and require a functioning nasal cavity and nasopharynx (11). The nasal cavity and sinuses condition the air that we breathe, humidifying and adjusting the temperature and by doing so protect the lungs (9). The nasal hairs, mucus and activity of the beating cilia of the columnar respiratory ciliated epithelium remove bacteria, viruses and particulate matter from air to purify it, prior to entry to the lower respiratory tract (12). Our sense of taste is dependent on the ability of olfactory receptors within the nose binding to odour molecules. Most of these abilities are dependent on a tight balance of physiology and anatomy that can be interrupted by inflammation, such as in the case of both acute and chronic rhinosinusitis (13).

1.3 Rhinosinusitis

Rhinosinusitis is defined by inflammation affecting the sinuses and the nasal cavity. This can be acute (lasting less than 12 weeks) or chronic (lasting more than 12 weeks) (14). Up to 15% of the UK population suffer from CRS. The diagnostic symptoms have been defined by the European Position Paper on Rhinosinusitis (EPOS) as requiring one symptom of nasal blockage / obstruction / congestion or nasal discharge including facial pain / pressure or reduction / loss of smell sensation for greater than 12 weeks with clinical features of CRS including oedema, mucopus or nasal polyps on examination or found on computed tomography (CT) scanning (14). Classically, CRS was broken into phenotypes of the condition including non-polyp and nasal polyp associated disease (CRSsNP / CRSwNP respectively) and allergic fungal rhinosinusitis (AFRS) with the belief that each represented different inflammatory signalling pathways (Figure 1-3). Treatment of CRS is typically managed with medical therapy in the first instance, consisting of oral / topical steroid sprays, nasal douches and macrolide or tetracycline antibiotics, with endoscopic sinus surgery reserved for refractory cases (14). Outcomes following medical or surgical treatment show similar improvements in symptom scores at 6 and 12 months, hence medical therapy is the preferred first line treatment as it is less invasive (15).

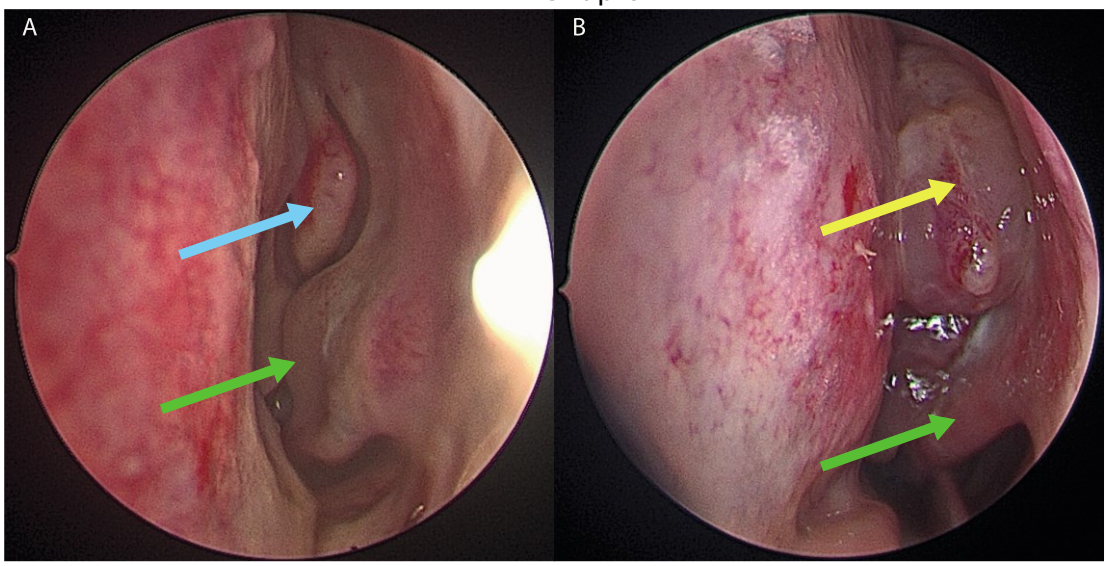


Figure 1-3 Endoscopic appearances of CRSsNP and CRSwNP nasal cavities

Endoscopic appearances of the left nostril in patients with CRSsNP and CRSwNP. A) CRSsNP patients typically demonstrate rhinitic nasal mucosa with mild oedema of middle (**blue arrow**) and inferior turbinates (**green arrow**). B) CRSwNP patients typically demonstrate nasal polyps (**yellow arrow**) that obscure the middle turbinate in this image, with the inferior turbinate (**green arrow**) partially obscured by the polyp. (Images taken by the author with subject's written consent).

In 2020, the most recent iteration of EPOS was released noting that development of treatments based on aetiopathogenesis had been limited and recommended that rhinologists divide patients into inflammatory endotypes, which have defined treatments, rather than phenotypes of CRSsNP and CRSwNP.

The condition has been divided into primary CRS, in which there is no clear predisposing factor and secondary CRS, where there is evidence of local pathology, mechanical defects, autoimmune conditions and immunodeficiency which drive development of the condition. Primary CRS can be further divided into localised or diffuse disease. It can also be separated by the cytokine inflammatory profile into T helper cell 2 (Th2) patterns or non-Th2 patterns (Figure 1-4) (13, 16). These guidelines were designed for the future where endotyping patients' tissue becomes common practice. At present, within the

NHS, this technology has not yet become widely available, and we continue to infer diagnoses based on the presence of polyps, eosinophil counts in mucus and blood, and associated comorbidities such as asthma, which suggest a Th2 pattern of disease.

Despite significant research efforts, a single common pathway for the development of CRS has not been identified. Currently, no singular causative factor has been determined as critical for the development of the condition, and it is likely that a combination of environmental and patient-specific factors contribute to the emergence of CRS and the specific endotypes.

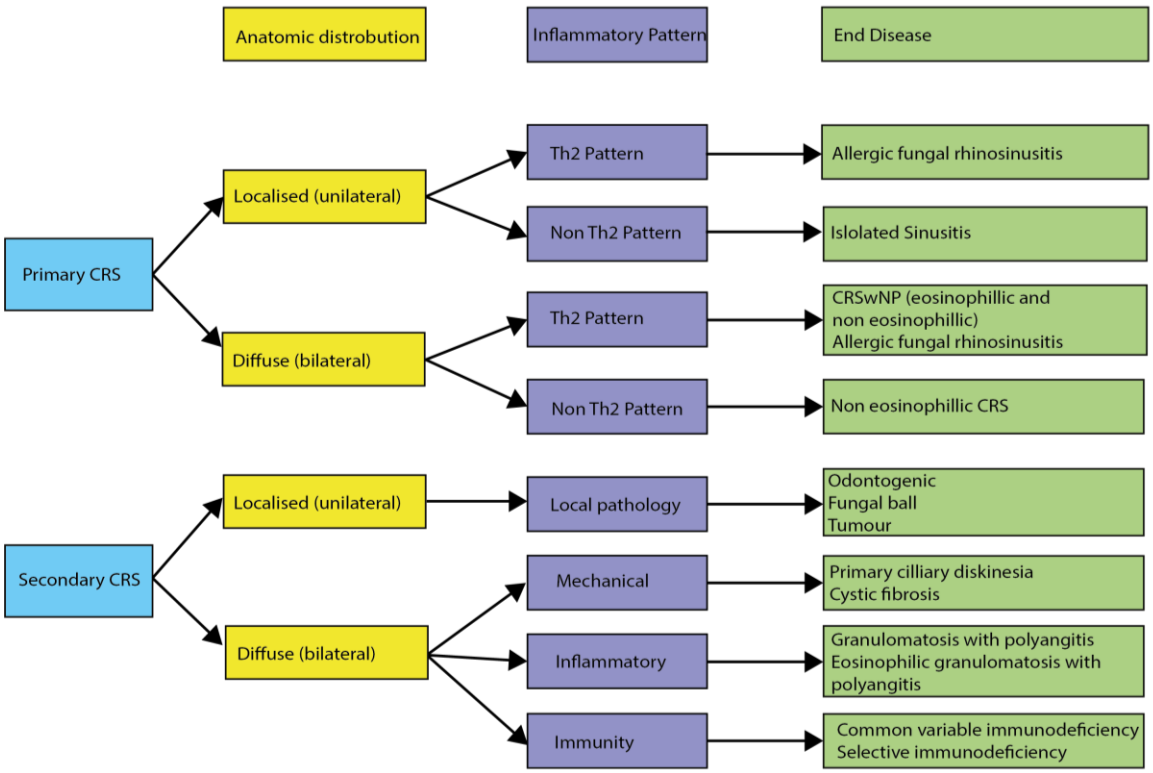


Figure 1-4 Classification of primary and secondary CRS

Classification of primary and secondary CRS. Primary CRS, where there are no clear predisposing factors to development of CRS, is broken into localised or diffuse disease and further divided by the presence of Th2 pattern cytokines. The CRSwNP phenotype is generally associated with Th2 cytokines, while CRSsNP is generally associated with non-Th2 disease, such as non-eosinophilic CRS and isolated sinusitis.
Secondary CRS is diagnosed where there is evidence of local pathology, mechanical, inflammatory or immunity-related causes and are divided by the presence of localised or diffuse disease and their associated cause as described by Grayson *et al.* (16).

1.4 Epidemiology and economic cost of chronic rhinosinusitis

1.4.1 Epidemiology of chronic rhinosinusitis

Calculating the actual epidemiology of CRS is complicated by the significant overlap in symptoms with allergic rhinitis and acute rhinosinusitis. Many extensive prospective studies rely on questionnaires, as examining and imaging large cohorts of patients is challenging. The addition of clinical examination can more accurately diagnose cases but often pick up asymptomatic cases of the condition. Estimates of prevalence of CRS range from 1.01% to 28.4% (Table 1-1). The Global Allergy and Asthma European Network (GA^2LEN) study, the largest and most comprehensive prospective study of CRS symptoms in Europe involving 12 countries and 57,128 participants, demonstrated an incidence of 10.9% (95% confidence interval (CI) of 6.9-27.1%) based on symptoms alone.

Table 1-1 Epidemiology of chronic rhinosinusitis

Author	Criteria used	Country	Incidence
Ostovar *et al*. (17)	EPOS	Iran	28.4%
Pilan *et al*. (18)	EPOS	Brazil	5.5%
Shi *et al*. (19)	EPOS	China	8.8%
Hirsch *et al*. (20)	EPOS	USA	12%
Hastan *et al*. (21)	EPOS	Europe	10.9%
Hoffmans *et al*. (22)	EPOS	Netherlands	16%
Min *et al*. (23)	Clinical symptoms and endoscopy	Korea	1.01%
Tomassen *et al*. (24)	EPOS and endoscopy	Netherlands	6.8%
Dietz *et al*. (25)	EPOS and radiological findings	Netherlands	3.0%
Hirsch *et a*l. (26)	Sinus symptom questionnaire and radiological findings	USA	Male = 7.5%, Female = 1.6%

Incidence of chronic rhinosinusitis demonstrated in each study and the location and diagnostic criteria used.

Interestingly, the prevalence of CRS varies significantly between countries, and even within individual countries. The GA^{2}LEN study demonstrated a low prevalence in Finland of 6.9% in contrast to a much higher rate of 27.1% in Portugal. Regional differences were also observed in Germany, where the town of Duisburg reported a 14.1% incidence compared to just 6.9% in Brandenburg. These disparities suggest that environmental factors might impact the prevalence of CRS (21). Tomassen *et al*. analysed a subgroup of Dutch participants who had filled in the GA^{2}LEN study questionnaire and invited 342

participants for nasal endoscopy. They discovered that while 61.7% of participants with CRS symptoms had positive endoscopic findings, a substantial 38.0% of individuals without reported symptoms showed endoscopic signs of the disease (24). Therefore, it is evident that questionnaire-based epidemiological research can provide a general indication of the CRS burden, but there is a risk of both false positives and false negatives.

Dietz *et al.* studied those undergoing CT or magnetic resonance imaging (MRI) of the head, excluding those undergoing dedicated CT sinus scanning, and asked them to fill out a GA^{2}LEN questionnaire assessing for symptoms of asthma and associated conditions. A total of 834 subjects responded, 6.4% had EPOS defined symptoms and radiological changes with a Modified Lund-Mackay score (MLMS) above 0 (Only 3.0% had a MLMS above 4). Furthermore, symptoms including nasal obstruction, rhinorrhoea and loss of smell were associated with a higher MLMS (expected count ratios of 2.01, 1.81 and 1.75 respectively) (25). Similarly, Hirsch *et al.* used low radiation CT sinus scans to study the MLMS of patients who had previously answered a questionnaire based on sinus symptoms and used weighting methods to account for sampling and participation rates. They demonstrated 7.5% of men and 1.6% of women had both symptoms of CRS and a MLMS ≥ 4. Furthermore, 11.1% of men and 15.6% of women demonstrated a MLMS of ≥ 4 regardless of symptoms in their study cohort, appearing to show that women are less symptomatic despite high levels of opacification within CT scans of the sinuses (26).

1.4.2 Economic cost of chronic rhinosinusitis

The cost of CRS can be broken into direct costs, describing those related to healthcare costs and indirect costs referring to the economic loss of productivity due to patients suffering the disease. The indirect costs of CRS are significant as the disease tends to affect those during working years (30-50). Most economic assessments are based on

small cross-sectional studies, particularly European ones, where costs are extrapolated to account for population size. The actual costs may be significantly different. The most recent study evaluating the economic cost of CRS in the UK has been published by Wahid and colleagues (1). Those with CRSsNP, CRSwNP and AFRS had significantly increased use of healthcare services when compared to non-diseased controls in primary care (4.14 vs 1.16 visits, P≤0.001) and secondary care (2.61 vs 0.40, P≤0.001). This equated to an annual cost of £2974 vs £547 per patient annually when comparing patients with CRS vs those without. When indirect costs were analysed, patients with CRS missed significantly more days of work (10.88 vs 2.92 days annually), equating to a difference in productivity cost of £1567 vs £453 between the groups. Given these costs and an estimate of prevalence of the condition of 11%, it was calculated that the incremental healthcare cost of CRS to the UK was above £16.8 billion (1). Similarly, Lourijsen *et al*. analysed the costs for Dutch patients with CRSwNP and estimated direct costs of €1501 per patient/year and substantially higher indirect costs for patients of €5659 per patient/year. The Netherlands study is more likely to have higher indirect costs due to a much deeper analysis of loss of productivity, in addition to presenteeism and absenteeism calculations. In summary, the direct economic cost to the Netherlands given a population size of 13.4 million and a prevalence of 2% for CRSwNP, was estimated at 402 million euros with indirect costs of 1.5 billion euros (27). Most cost analyses studies in the United States have been completed using administrative health databases which are likely to underestimate the prevalence of the condition but give an accurate cost analysis for the patients using healthcare services. The general trend appears to demonstrate direct costs in the USA are increasing from $8.6 billion in 2011(28) to $12.5 billion in 2016 (29).

Differences between phenotypes of the condition have demonstrated significant annual direct cost differences. Notably, patients with recurrent polyps after surgery had the highest direct cost difference, double that of patients with CRSwNP who did not require surgery, mainly due to increased postoperative costs associated with the surgery and follow up appointments. Patients with comorbidities including asthma, and patients taking corticosteroids or macrolides had increased costs when compared to others with CRSwNP (30).

Rudmik *et al.* demonstrated that patients who opted for surgical management suffered high preoperative indirect costs ($9097) in comparison with those who opted for medical management ($3464). Postoperatively, the surgical cohort had significantly reduced costs of $3301, while those opting for long term medical management tended to have no significant change in year-to-year costs ($3464). Interestingly, the reduction in indirect costs after functional endoscopic sinus surgery (FESS) was valued as higher than that of a total knee replacement (31, 32).

In summary, both the direct and indirect economic costs related to the management of CRS appear to be increasing. A better understanding of the disease drivers and development of novel treatment modalities are needed to reduce the financial strain on the NHS.

1.5 Aetiology of chronic rhinosinusitis

1.5.1 Asthma and allergy

The association of asthma with CRS is clear and suggests that inflammation within the ciliated, pseudostratified columnar respiratory epithelium in the lungs, nose and sinuses may share similar pathophysiological mechanisms. Moreover, according to the unified airway theory, inflammation of the lungs and sinuses are interconnected, with inflammation at one site promoting inflammation at the other. The GA^{2}LEN survey demonstrated an increased adjusted odds ratio (OR) of 3.47 for CRS sufferers also having asthma. In particular, those suffering from CRS and allergic rhinitis demonstrated a much increased OR of 11.85. Furthermore, CRS alone appeared to increase the risk of developing late onset asthma, with an increased relative risk ratio (RRR) of 3.09 (33).

The UK National Chronic Rhinosinusitis Epidemiology Study (CRES) used questionnaires to compare control patients with CRSsNP, CRSwNP, AFRS sufferers, demonstrating a significantly increased prevalence of asthma (9.95%, 21.16%, 46.9% and 73.3% respectively) and inhalant allergy (13.1%, 20.3%, 31.0% and 33.3% respectively). Interestingly, the incidence of house dust mite allergy was significantly higher for those with CRSwNP than CRSsNP phenotypes. Furthermore, patients suffering from CRSwNP and AFRS were increasingly likely to report aspirin sensitivity and require more sinus surgeries (34, 35).

In the USA, 23.4% of CRS patients suffer from asthma compared with 5% of the general population. These patients are more likely to demonstrate the CRSwNP phenotype (47% vs 22%), suffer from olfactory dysfunction (26% vs 6%) and experience nasal congestion (85% vs 60%). Patients with asthma also appear to require more revision sinus surgery procedures (2.9 vs 1.5 procedures) (36).

In the Netherlands, Hoffmans *et al.* demonstrated a significant association between CRS and asthma (OR 2.36), allergic rhinitis (OR 2.87) or an adverse response to painkillers, suggesting they suffered from aspirin-exacerbated respiratory disease (OR 2.34) in their study of 8,347 individuals. It also appeared to be significantly associated with those who classed themselves as descending from Latin-American (OR 3.56), African Creole (OR 2.53) and Hindustani (OR 2.04) backgrounds (22).

Treating the nasal cavity of CRSwNP patients with medical or surgical therapy has been shown to improve asthma control and symptoms, with a measured reduction in exhaled nitric oxide (a marker of lower airways inflammation) and an improvement in forced expiratory volume (37).

1.5.2 Atopic dermatitis

The association of atopic dermatitis (AD, also known as atopic eczema) and CRSwNP is much less clear, yet both seem to exhibit very similar pathophysiological mechanisms. It is estimated up to 9% of patients with CRSwNP also suffer from AD across Europe and the USA, compared with a prevalence of 3.61-10.2% in the general adult population (38, 39). Both conditions appear to have similar Th2-mediated cytokine patterns and involvement of *S. aureus* (40).

In AD, there are many similar histological observations to CRSwNP such as a damaged epithelium, B-cell proliferation and infiltration of Innate lymphoid type 2 cells (ILC2), mast cells and eosinophils. Furthermore, the condition also demonstrates high local and serum levels of IgE (40, 41).

Epithelial dysfunction leads to exposure of the immune system to allergens, microbes and irritants which leads to inflammation and tissue damage further weakening the epithelium. Thymic stromal lymphopoietin (TSLP), a potent pro-inflammatory cytokine,

polarises T cells towards $CD4^+$ Th2 cells producing Interleukin (IL)-4, IL-5 and IL-13 cytokines (40).

S. aureus also appears to be involved in AD with evidence it colonises inflamed skin, promoting the condition's development and severity (42). Recent evidence has shown that *S. aureus* cultured from AD promotes a Th2 cytokine pattern in the dermis of mice and directly activates mast cells using secreted toxins. It appears to significantly upregulate expression of TSLP and Th2 cytokines, as well as stimulate mast cell degranulation, further promoting tissue damage (42-44). Despite the similarities in pathophysiology, there appears to be no association in the prevalence of both conditions suggesting they share very similar but independent mechanisms.

1.5.3 Immune deficiency

It has long been established that immunodeficiency can lead to recurrent respiratory infections, particularly in the case of common variable immune deficiency (CVID) with a prevalence of 1 in 25,000. Evidence has demonstrated the importance of systemic immune deficiency as a cause for CRS, particularly in those with difficult-to-treat disease. Schwitzguebel *et al.* completed a meta-analysis of immune deficiencies in CRS patients, demonstrating an increased prevalence of CVID, selective immunoglobulin A, and unspecified immunoglobulin G deficiencies. They identified a prevalence of 12.7% (95% CI 8.6-18.3%) in those with recurrent CRS and a prevalence of 22.8% (95% CI 17.5-29.3%) in those with difficult-to-treat CRS, compared with a 1% incidence in the general population. The authors noted that many of the immunoglobulin deficiencies were not reported in sufficient detail or were not adequately investigated, which may underestimate the prevalence estimates compared to the true value (45). Research demonstrates that 10% of patients with CRS have symptoms and a diagnosis of CVID.

Pathologically low levels of immunoglobulin A can be found in 16.7% of individuals with CRS and selective immunoglobulin A deficiency represents 6.2% of cases (13, 46). In summary, there is no international standard for the diagnosis of immune deficiency, with wide ranges used for diagnosing many immune deficiencies. This lack of standardisation has made comparison of studies considerably more challenging.

1.5.4 Smoking and pollution

Tobacco smoke produces more than 4,000 harmful components, many of which are toxic to the nasal mucosa. Both active and passive smoke cause structural changes in the nasal mucosa, including an increase in the number and size of the glandular cells, a reduction in the viability of columnar cells, and low-grade inflammation. Furthermore, it reduces the beat frequency of the cilia and inhibits the regeneration of nasal mucosa after endoscopic sinus surgery (47). Unsurprisingly, the GA^{2}LEN study concluded both current and past smoking demonstrated a positive association with CRS, with OR of 1.91 (95% CI 1.77-2.05) and 1.28 (95% CI 1.18-1.38), respectively. A dose-response relationship was identified, with smokers having smoked 50 pack years showing an increased chance of developing CRS, OR 1.45, compared to 25 pack years, OR 1.19 (33). Furthermore, patients with CRS who are former smokers demonstrate worse Sino-Nasal Outcome Test-22 (SNOT-22) and quality of life (EQ-5D VAS) scores than those who have never smoked (48). Interestingly, cessation of smoking can reverse many of the histopathological changes observed in nasal mucosa. Cessation can allow for the regeneration of cilia, columnar and goblet cells and reduce mucosal oedema (34). Complete resolution of quality-of-life scores and histopathological changes to that of baseline are expected to take between 10-20 years (48).

Airborne pollutants also appear to correlate with the development of CRS. This was most clearly observed in the firefighters who developed CRS after the attacks on the World Trade Centre in 2001, with a relative rate of 1.99 (95% CI = 1.64-2.41) for high *vs* low levels of dust exposure and 1.52 (95% CI = 1.28-1.80) for moderate vs low levels of dust exposure (49). Occupational pollutants may increase the risk of developing CRS. Clarhed *et al*. conducted a large questionnaire-based study in Telemark, Norway involving 16,099 participants. Participants were asked about symptoms of CRS and exposure to occupational pollutants. A positive correlation was identified between exposure to paper dust (OR 1.3 95% CI 1.1-1.5), cleaning agents (OR 1.2 95% CI 1.0-1.3), metal dust (OR 1.3 95% CI 1.1-1.6), animals (OR 1.2 95% CI 1.0-1.5) and moisture / mould / mildew (OR 1.3 95% CI 1.3 95% CI 1.1-1.5) and physically strenuous work (OR 1.4 95% CI 1.2-1.7) (50). A similar study in China demonstrated an increased prevalence of CRS symptoms in those having a clearance-related job (OR 1.56), occupational exposure to dust (OR 2.32), and occupational exposure to poisonous gas (OR 2.75) (51).

1.5.5 Viruses

It is well accepted that 99% of acute rhinosinusitis infections are due to viral infection. Typically, the condition lasts for around two to four weeks due to the body's ability to mount an antiviral response. This antiviral response is often non-specific, involving cytokine production and innate immune recruitment, which leads to secondary damage including oedema, engorgement of blood vessels, fluid extravasation and mucus production. This innate immunity typically results in viral elimination and repair of damaged structures, with resolution of most symptoms within four weeks. Interestingly, 80% of patients with CRSsNP were found to have PCR-detectable viral RNA present in their nares, compared with 0% of control patients (52). Rhinovirus, human bocavirus and metapneumovirus are often detected in the nasal wash of CRS patients suffering acute

rhinosinusitis (53). It has also been shown that viral infections have an impact on the bacterial microbiome, promoting pathogenic species including *Neisseria subflava, Haemophilus influenzae* and *Staphylococcus aureus* species (54).

1.5.6 The bacterial microbiome

Early studies looking at bacteria involved in CRS were dependent on culture techniques and demonstrated an abundance of pathogenic species, including *S. aureus*, *Staphylococcus epidermidis (S. epidermis)* and anaerobic gram-negative bacilli including *Pseudomonas aeruginosa (P. aeruginosa)*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Enterobacter* species. These early studies suffered limitations in their detection of anaerobic bacteria and their ability to detect multiple bacterial species, mostly due to the culture techniques used (55).

Culture-independent sequencing techniques, including 16S ribosomal RNA sequencing, have introduced the concept of dysbiosis, a disruption of the delicate balance of bacterial flora known as the microbiome which can initiate and support inflammatory changes throughout the nasal passages of CRS patients (55). Indeed, patients with CRS and those which have poorer surgical outcomes have demonstrated less microbiome biodiversity than controls, where pathogenic organisms including *Streptococcus, Pseudomonas, Corynebacterium* and *Haemophilus* species are likely to outcompete controls (55, 56). A number of community state types (CSTs) which represent various patterns of bacterial biodiversity and organism abundance within the nasal microbiome have been described. To date, seven CSTs have been identified and characterised. Some types have very high rates of perceived pathological organisms, including *S. aureus, P. aeruginosa, and K. pneumoniae species (57)*.

Host and environmental factors such as genetics, sex, immunity, previous antibiotic use, occupation and season appear to influence CSTs (57 , 58). For example, dairy farmers have been demonstrated to have higher biodiversity in their nasal cavities when compared with non-farmers and tend to demonstrate significantly less *Staphylococcus* carriage in their nares, suggesting greater diversity can protect from over colonisation with pathogenic bacteria (59). Other risk factors for CRS, such as smoking have been shown to promote pathogenic organisms such as *Staphylococcus* in the microbiome of the nares (55). Human immunodeficiency virus sufferers have a higher burden of CRS than the general population and are more commonly colonised with high burdens of *S. aureus* in the nasal cavity.

The immature infantile nasal microbiome lacks some of the organisms seen in developed adults and is much less biodiverse. Development of biodiversity in the nasal microbiome is poorly understood but may be influenced by environment at this stage; for example, breastfeeding promotes a nasal microbiome dominated by *Corynebacterium* and *Dolosigranulum* species whereas formula-fed infants are more likely to harbour organisms such as *S. aureus* (60).

There is a complex interplay between bacteria in the microbiome which causes suppression and promotion of other bacteria. For example, those carrying *Corynebacterium* have much lower rates of *S. aureus* carriage as *Corynebacterium* has a higher affinity for nasal mucin. It has also been demonstrated to reduce the expression of various *S. aureus* virulence factors and may outcompete *S. aureus* in the nasal cavity (60). Similarly, those colonised with *S. epidermidis* were 80% less likely to be colonised with *S. aureus* at the same nasal site. The prevalence of *Dolosigranulum* species and

Corynebacterium in the nasal cavity was inversely correlated with the presence of *S. aureus (60)*.

Antimicrobial molecules are produced by bacteria under nutrient-limited conditions to inhibit competition. *S. aureus, Pseudopropionibacterium propionicum (P. propionicum), S. epidermis* and *Staphylococcus lugdunensis (S. lugdunensis)* have all been shown to produce antibacterial metabolites that may influence the surrounding microbiome (57). *S. aureus* has been shown to induce host inflammation by producing lipoproteins which activate Toll-like receptors (TLRs) on macrophages and dendritic cells, initiating an immune response. The resulting innate immune response is less damaging to *S. aureus* than other commensals, consequently S. *aureus* benefits from less competition for nutrients and space (57). *S. aureus* has been demonstrated to produce various adhesion molecules to bind to nasal mucosa, preventing clearance from mucociliary flow (57).

Biodiversity studies comparing the middle meatus and inferior meatus in patients with normal nasal mucosa, allergic rhinitis, CRSsNP and CRSwNP has revealed significant differences in the microbiome composition with low inter-subject variability. Patients with CRSsNP showed increased rates of *Haemophilus, Fusobacterium* and *Staphylococcus* species with depletion of non-pathogenic taxa. Patients with CRSwNP demonstrated dominance of *Staphylococcus, Alloiococcus* and *Corynebacterium* species (61 , 62). Functional endoscopic sinus surgery (FESS) and postoperative co-amoxiclav/clarithromycin appear to reduce the biodiversity of the ethmoid sinuses but this appears to recover at 6 weeks, showing a mild change in the microbial consortia with a similar biodiversity to that of the nasal vestibule or anterior ethmoids identified during surgery (63).

1.6 Cell types in CRS

1.6.1 Mast cells

Mast cells are sentinel cells which are best known for their acute production of histamine and degranulate in response to antigen stimulation in allergic and anaphylactic reactions. In recent years, we have discovered that they have a significant role in chronic inflammation and tissue remodelling.

In CRSwNP, mast cells typically reside in the epithelial layer. Regulated on activation, normal T cell expressed and secreted (RANTES), produced by eosinophils and mast cells, has been shown to be a potent chemoattractant factor for cultured human mast cells, which express C-C chemokine receptor (CCR) 3 and 5. These can be upregulated by histamine and tryptase release, leading to vasodilation, tissue oedema and production of mucin (64). Patients with CRSwNP have been shown to have an abundance of mast cells within the epithelial layer, which contain tryptase but not chymase (65, 66).

Activation and degranulation of mast cells can occur via antigen binding to the immunoglobulin E and high-affinity IgE receptor complex (IgE:FcεR1), resulting in cross-linking of IgE:FcεR1 complexes or substance P binding the Mas-related G protein-coupled receptor X2 (MRGPRX2). This typically leads to the immediate release of preformed mediators including β-hexosaminidase, chymase, histamine, tryptase, tumour necrosis factor alpha (TNF-α), prostaglandins and leukotrienes. Furthermore, their stimulation leads to the delayed expression of cytokines including IL-5, IL-6 and IL-7 as well as adhesion molecules, including vascular cell adhesion molecule (VCAM)-1, platelet aggregating factor and matrix metalloproteinase 9 which promote further accumulation of mast cells, basophils and eosinophils and tissue remodelling (31, 59, 67). Mast cells also express IL-1R, IL-33R and TSLP-R, and respond to their ligands, which

are produced by epithelial cells stimulated by TLR signalling or direct damage, and in turn produce cytokines (IL-1α, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13 and TNF-α and granulocyte-macrophage colony-stimulating factor (GM-CSF)) in response (68). Mast cells also appear to upregulate localised B-cell production of IgE via IL-4 and IL-13 secretion (69). B-cells tend to be presented with antigens by dendritic cells, antigen-presenting cells and helper T cells. The most potent antigens appear to be *S. aureus* enterotoxins, particularly staphylococcal enterotoxin B (SEB), which is the most commonly identified target of IgE in CRSwNP patients (70, 71). This likely leads to local activation and degranulation of mast cells and stimulates the cycle of B-cell IgE production and mast cell recruitment.

Mast cells also produce TLR1-7 and TLR9 which can allow bacterial and viral products to activate them directly, producing leukotrienes, IL-1β and GM-CSF (72, 73). They also play a role in innate immunity by phagocytosing *S. aureus*. Abel *et al.* have demonstrated bone-derived murine mast cells and human mast cell line-1 (HMC-1) cells secrete extracellular DNA traps and antimicrobial compounds that facilitate internalisation and antimicrobial killing. However, *S. aureus* has been shown to subvert these mechanisms in nasal tissue by increasing cell wall thickness and replicating within mast cells (74, 75). What remains unknown is how *S. aureus* subverts these phagocytic mechanisms in CRSwNP to remain within cells and replicate, thereby promoting inflammation.

1.6.2 Epithelial cells

A tight barrier between epithelial cells prevents environmental insults from interacting with the immune system and reinforces epithelial cell polarity. In many allergic and autoimmune diseases, including asthma, atopic dermatitis and allergic rhinitis, this barrier is found to be defective (76). The epithelial barrier relies on impermeable junctions

between epithelial cells, which create a barrier formed by tight junctions, adherens junctions, desmosomes and hemidesmosomes (77).

Tight junctions are the most apical structures, which inhibit solute and water flow through the paracellular space and establish cell polarity, determining the apical and basolateral aspect of the cells. They are formed by transmembrane proteins such as, claudin, occludin and junctional adhesion molecule proteins, which interact with the intracellular proteins known as zona occludens that connect to the actin cytoskeleton (78).

Adherens junctions, formed by cadherin and nectin, are located below the tight occludin junctions and connect to actin filaments via α and β catenin, further reinforcing barrier tensile strength (77).

Desmosomes and hemidesmosomes are the most basal intercellular junction complexes and are formed by the interaction of transcellular proteins desmoglein and desmocollin. These bind to intracellular desmoplakin and intermediate filaments such as cytokeratins, providing strong intracellular binding between cells and the basement membrane (77).

Many studies have reported epithelial barrier defects in the polyps of CRS patients. The epithelial layer appears to transition from an epithelial to a mesenchymal pattern, with a thin layer of unciliated, unpolarised epithelial progenitor stem cells in a process known as epithelial-mesenchymal transition (EMT) (79). Many of the proteins protecting the paracellular space, including claudin, occludin and zonula occludens proteins, are reduced in polyps, while more mesenchymal (basal stem cell) proteins are expressed. The result is a loss of cell polarity and epithelial barrier function, allowing bacteria, viruses and antigens to cause further inflammation (80 , 81).

It is likely EMT is driven by Th1 and Th2 cytokines, including interferon gamma (IFN-γ) and IL-4, as they have been shown to reduce the production of tight junction proteins in air-liquid interface (ALI) cells cultured from patients with CRSwNP (82). Furthermore, epithelial cells express all known TLRs and respond to various stimuli including viral RNA, bacterial pathogen associated molecular patterns (PAMPs), endotoxins, lipopolysaccharide, and flagellin (present in flagellated bacteria) (78). Activation of TLRs has been shown to reduce epithelial barrier integrity directly, diminishing occludin and zonula occludens protein production, as well as increasing production of pro-inflammatory cytokines including IL-6 and IL-8 (83).

Nasal epithelial cells have been shown to harbour intracellular *S. aureus* within the perinuclear space of CRSsNP and CRSwNP patients' nares, and this finding is associated with recalcitrant disease and a more severe disease phenotype (3, 84). To date, it remains unclear through which mechanism *S. aureus* is able to enter this cell type and what effects this has on the cytokine profile of infected epithelial cells. Furthermore, antibiotics, which are commonly used to treat CRS in therapeutic doses, have limited efficacy in eliminating intracellular *S. aureus*. This is due to poor diffusion across the cell membrane and *S. aureus* ability to adapt and survive intracellularly (85, 86).

1.6.3 Dendritic cells

Dendritic cells are antigen-presenting cells located both within the epithelial layer and the submucosa. They interact with naive T cells to induce differentiation into Th1, Th2, Th17 and T regulatory (Treg) cells. There are two known subsets of dendritic cells: myeloid and plasmacytoid dendritic cells. Myeloid cells function primarily as antigen-presenting cells, while plasmacytoid dendritic cells appear to be involved in response to viruses (87). Myeloid dendritic cells, removed from non-eosinophilic nasal polyps, appear to skew T

cells towards Th1 and Th17 phenotypes, while eosinophilic nasal polyp dendritic cells appear to skew naive T cells towards Th2 responses. They also express various receptors, including TLR9 and IgE receptors, which may influence Th1 and Th2 responses respectively. Myeloid dendritic cells appear significantly increased in nasal polyps when compared to non-inflamed mucosa, suggesting they have a significant role in disease pathogenesis (88).

1.6.4 T cells

Naive T helper cells have the capacity to differentiate into Th1, Th2, Th17 and Treg cells which are influenced by antigen-presenting cells such as dendritic cells. Early research divided CRSsNP and CRSwNP into separate phenotypes that elicit different T helper cell responses. CRSsNP demonstrates a Th1 inflammatory pattern (predominated by IFN-γ and TNF-α cytokines) while CRSwNP exhibits a Th2 inflammatory pattern (predominated by IL-4, IL-5 and IL-13 cytokines and eosinophils). Th1 responses are driven by dendritic cells and macrophage activation from environmental stressors, leading to innate lymphoid type 1 cell (ILC1) and Th1 cell differentiation, producing IFN-γ and TNF-α. This leads to the recruitment of neutrophils, resulting in tissue inflammation that does not appear to remodel the tissue to the same degree as Th2 responses. In contrast, Th2 responses can be induced by TSLP, released in response to epithelial cell damage in the presence of dendritic cells. This is more likely to result in tissue remodelling, with high levels of IL-4, IL-5 and IL-13 expression, intense eosinophil infiltration and nasal polyp formation (89).

Th17 responses, primarily characterised by IL-17 production, have been identified in subsets of patients with nasal polyps. These responses, also prevalent in autoimmune diseases and malignancies, are associated with widespread tissue remodelling. Treg

cells act to downregulate other T cell responses by limiting the differentiation of other T cell lineages and have been found to be elevated in nasal polyps (90).

1.6.5 Eosinophils

Eosinophils are granulocytes that play roles in parasitic, bacterial and viral infections, as well as mucosal defence. In the majority of CRSwNP patients, high numbers of eosinophils are found in the circulation and within nasal polyps. There are geographic differences; Asian polyps demonstrate a more Th1/Th17 pattern of inflammation with significant neutrophil infiltration within tissues, whereas Caucasian polyps appear to demonstrate a predominantly Th2 profile with high eosinophilic infiltration (70). Eosinophils are attracted to nasal polyps by T cell-secreted chemokines including RANTES, eotaxin 1-3, monocyte chemoattractant protein 4 (MCP-4), very late antigen (VLA)-4, and VCAM-1, promoted by IL-3, IL-4, and TNF-α cytokines (91-93). These cells are primed by GM-CSF and their survival is promoted by $CD4^+$ T cell-released IL-5, which has been shown to increase eosinophil survival in nasal polyps (94). Eosinophils can produce eotaxin and IL-5, leading to further recruitment and activation in a positive feedback loop (95). Once activated, eosinophils degranulate, producing granule proteins including major basic protein, eosinophil peroxidase, eosinophil cationic protein and eosinophil-derived neurotoxin which leads to the activation and recruitment of neutrophils, mast cells and basophils resulting in widespread tissue inflammation and remodelling (96).

1.6.6 Macrophages

Macrophages are typically found in both Th1 and Th2 endotypes and play very distinct roles in each. In Th1-related diseases, macrophages are influenced to differentiate into classical macrophages by IFN-γ and primarily phagocytose microbes and release

reactive oxygen species (97). However, little is known about their specific function in CRS.

In Th2 mediated CRS, macrophages develop into the M2 phenotype, stimulated by type 2 cytokines. They contribute to remodelling the extracellular environment by secreting factor XIIIA, which promotes fibrin deposition and tissue remodelling. Additionally, they secrete C-C chemokine ligands (CCL) 18 and 23, recruiting further macrophages which produce eotaxins and MCP-4, which are involved in the chemotaxis of eosinophils (98-100). Given that eosinophils also produce CCL-23, it is likely these cell types establish a positive feedback loop, leading to tissue eosinophilia, further macrophage recruitment and tissue remodelling.

1.6.7 Endotyping chronic rhinosinusitis

The complexity of inflammatory patterns in CRS cannot be fully explained by in vitro cell studies due to the variation of inflammatory pathways activated in each individual. Multiple inflammatory pathways are known to be active, presumably as a result of interactions between the immune system and environmental stressors (13).

Initial research categorised CRS into phenotypes of CRSsNP and CRSwNP, suggesting they were driven by Th1 and Th2 patterns of cytokines respectively. This simplistic model provided a conceptual basis to explore Th1 responses, which indicate dendritic cells and macrophages, activated by environmental stressors, lead to ILC1 and Th1 cell differentiation producing IFN-γ and TNF-α. These responses result in the recruitment of neutrophils. Both cytokines and neutrophils cause tissue inflammation and oedema, but do not appear to remodel the tissue to the same degree as in Th2-mediated responses. Conversely, Th2 responses are more likely to result in tissue remodelling with intense eosinophil infiltration and nasal polyp formation. It is suggested that damage to the nasal

epithelium triggers the production of epithelial-derived cytokines including TSLP, IL-33 and IL-1. These cytokines act on dendritic cells to induce naive $CD4^+$ T cells to differentiate into Th2 cells and prompt ILC2 cells to produce IL-4, IL-5 and IL-13. Epithelial damage facilitates the binding of IgE to environmental allergens on mast cells, inciting degranulation and increased production of IL-5, IL-13, prostaglandin D2 (PGD2) and leukotriene C4 (LTC4). This results in recruitment of eosinophils and M2 macrophages causing further inflammation. Macrophages and IL-13 promote fibrin cross-linking and fibrin deposition by expressing factor XIIIa and inhibiting tissue plasminogen activator in the subepithelial layer leading to structural changes (70, 101, 102).

The advent of "inflammatory endotyping" has revealed that the molecular pathology of CRS is considerably more complex, with ten distinct endotype clusters exhibiting a wide range of CRS phenotypes. Tomassen *et al*. demonstrated the multidimensional molecular pathology of the condition, finding IL-5 expression and IgE sensitivity towards *S. aureus* superantigens were significantly associated with polyp disease and asthma (72).

1.7 *Involvement of S. aureus and potential therapies*

1.7.1 *S. aureus* pathogenicity

S. aureus bacteria cause a wide array of disease in humans, including skin, chest, joint, bone, urinary and cardiac infections (103). *S. aureus* pathogenicity stems from a combination of factors, such as antimicrobial resistance genes, enterotoxin genes and immune evasion genes. Moreover, the bacteria have a superb ability to adapt to the environment by switching between different states: planktonic (free floating), biofilm (a polymicrobial cluster of bacteria held in a glycoprotein matrix), intracellular (hiding within host cells) and small colony variant (SCV, the bacterial equivalent of fungal spores).

Antimicrobial resistance is becoming an increasingly prevalent problem among *S. aureus* isolates, which form part of the ESKAPE pathogens group (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* species), recognised for causing serious human infections and multidrug resistance (104). In this context, 14.7% of *S. aureus* clinical isolates are now identified as methicillin-resistant (MRSA), and 7% as vancomycin-resistant (VRSA), with substantial geographical variances likely due to local antibiotic prescribing practices (105, 106). Genes supporting antimicrobial resistance are often carried on mobile genetic elements such as plasmids or transposons (for example the *blaI*, *blaR1*, *blaZ* genes involved in penicillin resistance) or the staphylococcal chromosome cassette *mec* (SCC*mec*). The SCCmec contains multiple mobile genomic islands and antimicrobial resistance genes, including (*mepA, fosB, tet(38), tet(M)*, *erm(C/T), ant(9)-la, mecC* and *mecI* which confer resistance to aminoglycosides, macrolides, lincosamides, streptogramin B, tetracyclines and

methicillin. This cassette facilitates the rapid recombination of genes and horizontal gene transfer between strains (107).

