Novel Topical Anti-biofilm Agents in the Treatment of Recalcitrant Chronic Rhinosinusitis

A thesis submitted for the degree of Doctor of Philosophy

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Dedication

To my parents who gave us the best of everything.

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Abstract

Novel Topical Anti-biofilm Agents in the Treatment of Recalcitrant Chronic Rhinosinusitis

Chapter 1 of this thesis reviews the current literature on Chronic Rhinosinusitis (CRS) including its aetiology and the role of bacterial biofilms in CRS. It also presents an updated review on all current and emerging topical anti-biofilm options in the management of CRS.

Chapter 2 describes the optimisation of an *in vivo* model using Chitogel (CG) as a drug delivery vehicle for anti-biofilm agents to treat *Staphylococcus aureus* biofilms. Budesonide and Mupirocin were incorporated as they have some efficacy in the management of recalcitrant CRS in the form of topical irrigations. This study showed that Chitogel- Budesonide-Mupirocin gel significantly reduces *S. aureus* biofilms *in vivo* and is the first study to suggest an alternative mode of topical drug delivery to the sinuses.

Chapter 3 furthers the study of topical anti-biofilm gel by incorporating novel antimicrobial agents Deferiprone and Gallium Protoporphyrin (DG) into Chitogel. DG has a synergistic anti-biofilm effect against *S. aureus* and Methicillin Resistant *S. aureus* (MRSA) biofilms by targeting the iron metabolism pathway that is crucial for bacterial growth and survival. This study has shown that Chitogel- Deferiprone-Gallium Protoporphyrin is safe and effective in eradicating *S. aureus* biofilms *in vivo*.

Chapter 4 explores the potential wound healing properties of Deferiprone *in vitro*. In this study Deferiprone was shown to delay primary nasal fibroblast migration without affecting epithelial migration, decrease collagen and reactive oxygen species (ROS) production and reduce interleukin 6 (IL-6) production. This anti-inflammatory potential and ability to limit scar tissue formation has wide prospective applications in the field of sinus surgery.

Chapter 5 explores the long-term safety of *S. aureus* bacteriophage (NOV012) *in vivo* as a novel treatment option in CRS. Bacteriophage (Phage) therapy was first proposed as an antibacterial treatment in the 1910s and has been recently revived in the face of a global antibiotic resistance crisis. In this study we have shown that *S. aureus* phage are safe as a topical sinus irrigation *in vivo* for up to 3 weeks.

Chapter 6 furthers the study of *Pseudomonas aeruginosa* phage (CT-PA) *in vivo*. Topical sinus irrigation of CT-PA was safe and effective in reducing *P. aeruginosa* biofilms *in vivo* over 3 weeks.

Chapter 7 explores the translation of our *in vivo* studies into the first phase-1 clinical trial investigating the safety and preliminary efficacy of phage in *S. aureus* CRS. It was safe and well tolerated up to 3×10^9 PFU twice daily for 14 days with no dose-limiting side effects. Preliminary efficacy indicated favourable outcomes across all cohorts with 2 out of 9 patients achieving eradication of infection.

Chapter 8 presents a pilot study investigating the evidence behind alternative treatments like colloidal silver (CS) in recalcitrant CRS. This is the first clinical study

looking at the safety and efficacy of CS as a topical sinus irrigation in the management of CRS despite it being widely available for purchase over-the-counter. Twice daily CS sinonasal rinses for 10 days is safe but not superior to culture-directed oral antibiotics. We believe that a larger study and further optimisation of treatment duration could further the potential of this therapy.

Chapter 9 investigates the role of manuka honey (MH) sinus irrigations with augmented methylglyoxal (MGO) in recalcitrant CRS. Twice daily MH rinses for 14 days have not been shown to be superior to culture-directed oral antibiotics and saline rinses in bacterial eradication but have shown significant improvement in endoscopic scores comparable to control.

In the face of a global antibiotic resistance crisis, now more than ever, clinicians are practising more judicious use of systemic oral antibiotics. With an eye to the future, we hope that this thesis on novel topical anti-biofilm therapies would spur further research and someday offer clinicians viable alternatives to systemic oral antibiotics in the management of recalcitrant CRS.

Declaration

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

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Publications arising from thesis

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Safety and Efficacy of Topical Chitogel- Deferiprone- Gallium Protoporphyrin in Sheep Model

Ooi ML, Richter K, Drilling AJ, Thomas N, Prestidge CA, James C, Moratti S, Vreugde S, Psaltis AJ, Wormald PJ

Frontiers in Cellular and Infection Microbiology, Special Edition, 2018 June

Deferiprone has Anti-inflammatory Properties and Reduces Fibroblast Migration *in vitro*: A New Anti-Adhesion Product?

Ramezanpour M, Smith J, Ooi ML, Gouzos M, Psaltis AJ, Wormald PJ, Vreugde S

Nature Scientific Reports, 2019 February

Long-Term Safety of Topical Bacteriophage Application to the Frontal Sinus Region

Drilling AJ, Ooi ML, Miljkovic D, James C, Speck P, Vreugde S, Clark J, Wormald PJ

Frontiers in Cellular and Infection Microbiology, Special Edition, 2017 February

Safety and Efficacy of a Bacteriophage Cocktail in an *in vivo* model of *Pseudomonas aeruginosa* Sinusitis

Fong SA, Drilling AJ, Ooi ML, Vreugde S, Psaltis AJ, Wormald PJ

Translational Research, 2018 December

Safety and Tolerability of Bacteriophage Therapy for Chronic Rhinosinusitis Due to *Staphylococcus aureus*

Ooi ML, Drilling AJ, Morales S, Fong SA, Moraitis S, Macias-Valle L, Vreugde S, Psaltis AJ, Wormald PJ

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Topical Colloidal Silver for the Treatment of Recalcitrant Chronic Rhinosinusitis

Ooi ML, Richter K, Bennett C, Macias-Valle L, Vreugde S, Psaltis AJ, Wormald PJ

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Manuka Honey Sinus Irrigations in Recalcitrant Chronic Rhinosinusitis: Phase 1 Randomized, Single-Blinded Placebo-Controlled Trial

Ooi ML, Jothi A, Bennett C, Ooi E, Vreugde S, Psaltis AJ, Wormald PJ

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A Phase 1 Clinical Trial to Evaluate the Safety, Tolerability and Preliminary Efficacy of Bacteriophages in Patients with Staphylococcus aureus Chronic Rhinosinusitis
Australian Society of Otolaryngology Head and Neck Surgery (ASOHNS) Annual Scientific Meeting, Adelaide Australia, March 2017

Topical Colloidal Silver for the Treatment of Recalcitrant Chronic Rhinosinusitis
American Rhinologic Society Meeting, Chicago USA, September 2017

Phase 1 Bacteriophage Trial in Patients with Recalcitrant *Staphylococcus aureus* Chronic Rhinosinusitis
Basil Hetzel Institute, Research Day Meeting, Adelaide Australia, October 2016

Viruses: Friend or Foe in Sinus Infection
Invited Speaker Clinical updates, South Australian and Northern Territory Australian Society of Microbiology, Adelaide Australia, April 2017

Emerging Treatment Options in Chronic Rhinosinusitis
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Phage Therapy for Staphylococcus aureus Chronic Rhinosinusitis: a Phase 1 First-in-Human Study

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Abbreviations

CRS	Chronic rhinosinusitis
ARS	Acute rhinosinusitis
ABRS	Acute bacterial rhinosinusitis
VRS	Viral rhinosinusitis
RARS	Recurrent acute rhinosinusitis
CRSwNP	Chronic rhinosinusitis with nasal polyps
CRSsNP	Chronic rhinosinusitis without nasal polyps
Th 1	Type 1 T helper
Th 2	Type 2 T helper
IL	Interleukin
IFN-γ	Interferon-γ
TGF-β	Transforming Growth Factor-β
Ig E	Immunoglobulin E
AERD	Aspirin exacerbated respiratory disease
CHES	Chronic hyperplastic eosinophilic sinusitis
AFRS	Allergic fungal rhinosinusitis
SE-Ig E	Staphylococcal Enterotoxin specific Immunoglobulin E
IgG	Immunoglobulin G
G-CSF	Granulocyte-colony stimulating factor
bFGF	Basic fibroblast growth factor
IP-10	IFN-induced protein 10
IgM	Immunoglobulin M
CF	Cystic fibrosis
HIV-AIDS	Human Immunodeficiency Virus- Acquired Immunodeficiency Syndrome
CD	Cluster of differentiation
IgA	Immunoglobulin A
Th17	T-helper cell 17
TLOs	Tertiary lymphoid organs
SLE	Systemic lupus erythematous
RA	Rheumatoid arthritis
ESS	Endoscopic sinus surgery
COX	Cyclooxygenase
RAST	Radioallergosorbent test
Alternaria sp	Alternaria species
Aspergillus sp	Aspergillus species
S. pneumoniae	*Streptococcus pneumoniae*
H. influenza	*Haemophilus influenza*
M. catarrhalis	*Moraxella catarrhalis*
S. aureus	*Staphylococcus aureus*
P. aeruginosa	*Pseudomonas aeruginosa*
K. pneumoniae	*Klebsiella pneumoniae*
P. mirabilis	*Proteus mirabilis*
Enterobacter sp	Enterobacter species
E. coli	*Escherichia coli*
SCV	Small colony variants
MIC	Minimum inhibitory concentrations
ISMS	International Sinonasal Microbiome Study

Corynebacterium sp	Corynebacterium species
S. epidermidis	*Staphylococcus epidermidis*
AMT	Appropriate medical therapy
PARI	Pulsating aerosol delivery system
SNOT-20	20-item Sinonasal outcome test
QoL	Quality of life
RCT	Randomized controlled trial
INCS	Intranasal corticosteroid
cAMP	Cyclic adenosine monophosphate
HPAA	Hypothalamic- pituitary- adrenal axis
SNOT-22	22-item Sinonasal Outcome Test-22
HPF	High-power field
LKS	Lund-Kennedy Score
MRSA	Methicillin-resistant *Staphylococcus aureus*
EDTA	Ethylene diamine-tetraactic acid
RNA	Ribonucleic acid
CT	Computed tomography
TEER	Transepithelial electrical resistance
ALI	Air-liquid interface cell cultures
MH	Manuka honey
MGO	Methylglyoxal
CS	Colloidal silver
ATP	Adenosine triphosphate
DNA	Deoxyribonucleic acid
MMP	Matrix metalloproteinases
MT	Metallothionein
TNF-α	Tumour necrosis factor-α
FDA	United States Food and Drug Administration
Phage	Bacteriophage
GRAS	Generally Recognised as Safe
WHO	The World Health Organization
BIM	Bacteriophage insensitive mutants
ICTV	International Committee on Taxonomy of Viruses
NT	No treatment
DG	Deferiprone and Gallium Protoporphyrin IX
Cip	Ciprofloxacin
VAS	Visual analogue scale
NO	Nitric oxide
CAZS	Citric acid/zwitterionic surfactant
S-CMC	S-Carboxymethylcysteine
ATCC	American Type Culture Collection
CFU	Colony forming unit
SEM	Scanning electron microscopy
PBS	Phosphate buffered solution
OsO_4	Osmium Tetroxide

Chapter 1: Introduction

1.1 Chronic Rhinosinusitis (CRS)

Definitions

Rhinosinusitis is defined as symptomatic inflammation of the nasal cavity and paranasal sinuses.

In 2015 the American Academy of Otolaryngology–Head and Neck Surgery Foundation published an updated clinical practice guideline on the management of adult rhinosinusitis[1]. In this published guideline, acute rhinosinusitis (ARS) was defined as ≤4 weeks of purulent nasal drainage (anterior, posterior or both) with associated nasal obstruction, facial pain/ pressure/fullness or both.

ARS was further classified as acute bacterial rhinosinusitis (ABRS) or viral rhinosinusitis (VRS). Clinically both entities can be hard to distinguish from the onset of symptoms, therefore differentiation was recommended based on illness pattern and duration. The body suggested that persistent symptoms beyond 10 days or worsening symptoms after an initial improvement (double worsening) indicates ABRS, whereas viral disease is self-limiting and usually lasts for less than 10 days.