Enterotoxins are superantigens, which are encoded by the enterotoxin gene cluster. They are highly potent T cell activators, and many are responsible for food poisoning. Superantigens are short proteins which are particularly resistant to degradation by proteolytic enzymes and bind to major histocompatibility complex (MHC) class II receptors on antigen-presenting cells (APCs) (108). These superantigens bind to the T cell receptor not at the antigen-presenting site, but rather at the common β-chain found in approximately 20% of T cell clones, leading to their widespread activation (109). Enterotoxins, such as SEB have been demonstrated to directly damage nasal polyp epithelial cells through TLR2 activation. Moreover, they appear to skew non polypoidal tissue of CRSwNP patients towards a Th2 cytokine pathway in explanted tissue (83, 110, 111). The role of other enterotoxins in Th2 mediated diseases is less well understood.

Immune evasion genes are located on the immune evasion cluster and include the staphylococcal complement inhibitor (*scn*) and a combination of staphylokinase (*sak*), chemotaxis inhibitory protein of *S. aureus* (*chp*), staphylococcal enterotoxin A (*sea*) or staphylococcal enterotoxin P (*sep*), with these genes typically harboured on β-haemolysin-converting bacteriophages. Nepal *et al.* have demonstrated a significant enrichment of prophages harbouring these genes in 96% of CRSwNP *S. aureus* isolates, in contrast to 64% of CRSsNP *S. aureus* isolates (112).

Quorum sensing is the ability of bacteria to detect their environment and cell population density. In *S. aureus,* the accessory gene regulator (*agr*) system is the most important for regulating toxin production and a host of other virulence factors. *S. aureus* produces autoinducing peptide (AIP), which accumulates as greater numbers of the bacteria

produce it. When the concentration of AIP is high, it activates the transmembrane receptor AgrC, which, upon phosphorylation binds to AgrA, leading to AgrA phosphorylation. Phosphorylated AgrA then binds to the *agr* region activating two bidirectional promotors, P2 and P3. The P2 promotor expresses RNAII, which includes genes such as the AIP precursor *agrD* and *agrB*, the latter of which encodes the extracellular transporter for AgrD. It also contains *agrA* and *agrC* setting up a positive feedback loop once activated. The P3 promoter expresses RNAIII, a regulatory RNA which also encodes a potent toxin known as delta-haemolysin within the sequence (113). Both activated AgrA and RNAIII influence various downstream targets that enhance virulence. The protein AgrA has been shown to promote production of phenol-soluble modulins. RNAIII triggers the expression of toxin genes including alpha-haemolysin, gamma-haemolysin, serine proteases (*splA-F*), leukocidins (*lukAB, GH*) as well as repression of the repressor of toxins gene (*rot*) (114-116). The Repressor of toxins (Rot) protein represses the synthesis of enterotoxins, such as enterotoxin B (117). Mutations of the *agr* operon have been shown to produce SCVs, low metabolism, smaller clones with thicker cell walls and an enhanced ability to survive within the intracellular compartment (118).

1.7.2 *S. aureus* immune interaction

Our knowledge of the inflammatory mediators driving chronic rhinosinusitis has advanced considerably in the past decade. Epidemiological data clearly indicate that environmental factors play a significant role in development of the disease. However, the mechanisms by which environmental stressors interact with the immune system to promote disease remain elusive. *S. aureus* is potentially the most extensively studied environmental stressor, with accumulating evidence suggesting it plays a substantial role in CRS.

S. aureus colonises the nasal cavity in 32.4% of the general population, compared with 60% of patients with CRSwNP (4, 119). Only 6.7% of the general population demonstrate serum IgE antibodies towards a small repertoire of known *S. aureus* enterotoxins, in contrast to 32.5% of patients with CRSwNP, many of which are directed towards SEB (73).

Indeed, SEB remains the most extensively researched virulence factor and has been shown to significantly increase pro-inflammatory cytokine production in ex vivo nasal polyp models (14). Patients with nasal polyps who have IgE antibodies targeting SEB exhibit a distinctive pro-inflammatory endotypes, characterised by the expression of IL-5, IL- 8, IL-6, IL-17A, IL-22 and myeloperoxidase, which is distinct from control tissue (72). Moreover, exoproducts from *S. aureus* have been shown to impede epithelial repair by altering epithelial cell polarity, as well as reducing the number and dynamic movement of lamellipodia (120).

Prior work by the Southampton Upper Airway Research Group has shown that nasal polyps contain an abundance of epithelial mast cells harbouring intracellularly localised *S. aureus* (121). Using HMC-1 and Laboratory of Allergic Diseases 2 (LAD2) cell lines, our group's previous work has demonstrated a CRS-specific, pathogenic *S. aureus* was able to internalise, replicate and escape into the extracellular space, repopulating eradicated bacterial populations (121, 122). This curious observation is not the case with all *S. aureus* strains, as a recent study demonstrated only 7 out of 23 clinical isolates survived internalisation by non-professional phagocytes (123, 124).

Intracellular bacteria are highly resistant to commonly used antibiotics and antiseptics (Figure 1-5) (85). Furthermore, the internalisation of bacteria has been proposed as a protective mechanism to evade the host immune system and provide nutrients for the organism to propagate. These mechanisms may explain why *S. aureus* persists as a very

common and serious pathogen, capable of causing life-threatening and challenging infections. It remains unclear why many individuals with *S. aureus* nasal colonisation show no evidence of CRS or mast cell infection, while patients with CRSwNP exhibit a plethora of mast cells with *S. aureus* infection. Given the fact that only a minority of clinical *S. aureus* isolates survive internalisation, it is probable that there are key differences in virulence factors between commensal strains colonising healthy individuals and those infecting patients with CRSwNP.

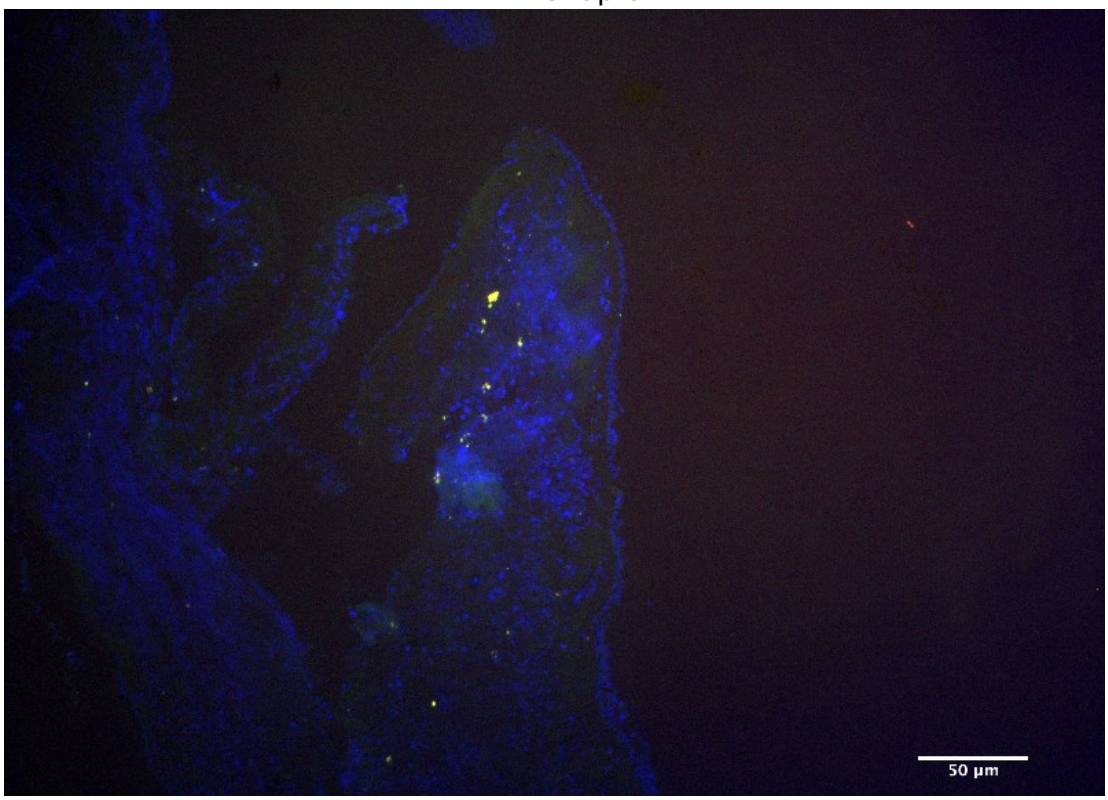


Figure 1-5 Antibiotic treated nasal polyp probed for the presence of intracellular S. aureus

A nasal polyp treated with penicillin/streptomycin to eliminate extracellular *S. aureus* and probed for the presence of intracellular *S. aureus* using a 16S ribosomal RNA probe specific for *S. aureus* (depicted in yellow). Nuclei of cells are stained with DAPI, appearing blue. Image taken from Goldie *et al.,* unpublished data.

1.7.3 Available antistaphylococcal treatments

There are limited treatments that have proven effective in eliminating *S. aureus* associated with CRS. Oral antibiotics have been widely used for treating acute-on-chronic exacerbations, with little evidence supporting their superiority over placebo or nasal steroid therapies. A two-week course of oral co-amoxiclav compared to placebo showed a modest reduction in *S. aureus* carriage between the co-amoxiclav-treated group (62% down to 23%) as opposed to the placebo group (remained at 36%), although the number of participants was small and this was not the primary analysis of the study (125).

Macrolides including clarithromycin, and tetracyclines such as doxycycline, are commonly used to treat CRS exacerbations, but there is limited evidence on their ability to specifically clear *S. aureus*. This is because many studies do not distinguish between positive cultures of various organisms, making it challenging to assess the efficacy of these drugs against *S. aureus*. Legent *et al.* conducted a large double-blinded study randomising patients to co-amoxiclav and ciprofloxacin, demonstrating clearance of various bacteria including *S. aureus* in 80% of cases for both treatment groups and significant improvements in patient symptoms and endoscopic findings by day 10 (126). Van Zele *et al.* carried out a double-blinded study comparing oral methylprednisolone or doxycycline in nasal polyp patients, observing reductions in nasal polyp size, nasal symptom scores and inflammatory markers in both groups (127). It is postulated that these oral antibiotics may exert a pleiotropic effect in CRS; however, it is possible that some of the observed benefit in patient outcomes is attributable to their antimicrobial activity against bacteria including *S. aureus*.

Topical mupirocin gel is routinely employed to decolonise the nasal cavity in patients with nasal MRSA colonisation. Jervis-Bardy *et al.* conducted a randomised double-blinded study comparing mupirocin sinus rinses with saline nasal rinses in CRS patients colonised by *S. aureus*. They observed a significant reduction in *S. aureus* culture (89%, $P \leq 0.01$) and an improvement in patient outcomes measured by visual analogue scale in the mupirocin group, as opposed to no change in the saline rinse group, after an assessment period of 28 days (128). Solares *et al.* treated 42 patients with exacerbations of CRS due to MRSA with mupirocin and noted significant symptom improvement in 41 patients. Only one case exhibited resistant disease at 6 weeks; however, the study's methodology did not include cultures from patients who improved (129). Neither study

conducted long term follow-ups and it remains possible that patients may have experienced recurrences at a later date.

Povidone-iodine topical treatment, when added to nasal washes, has been shown to improve patient nasal discharge as observed on endoscopy and to achieve reductions in eosinophil, neutrophil and IgE counts. However, it had minimal effect on *S. aureus* culture, with only a slight reduction following treatment (38% reduced to 23%) (130).

Currently, CRS is the most common reason for prescribing antibiotics in primary care in the USA (131). Furthermore, most antimicrobials fail to treat intracellular bacteria effectively, often due to their inability to penetrate the cell membrane. With antimicrobial resistance rising at an alarming rate, there is a pressing need to curtail the use of antimicrobials across medicine. Consequently, the development of new therapeutic approaches is becoming increasingly crucial.

1.7.4 The potential of statins as a CRS treatment

Two large-scale studies have recently reported a significantly reduced incidence of CRS in patients taking oral statins. Gilani *et al.* retrospectively studied 10,965 patients and demonstrated a reduction in the OR of being diagnosed with CRS to 0.716 (95% CI 0.612-0.838) for patients taking oral statins (5). Wilson *et al.* reported similar results using the National Ambulatory Medical Care Survey data from North America. In their review of over 10 million patient visits, they found reduced OR of being diagnosed with CRS in patients taking statins of 0.53 ($P<0.001$) for univariate and 0.79 ($P=0.03$) for multivariate logistic regression analysis (6).

Topical treatment of mouse wounds infected with MRSA with simvastatin has shown significant reductions in bacterial load and marked improvements in wound healing, alongside reductions in pro-inflammatory cytokines IL-6, TNF-α and IL-1β (132, 133).

The effects of statins are primarily attributed to inhibition of the mevalonate pathway, which is essential for isoprenoid synthesis in humans and certain bacterial species, including *S. aureus*, through the inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase). This inhibits cholesterol synthesis and the production of isoprenoids necessary for protein prenylation (134). Lipophilic statins, in vitro, diminish cholesterol production, which directly affects bacterial growth and alters protein manufacture by reducing signalling protein prenylation (132, 135). In mammalian cells, statins lower cholesterol levels in lipid rafts, which in turn decreases areas involved in bacterial translocation and intracellularisation, attenuating the resultant pro-inflammatory response. They also appear to modulate signalling in mast cells, reducing degranulation in response to IgE-dependant stimulation and safeguarding cells from the effects of bacterial toxins (83, 136-139).

Given the capacity of lipophilic statins to penetrate the cell membrane, alongside their observed reduction in the prevalence of CRS and their anti-inflammatory and bactericidal properties, there is a potential to repurpose them as a novel treatment for CRS, particularly for cases related to *S. aureus*.

1.8 Preliminary work

Our group's preliminary work in HMC-1 and LAD2 cell lines has demonstrated that infected mast cells can produce low-level pro-inflammatory effects via release of IL-5, IL-8, TNF-α and TGF-β1. The potential actions of these cytokines include mediating chronic inflammation, tissue oedema and polyp formation. The virulence factor and toxin SEB was found to markedly increase the ability of mast cells to internalise *S. aureus*. Furthermore, SEB independently reduced the expression of IL-1B, involved in recognition of PAMPs, potentially impairing the immune system's ability to recognise and respond to bacterial infections (122). These results have been corroborated in nasal epithelial explant models. Live *S. aureus* drastically increased mast cell recruitment to the epithelial and subepithelial layers in CRSwNP patients but not in control patients. When combined with SEB, the number of mast cells containing intracellular *S. aureus*, along with mast cell density and extent of degranulation, appeared to significantly increase in the subepithelial layer in CRSwNP tissue (75).

This earlier work was conducted with a single strain of *S. aureus* cultured from a patient with CRSwNP and it was unclear if *S. aureus* found in the nasal cavity of patients without the disease would exhibit the same behaviour or share similar virulence factors.

1.9 Hypothesis

CRSwNP-associated *S. aureus* harbours virulence factors that facilitate internalisation and replication within mast and epithelial cells. Simvastatin can decrease the viability of intracellular *S. aureus* and reduce the production of pro-inflammatory cytokines linked to CRSwNP-associated *S. aureus*.

1.10 Aims

1. To compare genome differences and virulence factors between *S. aureus* strains collected from control, CRSsNP and CRSwNP patients.

2. To ascertain whether CRSwNP *S. aureus* isolates have an enhanced capacity to internalise, survive and replicate within mast cells in comparison to control *S. aureus* isolates.

3. To investigate whether simvastatin can diminish the intracellular survival of CRSwNP-related *S. aureus* isolates within mast and epithelial cells, as well as mitigate the associated pro-inflammatory response.

Chapter 2 **Materials and methods**

2.1 Ethical approval

Tissue and bacterial swabs were provided by patients attending rhinology clinics, undergoing FESS for CRS (sNP/wNP) and those undergoing nasal surgeries including turbinoplasty, septoplasty and biopsies of nasal masses / skull base surgery. Full ethical approval was obtained for the receipt of tissue and bacterial specimens by the author from Southampton University Research Integrity and Governance Panel (**ERGO 54006)**, Hampstead Research and Ethics Committee and the Health Research Authority (Reference code: **20/PR/0183**) (Appendix A, B) for the protocol (Appendix C). Patients were provided with a participant information sheet in advance of their surgery (Appendix D) and verbally consented by the research team for study participation signing a consent form on the day of surgery (Appendix E). The study was adopted onto the National Institute of Health Research (NIHR) portfolio gaining support from the local Clinical Research Network. Ethical approval for the receipt of previously isolated strains of *S. aureus* was granted from Southampton and South West Hampshire Research and Ethics Committee (Reference code: **REC 09/HO501/74**).

2.2. Subjects

Subjects were approached preoperatively or in clinic, prior to their procedure, using a patient information sheet and verbal explanation of the study. Patients consenting to participate in the study had demographics recorded including age, sex, nasal disease, surgical or clinic procedure, use of antibiotics and oral steroids in the past month, allergy status, smoking status, coexisting medical comorbidities including immunosuppression, asthma and chronic obstructive pulmonary disease (COPD). Each participant was assigned a pseudonymised sample log ID, which served to minimise the risk of a

confidentiality breach. Bacterial nasal swabs (M40 Transystem, Copan, Brescia, Italy) and/or nasal tissue was taken from the participant and both samples and demographic information were linked to this code. A code break list was securely stored separately from the pseudonymised data on the Edge server at University Hospital Southampton. This ensured that patients could withdraw their consent at any point, allowing both their data and samples to be destroyed.

2.3 *S. aureus* bacteria receipt and confirmation testing

S. aureus cultured from study participants, a strain cultured from a CRSwNP patient (CRSwNP 1) kindly donated by Dr Timothy Biggs (Southampton Upper Airway Research Group) and a control strain (Control 1) provided by Dr David Cleary (Senior Research Fellow in Microbial Sciences, NIHR Biomedical Research Centre, University of Southampton) from a paediatric nasopharyngeal swab were utilised for further study. All strains underwent confirmation testing by growing cultures on Columbia blood agar (CBA) plates (Oxoid, Basingstoke, UK) at 37°C in 5% CO_2. Single colonies were aseptically selected from the plates using a sterile pipette tip and tested for production of catalase, DNase and coagulase.

2.3.1 Catalase test

Single colonies were tested for the presence of catalase, an enzyme produced by aerobes and facultative anaerobes which catalyses conversion of hydrogen peroxide to oxygen and water, utilising the well-established slide method (94). A 10 µL drop of 3% hydrogen peroxide (Sigma Aldrich, Poole, UK) was placed on a glass slide. Colonies were aseptically transferred from CBA plates with sterile pipette tips and placed into the hydrogen peroxide. A positive reaction was demonstrated by the formation of bubbles created by oxygen gas release via the reaction $2H_2O_2 \rightarrow 2H_2O + O_2$.

2.3.2 DNase test

Deoxyribonuclease enzymes, typically secreted by *Streptococcus* and *Staphylococcus* species, function to break down extracellular DNA traps. To test the presence of these enzymes, single colonies of *S. aureus* were incubated in Roswell Park Memorial Institute medium 1640 (RPMI 1640, Fisher Scientific, Loughborough, UK) at 37°C in the presence of 5% CO_2. When an optical density at 600nm (OD600) of 0.2 was achieved, 10 µL was pipetted and smeared onto dehydrated DNA agar plates (Oxoid), and incubated overnight for 18 hours following a well-established method (140). The plates were then exposed to 1 N hydrochloric acid (Sigma Aldrich) for 20 minutes which denatured the dehydrated DNA. This resulted in a visible halo around colonies producing DNase, indicating areas where DNA has been digested.

2.3.3 Coagulase testing

The enzyme coagulase, which distinguishes between *Staphylococcus* species, is produced by *S. aureus*. The enzyme binds to prothrombin and activates it, converting fibrinogen into fibrin (140). To test for the presence of coagulase, single colonies of *S. aureus* were incubated in RPMI 1640 at 37°C in a 5% CO_2 atmosphere. After reaching an OD600 of 0.2 as measured by spectrophotometry (Jenway 6300 visible spectrophotometer, Cole-Parmer, Cambridgeshire, UK) 10 µL of the broth was pipetted and spread onto chromogenic agar plates (Brilliance Staph 24 Agar, Oxoid). Plates were further incubated for 24 hours at 37°C in 5% CO_2. Blue colonies on the agar indicated the presence of coagulase in the samples (Figure 2-1).

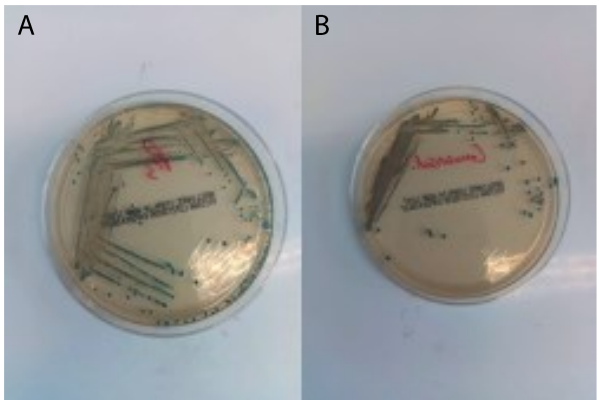


Figure 2-1 S. aureus isolates cultured on Brilliance Staph 24 Agar

S. aureus Control 1 and CRSwNP 1 isolates were cultured on Brilliance Staph 24 Agar. **A** – CRSwNP 1 *S. aureus* isolate demonstrating the presence of blue coagulase-positive colonies on the chromogenic agar. **B** – Control 1 strain of *S. aureus* also showing blue coagulase-positive colonies on chromogenic agar.

2.3.4 Storage of Samples

Isolated bacteria were tested as described above and stored if they exhibited enzymes characteristic of *S. aureus,* including catalase, DNase and coagulase. Four colonies were selected from Brilliance Agar and grown in 10 mL of RPMI 1640 at 37°C in a 5% CO_2 atmosphere until reaching log phase, with an OD600 between 0.2-0.4 as determined by spectrophotometry. Cultures were removed from the incubator and 750 µL of the culture was mixed with 250 µL of 100% glycerol (VWR, Lutterworth, UK) before being frozen at -80°C.

2.3.5 MALDI-TOF mass spectrometry of stored samples

Each isolate was grown on CBA plates at 37°C in the presence of 5% CO_2 for 16 hours. Isolates were selected and underwent matrix-assisted laser desorption/ionisation-time of flight (MALDI-TOF) mass spectrometry using a MALDI Biotyper Sirius system (Bruker, Cambridge, UK). The time-of-flight patterns were compared with those in the MALDI Biotyper Library RUO (Bruker) at the Public Health England laboratory based in Southampton General Hospital.

2.3.6 Short-read, paired-end Illumina sequencing of microbial genomes

Samples were recovered from the -80°C freezer and thawed at room temperature. These samples were then plated on CBA plates and incubated at 37°C in a 5% CO_2 environment for 16 hours. Single colonies were cultivated to logarithmic growth as previously described and spread across two CBA plates using a sterile loop; they were then incubated at 37°C for 16 hours. The bacterial cultures were harvested into 20 mL of phosphate-buffered saline (PBS) without calcium or magnesium (Sigma Aldrich) and assessed for an OD600 value. A volume corresponding to 10 OD600 units was centrifuged at 12,000 g (Fresco 21 Microfuge, Thermo Fisher, Loughborough, UK) and resuspended in 1 mL of PBS. The samples were then washed in PBS and re-suspended in 0.5 mL of DNA/RNA shield buffer (Zymo Research, Freiburg im Breisgau, Germany). The prepared samples were sent to MicrobesNG (Birmingham, UK) for short-read (30x), paired-end Illumina sequencing.

2.3.7 Bioinformatics approach

The Bactopia pipeline facilitates comprehensive analysis of bacteria that have been shotgun or Illumina-sequenced, offering an array of tools for assembling draft genomic sequences and analysing sequences to classify them and predict virulence-related genes. Additionally, it incorporates quality checks to confirm that samples meet the requisite standards for subsequent examination (Figure 2-2). Analysis of the short-read, paired-end Illumina sequences was conducted utilising the Bactopia pipeline by the author.

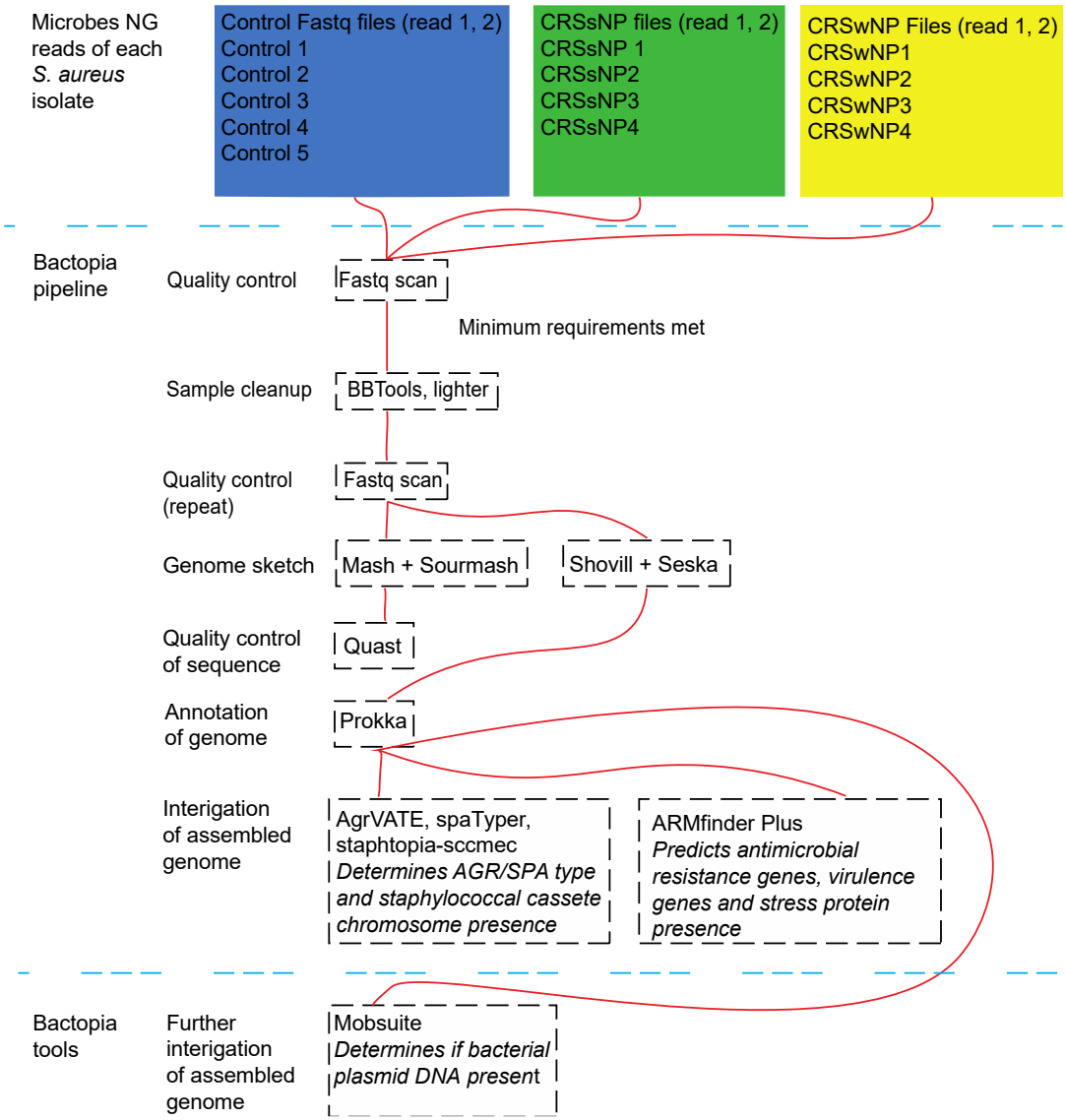


Figure 2-2 Schematic depicting S. aureus genome assembly and analysis

The Bactopia pipeline overview. Bactopia accepts FASTQ file inputs and performs quality assurance on the paired-end reads. The Fastq-scan programme ensures that each contig meets a minimum read length requirement of 35 and 250 base pairs, and it estimates the number of base pairs for each contig using Mash. Following this, the pipeline employs BBTools and Lighter to remove Illumina adapters, phiX contaminants and to correct sequencing errors, ultimately providing quality controlled paired FASTQ files.

Bactopia (version 2.2.0) was installed using Conda (version 4.13.0) on an Apple MacBook Pro. The datasets for *S. aureus* and *S. pseudintermedius* multi-locus sequence type (MLST) schemes, as well as completed reference sequence genomes and other completed genomes from *Staphylococcus* species were downloaded using the following terminal commands:

```
-- Conda activate bactopia
-- species “staphylococcus aureus, staphylococcus pseudintermedius”
-- include_genus
```

Paired-end DNA sequencing reads (contigs) were received as FASTQ files from MicrobesNG. Each paired-end read sequence was allocated to the corresponding files and saved within the ‘results’ parent directory. Subsequently, a manifest file listing the location of each sequence was generated and named ‘prepareoutput.txt’

```
sample runtype r1 r2 extra
1006 paired-end
     /Users/simongoldie/sequecingdata/samplefastq/1006_R1.fastq.gz
     /Users/simongoldie/sequecingdata/samplefastq/1006_R2.fastq.gz
1007 paired-end
     /Users/simongoldie/sequecingdata/samplefastq/1007_R1.fastq.gz
     /Users/simongoldie/sequecingdata/samplefastq/1007_R2.fastq.gz
1012 paired-end
     /Users/simongoldie/sequecingdata/samplefastq/1012_R1.fastq.gz
     /Users/simongoldie/sequecingdata/samplefastq/1012_R2.fastq.gz
1015 paired-end
     /Users/simongoldie/sequecingdata/samplefastq/1015_R1.fastq.gz
     /Users/simongoldie/sequecingdata/samplefastq/1015_R2.fastq.gz
1016 paired-end
     /Users/simongoldie/sequecingdata/samplefastq/1016_R1.fastq.gz
     /Users/simongoldie/sequecingdata/samplefastq/1016_R2.fastq.gz
1020 paired-end
     /Users/simongoldie/sequecingdata/samplefastq/1020_R1.fastq.gz
     /Users/simongoldie/sequecingdata/samplefastq/1020_R2.fastq.gz
2004 paired-end
     /Users/simongoldie/sequecingdata/samplefastq/2004_R1.fastq.gz
     /Users/simongoldie/sequecingdata/samplefastq/2004_R2.fastq.gz
2008 paired-end
     /Users/simongoldie/sequecingdata/samplefastq/2008_R1.fastq.gz
     /Users/simongoldie/sequecingdata/samplefastq/2008_R2.fastq.gz
```

2010 paired-end
/Users/simongoldie/sequecingdata/samplefastq/2010_R1.fastq.gz
/Users/simongoldie/sequecingdata/samplefastq/2010_R2.fastq.gz
2011 paired-end
/Users/simongoldie/sequecingdata/samplefastq/2011_R1.fastq.gz
/Users/simongoldie/sequecingdata/samplefastq/2011_R2.fastq.gz
3019 paired-end
/Users/simongoldie/sequecingdata/samplefastq/3019_R1.fastq.gz
/Users/simongoldie/sequecingdata/samplefastq/3019_R2.fastq.gz
3021 paired-end
/Users/simongoldie/sequecingdata/samplefastq/3021_R1.fastq.gz
/Users/simongoldie/sequecingdata/samplefastq/3021_R2.fastq.gz
3022 paired-end
/Users/simongoldie/sequecingdata/samplefastq/3022_R1.fastq.gz
/Users/simongoldie/sequecingdata/samplefastq/3022_R2.fastq.gz
P3strain paired-end
/Users/simongoldie/sequecingdata/samplefastq/P3strain_R1.fastq.gz
/Users/simongoldie/sequecingdata/samplefastq/P3strain_R2.fastq.gz
commensal paired-end
/Users/simongoldie/sequecingdata/samplefastq/commensal_R1.fastq.gz
/Users/simongoldie/sequecingdata/samplefastq/commensal_R2.fastq.gz

The Bactopia tool was used to align each paired-end read sequence employing the following command in the terminal:

```
bactopia --samples prepareoutput.txt
```

The quality controlled FASTQ files are converted into genome sketches using Mash and Sourmash, referencing RefSeqMashSketch and GenBank Sourmash Signatures (Figure 2-3). The sequence reads are also assembled *de novo* by Shovill and Seska (141, 142). The resulting assemblies undergo quality assessment with QUAST, which evaluates genome coverage and base pair discrepancies between contigs.

Subsequently, genome annotation is performed with Prokka, yielding predicted genes and proteins. These annotations are further examined using various command-line scripts, which include:

- AgrVATE, spaTyper, staphopia-sccmec was employed to ascertain the *agr* locus type and operon variants, typing of staphylococcal protein A, and the detection of the staphylococcal cassette chromosome element carrying the methicillin-resistance gene.
- AMRFinderPlus was utilised, leveraging the AMRFinderPlus database, which encompasses information on antimicrobial resistance genes, virulence factors and stress-response proteins.
- MOB-suite was engaged to identify the presence of bacterial plasmid DNA within the draft genome assemblies.

Figure 2-3 Fastq-scan quality score of each paired-end read

Quality control assessment of the FASTQ sequences used for assembling draft genome assemblies post sample clean-up. The Y-axis represents the sequencing quality score (Phred score), while the X-axis indicates the number of base pairs into the contig read. The blue line indicates the calculated sequence quality. The yellow box denotes the 25th to 75th percentiles and the error bars represent the 10th to 90th percentiles. Each paired-end read fell within the Phred score threshold for analysis, depicted by the green area.

2.3.8 *S. aureus* bacterial culture

S. aureus cultures, stored at the logarithmic phase in RPMI 1640 with glycerol were recovered from -80°C storage and allowed to warm to room temperature. The isolates were plated on CBA plates (10 µL/plate) and incubated at 37°C in the presence of 5% CO_2. Individual colonies were selected and inoculated in 10 mL of RPMI 1640 and incubated at 37°C. Serial OD600 measurements were taken using a spectrophotometer at 600nm and graphed against colony-forming unit (CFU) formation on CBA plates for each isolate (Figure 2-4). These experiments were replicated four times, and the exponential growth phase was correlated with CFU counts to determine the line of best fit and R^2 value (Figure 2-5).

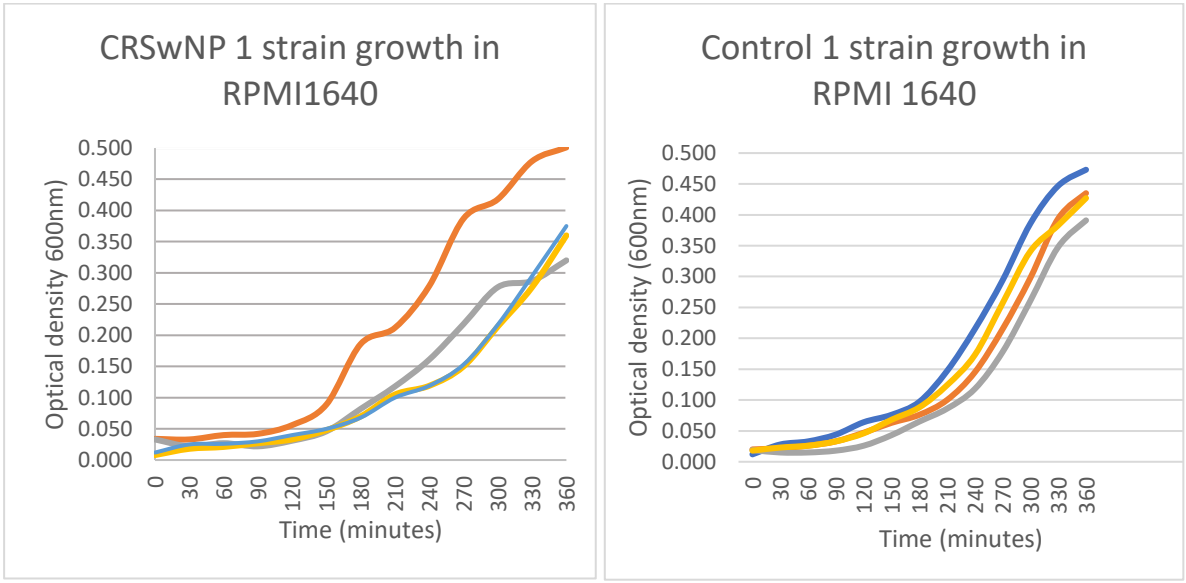


Figure 2-4 S. aureus growth curves over time for the Control 1 and CRSwNP1 strains

S. aureus was cultured over time in RPMI 1640 and the growth of the Isolate was measured by spectrophotometry at 600nm. Both strains were observed to enter the exponential growth phase between 180 and 360 minutes before reaching a stationary phase. Each colour (blue, yellow, orange and grey) represents an experimental replicate.

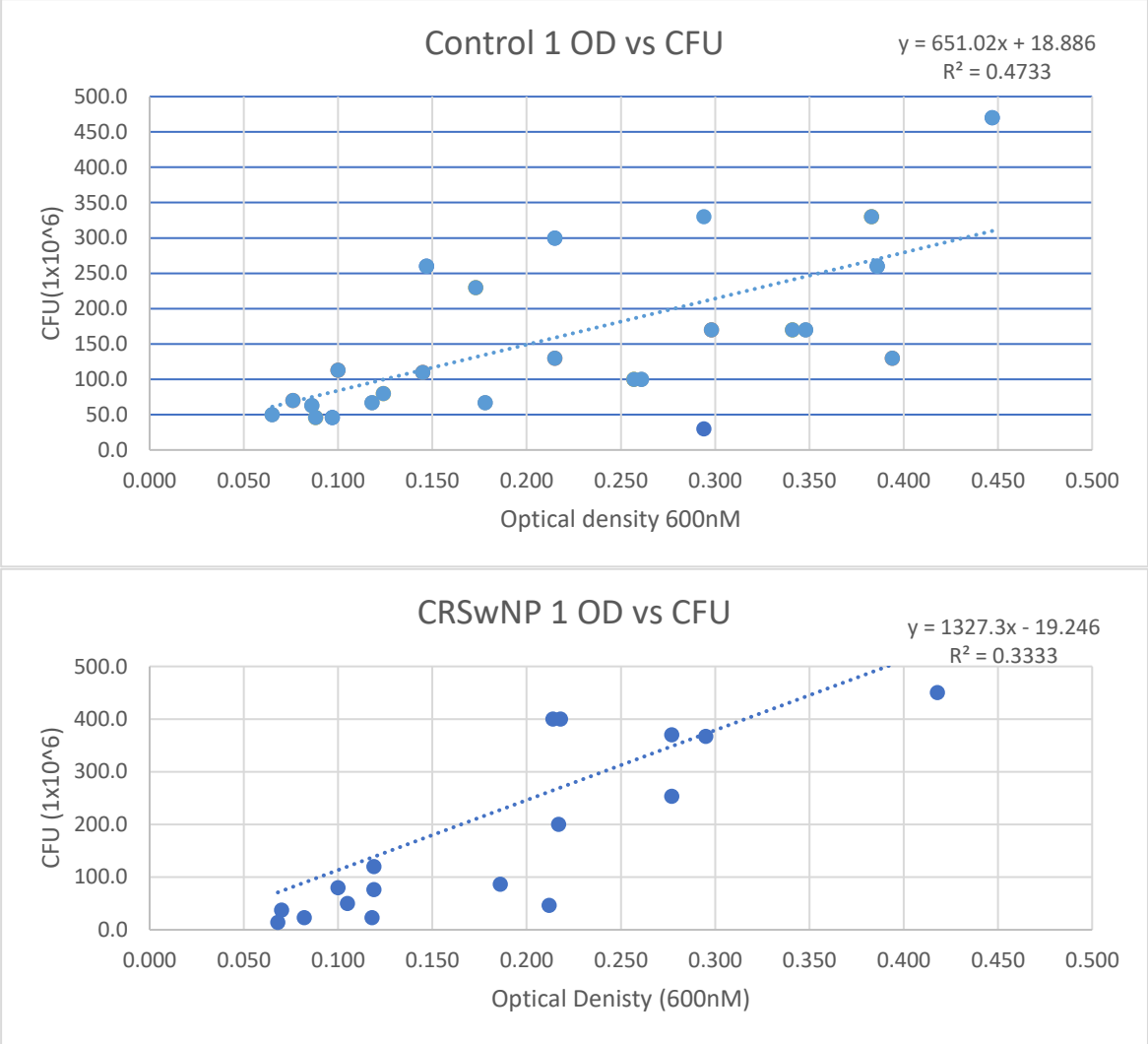


Figure 2-5 Serial optical density measurements vs colony forming unit enumerations for S. aureus isolates

Serial optical density measurements for *S. aureus*, taken using spectrophotometry at 600nm were compared with colony-forming unit counts on CBA plates. Blue dots depict each absorbance value and the corresponding CFU enumeration. The dashed line represents line of best fit.

2.4 Cell and tissue culture

2.4.1 HMC-1 mast cell line

Human mast cell line 1 (HMC-1) cells represent immortal, immature mast cells derived from a patient with mast cell leukaemia and were originally isolated by Dr. J.H. Butterfield at the Mayo Clinic in Rochester, Minnesota, USA. Due to activating mutations in the tyrosine kinase receptor KIT, the HMC-1 cell line exhibits growth factor-independent proliferation with a rapid doubling time of 18 hours. However, these cells have certain limitations, including the expression of a non-functional high-affinity IgE receptor (FcεRI) and poor granularity; namely, they lack significant quantities of histamine, heparin, tryptase and chymase (143). HMC-1 cells were cultured in 250 mL cell culture flasks (Greiner Bio-One International, Kremsmunster, Austria) using Iscove's Modified Dulbecco's Medium (IMDM, Sigma Aldrich) supplemented with 10% foetal bovine serum (FBS, Sigma Aldrich) and 0.2% penicillin/streptomycin (Sigma Aldrich). The medium was refreshed every 3 days by centrifuging the cells (250 g for 5 minutes, MSE Mistral 3000, Rue du Vieux Bourg, France) and resuspending them in a suitable volume of fresh medium to maintain a cellular density below 5 million cells per millilitre.

2.4.2 LAD2 cell Line

Laboratory of Allergic Diseases 2 (LAD2) cells were established from a $CD34^{+}$ bone marrow aspiration of a patient diagnosed with aggressive mastocytosis and possess a functioning KIT receptor (144). These cells exhibit a typical doubling time of two weeks and represent a significantly more differentiated cell type when compared with HMC-1 cells. They exhibit characteristics more akin to mature mast cells, including the possession of granule components such as tryptase, heparin and chymase. Moreover, they demonstrate markedly improved histamine release capability due to a functional

FcεRI. LAD2 cells were cultured in StemPro–34 media (Life Technologies, Paisley, UK) in the presence of 0.1 µM stem cell factor (SCF, PeproTech, London, UK), and 0.2% penicillin/streptomycin and 4 mM L-glutamate (Sigma Aldrich). LAD2 cells received fresh media and mechanical dislodgement from 250 mL cell culture flasks every week to keep cell numbers below a maximum of 5 x 10^5 cells /mL. Complete media exchange was performed every 4 weeks by centrifugation at 250 g for 10 minutes with resuspension in fresh media.

2.4.3 Primary nasal epithelial cell ALI cultures

Collagen-coated T-75cm^2 cell culture flasks (Scientific Lab Supplies, Nottingham, UK) were coated with 50 mL of 1:100 PureCol:ddH_2O (Advanced Biomatrix, Carlsbad, CA, USA). Inferior turbinate tissue obtained from patients undergoing inferior turbinate reduction surgery was placed in Hank's balanced salt solution (HBSS, Sigma Aldrich) and sectioned into 3 mm squares. Subsequently, 2-3 squares of turbinate tissue were cultured in 10 mL of PneumaCult™-EX Plus media. This medium comprised PneumaCult™ EX Basal Medium, 1X PneumaCult™ EX Supplement, 1X Hydrocortisone (STEMCELL Technologies), 0.125 µg/mL amphotericin B (Sigma Aldrich) and 25 µg/mL gentamycin (Gibco, Paisley, UK) within the prepared collagen coated T-75 cm^2 flasks. The cultures were incubated at 37°C in a 5% CO_2 atmosphere. Media changes were conducted daily, with volumes of 5 or 10 mL alternated each day until the epithelial cells achieved 80% confluence.

Once the epithelial cells reached 80% confluence, they were rinsed twice with 10 mL PBS. The monolayer was then treated briefly with 2.5 mL 1X trypsin-EDTA solution (Sigma Aldrich), which was promptly removed. Subsequently, an additional 1 mL of 1X trypsin-EDTA solution was added to the cells and incubated for 3-5 minutes at 37°C. To inhibit

trypsin activity, 10mL of neutralisation medium containing 10% FBS, 0.2% penicillin/streptomycin/amphotericin (Sigma Aldrich), 25 µg/mL gentamycin (Gibco) and 4 mM L-glutamate in RPMI1640 was added to the flask. The mixture was then transferred to a universal tube. Cells were centrifuged at 400 g for 5 minutes at 4°C and subsequently resuspended in 1 mL PneumaCult™ -EX Plus medium.

Cells were seeded onto 0.33 µm pore size cell culture inserts (Corning Inc, Wiesbaden, Germany), at a density of 1 x 10^5 cells per insert within a 24-well cell culture plate and suspended in 800 µL basolateral and 200 µL apical media composed of PneumaCult™ EX Basal Medium, 1X PneumaCult™-EX Supplement, 1X Hydrocortisone, 0.125 µg/mL amphotericin B and 25 µg/mL gentamycin. To maintain humidity, 1 mL PBS was added to 12 empty wells in the cell culture plate. Fresh media was provided every two days until cells achieved 70% confluence, at which point the media was exchanged for PneumaCult ALI Maintenance Medium containing PneumaCult™ ALI Basal Medium, 1X PneumaCult™ ALI Supplement, 1X PneumaCult™ ALI Maintenance Supplement, 1X Hydrocortisone, Heparin solution (STEMCELL Technologies), 100 U/mL penicillin (Sigma Aldrich) and 100 µg/mL streptomycin (Sigma Aldrich). After two days, the apical media was removed to allow the cells to form an ALI. This protocol was continued for at least four weeks until a pseudostratified respiratory epithelium was discernible with a high trans-epithelial electrical resistance (TEER) exceeding 600 Ω.cm^2 (Figure 2-6). Excess mucus was cleared weekly from the apical surface of the ALI culture by addition of 200 µL of HBBS and a 30-minute incubation before aspiration.

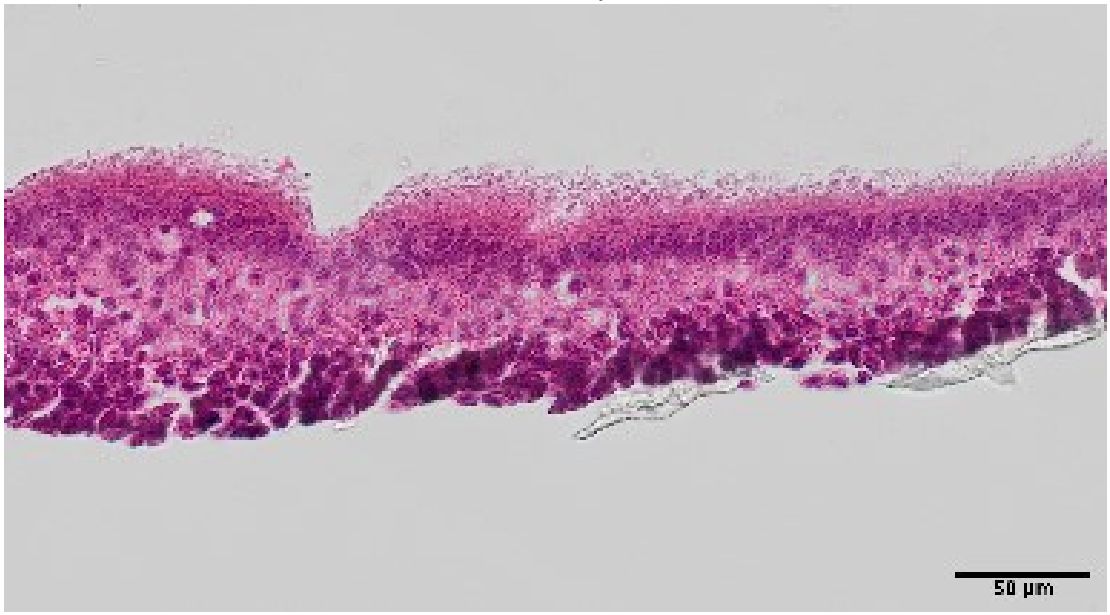


Figure 2-6 Primary nasal epithelial cells cultured in ALI

Example of a mature primary nasal epithelial cell ALI culture embedded in paraffin and sectioned at 5 µm stained with haematoxylin and eosin. The epithelium demonstrates a ciliated pseudostratified respiratory morphology with a discernible clear plastic insert base.

The TEER of each ALI culture was assessed weekly before each experiment. Measurements were conducted using a Millicell ERS-2 Voltohmmeter (Millipore, Watford, UK) equipped with a 'chopstick' electrode. Prior to measurements, the electrode was sterilised in 70% ethanol for 15 minutes and then calibrated in Ca^{2+} and Mg^{2+} deficient PBS (Sigma Aldrich) for 10 minutes. Before measuring TEER, basal media of each ALI culture was aspirated and replaced with pre-warmed (37°C) Ca^{2+} and Mg^{2+} deficient PBS (800 µL in the basolateral compartment and 200 µL in the apical). The longer electrode was immersed in the basolateral side, and the shorter electrode was inserted into the apical side. TEER values, in ohms (Ω), were measured twice for each ALI insert and averaged. These values were converted into values of $\Omega.cm^2$ using the following formula:

TEER in $\Omega \times cm^2$ = resistance in Ω x surface area of insert (0.33 cm^2)

2.5 Molecular assays

2.5.1 Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) is a cytosolic enzyme present in most eukaryotic cells, which is released into the extracellular space in response to plasma membrane damage. A colorimetric LDH cytotoxicity assay kit (ScienCell Research Laboratories, Carlsbad, CA, USA) was utilised in accordance with the manufacturer's instructions. To construct an LDH standard curve, a known quantity of HMC-1 and LAD2 cells was lysed with 0.5% Triton X-100 (Sigma Aldrich) followed by vortexing. The cell lysate was used then used to establish a standard curve correlating cell death with absorbance (SpectraMax 2, Molecular Devices, San Jose, USA), aiding in the determination of the optimal number of cells for further experiments (

Figure 2-7).

For each assay, control samples containing the same number of cells as the experimental sets were used to calculate both spontaneous and maximum LDH release. Spontaneous release was calculated by incubating cells in media alone, centrifuging at 250 g for 10 minutes, and collecting the supernatant. Maximal LDH release was calculated by lysing cells in 0.5% Triton X-100-containing media, subjecting them to vigorous vortexing and rolling for 30 minutes, followed by centrifugation at 250 g for 10 minutes and supernatant collection. Experimental values were obtained by centrifuging the treatment groups similarly at 250 g for 10 minutes and analysing the collected supernatant.

150 µL of LDH controls and culture supernatants were dispensed into flat-bottom 96-well plates (Greiner Bio-One). Following this, 60 µL of reaction mixture was added according to the manufacturer's instructions. The plates were incubated at room temperature in darkness for 20 minutes, after which the reaction was halted by adding 20 µL of sodium

oxamate solution to each well. The absorbance was measured with a spectrophotometer at 490 nm. All experiments were performed in triplicate and results expressed as net LDH release using the following equation:

$$\text{Net LDH release (\%)} = \frac{\text{absorbance of experiment} - \text{spontaneous release absorbance}}{\text{Total release absorbance} - \text{spontaneous release absorbance}} \times 100$$

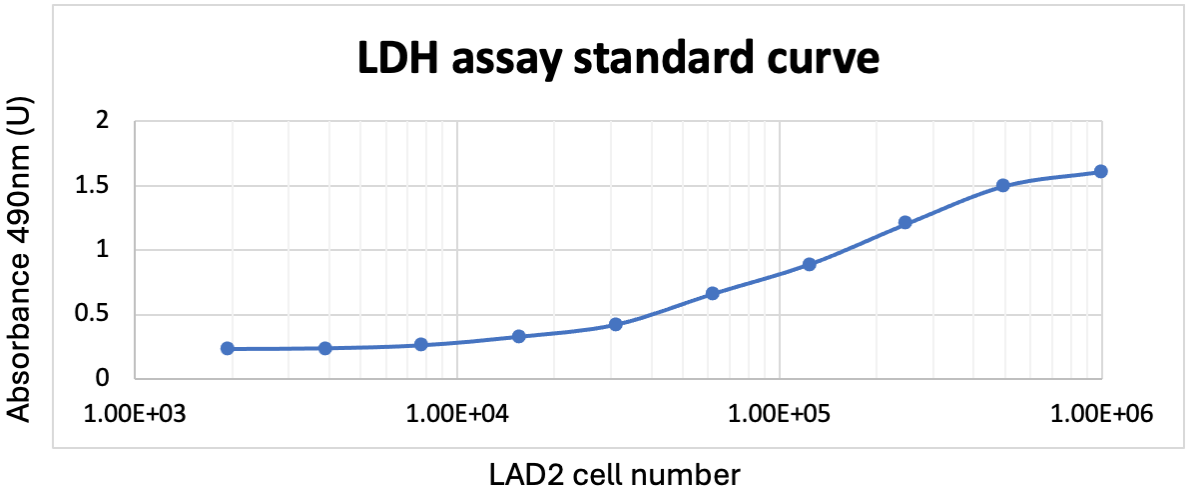


Figure 2-7 Lactate dehydrogenase standard curve

Standard curve to determine the optimum LAD2 cell density offering the greatest sensitivity for LDH release. The number of cells lysed was plotted against the absorbance at 490 nm, showing an optimum sensitivity above 1 x 10^4 cells and below 1 x 10^6.

2.5.2 β-Hexosaminidase assay

β-Hexosaminidase is a lysosomal enzyme stored in the granules of mast cells and is released in response to mast cell activation, indicating mast cell degranulation. A colorimetric β-hexosaminidase assay was devised using a known number of HMC-1 and LAD2 cells which were lysed with 0.5% Triton X-100 followed by vortexing. The lysate was utilised to create a standard curve with a plate reader that described substrate breakdown vs absorbance, thereby enabling calculation of the optimal cell concentration for use in the assays.

For each experiment, controls containing the same number of cells as those used in the experiment were prepared to calculate spontaneous and maximum β-hexosaminidase

release. Spontaneous release was quantified by incubating cells in media alone, then centrifuging at 250 g for 10 minutes and removing the supernatant. Maximal β-hexosaminidase release was determined by lysing the cells with 0.5% Triton X-100 in the medium, followed by vigorous vortexing and rolling for 30 minutes. The lysate was then centrifuged for 10 minutes at 250 g, with the supernatant subsequently extracted. Each experimental value was obtained by centrifuging the cells for 10 minutes at 250 g and collecting the supernatant for analysis.

A 30 µL aliquot of cell supernatant or control was transferred to a flat bottomed 96-well plate and combined with 50 µL of naphthol AS-BI N-acetyl-ß-D-glucosaminide reaction substrate (Sigma Aldrich) and left in darkness at 37°C for 1 hour. The reaction was suspended by the addition of 100 µL of glycine solution (Sigma Aldrich). The results were calculated as percentage β-hexosaminidase release using the equation below:

$$\beta\text{-hexosaminidase release (\%)} = \frac{\text{Absorbance of experiment}}{\text{Total (maximal) release absorbance}} \times 100$$

2.6 *S. aureus* co-culture experiments

2.6.1 *S. aureus* mast cell and co-culture models

S. aureus was co-cultured with both HMC-1 and LAD2 cell lines to investigate the differences in intracellular translocation between the strains. *S. aureus* was grown to mid-log phase, and the OD600 was measured; the CFU count was extrapolated using the equation y=mx + c (Figure 2-5). A known quantity of *S. aureus* was resuspended in a specific quantity of HMC-1 or LAD2 cells that had been washed in RPMI 1640, which was free from antibiotics, at various multiplicity of infection (MOI) ratios.

2.6.2 HMC-1: *S. aureus* lysostaphin protection assay

Both CRSwNP 1 and Control 1 strains of *S. aureus* were cultured in RPMI 1640 at 37°C in a 5% CO_2 environment until the exponential growth phase was reached. Absorbance at 600nm was measured and the results were extrapolated using absorbance vs CFU enumeration graphs. The bacteria were diluted in RPMI 1640 medium to achieve a known concentration, which could be pipetted accurately for further experimentation.

Simultaneously, a known quantity of HMC-1 cells was washed in antibiotic-free medium containing IMDM and 10% BSA three times. Co-culture was conducted by adding 4 x 10^6 of *S. aureus* bacteria to 2 mL of HMC-1 cells at a concentration of 1 x 10^6 / mL providing an MOI of 2:1 in 15 mL conical tubes (Greiner Bio-One). Samples were incubated for 2, 4 or 6 hours at 37°C in a 5% CO_2 environment. After each timepoint, samples were centrifuged at 250 g for 10 minutes and the supernatant was removed for analysis (CFU, LDH and β-hexosaminidase release). The supernatant was plated onto CBA plates (Oxoid) at 1:10 serial dilutions to quantify extracellular *S. aureus* survival.

The remaining cell pellet was resuspended in 1 mL of IMDM medium containing 20 µg/mL lysostaphin (Sigma Aldrich) supplemented with 10% BSA and incubated for 30 minutes. After lysostaphin treatment, the cells were washed three times in lysostaphin-free medium using multiple centrifugations at 250 g for 10 minutes each. A streak plate on CBA was performed to ensure the absence of bacterial growth in the washed medium. The final pellet was resuspended in 0.5% Triton X-100 and vortexed for 10 minutes. The lysate was then set aside for further analysis, and serial CFU enumerations were performed (Figure 2-8).

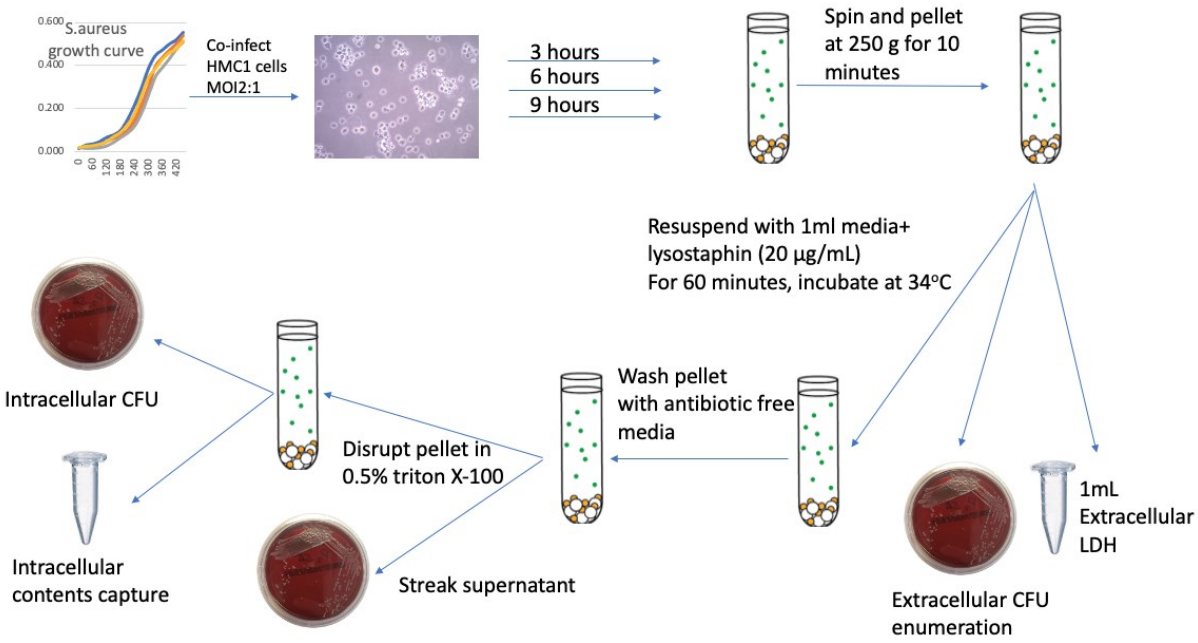


Figure 2-8 Diagrammatic representation of a lysostaphin protection assay

S. aureus was grown to exponential growth phase and used to co-infect HMC-1 cells at a ratio of 2:1. The co-cultures were incubated at 37°C in a 5% CO_2 atmosphere for various time periods. Subsequently, the cells and supernatant were separated by centrifugation. The extracellular CFU values were determined using CFU enumerations, and the cell pellet was washed in medium containing lysostaphin to eradicate any extracellular bacteria. After removing the lysostaphin, the absence of extracellular bacteria was confirmed using a streak plate to ensure sterility. The remaining cells were lysed with 0.5% Triton X-100 and the intracellular CFU concentration was ascertained.

2.6.3 LAD 2: *S. aureus* lysostaphin protection assay

Both CRSwNP 1 and Control 1 strains of *S. aureus* were cultured in RPMI 1640 at 37°C in the presence of 5% CO_2 until exponential growth phase was reached. Absorbance at 600 nm was measured and extrapolated using absorbance vs CFU enumeration graphs. The bacteria were diluted in RPMI 1640 medium to provide a known quantity of bacteria that could be pipetted accurately.

LAD2 cells were cultured to known concentrations and washed thrice in antibiotic-free StemPro-34 medium (Life Technologies) containing 0.1 mM SCF (PeproTech). A known quantity of *S. aureus* was added at an MOI ratio of 1:1 to the LAD2 cells in 2 mL of medium. The co-cultures were then incubated for 3, 6 and 9 hours, followed by centrifugation at 250 g for 10 minutes. Supernatants were collected for LDH assay, β-hexosaminidase assay and CFU enumeration on CBA plates. The cell pellet was subsequently resuspended in 1 mL of medium containing 20 µg/mL lysostaphin and 0.1 mM SCF for 60 minutes.

After incubation, LAD2 cells were pelleted at 250 g for 10 minutes and washed thrice with 1 mL antibiotic-free medium. The washed LAD2 cells were pelleted again at 250 g for 10 minutes and the supernatant was streaked onto a CBA plate to confirm sterility. The remaining pellet was either resuspended in StemPro-34 for imaging or resuspended in StemPro-34 medium containing 0.1mM SCF and 0.5% Triton X-100 and vortexed for 10 minutes. The final supernatant was utilised to conduct serial CFU enumerations using CBA plates.

Analysis of intracellular survival at 24 hours was conducted by infecting LAD2 cells and co-culturing for 6 hours, followed by the eradication of extracellular bacteria as previously described and washing the cells three times in antibiotic-free medium. A

streak plate was employed to confirm that the extracellular medium had no *S. aureus* growth, and the co-culture was continued until the 24-hour timepoint. At this point, the cells were resuspended in 0.5% Triton X-100 medium and vortexed for 10 minutes. The lysate was then used in serial CBA plate enumerations as outlined earlier.

An alternative method was also utilised, in which the bacterial co-culture was maintained as above for 6 hours, and then 1.32 µg/mL of lysostaphin was added to the extracellular medium until the 24-hour mark. The cells were subsequently washed three times in medium, and a streak plate was performed to ensure the absence of growth in the supernatant. The cells were then lysed in medium containing 0.5% Triton X-100 and vortexed for 10 minutes. The lysate from this process was similarly used in serial CBA plate enumerations as previously described.

2.6.4 Intracellular survival of CRSwNP1 *S. aureus* in LAD 2 cells when cultured with simvastatin

CRSwNP 1 *S. aureus* was grown in RPMI 1640 at 37°C in a 5% CO_2 atmosphere until exponential growth phase was attained. Absorbance at 600nm was measured and the data extrapolated using absorbance vs CFU enumeration graphs. The bacteria were diluted in RPMI 1640 medium to achieve a known concentration that could be pipetted accurately for further experiments.

LAD2 cells were cultured in antibiotic-free conditions in StemPro-34 medium containing 0.1 mM SCF. LAD2 cells (5×10^5) in 1 mL were preincubated with simvastatin (Sigma Aldrich) at various concentrations for 2 hours. Each concentration of simvastatin was followed by the addition of RPMI 1640 (control) or CRSwNP 1 *S. aureus* (5×10^5 CFUs) as a challenge. The co-cultures were then incubated for 6 hours before being centrifuged at 250 g for 10 minutes. Supernatants were collected for the LDH assay.

Subsequently, the cell pellet was resuspended with 1 mL of 20 µg/mL lysostaphin in StemPro-34 media with 0.1 mM SCF and incubated for 60 minutes. Afterwards, LAD2 cells were pelleted at 250 g for 10 minutes and washed in 1 mL antibiotic-free medium three times. The washed LAD2 cells were then pelleted again at 250 g for 10 minutes, and the supernatant was streaked onto a CBA plate to confirm sterility.

The remaining pellet was resuspended in StemPro-34 medium containing SCF and 0.5% Triton X-100 and underwent 10 minutes of vortexing. The resultant supernatant was used to perform serial CFU assessments on CBA plates.

2.6.5 Inflammatory response to *S. aureus* infection in LAD2 cells and the effect of simvastatin treatment

LAD2 cells were cultured in antibiotic-free conditions in StemPro-34 medium supplemented with 0.1 mM SCF. Antibiotic-free LAD2 cells were subjected to either no treatment (untreated) or preincubation with 30 µmol/L simvastatin for 16 hours. Concurrently, CRSwNP 1 *S. aureus* was grown in RPMI1640 medium at 37°C within a 5% CO2 incubator until the exponential growth phase was reached. OD600 values were measured to assess bacterial growth, and values were converted to CFUs using established absorbance vs CFU enumeration curves.

The bacteria were subsequently diluted in RPMI 1640 to attain a concentration amenable to precise pipetting. Three experimental conditions were established: a control group containing $5x10^5$ untreated LAD2 cells, a *S. aureus* infection group consisting of $5 x 10^5$ untreated LAD2 cells exposed to CRSwNP 1 *S. aureus* at an MOI of 1 and a treatment group comprising $5x10^5$ simvastatin preincubated LAD2 cells also infected with CRSwNP 1 *S. aureus* at an MOI of 1. These groups were incubated for 6 hours at 37°C in a 5% CO_2 atmosphere using 24-well plates (Greiner Bio-One).

Post-incubation, LAD 2 cells were centrifuged at 250 g for 10 minutes, and the resultant cell pellets were washed three times with Ca^{2+} and Mg^{2+} deficient PBS. The cells were resuspended in 1mL of QIAzol (Qiagen, Aarhus, Denmark) and stored for subsequent RNA extraction and analysis of pro-inflammatory cytokines.

2.6.6 ALI trans-epithelial electrical resistance in response to CRSwNP 1 *S. aureus* and simvastatin treatment

ALI cultures were established as previously described in subchapter 2.4.3 and employed to investigate the protective effects of simvastatin treatment against apical infection with CRSwNP 1 *S. aureus*. The apical and basolateral aspects of ALI cultures derived from patients were rinsed twice in HBSS. The basal medium was replaced with PneumaCult™ ALI infection medium, consisting of PneumaCult™-ALI basal media and 1X PneumaCult™-ALI supplement (STEMCELL Technologies), supplemented with 0.02% BSA, for 24 hours. *S. aureus* was cultured to exponential growth in RPMI 1640 and diluted to a specified quantity of CFUs.

TEER was measured by removing the basolateral medium and adding Ca^{2+} and Mg^{2+} free PBS to both the basal and apical aspects of the well at a temperature of 37°C. A probe electrode and volt-ohmmeter were positioned, and the resulting resistance measured in ohms (Ω). The basal medium was replaced with fresh PneumaCult™ ALI infection medium. Wells treated with simvastatin received 10 µmol/L simvastatin for 16 hours prior to infection or treatment with RPMI 1640. After incubation, both simvastatin-free and simvastatin-treated ALI had either RPMI 1640 alone or 5 x 10^5 CRSwNP *S. aureus* added to the apical chamber reaching a total volume of 100 µL. This created the experimental conditions: RPMI 1640 (Control), 5 x 10^5 CRSwNP 1 *S. aureus,* 10 µmol/L simvastatin alone or a combination of 5 x 10^5 CRSwNP 1 *S. aureus* and 10 µmol/L simvastatin.

Subsequently, the ALI cultures were incubated at 37°C in a 5% CO_2 atmosphere for 6 hours.

Following this, the basal medium was removed and stored at -80°C for analysis. TEER was re-measured using the aforementioned method. Cells from the ALI were lysed in QIAzol for downstream analysis via two-step quantitative real-time polymerase chain reaction (qRT-PCR). The change in TEER was calculated by dividing the final TEER value in $\Omega.cm^2$ by the initial TEER in $\Omega.cm^2$, yielding a fold-change.

2.7 Antimicrobial compound activity calculation

2.7.1 Minimal inhibitory concentration calculation of prodrug simvastatin and activated simvastatin

Simvastatin was activated chemically by dissolving 4 mg of simvastatin in 100 µL of ethanol and 150 µL of 0.1 M NaOH (Sigma Aldrich) and was incubated for 2 hours at 50°C, Subsequently, the pH was adjusted to 7 and the total volume was made up to 1 mL, providing a 4 mg/mL solution as per McKay *et al*. (145).

The minimal inhibitory concentration (MIC) of simvastatin against the CRSwNP 1 strain of *S. aureus* was calculated using the international standard broth microdilution method (146). CRSwNP 1 *S. aureus* was cultured to exponential growth and diluted to a stock concentration of 1 x 10^6 CFU/mL in Müller Hinton broth, pH7 (Sigma Aldrich). Every well received 90 µL of Müller Hinton broth with a decreasing concentration of simvastatin. To each well, 1 x 10^5 CFU of CRSwNP 1 *S. aureus* were added, and the plate was incubated at 37°C in a 5% CO_2 atmosphere for 16 hours. Each plate was analysed using a microplate reader at 600nm absorbance.

2.8 Cell signalling experiments

2.8.1 Substance P/ Compound 48/80 stimulation of LAD2 mast cells at various concentrations of simvastatin

LAD2 cells were cultured in StemPro-34 supplemented with 0.1 µM SCF and 0.2% penicillin/streptomycin. The cells were washed in Ca^{2+} and Mg^{2+} free PBS three times. The LAD2 cells were resuspended in Tyrode's buffer (Fisher Scientific), and 1 x 10^5 cells were allocated to each well in a cone-bottomed 96-well cell culture plate (Greiner Bio-One) to achieve a total volume of 180 µL. A total cell lysate was obtained by removing the contents of a well, centrifuging the cells at 250 g for 10 minutes, resuspending in Tyrode's buffer containing 0.5% Triton X-100 for 60 minutes, and centrifuging again for 10 minutes at 500 g.

Simvastatin was preincubated with the cells between concentrations ranging from 0 to 30 µmol/L for 30, 60, 120 minutes at 37°C in a 5% CO_2 atmosphere. Following preincubation, each concentration of simvastatin was challenged with 20 µL of Tyrode's buffer alone, 1 µM substance P (Sigma Aldrich) or 3 µM compound 48/80 (C48/80; Sigma Aldrich) for 60 minutes at 37°C in a 5% CO_2 environment.

The plate was then removed and centrifuged at 250 g for 10 minutes at 5°C. Next, 30 µL of the total and each supernatant was transferred to a flat-bottomed plate with 50 µL of 1.3 mg/mL p-nitrophenyl-N-acetyl-β-D-glucosaminide and incubated at 37°C for 1 hour. The reaction was terminated by adding 0.2 M glycine solution and analysed at 410 nm using a spectrophotometer plate reader. β-hexosaminidase release was calculated using the following equation:

$$\text{Total release (\%)} = \frac{\text{sample absorbance}}{\text{total absorbance}} \times 100$$

2.8.2 Substance P/ Compound 48/80 stimulation of LAD2 mast cells after preincubation for 24 hours with 10 µmol/L simvastatin

LAD2 cells were cultured in StemPro-34 medium with 0.1 mM SCF (PeproTech), 0.2% penicillin/streptomycin, and in the presence or absence of 10 µmol/L simvastatin for 24 hours at 37°C in a 5% CO_2 atmosphere. The cells were then washed three times in Ca^{2+} and Mg^{2+} free PBS and resuspended in Tyrode's buffer, either with or without 10 µmol/L simvastatin. The cells were stimulated with substance P and C48/80 at varying concentrations from 0 to 10 µM for 60 minutes at 37°C. The reaction was terminated using 0.2 M glycine and absorbance was measured at 410 nm using a spectrophotometer plate reader.

2.9 qRT-PCR

2.9.1 ALI/LAD2 RNA extraction and cDNA library production

ALI cultures and LAD 2 cells suspended in QIAzol had 0.2 mL chloroform (MP Biomedicals, Ohio, USA) added, were shaken vigorously for 15 seconds, and left to settle for 10 minutes at room temperature. The samples were centrifuged at 12,000 g at 4°C for 15 minutes. The aqueous RNA-containing phase was carefully removed and transferred to a fresh Eppendorf tube. RNA precipitation was initiated by adding 10 µg of glycogen and 0.5 mL of molecular biology grade isopropanol (Thermo Fisher Scientific) and vortexing the solution. Samples were left on ice for 30 minutes and subsequently centrifuged for 20 minutes at 4°C at 12,000 g. The supernatant was discarded, and the RNA pellet was washed in 75% ethanol, vortexed, and centrifuged at 12,000 g at 4°C. This step was repeated, but with the ethanol pre-chilled for 2 hours at -20°C before repeating the centrifugation at 12,000 g.

The ethanol was decanted, and the pellet was air-dried for 5 minutes. RNA was resuspended in 40 µL RNase-free H_2O (Thermo Fisher Scientific), and RNA concentration and purity were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific).

RNA samples were maintained on ice at 5°C and converted into complementary DNA (cDNA) libraries using the MultiScribe™ Reverse Transcriptase Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. A master mix was prepared containing 2 µL of 10X buffer, 0.8 µL of 25X dNTPs, 2.0 µL of 10X random primers, 1 µL of MultiScribe™ Reverse Transcriptase, and 1 µL of RNase inhibitor. RNA was diluted to yield 250 ng RNA in 13.2 µL of RNase-free H_2O per PCR tube (Alpha Laboratories, Eastleigh, UK) and combined with 6.8 µL of the master mix. The reaction mixture was then processed in a thermal cycler (MJ Research Tetrad 2, Bio-Rad, Hertfordshire, UK) using a

programme of 25°C for 10 minutes, 37°C for 120 minutes and 85°C for 5 minutes. The resultant cDNA library was adjusted to a final volume of 200 µL by the addition of RNase-free H_2O.

2.9.2 Quantitative PCR of cDNA library

Each cDNA library was assessed for reference genes and genes of interest. Quantitative RT-PCR was performed using TaqMan™ Universal Master Mix II (Thermo Fisher Scientific) according to the manufacturer's instructions. A master mix containing TaqMan™ enzyme, TaqMan™ primers (Table 2-1) and RNase-free H_2O was prepared, and 4 µL of this mix was added to 1 µL of cDNA in duplicate to a 384-well plate (VWR). The analysis was conducted using the 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific) following a cycle protocol consisting of a 95°C initialisation step for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Gene expression results were normalised to the housekeeping gene beta-2-microglobulin, which was identified as having the most consistent expression across ALI cultures. Results were presented as fold changes, calculated using the delta-delta cycle threshold (ΔΔCt) method:

2^[(Ct of interest gene - Ct of housekeeping gene) – (Ct of untreated interest gene – Ct of untreated housekeeping gene)]

Duplicates for each reaction were performed, and the average of the ΔΔCt values was used for analysis.

Table 2-1 List of TaqMan™ primers

Gene	Accession number
B2M	Hs00984230_m1
IL6	Hs00174131_m1
CXCL8	Hs00174103_m1
TNF	Hs00174128_m1
IFNG	Hs00989291_m1
IL33	Hs04931857_m1
GMCSF	Hs00738432_g1
ACTB	Hs99999903_m1
IL5	Hs01548712_g1

List of TaqMan™ primers (Thermo Fisher Scientific) used in qRT-PCR, labelled with fluorescein amidite (FAM) reporters.

2.10 Imaging

2.10.1 LAD2: *S. aureus* lysozyme protection assay imaging

LAD2 cells were cultured to known concentrations and washed three times in antibiotic-free StemPro-34 medium containing 0.1 mM SCF. A known quantity of CRSwNP 1 and Control 1 *S. aureus* was added at an MOI ratio of 1:1 million LAD2 cells in 2 mL medium. The co-cultures were incubated for 3, 6 or 9 hours. 100 µL was removed for further analysis.

Both co-cultures, treated with and without antibiotics, and controls consisting of LAD2 cells and *S. aureus* alone, were centrifuged at 500 rpm for 10 minutes (Shandon Cytospin 3, Fisher Scientific) to attach the cells to 3-aminopropyltriethoxysilane (APES) coated slides (Sigma Aldrich). The slides were air-dried for 12 hours then fixed in 100% methanol for 10 minutes. Slides were stained in 1:20 dilution of Giemsa stain (Sigma Aldrich) for 10 minutes, then washed in sterile H_20 for 10 minutes. Imaging was performed with a dotSlide light microscope and analysed using OlyVIA software (Olympus Essex, UK)

2.10.2 Embedding of nasal polyp/ ALI tissue

Nasal polyp tissue or ALI inserts were processed by the Immunohistochemistry Department at Southampton General Hospital and embedded into paraffin wax. Sections of 5-micron thickness were taken and placed onto APES-coated slides using a Thermo Shandon Finesse ME Microtome (Thermo Fisher Scientific).

2.10.3 Haematoxylin and eosin staining

APES slides bearing ALI tissue were placed in a staining rack and subjected to two 10-minute immersions in Tissue-Clear (Sakura, Newbury, UK), followed by two 5 minutes immersions in 100% industrial methylated spirit (IMS; Sigma Aldrich). Subsequently, slides were immersed for 5 minutes in 70% IMS and then rinsed for 5 minutes in running

H_2O. The slides were stained with Mayer's haematoxylin (Sigma Aldrich) for 5 minutes and washed again in running H_2O for 5 minutes. Further staining was conducted in eosin (Sigma Aldrich) for 5 minutes, after which the slides were dipped three times in H_2O. Slides were then returned to 100% IMS for 2 minutes and Tissue Clear for 9 minutes. Slides were mounted with ExPert XTF (CellPath, Newtown, UK). Imaging was performed using a dotSlide microscope at 40x magnification. Images were processed with OlyVIA software and ImageJ.

2.10.4 FISH of ALI cultured epithelial cells

Tissue/ ALI slides were briefly washed in H_2O and underwent serial dehydration in 70%, 90% and 100% ethanol, spending 5 minutes in each solution. They were air-dried and stored at -20°C in the presence of silica gel.

Stock solutions of hybridisation buffer were produced containing 360 µL of 5 M NaCl, 40 µL of 1 M Tris pH8 (Sigma Aldrich), 999 µL of H_2O, 1 µL of 20% SDS (Sigma Aldrich) and 600 µL of formamide. To this, 1 µL of 50 ng/µL bacterial 16S ribosomal RNA probe (Table 2-2; Eurofins, Camberley, UK) was added to 19 µL of the hybridisation buffer. A total of 20 µL of the hybridisation buffer with the 16S probe was placed onto each APES-coated glass slide, and a cover slip was applied. Each slide was incubated at 46°C in a FISH hybridiser (Dako Statspin, Cheshire, UK) for 2 hours. Slides were then placed in a washing buffer (0.1 M NaCl, 0.2 M Tris pH8, 5 mM EDTA, 0.01% SDS in H_20) for 15 minutes at 48°C, during which the cover slips were removed. The samples were then rinsed in PBS (Sigma Aldrich), washed in H_2O, dehydrated in 70%, 90%, 100% ethanol, and stained with DAPI (Thermo Fisher Scientific).

Autofluorescence was quenched by applying 10 µL of Vector® TrueVIEW® (Vector Laboratories, Newark, CA USA) over the tissue for 5 minutes, then glass cover slips were mounted using ExPert XTF.

Plates were stored in a chamber slide box covered in tin foil at 5°C. Images were obtained using a Zeiss Axioskop 2 Mot fluorescence microscope with a 40x magnification objective. Images were acquired using Micro-Manager and analysed with ImageJ. Controls were used to confirm that the *S. aureus*-specific probe and ubiquitous probes specifically bound their respective targets.

Table 2-2 DNA sequence and fluorophore details for FISH 16S ribosomal RNA probes.

Probe name	Fluorophore	Sequence (5'-3')	Melting temp (Tm)
Ubiquitous to aerobic bacteria	5' Rhodamine	GCT GCC TCC CGT AGG CAG C	65.3°C
Specific to *S. aureus* probe	5' Alexa Flour 488	GAA GCA AGC TTC TCG TCC G	58.8°C

Probes used in fluorescence in situ hybridisation experiments with their fluorescent fluorophore reporter, sequence, and estimated melting temperature.

2.11 Statistical analysis

Patient demographic data were analysed using SPSS (IBM, Portsmouth) applying the appropriate statistical tests. The Pearson chi-squared test was employed for categorical data, and one-way ANOVA was used for parametrically distributed nominal age values. Experimental data were analysed using GraphPad Prism 8 software (GraphPad Software Inc., San Diego, CA, USA). Data were assessed for normality using histogram plots and normality tests. For datasets with more than five data points, the D'Agostino-Pearson test was used; for those with fewer than five data points, the Shapiro-Wilk test was applied. Parametric tests were employed where data were confirmed to demonstrate a normal distribution. Paired t-tests were used to compare differences between *S. aureus* strain co-cultures. LDH and β-hexosaminidase experiments also utilised paired t-tests and ANOVA with Tukey's multiple comparison test. A two-way ANOVA, using either Tukey's or Šidák multiple comparison tests, was conducted to appraise the significance of substance P and C48/80 degranulation in relation to time and concentration. Quantitative RT-PCR experiments were analysed using a non-parametric Kruskal-Wallis test.

Chapter 3 **Enhanced pathogenicity of chronic rhinosinusitis-related *S. aureus* strains may mediate disease recalcitrance and treatment resistance**

3.1 Introduction

One of the primary objectives of this study was to isolate *S. aureus* from non-CRS, CRSsNP and CRSwNP patients. The goal was to determine whether there are differences in quorum sensing genes and virulence genes across these groups that might explain variations in *S. aureus* pathogenicity and phenotypic characteristics between the disease states. These phenotypic characteristics include antimicrobial resistance, SCV formation, intracellular survival and biofilm formation (3, 86, 147). Furthermore, to deepen our understanding of factors related to *S. aureus* colonisation, we collected specimens and demographic data from non-CRS (control) patients, as well as those afflicted with CRSsNP and CRSwNP. This approach enabled us to compare our findings with those of extensive epidemiological studies, ensuring that our cohort was representative (33, 148).

Our Initial goal was to collect five samples of *S. aureus* from the middle meatus of control, CRSsNP and CRSwNP patients. We anticipated a frequency of culture of approximately 20% in controls, 33% in those with CRSsNP, and 60% in patients with CRSwNP, as demonstrated in similar studies (149, 150). However, the observed frequency of culture in our study was significantly lower than expected.

A challenging factor for genome sequencing is that bacterial clinical samples are likely to exhibit diversity at the genomic level, which could interfere with sequencing efforts. To address this, single clones from each patient's nasal swab were selected for Illumina paired-end sequencing. Fortuitously, the genome of *S. aureus* has been well curated; numerous virulence factor, antimicrobial resistance and stress-response genes, plasmid

cassettes and mobile genetic elements have been identified. These are accessible through various databases which the Bactopia pipeline utilises (141). This chapter reviews the demographics of each cohort, provides a subanalysis of demographic risk factors that could increase the likelihood of culturing *S. aureus,* and analyses the differences between the genomes of *S. aureus* cultured in each disease subtype.

3.2 Results

3.2.1 Patient demographics and subgroup analysis of those growing *S. aureus*

Nasal swabs were taken from 44 patients enrolled in the study. In total, 17 control, 10 CRSsNP, and 17 CRSwNP patients participated. *S. aureus* was cultured in 4 control patients, 4 CRSsNP patients, and 3 CRSwNP patients. There was a higher proportion of asthmatic patients within the CRSwNP group, which was found to be statistically significant at 58.8% compared with 17.6% in the control group and 20% in the CRSsNP group ($P \leq 0.05$). The overall mean MLMS was higher in the CRSwNP group (15.6), than in the control (4) or CRSsNP (5.1) groups ($P \leq 0.001$) (Table 3-1).

There was a larger number of smokers in the control group (23.5%), with no smokers in the CRSsNP or CRSwNP groups ($P \leq 0.05$). Other demographic factors showed no statistically significant differences between the groups (Table 3-1). A subgroup analysis performed on subjects from whom *S. aureus* was cultured revealed no significant difference in demographics, including age, procedure, antibiotic use, steroid use, airborne allergies, asthma prevalence and smoking status (Table 3-2).