Recurrent acute rhinosinusitis (RARS) is another clinical entity important to be distinguished from chronic rhinosinusitis (CRS). Recurrent acute rhinosinusitis (RARS) is defined as ≥4 episodes of acute rhinosinusitis over 12 months, with the clear

distinction that patients are without signs or symptoms between episodes. Whilst to make a diagnosis of CRS, patients must have persistent subjective symptoms over 12 weeks and meet objective clinical criteria below:

≥12 weeks of 2 or more of the following subjective symptoms

- Nasal obstruction (congestion)
- Mucopurulent drainage
- Facial pain, pressure or fullness
- Reduced sense of smell

AND inflammation observed as

- Purulent mucus or edema in the middle meatus or anterior ethmoid, and/or
- Polyps in the nasal cavity or middle meatus, and/or
- Radiographic imaging showing paranasal sinus inflammation

Epidemiology and burden of disease

In the United States 1 in 7 adults are affected by rhinosinusitis, resulting in approximately 30 million cases diagnosed annually[2], and costing over $8.6 billion per year in national health-care expenditure[3] and 11.5 million work days lost[4].

Similarly, in Australia, it is one of the most common primary care presentations. 1.4 of every 100 general practice encounters are for sinusitis. In 2011 and 2012, approximately 1.9 million Australians were diagnosed with CRS.[5]

Apart from being a significant economic burden, there is evidence that CRS has substantial adverse effect on patients' quality of life; with the greatest impact on their physical domains, followed by functional and emotional domains.[6] CRS patients have reported lower quality of life index than chronic heart failure, chronic obstructive pulmonary disease, angina and back pain.[7]

Changing paradigm: Phenotyping vs Endotyping CRS

Although the definition of CRS has remained unchanged over the years, our understanding of the disease process has been steadily shifting**.** Historically CRS has been grouped phenotypically into CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP). It was once believed that CRSwNP and CRSsNP are on different spectrums of the same disease but recent studies have suggested they are quite distinct entities with completely different pathomechanisms.

The molecular differences in CRSwNP and CRSsNP was first demonstrated when a larger preponderance of Type 1 T helper (Th1) cells was noted in patients with CRSsNP and Type 2 T helper (Th2) cells and eosinophils in patients with CRSwNP.

Non eosinophilic sinusitis is believed to be caused by chronic obstruction of sinus outflow tract from protracted rhinitis[8]. This spurs a remodeling process mainly driven by Th1 pathways, with characteristic elevation of IL-17, IFN-γ and TGF-β[9]. This

remodeling is predominantly a fibrotic process. It initiates changes to sinonasal epithelium which includes ciliary denudation, increased mucus production from goblet cell and glandular hyperplasia, increased viscocity of sinonasal mucus from excessive collagen deposition in the extracellular matrix[10] and ultimately hyperreactive and thickened respiratory lining from chronic infiltration of mononuclear cells and neutrophils[8].

Eosinophilic sinusitis is predominantly characterized by Th2 driven inflammation with elevated Ig E, IL-4, IL-5, IL-13 and eotaxin which is responsible for allergic sensitization and eosinophilic infiltration and activation[11, 12]. The degranulation of activated eosinophils is believed to drive polypoidal formation seen in CRSwNP. It also encompasses aspirin exacerbated respiratory disease (AERD)[13], chronic hyperplastic eosinophilic sinusitis (CHES)[14] and allergic fungal rhinosinusitis (AFRS) which has prominent eosinophilic expressions[8].

However, recent data has suggested that the inflammatory profiles in CRS patients may be more diverse than previously believed and have important prognostic and therapeutic value. In fact, Tomassen et al[15] reported that patients with CRS could be differentiated into 10 clusters based on characteristic groups of cytokines identified. The presence of IL-5 seems to be the main indicating factor and together with the presence of Staphylococcal Enterotoxin (SE)-IgE and Ig E, are strongly associated with CRSwNP and comorbid asthma. Interestingly, 3 clusters of cytokine profiles were associated almost exclusively with CRSsNP and 3 clusters were associated exclusively with CRSwNP and asthma as shown below (Figure 1).

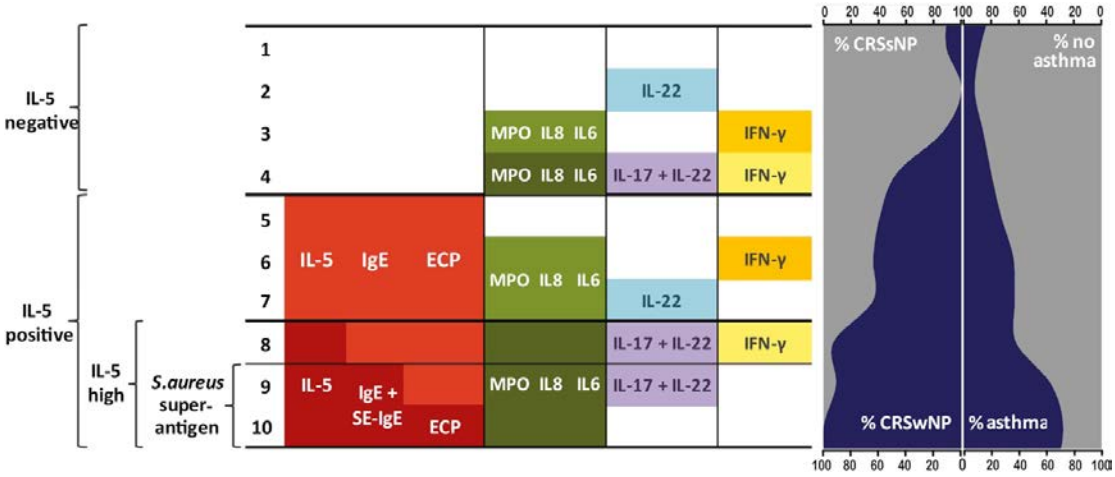

Figure 1. Different clusters of CRS endotypes based on distinct cytokine expressions and its corresponding relationship with CRS phenotypes. Adapted from Tomassen et al (2015)[15]

This finding has been consistent with Bachert et al[16] who was first to suggest IL-5 positive polyps and increased serum SE-IgE patients were strongly associated with comorbid asthma and poorer prognosis. Bachert et al[16] described the clinical significance of identifying these 3 main endotypes 1) non-type 2, 2) moderate type 2, 3) severe type 2 reactions for its prognostic value and for identifying subsets of patients who may be able to benefit from endotype- specific biologics therapy ie. anti-IgE, anti-IL 5, anti-IL 5 receptor α, and anti-IL 4 receptor α (Figure 2).

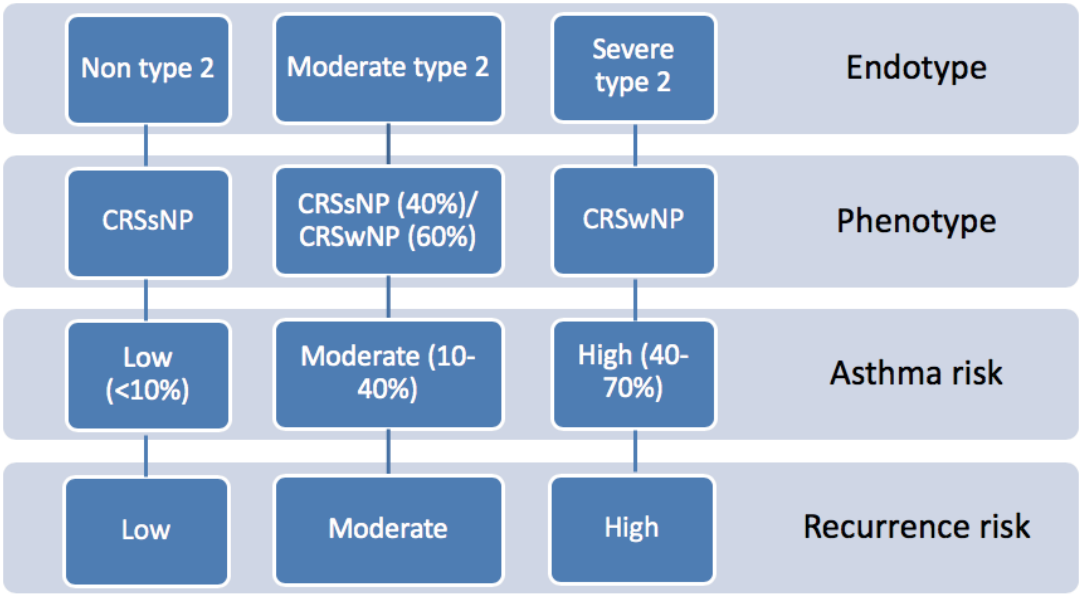

Figure 2. Comorbid condition and prognosis based on patient stratification. Adapted from Bachert et al (2016)[16]

The framework which Bachert et al[16] proposes for patient stratification to differentiate between non-type 2 and type 2 driven inflammatory endotype patients is shown below (Table 1).

Biomarkers	**Clinical assessment**
Serum eosinophils > 300/µL	Comorbid late onset asthma
Total serum IgE > 150kU/L	Recalcitrance after adequate sinus surgery
Other biomarkers: Presence of serum SE-IgE, Increased serum periostin level	

Table 1. Patient stratification between non-type 2 and type 2 driven inflammatory endotypes based on serum biomarkers and clinical assessment. Adapted from Bachert et al (2016)[16]

Since then, several studies have attempted to perform cluster analyses with the inclusion of several other known inflammatory markers in CRS as well as in accordance to patients' treatment response.

Lou et al[17] further stratified CRSwNP patients into 5 endotype clusters based on dominant inflammatory cell pathways; 1) plasma cell dominant, 2) lymphocyte dominant, 3) neutrophil dominant, 4) eosinophil dominant and 5) mixed inflammatory. Plasma cell dominant and lymphocyte dominant patients had good prognosis while eosinophil dominant patients, consistent with current literature, had the highest risk of polyp recurrence.

Liao et al[18] reported 7 CRS clusters amongst Chinese patients with the inclusion of 28 clinical parameters and 39 biomarkers taking into account patients' treatment response (Table 2).

	Main features	Polyp status	Difficult to treat cases*	Biomarkers
Cluster 1	Type 2 response dominated eosinophilic CRS; High frequency of comorbid asthma and allergic rhinitis	All CRSwNP	50%	Highest serum eosinophil, mucosal eosinophil, mucosal level of IL-5, IL-13, eotaxin, IgG4, Ig E. Low tissue IL-8, mucosal neutrophil, Granulocyte-colony stimulating factor (G-CSF), basic fibroblast growth factor (bFGF)
Cluster 2	Severe atopy CRS	Predominant CRSsNP	35%	Mild eosinophilic inflammation: Slight increase in IL-5 and IL-4 and eosinophil
Cluster 3	Neutrophilic CRS	Predominant CRswNP	44%	Highest mucosal neutrophil, Highest tissue IL-1β, IL-1Ra, IL-6, IL-8, IL-10, interferon (IFN)-γ, IFN-induced protein 10 (IP-10), G-CSF, monocyte chemoattractant protein-1, macrophage inflammatory protein-1β, IgG1, and IgM
Cluster 4	Young male CRS	Equal CRSwNP and CRSsNP	21%	Highest serum neutrophil leukocyte and monocyte count, highest mucosal IgG3
Cluster 5	High IL-10 CRS	Predominant CRSwNP	Good outcomes	
Cluster 6	Long disease duration, previous surgeries and moderate neutrophilic CRS	All CRSwNP	46.15%	Moderate levels of IL-8, neutrophilic inflammation
Cluster 7	Non-eosinophilic, non-neutrophilic CRS	Predominant CRSwNP	10%	Low cellular and molecular biomarkers

Table 2. CRS clusters based on biomarkers and clinical responses. Summarized from Liao et al (2018)[18].

*Difficult-to-treat cases, as defined by the European Position Paper on Rhinosinusitis and Nasal Polyps, are subset of patients with poor disease control despite acceptable surgery, intranasal corticosteroids, and up to 2 short courses of antibiotics or systemic corticosteroids in the last year.

Liao at al[18] demonstrated that neutrophilic-driven CRSwNP cohorts are more often seen in Asian populations[19, 20], but that these too can behave like the eosinophilic-driven Caucasian cohorts associated with poorer disease outcomes and higher risk of disease recalcitrance. This illustrates that both eosinophilic and neutrophilic pathways can drive recalcitrant nasal polyposis with poor prognosis and suggests potential variations in developing clinical endotypes within different communities or geography.