Table 3-1 Demographic profile of study population

Patient group	Control	CRSsNP	CRSwNP	P-value
Number of subjects	17(38.6%)	10(22.7%)	17(38.6%)	-
Gender (M:F)	10:7	6:4	9:8	.917 *
Mean age (SD)	52.4 (15.7)	50.8 (17.4)	46.5 (16.0)	.563 †
Clinic review/ procedure performed (n)	Clinic review (1) Biopsy (3) Septoplasty/PETS (9) Limited FESS (4) (for isolated mucoceles) Full house Fess (0)	Clinic review (2) Biopsy (0) Septoplasty/PETS (1) Limited FESS (5) Full house FESS (2)	Clinic review (1) Biopsy (0) Septoplasty/PETS (0) Limited FESS (0) Full house FESS (16)	N/A
Antibiotic use in past month	1 (5.88%)	1 (10%)	4 (23.5%)	.302*
Steroid use in past month	1 (5.88%)	0	4 (23.5%)	.117*
S. aureus culture	4 (23.5%)	4 (40%)	3 (17.6%)	.563*
Airborne allergies	6 (35.2%)	3 (30%)	8 (47.0%)	.637*
Asthma	3 (17.6%)	2 (20%)	10 (58.8%)	.023*
Ex-smokers	2 (11.8%)	0	4 (23.5%)	.243*
Current smokers	4 (23.5%)	0	0	.034*
Mean Modified Lund-Mackay score	4	5.1	15.6	<.001†

Study Population Demographics (*Pearson chi-squared test for categorical data, †one-way ANOVA for numerical data). The P-value for the Pearson chi-squared test quantifies the association of categorical variables (e.g., antibiotic use) between control, CRSsNP and CRSwNP groups. The P-value for one-way ANOVA test quantifies the association of numerical data (e.g., age) between control, CRSsNP and CRSwNP groups.

Table 3-2 Sub-group analysis of patients culturing S. aureus

Culture of *S. aureus*	No growth	Growth	P-value
Number of subjects	33 (75%)	11 (25%)	-
Gender (M:F)	21:12	4:7	.054*
Age	49.3	51.0	.75†
Clinic review/ procedure (n)	Biopsy (1) Septoplasty/PETS (6) Limited FESS (9) Full House Fess (14) Clinic review (3)	Biopsy (2) Septoplasty/PETS (4) Limited FESS (0) Full House Fess (4) Clinic review (1)	.99*
Antibiotic use in past month	5	1	.53*
Steroid use in past month	4	1	.69*
Airborne allergies	12	5	.80*
Asthma	11	4	.948*
Ex-smokers	5	1	.508*
Current smokers	3	1	.89*
Mean Modified Lund-Mackay score	8.64	11.25	.403†

Subgroup analysis of patients culturing *S. aureus* and their demographics (*Pearson chi-squared test, †one-way ANOVA). The P-value resulting from the Pearson chi-squared test quantifies the association of the categorical variable (e.g., antibiotic use) among control, CRSsNP and CRSwNP groups. The P-value from one-way ANOVA test quantifies the association of numerical data (e.g., age) between the control, CRSsNP and CRSwNP groups.

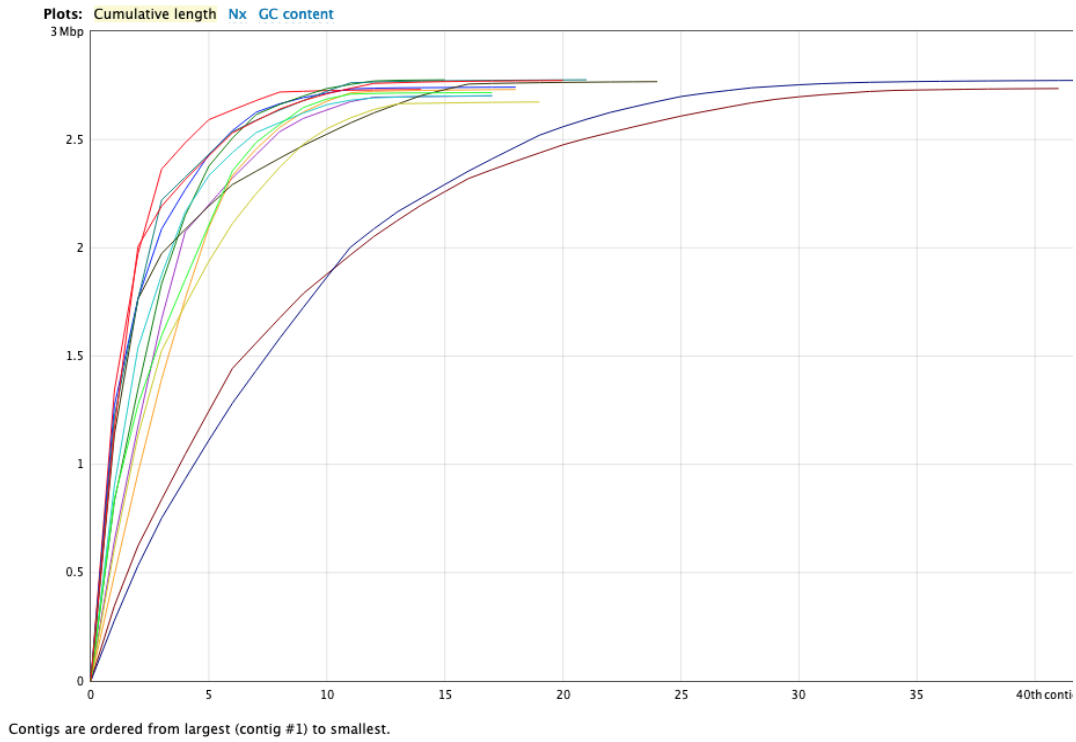
3.2.2 *S. aureus* genome sequencing

Illumina short-read sequencing was completed for 11 *S. aureus* strains identified from 4 control subjects, 4 CRSsNP patients, and 3 CRSwNP patients. Two previously collected, well-characterised *S. aureus* strains from a patient without CRS (Control 1) and a CRSwNP strain (CRSwNP 1) were also sequenced (Table 3-3). Each paired-end read sequence was reconstructed and aligned using bacterial alignment and analysis pipeline, Bactopia (141). Quality assurance of the assembled genomes was carried out, demonstrating each isolate spanned between 2.67 and 2.77 megabase pairs (reference genome size 2.89 megabase pairs), representing 92- 96% of the genome sequenced (Figure 3-1).

Table 3-3 Demographics of subjects from which S. aureus was cultured

S. aureus specimen	Cultured in study	Gender	Age	Antibiotic use in past month	Steroid use in past month	Airborne allergies	Asthma	Previous smoker	Current smoker	Comments
Control 1	No									*S. aureus* received from a paediatric nasal swab with no evidence of CRS.
Control 2	Yes	F	68	No	No	No	No	No	No	
Control 3	Yes	F	24	No	No	Yes	No	No	No	
Control 4	Yes	F	66	No	No	Yes	No	No	No	
Control 5	Yes	M	36	No	No	Yes	No	Yes	No	
CRSsNP 1	Yes	F	67	No	No	No	No	No	Yes	
CRSsNP 2	Yes	F	79	No	No	No	Yes	No	No	
CRSsNP 3	Yes	F	77	No	No	Yes	No	No	No	
CRSsNP 4	Yes	M	48	No	No	No	No	No	No	
CRSwNP 1	No									*S. aureus* previously cultured from subject with CRSwNP.
CRSwNP 2	Yes	F	62	No	No	No	Yes	No	No	
CRSwNP 3	Yes	F	34	Yes - Doxycycline	Yes	Yes	Yes	No	No	
CRSwNP 4	Yes	M	28	No	No	No	No	No	No	

Details of the subjects from whom *S. aureus* was cultured. Control 1 was isolated from a nasal swab of a subject with no evidence of CRS from a separate study. The CRSwNP 1 strain was cultured from a nasal polyp and has been used to represent CRS-associated *S. aureus* in numerous publications (75, 111, 151).



Legend:

Control 1	CRSsNP1	CRSwNP1
Control 2	CRSsNP2	CRSwNP2
Control 3	CRSsNP3	CRSwNP3
Control 4	CRSsNP4	CRSwNP4
Control 5		

Figure 3-1 QUAST output of assembled contigs

Assessment of genome coverage was conducted for each isolated sequenced contig (a fragment of the genomic sequence) using the QUAST program. The y-axis represents the calculated genome length plotted against the number of contigs, which is displayed on the x-axis. The legend outlines the colour of each isolate plotted on the graph.

3.2.3 *S. aureus* typing

Prior to the advent of whole-genome sequencing, *S. aureus* was classified using accessory gene regulator (*agr*) sequencing and staphylococcal protein A (*spa*) gene sequencing. This classification would provide some indication of a particular *S. aureus* strain's virulence and remains useful for comparison with data from before the widespread use of whole-genome sequencing methods. In our study, the sequences demonstrated that the predominant *agr* locus type was type I, present within 69% of the isolates. The *agr* groups II and III each accounted for 15% of the isolates. No *agr* type IV isolates were noted. *Spa* typing, which analyses the highly variable region of the *spa* gene, is useful for determining outbreaks of disease from an epidemiological standpoint. Our results demonstrated three duplicated *spa* types: t008, t015, t571, two of which belonged to the control and CRSwNP groups, and one pair which belonged to the CRSsNP group (Table 3-4).

Table 3-4 Accessory gene regulator locus and spa typing of isolates

Isolate	*agr* locus type	*spa*-type
Control 1	I	t015
Control 2	I	t008
Control 3	II	t084
Control 4	I	t065
Control 5	III	t338
CRSsNP 1	I	t1451
CRSsNP 2	I	t571
CRSsNP 3	I	t571
CRSsNP 4	I	t340
CRSwNP 1	I	t008
CRSwNP 2	III	t127
CRSwNP 3	II	t6292
CRSwNP 4	I	t015

S. aureus isolates cultured from control (**blue**), CRSsNP (**green**) and CRSwNP (**yellow**) group patients are presented. The accessory gene regulator (*agr)* locus types and staphylococcal protein A (*spa*) types for each group are displayed. All isolates from the CRSsNP group belonged to *agr* group I, while the isolates from the control and CRSwNP groups varied between *agr* groups I-III. Significant heterogeneity in *spa* types was observed among the groups.

3.2.4 Stress proteins

The inference of virulence and resilience in organisms, previously done through other methods, is now increasingly being replaced by whole-genome sequencing. This allows for in-depth analysis of genes and proteins that contribute *S. aureus* resilience and pathogenicity. To perform this analysis, the Bactopia tool, AMRFinderPlus was utilised to detect stress proteins. AMRFinderPlus compares the assembled sequences to the NCBI's curated reference gene database, which contains the currently known stress, virulence and antimicrobial resistance genes as of the date of access (5 June 2022).

Upon reviewing the genes predicted from our sequences that support bacterial stress responses, we noted that the multidrug efflux transporter gene [*lmrS*] was highly conserved across all groups, appearing in 92% of the isolates. The cadmium resistance transporter gene [*cadD*] was present in 100% of CRSsNP group, 75% of the CRSwNP group, but only in 40% of the control group. Less conserved genes such as the quaternary ammonium compound efflux MFS transporter gene [*qacA*] and the multidrug transcriptional regulator gene [*qacR*] were identified in 25% of CRSwNP isolates. Genes involved in arsenite reduction and efflux exhibited variable presence: the *arsC* gene was observed in 40% of control isolates and 25% of CRSwNP isolates, while the *arsR* gene was seen in 25% of CRSwNP isolates and was absent in other groups. The *arsB* gene and the multi coper oxidase gene [*mco*] were each identified in 20% of the control group (Figure 3-2).

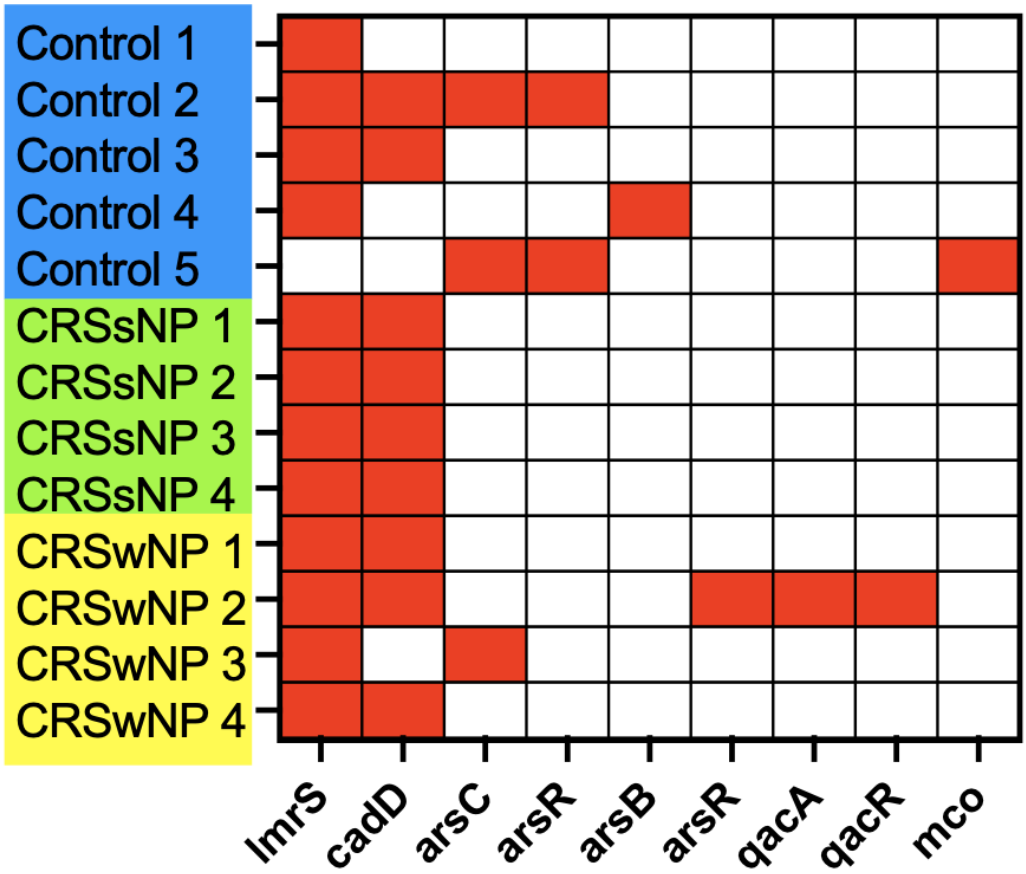


Figure 3-2 Stress gene presence in control, CRSsNP and CRSwNP S. aureus isolates

Hierarchical cluster heatmap illustrating the presence (red) or absence (white) of stress genes as identified by AMRFinderPlus in each *S. aureus* isolate.

3.2.5 Antimicrobial resistance genes

A variety of genes involved in resistance to tetracycline [*tet(M)*, *tet(38)*], beta-lactam [*blaI, blaZ, blaR1*, *blaPC1*], macrolide [*erm(A), erm(C), erm(T)*], aminoglycoside [*ant(9)-la*], methicillin [*mecC, MecI*], fosfomycin [*fosB*] and the multidrug efflux MATE transporter [*mepA*] were identified. Overall, similar levels of antimicrobial resistance genes were noted across all groups, with most isolates harbouring the tetracycline resistance gene *tet(38)* (92%) and the multidrug efflux MATE transporter gene *mepA* (92%). Notably, fosfomycin resistance genes were more prevalent in the control and CRSwNP groups (60% and 25%, respectively) and not observed in the CRSsNP group. The *S. aureus* β-lactamase gene [*blaZ*] was less frequently detected in the control group (20%) compared to the CRSsNP (75%) and CRSwNP (50%) groups. Macrolide resistance genes [*ermT, ermA, ermC*] were more common in the CRSsNP group (75%), as opposed to the control (20%) and CRSwNP (25%) groups. Methicillin resistance genes [*mecC/mecI*] and the aminoglycoside resistance gene [*ant(9)-la*] were infrequently found, observed in just one CRSwNP patient and one control patient, respectively (Figure 3-3).

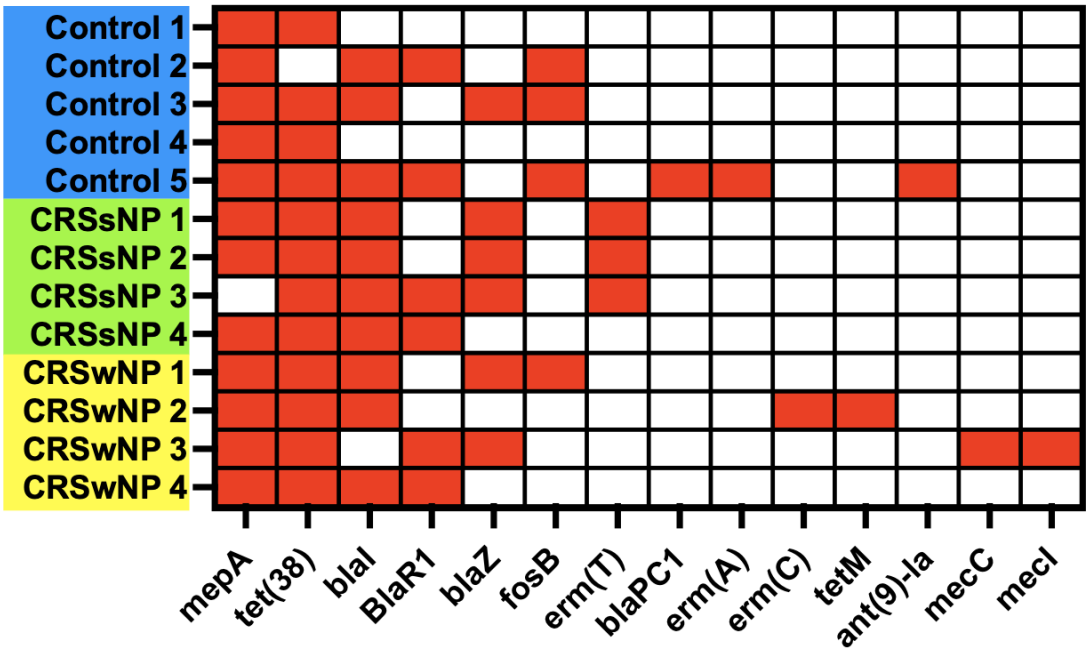


Figure 3-3 Antimicrobial resistance gene presence in control, CRSsNP and CRSwNP S. aureus isolates

Hierarchical cluster heatmap demonstrating the presence (red) or absence (white) of antimicrobial resistance genes as identified by AMRFinderPlus from each isolate.

3.2.6 Virulence genes

Bi-component gamma-haemolysin genes [*hlgA, hlgB* and *hlgC*], the polysaccharide intracellular adhesin biosynthesis/export protein gene [*icaC*], and the aureolysin gene [*aur*] were ubiquitous throughout our cohort. A reduced presence of delta-haemolysin [*hld*] and staphylococcal complement inhibitor [*scn*] genes were observed in the CRSwNP (50%) cohort compared with the control (100%) and CRSsNP (100%) cohorts. CRSsNP strains exhibited a reduced repertoire of virulence genes; none exhibited leukocidin E/D [*lukE/D*] or serine protease A/B [*splA, splB*]. Additionally, CRSsNP strains harboured few enterotoxins from the enterotoxin gene cluster [*sei, sem, sen and seo*] or the enterotoxin-like protein X [*selX*], with only one isolate showing *sei/m*. Both control and CRSwNP strains exhibited either a complete enterotoxin gene cluster or *splA, splB and lukE/D*; however, no isolate contained both profiles. In the control group, 40% carried *lukE/D* and *splA/B,* compared with 75% of the CRSwNP group (Figure 3-4). A comparison of the *agr* group with the presence of *lukE/D*, *splA, splB* or the enterotoxin gene cluster revealed no significant association (Table 3-5).

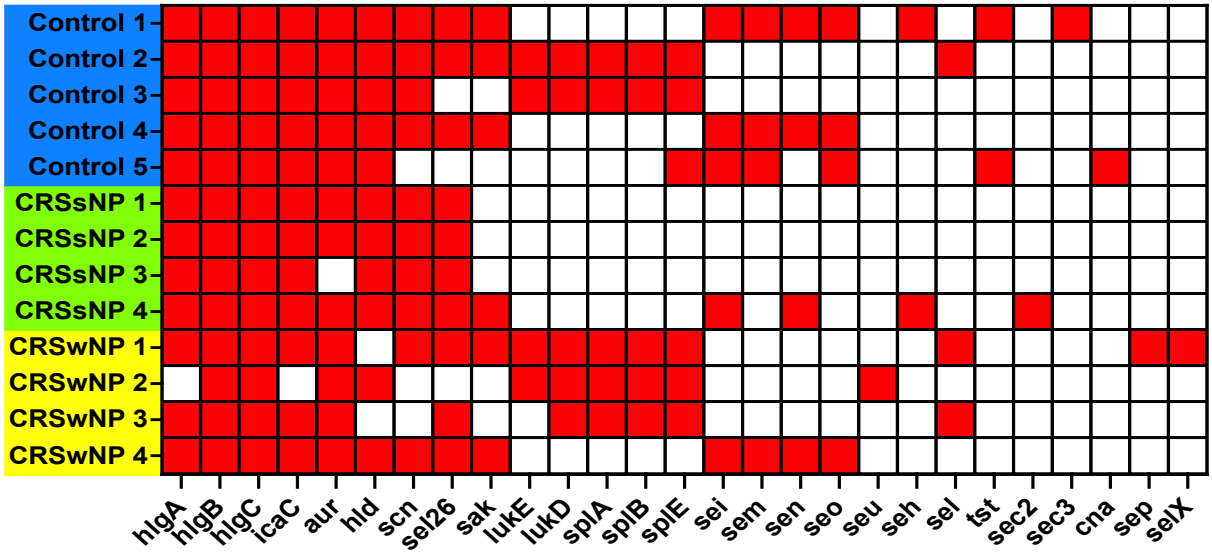


Figure 3-4 Virulence gene presence in control, CRSsNP and CRSwNP S. aureus isolates

Hierarchical cluster heatmap demonstrating the presence (red) or absence (white) of virulence genes as identified by AMRFinderPlus from each isolate.

Table 3-5 Accessory gene regulator locus type and presence of virulence gene patterns

agr locus type	*lukE/D* and *splA/B*	Enterotoxin gene cluster	Total number of isolates
1	2 (22%)	3 (33%)	9 (100%)
2	2 (100%)	0 (0%)	2 (100%)
3	1 (50%)	1 (50%)	2 (100%)
4	-	-	0

The prevalence of specific virulence factor patterns - leukocidin E/D (*lukE/D*) with serine protease A/B (*splA/B*) genes or the enterotoxin gene cluster across *S. aureus* isolates grouped by their accessory gene regulator (*agr*) locus types. The table shows the count and percentage of isolates within each agr type that exhibit these virulence factor combinations.

3.3 Discussion

Our study set out to determine whether there were discernible differences in the genomic profiles of *S. aureus* isolates between control subjects and those with CRS, potentially accounting for the heightened pathogenicity observed in CRSwNP patients. The data indicated that variations exist in the profiles of antimicrobial resistance and virulence factor genes between *S. aureus* strains derived from control individuals and patients with CRSsNP or CRSwNP. These distinctions could confer a bacterial survival advantage that is specific to the pathophysiological environment of each CRS disease state.

The cohort of patients from whom we studied *S. aureus* exhibited similar demographic characteristics as have been reported in other studies. Notably, the CRSwNP group showed a significantly higher prevalence of asthma compared to the CRSsNP and control groups, with respective rates of 58.8%, 20.0% and 17.6%. These findings align with data from the UK CRES epidemiology study, which reported a prevalence of 46.9%, 21.2% and 10.0%, and are also consistent with the GA^{2}LEN studies (33, 148). Furthermore, the incidence of inhalant allergy was greater in the CRSwNP group (47.0%) compared to the CRSsNP (30.0%) and control (35.2%) groups in our cohort. This reflects the trend observed in the UK CRES study (31.0%, 20.3% and 13.1%). The increased rates of inhalant allergy across all groups in our study could partly be ascribed to the different recruitment settings, which included surgical lists and rhinology clinics (148). Moreover, the control group comprised individuals with isolated allergic rhinitis, who were more inclined to undergo powered endoscopic turbinoplasties rather than endoscopic sinus surgery, a procedure more commonly required by the CRSsNP or CRSwNP groups. The MLMS were notably higher in the CRSwNP group compared to the CRSsNP and control groups, with average scores of 15.6, 5.1, and 4.0, respectively). This outcome aligns with the EPOS

diagnostic criteria for CRS, which stipulates a MLMS threshold of more than 4 on CT imaging of the sinuses. Comparable MLMS scores have been reported in other studies (13, 152). Surprisingly, a higher prevalence of active smokers was found in the control group, a finding which approached statistical significance. Smoking is frequently identified as a contributing factor for the development of CRS, and it is conceivable that the reduced incidence of smoking among patients with asthma and CRS could be attributed to healthcare advice, potentially influencing the demographic features of our cohort (33, 48).

We observed a lower-than-expected colonisation rate of *S. aureus* in patients with CRSsNP (40%) and CRSwNP (17.6%). These rates contrast sharply with those reported by other researchers, who have documented very high rates of *S. aureus* nasal carriage using 16S ribosomal RNA sequencing of the variable V3-V4 region among control subjects (92.9%), CRSsNP (83%) and CRSwNP (81.8%) patients (153). It is well documented that culture methods tend to yield a lower incidence of positive bacterial cultures compared to molecular techniques. For example, Thunberg *et al.* identified *S. aureus* cultures in 58% of CRSwNP patients, 56% of CRSsNP patients and 28% of control subjects (154). This project was conducted during and in the wake of the COVID-19 pandemic, a period that likely reduced patient contacts due to social distancing, encouraged mask usage and may have led to increased antimicrobial consumption. Many CRS patients seen in theatres and clinics had undergone one or more trials of medical therapy in the preceding year, often including antibiotic treatment, which could have lessened *S. aureus* loads. We reviewed the prescription of antibiotics in the month before inclusion in the study and established that 5.9% of control subjects, 10% of CRSsNP patients and 23.5% of CRSwNP patients had received antibiotics prior to their participation in the study; this factor could have impacted our findings.

Subgroup analysis showed no significant demographic differences among patients who cultured positive for *S. aureus*. However, there was a trend toward higher *S. aureus* culture positivity in female subjects compared to males, with this observation nearing statistical significance. Similar associations between *S. aureus* colonisation and gender have been reported in larger studies (119, 155).

The *S. aureus agr* locus orchestrates the production of RNA II and RNA III and stimulates downstream virulence factor synthesis, with both RNA II and RNA III functioning as quorum-sensing genes. Numerous studies categorise the *agr* locus into groups I-IV based on polymorphisms in the *agrB*, *agrC* and *agrD* genes, each group influencing downstream virulence factor production (156). Distinct disease patterns are associated with each group: Group I is predominantly linked to commensal carriage, urinary infections and bacteraemia; Group II is associated with hospital-acquired infections, invasive diseases and nasal carriage; Group III is commonly connected to respiratory infections and toxic shock syndrome; and Group IV is often related to cutaneous infections, including staphylococcal scalded skin syndrome (157, 158). In our collection of isolates, the majority corresponded to group I, with no significant differences observed across the study cohorts.

Typing of the *spa* gene is commonly employed to detect outbreaks of *S. aureus* isolates among populations. Additionally, certain *spa* types are disproportionately represented in specific demographics and clinical conditions, such as *S. aureus* bacteraemia and vascular access infections (119, 155, 159, 160). In our analysis, a variety of *spa* types were identified, with only three duplicates, suggesting that an epidemic model of *S. aureus* transmission in CRS is unlikely. However, given the limited number of participants

in our study, the possibility of an undetected, subtle association cannot be entirely discounted.

To elucidate the virulence genes potentially associated with CRS, we examined our assembled sequences for the presence of stress, antimicrobial resistance and virulence genes as catalogued by the AMRFinderPlus resistance gene database (161).

With respect to stress genes, the lincomycin resistance protein of *S. aureus* [*lmrS*] was highly conserved across the groups, with presence confirmed in 92% of isolates. This gene is a well-preserved member of the major facilitator superfamily, which plays a role in the transport of multidrug and essential solutes across the cell membrane (162). The cadmium resistance gene, *cadD,* was identified more frequently in CRSsNP and CRSwNP patients than control subjects. Despite being a poorly characterised gene known to endow cadmium resistance, *cadD* has not been associated with resistance to other heavy metals or drug toxicity (163). The role of *cadD* in CRS could suggest additional, unidentified targets of this transporter that may confer an advantage to *S. aureus* in the context of CRS. The occurrence of other heavy metal resistance genes was rare and did not seem to correlate with any specific disease state.

We observed that antimicrobial resistance genes, specifically the multidrug efflux transporter gene *mepA* and the tetracycline efflux transporter gene *tet(38),* were highly conserved across almost all *S. aureus* isolates. The β-lactamase gene *blaZ* was detected in only six isolates, constituting 20% of the controls and 62.5% of the CRS isolates, with each case exhibiting either the upstream regulator *blaR1* or the repressor *blaI* (164). Notably, the macrolide resistance gene *ermT* was found in 75% of isolates from patients with CRSsNP. Other macrolide resistance genes, such as *ermA* and *ermC,* were identified in only one isolate each from the control and CRSwNP group. This observed

increase in macrolide resistance could be attributed to selection pressures due to increased prescribing of macrolides within the CRSsNP patient group (14). Notably, other studies have documented that *S. aureus* isolates from osteomyelitis patients exhibit a high prevalence of β-lactamase (66.2%) and a relatively low incidence of macrolide resistance (8.6%), trends that might reflect condition-specific antimicrobial prescribing practices and the resultant selection pressures (165). Interestingly, in our study, the fosfomycin resistance gene *fosB* was detected in 60% of the control group isolates, yet it was found in only one other additional isolate across the other groups.

Phenol-soluble modulins, such as delta-haemolysin, are unique to *S. aureus* and play a role in the lysis of neutrophils and erythrocytes (166). We observed the absence of the delta-haemolysin gene (*hld*) gene in 50% of CRSwNP isolates, although it was present in all other isolates examined. Previous research has demonstrated that deletion of the *hld* gene, which resides within the *agr* operon, can substantially reduce the transcription of *agrA* and, consequently, the expression of downstream virulence factors, including alpha toxin, beta-haemolysin, enterotoxin B and gamma-haemolysin (167). Mutations in the accessory gene regulator locus are known to give rise to SCVs with attenuated virulence but enhanced ability to translocate intracellularly in epithelial cells. Therefore, the lack of the *hld* gene may promote a pathogenic strategy in *S. aureus,* potentially leading to the establishment of an intracellular reservoir of infection (168-170).

Immune modulators, including the aureolysin gene (*aur*), which is known to cleave the C3 component of the classical and alternative complement pathways, were highly conserved across our sample set. Conversely, the staphylococcal complement inhibitor (*scn*) gene was found in only 50% of the CRSwNP isolates, while present in 80% of control isolates and all of the CRSsNP isolates. The staphylococcal complement inhibitor (SCIN)

protein is an effective inhibitor of C3 convertase enzymes in vitro, which are crucial for the conversion C3 to C3b (171). A cleavage product of C3b known as C3d[g], acts as a potent opsonin which is bound to the *S. aureus* cell wall and has been shown to activate B-cell CD21 receptors in B-cell lymphoma cell lines, enhancing antibody formation towards *S. aureus* cell components; hence the absence of SCIN could lead to greater antibody formation towards *S. aureus* as is seen in CRSwNP (150, 172, 173).

Exfoliative virulence factors that contribute to pore formation in host cell membranes, specifically the bi-component-gamma-haemolysin components (*hlgA, hlgB and hlgC*) showed high conservation across all isolate groups. In contrast, *lukE/D,* which operates independently of the *agr* regulatory locus, was detected in 40% of control isolates and 75% of CRSwNP isolates but was absent in CRSsNP isolates. Both gamma-haemolysin and leukocidin E/D are capable of forming pores in neutrophils and erythrocytes. Additionally, leukocidin E/D can trigger calcium channel activation in neutrophils, leading to cell death, in a process independent of the toxin's pore-forming ability (174). Leukocidin E/D also undermines the adaptive immune response by lysing polymorphonuclear neutrophils, which can compromise local immunity and has been deemed crucial for bacterial proliferation and dissemination in a mouse model of bacteraemia (175). Serine proteases A and B (SplA, SplB*)*, commonly associated with invasive *S. aureus* infections, interact with the host immune system in distinct ways. SplA protein, has the capability to cleave mucin 16 glycoprotein, a key constituent of the nasal mucosal barrier. In contrast SplB protein, influences the immune response by polarising T helper cells towards a Th2 cytokine profile, which is associated with increased IgE production. This effect was demonstrated in studies where SplB was injected into the peritoneal cavities of mice (176).

We found a high prevalence of serine protease genes (*splA* and *splB*) in the CRSwNP group, with 75% of the isolates carrying these genes. In stark contrast, these protease genes were absent in the CRSsNP isolates and were present in only 40% of the control group (177). The concurrent presence of the *splA*, *splB* and *lukE/D* genes was notably absent in strains containing the enterotoxin gene cluster, suggesting a potential phenotypic differentiation among the strains. These results are consistent with the findings of Nepal *et al*. who found that the Sa3int prophage - which harbours *lukE/D* genes – was more prevalent in isolates from CRSwNP patients when compared to controls and CRSsNP patients (112). Interestingly, our analysis did not reveal any clear link between the simultaneous presence of *splA*, *splB* and *lukE/D* or the enterotoxin gene cluster with any specific *agr* group categorisation.

Superantigens, including the enterotoxin gene cluster comprising genes *sei, sem, sen and seo,* were identified in 60% of control isolates, 25% of CRSwNP isolates and were only partially present in a single CRSsNP isolate. These enterotoxins have been linked to persistent colonisation in diverse environments such as the nasal airways, the lungs of cystic fibrosis patients and atopic dermatitis wounds. Our findings of their prevalence in the control group are in alignment with previous studies (178, 179). Despite their capacity to stimulate T-cell proliferation, these toxins surprisingly elicit low levels of specific immunoglobulins in human serum relative to other superantigens (179, 180). Nonetheless, the precise role of these enterotoxins in *S. aureus* pathogenicity and host interaction remains largely elusive.

In the CRSwNP environment, *S. aureus* may benefit from shedding virulence genes such as the *hld* gene and those in the enterotoxin gene cluster. The loss of these genes could enable a more senescent phenotype with a concomitant reduction in virulence factor

production, potentially enhancing the bacteria's intracellular survival abilities. Additionally, the presence of genes such as *lukE/D* and *splB* could further reinforce the Th2-dominated immune milieu. This shift is thought to downregulate the immune response by depleting memory T lymphocytes, resulting in decreased production of IL-17 and IFN-γ and subsequently impaired bacterial clearance (181, 182). In contrast, the genomes of *S. aureus* isolates from the CRSsNP group generally lacked *lukE/D* and *splA/B* genes, with only a single isolate carrying a partial enterotoxin gene cluster. The scarcity of certain virulence and immunomodulatory genes in CRSsNP isolates could account for the lower IgE levels observed in CRSsNP patients, which are akin to those found in non-CRS individuals.

During the time of our study, we had established growth curves and OD600 measurements that corresponded to CFU counts for the Control 1 and CRSwNP 1 strains; the other strains had not yet been fully characterised. The Control 1 strain possessed an *hld* gene and the enterotoxin gene cluster, while lacking the *splA, splB* and *lukE/D* genes - a genomic profile commonly observed in this group. In contrast, the CRSwNP 1 strain was defined by the presence of *splA, splB* and *lukE/D* genes and the absence of the *hld* gene and the enterotoxin gene cluster, mirroring the gene patterns typically found in the CRSwNP cohort. Given the detailed information available on these two strains, we selected them for extensive analysis in chapter 4.

The research reported in this chapter is subject to several limitations. One major constraint is the small number of isolates cultured and sequenced from each group. Additionally, the genetic diversity inherent in sequencing a large array of virulence factors introduces significant heterogeny. This variability complicates the attainment of statistical significance, particularly in the absence of a large cohort for analysis. Despite

these challenges, our study's findings provide valuable contributions to the current sequencing landscape and enhance our insight into the mechanisms by which *S. aureus* may exhibit enhanced pathogenicity in cases of CRS.

3.4 Summary

Compared to non-CRS controls, *S. aureus* strains associated with CRSwNP display a higher prevalence of *splA, splB* and *lukE/D* virulence genes, while exhibiting reduced carriage of the enterotoxin gene cluster and the *hld* gene. Remarkably, strains from CRSsNP cases exhibit a starkly limited array of virulence factors, lacking *splA, splB* and *lukE/D,* harbouring only sparse elements of the enterotoxin gene cluster. These variations in virulence gene profiles may contribute to *S. aureus* persistence within each specific disease milieu or influence the distinct cytokine responses characteristic of each CRS state. In particular, the scarcity of the *hld* gene in CRSwNP strains could be an attribute that favours the development of SCVs, providing *S. aureus* with a strategic advantage for survival and enabling it to elude immune detection and infiltrate intracellular environments.

Chapter 4 **Intracellular survival of *S. aureus* strains cultured from control and CRSwNP patients**

4.1 Introduction

Building on the findings of the previous chapter, which revealed genomic differences in *S. aureus* strains isolated from control subjects, as well as CRSsNP and CRSwNP patients, this chapter aims to further investigate these distinctions. Specifically, we will examine the ability of these strains to internalise, survive within, and replicate in the intracellular environment. Our past research has highlighted the significance of intracellular *S. aureus* in promoting CRS and the activation of the adaptive immune response. We have observed that a strain of *S. aureus* obtained from a CRSwNP patient can be internalised effectively by HMC-1 and LAD2 mast cell lines. This internalisation leads to the release of pro-inflammatory mediators, including IFN-γ, IL-17a, TNF-α, IL-1b and IL-6, mimicking the raised inflammatory markers seen in both CRS phenotypes (111). Moreover, *S. aureus* has been implicated in the recruitment and accumulation of mast cells in the subepithelial layer in CRSwNP patients - a phenomenon not observed in controls. When these mast cells are recruited by *S. aureus*, the bacteria have been shown to internalise, thereby engaging the adaptive immune system and fostering inflammation in affected individuals (75).

S. aureus internalisation and subsequent intracellular survival require the expression of various genes, notably the fibronectin-binding proteins A and B (FnBPs). These proteins interact with $\alpha_1\beta_5$ integrins on the cell surface membrane and facilitate uptake via a zipper-type mechanism (183). Beyond this well-characterised pathway, other less thoroughly investigated mechanisms facilitate *S. aureus* to anchoring to host cells. These include the binding of FnBPs' to heat shock protein (HSP) 60, autolysin's interaction with

HSP70, the adherence of clumping factor β to extracellular matrix proteins, and the direct interaction of staphylococcal protein A with TNF-α. Evaluating the significance of these internalisation pathways presents a challenge, as they have been demonstrated across a wide variety of cell types (184-187).

Following internalisation, *S. aureus* strains must evade cellular defences, particularly the phagosome. Studies have demonstrated that both alpha-haemolysin and delta-haemolysin independently allow escape from phagocytic vesicles in diverse cell types, providing redundancy of function (188, 189). Once inside the cell, *S. aureus* strains associated with CRS have been observed to transition into SCVs, a metabolic alteration that decreases cellular activity. This metabolic shift is typically driven by non-functioning mutations in the *agr* locus or through deletion of the *hld* gene (118). Furthermore, as detailed in the preceding chapter, mutations within the *agr* locus and the absence of delta-haemolysin result in decreased expression of virulence factors by downregulating the main effector RNAIII. This reduction affects heme biosynthesis and oxidative phosphorylation pathways while increasing the thickness of the bacterial cell wall, creating resistant quiescent clones similar to fungal spores (86). Further investigation is needed to determine whether there are distinctions in intracellular survival rates between *S. aureus* strains isolated from individuals with CRS and strains residing in healthy individuals' microbiomes.

This part of the study aimed to investigate these distinctions further by utilising well-characterised strains that best represent a control and CRSwNP strain. Control 1 (possessing a functional *hld* gene and the enterotoxin gene cluster but lacking *splA, splB* or *lukE/D* genes) was compared in mast cell intracellularisation models with CRSwNP 1

(carrying *lukE/D* and *splA, splB* genes but lacking the *hld* gene and the enterotoxin gene cluster).

4.2 Results

4.2.1 HMC-1 lysostaphin protection assay

HMC-1 cells, which are immortal immature mast cells, were used to model mast cells in co-culture with *S. aureus*. Our aim was to determine if there was any difference in extracellular and intracellular survival between the two strains. HMC-1 cells (2×10^6) were co-cultured with *S. aureus* bacteria at a multiplicity of infection (MOI) of 2. We observed that the extracellular concentration of *S. aureus* from both CRSwNP 1 and Control 1 strains increased exponentially in the extracellular space. However, there appeared to be a difference in the degree of viable bacteria increase between the strains; the Control 1 strain's Colony forming unit (CFU) count increased by 200-fold between 2-6 hours, vs the 1300-fold increase observed in the CRSwNP 1 strain's co-culture. At each timepoint, the Control 1 strain exhibited significantly fewer CFUs when compared with the CRSwNP 1 strain. Specifically, at 2, 4 and 6 hours the CRSwNP strain demonstrated 4 ($P \leq 0.01$), 12,044 ($P \leq 0.0001$) and 20-fold ($P \leq 0.05$) increases in CFU values when compared with the Control 1 strain (Figure 4-1).

Intracellular survival, determined by lysostaphin-treated HMC-1 mast cells, demonstrated no significant uptake or survival at 2 hours, but appeared to increase dramatically at 4 hours before decreasing at 6 hours. There was a significant difference in intracellular CFUs between strains at 4 and 6 hours, with the CRSwNP 1 strain demonstrating a 500-fold ($P \leq 0.05$) increase at 4 hours and 20-fold ($P \leq 0.01$) increase at 6 hours, above that of the Control 1 strain (Figure 4-2).