Different proposals of CRS clustering are likely to continue to emerge with inclusion of different clinical and biomarker parameters in the near future, however its clinical significance remains to be defined in parallel with the emerging role and impact of biologic therapeutics.

This finding

1) Provides a novel understanding of the various CRS endotypes which exists within previously conformed phenotypical subgroups,

2) Challenges our understanding of what constitutes best medical management for each individual patient and confronts our previous paradigm of a one-size-fits-all treatment,

3) Raises new potential for therapeutic agents with immunomodulatory functions.

4) Spurs future research in the discovery of other endotypes in CRS

Aetiology of CRS

In the 1980s, the causal principles of CRS had been based mainly on conceptual understanding of sinus ostia obstruction resulting in impaired sinus drainage, altered sinonasal ventilation and inadequate mucociliary clearance which resulted in stagnation of sinonasal secretions that precipitated chronic infection[21].

However, since then our understanding of the causal factors of CRS has improved significantly with integration of our renewed understanding of distinct inflammatory processes driving CRS. It is now believed that CRS is a multifactorial inflammatory process (Table 3).

Systemic host response	Local host factors	Environmental factors
Mucociliary dysfunction Immune Dysregulation Allergy Aspirin intolerance	Anatomic Neoplasm Previous trauma or surgery	Noxious stimuli (tobacco) Microbiological trigger (viral, fungi, bacteria, small colony variants) Microbial dysbiosis

Table 3. Aetiology of CRS

Mucociliary dysfunction

Mucociliary clearance constitutes an integral line of our defense mechanism. Cilia on sinonasal mucosa traps foreign particles and transports mucus to the postnasal space then into the oesophagus. Impaired cilia is thought to result in respiratory secretion stagnation which promotes bacterial propagation[22] and chronic inflammation[23]. Primary ciliary dyskinesia disorders which includes Kartagener's syndrome and disorders with

secondary ciliary dysfunction such as cystic fibrosis (CF) are usually associated with CRS.

Immune Dysregulation

Abnormalities in immune functions have been associated with recalcitrant CRS. However, our understanding has been previously focused on the role of immune deficiency in CRS. Several immunodeficiency disorders such as HIV-AIDS with CD4+ deficiency are associated with recalcitrant CRS[24, 25]. Primary humoral deficiency such as common variable immunodeficiency has up to 36% of patients presenting with initial symptoms of CRS[26]. Patients with CRS have also been associated with a number of immunoglobulin deficiencies i.e. IgA deficiency[27], IgG subclass deficiency[28] and dysfunctional IgG responses[27]. The inability to mount an effective immune response against infectious onslaughts due to weakness in innate and adaptive immunity has been implicated as the driver of disease recalcitrance.

In recent years, there has been increased understanding and insight into the relationship between immune dysregulation and disequilibrium with CRS. Miljkovic et al[29-31] demonstrated that CRSwNP patients have distinctly different immune regulatory cells compared to CRSsNP and healthy controls. CRSwNP patients were found to have increased mucosal B cell subtypes[30], differing B cell expression of CD180[32], increased T regulatory cells[29], Th17 cells and its associated cytokines (ie. IL-17A, IL-17F, IL-21, and IL-22) within their polyps[31]. This perspective of immune hyperactivity driving disease continuity challenges our previous understanding of the role of our immune system in CRS. This also further supports our current understanding of CRSwNP and

CRSsNP being distinctly different disease entities driven by distinctly different pathophysiology.

Tertiary Lymphoid Organs

Tertiary lymphoid organs (TLOs) are an organized accumulation of T and B cell lymphoid follicles outside of secondary lymphoid organs, induced de novo from non-lymphoid tissues. They are found in chronic inflammatory sites of autoimmune diseases such as SLE and RA[33]. Thought to form in response to a persistent antigen or chronic immununological stimulation, it provides continuous extravasation of leukocytes and is commonly observed at sites of infection, transplantation, and autoimmunity. During an infection, it is believed that TLOs are established to provide local protective immunity and usually resolves in conjunction with the infection. However in recalcitrant or recurrent infections, it has the potential to produce ongoing humoral immune responses which could be damaging to surrounding tissues. Although this phenomenon has long been associated with autoimmune diseases, Lau et al[34] was first to demonstrate the presence of TLOs in CRS patients and its association with poorer outcomes. The study was able to demonstrate a positive association of TLOs with increased number of previous surgeries.

Lau et al[34] also examined hematoxylin and eosin-stained sinonasal mucosa sections of 158 patients for the presence of TLOs and eosinophilia. TLOs were not observed in control patients and were found exclusively in CRS patients; of that 10% were seen in CRSsNP subset compared to 37% observed in patients with CRSwNP. TLOs were also found to be positively associated with tissue eosinophilia, which is consistent with our current understanding of CRSwNP being driven by eosinophilic inflammatory

responses. The study elucidated that CRSwNP patients have significantly upregulated genes related to TLO formation and maintenance.

Paramasivan et al[35] also reported significant upregulation of inflammatory genes seen in TLO-positive patients. It is believed that chronic antigen presentation acts as a catalyst for TLO formation initially to confer tissue protection but subsequently becomes a source of immunoglobulin production[36] perpetuating local damage and inflammation.

Interestingly, T helper cells and their effector cytokines, in particular Th17 cells and associated IL-17 cytokines, have been recently identified as key initiators of TLO formation.[37] As described by Miljkovic et al[31] in earlier sections of our discussion, Th17 cells and its associated cytokines (ie. IL-17A, IL-17F, IL-21, and IL-22) have been found with increased prevalence within the polyps of patients with CRSwNP. It has also been identified as the dominant pathway driving CRSwNP disease in Asian cohorts.[38, 39] This raises the possibility that patients with Th17 driving CRSwNP disease if found to have concurrent TLOs, may respond better to biologic therapies targeting Th17 pathways. TLOs could potentially serve additionally as a prognostic factor to help select patients who would benefit from such targeted biologic therapies.

Neo-osteogenesis

Osteitic bone has been shown to be associated with recalcitrant CRS although its role as a cause or effect has yet to be ascertained[40]. The radiological severity (Lund Mackay Score) of CRS and number of revision surgeries are positively associated with the extent of osteitis[41], implicating its prognostic value. However its pathomechanics have

yet to be elucidated. Oue et al[42] was first to suggest that patients with neo-osteogenesis had a higher IL-13 expression. IL-13 has been found to increase calcium rich mineralisation and if IL-13 is denatured prior to osteoblast activation this effect could be reversed. This finding was first to illustrate a relationship between neo-osteogenesis with CRS and have shown that if treated the process of osteitis could potentially be reversed.

Allergy

The role of allergy in the pathogenesis of CRS remains a debate. Allergic patients have been associated with more severe disease[43] and higher prevalence of recalcitrance after endoscopic sinus surgery (ESS)[44], but there has also been studies showing no prognostic association with CRS[45]. Allergic rhinitis is currently defined as a cluster of allergic reactions such as nasal itchiness/ congestion/ rhinorrhea, sneezing and itchy eyes following exposure to allergen(s) together with an objective demonstration of allergic sensitization with positive skin prick test and/or increased serum specific IgE test. In contemporary practice, investigation for atopy/ allergy continues to be part of the work up for recalcitrant CRS and managed concurrently with other treatment modalities.

Aspirin intolerance

Samster's triad are patients with impaired COX regulation pathways resulting in a triad of symptoms i.e. sensitivity to aspirin or other COX-1 inhibitors, CRSwNP, and asthma. Bronchospasm and nasal polyposis are believed to be due to increased leukotriene levels secondary to increased arachidonic acid metabolism via 5-lipoxygenase pathway. Patients with Samster's triad are known to have severe CRSwNP with high recalcitrance rates after ESS. Success rates of ESS have been estimated to be 80%, but

recurrence of nasal polyps has been reported to be as high as 40%. Therefore, patients with Samster's triad should receive concurrent aspirin desensitization therapy following ESS to prevent recurrence of CRS with nasal polyps[46].

Environmental factors

Tobacco smoking

Hastan et al[47] showed a strong positive association of CRS with active smokers and a significant association of CRS with ex-smokers. There was also a dose-dependent correlation with patients with higher pack years more likely to report CRS than non-smokers. Ericksson et al[48] supported this by finding that smokers, regardless of gender, had a higher prevalence of CRS.

Microbiology

Viruses

The role of viruses in the pathogenesis of CRS has yet to be determined. Previous studies have found that viral detections are associated with increased prevalence of sinus disease. Gwaltley et al[49] found a high prevalence of osteomeatal occlusion and sinus abnormality in patients with viral upper respiratory tract infections. Cho et al[50] and Jang et al[51] suggested that there is significantly increased viral detections in CRS cohorts compared to controls, specifically noting the higher incidence of rhinovirus. Most recently Goggin et al[52] reported that the presence of viruses has been found to be prognostic of CRS severity. These studies strongly suggest a relationship between viruses and CRS and have prompted renewed interests in elucidating the role of viruses in CRS.

Goggin et al[53] have also demonstrated that different viral species were found at different sampling sites within the nasal cavity which implicated the potential complexity of the relationship between viruses and CRS and highlighted the importance of standardization in the method of sampling in this area of research. These new discoveries will hopefully propel further study in this area and someday help to elucidate the pathomechanics of how self-limiting viral illness can progress into CRS.

Fungi

The role of fungi in CRS is not completely understood. Studies have suggested that fungi provoke a local inflammatory response and eosinophilic infiltration of sinonasal tissue[54] in a certain subset of patients. Bent and Kuhn[55] outlined the entity of allergic fungal rhinosinusitis (AFRS), with the diagnostic criteria below:

Major criteria

1. History of type 1 hypersensitivity (history, IgE/ RAST/ skin prick test)
2. Eosinophilic mucus
3. Positive noninvasive fungal hyphae on staining
4. Nasal polyposis
5. Characteristic CT findings (hyperattenuation/ double signal density)

Minor criteria

1. Asthma
2. Charcot Leyden crystals
3. Serum eosinophilia
4. Unilateral disease
5. Bone erosion and expansion
6. Fungal cultures

To meet the diagnosis of AFRS, patients must meet all major criteria, while minor criteria serve to support the diagnosis.

Emerging evidence suggests that AFRS rather than a form of infection is driven mainly by IgE and IgG immune mediated hypersensitivity response leading to an eosinophilic inflammatory reaction[56]. This is found to be mostly associated with Alternaria sp and Aspergillus sp. However, fungi have been successfully cultured from both control and CRS patients[57]. Furthermore, with increasing evidence showing patients having no benefit from systemic and oral antifungal therapy, the diagnosis of AFRS has been less emphasized.

Bacteria

The role of bacteria in CRS remains uncertain. Historically CRS was largely believed to be infectious in etiology but it is now mainly recognized as an inflammatory process[58]. The inherent difficulty in ascertaining the role of bacteria in driving the pathogenesis of CRS makes it difficult to define the role of antibiotics in clinical management.

Brook et al[59] reported that *S. pneumoniae*, *H. influenza* and *M. catarrhalis* are associated with acute sinusitis while anaerobic bacteria and *S. aureus* are commonly associated with CRS. CRS with gram-negative bacilli are also an emerging clinical entity, especially in patients with recalcitrant disease, such as *P. aeruginosa, K. pneumoniae, P. mirabilis,* Enterobacter sp and *Escherichia coli*[60]. As such, empirical selection of standard antimicrobial agents is not always successful in eradicating these infections and has instead served to propagate antibiotic resistance. Foreman et al[61] also

showed that over 50% of CRS patients exhibit polymicrobial bacterial growth which further complicates treatment decisions in clinical management.

Small colony variants

Small colony variants (SCVs) are subpopulation of bacteria derived from a parent strain characterized by small colony morphology. Proctor et al[62] was first to describe the presence of *S. aureus* SCVs in persistent purulent sinusitis not responding to intensive treatment[63, 64].

SCV-associated infections are challenging to diagnose due to the difficulty in recovering and culturing SCVs[65] and they are even more difficult to eradicate due to several of their innate properties. They are commonly characterised by reduced colony size, reduced virulence expression, slow growth rate and has biofilm forming properties. SCVs ability to be internalised by phagocytes and survive intracellularly in fibroblasts, osteoblasts, keratinocytes, endothelial and epithelial cells, allows it to evade host immune responses[66] and are associated with increased antibiotic tolerance and resistance.

It has been found that SCVs can be induced by exposure to antibiotics such as gentamicin[67] or antiseptics such as triclosan[68]. Sub-therapeutic antibiotic exposure can also trigger biofilm formation by *S. aureus* SCVs and their parent strains[69]. Further challenging treatment is the ability of aminoglycoside resistant SCVs in aiding the survival of sensitive variants[70]. Prolonged antibiotic regimens with higher MICs are often required to treat SCV-associated infections, concurrent with surgical management.

However, in spite of this, clinical outcomes are still less than satisfactory with frequent relapse and recurrence[71].

A theorem shift: Microbiome dysbiosis and CRS

With the rapid advancement of microbiome gene sequencing techniques, we are now beginning to better appreciate microbiota composition of healthy and diseased sinuses. Recently, emerging data has proposed the concept of sinonasal microbiome dysbiosis in perpetuating disease recalcitrance.

Although there is no consensus on what constitutes healthy microbiota composition, a number of studies have consistently demonstrated that CRS patients have less bacterial diversity than healthy controls[72, 73].

The study of sinonasal microbiome to date has mostly been small and heterogeneous with conflicting results. In a recent multi-national, multi-center International Sinonasal Microbiome Study (ISMS), Bassiouni et al[74] proposed that sinonasal microbiome could be 'microbiotyped' into 3 groups: (1) Corynebacterium-dominated, (2) Staphylococcus-dominated, and (3) other core genera dominated (ie. *S. pneumoniae*, *H. influenzae*, *M. catarrhalis*, and *P. aeruginosa*). There were geographical differences in microbiotype predominance but not one microbiotype is associated with a more diseased state. Within the same geography, they found similar microbiotype predominance between healthy and diseased sinuses, and between patients with CRSwNP and CRSsNP. The authors attributed this to be a result of local prescribing practices, variable diet, lifestyle and environmental exposures[75].

Interestingly, they have highlighted an antagonistic relationship between *S. aureus* and Corynebacterium species (Corynebacterium sp) consistent with other literature[76]. Bassiouni et al[74] also proposed that both *S. epidermidis* and Corynebacterium sp may have potential inhibitory effects on *S. aureus*. This had been consistent with earlier murine *in vivo* models reported by Cleland et al[77] who demonstrated that *S. epidermidis* competitively inhibits *S. aureus* resulting in reduced goblet cell counts compared to *S. aureus* inoculated sinuses. Abreu et al[72] demonstrated *in vivo* that the administration of antibiotics (which depletes sinus microbiota) followed by administration of *Corynebacterium tuberculostearicum* significantly increased goblet cell hyperplasia. However, when *Lactobacillus sakei*, which competitively inhibits *Corynebacterium tuberculostearicum*, was administered the effect of goblet cell hyperplasia was nullified. In a separate study, Ramsey et al[78] proposed that this phenomenon could be attributed to potential mechanisms of *Corynebacterium striatum* in shifting the virulence profile of *S. aureus* towards commensalism.

This represents an exciting concept that could challenge our current interpretation of CRS infections. If this observation intimates that microbial composition and interaction supersedes the implication of microbial predominance, should *S. aureus* positive swab be recognised as Corynebacterium sp deplete swab? Can we identify other microflora that competitively inhibits other microbiotypes? In different geographical regions should there be a different set of personalised sinonasal probiotic treatment for different microbiotypes? Will we be able to derive a bank of probiotic treatment that evolves along with changes within the local demographic?

New concerns have also been raised about the effects of repeated oral antibiotic treatment in the management of CRS which had been known to have minimal effect against bacterial biofilms that are commonly associated with recalcitrant CRS. From what we understand based on current knowledge of gut microflora, oral amoxicillin could shift gut microbial composition for an average of 30 days up to 2 months[79], oral ciprofloxacin and second-generation cephalosporins like cefprozil[80] had more persistent effects lasting up to several weeks with highly subject-dependent restoration to baseline[81]. Although several studies have purported that the restoration of microbial diversity is quicker in saliva[82] and throat[83] which may extrapolate to the sinuses, the effect of microbial dysbiosis driven by frequent prescribing of oral antibiotics have yet to be characterised.

Bacterial biofilms and CRS

Biofilms were first described in CRS in 2004[84] however its role in the pathogenesis of CRS continues to be a topic of contention. It is believed that in CRS, altered sinus epithelium, ciliary dysfunction, mucus hypersecretion and reduced innate immunity promotes bacterial overgrowth and formation of biofilms. It has been reported that bacterial biofilms can be found in 29-72% of CRS patients. For these patients antibiotics often improve symptoms during acute exacerbations but fail to remove the biofilm nidus, resulting in a relapsing course of disease as the biofilm sporadically sheds planktonic organisms[85].

Biofilms are clusters of microorganisms encased within an extracellular polymeric matrix. Formation of biofilms aids survival in a hostile environment and propagation to new niches[86]. Biofilms have been found to confer 1000 folds more resistant to antibiotics[87].

There are several theories on how biofilms confer its mechanism of protection

1. Polymeric substances of biofilm matrices prevent penetration of antibiotics[88]
2. Bacteria within biofilm matrices are less metabolically active due to nutrient limitation which renders antibiotics dependent on bacterial replication ineffectual and helps evade innate immunity (polymorphonuclear neutrophils and complement-mediated cytotoxicity)[89]
3. Quorum sensing, a cell to cell gene regulatory mechanism based on cell density. It regulates genes involved in biofilm formation, biofilm dispersion and upregulates synthesis of surfactant molecules[90]. Synergistic interactions between multispecies biofilms also enhances antimicrobial resistance via interspecies gene transfers[91].

Bendouah et al[92] was the first study to demonstrate an association between the presence of *S. aureus* and *P. aeruginosa* biofilm with poorer post-surgical outcome. This was consistent with Psaltis et al[93] who reported that patients with biofilms have significantly more subjective postoperative symptoms and objective mucosal inflammation at 8 months post ESS.

Due to the strong association of bacterial biofilms with poorer surgical outcome, the role of topical anti-biofilm agents in eliminating bacterial biofilms is hoped to improve patients' outcome.

Current Management of CRS

The management of CRS begins with medical therapy. According to the International Consensus Statement on Allergy and Rhinology: Rhinosinusitis[94], the term 'maximal medical therapy' is now replaced with 'appropriate medical therapy'(AMT) to emphasize that surgery should not be delayed if deemed appropriate. Due to the lack of adverse effects, saline irrigations and intranasal corticosteroid are widely accepted to be part of AMT protocol. A short tapering course of oral prednisolone is generally accepted in patients with CRSwNP however its role in CRSsNP is still uncertain but could be considered. Long term macrolide therapy is seen to be appropriate in CRSsNP which is mainly neutrophilic driven while doxycycline has been shown to improve symptoms in patients with CRSwNP which is mainly eosinophilic driven. Culture directed oral antibiotics is often prescribed at a clinician's discretion. In the absence of available culture and antibiotic sensitivity results broad spectrum amoxicillin with clavulanic acid is considered. Patients with allergic rhinitis are often treated with concurrent topical or oral antihistamines. It is also accepted that 3-4 weeks treatment of medical therapy is an appropriate duration, failing which surgery should be deemed an appropriate intervention.

When patients have failed the trial of appropriate medical therapy, ESS is indicated. ESS helps to 1) reventilate sinuses through its natural ostia and restore mucociliary drainage 2) enable penetration of topical irrigations. Overall the success of ESS ranged from 83-92%[95, 96]. A subset of patients that continue to have persistent symptoms of CRS despite adequate medical and surgical treatment are known to have recalcitrant CRS and they continue to present as a challenging entity to manage.

At present there is no consensus on what constitutes appropriate management of recalcitrant CRS and there is insufficient evidence in the literature to provide clinicians with available management options. To date there is some evidence suggesting topical treatments can be effective but paucity and heterogeneity of literature has limited its progressive application.

Topical Delivery Devices

Topical treatments confer several advantages over systemic agents. Topical delivery allows the use of higher concentration of agent, directly to the affected area. This circumvents first-pass clearance and in the case of agents that are poorly absorbed from the nasal mucosa, results in much lower systemic side effects.

The effective delivery of topical treatments into sinus cavities remains the main obstacle. The efficacy of topical therapies is limited by the ability of topical medications

to reach the site of interest. The ideal topical delivery modality achieves complete sinus distribution, prolonged mucosal contact time to increase local absorption and minimal waste[97]. At present there is no single method that has all the desirable characteristics of optimum sinonasal delivery but some modalities confer more advantages than others. Currently, topical treatments are administered via sprays, nasal drops, turbuhalers, breath-actuated insufflators, nebulizers, atomizers, and nasal irrigations. Patients receive highly variable drug penetration and retention influenced by patient condition, sinus surgery, delivery devices, irrigation volume and pressure and patient positioning[97-100].

Below includes a review of various topical sinonasal drug delivery modalities.

Methods of topical delivery

For ease of discussion topical sinonasal delivery devices have been grossly divided into

1) Low volume devices: (μLs) Nasal sprays and turbuhalers; (mLs) nasal drops, atomizers or nebulizers (mLs)
2) High volume devices: (50-240mLs) Squeeze bottles and Neti pots

Nasal sprays

Commercially available nasal sprays generate particles between 50-100 μm which ranges from 25-200 μL per puff. Moller et al[101] reported excellent particle deposition from nasal sprays in the anterior nasal cavity however with less than 1% penetrating into sinuses. This finding is consistent with human nasal replica models with majority of particles found deposited at nasal vestibule, nasal valve, and anterior turbinates[102].

Furthermore, Moller et al[101] have shown high clearance rate from nose with nasal sprays, with less than 50% of activity remaining after 15 minutes and less than 5% activity retained in the nose after 6 hours. This is similar with reports by Suman et al[103] showing 42% clearance rate of nasal spray particles within 30 minutes. It is believed that the high clearance rate from aerosol sprays may be due to larger particles deposited at densely ciliated regions of nasal cavity which physiologically acts as an efficient filter and clearance of aerosol pollutants, however presents as a limiting factor for aerosolized particle delivery.

Laube et al[104] elegantly described the challenges in the effective delivery of aerosolized particles to the middle and superior turbinates and sinuses; 1) need to overcome the anatomy of the anterior 1/3 of the nasal cavity, 2) aerosol distribution is dependent on site of particle release which follows physiologic streamline of nasal airflow, 3) drugs need to exert its effect before mucociliary clearance removes drug particles along the floor of the nose and inferior turbinates to the nasopharynx, 4) diseased sinus ostia are commonly narrowed with oedematous mucosa or polypoidal tissue obstructing the ostiomeatal complex. The study also concluded that smaller particles and slower dispersion optimizes drug particle delivery to the middle meatus and into sinuses.

Hyo et al[105] also reported that for effective delivery of aerosolized particles, it is dependent on 3 main factors; 1) size of sinus ostia, 2) aerosol pressure/ flow rate and 3) particle size with the ideal particle size effective for aerosol delivery into maxillary sinuses between 3 – 10 μm. It is reported that in both healthy subjects and a nasal cast model only 3% of particles between 3 to 10 μm can be found to penetrate the maxillary

sinus.

Saijo et al[106] further supported this hypothesis by demonstrating that higher flow rate, smaller particle size and larger ostia diameter results in better penetration into the maxillary sinus. A 45 degree nozzle insertion angle also significantly increased particle deposition at ostiomeatal complexes compared to 30 degree insertion, which was consistent with the findings of Weber et al[107].

Itoh et al[108] demonstrated similarly that radioaerosol particles that are larger than 4 μm are mainly deposited at ostium internum (where turbulent eddies develop) and the anterior region of middle turbinate (where airflow changes from upward to horizontal), as illustrated in Figure 3.