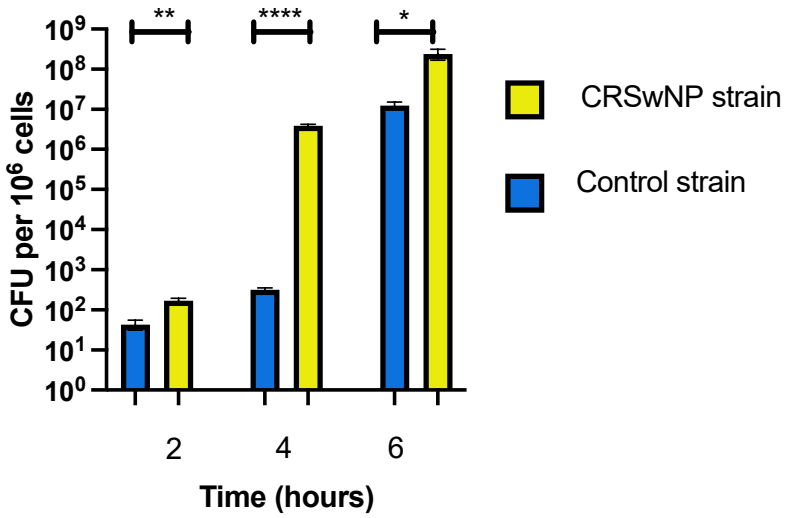


Figure 4-1 Extracellular survival of Control 1 and CRSwNP 1 S. aureus in co-culture with HMC-1 cells

Extracellular survival of *S. aureus* in co-culture with HMC-1 cells at 2, 4 and 6 hours. In each condition, 2 x 10^6 HMC-1 cells were co-cultured with 4 million Control 1 and CRSwNP 1 *S. aureus* bacteria. The supernatant was extracted and used to calculate the number of CFUs per 1 x 10^6 HMC-1 cells. The bars represent the mean of nine experimental repeats with the standard error of the mean depicted by error bars. Statistical analysis was performed using a paired t-test (*P≤0.05, **P≤0.01, ****P≤0.0001).

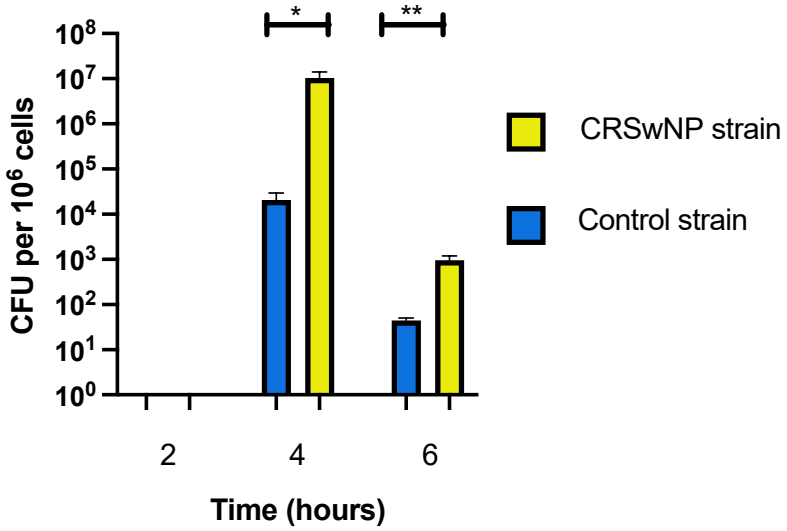


Figure 4-2 Intracellular survival of Control 1 and CRSwNP 1 S. aureus in co-culture with HMC-1 cells

Intracellular survival of *S. aureus* in co-culture with HMC-1 cells was assessed for the Control 1 and CRSwNP 1 strains. The HMC-1 cells were centrifuged and washed in lysostaphin-containing media to eradicate extracellular bacteria. The intracellular compartment was extracted, and *S. aureus* CFUs were quantified per 1 x 10^6 HMC-1 cells. The bars represent the mean of nine experimental repeats, with the standard error of the mean depicted by error bars. Statistical analyses were performed using a paired t-test (*P≤0.05, **P≤0.01).

4.2.2 LAD2 lysostaphin protection assay

We next utilised LAD2 cells which represent another available mast cell line – reported to exhibit greater differentiation than HMC-1 cells - in our co-culture model. LAD2 cells ($1x10^6$ per well) were cultured in antibiotic-free medium with the Control 1 and CRSwNP 1 strains at an MOI of 1. Both strains displayed exponential extracellular growth over the 9-hour experimental period. A significant increase in extracellular survival was observed at 3 hours (2.9-fold, $P \leq 0.001$) and 6 hours (1.65-fold $P \leq 0.05$) for the CRSwNP 1 strain in comparison to the Control 1 strain (Figure 4-3).

Intracellular survival showed an increase up until 6 hours, after which the number of viable bacteria decreased. Again, marked differences were observed; no viable bacteria were detected at 3 hours in the Control 1 strain, whereas growth was noted in the CRSwNP 1 strain. No significant difference was observed at 6 hours between the strains; however, the CRSwNP 1 strain demonstrated a 9.8-fold increase in survival at 9 hours ($P \leq 0.05$) (Figure 4-4).

At the 9-hour timepoint, the number of viable intracellular *S. aureus* appeared to decrease when compared to the 6-hour values, likely due to LAD2 cell death (as confirmed in subchapters 4.2.3 and 4.2.4). Consequently, we eliminated extracellular *S. aureus* at 6 hours to focus on the observation of intracellular *S. aureus* survival and replication. We observed persistence and an increase in intracellular CFUs for both the Control 1 and CRSwNP 1 strains beyond what is typically seen at 6 hours. The CRSwNP 1 strain displayed an 8.7-fold increase in CFUs in comparison to the Control 1 strain, following treatment with 20 µg/mL lysostaphin for 60 minutes. Moreover, when the co-culture was treated with 1.32 µg/mL lysostaphin between 6- 24 hours, an 11-fold increase

in intracellular survival was recorded for the CRSwNP 1 strain relative to the Control 1 strain ($P \leq 0.05$) (Figure 4-5).

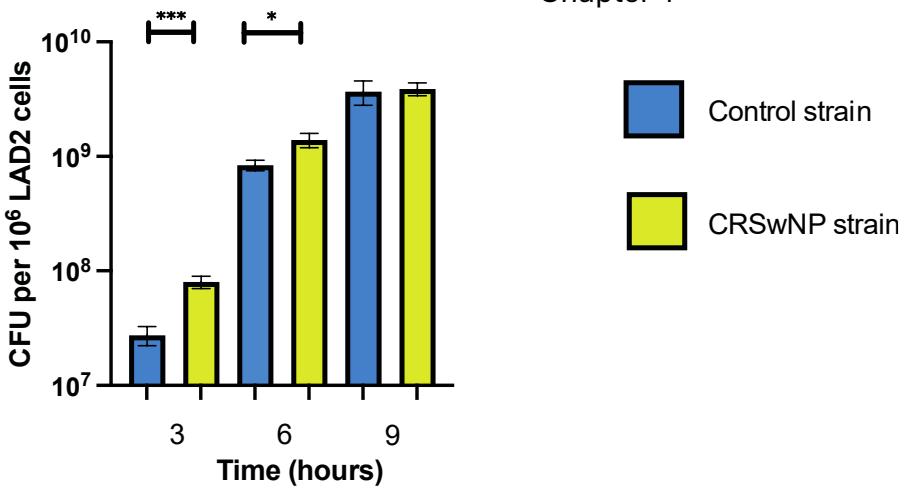


Figure 4-3 Extracellular survival of Control 1 and CRSwNP 1 S. aureus in co-culture with LAD2 cells.

Extracellular survival of *S. aureus* in co-culture with LAD2 cells was assessed; 1 x 10^6 LAD2 cells were co-cultured with 1 x 10^6 Control 1 and CRSwNP 1 *S. aureus* bacteria. The supernatant was extracted and analysed to determine the number of CFUs per 1 x 10^6 LAD2 cells. The bars indicate the mean of nine experimental repeats, and the standard error of the mean is illustrated by the error bars. Statistical analysis was conducted using a paired t-test (*P≤0.05, ***P≤0.001).

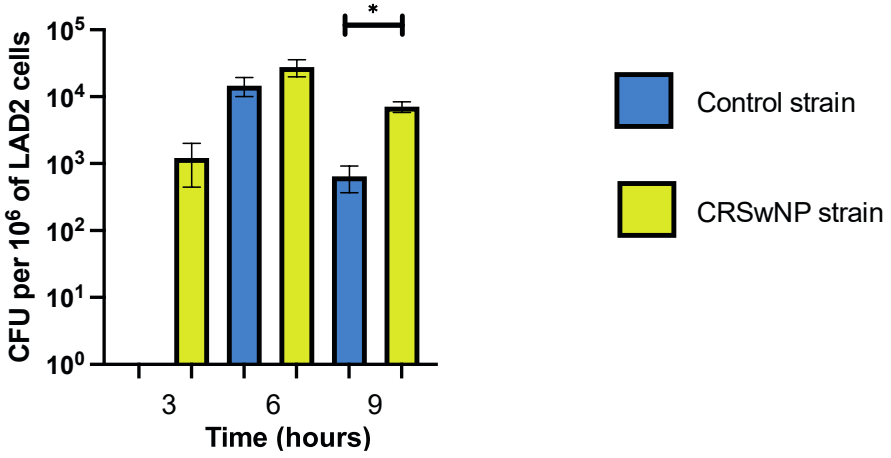


Figure 4-4 Intracellular survival of Control 1 and CRSwNP 1 S. aureus in LAD2 mast cell co-culture over time.

Intracellular survival of *S. aureus* in a LAD2 mast cell co-culture was monitored over time. The Control 1 *S. aureus* strain was compared with the CRSwNP 1 *S. aureus* strain at 3, 6 and 9-hour timepoints. The intracellular compartment was extracted, and *S. aureus* CFUs were quantified per 1 x 10^6 LAD2 cells. The bars reflect the mean of nine experimental repeats, and the standard error of the mean is indicated by error bars. Statistical analysis was conducted using a paired t-test (*P≤0.05).

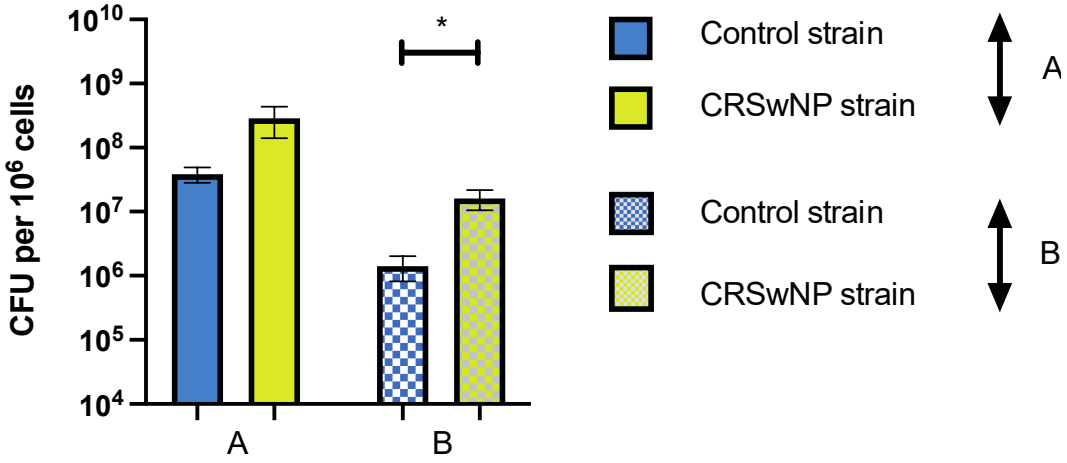


Figure 4-5 Intracellular survival of Control 1 and CRSwNP 1 S. aureus strains at 24 hours after treatment with lysostaphin at 6 hours.

Intracellular survival of *S. aureus* in LAD2 mast cell co-culture was assessed at 24 hours after the eradication of extracellular bacteria at 6 hours. **A)** Six experimental repeats comparing the intracellular survival of the Control 1 *S. aureus* strain with the CRSwNP 1 *S. aureus* strain following treatment with 20 µg/mL lysostaphin at 6 hours for 60 minutes. **B)** Six experimental repeats comparing the Control 1 *S. aureus* strain with the CRSwNP 1 *S. aureus* strain following the eradication of extracellular bacteria at 6 hours with 1.32 µg/mL lysostaphin for the duration of culture until 24 hours. Each bar represents the mean of the experimental values, and the standard error of the mean is indicated by the error bars. Statistical analysis was conducted using a paired t-test (*P≤0.05).

4.2.3 LAD2 cell death

Lactate dehydrogenase (LDH) is a ubiquitous enzyme found in the cytoplasm of all mammalian cells and catalyses the conversion of lactate to pyruvate. The analysis of LDH has become a widely accepted method for determining cell viability. To investigate potential differences in cell death between the co-cultures - which may indicate virulence distinctions and could affect the viable number of LAD2 cells available for *S. aureus* internalisation - we examined LDH release in LAD2 co-cultures. We assessed supernatant levels of the enzyme against a standard where 1 million LAD2 cells were lysed with 0.5% Triton X-100. LDH release escalated over the 9-hour period, from a mean net release of 39% at 3 hours to a mean net release of 92% at 9 hours in both groups. At each timepoint, no significant difference between the groups was noted. Heat-killed *S. aureus* showed no significant increase in LDH release during the 9-hour period in either group. Heat-killed *S. aureus* bacteria (1 x 10^6 in total) from both strains were used to establish baseline LDH release, which remained stable at 37.5% over the 9-hour experimental period (Figure 4-6).

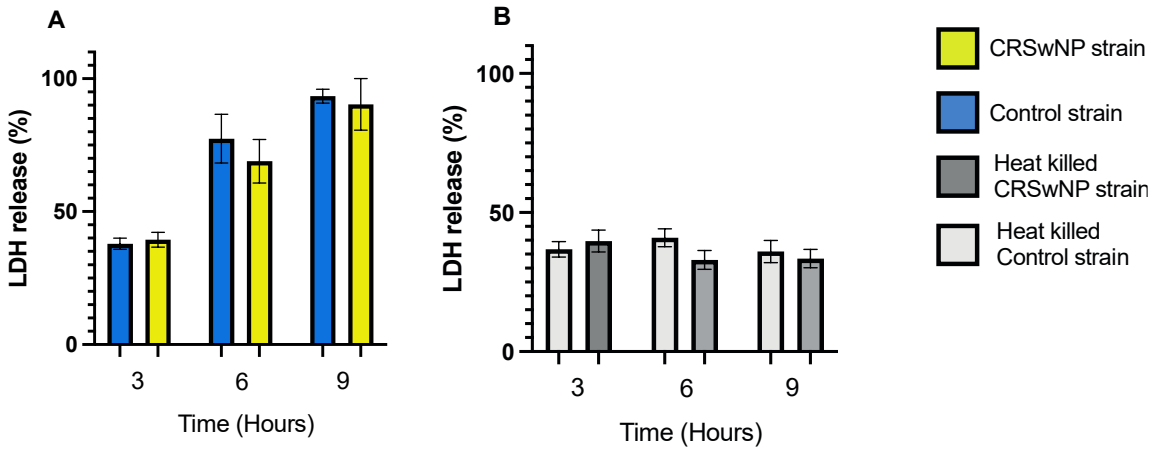


Figure 4-6 LDH release from LAD2 cells co-cultured with live and dead S. aureus isolates

A) Net release of LDH from LAD2 cells upon exposure to Control 1 and CRSwNP 1 strains of *S. aureus*. Nine experimental repeats were performed; the mean is depicted by the bar, with error bars representing the standard error of the mean. No significant differences were observed between the groups at each timepoint. **B)** Heat-killed Control 1 and CRSwNP 1 *S. aureus* bacteria (1 x 10^6 in total) were cultured with 1 x 10^6 LAD2 cells. Following nine experimental repeats, the mean LDH release is illustrated by the bar, with error bars indicating the standard error of the mean. Over the 9-hour experimental time period, no significant change in LDH release was observed, and no significant differences between the groups were detected at each timepoint using a paired t-test.

4.2.4 Imaging of intracellular survival

To further explore the relationship between each *S. aureus* strain and their capacity for intracellular survival, we co-cultured LAD2 cells with both Control 1 and CRSwNP 1 *S. aureus* strains at an MOI of 1 for 3, 6 and 9 hours, subsequently eradicating extracellular bacteria with lysostaphin. The remaining bacteria represented those shielded by docking or localised intracellularly, facilitating the visualisation of intracellular bacteria between strains. This allowed us to observe their abundance and distribution within the cytoplasm (Figure 4-7). The images suggest the CRSwNP 1 strain may more effectively translocate to the intracellular space within LAD2 cells, whereas the Control 1 strain appeared predominantly at the periphery of the cells, potentially in the docking phase prior to intracellularisation. However, due to the two-dimensional nature of these images, accurately pinpointing the bacterial location on the cell surface presents challenges.

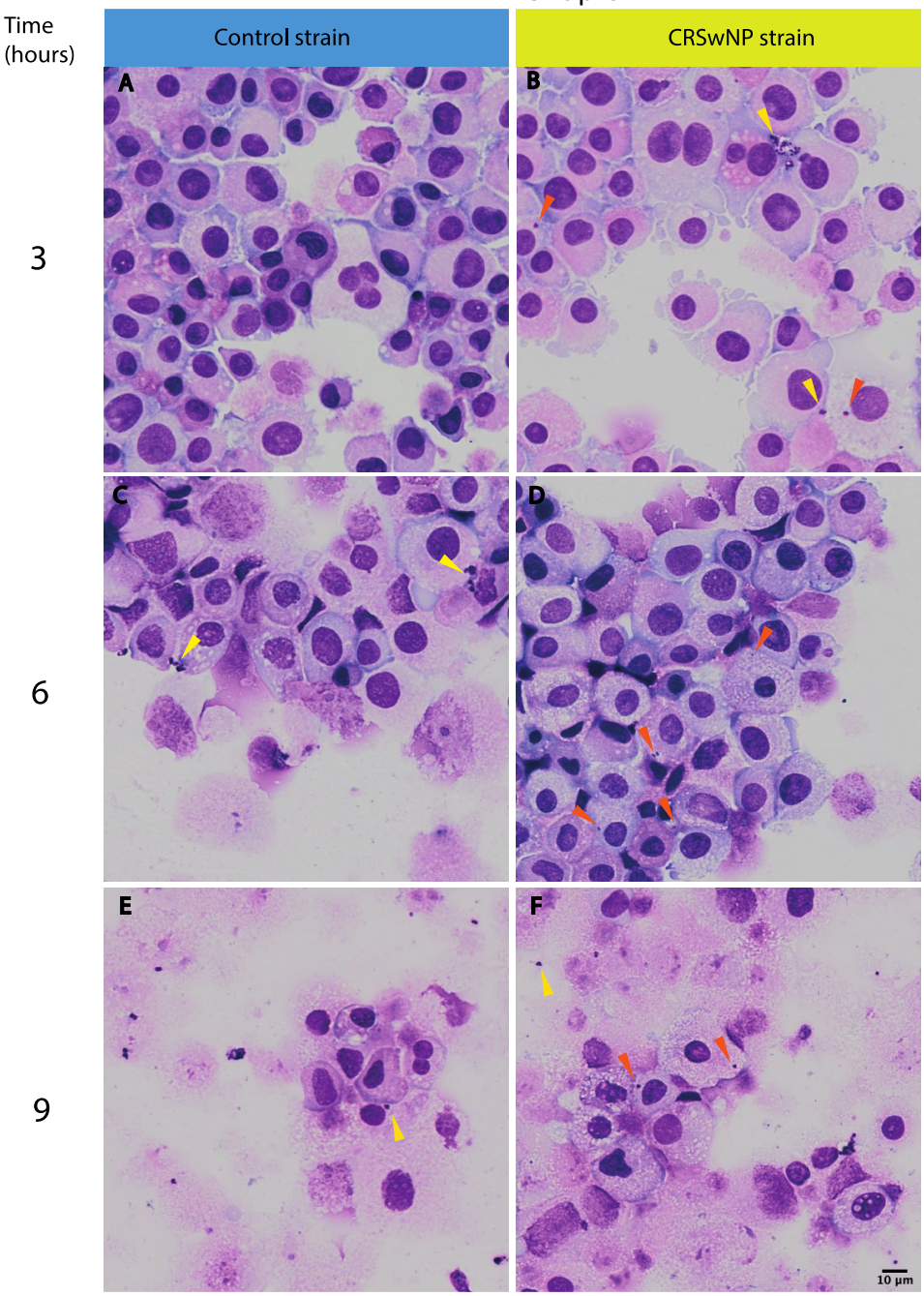


Figure 4-7 Giemsa-stained cytospins of LAD2 cells co-cultured with Control 1 and CRSwNP 1 S. aureus strains.

A) 3-hour co-culture with Control 1 strain: LAD2 cells appear healthy with no intracellular bacteria observed.
B) 3-hour co-culture with the CRSwNP 1 strain: Intracellular bacteria visible (indicated by orange arrows) and some bacteria appear docked on the cell wall (yellow arrows).
C) 6-hour co-culture with Control 1 strain: Minimal surface-bound *S. aureus* present.
D) 6-hour co-culture with the CRSwNP 1 strain: Multiple LAD2 cells show classical *S. aureus* diplococci within their cytoplasm.
E) 9-hour co-culture with Control 1 *S. aureus*: extensive cell death observed, with few cells remaining intact.
F) 9-hour co-culture with CRSwNP 1 *S. aureus*: A few surviving cells are present with persistent intracellular bacteria.
Scale bar signifies 10 µm.

4.2.5 β-hexosaminidase release

β-hexosaminidase is stored within the granules of mast cells and is expelled upon their activation and degranulation, which can disrupt the cell walls of invading bacterial pathogens. Mast cell activation may be triggered by several mechanisms, including IgE crosslinking to the FcεR1 receptor, bacterial toxins binding to TLR2 and TLR4 or complement receptor activation (67). Our previous research has shown that a CRSwNP-associated *S. aureus* strain is capable of internalising within mast cells and recruiting additional mast cells to the subepithelial layer (75). In light of this, we examined if this phenomenon was dependent on the degranulation of preformed mediators and whether there were any differences in behaviour of the strains tested. We aimed to identify if there were any variations in each *S. aureus* strain's ability to activate LAD2 cells across time points ranging from 3 to 9 hours. Net β-hexosaminidase release was quantified by calculating the total release of 1 million LAD2 cells lysed with Triton X-100, and this data was used to ascertain the percentage release at each timepoint. β-hexosaminidase release escalated from 10.9% to 18.9% for the Control 1 strain and from 9.69% to 20.7% for the CRSwNP 1 strain between the 3 and 9-hour intervals, respectively. No significant differences in β-hexosaminidase release were observed between the two groups at any timepoint (Figure 4-8).

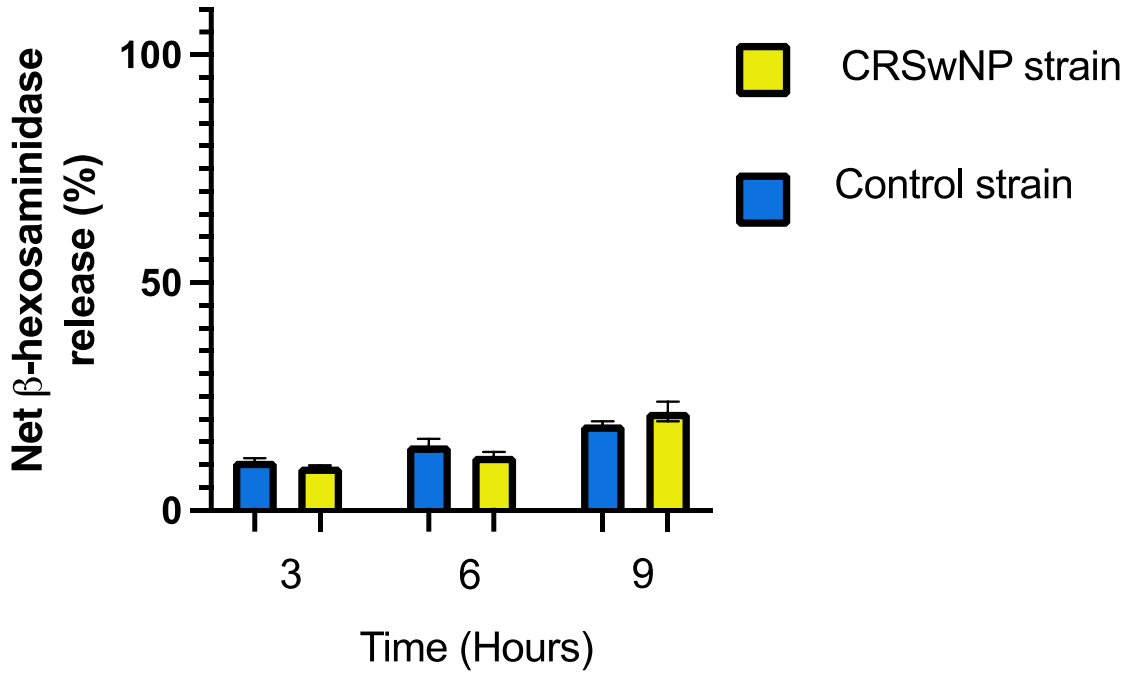
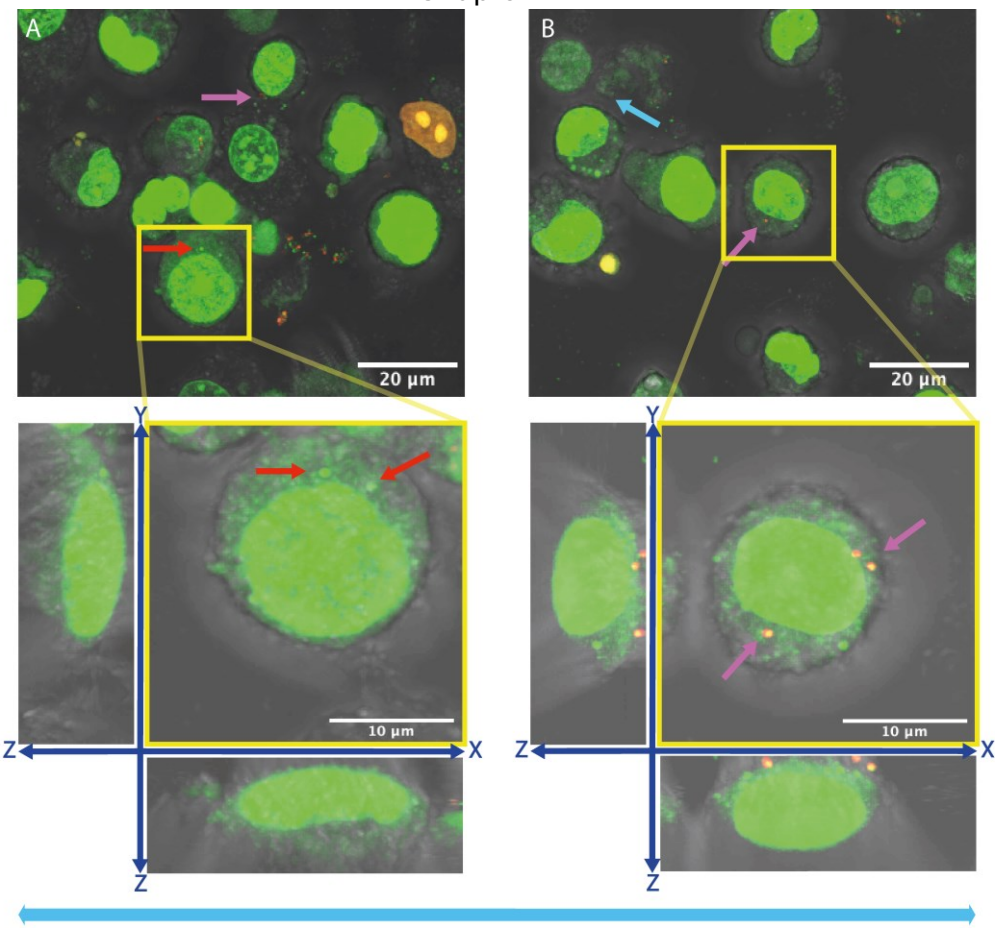


Figure 4-8 β-Hexosaminidase release from LAD2 co-cultures with Control 1 and CRSwNP 1 S. aureus strains

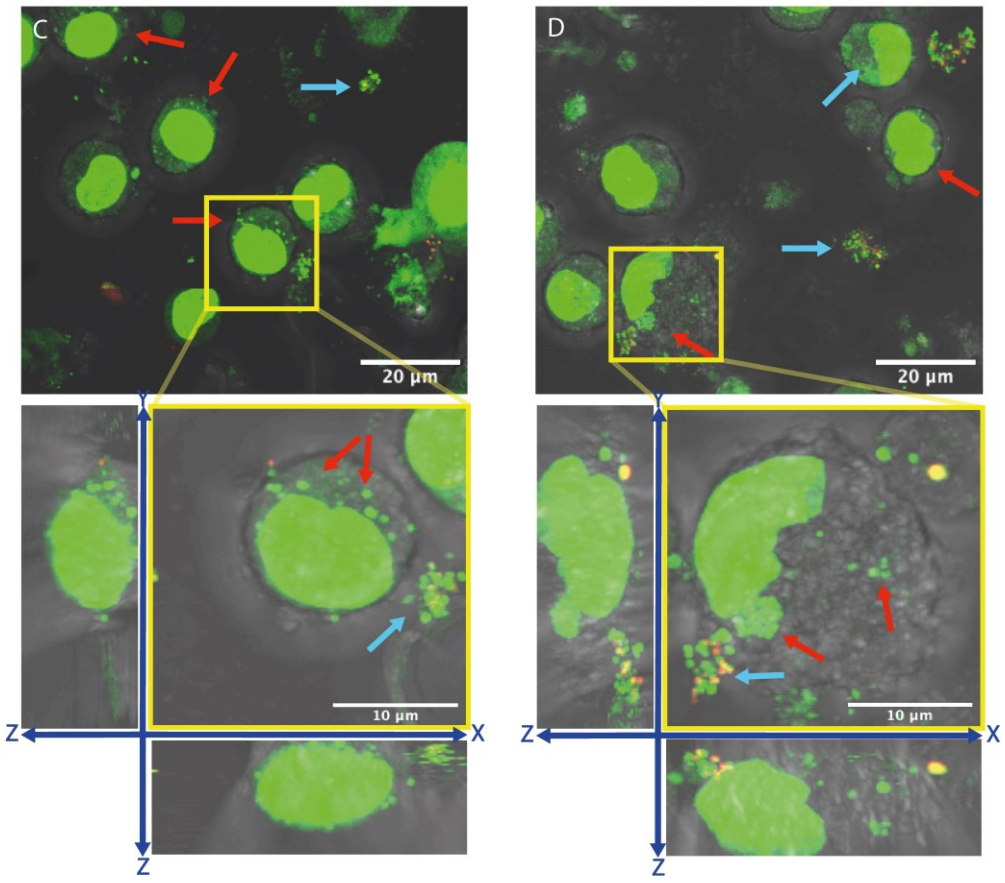
Net β-hexosaminidase release from LAD2 cells co-cultured with 1 x 10^6 Control 1 and CRSwNP 1 *S. aureus* strains at an MOI of 1. The bars represent the mean for nine experimental repeats, with error bars indicating the standard error of the mean. No significant differences in β-hexosaminidase release between the strains were observed at each timepoint, as determined by a paired t-test.

4.2.6 Confocal microscopy

Consistent with our previous observations, a 6-hour incubation period was determined to be optimal for *S. aureus* co-cultures to ensure maximal internalisation within LAD2 cells. Consequently, we co-cultured LAD2 cells with *S. aureus* for 6 hours, after which we applied lysostaphin to eliminate extracellular bacteria. After staining with BacLight™ LIVE/DEAD™ stain, we utilised confocal microscopy to visualise the cells. These images verified that the bacteria were indeed localised within the mast cell cytoplasm. Notably, the CRSwNP 1 strain-infected cells contained a higher number of intracellular bacteria and featured apoptotic cell bodies lacking a nucleus, with an abundance of *S. aureus* present within these cell remnants, in comparison to the Control 1 strain (Figure 4-9). The Control 1 *S. aureus* strain was observed infecting between 14-25% of cells, whereas the CRSwNP 1 strain infected 52-57% of cells (P=0.18).



Control *S. aureus* strain



CRSwNP *S. aureus* strain

Figure 4-9 Confocal imaging with BacLight™ LIVE/DEAD™ staining of LAD2 co-cultures with Control 1 and CRSwNP 1 S. aureus strains.

Live bacteria and intact cell nuclei are stained green, whereas dead bacteria and nuclei are stained red. The images provided are Z-stacks complimented with orthogonal projections of single cells below.
A,B) Co-culture of LAD2 cells with the Control 1 *S. aureus* strain: A limited number of *S. aureus* bacteria are seen within the intracellular compartment (indicated by red arrows), with occasional dead bacteria present within cells (indicated by purple arrows).
C,D) Co-culture of LAD2 cells with the CRSwNP 1 *S. aureus* strain: A substantial quantity of *S. aureus* bacteria is visible inside live cells (indicated by red arrows), alongside numerous apoptotic cell bodies harbouring both live and dead bacteria (indicated by blue arrows).

4.3 Discussion

The intracellular survival of *S. aureus* in host cells is a relatively understudied area, few clinical isolates have demonstrated this ability to date. Krut *et al.* demonstrated that among 23 *S. aureus* clinical isolates only 7 appeared to internalise and exhibit cytotoxicity in fibroblasts and keratinocyte cell lines (124). Remarkably, these cytotoxic isolates appeared capable of avoiding lysosomal degradation, contrasting starkly with the non-cytotoxic isolates, which were predominantly sequestered within phagosomes that stained positive for lysosomal markers (124). These findings, in conjunction with our observations, suggest that the pathogen's ability to bypass normal cellular processing routes may be associated with increased virulence.

We utilised two mast cell lines to investigate the intracellular survival capabilities of Control 1 and CRSwNP 1 *S. aureus* strains. The HMC-1 cell line represents a poorly differentiated mast cell model with two-point mutations in the c-KIT oncogene, causing constitutive phosphorylation and activation. These cells resemble mast cells by producing vesicles containing β-tryptase, histamine, heparin, chondroitin sulphate and TNF-α. However, they lack vesicles with chymase, myeloperoxidase, extracellular cationic protein, lysozyme, elastase and do not express the high-affinity (FcεRI) or low-affinity IgE receptors (FcεRII) (143). In comparison, the LAD2 cell line more accurately reflects mature human mast cells. LAD2 cells are a phenotypically mature mast cell line without c-KIT mutations. They proliferate at a slower rate and require the c-KIT ligand stem cell factor (SCF) for growth. Furthermore, they express the high affinity IgE receptor and undergo degranulation when IgE bound to this receptor is crosslinked by an antigen, leading to downstream signalling. They demonstrate more stable degranulation than the HMC-1 cell line, with larger granules which release antimicrobial defences (190).

To assess potential differences in intracellular survival between CRS-associated *S. aureus* strains and those isolated from nares of individuals without chronic rhinosinusitis, we employed a lysostaphin protection assay. Lysostaphin is a 27kDa glycylglycine endopeptidase known to hydrolyse the polyglycine bridges that cross-link glycopeptide chains, which constitute the cell wall of *S. aureus*. The use of lysostaphin in protection assays is preferred over gentamycin protection assays due to lysostaphin's specificity for *S. aureus,* rapid bactericidal action and inability to penetrate eukaryotic cell membranes, thereby ensuring targeted destruction of extracellular bacteria without harming host cells (191).

Our initial data, comparing the CRSwNP 1-related *S. aureus* strain with the Control 1 strain, suggest that both are capable of surviving in the extracellular environment of HMC-1 and LAD2 cells. From an extracellular perspective, the CRSwNP 1 strain demonstrated a modest yet statistically significant increase in survival and proliferation compared to the Control 1 strain in both HMC-1 and LAD2 mast cell environments. Intracellularly, survival rates were markedly higher for the CRSwNP strain, with a 500-fold increase noted in HMC-1 cells at 4 hours and a 9.8-fold increase in LAD2 cells at 9 hours, relative to the Control 1 strain.

These results are likely attributable to strain-specific differences that enhance intracellularisation, survival and replication of CRSwNP 1 *S. aureus* within mast cells. The absence of the *hld* gene and presence of *splA, splB* and *lukE/D* genes in the CRSwNP 1 strain may confer advantages for survival both within and alongside mast cells. The lack of the *hld* gene is thought to impair the *agr* locus functionally, leading to diminished production of various virulence factors, including alpha-haemolysin, gamma-haemolysin, serine proteases (*splA-F*) and leukocidins (*lukAB, GH*). This alteration may

also entail repressed enterotoxin production through *rot* gene transcription regulation (114-116). Moreover, the *hld* gene product is known to induce lysis and degranulation in HMC-1 cells, which would potentially reduce the available mast cells for *S. aureus* internalisation (192). The *splB* gene encodes SplB, a protein that disrupts complement function and opsonophagocytosis by cleaving and inactivating complement pro-enzymes C3-9 and activated components C3b and C4b, therefore inhibiting C3b-facilitated neutrophil-mediated opsonophagocytosis. Additionally, it inhibits the alternative complement pathway, preventing the assembly of the membrane attack complex on the surface of *S. aureus* (193).

Overall, LAD2 cells demonstrated similar levels of intracellular Control 1 *S. aureus* as those observed in HMC-1 cells, albeit at a 350-fold lower concentration with respect to the CRSwNP 1 strain. This finding aligns with my predecessor, Dr Timothy Biggs, and could be attributed to the more sophisticated host defence mechanisms of the better-differentiated LAD2 cell line (122). Notably, the HMC-1 cell line is deficient in lysozyme, a critical component of cellular defence involved in bacterial killing (143). Therefore, HMC-1 cells may internalise *S. aureus,* allowing the bacteria to remain viable, whereas LAD2 cells are potentially more proficient in eradicating *S. aureus*.

For the reasons mentioned, further investigation was focused on the LAD2 cell line. Both the Control 1 and CRSwNP 1 *S. aureus* strains demonstrated the capacity for internalisation and subsequent intracellular viability, despite a 60-minute lysostaphin treatment intended to eliminate extracellular bacteria. Notably, the CRSwNP 1 strain showed superior internalisation efficiency at each measured timepoint when compared with the Control 1 strain. To evaluate whether *S. aureus* strains could not only survive but also proliferate within the intracellular environment, extracellular bacteria were

eliminated after 6 hours of co-culture by a 60-minute lysostaphin treatment, followed by washing and resuspension of the LAD2 cells in fresh media without antibiotics. The subsequent quantification of intracellular CFUs revealed a substantial rise between the 6-hour and 24-hour timepoints: an 1820-fold increase for the Control 1 strain and an 8420-fold increase for the CRSwNP 1 strain. These increases in CFUs unequivocally suggest that both strains are capable of surviving and proliferating within the intracellular space. Additionally, at 24 hours post-infection, the CRSwNP 1 strain exhibited a 9-fold increase in CFUs compared to the Control 1 strain. In our experimental approach, we used lysostaphin at higher concentrations and for a longer duration than typically used in lysostaphin protection assays (191). While extracellular *S. aureus* was effectively eradicated at the 6-hour mark, we could not control for the extracellular leak of *S. aureus* from lysed LAD2 cells during the subsequent 18-hour period. To mitigate this, we adapted the protocol of Kim *et al.* and maintained co-cultures in 1.32 µg/mL lysostaphin from 6 to 24 hours (191). Despite this modification, our results consistently showed significant increases in intracellular survival between 6 and 24 hours, with a 97-fold increase for the Control 1 strain and 580-fold increase for the CRSwNP 1 strain. Moreover, the CRSwNP 1 strain's CFUs were 11 times higher than those of the Control 1 strain at the 24-hour timepoint. Collectively, these findings indicate that while both strains have the capacity to internalise, survive and replicate within mast cells, the CRSwNP 1 strain demonstrates a markedly superior potential in these regards.

Live CRSwNP 1 and Control 1 strains both exhibited comparable cytotoxic effects, with net LDH release increasing from 32% to 92% between 3 to 9 hours, and no statistically significant differences observed between the two groups. Heat killed *S. aureus,* both Control 1 and CRSwNP 1 *S. aureus,* failed to induce cytotoxicity, indicating that metabolic activity or gene expression is necessary for eliciting cell death. Contrary to our

expectations, the Control 1 strain, possessing an intact *hld* gene, did not display elevated cell stress or death relative to the CRSwNP1 strain, which has no *hld* gene. Surprisingly, despite the differing virulence factor profiles, we observed no marked difference in mast cell survival between the strains. This result assured us that an equal number of mast cells were present across the same experimental timepoints, suggesting that the observed variations in intracellular survival within the CRSwNP 1 group were not due to differential mast cell availability for infection. A possible explanation for the equivalent cytotoxicity levels is the presence of the *lukE/D* gene in the CRSwNP 1 strain. This gene encodes a pore-forming toxin capable of lysing erythrocytes and polymorphonuclear neutrophils, and it operates independently of the *agr* regulatory system, potentially compensating for the absence of the *hld* gene (194, 195).

Mast cells play a pivotal role in adaptive immunity; they actively degranulate in response to *S. aureus* cell wall components, a process mediated by TLR2 and TLR4 signalling, as well as activation of the complement cascade. Uniquely, mast cells are among the few extrahepatic cell types capable of producing both classical (C1q/r/s, C2, C4) and alternative (C3) complement components. Mast cell-secreted products such as tryptase, chymase and various other zymogens serve as activators for these complement pro-enzymes. Additionally, mast cells express receptors for complement fragments C3a and C5a, which further stimulate chemotaxis and degranulation (196).

Consequently, we postulated that the CRSwNP 1 strain may reduce mast cell activation due to the presence of *splB,* a potent complement inhibitor, which could diminish bacterial cell death, decrease degranulation, and reduce the release of bactericidal products, potentially contributing to the increased internalisation observed with this strain. Contrary to our expectations, both the Control 1 and CRSwNP 1 strains induced

comparable levels of β-hexosaminidase release at each timepoint, reaching a peak of 20.7% net release. Intriguingly, β-hexosaminidase release did not mirror that of LDH release, which would typically correspond to cellular death or high levels of activation. A possible explanation for this divergence could be the capacity of both *S. aureus* strains to circumvent traditional mast cell activation and subsequent degranulation. Alternatively, β-hexosaminidase production may have been suppressed as a consequence of bacteria-induced autophagy. In such a scenario, the *S. aureus* bacteria could hijack cellular signalling substrates necessary for both degranulation and β-hexosaminidase release. This phenomenon has been observed in HeLa cells infected with USA300 *S. aureus*, where bacterial invasion prompts starvation responses mediated by AMP–activated protein kinase and extracellular signal-regulated kinase (ERK) pathways, leading to autophagy that provides nutrients to support bacterial growth and replication (197).

Our data indicate that LAD2 cells remain largely unactivated following infection, with the observed modest increase in β-hexosaminidase release potentially resulting from gradual cell death rather than degranulation in response to the pathogen. Giemsa staining of LAD2 cells revealed a higher presence of CRSwNP 1-associated *S. aureus* within the cytoplasm compared to the Control 1 strain. However, interpreting cytospin preparations can be challenging; distinguishing definitively between internalised bacteria and those in the docking phase at the cell surface can be difficult. To address this limitation, we employed three-dimensional confocal microscopy and noted that almost twice as many mast cells were infected with CRSwNP 1 *S. aureus* compared to Control 1 *S. aureus*. Notably, many of the apoptotic mast cell bodies were filled with bacteria. Yet, it is important to note that these differences did not reach statistical significance, likely due to the small number of replicates used in the study.