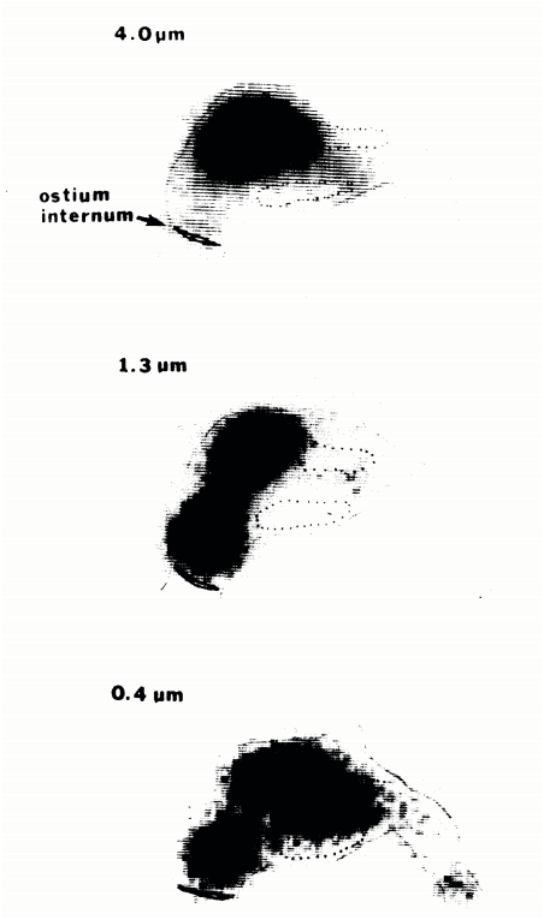

Figure 3. Image of radioaerosol deposition sites within nasal cavity based on different particle sizes. Adapted from Itoh et al (1995)[108].

Based on these studies, we conclude that nasal sprays effectively delivers topical medications to anterior nasal cavity however have limited penetration into sinuses especially prior to endoscopic sinus surgery.

Nasal drops

One of the oldest form of nasal drug delivery, nasal drops are instilled using a pipette or glass dropper, however efficacy can be highly position and technique dependent.

In a randomised, placebo controlled study, Penttila et al[109] reported patients on 400μg of fluticasone propionate nasal drops twice a day over 12 weeks had significant improvement in clinical symptoms and reduction in polyp size when compared to patients on placebo. In a multicentre randomised double blinded study, Keith et al[110] also reported patients on daily 400 μg of fluticasone propionate nasal drops had significant improvement in clinical symptoms and, although did not achieve statistical significance, demonstrated greater reduction in polyps size compared to placebo. Both studies required patients to instil nasal drops in the head down forward position/ Mecca position and may not be amenable to all patients.

Although studies have shown clinical improvements with administration of intranasal steroid via drops, however these studies were compared with non-treatment in patients with CRSwNP thus making it difficult to compare with other intranasal steroid delivery modalities.

Inhalers (ie. Rhinocort turbuhaler)

Tos et al[111] reported that Rhinocort turbuhaler delivering budesonide powder in a multi-dose inhaler device and Rhinocort nasal spray (both administered twice daily for 6 weeks) significantly reduced polyp size when compared to placebo group, but did not show any differences between both delivery modalities. However, Rhinocort nasal spray had significantly better clinical symptoms compared to the turbuhaler group.

Similarly, Andersson et al[112] reported that daily budesonide powder turbuhaler and fluticasone propionate aqueous spray, are both safe and effective in reducing symptoms

of perennial allergic rhinitis compared to placebo, but once again there were no differences between both delivery modalities.

This suggests that while there are some clinical benefits observed in the delivery of intranasal steroid via a nasal turbuhaler device, the clinical effect is not superior to nasal sprays which in previous discussion demonstrated deposition of aerosolized particles mainly in the anterior nasal cavity.

Breath-actuated bidirectional insufflators (ie. OptiNose, OptiMist)

Figure 4. OptiNose breath-actuated bidirectional insufflator device.

Breath-actuated bidirectional insufflator device comes with a flexible mouthpiece and an asymmetrically shaped nosepiece. As the patient inserts the nosepiece into one nostril and starts blowing through the mouthpiece, the device uses the patient's exhaled breath to naturally close the soft palate (obstructing drug entry into the lungs) and to facilitate delivery of drug to the nasal passages. The nosepiece is designed to seal the nostril and also expand and stent the nasal valve, which is commonly the narrowest part

of the nasal passage. The patient completes use by actuating the device to deliver aerosolized particles of 43 μm into one nostril and out of the contralateral nostril achieving bidirectional delivery.

Djupesland et al[113] reported significantly better deposition of radiolabelled particles in the middle meatus sinus ostia and olfactory region by using bidirectional device OptiNose and less deposition in anterior nasal cavity when compared to nasal spray.

In a prospective, randomised placebo controlled trial Hansen et al[114] reported twice daily 400 μg fluticasone dipropionate delivered using OptiNose was found to demonstrate significant improvement in clinical symptoms and nasal endoscopy scores when compared to placebo in recalcitrant CRSsNP.

In a multicenter, randomized, double-blind, placebo-controlled, parallel-group study, Djupesland et al[115] also reported 400 μg of twice daily fluticasone dipropionate delivered using OptiNose device for 12 weeks compared to placebo showed significant and progressive reduction of polyp size in all polyp size subgroups.

These studies suggest that intranasal steroid delivery via bidirectional insufflators have demonstrable clinical benefits when compared to no treatment and may be superior to nasal sprays but is indeterminate in its penetration into sinuses.

Medical nebulizers

Passive diffusion nebulizer (ie. SinuNeb, RinoFlow)

Nebulizers facilitate the breakup of therapeutic suspensions into aerosolized droplets using compressed gasses, mechanical or ultrasonic power and deliver medicated particles via direct inhalation or dispersion into nasal or oral cavity. Passive-diffusion nebulizers (compared to vortex atomizers) produces smaller particles delivered at a slower velocity in a fixed direction.

Although Suman et al[103] reported improved distribution of nebulized radioaerosol particles past anterior nasal cavity when used with a nebulizer fitted with a nasal adaptor when compared to nasal sprays, majority of studies investigating passive diffusion nebulizers have reported inconsistent and minimal penetration into sinuses.

Passive-diffusion nebulizer SinuNeb device produces aerosolized particles of 3 μm and was found by Miller et al[116] to be inferior to bulb irrigation in particle distribution to the anterior nasal cavity, posterior nasal cavity, middle turbinate, ethmoidal and maxillary sinus in patients post ESS.

Other passive diffusion nebulizers like RinoFlow device delivers aerosolized particles between 20-30 μm with recommendation to commence irrigation with micronized saline jet at turbulent flow of 8 L/min followed by laminar flow of 4 L/min for best results. However, Negley et al[117] reported incomplete and inconsistent technetium Tc^{99m} deposition in frontal and maxillary sinuses by RinoFlow at laminar flow of 4 L/min.

These studies suggest that passive diffusion nebulizers have unreliable penetration into the sinuses and limited deposition past anterior nasal cavity.

Vortex propelled nebulizer/ atomizer (ie. ViaNase)

Figure 5. ViaNase vortex propelled nebulizer/ atomizer device.

Handheld battery operated atomizers (ViaNase) produces a vortical flow on exiting particles between 9–14 μm (larger particles than passive diffusion nebulizers). This centrifugation of particles to the outer edge of the vortex disrupts inherent nasal airflow streams and allows aerosolized particles to penetrate deeper into the nasal cavity. Giroux et al[118] examined the effects of vortical flow on aerosol deposition in the nasal cavity of healthy subjects. When compared to passive diffusion nebulizer, the ViaNase device demonstrated enhanced distribution of particles within the nasal cavity in the region of the middle meatus and the superior and posterior nasal cavity.

In a radionuclide study Hwang et al[119] demonstrated that in healthy unoperated individuals, vortex nebulization only had selective and intermittent aerosol penetration into sinuses. This was still superior to nasal spray and passive diffusion nebulizers which had no evidence of sinus penetration. In patients post ESS, both vortex and passive nebulizer did not show significant sinus penetration. Nasal sprays had more

pharyngeal and stomach penetration while vortex and passive diffusion nebulization had more pulmonary deposition.

These studies suggest that while vortex nebulizers may have better nasal delivery when compared to passive diffusion nebulizers and nasal sprays, it is inconsistent in its delivery of particles to sinuses.

Pulsating aerosol delivery (ie. PARI Sinus Pulsating Aerosol System)

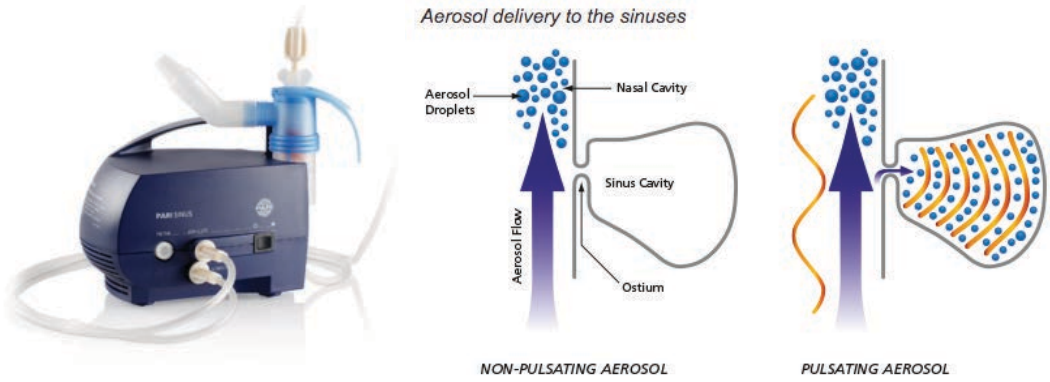

Figure 6. PARI Sinus pulsating aerosol delivery system.

A pulsating aerosol delivery system (PARI Sinus) combines nebulization with a vibrating pulse to deliver aerosolized particles of 3.2 μm directly to the sinuses. The mechanism of penetration for the PARI device is based on generating variable pressure differentials with pulsation, instead of the constant pressure applied from irrigation, to deliver aerosols into sinuses

Moller et al[120] reported that 6.5% of nasally administered drug was delivered into sinuses via the PARI device in healthy volunteers while none was delivered using nasal

sprays. In a separate study, the same author reported a significant increase in deposition in the sinuses via PARI device in post ESS with CRSsNP[121] compared to before ESS.

Valentine et al[122] compared PARI sinus nebulizer vs high volume nasal irrigation in a cadaveric model that had complete sphenoethmoidectomy and a frontal sinus drillout (Draf III). High volume nasal irrigation demonstrated reliable sinus penetration (stained 96% of all sinuses) and is superior to the PARI sinus nebulizer (stained ethmoids consistently at 92%; less reliable staining of frontals at 43%, maxillaries at 46%, and sphenoids at 54%).

These studies suggest that while PARI was able to achieve some penetration into sinuses, it is inferior to high volume nasal irrigations.

High volume nasal irrigations (ie. Neti pot [NasaFlo], squeeze bottle [Sinus Rinse])

Although at present there is no single topical delivery modality with all the desirable characteristics of optimum sinus penetration, high volume nasal irrigations have remained consistently the preferred modality over others. High volume nasal irrigation devices include Neti pots and squeeze bottles. Neti pots are traditionally an ayurvedic practice for nasal cleansing which delivers 240 mLs of solution; patients tilt their heads sideways and insert the spout of the Neti pot into the upper nostril then pour the solution into the nostril. Similarly squeeze bottles are used to deliver 240mL of irrigation; patients perform the rinses by squeezing the bottle gently to deliver solution into each nasal cavity.

In several post ESS cadaveric studies presented below high volume irrigation has been shown to be superior to other modalities.

Abadie et al[123] demonstrated that in maximally operated sinuses (minimum ostia size of 4mm) with frontal drill out (Draf III), wide maxillary antrostomy and complete sphenoethmoidectomy, Sinus Rinse high volume irrigator showed consistent penetration into sinuses when compared to squeeze/ pump or mechanical atomizers.

Wormald et al[124] demonstrated high volume irrigation (squeeze bottle) had better sinus penetration compared to nasal sprays and nebulizers.

Harvey et al[125] reported that Neti pot irrigations had the best sinus distribution followed by squeeze bottle irrigation and pressurized spray in all post-surgical states, ESS (frontoethmosphenoidectomy + maxillary antrostomy) and ESS + modified medial maxillectomy. The authors also reported that the residual fluid remaining within sinuses after a standard squeeze bottle irrigation to be less than 6% of total solution, suggesting that delivery and retention of pharmaceutical agents to the sinuses is limited at best.

Several clinical studies investigated the efficacy of high volume nasal irrigations have reported consistent and reliable sinus penetration. Olson et al[126] conducted a radiocontrast irrigation study with 3 dimensional CT reconstruction and found that both negative pressure (inhaled/sniffed contrast solution from palm of hand) and positive pressure irrigation (Sinus Rinse, Neilmed) had reliable distribution to ethmoid sinuses, limited but comparable distribution to frontal and sphenoid sinuses. However, positive pressure irrigations had better distribution to bilateral maxillary sinuses and retained

larger volume of contrast solution with a mean of 1.1 mL. Passive diffusion nebulisation using Rinoflow device was found to have the poorest distribution beyond nasal cavity and retention of contrast materials.

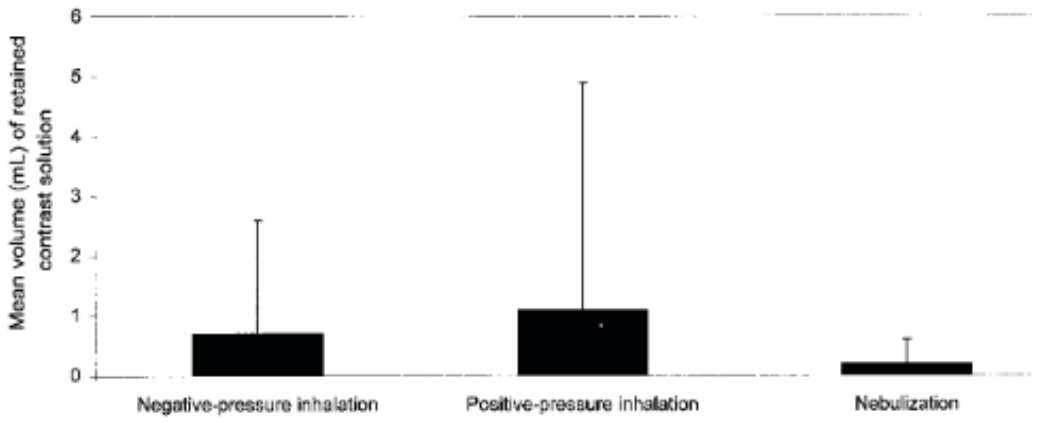

Figure 7. Mean volume of contrast solution retained in sinuses after each of three sinus irrigation method, negative- pressure inhalation, positive- pressure inhalation, and nebulization. Adapted from Olson et al (2002)[126].

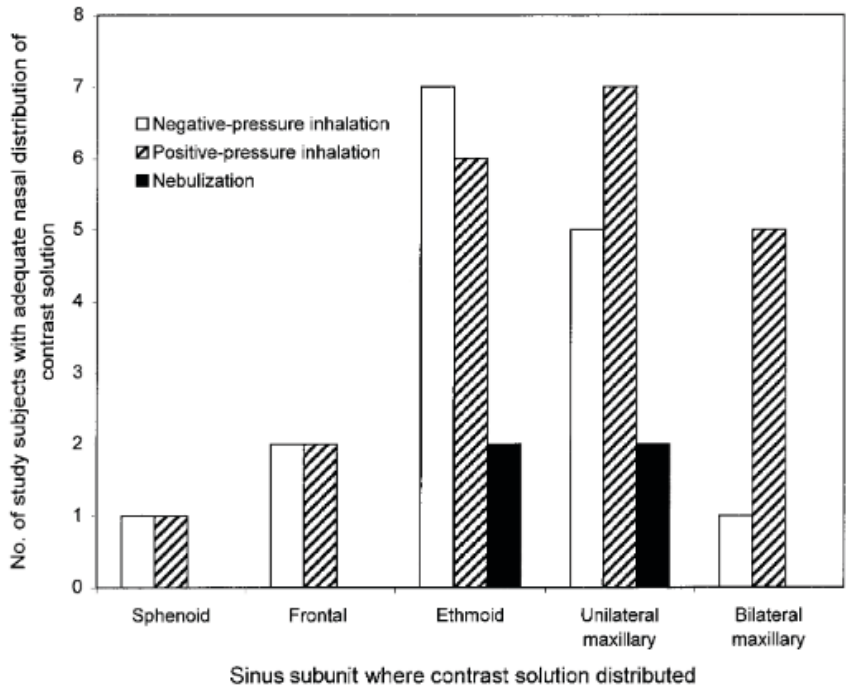

Figure 8. Distribution of contrast solution in sinus subunits after each of three sinus irrigation method. Adapted from Olson et al (2002)[126].

In the only level 1b evidence study, Pynnonen et al[127] compared high volume saline irrigation (squeeze bottle) to nasal saline spray and found that both groups demonstrated improvement in symptoms and SNOT-20 scores at 8 weeks but high volume irrigation conferred significantly larger improvements.

In a comprehensive evidence based review, Thomas et al[128] included 15 studies examining nasal spray distribution, 12 prospective human studies and 3 cadaveric studies and concluded that low volume devices (sprays, drops, nebulizers) were inferior to high volume irrigations in sinus penetration.

Due to limited availability of high level evidence, the International Consensus Statement on Allergy and Rhinology: Rhinosinusitis[94] concluded that high volume irrigations (>200 mL) appear to be superior to low volume nasal sprays, but further trials are required.

Before vs after ESS

Multiple studies have consistently demonstrated that in unoperated patients there is limited sinus penetration regardless of head position, delivery modalities and delivery volume[100, 125, 129, 130]. In post ESS patients, the larger the sinus ostia the better the effects of sinus irrigation. Grobler et al[100] reported that for high volume nasal irrigations to reliably penetrate the maxillary sinus a minimum of 3.95 mm ostia diameter is required. Singhal et al[131] also reported that a minimum ostia diameter of 4.7 mm is required for maximum penetration of maxillary and sphenoid sinuses.

Positions of delivery

The ideal position for intranasal drug delivery varies depending on the different modalities of instillation. Below we attempt to discuss the various positions available and their delivery efficacy.

Figure 9. Instillation of nasal drops in the Mygind's position (left) and corresponding flow of white liquid latex in a cadaveric nasal model (right). Adapted from Raghavan et al (2000)[132].

In a cadaveric model, Raghavan et al[132] reviewed several positions of nasal drops instillation and found that the best position to deliver nasal drops to the middle meatus is the Mygind's position and a newly proposed Ragan's position. In the Mygind's position patients lie supine with head extended off the end of the bed, patients' head is turned to either side while nasal drops are instilled and each position is maintained for 30 seconds before sitting up (Figure 9). Other studies have also shown good distribution of radiolabelled nasal drops in the Mygind position[133].

Kayarkar et al[134] also reported that the lying 'head back position'/ Mygind's position had the highest distribution of nasal drops to middle meatus (55.5%) followed by the head forward and down/ Mecca position which has been described as the most uncomfortable position (31.6%).

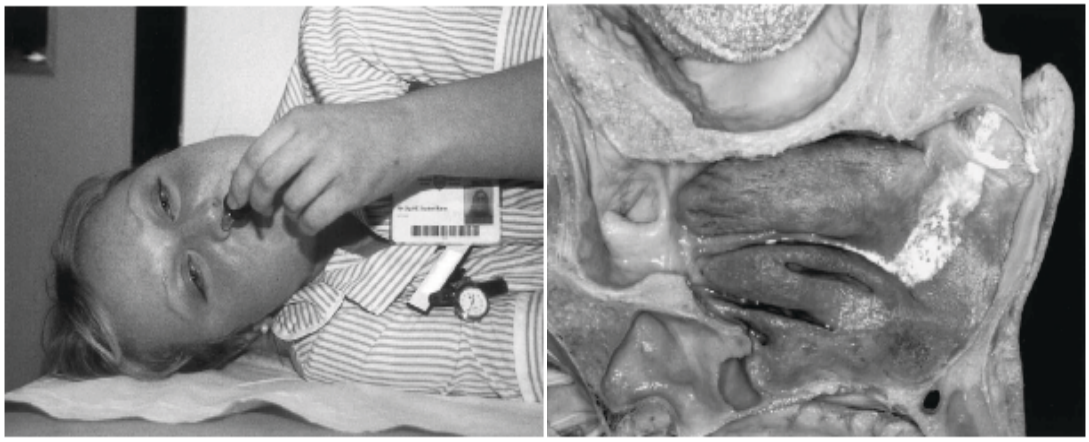

Figure 10. Instillation of nasal drops in the Ragan's position (left) and corresponding flow of white liquid latex in a cadaveric nasal model (right). Adapted from Raghavan et al (2000)[132].

In the Ragan position, patients lie on their side with parietal eminence resting on a flat bed at the level of their shoulder with their nose turned towards their upper shoulder. Nasal drops are then instilled and this position maintained for 30 seconds before doing the same for the opposite side (Figure 10). Both the Mygind and Ragan position showed consistent delivery of nasal drops to the middle meatus, but the authors concluded that the Ragan position is more tolerable[132].

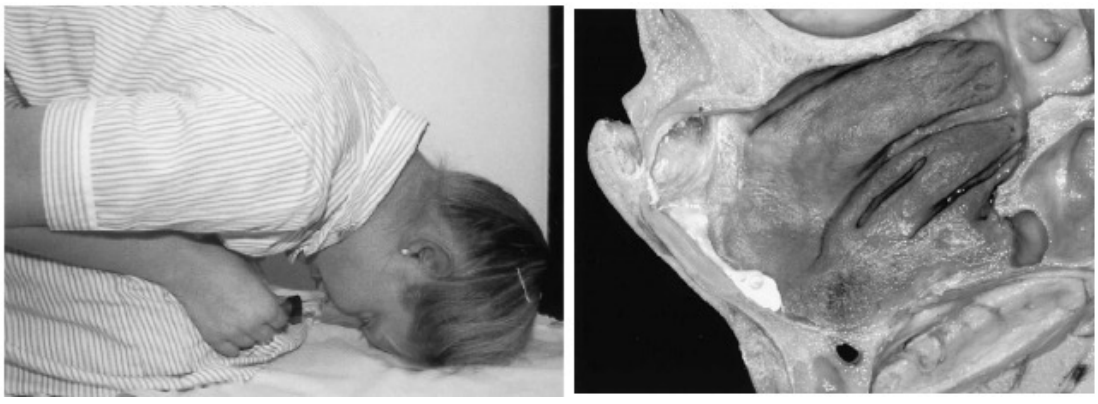

Figure 11. Instillation of nasal drops in the Mecca position (left) and corresponding flow of white liquid latex in a cadaveric nasal model (right). Adapted from Raghavan et al (2000)[132].

The traditional 'praying to mecca position', 'vertex to floor' or 'head down forward position' (Figure 11) had demonstrated favourable clinical outcomes[135] but was described as the most uncomfortable position with nasal drops mainly directed at the atrium of nasal cavity towards olfactory cleft.

In a post ESS cadaveric study Beule et al[136] reported that 'vertex to floor' position to be superior to the 'head forward position' for better penetration into frontal sinuses. However if this position proves challenging to patients, Singhal et al[131] reported that 'head over sink' position was still superior to 'vertex to ceiling' position when using high volume nasal irrigation targeting the frontal sinuses. Head position was found to be non-significant when targeting maxillary and sphenoid sinuses.

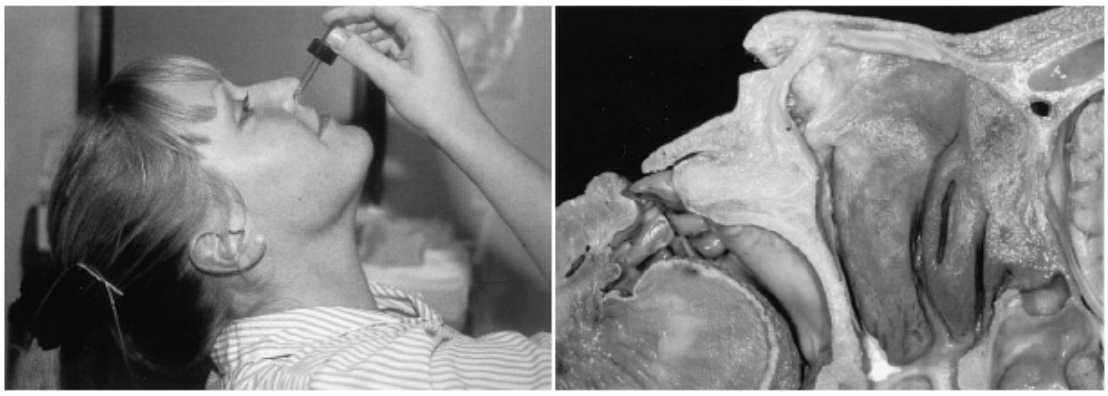

Figure 12. Instillation of nasal drops in the head back position (left) and corresponding flow of white liquid latex in a cadaveric nasal model (right). Adapted from Raghavan et al (2000)[132].