4.4 Summary

This chapter reveals that the CRSwNP 1 *S. aureus* strain has a notably enhanced capacity for internalisation and replication within mast cells compared to the Control 1 strain. Despite this, the level of LDH release was similar between both strains, indicating a comparable propensity to elicit cell death. Interestingly, we did not observe mast cell activation as would be indicated by increased β-hexosaminidase release in response to either *S. aureus* strain. Advanced imaging techniques confirmed internalisation by both isolates; yet the CRSwNP 1 strain showed a tendency to infect a larger number of cells. It also seemed to exploit apoptotic cell bodies as a strategy to circumvent lysostaphin-mediated extracellular killing more effectively than the Control 1 strain. A deeper comprehension of the bacterial internalisation mechanisms highlighted by our study can potentially contribute to the development of innovative therapeutic interventions. Such strategies could specifically target internalising bacteria, aiming to diminish the infective load and enhance clinical outcomes for patients with CRS.

Chapter 5 **Pleiotropic and antistaphylococcal activity of simvastatin**

5.1 Introduction

As detailed in preceding chapters, both a representative control and CRSwNP-related *S. aureus* strain - internalised within mast cells. Intracellular *S. aureus* tends to be difficult to treat as the mammalian cell wall prevents diffusion of many commonly used antibiotics (85). Furthermore intracellular *S. aureus* often forms SCVs, reducing their metabolism and increasing cell wall thickness. As a consequence, anti-metabolic antibiotics show little effect on these resistant variants (170).

In CRS, *S. aureus* appears to exist extracellularly and can alter phenotype to behave as an intracellular SCV within epithelial and mast cells of the nasal mucosa (75, 86). Interestingly, both *S. aureus* cultured from antibiotic-treated tissue and nasal swabs of the middle meatus demonstrate identical genotypes, suggesting the extracellular bacteria can switch phenotype to thrive within cells (86). Furthermore, a significant association has been observed between the presence of intracellular *S. aureus* in the nasal mucosa and the need for repeat endoscopic sinus surgery, with patients harbouring intracellular *S. aureus* at a higher risk of requiring additional surgery compared to those without intracellular *S. aureus* (85% vs 33%, P=0.0083) (3). Thus, intracellular *S. aureus* in CRS is commonly linked with recalcitrant disease and antibiotic resistance resulting in the need for multiple surgical procedures.

Lipophilic statins, such as simvastatin, atorvastatin, lovastatin and fluvastatin have the capacity to cross cell membranes and have exhibited antistaphylococcal properties both outside and inside cells. In vitro studies have shown these statins to be active against *S. aureus* at various concentrations, with simvastatin demonstrating particularly potent

activity characterised by the lowest observed minimal inhibitory concentration (MIC) ranging from 16 to 63 mg/L. However, it is important to note that the MICs required for antistaphylococcal efficacy in these experiments – ranging from 10 to 500 mg/L - far exceeds the serum levels typically achieved through oral administration of these drugs, which range from 8 to 13 µg/L (198, 199).

Despite in vitro MIC levels suggesting limited direct antimicrobial activity within the therapeutic range of oral administration, statins have been linked to a significant reduction in the risk and mortality associated with severe sepsis, an effect attributed to their pleiotropic properties (200-202). Furthermore, recent large-scale epidemiological studies have indicated that statin use may contribute to a decreased incidence of CRS. In a retrospective analysis of 10,965 patients, Gilani *et al.* reported that statin use was associated with a reduced OR for CRS diagnosis, estimated at 0.716 (95% CI 0.612-0.838) (5). In an even more extensive evaluation of over 10 million patient visit records from the National Ambulatory Medical Survey of North America, Wilson *et al.* observed similar trends. Their findings indicated a reduction in risk for CRS, with an OR of 0.53 ($P<0.001$) on univariate logistic regression analysis and 0.79 ($P=0.03$) on multivariate analysis (6).

In vivo research has shown that topical simvastatin treatment of wounds infected with Methicillin-resistant Staphylococcus aureus (MRSA) not only reduces the bacterial load but also markedly improves wound healing. This improvement is accompanied by decreased concentrations of pro-inflammatory cytokines including IL-6, TNF-α and IL-1β (132, 133). Statins are known to mediate their therapeutic effects by inhibiting the mevalonate pathway, which is critical for isoprenoid synthesis in both human and bacterial cells, including *Staphylococcus*. This inhibition occurs via the target enzyme 3-

hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase), ultimately leading to reduced synthesis of cholesterol and the isoprenoids required for post-translational modification of proteins (134). When studied in vitro, lipophilic statins have been observed to decrease cholesterol production, directly influencing bacterial growth and protein synthesis by impairing the prenylation of signalling proteins (132, 135). In mammalian host cells, statins can lower the abundance of cholesterol within lipid rafts, key areas involved in bacterial internalisation and subsequent pro-inflammatory responses. This increases the energy cost in transporting bacteria across the cell membrane, reducing bacterial internalisation. Furthermore, statins appear to modulate mast cell signalling, reducing degranulation in response to IgE-dependant stimuli and offer protection against bacterial toxin effects (83, 136-139).

Simvastatin is one of the most commonly used statins with a widely understood side-effect profile and has demonstrated multiple antibiotic effects when directly applied to bacteria. Even the most virulent of *Staphylococcus* strains, including methicillin- and vancomycin-resistant strains, demonstrate sensitivity towards simvastatin (132, 134, 203). Given the above findings, we postulated whether simvastatin, with a low MIC, well-characterised pharmacokinetics, and low cost, might reduce the number of intracellular *S. aureus* within mast and epithelial cells. This could represent an exciting opportunity to develop a novel targeted therapy against intracellular *S. aureus* in patients with *S. aureus*-related chronic inflammatory conditions such as CRS.

5.2 Results

5.2.1 Minimal inhibitory concentration of prodrug simvastatin for CRSwNP *S. aureus*

To evaluate the concentration of simvastatin needed to inhibit the growth of CRSwNP *S. aureus* we performed an MIC calculation in accordance with the internationally agreed method (ISO 20776-1:2019 standard) utilising the CRSwNP 1 *S. aureus* strain (75, 146). Our results indicate an MIC of between 25-50 µmol/L (Figure 5-1A). We further refined this MIC to 40 µmol/L (Figure 5-1B). Simvastatin had to be dissolved in DMSO, which has been shown to inhibit biofilm formation and bacterial growth. To ensure DMSO was not contributing to the antistaphylococcal effect observed, we performed a control experiment which demonstrated no significant bactericidal effects between concentrations of 0 and 0.418% v/v DMSO, which was utilised to dissolve 100 µmol/L of simvastatin (Figure 5-1C).

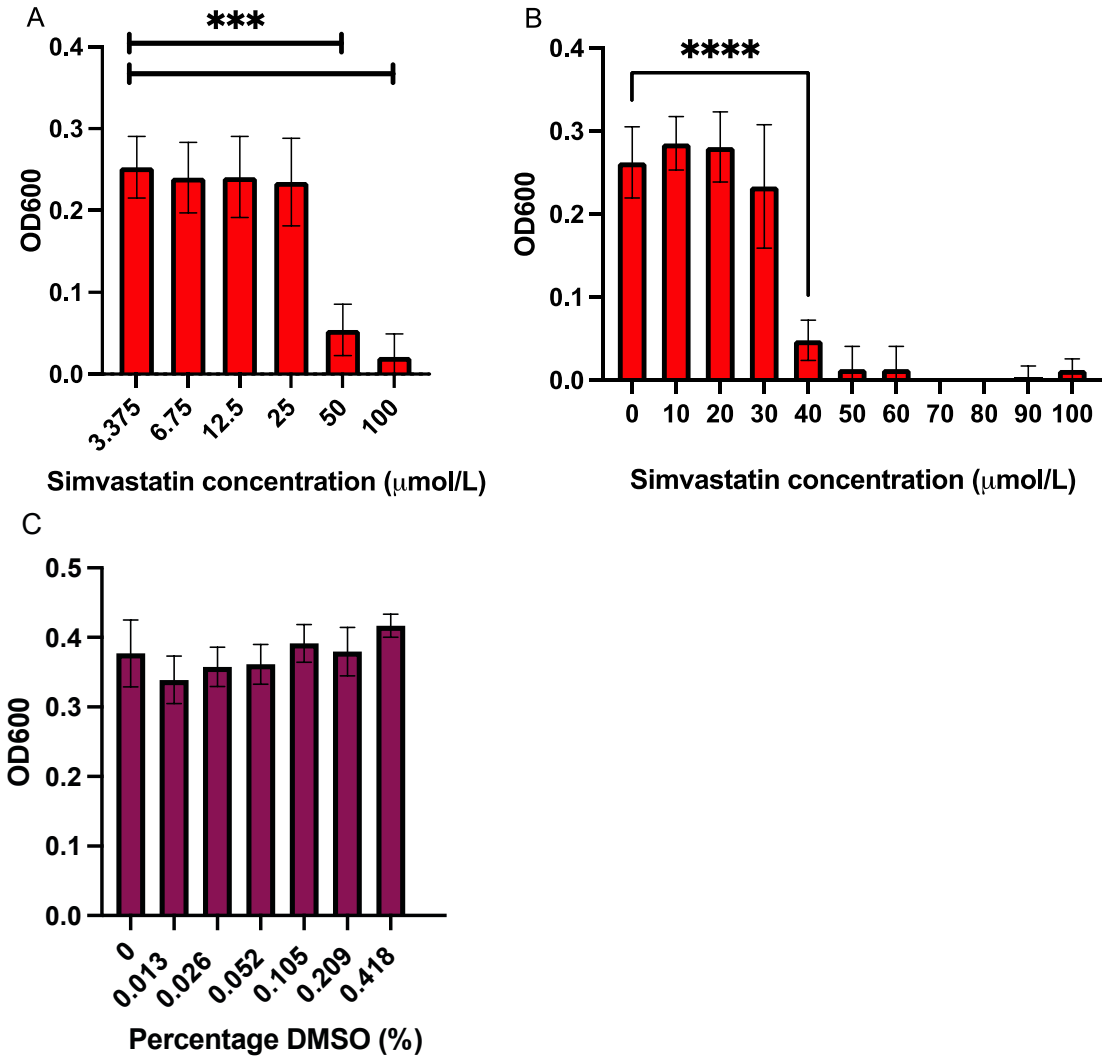


Figure 5-1 Minimal inhibitory concentration of prodrug simvastatin

Serial dilutions of simvastatin (**A+B**) and DMSO (**C**) were created, and an inoculum of 1 x 10^5 CFU of the CRSwNP 1 strain was added to each well to create a total volume of 200 µL. Plates were incubated at 37°C for 16 hours and then analysed using a plate spectrophotometer at 600nm to determine bacterial density. **A**) The results demonstrate an initial MIC of 25-50 µmol/L. **B**) A refined MIC of 40 µmol/L is calculated. **C**) Experimental DMSO concentrations present in figure used to dissolve simvastatin. DMSO at concentrations of 0.013, 0.026, 0.052, 0.105, 0.209, 0.418 v/v were used to dissolve 3.375, 6.75, 15.5, 25, 50, 100 µmol/L simvastatin, respectively. Statistical analysis was performed utilising one-way ANOVA and Tukey's multiple comparison test (for each graph, nine experimental repeats were completed; bars represent mean and standard deviation is represented by error bars. (***P≤0.001 ****P≤0.0001)).

5.2.2 Minimal inhibitory concentration of activated simvastatin for CRSwNP *S. aureus*

Most simvastatin is present in the serum in the inactive prodrug form (68-77%), with 95% bound to proteins. However, a proportion is available in the hydroxylated “active form”, simvastatin, hydroxy acid. Several mechanisms have been proposed to explain how statins exert their antibacterial effects, but few studies have utilised the activated form of simvastatin (199). Therefore, we questioned whether active simvastatin would have a lower MIC than the prodrug form. Simvastatin was converted to the active form using the methods described by McKay *et al*. (145). Activated simvastatin demonstrated no antimicrobial activity against the CRSwNP 1 strain of *S. aureus* (Figure 5-2A,B).

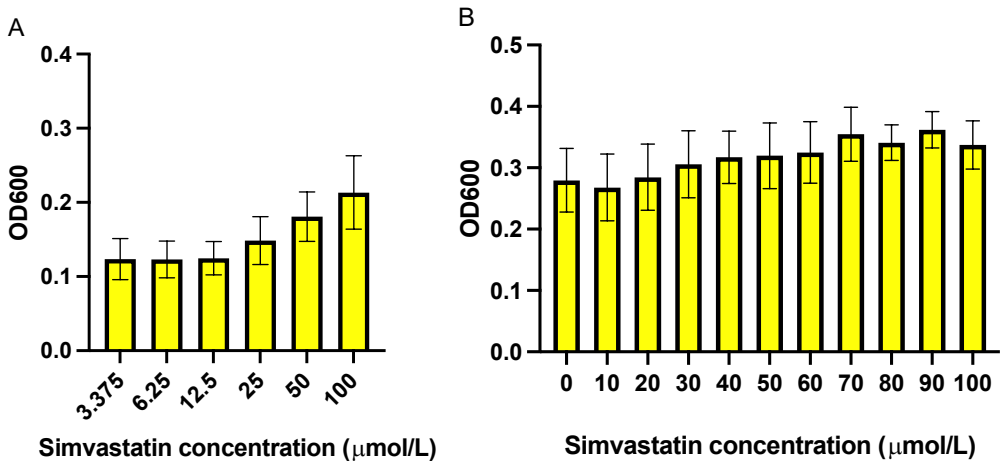


Figure 5-2 Minimal inhibitory concentration of activated simvastatin

Serial dilutions of activated simvastatin were prepared and an inoculum of 1 x 10^5 CFU of the CRSwNP 1 strain was added to achieve a total volume of 200 µL. Plates were incubated at 37°C for 16 hours then analysed using a plate spectrophotometer at 600nm to ascertain bacterial density. **A+B**) The results showed no significant inhibition of *S. aureus* growth within the 0 -100 µmol/L range of simvastatin. Statistical analyses were conducted using one-way ANOVA and Tukey's multiple comparison test. Nine experimental repeats were conducted for each condition. Bars represent mean value, with standard deviation represented by error bars.

5.2.3 Simvastatin-treated LAD2 intracellular protection assay using typical serum concentrations

The concentrations of simvastatin used above were significantly higher than those observed in human serum. Despite this, we considered whether simvastatin would exert activity at these concentrations in co-culture with mast cells, as the hydrophobic and lipophilic nature of simvastatin might favour localisation to the cell membrane and result in an increased concentration in this vicinity. It has been proposed that statins can stabilise lipid rafts, which permit entry of bacteria, and inhibit downstream signalling of bacterial toxins (198). To ascertain if there was any difference in intracellular *S .aureus* survival at serum concentrations, we pre-treated LAD2 mast cells for 16 hours with simvastatin concentrations typically observed in the human serum of patients taking the drug orally [19-31 nmol/L (199)] and inoculated them with the CRSwNP 1 strain of *S. aureus* at an MOI of 1. The results showed no difference in intracellular survival between simvastatin concentrations from 0-40 nmol/L (Figure 5-3).

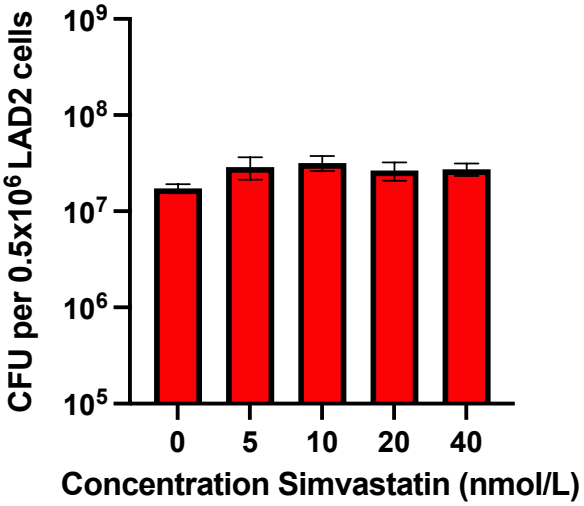


Figure 5-3 Simvastatin treated LAD2 cells and recovery of intracellular CFUs after S. aureus infection

LAD2 cells were pre-treated with typical serum concentrations of simvastatin for 16 hours and co-cultured with CRSwNP 1 *S. aureus*. No significant difference in intracellular survival was observed at simvastatin concentrations ranging from 0 to 40 nmol/L. Statistical analysis conducted using a one-way ANOVA revealed no significant differences. Eighteen experimental repeats were completed; bars indicate the mean value, with error bars representing the standard error of the mean.

5.2.4 Simvastatin-treated LAD2 intracellular protection assay using simvastatin concentrations above typical serum concentrations

Given that simvastatin did not demonstrate any reduction in intracellular survival at concentrations recorded in serum, we increased the concentration of simvastatin to doses shown to inhibit intracellular growth by Horne *et al.* and Thangamani *et al.,* while simultaneously assessing cellular toxicity (132, 204). There was a sequential reduction in intracellular survival of the CRSwNP 1 strain from 2.1×10^6 to 5.1×10^3 CFU at simvastatin concentrations ranging from 1 to 100 µmol/L after 6 hours, and this reduction was significant at simvastatin concentrations between 10-100 µmol/L (Figure 5-4A). To determine if simvastatin had any protective effect against cytotoxicity, we performed a LDH assay on the supernatant. We identified that increasing concentrations of simvastatin up to 30 µmol/L appeared to result in a serial reduction in LDH release, suggesting a protective effect. However, higher concentrations led to increases in LDH release, suggesting toxicity, although these results failed to reach significance (Figure 5-4B). To ensure that these results were not simply attributable to simvastatin-inducing cellular toxicity, we performed a parallel experiment to assess LDH release from uninfected LAD2 cells cultured with simvastatin for 6 hours. We observed no significant difference in cellular toxicity at concentrations below 30 µmol/L, suggesting that the reduction in intracellular survival was due to the effects of simvastatin in reducing the internalisation of CRSwNP 1 *S. aureus,* rather than a reduction in the cell number capable of supporting intracellular *S. aureus* survival (Figure 5-4C). However, LDH release was unexpectedly high at all concentrations, leading us to suspect that the cell handling methods employed may have caused significant cell stress.

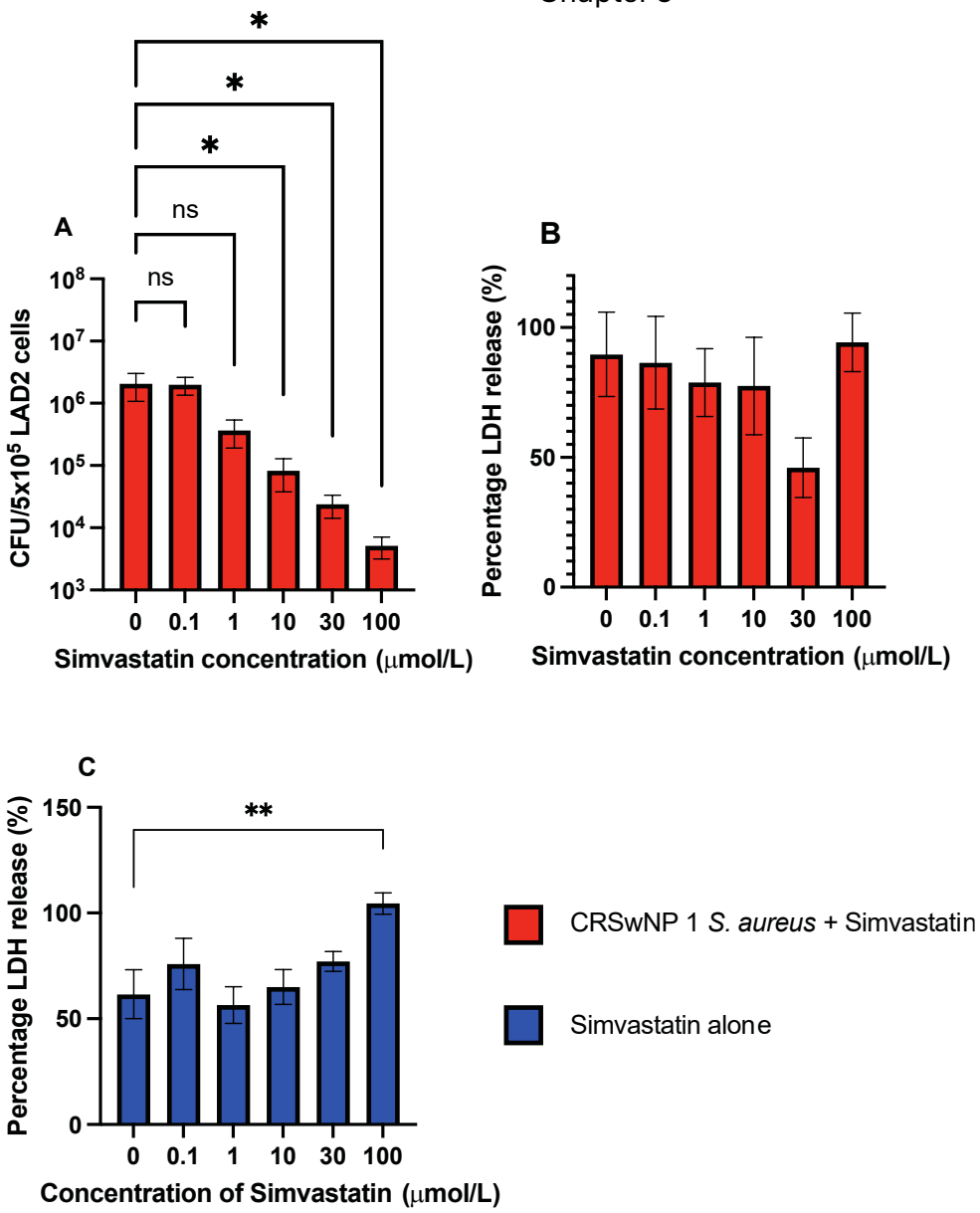


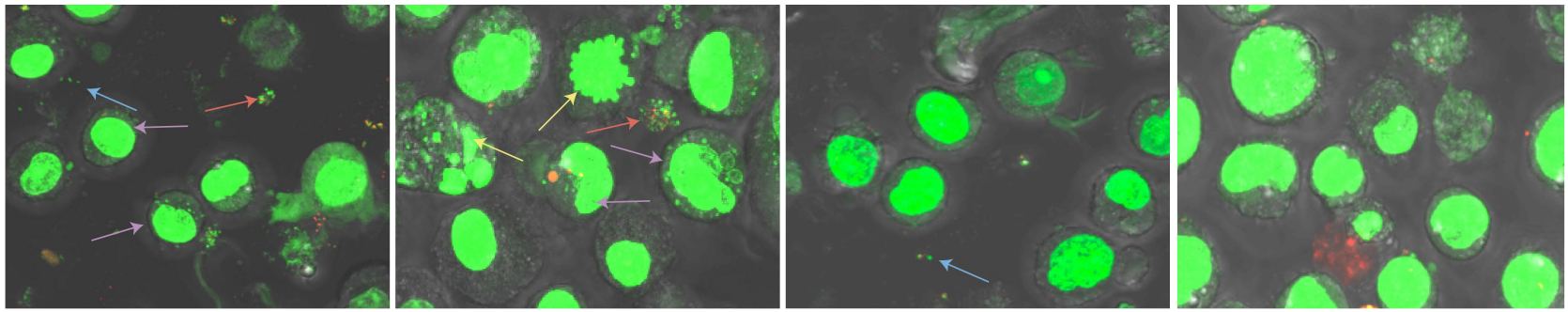
Figure 5-4 Intracellular survival of CRSwNP S. aureus at various concentrations of simvastatin

Mean intracellular survival of CRSwNP 1 *S. aureus* in co-culture with LAD2 cells pre-treated with simvastatin for 16 hours.
A) Intracellular CRSwNP 1 *S. aureus* survival in 5 x 10^5 LAD2 cell co-cultures, cultured at an MOI of 1 and treated with simvastatin at concentrations ranging between 0 and 100 µmol/L. Bars represent the mean of nine experimental repeats, with the standard error of the mean demonstrated by error bars. One-way ANOVA and Dunnett's multiple comparison test used for statistical analysis (*P≤0.05) **B)** LDH release from infected cells treated with simvastatin concentrations ranging between 0 to 100 µmol/L. The mean is represented by bars, with the standard error of the mean depicted by error bars. One-way ANOVA and Dunnett's multiple comparison test revealed no significant difference **C)** LDH release from LAD2 cells in response to various simvastatin concentrations ranging from 0 to 100 µmol/L. Nine experimental repeats were conducted, with bars representing the mean and error bars indicating the standard error of the mean. Multiple comparisons between the groups performed using one-way ANOVA and Dunnett's multiple comparison test (**P≤0.01).

5.2.5 Confocal microscopy of LAD2 simvastatin co-culture with CRSwNP *S. aureus*

To corroborate the above findings using an alternative technique and determine if the reduction in intracellular CFUs was attributable to an increased number of infected mast cells, we employed confocal microscopy and BacLight™ LIVE/DEAD™ imaging across varying concentrations of simvastatin. Our results indicate that at simvastatin concentrations of 0 and 1 µmol/L, 32.8-33.9% of LAD2 cells were infected, while at concentrations of 30 and 50 µmol/L, only 15.3-17.1% of cells were infected ($P \leq 0.01$). Consequently, we concluded that simvastatin conferred protection to LAD2 cells against intracellular CRSwNP 1 *S. aureus* infection (Figure 5-5).

A



Simvastatin Concentration	0 µmol/L	1 µmol/L	30 µmol/L	50 µmol/L
% cells infected	32.8%	33.9%	17.1%	15.3%

B

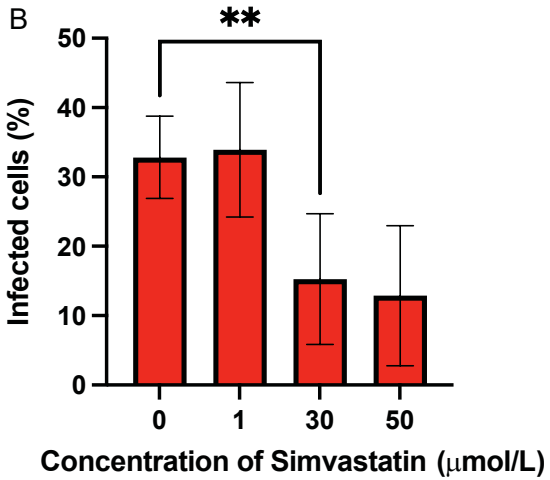


Figure 5-5 LAD2 infection with CRSwNP S. aureus at various simvastatin concentrations

LAD2 cells were pre-treated with simvastatin for 16 hours at concentrations of 0, 1, 30 and 50 µmol/L and subsequently cultured with CRSwNP 1 *S. aureus* for 6 hours, followed by staining with BacLight™ LIVE/DEAD™ stain. **A)** Representative confocal z-stacks of each experimental condition are displayed. The percentage of infected cells was calculated from 6 separate z-stack images, each containing between 74-152 cells. **Purple** arrows indicate infected cells, **blue** arrows highlight extracellular bacteria, **yellow** arrows denote apoptotic cells, and **red** arrows show infected apoptotic cell bodies. **B)** A graphical representation of the percentage of infected cells is provided. Bars represent the mean number of cells infected, with standard deviation denoted by error bars. Statistical analyses were performed using one-way ANOVA and Tukey's multiple comparison test (**P≤0.01).

5.2.6 Effect of simvastatin treatment on pro-inflammatory cytokine expression in LAD2 cells

In light of the observed reduction in intracellular infection, we investigated whether simvastatin could attenuate the production of pro-inflammatory cytokines associated with CRSwNP *S. aureus* infection in mast cells. To this end, we cultured 1 x 10^6 LAD2 cells with or without 30 µmol/L simvastatin and subjected each group to control conditions or infection with CRSwNP 1 *S. aureus* at an MOI of 1. Housekeeping genes beta-2-microglobulin (*B2M*) and actin-beta (*ACTB*) were utilised to confirm consistent expression levels across all experimental groups. We assessed gene expression of *IL5*, *IL6*, granulocyte-macrophage colony-stimulating factor (*GMCSF*) and tumour necrosis factor alpha (*TNF*). While housekeeping genes were robustly expressed, there was a notably low expression of the *IL5* gene and an even poorer expression of the *IL6*, *GMCSF* and *TNF* genes (Table 5-1). Given the stable cycle threshold (Ct) observed for the housekeeping genes and the expression of genes of interest around the 37 Ct cycle threshold set on the analyser, we concluded that the LAD2 cell line was unlikely to express significant levels of these genes. This was potentially due to multiple mutations that had accumulated over time as the cell line grew and matured. Therefore, we recommenced the culture with a new batch of LAD2 cells and obtained similar results. We concluded that the LAD2 culture produced negligible levels of the cytokines of interest, which was likely attributable to their deteriorating cell signalling network as the cells reach their 20th year since isolation (190).

Table 5-1 Cytokine gene expression of LAD2 cells exposed to CRSwNP S. aureus in the presence and absence of simvastatin

	ACTB	*B2M*	*IL5*	*IL6*	*GMCSF*	*TNF*
Control 1	20.667	21.836	N/A	N/A	N/A	N/A
Control 2	22.094	22.439	34.865	N/A	N/A	34.995
Control 3	21.698	21.899	34.158	N/A	N/A	N/A
Control 4	21.599	21.568	33.802	N/A	N/A	N/A
Control 5	22.601	21.796	33.682	N/A	N/A	36.983
Control 6	21.611	21.591	34.649	N/A	N/A	N/A
Control 7	22.326	22.282	35.139	N/A	N/A	35.145
S. aureus 1	23.115	22.397	N/A	N/A	N/A	35.470
S. aureus 2	22.295	22.105	34.926	N/A	N/A	34.235
S. aureus 3	22.365	22.281	33.519	N/A	N/A	33.456
S. aureus 4	22.470	22.639	36.052	N/A	N/A	34.872
S. aureus 5	22.130	22.371	N/A	N/A	N/A	35.315
S. aureus 6	22.196	22.512	36.052	N/A	N/A	36.182
S. aureus 7	21.794	21.957	34.881	N/A	N/A	N/A
Treatment 1	22.202	22.633	35.335	N/A	N/A	36.485
Treatment 2	21.842	22.156	35.074	N/A	N/A	N/A
Treatment 3	21.911	22.340	N/A	N/A	N/A	N/A
Treatment 4	22.556	22.208	N/A	N/A	N/A	N/A
Treatment 5	22.580	22.477	35.217	N/A	N/A	N/A
Treatment 6	22.831	22.348	34.200	N/A	N/A	N/A
Treatment 7	22.958	21.997	N/A	N/A	N/A	N/A

Quantitative RT-PCR cycle threshold (Ct) outputs for actin beta (*ACTB*), beta-2-microglobulin (*B2M*), interleukin 5 (*IL5*), interleukin 6 (*IL6*), granulocyte-macrophage colony-stimulating factor (*GMCSF*) and tumour necrosis factor alpha (*TNF*) genes in 1 x 10^6 LAD2 cells cultured for 6 hours under three experimental conditions: in the absence of bacteria (Control), with 1 x 10^6 CRSwNP 1 *S. aureus* (*S. aureus* group) and with 1 x 10^6 CRSwNP 1 *S. aureus* in the presence of 30 µmol/L simvastatin (Treatment group). N/A indicates no detectable signal below the maximum cycle threshold setting of 37.

5.2.7 Simvastatin effect on mast cell degranulation

Substance P has been found at significantly higher concentrations in the nasal lavage of patients with CRSwNP and aspirin-exacerbated respiratory disease (AERD). Growing evidence suggests that substance P plays a role in the formation of nasal polyps, nasal allergies and sinogenic pain. This neuropeptide is classically released from trigeminal nerve's unmyelinated afferent C fibres, located in the nasal mucosa near the blood vessels of the lamina propria. It has also been shown to be released by macrophages and eosinophils, particularly in response to bacterial lipopolysaccharide (205-207). Mast cells are among the primary responders to substance P, through the Mas-related G protein-coupled receptor X2 (MRGPRX2) receptor, exhibiting both chemotaxis and degranulation in response. This significantly contributes to the Th2-type inflammation observed in atopic dermatitis (208). Given these findings, we aimed to investigate whether simvastatin plays a role in attenuating intracellular signalling downstream of the MRGPRX2 receptor and subsequent degranulation, potentially contributing to the reduced prevalence of CRS in patients taking statins.

To assess whether simvastatin might interfere with signalling related to substance P and affect degranulation, we investigated the effects of substance P and compound 48/80 (C48/80, a potent MRGPRX2 activator) on LAD2 cells, with varied simvastatin concentrations and incubation durations. To ascertain that simvastatin alone was not responsible for degranulation, we first tested the effect of simvastatin on LAD2 cells in isolation. Our observations indicated that simvastatin alone exerted no significant effect on degranulation at concentrations from 0.1 to 10 μmol/L. However, at a concentration of 30 μmol/L, a significant increase in β-hexosaminidase release was noted (14.5% and 8.5% respectively) at 30 minutes and 2 hours, suggesting that degranulation is likely due

to toxicity at this elevated concentration (P≤0.001) (Figure 5-6A). Aside from this finding, there was no apparent induction of degranulation by simvastatin.

Cells incubated with simvastatin for 0.5, 1 and 2 hours and stimulated with 1 µmol/L substance P displayed no significant variation in degree of degranulation at simvastatin concentrations of 0 to 10 µmol/L. Elevated concentrations of simvastatin led to a gradual increase in degranulation when exposed to 1 µmol/L substance P, which was significant at 30 µmol/L compared to the absence of simvastatin (Figure 5-6B). Moreover, LAD2 cells stimulated with C48/80 demonstrated no significant change in degranulation when exposed to increased pre-incubation times or doses of simvastatin (Figure 5-6C).

To examine whether prolonged pre-incubation with simvastatin could affect MRGPRX2-mediated degranulation more significantly, we incubated LAD2 cells with 10 µmol/L simvastatin for 24 hours and assessed their response to various doses of substance P (Figure 5-7A) and C48/80 (Figure 5-7B). As anticipated, higher concentrations of substance P and C48/80 resulted in increased β-hexosaminidase release. No significant differences in β-hexosaminidase release were observed between the simvastatin-treated and untreated groups at any tested concentration. Cells exposed to C48/80 displayed significant differences at 0.1 µmol/L and 3 µmol/L upon C48/80 stimulation. However, this finding is likely to represent a Type 1 error due to the low number of replicates.

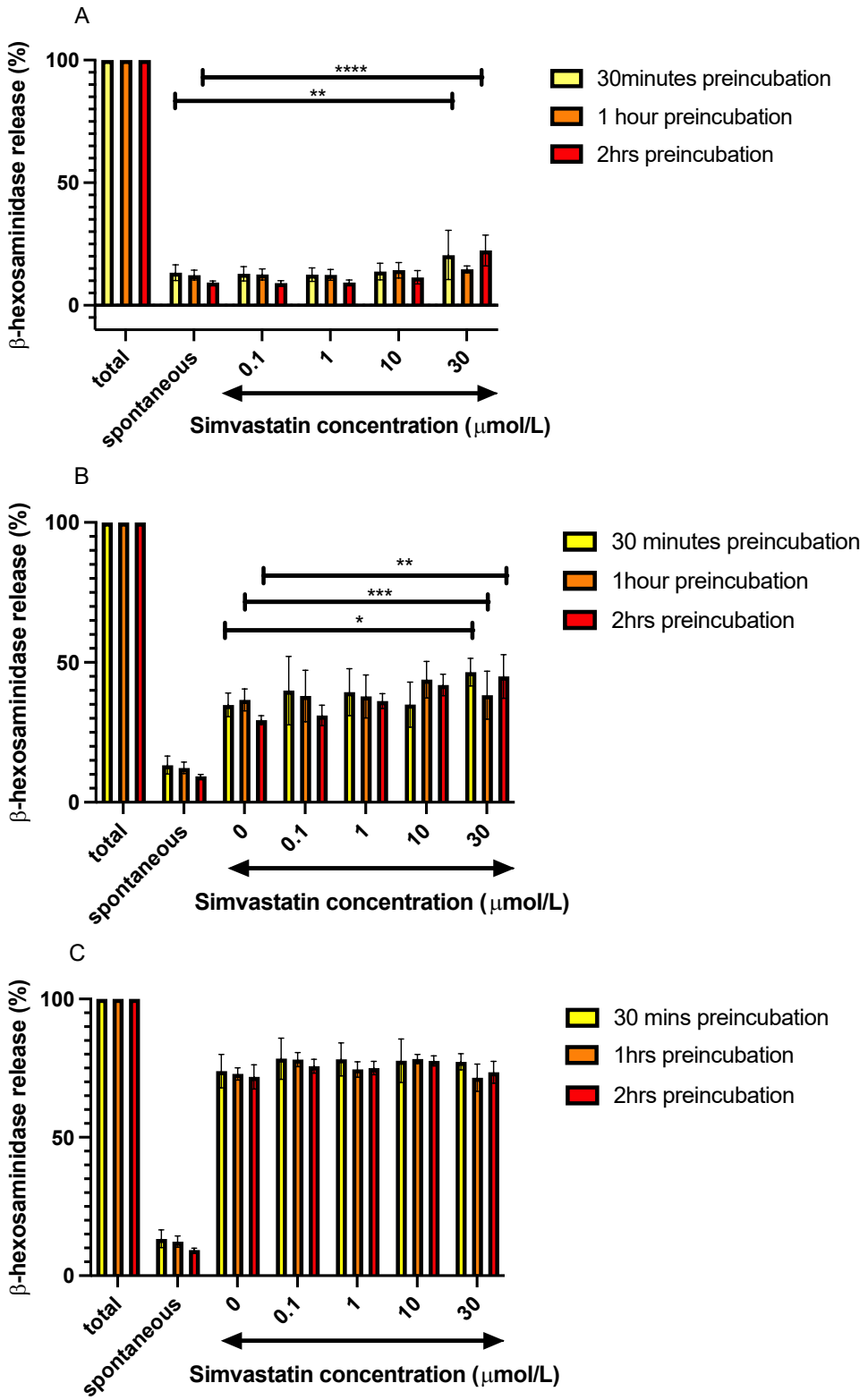


Figure 5-6 Effect of simvastatin treatment on mast cell degranulation

Effect of simvastatin treatment alone on β-hexosaminidase release in the absence of any stimulus **(A)** and following stimulation with substance P (10 μmol/L) **(B)** and compound 48/80 (3 μmol/L) **(C)** after 30 minutes, 1 hour and 2 hours of pre-incubation with various concentrations of simvastatin. Bars represent the mean of six experimental repeats with error bars indicating one standard deviation. Statistical analyses were carried out with two-way ANOVA and Tukey's multiple comparisons test (*P≤0.05, **P≤0.01, ***P≤0.001).

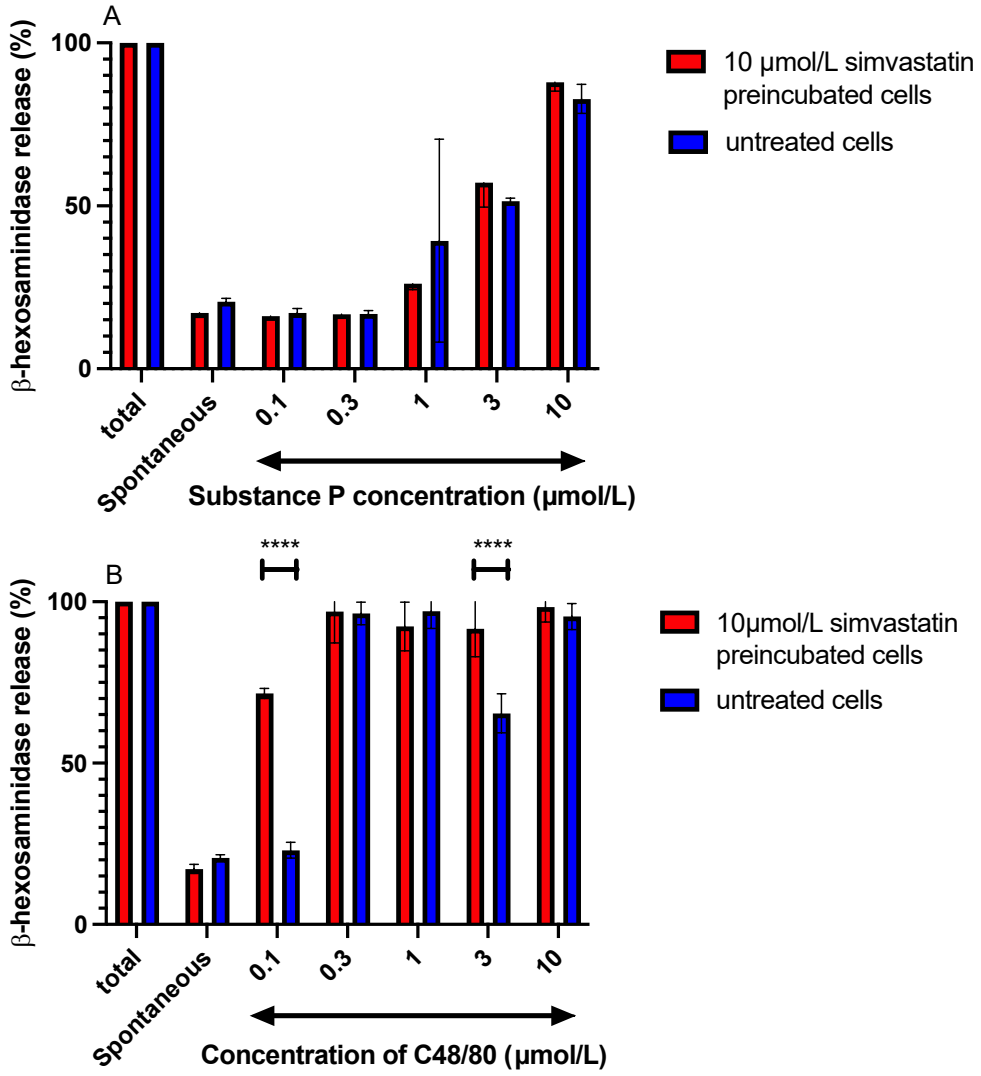


Figure 5-7 LAD2 cells pre-incubated with 10 µmol/L simvastatin for 24 hrs exposed to substance P and C48/80 at varying concentrations

Mast cells pre-incubated with 10 µmol/L simvastatin for 24 hours and stimulated with increasing concentrations of substance P (A) and C48/80 (B). Bars indicate the mean of three experimental repeats, with error bars representing one standard deviation. Statistical analyses were conducted using two-way ANOVA and Šidák's multiple comparisons test (****P≤0.0001).