In the head back position (Figure 12) nasal drops did not reach middle meatus but ran along the floor of the inferior meatus into the nasopharynx.

Based on the review above, we conclude that high volume nasal irrigations have demonstrated reliable and consistent penetration into post ESS sinuses and efficacy is improved with good patient positioning (Table 4). Some low volume nasal delivery devices like PARI may have minimal sinus penetration for patients unable to tolerate high volume irrigations.

Intervention	**Recommendations**
Sinus Surgery	Recommend post ESS for better sinus penetration
Device	Recommend for high volume delivery system for reliable sinus penetration
Position	**High volume devices:** Mecca/ vertex to floor/ head down forward position **Low volume devices:** Mygind/ lying head back and Ragan position (for nasal drops), nozzle aimed at 45 degrees (for nasal sprays)

Table 4. Summary of recommendations for effective sinonasal irrigation.

1.2 Current and Emerging Topical Therapies for Chronic Rhinosinusitis

Saline

A number of systematic reviews evaluating the use of saline irrigation in CRS have reported an overall benefit in terms of symptom reduction and quality of life (QoL). Saline irrigation is believed to confer several advantages; 1) decrease viscosity of sinonasal mucus 2) increase efficiency of mucociliary clearance, 3) reduce mucosal edema, 4) reduce microbial burden, inflammatory cells and aeroallergens and 5) provide mechanical debridement by disrupting biofilm matrixes.

Harvey et al[137] performed a systematic review which included 8 randomized controlled trials (RCTs) comparing saline irrigation vs placebo, saline irrigations as an adjunct, saline irrigation vs intranasal corticosteroid (INCS) and hypertonic vs isotonic saline. This review reported that saline irrigations are beneficial in CRS as a sole modality of treatment and as an adjunct treatment. Although when compared with INCS, the latter demonstrated better outcomes. There is some evidence that hypertonic saline irrigations improve CT scores over isotonic saline.

However, Chong et al[138] conducted a Cochrane systematic review update on the role of nasal saline irrigation in the management of CRS which included 2 RCTs. The review showed that there may be some benefit of daily large volume 150 mL hypertonic saline irrigation compared to placebo but no benefit of low volume nebulized saline over INCS.

The International Consensus Statement on Allergy and Rhinology: Rhinosinusitis[94] recommends the use of saline irrigations in CRS and encourages its use as an adjunct to other topical treatments.

Saline tonicity

Formulation of saline irrigations can vary from physiologic isotonic (0.9%) to hypertonic (variable concentrations up to 7%). Several *in vitro* studies have suggested that hypertonic saline confers increased benefit over isotonic saline. Hypertonic solution is believed to reduce mucosal oedema by osmotic pressure, increase hydration and reduce mucoadhesiveness of sinonasal mucus which aids mucociliary clearance[139] and increased intracellular Ca^{2+} and cAMP[140] helps stimulates ciliary beat frequency[141]. Whilst *in vitro* studies support the effect of hypertonic solution, clinical evidences have been heterogeneous.

Bachmann et al[142] found that twice daily high volume irrigation of Ems salt (1.1%) vs isotonic saline (0.9%) over 7 days significantly improved clinical symptoms and endoscopy scores. Although not statistically significant, patients using Ems salt irrigation had more sustained improvement with nasal obstruction, better mucociliary clearance and improved rhinomanometry when compared to isotonic saline group.

Although in Hauptman and Ryan's[143] randomised, double-blind study, they found that hypertonic (3%) and isotonic saline (0.9%) sprays both significantly improved mucociliary clearance, the isotonic saline group showed significant improvement in nasal patency on rhinomanometry compared to hypertonic saline group.

In several randomized double-blind studies Talbot et al[144], Homer et al[145], and Ural et al[146] found that a hypertonic saline nasal irrigation improves the mucociliary clearance time compared to isotonic saline in healthy subjects.

In a systematic review, Kanjanawasee et al[147] included 9 studies which compared hypertonic saline vs isotonic saline delivered by any method, concentration, frequency, and duration of treatment. They found that although there was no significant difference in SNOT-20 between both groups, hypertonic saline conferred greater improvement in nasal symptoms, with greatest improvement observed when administered as high volume irrigations than low volume irrigation.

Hypertonic saline was also found to confer greater symptom improvement in non-operated patients when compared to isotonic saline and found no significant difference in symptom improvement between both groups in post-operative patients. Hypertonic saline at tonicity of under 5% was found to improve symptoms greater than isotonic saline but there was no observable difference between both groups when tonicity is greater than 5%. Although there is a higher risk of nasal irritation and burning sensation reported, all adverse symptoms reported were self-limiting.

In a systematic review which included 29 studies (12 meta-analyses, 13 systematic reviews, 4 RCTs) Rudmik et al[148] concluded both isotonic and hypertonic saline solutions confers similar improvement in clinical symptoms and QoL.

Similarly in a Cochrane meta-analysis by Harvey et al[137] which looked at 2 RCTs, the author concluded that there was no significant difference in improvement of symptoms and endoscopic scores between hypertonic and isotonic saline groups, although in a paediatric population there was significant improvement in cough reduction and radiological scores.

In view of these findings, The International Consensus Statement on Allergy and Rhinology: Rhinosinusitis[94] concluded that isotonic and hypertonic saline irrigations provide similar subjective outcomes and high-volume saline irrigation appears to be superior to low-volume nasal spray techniques and should be implemented as an adjunct to other topical therapy strategies.

Steroids

Intranasal Corticosteroids (INCS) sprays

Several systematic reviews and clinical guidelines provide strong recommendations for the use of INCS in CRSwNP and CRSsNP, showing significant symptom and endoscopic improvements.

Joe et al[149] performed a meta-analysis of 6 RCTs looking at INCS in CRSwNP and found that there was significant improvements in polyp size compared to control.

Rudmik et al's[150] meta-analysis of 12 studies found that topical nasal steroid (fluticasone, mometasone and budesonide) resulted in significant symptom improvements in CRwNP.

Kalish et al[151] conducted a review on the role of intranasal corticosteroid in CRSwNP involving 40 studies and concluded that INCS improves symptoms, reduces polyp size and recurrence and there was no evidence of significant adverse effects.

Following that, Chong et al[152] performed an updated Cochrane review. 18 RCTs were reviewed comparing intranasal corticosteroids (beclomethasone dipropionate, triamcinolone acetonide, flunisolide, budesonide) against placebo or no treatment in CRSwNP and found that there was improvement in overall symptoms especially nasal blockage and post nasal drip but with an increased risk of epistaxis.

Chong et al[153] did a further Cochrane review on different types of intranasal corticosteroids in CRS and found that there was no difference between different types of INCS and methods of delivery (spray vs aerosols). Higher dose INCS is associated with increased risk of adverse effects (epistaxis) with not associated improvement in symptoms.

Snidvogs et al[154] did a Cochrane review on the role of topical steroids in CRSsNP. The evidence showed that patients on INCS experienced a significant improvement in symptoms compared to placebo with no evidence of significant adverse effects.

Consistent with these findings, The International Consensus Statement on Allergy and Rhinology: Rhinosinusitis[94] recommends the use of standard INCS sprays in CRSsNP and CRSwNP (before and after surgery).

Budesonide

The role of topical intranasal corticosteroid sprays in the management of CRS is well established and its safety profile has been well known, however these low-volume sprays have been shown to have limited penetration into the sinuses. High-volume irrigation techniques have been utilised to optimise drug delivery into the sinuses and offers the added benefit of mechanical irrigation. The use of Budesonide in high volume saline irrigation has been an off-label practice in the management of CRS over the last decade. Although it is widely accepted as an adjunct topical treatment in clinical practice, the evidence for its use has been mixed. The International Consensus Statement on Allergy and Rhinology: Rhinosinusitis[94] concluded that although there is some evidence that it improves QoL, symptoms and endoscopic scores, it may be most beneficial in post-operative patients.

Budesonide is a potent topical corticosteroid that has 1000 times more anti-inflammatory potency than cortisol. After binding with the glucocorticoid receptor it exerts its anti-inflammatory properties via several mechanisms; it alters the release of arachidonic acid metabolites, inhibits leukocyte accumulation, reduces vascular permeability, down-regulates neuropeptide-mediated responses, and reduces glycoproteins secretion from submucosal glands[155].

Multiple studies have shown that off-label use of budesonide irrigations are well tolerated and in the short term (less than 8 weeks)[156-158] and does not have hypothalamic- pituitary- adrenal axis (HPAA) suppression sequelae. Smith et al[159] investigated the safety of long term twice daily high volume budesonide irrigation (1mg per irrigation with a total daily dose of 2 mg) and was found to be safe over a mean

duration of 38.2 months (2.9 years) and found no evidence of HPAA suppression even though 62% of patients were on concurrent intranasal corticosteroid for asthma.

However, in a conflicting report Soudry et al[160] showed that 23% of patients developed asymptomatic HPAA suppression when received an average budesonide of 0.75 mg daily dose over 22 months. This effect was found to be transient in 3 out of 4 patients who repeated Synacthen/ cosyntropin stimulation stimulation test after 1 month of budesonide cessation. All 4 patients were evaluated by endocrinologist and resumed on budesonide irrigations due to symptomatic benefit gained from treatment and lack of adverse clinical adrenal suppression symptoms (ie. weakness, fatigue, dizziness, muscle aches, nausea, vomiting, and diarrhea). Concurrent use of INCS sprays and pulmonary steroid inhalers were found to increase the risk of HPAA suppression.

Author	Study Group	Study design	Treatment	End Points	Outcomes
Rotenberg et al (2011)[161]	Samsters Triad (CRSwNP)	RCT double blind placebo controlled trial	Budesonide saline irrigation vs Saline irrigation + budesonide spray vs Saline irrigation	Radiologic QoLs	No difference between all groups
Snidvogs et al (2012)[162]	Postsurgical	Prospective	Budesonide saline irrigation (1mg) vs Bethamethasone irrigations (1mg) daily	QoLs Symptoms Endoscopic	Improved symptoms, SNOT-22 and endoscopic scores. Patients with high tissue eosinophilia (>10 high-power field) demonstrated better response.
Rawal et al (2015)[163]	Postsurgical CRSwNP	Blinded RCT	Normal saline irrigations vs Budesonide saline irrigation	QoLs Olfaction	Both groups showed improvement in QoL and no improvement in olfaction. Neither group is more superior.
Kosugi et al (2016)[164]	Recalcitrant CRS	Prospective	Budesonide saline irrigations (1mg) over 3 months	QoL Symptom Endoscopic	75% of patients had improved SNOT-22 and LKS
Kang at el (2017)[165]	CRSwNP and Asthma	Prospective	Budesonide saline irrigation (0.5mg) daily	QoLs Endoscopic Oral steroid requirements	Improved SNOT-22 and endoscopic scores. Reduced oral steroid intake.
Tait et al (2019)[166]	CRS	Prospective RCT double blind placebo controlled trial	Budesonide irrigation (1mg) daily vs saline irrigations over 30 days	SNOT-22	Improved SNOT-22
Soudry et al (2015)[160]	Postsurgical	Retrospective	Long term Budesonide saline irrigations (0.5mg) daily or BD over 6 months	Safety	Safe but asymptomatic HPAA suppression occurred in patients using concomitant pulmonary steroid inhalers. No intraocular pressure increase.
Smith et al (2016)[159]	Postsurgical	Prospective	Long term Budesonide saline irrigations (1mg) BD over 12 months	Safety	No evidence of HPA axis suppression

Table 5. Summary of clinical trials investigating the safety and efficacy of off-label budesonide use.