5.2.8 Simvastatin effects on *S. aureus*-infected ALI trans-epithelial electrical resistance.

The presence of *S. aureus* within nasal epithelial cells at the time of surgery is associated with early disease recurrence and recalcitrant disease (3). In light of our findings in LAD2 cells, that simvastatin can induce a reduction in intracellularly viable bacteria, we sought to determine if it exhibits a similar capacity to reduce intracellular *S. aureus* within epithelial cells and whether it offers protection against bacterial damage. Our aim was to evaluate the effect of live *S. aureus* on air-liquid interface (ALI) culture trans-epithelial electrical resistance (TEER) in the presence of simvastatin. ALI cultures were derived from two control patients and one CRSwNP patient (Table 5-2). The apical surface was treated with four conditions: RPMI 1640 medium alone, inoculated with 5 x 10^5 CFUs of CRSwNP 1 *S. aureus*, exposed to 10 µmol/L simvastatin alone, and a combination of 5 x 10^5 CRSwNP 1 *S. aureus* and 10 µmol/L simvastatin for a duration of 6 hours. The TEER decreased by 0.76-fold in the control group and 0.72-fold in the simvastatin-treated group. For the *S. aureus*-infected and the group treated with both CRSwNP 1 *S. aureus* and simvastatin, the TEER fell by 0.49-fold and 0.55-fold, respectively. No significant differences were noted between the groups in terms of TEER change (Figure 5-8).

Table 5-2 Demographics of subjects from whom ALI cultures were derived

Primary nasal epithelial cell donor	1	2	3
Patient group	Control	Control	CRSwNP
Gender (M:F)	M	F	M
Age	59	65	26
Clinic review/ procedure	Septoplasty/PETS	Limited FESS for mucocele	Full house FESS
Antibiotic use in past month	No	No	Yes
Steroid use in past month	No	No	Yes
S. aureus culture	No	No	No
Airborne allergies	No	No	No
Asthma	Yes	No	No
Ex-smokers	No	No	No
Current smokers	No	No	No
Mean modified Lund-Mackay score	0	4	10

Demographics of subjects from whom primary nasal epithelial cells were obtained and subsequently cultured into mature air-liquid interface epithelial cells.

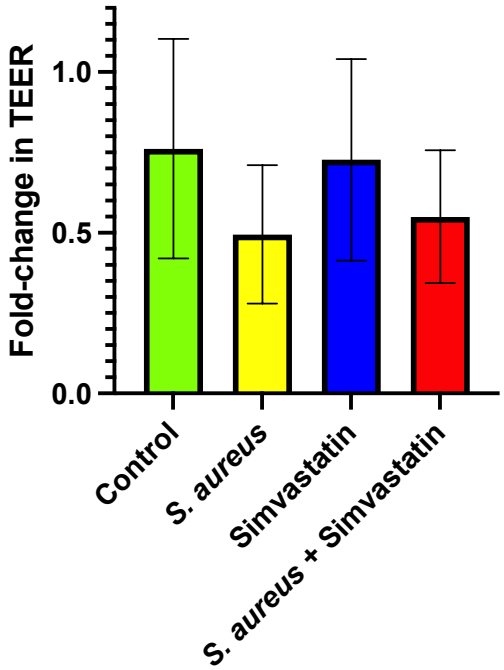


Figure 5-8 Trans-epithelial electrical resistance of ALI cultures exposed to CRSwNP 1 S. aureus and simvastatin

Trans-epithelial electrical resistance fold change from baseline to post-treatment after a 6-hour exposure to RPMI 1640 medium alone (green), 5 x 10^5 CRSwNP 1 *S. aureus* (yellow), 10 µmol/L simvastatin (blue) and a combination of 10 µmol/L simvastatin with 5 x 10^5 CRSwNP 1 *S. aureus* (red). Each condition was conducted nine times, with the mean of the results illustrated by a bar and error bars delineating one standard deviation. No significant variances were found utilising one-way ANOVA with Tukey's multiple comparisons test.

5.2.9 Simvastatin effects on *S. aureus* infected ALI structure and intracellular *S. aureus* presence

Following the finding of a reduction in TEER, we wished to examine the structure of the ALI and presence of CRSwNP 1 *S. aureus* in each treatment condition. We employed fluorescence in situ hybridisation (FISH) using a Cy3 fluorophore-tagged 16S ribosomal RNA probe, coupled with 4',6-diamidino-2-phenylindole (DAPI) staining of the cell nuclei, and imaged the specimens with a 40x objective lens. It was observed that the application of CRSwNP 1 *S. aureus* alone resulted in a film of bacteria forming over the ALI surface, which was discernible under the DAPI and Cy3 filters. However, due to the limited resolution of the detector, only aggregates of bacteria with sufficient signal intensity were visible on the epithelial surface; individual small bacteria could not be clearly distinguished. Intracellular bacteria were not detected within the primary epithelial cell ALI cultures. In ALI cultures treated with both simvastatin and CRSwNP 1 *S. aureus,* the epithelial layer surface appeared devoid of any bound *S. aureus* bacteria. The control ALI samples treated with RPMI 1640 maintained a healthy structure, with the absence of any observable *S. aureus* on the epithelial surface (Figure 5-9).

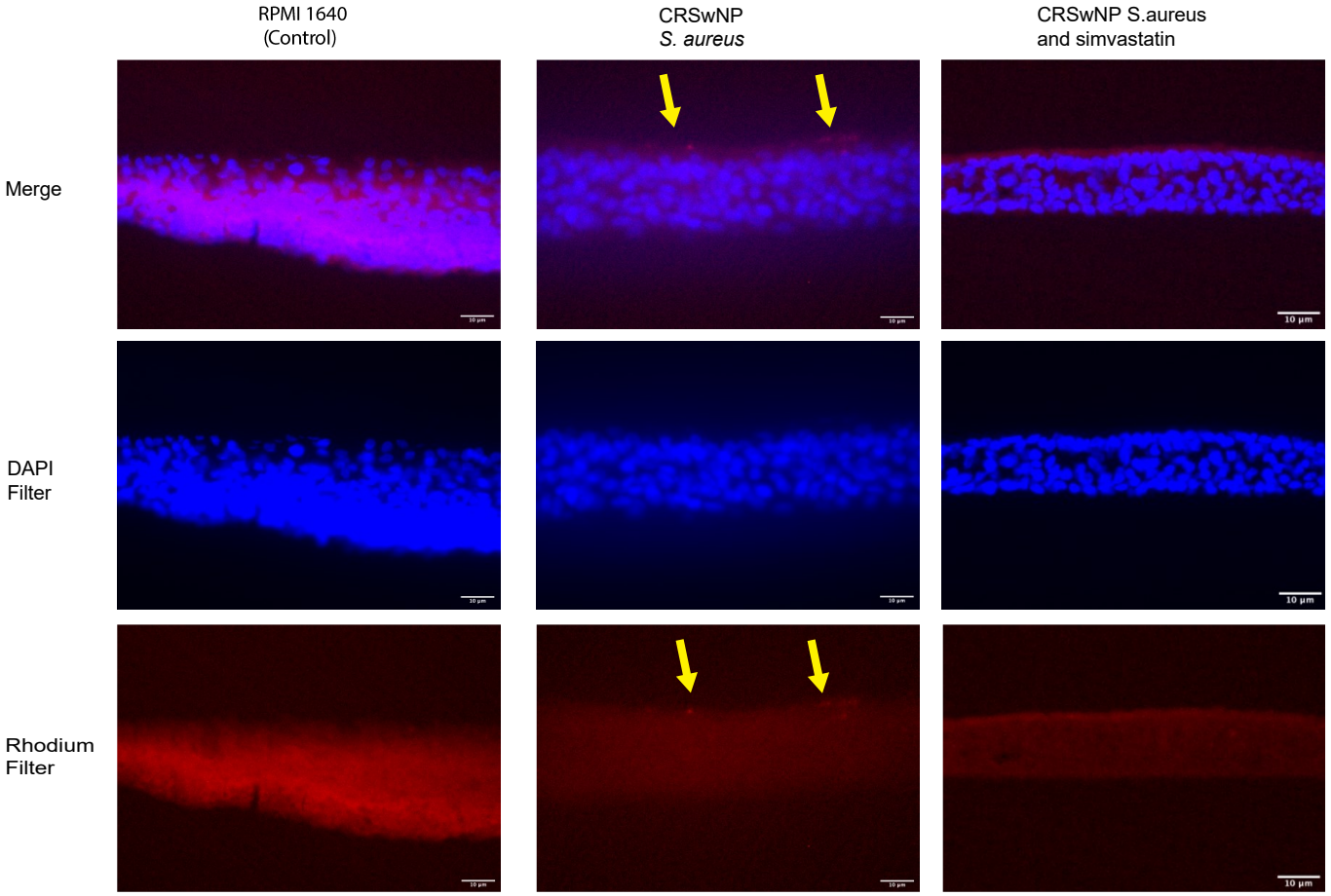


Figure 5-9 16S Fluorescence In Situ Hybridisation of ALI treated with CRSwNP 1 S. aureus and simvastatin

Light microscopy of ALI cultures following treatment with RPMI 1640 (control), 5 x 10^5 CRSwNP 1 *S. aureus* and a combination of CRSwNP 1 *S. aureus* with 10 µmol/L simvastatin). The cells were stained with DAPI to visualise the nucleus and probed with a ubiquitous 16S ribosomal RNA probe for bacterial detection. Merged images portray the overlay of DAPI and Cy3 signals, with separated channel displays provided below. A scale bar indicating a 10 µm reference is located in the bottom right of the images.

5.2.10 Expression of epithelial expressed cytokines in response to CRSwNP *S. aureus* and simvastatin treatment.

Infection of the epithelium by *S. aureus* has been linked to the induction of pro-inflammatory cytokine transcription. Considering the known antistaphylococcal and pleiotropic effects of simvastatin, we investigated whether it could modulate the production of pro-inflammatory cytokines. For this purpose, we pre-treated ALI cultures with 10 µmol/L simvastatin for 16 hours. The apical surface of simvastatin free and simvastatin pre-treated ALI cultures were then treated with RPMI 1640 or 5 x 10^5 CRSwNP 1 *S. aureus* for 6 hours. We used two-step quantitative real-time polymerase chain reaction (qRT-PCR) to assess the expression of pro-inflammatory cytokine genes in each condition. Our results showed a non-significant increase in the expression of the *TSLP, TNF and CXCL8* (*IL8*) genes, as well as a decrease in *IL6* and *IL33* RNA expression upon exposure to *S. aureus*. Simvastatin treatment alone tended to reduce the baseline RNA production of all the studied cytokines, although not significantly when compared to the controls. ALI cultures treated with both CRSwNP 1 *S. aureus* and simvastatin exhibited a non-significant decrease in gene expression for *TNF, CXCL8 and TSLP* (by 0.95, 0.79 and 0.69-fold respectively), but there was a minor but non-significant increase in *IL6 and IL33* expression (by 1.64, 1.13-fold respectively) when compared to treatment with *S. aureus* alone (Figure 5-10).

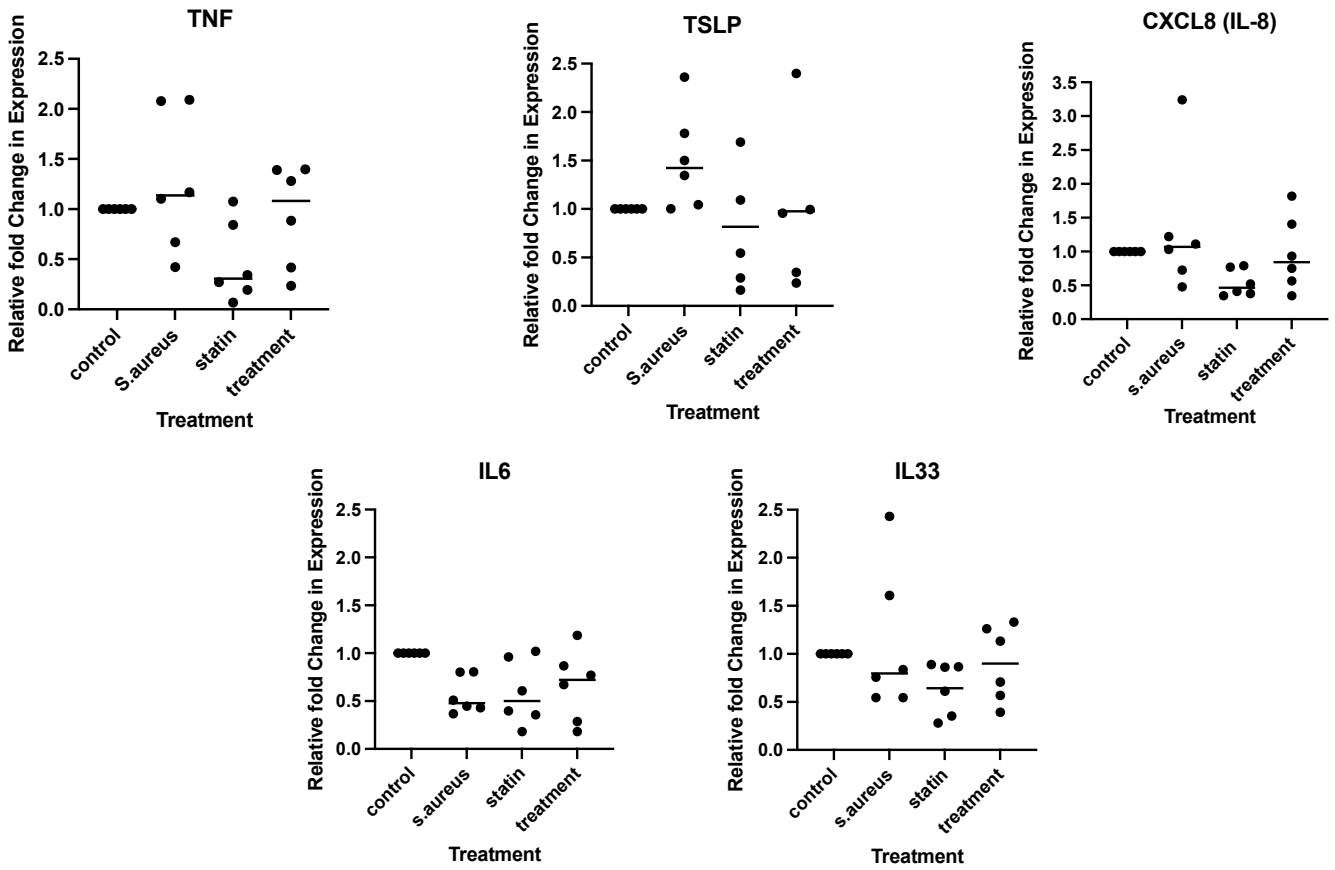


Figure 5-10 Quantitative RT-PCR of cytokine genes expressed in ALI epithelial cell cultures in response to S. aureus and simvastatin challenge

Relative fold-change in gene expression of cytokines (*TNF*, *TSLP*, *CXCL8* (*IL8*), *IL6 and IL33*) expressed by epithelial cells in response to a 6-hour treatment with RPMI 1640 (control), 5 x 10^5 CRSwNP 1 *S. aureus* (*S. aureus*), 10 µmol/L simvastatin (statin) and a combination of both (treatment). A total of six experimental repeats were performed for each condition. *B2M* was utilised as the reference gene, which displayed stable expression across all test conditions. The Δ ΔCT method was applied to calculate the relative changes in gene expression as compared to the control condition. Each bar on the graph represents the median of the values, with individual data points plotted. Statistical significance was determined using the Kruskal-Wallis test.

5.3 Discussion

Previous research has indicated that patients taking oral statin therapy exhibit a significantly reduced OR of being diagnosed with CRS (5, 6). However, the mechanisms underlying this antibacterial effect are not well understood, and it remains to be determined whether this could lead to the development of novel strategies for treating intracellular *S. aureus* in conditions such as CRS. To study these mechanisms further we used simvastatin, the most commonly prescribed statin, which demonstrates a lower MIC against *S. aureus,* in contrast to other statins (209). Past research has primarily utilised the prodrug form of simvastatin to determine the MIC. To our knowledge, there are no studies that have tested the activity of simvastatin's hydroxylated, active metabolite form as an antistaphylococcal agent (7, 132, 134, 198, 203, 210).

We determined that the prodrug form of simvastatin inhibits the growth of virulent CRSwNP 1 *S. aureus* isolate at an MIC below 40 µmol/L, which aligns with findings from other studies that reported typical MIC values ranging between 38.1 to 398 µmol/L (equivalent to 16-167 mg/L) (7, 132, 134, 198, 203, 210). However, these in vitro concentrations surpass those found in the serum of patients taking oral simvastatin, which typically ranges from 19 to 31 nmol/L (199). Contrary to expectations, we observed no direct antibacterial effect of the hydroxylated 'active' form of simvastatin against a CRSwNP-related *S. aureus* isolate. Although *S. aureus* possesses an HMG-COA reductase enzyme (*mvaA*) crucial for survival, and fluvastatin has been shown to inhibit this enzyme, the lack of effect from simvastatin was unanticipated. Interestingly, statins have been found to exert bactericidal effects with similar MICs in organisms lacking an HMG-CoA reductase enzyme (198). While oral simvastatin may not suffice as a nasal antistaphylococcal treatment due to subtherapeutic serum concentrations, alternative methods such as topical delivery could increase the local concentration in the nasal

passages and potentially harness simvastatin's antistaphylococcal properties to treat patients with *S. aureus*-related CRS.

Our prior research established that *S. aureus* can recruit mast cells to the subepithelial layer and survive within these cells, leading to the production of pro-inflammatory mediators and degranulation. This phenomenon was observed both in inferior turbinate nasal explant models and in the LAD2 mast cell line, with resulting recruitment of additional mast cells and other inflammatory cell types (75). Other studies have shown that simvastatin can decrease intracellular translocation and survival of *S. aureus* in HEK293A epithelial cells at concentrations ranging from 0.1 to 1 µmol/L. Intriguingly, this effect could be reversed by addition of HMG-CoA reductase products including mevalonate, geranylgeraniol and to a lesser extent, farnesyl pyrophosphate (204). For this reason, we studied simvastatin's effect on intracellular translocation in mast and epithelial cells.

In LAD2 mast cells pretreated with simvastatin for 16 hours, we found no effect of *S. aureus* intracellular presence at 6 hours with typical serum concentrations of simvastatin ranging from 1-40 nmol/L. However, we noted a significant reduction in intracellular *S. aureus* at simvastatin concentrations between 1-100 µmol/L. Subsequent analyses showed that simvastatin concentrations of 100 µmol/L were toxic to LAD2 cells. Interestingly, simvastatin appeared to have a protective effect, with a reduction in LDH release at progressively higher simvastatin concentrations from 1-30 µmol/L, although this difference was not statistically significant. Our data suggest that at concentrations beyond that observed in the serum, simvastatin can impede *S. aureus* translocation and intracellular survival in LAD2 cells in a concentration-dependent manner.

There are several potential explanations for the observed effects of simvastatin on intracellular *S. aureus* translocation and survival. One hypothesis is that simvastatin reduces lipid raft formation on LAD2 cells, reducing the translocation of *S. aureus*. Another possibility is that simvastatin impacts production of toxins by *S. aureus*. Simvastatin is known to inhibit the enzyme HMG-CoA reductase, the enzyme responsible for both mammalian and bacterial isoprenoid and cholesterol synthesis (198). Cholesterol is a vital constituent of lipid raft domains, which are areas of disorganised lipids which serve as docking areas for foreign material, including bacteria, thereby lowering the energy requirements associated with endocytosis (21). It has been shown that other organisms interact with and internalise through these domains, suggesting that *S. aureus* might employ a similar mechanism for entry into host cells (211).

Moreover, *S. aureus* also appears *to* activate small GTPases including CDC42 and Rac, which lead to the activation of p85 and PI3K, culminating in actin-mediated caveolae formation and endocytosis (212). Simvastatin could impede the association of these GTPases with the cell membrane by inhibiting their prenylation, resulting in their sequestration in the cytoplasm and effectively prevent *S. aureus* endocytosis (204, 213).

Other studies have highlighted the potential of simvastatin to reduce production of proteins, including *S. aureus* toxins such as Panton-Valentine leukocidin (PVL) and alpha-haemolysin, at similar concentrations to those we have tested (7). Alpha-haemolysin has been long implicated in facilitating intracellular translocation by aiding in the escape from phagosomes (188, 189).

Simvastatin has been shown to markedly reduce IgE-mediated release of IL-6, TNF-α and IL-13 release from mast cells (137). Similarly, fluvastatin, a closely related statin, has demonstrated a reduction in degranulation of β-hexosaminidase and histamine in

response to IgE stimulation. This effect is attributed to decreased phosphorylation of Protein Kinase B (PKB), extracellular signal-regulated kinase (ERK) and Rab2b in the rat basophilic leukaemia cell line RBL-2H3. It is believed that this reduction stems from reduced production of isoprenoid precursors essential for the prenylation of signalling molecules which aid localisation of downstream IgE components to the cell membrane (137, 214). The LAD2 cell line utilised in our laboratory expresses the high-affinity IgE receptor (FcεRI) but fails to degranulate on activation, suggesting a potential loss-of-function mutation in the activation pathway. Despite this, it seems to retain a functional MRGPRX2 receptor, which responds to substance P or C48/80 and shares similar signalling pathways involving PKB and ERK with IgE signalling pathways (215). We observed no significant effect of simvastatin on degranulation using substance P or C48/80, which was neither dependent on concentration nor pre-incubation time. This lack of effect could be due to additional redundant signalling pathways used by the MRGPRX2 receptor to stimulate degranulation, which may be less susceptible to disruption by statin treatment compared to the more statin sensitive IgE signalling pathways.

Patients with CRSsNP and CRSwNP are known to exhibit defective nasal epithelial barriers. This defect involves patchy and irregular expression of tight junction proteins including zonula occludens and occludin 1, which leads to the adaptive immune system being exposed to bacteria, enterotoxins, allergens and viruses (82). Studies have also indicated that the cell lysate of *S. aureus* can delay the healing of wounds in ALI cultured primary nasal epithelial cells, and it has been shown that the enterotoxin SEB can disrupt the formation of tight junctions, decreasing TEER due to activation of TLR2 (83, 120). Furthermore, CRS patients with nasal epithelial cells harbouring intracellular *S. aureus* tend to experience early disease recurrence and show a persistent, recalcitrant form of

the disease (86). We explored the impacts of CRSwNP-related *S. aureus* and simvastatin on patient-derived epithelial cells cultivated into ALI. We discerned no significant differences in TEER after 6 hours among the control, CRSwNP 1 *S. aureus* treated, simvastatin treated and the simvastatin and CRSwNP 1 *S. aureus* treated groups. The ALI cultures maintained a healthy mean cell depth of approximately 6-8 cells, and this did not appear to be impaired by any treatment. Visualisation of *S. aureus* was challenging; increasing magnification simultaneously reduced the focal depth through the specimen and decreased the observable number of *S. aureus*. Conversely, at lower magnifications, while the focal depth was broader allowing more *S. aureus* to be visible, the resolution suffered. A 40x objective lens emerged as the most effective for observing bacterial density on ALI cultures, although the images captured do not clearly reflect the image seen through the eyepiece, owing to limitations in detector pixel size. Regardless, pre-treatment with 10 µmol/L simvastatin followed by exposure to CRSwNP 1 *S. aureus* appeared to result in sparse presence of bacterial cells on the ALI epithelial surface. Intracellular *S. aureus* was not observed in any specimens, possibly due to the short exposure time. Extending incubation times resulted in widespread ALI death, rendering qRT-PCR unfeasible. Kiedrowski *et al.* reported similar results with Calu-3 ALI cultured epithelial cells, noting extensive cell death within 24 hours, likely induced by *S. aureus* alpha toxin (216). Our primary epithelial cells may have been more susceptible to damage than the immortalised Calu-3 line, rendering them more prone to apoptosis and necrosis. Moreover, quantifying the levels of alpha-toxin and other toxins secreted by our CRSwNP 1 *S. aureus* strain proved challenging.

We noted a non-significant increase in pro-inflammatory cytokine genes *TSLP, TNF* and *CXCL8,* along with a decrease in *IL6* gene expression, following exposure of the ALI culture to CRSwNP 1 *S. aureus*. Typically, IL-6 is a pleiotropic cytokine that signals

epithelial tissue damage. Nasal explant models challenged with *S. aureus* have exhibited elevated IL-6 release (72, 217). Murphy *et al.* reported a similar phenomenon in primary cultured nasal epithelial cells in which *S. aureus* conditioned media led to a reduction in IL-6 protein, an effect attributed to the direct cleavage of IL-6 by the *S. aureus* V8 protease. In contrast, our findings would hint at an alternative mechanism, considering that V8 protease was absent in our isolate. Furthermore, our isolate appeared to suppress the expression of the *IL6* gene in the ALI cultured cells. Given the crucial role of IL-6 in chemoattraction of monocytes and T cells, a diminution in this cytokine's expression could potentially weaken local immune responses at the epithelial surface (92, 218).

Statin treatment also appeared to reduce baseline expression of *TSLP, TNF, IL33, CXCL8* and *IL6* genes although these changes were not statistically significant. When ALI cultures, pre-incubated with simvastatin were exposed to CRSwNP1 *S. aureus* we observed a slight, non-significant decrease in expression of *TSLP, TNF* and *CXCL8* genes, along with a small increase in *IL6* and *IL33* expression.

In summarising our findings, the minimal alterations in relative expression, combined with the substantial variation among treatment groups, presented challenges. This complexity was further compounded by the heterogeneity inherent in the primary cultured nasal epithelial cells - sourced from two control individuals and one CRSwNP patient - and the limited number of replicates.

We theorise that primary nasal cultured epithelial cells in an ALI model lack the robustness of epithelial cells that are strengthened by a basement membrane and supporting cells. Such a setup is highly susceptible to damage from CRSwNP 1 *S. aureus*. This makes it difficult to titrate exposure time and bacterial density conditions that allow

ALI cultures to remain viable while still yielding observable cytokine responses and structural changes in the model.

Moreover, variations arising from the heterogeneity of our primary epithelial cell cultures likely influenced both bacterial-cell interactions and cytokine expression levels, introducing confounding factors. Consequently, we concluded that this model was not viable for our research purposes, given the substantial number of variables that hindered interpretation of our results.

5.4 Summary

In summary, at typical serum concentrations present in patients taking oral simvastatin therapy, we did not detect any significant antistaphylococcal effects for either the prodrug or activated simvastatin forms. When tested at higher concentrations, achievable through topical administration, prodrug simvastatin exhibited direct antistaphylococcal activity, whereas activated simvastatin demonstrated negligible effects. Treatment with simvastatin reduced the intracellular presence of *S. aureus* in mast cells and potentially the number of cells infected. However, simvastatin did not significantly impact MRGPRX2 signalling pathways, suggesting that it does not inhibit mast cell degranulation. We were unable to demonstrate a significant antistaphylococcal effect of simvastatin in ALI cultures.

Given the accessibility of the nose to topical treatments, including the use of creams, ointments, sprays and drops, administering simvastatin at the concentrations used in our experiments seems both achievable and practical. Nonetheless, it is important to note that these are preliminary findings. Any potential therapeutic use of simvastatin would require validation through larger-scale studies that assess safety and tolerability in addition to efficacy.

Chapter 6 **Conclusions and further work**

6.1 Summary of findings

6.1.1 Introduction

Chronic rhinosinusitis (CRS) is an under-appreciated and debilitating condition representing the second most prevalent chronic disease after arthritis. The condition has been demonstrated to have a greater effect on quality of life than back pain or angina and costs the UK economy £16.8 billion annually in medical treatment and reduced productivity due to absenteeism (1). It is the most common reason for an antibiotic prescription in primary care in the US (129). Despite the significant impact on quality of life and treatment requirements, including antibiotics and multiple surgical procedures, research investment in the condition remains relatively low with an urgent and unmet need for new therapeutic strategies.

S. aureus has been shown to be intimately related with CRS, particularly the recalcitrant form of the condition. Patients with CRSwNP have a higher frequency of *S. aureus* cultured from their nasal tissues, and the prevalence of IgE antibodies specific to *S. aureus* enterotoxins is fivefold higher in these patients compared to controls (71, 73, 119). The presence of Intracellular *S. aureus* within the tissues of CRS patients is linked with disease recalcitrance, treatment resistance, and an increased likelihood of needing additional surgical interventions (3). Additionally, *S. aureus* appears to promote inflammation and prevent epithelial barrier repair through production of pro-inflammatory cytokines and recruitment of other inflammatory cell types including mast cells and neutrophils (75). Within host cells, *S. aureus* shows greater resistance to the effects of antimicrobials and the actions of the immune system, making it a challenging reservoir of infection to eliminate (85, 170).

As *S. aureus* colonises the nares of patients without the condition, we hypothesised that *S. aureus* will have a different virulence factor profile in CRS patients compared to non-diseased controls. Our research also examined the impact of such a differential virulence factor profile on the intracellular survival of *S. aureus*. Additionally, we investigated simvastatin as a potential therapeutic agent capable of eradicating intracellular *S. aureus,* as well as the broader therapeutic implications of such a treatment. This chapter aims to consolidate and discuss the main findings presented in this thesis while contemplating their broader significance in relation to our current understanding of the pathophysiological basis of this disease.

6.1.2 Differences in *S. aureus* virulence factors between controls and CRS phenotypes.

Virulence factors are essential and drive the pathogenicity of *S. aureus* in many diseases. Some virulence factors have known associations with specific medical conditions; for example, staphylococcal toxic shock syndrome toxin 1 is associated with menstrual toxic shock syndrome, while delta-haemolysin is linked to atopic dermatitis (219, 220).

In Western populations with CRSwNP, the immune response generally skews towards a Th2-type profile. This is characterised by elevated IgE levels, raised towards *S. aureus* enterotoxins, alongside elevated cytokine levels such as thymic stromal lymphopoietin (TSLP), IL-4, IL-5, IL-6, IL-8, IL-13, IL-33 and myeloperoxidase (MPO). The polypoid nasal tissue in these patients often shows increased levels of innate lymphoid type 2 cells (ILC2), along with dendritic cells, basophils, mast cells, eosinophils and M2 macrophages. By contrast, patients with CRSsNP exhibit a more heterogeneous inflammatory profile. Some may exhibit a Th1-type of inflammation, marked by high levels of IFN-γ and tissues dominated with Th1 cells, cytotoxic $CD8^{+}$cells, NK cells and antigen-presenting cells (APCs). Meanwhile, other CRSsNP patients might exhibit a Th17-type

inflammatory response, characterised by high levels of IL-17 and an increased presence of Th17 cells, neutrophils, B-cells and APCs (221).

The observed differences in *S. aureus* virulence factor profiles between patients with CRSsNP and the other groups might contribute to the variability in pro-inflammatory cytokine production and the histopathological differences identified between the phenotypes. Specifically, in the CRSsNP-related *S. aureus* isolates, the presence of the delta-haemolysin (*hld*) gene alongside a presumably functional *agr* locus could correlate with a lower bacterial virulence. This phenotype may result in a more sessile form of *S. aureus*, that does not produce small colony variants (SCVs) – a form recognised for immune evasion capabilities (167).

Furthermore, the lack of genes encoding serine proteases A and B (*splA*, *splB*) in these isolates may have implications for the bacteria's interaction with the host. Serine protease A (SplA) degrades mucin 16, which is the most abundant glycoprotein protecting the epithelial surface, while serine protease B (SplB) acts as an inhibitor to the complement system. The absence of these proteases means that *S. aureus* in CRSsNP might be less effective in penetrating mucosal barriers and evading the complement-mediated immune response.

Additionally, these *S. aureus* isolates do not contain *lukE/D* genes responsible for producing leukocidin E/D, which targets neutrophils and macrophages, possibly explaining why these immune cells accumulate in Th17-type inflammatory responses (175, 181). Also, the presence of the gene that encodes the staphylococcal complement inhibitor (SCIN), which blocks C3 convertase enzymes, may decrease the activation of complement elements including C3b and C3d. Such elements play a role in IgE production by engaging complement receptor 2 on B-cells; therefore, a reduction in

complement activation might lower IgE responses to *S. aureus* toxins and diminish mast cell recruitment and activation (171, 196, 222).

This weakened virulence profile of *S. aureus* in CRSsNP could foster a tissue environment that favours M1 macrophages and neutrophils. These immune cells produce IL-12, which promotes T-cell differentiation and natural killer T (NKT) cell proliferation into mixed Th1 and Th17 patterns (Figure 6-1) (221).

The analysis of *S. aureus* isolates from patients with CRSwNP reveals a diverse profile of virulence factor genes, including *splA, splB and lukE/D* genes. However, only half of these isolates possess *hld* or *scn* genes.

The serine protease SplA, cleaves mucin 16, and likely facilitates bacterial attachment of *S. aureus* to the epithelium and potentially enhances colonisation and infection (177). Our studies show *S. aureus* infection of the nasal epithelium leads to upregulation of genes responsible for TSLP and TNF-α production. Elevated TSLP and TNF-α may activate ILC2 and promote a Th2 immune response. This, in turn, leads to the recruitment of eosinophils via IL-5 production, a process that can exacerbate the inflammatory and allergic responses characteristic of CRSwNP (70).

The gene producing SCIN, which impedes complement system activation by inhibiting C3 convertase enzymes, was absent in many *S. aureus* isolates from CRSwNP patients. In the absence of this inhibitor, SplB may compensate by cleaving activated complement components including C3, C5, C6, C7, C8 and C9. Notably, SplB targets C3 at the region that is also cleaved by endogenous factor I, resulting in C3b and C3d fragments. Of these, C3d is recognised as a potent activator of B-cells and can act as a chemotactic factor for mast cells, thereby intensifying immune responses (6, 223).

While additional experimental investigation is required for confirmation, it is possible that the concurrent presence of *splB* and absence of *scn* genes could promote B-cell activation, due to an increased production of the activated complement component C3d. This could potentially lead to an increase in both local and systemic IgE levels and further drive the recruitment and activation of mast cells. Once activated, these mast cells can release a cascade of prostaglandins, leukotrienes and cytokines such as IL-5 and IL-13. Moreover, SplB may also facilitate *S. aureus* survival by inhibiting other elements of complement activation, including formation of the membrane attack complex, and by stalling neutrophils from phagocytosing opsonised *S. aureus* (193) (Figure 6-1).

The absence of the delta-haemolysin (*hld*) gene in half of the CRSwNP isolates may lead to a reduction in the production of virulence factors regulated by the *agr* system, including enterotoxins, and promote the formation of SCVs (167). SCVs are known to enhance bacterial survival within host cells, particularly epithelial and mast cells. Within mast cells, the presence of *S. aureus* bacteria can promote the release of IL-5, IL-8, TNF-α and transforming growth factor-beta (TGF-β) (151).

Additionally, the presence of the *lukE/D* gene, which produces the pore-forming toxin leukocidin E/D, that lyses polymorphonuclear neutrophils is likely to lead to their depletion from nasal tissue (174, 175). The subsequent accumulation of immune components such as C3d, TNF-α and TGF-β will likely attract M2 macrophages, which promote tissue remodelling and fibrin deposition (Figure 6-1).

It is plausible that *S. aureus* associated with CRSwNP drives a Th2 disease phenotype, amplifying the response rather than being the causative agent. Tomassen *et al.* identified that only two out of ten endotypes demonstrated elevated IgE levels against *S. aureus* enterotoxins. Correspondingly, we demonstrated a low culture rate of 23.5% in the

CRSwNP group, comparable to that of controls (72). Additionally, other risk factors for the development of CRSwNP, including asthma, allergic rhinitis, environmental pollutants and ethnicity, are likely to be unrelated to *S. aureus* nasal carriage. Regardless, *S. aureus* appears to play a significant role in the most severe and treatment-resistant cases.

It must be acknowledged that these hypothesis regarding *S. aureus* interactions with the immune system are largely speculative and require validation. Conducting such validation is challenging in isolated cell culture models due to their inability to replicate the complex intercellular signalling observed in vivo among different nasal cell types. Despite this limitation, a more robust approach to investigating these theories could involve the use of explanted nasal tissue from CRSsNP and CRSwNP patients. By introducing various strains of *S. aureus,* each with a unique pattern of virulence factors, onto these tissue samples, researchers could gain insights into the resultant cytokine profiles and develop an understanding of how virulence factor patterns alter the immune profile of nasal tissue.

The proposed methodology allows for a closer approximation to the actual cellular interactions and conditions present in human nasal mucosa. The current research constitutes the initial effort to systematically categorise *S. aureus* virulence factors across control, CRSsNP and CRSwNP patients' middle meatuses, thereby establishing an invaluable bank of isolates to facilitate future experimental inquiries.

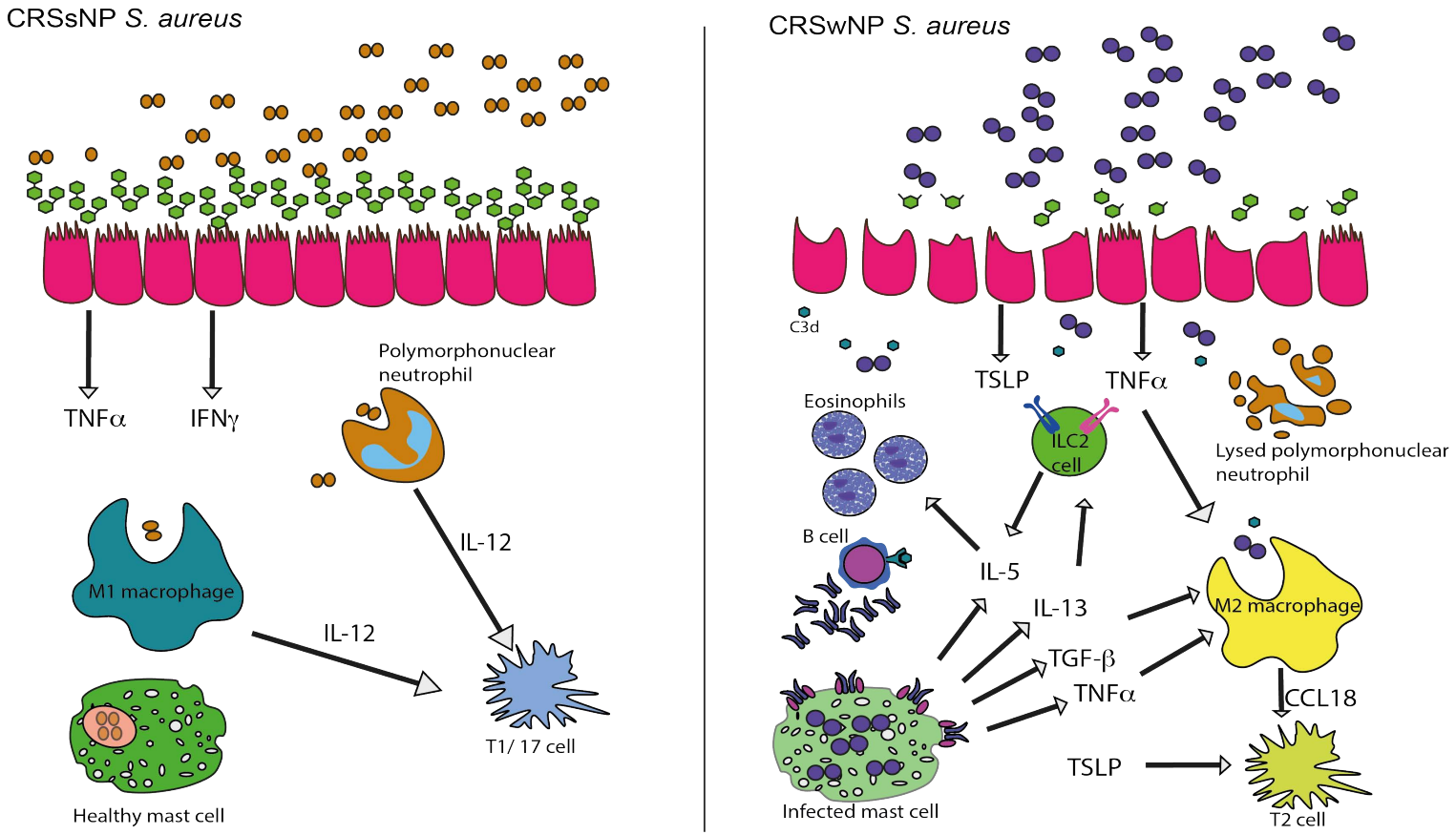


Figure 6-1 Proposed significance of S. aureus virulence factor differences and their relevance to endotype inflammatory patterns in CRSsNP and CRSwNP

CRSsNP strains carry fewer virulence factors but have a functioning *hld* gene, making them less capable of forming SCVs and residing within cells. The absence of *lukE/D* in these strains allows neutrophil and M1 macrophage accumulation, promoting IL-12 accumulation and enhancing the Th1/17 response. Additionally, the presence of SCIN reduces complement activation, minimising B-cell activation.
In contrast, CRSwNP strains of *S. aureus* damage the epithelium, increasing TSLP, TNF-α and IL-5 production by epithelial and ILC2 cells. The absence of SCIN allows C3 convertase enzymes to activate C3 components, attracting B-cells and mast cells. However, SplB prevents the formation of the membrane attack complex, moderating the immune response. *S. aureus*-infected mast cells harbour SCVs, producing IL-5, which attracts eosinophils that produce IL-13. This pro-inflammatory cytokine pattern, promotes the recruitment of M2 macrophages, reinforcing the Th2 pattern of inflammation.

6.1.3 Intracellular survival differences between control and CRSwNP *S. aureus* isolates

Our group's previous work has demonstrated that *S. aureus* cultured from CRSwNP patients appears to reside within mast cells, thereby recruiting them to the epithelium and triggering the release of pro-inflammatory cytokines that exacerbate inflammation in CRSwNP disease states (75). Within mast cells, the *S. aureus* is shielded from the local immune system and the effects of antimicrobials (85). We hypothesised that *S. aureus* cultured from CRSwNP would exhibit enhanced capabilities to internalise, survive and replicate within mast cells when compared with control isolates.

Our findings confirmed that the CRSwNP 1 *S. aureus* strain has a significantly increased ability to internalise and survive within mast cells, and it also appeared to thrive within apoptotic cell bodies. Additionally, this strain demonstrated increased replication rates within mast cells compared to the Control 1 isolate. These observations could be attributed to differences in virulence factors outlined in Chapter 3. Notably, the CRSwNP 1 strain lacks the *hld* gene but contains *spl*A, *splB* and *LukE/D* genes, while the Control 1 strain possesses the enterotoxin gene cluster, including a complete *hld* gene. The absence of the *hld* gene, which is associated with suppression of the *agr* operon function, may facilitate SCV formation and reduce the expression of *agr*-controlled cytotoxic virulence factors, potentially manifesting with enhanced intracellular survival (167).

We investigated whether treatment with CRSwNP 1 *S. aureus* resulted in less mast cell death when compared to the Control 1 strain. Both strains, however, demonstrated similar cell death rates at each timepoint. Delta-haemolysin, encoded by the *hld* gene, has been shown to activate mast cells and cause degranulation though an as-yet-unidentified pathway unrelated to IgE signalling in foetal skin-derived mast cells and causes widespread mast cell degranulation seen in atopic dermatitis (220). Despite the

Control 1 strain possessing the *hld* gene and the CRSwNP 1 strain lacking a functional *hld* gene, we did not witness a significant difference in degranulation between the strains in our model. This may be due to either the mast cell model not responding to delta-haemolysin, or the control strain not expressing the gene during infection.

Not all strains of *S. aureus* possess the ability to survive and replicate within cells. Krut and colleagues demonstrated that only seven out of twenty-three clinical isolates had the capacity to internalise and survive within keratinocytes and fibroblasts (124). This variability is likely due to presence and expression of virulence factor genes. Surprisingly, the control strain internalised and replicated to a greater extent than anticipated. Regardless, our results suggested that the CRSwNP 1 strain, which closely resembled the virulence factor pattern of other CRSwNP strains, showed enhanced survival compared with the Control 1 strain, representative of the control group.

It is feasible that this enhanced survival observed in CRSwNP *S. aureus* could lead to increased bacterial accumulation in mast cells and further recruitment of mast cells via the release of chemotactic factors including leukotrienes, histamine, tryptase and TNF-α (122). The subsequent activation of ILC2 propagates inflammation and the secretion of IL-5, IL-4 and IL-13, which, in turn, prompts further recruitment of Th2 pattern cell types (221).

Regrettably, much of our strain collection and sequencing data were unavailable until the final stages of this PhD project, delayed as a consequence of the COVID-19 pandemic. It would have been desirable to test intracellular survival in more isolates and our CRSsNP-cultured *S. aureus* isolates which exhibit significantly fewer virulence factors.

6.1.4 Simvastatin as a potential therapy in *S. aureus*-related CRS

Recent large-scale studies have indicated that patients on statins have a significantly lower risk of being diagnosed with CRS (5, 6). Furthermore, in vitro studies have shown that statins possess antistaphylococcal activities (132-136, 138, 139) . Consequently, we investigated the potential of repurposing simvastatin as a treatment for *S. aureus*-related CRS, which could reduce our reliance on steroids, antimicrobials and repeat surgeries. We discovered that simvastatin in the prodrug form was effective as an antistaphylococcal treatment; however, the activated form, simvastatin hydroxy-acid had no effect. Intriguingly, much of the available literature did not specify whether statins were utilised in their activated forms. However, upon closer inspection, it appears that most studies employed the prodrug form. We determined that prodrug simvastatin, at concentrations 1000 times higher than those present in the serum of those taking the drug orally, was capable of killing planktonic CRSwNP 1 *S. aureus*. Additionally, at these elevated concentrations, simvastatin was able to reduce both the intracellular survival of the bacteria and the number of mast cells infected.

Statins have been shown to reduce protein prenylation and IgE-mediated degranulation (204, 215). Fluvastatin, a similar lipophilic statin, has been shown to reduce IgE-mediated expression of *IL6*, *TNF* and *IL13* in mast cells (137). Our LAD2 mast cell model exhibited minimal degranulation in response to IgE, suggesting that the FcεRI may have become decoupled, a common issue with the LAD2 mast cell line. However, we did identify a functioning MRGPRX2 receptor responsive to substance P. Substance P has been found at high levels in nasal lavage fluid and shares a similar pathway to IgE-mediated degranulation (205). We therefore investigated whether simvastatin could attenuate degranulation via the MRGPRX2 receptor. However, our findings indicated no difference in degranulation across various incubation times and concentrations of simvastatin,

suggesting that simvastatin does not affect substance P signalling or the pathways it shares with IgE signalling.

We infected LAD2 mast cells and pre-treated mast cells with 30 µmol/L simvastatin to examine cytokine transcription. We noted stable expression of housekeeping genes, actin-beta and beta-2-microglobulin. However, the expression of pro-inflammatory cytokines was notably weak, even in control groups and remained unquantifiable. We suspect this was due to mutations in the cell line; nevertheless, we were unable to obtain different results on a newly recovered batch of LAD2 cells.

We finally tested the effects of simvastatin treatment on primary nasal epithelial ALI cells. We found that simvastatin non-significantly reduced the expression of *TNF, TSLP*, *CXCL8* (IL-8), *IL6 and IL33* cytokines from baseline. Furthermore, simvastatin-pre-treated, *S. aureus*-infected ALI cultures showed reduced expression of *TNF, TSLP* and *CXCL8* (IL-8), while increasing expression of *IL6* and *IL33* in comparison to cultures infected with *S. aureus* alone. Simvastatin also reduced the abundance of *S. aureus* on the apical surface of the ALI cultures and did not appear to disrupt the epithelial structure. Additionally, there were no significant differences in TEER between each treatment group.

In summary, prodrug simvastatin effectively reduces the extracellular staphylococcal burden at concentrations exceeding those typically present in the serum of orally medicated patients. It also demonstrates a significant potential to prevent intracellular infection with *S. aureus,* likely attributable to the lipophilic properties of the drug. This introduces a novel treatment approach that could specifically target and eliminate the intracellular reservoir of *S. aureus* within CRS tissues. By reducing both the extracellular burden and intracellular reservoir of *S. aureus,* there is a high likelihood of diminishing

the Th2 inflammatory pattern associated with *S. aureus*-related CRSwNP, thereby reducing disease severity and improving treatment outcomes (Figure 6-2).

Our results did not demonstrate any anti-inflammatory effects of simvastatin on mast cells, which was likely due to the limitations of our model, necessitating further testing. Although simvastatin treatment tended to reduce the transcription of pro-inflammatory cytokine genes in both non-infected and CRSwNP *S. aureus*-infected ALI, these reductions did not reach statistical significance.

The substantial heterogeneity in these results can be attributed to the cultured nasal cells being sourced from individual subjects, complicating the analysis. Additionally, other studies have reported that *S. aureus* can be particularly damaging to ALI cells unless the bacteria's virulence is weakened by the knock-down of virulence factors such as alpha toxin, which aligns with our findings (216). Given that our objective was to identify treatments for highly virulent, clinically relevant strains of *S. aureus,* weakening the strains was deemed counterproductive. Therefore, we plan to test simvastatin treatment on *S. aureus*-infected nasal explants, which are likely to be more robust and tolerate virulent strains for extended periods. This approach will also allow for examination of the crosstalk between cells involved in CRS, helping us explore the presence of intracellular bacteria in mast and epithelial cells and the cytokine response patterns that each tissue subtype generates in response to various isolates.

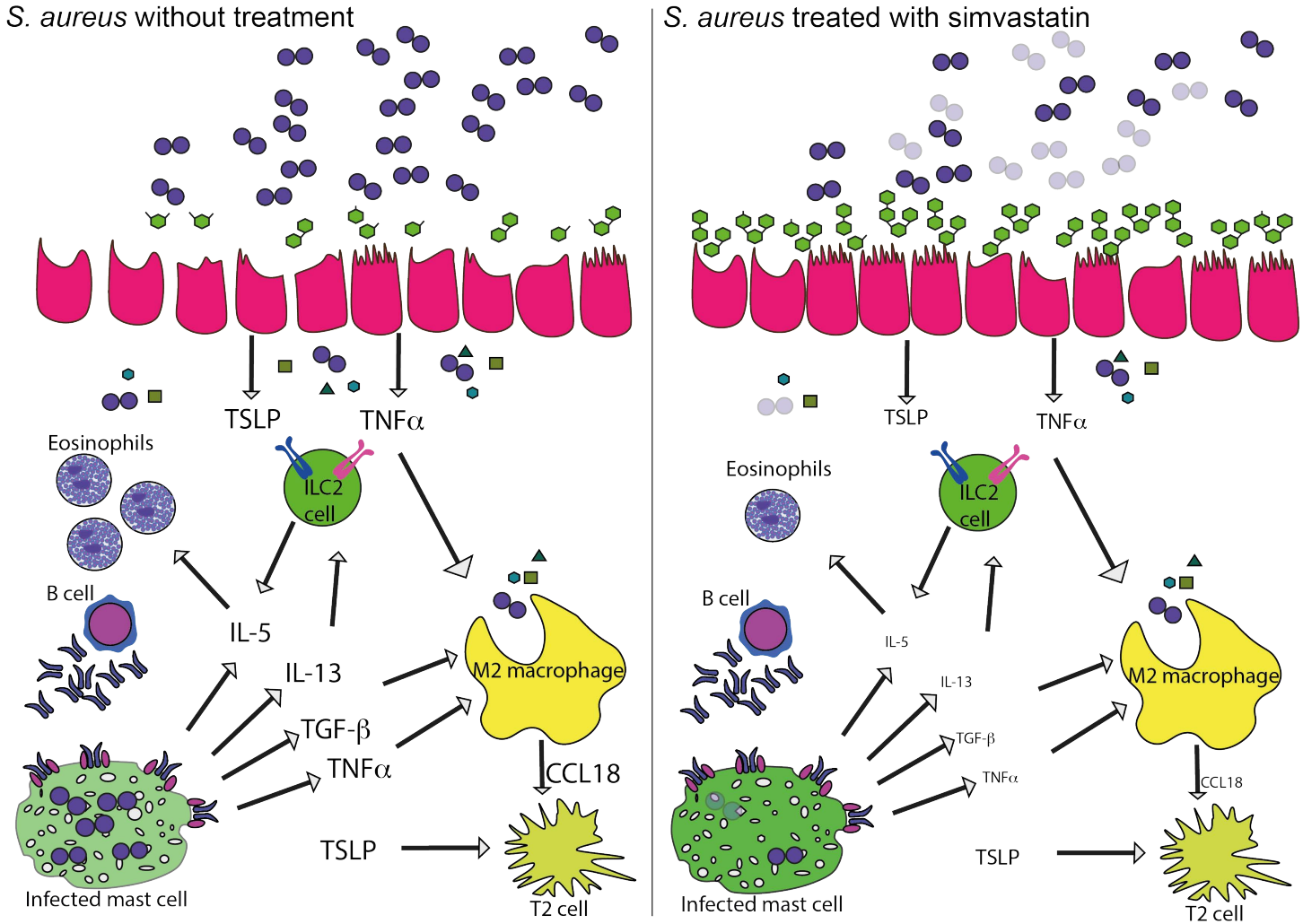


Figure 6-2 Potential effects of topical simvastatin treatment on CRSwNP-associated S. aureus

Simvastatin has the potential to eradicate extracellular *S. aureus* and diminish the intracellular burden of *S. aureus*. This action is likely to decrease epithelial damage and mast cell infection, thereby reducing the burden of pro-inflammatory cytokines such as TSLP, IL-5 and IL-13. As a result, there may be a reduction in the activation of eosinophils, B-cell and mast cells which may reduce disease severity. These effects require further testing to confirm their significance and mechanism.

6.1.5 Conclusion

This research set out to investigate the hypothesis that *S. aureus* associated with CRSwNP harbours virulence factors that facilitate internalisation and replication within mast and epithelial cells. We also hypothesised that simvastatin could diminish the viability of intracellular *S. aureus* and curtail the production of pro-inflammatory cytokines related to CRSwNP *S. aureus*.

We demonstrated that a CRSwNP related *S. aureus* possesses a distinct virulence factor pattern compared to *S. aureus* isolates from control and CRSsNP patients. These virulence factors enhanced CRSwNP *S. aureus* ability to internalise, replicate and survive within the intracellular milieu of mast cells, thus confirming our hypothesis in this cell type. Regrettably, we were unable to thoroughly test this hypothesis in epithelial cells due to the pronounced cytotoxicity of *S. aureus* infection on ALI-cultured primary epithelial cells. Nevertheless, it is plausible that the virulence factor patterns observed in CRSwNP *S. aureus* isolates support intracellularisation in more resilient, tissue-resident epithelial cells.

Moreover, the hypothesis that simvastatin can mitigate intracellular *S. aureus* infection in mast cells has been affirmed. However, we were unable to observe a corresponding decrease in the release of pro-inflammatory cytokines, a finding likely due to the deteriorating quality of our mast cell model.

6.2 Limitations of the work

This research project, like many others, was significantly impacted by the COVID-19 pandemic, particularly in relation to limitations on experimental work and funding availability as resources were redirected towards COVID-19 related research. Additionally, the number of elective rhinology surgical procedures was significantly reduced, impacting the recruitment of subjects and tissue collection. Supply chain issues, particularly concerning the availability of LAD2 StemPro-34 medium and plastic pipette tips further hampered work with the LAD2 cell line.

The *S. aureus* cultured from the middle meatus of control, CRSsNP and CRSwNP may not represent strains that have been internalised within epithelial cells and mast cells, as the swabs only capture extracellular bacteria. This limitation was recognised at study set up. Our main concern with harvesting intracellular *S. aureus* was the need to biopsy patients with no disease (controls), which we considered unethical and could potentially result in iatrogenic sinusitis. Consequently, we opted to utilise nasal swabs, which would be easily performed in all groups, including patients seen in the clinic, thus reducing selection bias. We also evaluated the use of steroids and antibiotics on the cultures, particularly whether these treatments might select for more pathogenic strains with increased antimicrobial resistance. Given the goal of this study was to culture clinically relevant strains, we included those patients, as this ensured a fairer representation of patients who attend specialist rhinology clinics and those needing surgery.

We used short-read genome sequencing as opposed to long-read sequencing. The pitfall of short-read sequencing was that we may not achieve adequate sequence coverage and the accuracy of the base pairs would be less than that seen in long-read sequencing. Despite this we observed 92-96% genome coverage with most strains sequenced within

15 contigs. This represented accurate genome coverage and will allow for accurate detection of base pairs and frameshift mutations within promoters and genes.

Our analysis of virulence factors, stress genes and antimicrobial resistance genes using the AMRFinderPlus database is limited by the genes currently identified in the database. As research progresses, more genes supporting *S. aureus* survival and infection will likely be identified, and we plan to revisit our virulence factor analysis accordingly. Moreover, the presence of virulence factor genes does not necessarily equate to expression; thus, our results only indicate the potential infection mechanisms the bacteria utilise to induce infection and enhance survival. Performing gene expression analysis using qRT-PCR at various infection timepoints would correlate bacterial virulence genes with actual gene expression levels, making the findings more clinically relevant. Furthermore, correlating gene expression with protein production levels through ELISA would have been desirable.

Experimental models have some inherent limitations in exploring CRS. Our mast cell models were deficient in FcεRI signalling, and as such may not reflect the behaviour of resident tissue mast cells. Although LAD2 cells represent the most differentiated cell line available to study human mast cells, the feasibility of procuring primary mast cells from nasal polyp tissue was considered, but the yield would be small and insufficient for infection experiments. Furthermore, there may be significant heterogeneity amongst isolated primary mast cells, as observed in our ALI cultures due to differing inflammatory pathways activated in each individual. Given these challenges we plan to compliment this data with nasal explant models, to further validate and support our data in future experiments.

ALI cultures appeared to be particularly sensitive to *S. aureus* infection and simvastatin treatment, demonstrating highly variable responses in cytokine expression. These

differences may well be significant in the context of different endotypes of Th2, Th1 and Th17 diseases, with genetic differences orchestrating different patterns of cytokine release in each tissue. However, to unpick these differences would have required endotyping of each individual from whom primary nasal cells were cultured as well as a high number of individuals to create the ALIs. This would have also been financially unviable.

6.3 Further work and future directions

Further *S. aureus* specimens from patients have been collected since the initial analysis and require sequencing to determine their virulence factor patterns. Given the redundancy and diversity of these factors, it is unlikely we will reach significance in terms of specific virulence factor carriage between groups. Regardless, we hope to gain some further insight into the genes that make CRSwNP *S. aureus* more virulent and to test these strains in improved experimental models. This will not only help us understand how they propagate the Th2 response, but also to enable testing of future potential therapies.

Regarding sequencing, investigating *agr* frameshift mutations, which are widely reported in intracellular *S. aureus* strains, would be beneficial. Our initial efforts faced obstacles as the Bactopia script 'AgrVATE' was unable to open Mackintosh zcat compressed files with the .gz.z extension, under which our assemblies were categorised. Despite several attempts to adjust the script, the analysis could not be executed. Having now obtained additional *S. aureus* strains from each patient group, our next step is to reconfigure the Bactopia pipeline on a computer with a Linux operating system to facilitate further analysis of *agr* frameshift mutations. Beyond this, we intend to investigate other aspects of the *S. aureus* genome, including the staphylococcal cassette chromosome mec (SCCmec, responsible for methicillin resistance), as well as patterns of antimicrobial resistance and the annotation of plasmid assemblies. Conducting such comprehensive genomic analysis will enhance our understanding of the *agr* quorum sensing operon, defining crucial differences among the strains and support the development of potential therapeutics.

Furthermore, we have laid out plans to perform qRT-PCR and proteomics analyses to investigate the expression and interplay of virulence factors in *S. aureus* isolates when

infecting explanted nasal tissues. In parallel, we will examine the cytokine responses of nasal explants using a custom MSD V-plex assay capable of detecting Th1, Th17 and Th2 cytokines. These simultaneous analyses aim to yield a more comprehensive understanding of virulence factor dynamics and their subsequent immune interactions, thereby offering insights into disease pathophysiology and potentially unveiling new antimicrobial targets.

Finally, our goal is to fine-tune our investigation into the antistaphylococcal efficacy of simvastatin by using nasal explant models. This strategy is intended to broaden our understanding of the drug's potential in modulating the effects of *S. aureus* and in reducing Th2 cytokine production. Armed with the findings from such data, we may apply for funding and ethical approval to conduct a clinical trial. This trial will assess the efficacy of topically administered simvastatin as a treatment for patients with *S. aureus*-related recalcitrant disease.

Appendix A Research and Ethics Committee approval

Health Research Authority

London - Hampstead Research Ethics Committee
Barlow House
3rd Floor
4 Minshull Street
Manchester
M1 3DZ

Telephone: 0207 104 8340

Please note: This is the favourable opinion of the REC only and does not allow you to start your study at NHS sites in England until you receive HRA Approval

21 July 2020

Professor Rami Salib
Associate Professor of Rhinology & Honorary Consultant Otorhinolaryngologist
University Hospital Southampton
ENT Department
Royal South Hants Hospital
Brintons Terrace
SO14 0YD

Dear Professor Salib

Study title:	**Bacterial regulation of inflammation and markers of neurodegeneration in the upper airways**
REC reference:	**20/PR/0183**
Protocol number:	**1.4**
IRAS project ID:	**275758**

Thank you for your letter received on the 20th July 2020, responding to the Proportionate Review Sub-Committee's request for changes to the documentation for the above study.

The revised documentation has been reviewed and approved by the PR sub-committee's Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Appendix B Health Research Authority approval of study

Ymchwil Iechyd a Gofal Cymru
Health and Care Research Wales

NHS
Health Research Authority

Professor Rami Salib
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24 July 2020

Dear Professor Salib

HRA and Health and Care Research Wales (HCRW) Approval Letter

Study title:	**Bacterial regulation of inflammation and markers of neurodegeneration in the upper airways**
IRAS project ID:	**275758**
Protocol number:	**1.4**
REC reference:	**20/PR/0183**
Sponsor	**Research Integrity and Governance, University of Southampton**

I am pleased to confirm that **HRA and Health and Care Research Wales (HCRW) Approval** has been given for the above referenced study, on the basis described in the application form, protocol, supporting documentation and any clarifications received. You should not expect to receive anything further relating to this application.

Please now work with participating NHS organisations to confirm capacity and capability, in line with the instructions provided in the "Information to support study set up" section towards the end of this letter.

How should I work with participating NHS/HSC organisations in Northern Ireland and Scotland?
HRA and HCRW Approval does not apply to NHS/HSC organisations within Northern Ireland and Scotland.

If you indicated in your IRAS form that you do have participating organisations in either of these devolved administrations, the final document set and the study wide governance report

Appendix C Protocol for Bacterial regulation of Inflammation and markers of neurodegeneration in the upper airways

Protocol
Bacterial regulation of inflammation and markers of neurodegeneration in the upper airways

Investigators

Chief Investigator: **Professor Rami Salib** (Associate professor and Consultant in Rhinology)

Investigators:	Mr Simon Goldie	(ENT registrar)
	Mr Mohammad Alsalem	(ENT registrar)
	Prof Roxana Carare	(Professor Clinical Neuroanatomy)

Background information

Chronic rhinosinusitis (CRS) otherwise known as sinusitis is a painful, under-researched condition causing regular facial pains, nasal blockage, smell and taste disturbance. It is the second most common disease in Europe and has a greater effect on quality of life than back pain or angina. Sufferers have much higher rates of asthma and airborne allergy with severe forms of CRS causing dramatically worse asthma control. Despite the significant impact that the condition has, research investment remains low and there remains a desperate unmet need for new therapeutic strategies.
There is growing evidence to suggest that bacteria (Staphylococcus aureus) are involved in severe forms of the disease. Recently the Upper Airways Group at the University of Southampton made the unexpected finding that bacteria can live inside mast cells, a type of immune cell involved in allergy. This is not the case with all forms of the bacteria only the very infective forms. We have shown that bacteria living inside mast cells can alter the production of molecules that are involved in inflammation. Also by preventing the immune system and antibiotics from killing the bacteria they may increase the degree of inflammation.
We aim to look at the DNA from bacteria in the noses of healthy people and make comparisons with that in patients with CRS. We shall seek to identify the genes that make the bacteria particularly infective and indicate means whereby they survive within the mast cell and host tissues causing inflammation. These studies will advance understanding of disease processes in chronic rhinosinusitis and may lead to desperately needed therapies.
Simultaneously, we will also look to determine if proteins and molecules present in fluid surrounding the brain are detectable in the nasal cavity using the same nasal tissue collected above. Recent evidence has suggested previously unknown drainage channels for fluid surrounding the brain may be present throughout the nasal cavity. If these molecules are detectable in nasal tissue this may represent a much less invasive method of investigating these molecules than the presently available technique of lumbar puncture.

Aims and objectives

The primary aims of the study include:

• Identifying the genomic sequence of S. aureus present in patients with no evidence of chronic rhinosinusitis and those with non-polyp and polyp disease.

• To determine whether it is possible for proteins and molecules present in the CSF can be detected in the nasal mucosa/ nasal sinus tissue.

Secondary aims include:

• Examine the surrounding nasal microbiome as this may point towards sources of mobile genetic elements (DNA shared between organisms).

• Evaluation of bacterial presence in the clinical samples and its impact on the inflammatory reaction.

• Determine the role of specific cells, including immune cells, in determining the magnitude of Inflammation.

Study Design

The study group will consist of patients with and without CRS undergoing nasal, sinus or skull base surgery (through an endoscopic transnasal route), where the removed tissue is not required for diagnostic analysis and would be otherwise discarded as clinical waste. The use of this tissue in the laboratory assays will not affect the medical care of the patients.

Inclusion criteria

• Patients undergoing nasal, sinus or skull base surgery (through endoscopic transnasal route) where the removed tissue is not required for diagnostic analysis.

Exclusion criteria

• Patients who are unable to give informed consent.

• Patients who have a known infectious disease which would place the researcher at risk, such as Hepatitis B, C or HIV.

Screening

The study will be co-ordinated with ENT surgeons/ neurosurgeons who are part of the research team. A list of weekly surgical procedures will be obtained from theatre and patients undergoing suitable procedures will be identified. Patients who fit the inclusion/exclusion criteria will be sent a copy of the patient information sheet via post describing the nature of the study and will have the opportunity to contact us to discuss this further if they wish to participate or discuss any questions prior to consenting on the day of surgery. Patients have the opportunity to discuss the study with friends, relatives or the clinical team. Patients will be re-approached when they are admitted for their surgery, and signed informed consent will be obtained from patients willing to participate in the study.

Subject withdrawal criteria

• Subjects have the right to withdraw from the study at any time for any reason.

• The investigator also has the right to withdraw subjects from the study in the event of intercurrent illness, protocol deviations, administrative reasons or other reasons.

Study size

This is a pilot study, hence we are unable to perform power calculation. It is anticipated that we will need to recruit approximately 150 patients. A power calculation will be performed at 50 patients to determine how many samples we require to reach statistical significance to answer our primary objectives.

Investigator training

The PI will ensure each member of the team is competent to perform each task for which they are responsible/ delegated on the delegation log. All participating Investigators will be required to demonstrate up to date GCP certification.

Obtaining the tissue sample

• Signed Informed consent will be taken as above following issue of the detailed explanation of the study to participants and issue of patient information leaflet.

• The surgical procedure, which is part of routine medical care and not part of the study, will be carried out as per usual surgical practice. Following the surgery, excised tissue (which would usually be treated as surgical waste) and nasal swabs (taken specifically for research purposes) will be provided to the researcher for use in laboratory experiments. This will be collected from theatre with a code allocated, by which it will be referred from this point onwards. A separate codebreak list will exist and be kept by the research team in an encrypted file on the NHS network in the surgical drive. This will allow patients to withdraw consent to participation at any point.

• In the laboratories, the nasal swab/tissue sample will only contain the code. Tissue will be processed on site in laboratories adjacent to surgical theatres at University Hospital Southampton. Nasal swabs/cultures containing bacteria will be prepared and sent off site to companies specialising in 16s ribosomal RNA sequencing and whole genome sequencing. **No patient data will be sent to external organisations only the anonymised code.**

Patient Demographics

If patients agree to participate, we will verbally ask the patient demographic information including age, sinus disease, surgical procedure performed, allergy status, past medical history, smoking status and previous antibiotic therapy. A code will be provided to both the tissue and the demographic information (which will be stored in pseudoanonymised form on the University of Southampton research filestore).

Laboratory assays on nasal swabs/tissue provided following surgery

Nasal Swabs

Non-pathogenic and pathogenic strains of *S. aureus* will be collected using three intraoperative nasal swabs of patients undergoing pituitary surgery with no evidence of Chronic rhinosinusitis (as control) and functional endoscopic sinus surgery with CRSsNP and CRSwNP. Nasal swabs will be cultured on Chromogenic agar plates, colonies will be selected and tested using catalase and DNase agar plates before formal mass spectrometry ensuring *S. aureus* is cultured. The remainder of the nasal swab will be used for Ribosomal 16s RNA sequencing to catalogue the nasal microbiome in each patient.

Following harvesting of suitable strains, stock solutions will be created and stored in 25% glycerol at -80°C. Genomic DNA will be prepared and stored for whole genome sequencing. HMC-1 mast cells, cultured in Iscove's Modified Dulbecco's Medium supplemented with 1% FCS and 0.2% gentamycin, penicillin and streptomycin will be co-cultured with *S. aureus* isolates, grown to the mid-log phase, at a multiplicity of infection (MOI) of 1:1 over 2, 6 and 24 hours. LAD-2 mast cells will be cultured in Stempro – 34 SFM media with stem cell factor and 0.2% gentamycin, penicillin and streptomycin and co-infected as described above. Bacterial survival experiments will be conducted by co-infecting HMC1 and LAD2 mast cell lines, measuring extracellular bacterial growth in the supernatant and intracellular bacterial growth after permeabilization of the cell pellets in 1% triton-x. Colony forming units from each group will be performed as well as β-hexosaminidase and lactate dehydrogenase assays.

Data, mapping the clinical phenotype with the surrounding microbiome, mast cell cytokine expression, mast cell survival and the in vitro virulence of the organism will be generated. Whole genome sequences will be analysed using high throughput computing techniques

for virulence factor gene presence, mobile genetic elements, frame shift mutations and phylogenetic analysis.

Tissue Samples
A variety of experimental procedures will be performed on the tissue provided following surgery. Some samples will be fixed for immunohistochemistry to enable us to determine which cells are involved in the release of inflammatory mediators, and mRNA for inflammatory proteins will also be extracted. Tissue will be cultured, either as explants or as individual cell types (such as eosinophils) and the effects of various inflammatory stimuli investigated. Tissue or cells will have a stimulus or buffer control added and after a defined incubation period, supernatants will be removed. The effects of stimulation will be measured using a range of experimental techniques (flow cytometry, western blotting, ELISA, microarray, RTPCR). We will use these techniques to detect activation markers on inflammatory cells and to measure levels of cytokines and other soluble mediators that may be involved in the inflammatory response. All results will be analysed using parametric and/or non-parametric statistical tests, where appropriate. For investigations looking at the influence of genetic polymorphisms on inflammation, DNA/ mRNA will be extracted from the tissue or cells and relevant genes analysed by PCR and/or DNA sequencing. When DNA is extracted it will only be used to study genes that may be relevant in inflammation, and not for any diagnostic purpose.
Samples will be fixed and processed for immunohistochemistry. Markers of neurodegeneration can be measured using a range of experimental techniques (immunocytochemistry, Western blotting, ELISA) to establish the presence of proteins such as Aβ, tau or neurofilament light in the nasal mucosa. All results will be analysed using parametric and/or non-parametric statistical tests, where appropriate.

Patient confidentiality
Patient confidentiality will be maintained as a code will be assigned before the samples leave theatre which can only be identified using the codebreak list. This list will be kept on the NHS network in the surgical folder in an encrypted file. Any other demographic data will be stored in an encrypted file on the University of Southampton research filestore. Consent forms will be kept within the site file in a locked room in the research and development office at University Hospital Southampton. Personal data will be destroyed at the end of the study but any anonymised data used for publications will be kept on the University of Southampton institutional data repository for a minimum of 10 years.

Disposal of Clinical material
Nasal Swabs will be kept until completion of the study to allow simultaneous processing for 16S ribosomal RNA sequencing. A Material Transfer Agreement (MTA) will be agreed with any external company that we use to process our samples. The MTA will be clear that any samples sent for external analysis will be used up/ destroyed after analysis by the company. Tissue specimens will be kept in accordance with the Human Tissue Authority regulations and disposed of on completion of the study. If patients withdraw consent for tissue to be kept the clinician and investigator delegated as responsible for the codebreak list will identify the patient and dispose of their tissue/ nasal swabs and erase their personal data which we hold.

Appendix D Patient information sheet

Bacterial control of inflammation & neuromarkers in the upper airways

Researcher: Professor Rami Salib, Mr Simon Goldie, Mr Mohammad Alsalem, Professor Roxana Carare

ERGO number: 54006

You are due to have nasal, sinus or skull base surgery (e.g. pituitary gland, repair of brain fluid leak, with access for the surgery through your nose and sinuses). We would like your help in our research investigating the causes of chronic sinusitis and in determining if proteins and molecules in the fluid, surrounding the brain can be identified in the nasal cavity. Chronic infection of the sinuses (rhinosinusitis) with or without polyps is an extremely common clinical condition affecting up to 15% of the population within the United Kingdom. It is more common than arthritis or high blood pressure. The impact of the disease is often under-appreciated with afflicted patients having a significantly impaired quality of life. Recent evidence demonstrates bacteria and fungus present on the surface of the sinuses can stimulate inflammation of the lining of the nose. Through a better understanding of the mechanisms of inflammation in chronic rhinosinusitis (with and without polyps), it is anticipated that improved treatments and better-targeted surgery will be possible in the future. To that end we wish to compare the inflammatory molecules from patients with rhinosinusitis with patients that have no evidence of sinus disease (those undergoing neurosurgical procedures) to detect any differences which may suggest why patients with chronic rhinosinusitis have greater levels of inflammation.
Fluid surrounding the brain, contains a number of proteins and molecules that can tell doctors about infections and overall brain health. To date the most widely used method for sampling this fluid is a lumbar puncture in which a needle is inserted into the lower spine. This is uncomfortable for patients and risks introducing infection. Recent MRI evidence has shown previously unknown drainage pathways for this fluid exist throughout the nasal and sinus cavities. If we can detect these proteins and molecules in sinus tissue, we may be able to develop a new less invasive method to investigate them. This would improve our understanding of brain disease and may improve patient care.
You are being invited to take part in a research study. Before you make your decision it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your doctors if you wish. Please ask us for further information if anything is unclear. Take time to decide whether or not you wish to take part.

Part one
What is the purpose of the study?
The purpose of this study is to help us understand the mechanisms that control inflammation in the nose and upper airways and determine if proteins and molecules present in fluid surrounding the brain can be detected in the sinus cavities. The causes of chronic rhinosinusitis and nasal polyps are not completely understood. Therefore, current treatments tend to be very non-specific and may not be as effective as would be desirable. In this study we want to carry out experiments in the laboratory on the tissue

(removed as part of normal surgery) and nasal swabs (taken during the operation as part of the research project) which we hope will help us to improve our understanding of how inflammation is controlled and what organisms (bacteria or fungi) are involved in driving the inflammatory reaction leading to chronicity of the disease process, with the ultimate aim of developing new drugs to treat polyps and/or rhinosinusitis. Furthermore, we also wish to test nasal sinus tissue for proteins and molecules typically found in fluid surrounding the brain with a view to developing a new sampling technique which is less invasive than those currently available. This would allow investigation of these proteins and molecules to help us better understand brain diseases.

Why have I been chosen?
You have been asked to take part in this study because your surgery involves removal of tissue from your nose due to sinus disease or as part of your neurosurgical procedure. The tissue which will be removed would usually be destroyed or thrown away. We are asking patients if we can use this tissue in laboratory experiments described above.

Do I have to take part?
No, it is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You will still be free to withdraw from the study at any time without giving a reason. This will in no way affect the surgery you will be undertaking or the standard of care you receive as a patient.

What will happen to me if I take part?
Taking part in this study will in no way change what happens during the surgery or your subsequent medical care. We will only use tissue that has been removed as part of the normal course of your operation. No material will be removed specifically for the purpose of this study and your operation will be exactly the same whether you decide to participate in the study or not. We will take nasal swabs in the operation which are purely for research purposes and this will not in any way affect the result of your surgery. We will ask you a few simple questions about your history of allergy or other diseases to aid us with our studies.
Some of the nasal swabs will also be sent to specialist centres to get the best information possible with respect to the assessing the presence of bacteria and fungi.

What are the possible side effects of taking part?
There are no side effects as a result of allowing us to take your nasal tissue that has already been removed by the surgeon as part of your routine surgical treatment. There is also no risk of harm due to nasal swab use.

What are the possible benefits of taking part?
Agreeing to participate in this study will be of no direct benefit to you. The information we gain from the study will hopefully lead to a better understanding of the inflammation that occurs in sinusitis and drainage of fluid surrounding the brain, that may lead to the development of new therapies.

What if there is a problem?

We do not anticipate any problems as entering into our study will not alter your normal course of care. However, any complaint about the way you have been dealt with during the study will be addressed; the detailed information on this is given in part two.

Will my taking part in the study be kept confidential?

Yes. All the information about your participation in the study will be kept confidential. Details are included in part two.

Study contact details

Mr Simon Goldie
Rhinology Research registrar
Telephone: 02380 796364
Email: s.goldie@soton.ac.uk

This completes part one of the information sheet. If the information in part one has interested you and you are considering participation, please continue to read the additional information in part two before making any decision.

Part two

What if there is a problem?

There are unlikely to be side effects from you allowing your nasal tissue to be used for research. This will not alter your surgery, or your treatment afterwards. If you have concerns regarding this contact the Head of Research Integrity and Governance rgoinfo@soton.ac.uk.

This study is covered by the professional indemnity insurance of the University of Southampton. If you are harmed by taking part in this research study due to someone's negligence, then you may have grounds for legal action against University of Southampton but you may have to pay for it. There are no arrangements for no-fault compensation in this study. University Hospital Southampton NHS Foundation Trust are responsible for any clinical negligence arising as part of your routine clinical care. Regardless of this, if you wish to complain about any aspect of the way you have been approached or treated during this study, the normal National Health Service complaints process will still be available to you

Will my taking part in this study be kept confidential?

All tissue received during this study will be treated confidentially. The sample of tissue that we receive from you will be assigned a code on collection, by which it will be referred from that point onwards. It will not be possible for anyone to identify you from the results of this study. Any samples which leave the hospital, including samples transferred to other centres, will have your name and address removed so that you cannot be recognised by it.

Storage of samples and genetic material

Some of the tissue you donate may be stored in our laboratory while awaiting sample processing. All samples will be coded and will not carry your name or other identifiable information. Samples will be stored until the end of the study, after which any unused material will be destroyed. At the end of the study all results and data will have all personal information removed and be published in the Southampton University institutional filestore to allow scrutiny of any publications. No information about you will be revealed to third parties without your written consent.

What will happen to the results of the research study?

It is anticipated that the results of the study will be published in a scientific journal. It will not be possible for anyone to identify you in any report or publication.

Who is organising and funding the research?

This research is organised by investigators in the University of Southampton and is funded by research grants to the University. None of your doctors will receive extra payment as a result of you agreeing to be involved in this study.

Who has reviewed the study?

This study has been reviewed by the University of Southampton and Hampstead regional ethics committee.

How will we use information about you?

We will need to ask you some information including your age, any diagnosis of sinus or neurological diseases, surgical procedure performed, allergy status, past medical history, smoking status and previous antibiotic therapies. This information will be

designated a code and kept on an encrypted database on the University of Southampton network. People who do not need to know who you are will not be able to see your name or contact details.
Nasal tissue and swabs will collected intraoperatively and given a anonymised code for analysis which corresponds to the above data. The nasal tissue and swabs will be stored in a freezer in a locked laboratory at Southampton University. The tissue will be used to look at inflammatory proteins and the effects that various bacteria have on inflammatory protein production and molecules typically found within fluid surrounding the brain. The nasal swabs will be used to look at what bacteria is present within the nasal cavity. Anonymised nasal swabs will also be sent to an external laboratory for analysis. When sample analysis is complete we will destroy the remaining samples.
The information collected will be stored in an encrypted database on the University of Southampton computer network using the code designated to the samples. A separate codebreak list will exist which links your name with this code to allow you to withdraw consent at any point. This list will be encrypted and password protected on the NHS computer network.
People will use this information to do the research or to check your records to make sure that the research is being done properly.

We will keep all information about you safe and secure.
Once we have finished the study, we will keep some of the data so we can check the results. We will write our reports in a way that no one can work out that you took part in the study.
What are your choices about how your information is used?
You can stop being part of the study at any time, without giving a reason by contacting us.
You can ask to view your own data we hold about you by contacting the individual named as study contact in part one.
We need to manage your records in specific ways for the research to be reliable. If you feel any data we hold for you is incorrect please contact the study contact above.
Where can you find out more about how your information is used?
You can find out more about how we use your information
at www.hra.nhs.uk/information-about-patients/
our leaflet available from:
http://www.southampton.ac.uk/assets/sharepoint/intranet/ls/Public/Research%20and%20Integrity%20Privacy%20Notice/Privacy%20Notice%20for%20Research%20Participants.pdf
by asking one of the research team
or by sending an email to rgoinfo@soton.ac.uk
by ringing us on 020 8120 4743

Data Protection Privacy Notice

The University of Southampton conducts research to the highest standards of research integrity. As a publicly-funded organisation, the University has to ensure that it is in the public interest when we use personally-identifiable information about people who have agreed to take part in research. This means that when you agree to take part in a research study, we will use information about you in the ways needed, and for the purposes specified, to conduct and complete the research project. Under data protection law, 'Personal data' means any information that relates to and is capable of identifying a living

individual. The University's data protection policy governing the use of personal data by the University can be found on its website (https://www.southampton.ac.uk/legalservices/what-we-do/data-protection-and-foi.page).

This Participant Information Sheet tells you what data will be collected for this project and whether this includes any personal data. Please ask the research team if you have any questions or are unclear what data is being collected about you.

Our privacy notice for research participants provides more information on how the University of Southampton collects and uses your personal data when you take part in one of our research projects and can be found at http://www.southampton.ac.uk/assets/sharepoint/intranet/ls/Public/Research%20and%20Integrity%20Privacy%20Notice/Privacy%20Notice%20for%20Research%20Participants.pdf

Any personal data we collect in this study will be used only for the purposes of carrying out our research and will be handled according to the University's policies in line with data protection law. If any personal data is used from which you can be identified directly, it will not be disclosed to anyone else without your consent unless the University of Southampton is required by law to disclose it.

Data protection law requires us to have a valid legal reason ('lawful basis') to process and use your Personal data. The lawful basis for processing personal information in this research study is for the performance of a task carried out in the public interest. Personal data collected for research will not be used for any other purpose.

For the purposes of data protection law, the University of Southampton is the 'Data Controller' for this study, which means that we are responsible for looking after your information and using it properly. The University of Southampton will retain the data for a minimum of 10 years from the end of the project. Any data used for publications will be anonymised and held in the institutional repository.

To safeguard your rights, we will use the minimum personal data necessary to achieve our research study objectives. Your data protection rights – such as to access, change, or transfer such information - may be limited, however, in order for the research output to be reliable and accurate. The University will not do anything with your personal data that you would not reasonably expect.

If you have any questions about how your personal data is used, or wish to exercise any of your rights, please consult the University's data protection webpage (https://www.southampton.ac.uk/legalservices/what-we-do/data-protection-and-foi.page) where you can make a request using our online form. If you need further assistance, please contact the University's Data Protection Officer (data.protection@soton.ac.uk).

Thank you for taking the time to read this information sheet. If you decide to participate in the study, please keep this sheet with your copy of the signed consent form.

Appendix E Patient consent form

IRAS Number: 275758 ERGO Number: 54006 Study Sample Number: .....

PATIENT CONSENT FORM

Bacterial control of inflammation & neuromarkers in the upper airways

Mr S Goldie, Mr M Alsalem, Prof R Carare, Prof. R Salib

Please initial box

I confirm that I have read and understand the information sheet dated 10/01/2020 (version 1) for the above study. I have had an opportunity to consider the information, ask questions and have had these questions answered satisfactorily. ☐ 1

I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐ 2

I agree to being asked questions regarding my medical history as part of the research project and consent to this data being kept for the duration of the study. ☐ 3

I agree to my nasal tissue, taken as part of my surgery, being stored for the duration of this study for use in this research project into inflammatory processes. ☐ 4

I agree to nasal swabs being taken intraoperatively and there storage for the duration of this study for use in this research project. ☐ 5

I agree to my nasal tissue, taken as part of my surgery, to be transferred securely and anonymously to those centres to get the best information possible with respect to the assessing the presence of bacteria and fungi. ☐ 6

I agree to the use of my data as outlined in the patient information sheet dated 15/07/2020 (version 1.5). ☐ 7

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent (if different from researcher)	Date	Signature
_____	_____	_____
Name of Researcher	Date	Signature

When completed, 1 copy for patient; 1 copy for researcher site file

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