There are a number of studies that have investigated the efficacy of budesonide irrigations and have all demonstrated improved outcomes[162, 164, 165]. However in studies that compared budesonide irrigations with saline irrigations Rotenberg et al[161] and Rawal et al[163] reported improved symptoms but no significant differences observed between both groups. This was similarly reflected in a systematic review performed by Yoon et al[167] who investigated the safety and efficacy of budesonide in post-surgical patients. From the 12 studies which were reviewed they found that although the addition of budesonide in saline irrigations over 3 months was shown to improve symptoms, endoscopic score and QoL post-surgery, but they concluded no differences when compared to saline irrigations alone. Although the authors recommended that it could still be considered as an adjunct to saline irrigations based on robust data on its safety.

Following that a recent prospective, double-blinded, placebo-controlled, randomized clinical trial by Tait et al[166] have demonstrated contrary results. In that study, patients on daily budesonide irrigations for 30 days had significantly improved SNOT-22 scores when compared to patients on saline irrigations. Interestingly, they reported greater benefit of budesonide irrigations seen amongst patients with no previous surgery than those who had undergone surgery, suggesting that patients may also benefit from topical budesonide irrigations without prior history of sinus surgery.

In recalcitrant CRSwNP, budesonide irrigations are still preferred as an alternative to systemic corticosteroids due to less systemic adverse effects. It has been shown that budesonide, commercially available under the tradename Pulmicort®, has direct antibacterial effect on planktonic and biofilm forms of *S. aureus* and MRSA[168]. This is

likely attributed by the excipients of Pulmicort® ethylene diamine-tetraactic acid (EDTA) which even at low concentrations have been shown to significantly reduce growth of *S. aureus* and MRSA biofilms, and has potential additive/synergistic effect with amoxicillin in reducing MICs. This *in vitro* data suggests that Pulmicort® may have potential for prophylactic antibacterial properties and may enhance bacterial eradication in recalcitrant CRS when on concurrent antibacterial treatment.

Antibiotics

The role of topical antibiotic therapies in the management of recalcitrant CRS remains unclear due to heterogeneity of data available.

In EPOS 2012, the authors concluded that the use of topical antibiotics in CRSwNP and CRSsNP is not recommendation due to the paucity of literature.

Rudmik et al[169] included 3 RCTs and a systematic review, in all 3 RCTs patients received topical antibiotics delivered via nebulizers or sprays and had demonstrable improved clinical outcomes however there was no significant difference when compared to placebo. Therefore the authors have recommended against the use of topical antibiotic therapy delivered via low volume devices in CRS, but suggested there may be a role in select CRS cases for culture directed topical antibiotics delivered using high volume sinus irrigations.

Lee et al[170] in a retrospective study collated 58 patients with post-ESS recalcitrant infections who received 1 month of twice daily culture directed topical antibiotics over 30 days; which included tobramycin (100 mg/100 mL), vancomycin (200 mg/100 mL),

levofloxacin (100 mg/100 mL), mupirocin (15 mg/100 mL), gentamicin (80 mg/100 mL), ceftriaxone (200 mg/100 mL), and ceftazidime (600 mg/100 mL). In the non-CF group, 72% had eradication of bacteria on culture with corresponding significant improvements observed in LKS, and trends of improved SNOT-20 scores but not achieving significance. In the CF group, 29% of patients had eradication of bacteria on culture, no change in SNOT-20 scores and non-statistically significant improvement on LKS. The authors conclude that there is a role for topical antimicrobial delivered with high volume irrigations in post ESS recalcitrant CRS given the lack of viable alternatives.

However at present the International Consensus Statement on Allergy and Rhinology: Rhinosinusitis[94] concluded that although there may a role for topical antibiotics in recalcitrant cases, their routine use is not recommended due to the lack of consistent benefits demonstrated from high quality studies. They also suggested for patients with infections resistant to oral antibiotics, topical therapy is preferred over intravenous antibiotics.

Author	Study Group	Antimicrobial	Study design	Treatment	End Points	Outcomes
Videler et al (2008)[171]	Recalcitrant CRS 14 patients	Bacitracin/ colimycin + oral levofloxacin vs Saline (placebo) + oral levofloxacin	RCT, double blind, placebo controlled, cross-over pilot study	Rhinoflow nebulizer BD with oral levofloxacin	Endoscopic QoLs Symptoms	Significant improvements in both groups
Uren et al (1997)[172]	Recalcitrant CRS 16 patients	0.05% mupirocin vs Saline	Open label pilot study	Nasal lavage BD for 3 weeks	Endoscopic QoLs Symptoms	94% had eradication of infection and improved endoscopy, 75% had improvement in symptoms
Solares et al (2006)[173]	MRSA exacerbation CRS 42 patients	2% mupirocin with oral antibiotics given to majority of patients	Retrospective chart review	Nasal lavage BD for 4 weeks	Endoscopic QoLs Symptoms	39% had eradication of infection, 67% patients had improved symptoms
Bardy et al (2012)[174]	Recalcitrant CRS 57 patients	0.05% mupirocin	Retrospective chart review	Nasal lavage BD for 4 weeks	Reculture rate Posttreatment resistance	73.7% reculture rate in 4.8 months, 2.8% posttreatment resistance rate
Bardy et al (2012)[175]	Recalcitrant CRS 25 patients	0.05% mupirocin vs Saline	RCT, double blind, placebo controlled	Nasal lavage BD for 4 weeks	Endoscopic QoLs Symptoms	88.9% eradication of infection but not sustained
Seiberling et al (2013)[176]	Maxillary CRS 16 patients	0.25mg/mL mupirocin vs Saline	Prospective	Intraoperative maxillary irrigation	qPCR	Significantly reduce *S. aureus* 10 days post op compared to saline alone
Desrosiers et al (2001)[177]	Recalcitrant CRS 20 patients	Tobramycin-saline vs Saline only	RCT, double blind, placebo-controlled study	Intranasal large particle nebulizer TDS for 4 weeks	Symptoms Endoscopic QoLs	No significant difference between groups
Di Cicco et al (2007)[178]	CF-associated CRS 27 patients	0.2% sodium hyaluronate and 3% Tobramycin vs 0.2% sodium hyaluronate only	RCT, double blind, placebo-controlled pilot study	2 week nasal spray	Symptoms Endoscopic	No difference in eradication of infection but significant improvements in symptoms and endoscopy in tobramycin group
Ezzat et al (2015)[179]	Recalcitrant CRS 12 patients	0.3% ofloxacin eye drops vs no treatment	Controlled study	3 drops each nostril intranasal drops TDS for 12 weeks	Middle meatal biopsy	80% eradication of infection and improvement on mucosal biopsy

Table 6. Summary of clinical trials using topical antibiotics in CRS.

Tobramycin

Antunes et al[180] demonstrated *in vivo* that the tobramycin concentration to eradicate pseudomonas was 5 times the recommended human dose (80 μg/mL). Higher tobramycin concentration increased bacterial elimination and improved mucosal and bone inflammation but did not eradicate infection . This supports the advantages of topical lavage which allows for higher tobramycin concentration without concerns of systemic toxicity.

Moss et al[181] reported that combined endoscopic surgery with serial tobramycin lavage in CF patients with positive *P. aeruginosa* demonstrated reduced nasal polyposis and need for revision surgery. Patients received 7-10 days of 40 mg tobramycin direct irrigation per maxillary sinus three times daily, serum tobramycin detected was less than 2 μg/L after lavage and dropped to undetectable levels prior to next lavage. This was repeated monthly and for recalcitrant patients this regime was increased in frequency or patients resumed on 7-10 days tobramycin lavage.

In a randomized, double-blind, placebo-controlled pilot study Mainz et al[182] investigated the efficacy of daily tobramycin (80 mg/2 mL) vs placebo (isotonic saline) over 28 days in cystic fibrosis patients. Both treatments were delivered via vibrating aerosol (Pari Sinus). The study found that 67% of patients in the tobramycin group had decreased *P. aeruginosa* growth whilst there was no reduction in the placebo group and a significant improvement in SNOT-20 scores when compared to placebo. Topical tobramycin was well tolerated with a serum detection of less than <0.5 mg/L in all patients.

Berkhout et al[183] also reported minimal systemic absorption of nasally administered 300 mg tobramycin in patients with cystic fibrosis. Only 2 out of 10 patients had detectable serum tobramycin levels, highest being 0.054 mg/L, well below toxic levels.

However Desrosier et al[177] in a randomized, double-blinded trial reported no significant differences in nebulized tobramycin compared to aerosolized saline solution, both groups demonstrated improvements in QoL, symptoms and endoscopic scores.

At present for patients with recalcitrant *P. aeruginosa* CRS, especially for patients unresponsive to or intolerant of oral antibiotics, there are limited alternative antimicrobial therapies available. *P. aeruginosa* known as a phenomenon of bacterial resistance[184] is increasingly prevalent in clinical practice and has become a challenging entity to manage. Although data on the efficacy of tobramycin irrigations in recalcitrant CRS are still lacking, due to evidence of minimal systemic absorption, tobramycin irrigations may have a role in patients who has failed oral antimicrobials.

Mupirocin

Mupirocin exerts its antimicrobial activity by irreversibly binding to the bacterial enzyme isoleucyl-transfer RNA, which prevents the incorporation of isoleucine during bacterial synthesis[185-187]. It has excellent antimicrobial activity against staphylococci including MRSA, most streptococci, and against certain gram-negative bacteria including *H. influenzae*[186].

Uren et al [172]reported in patients with positive *S. aureus* recalcitrant CRS, 94% had eradication of infection after 3 weeks of 0.05% mupirocin in 0.9% lactated ringers salt solution administered twice daily as nasal lavage.

In a retrospective study by Solares et al[173], 2% mupirocin irrigations were given alone or concurrently with oral antibiotics in recalcitrant MRSA CRS patients and had 39% eradication of MRSA with 67% of patients reported improved symptoms. 50% of patients had at least 1 episode of recurrence of MRSA exacerbation over a mean follow up duration of 11.8 months.

In a randomised, double-blind, placebo-controlled trial, Jervis Bardy et al[175] compared mupirocin irrigations to oral amoxycillin and clavulanic acid in *S. aureus* positive patients with recalcitrant CRS. Patients receiving twice daily 0.05% mupirocin irrigations over 4 weeks, 8/9 had negative bacterial cultures (88.9%) compared to the oral antibiotic group 0/13 had negative culture (0%). The mupirocin group also showed significant improvement in clinical symptoms and LKS compared to oral antibiotic treated patients. These improvements were not sustained, with the majority of patients having a relapse of their symptoms and endosopcic findings within 2 - 6 months post treatment. In a separate retrospective study Jervis Bardy et al[174] found that 74% of patients had relapse of *S. aureus* infection at a mean time of 144 days (approximately 5 months) post treatment and found that post treatment *S. aureus* resistance rate to mupirocin to be 2.4%.

Seiberling et al[176] performed intraoperative maxillary irrigations of 0.25 mg/mL mupirocin vs saline irrigations in contralateral maxillary sinus with patient as their own control showed significantly reduced *S. aureus* presence 10 days post op.

In a meta-analysis Kim and Kwon[188] included 6 studies (2 RCTs, 2 prospective studies and 2 retrospective studies) on mupirocin irrigations for *S. aureus* recalcitrant CRS and found a relative risk of 0.13 for residual infection after 1 month of mupirocin irrigations which increased to 0.53 at 6 months. The authors concluded that mupirocin is an effective short term intervention in the setting of recalcitrant CRS with *S. aureus* infection but will require longer term follow up to adequately assess its long term effects.

Recently Carr et al[189] reported evidence that 1 week of mupirocin irrigations resulted in a significant shift from gram-positive bacteria to gram-negative bacteria. There was an increased post treatment culture of *P. aeruginosa* and gram positive *Corynebacterium sp* (19 gram-positive vs 3 gram-negative before therapy, 9 gram-positive vs 13 gram-negative after therapy).

In patients with recalcitrant CRS where oral antibiotics have failed to be effective the option of topical mupirocin irrigations can be considered over intravenous antibiotics for exacerbations of CRS. However there should be increased awareness that current evidence lends support to the use of mupirocin for short term improvements with high relapsing rate and may put patients at risk of developing resistance and select for more gram-negative bacteria infections.

Antifungals

Around the emergence of the fungal hypothesis for CRS, early studies have suggested that antifungals conferred significant benefit to CRS patient. Since then there have been several well performed RCTs that have consistently demonstrated no added benefit from the use of these agents.

The role of systemic antifungals in recalcitrant CRS has been investigated by Kennedy et al[190] in a double-blind placebo-controlled trial. Patients were given high dose oral terbinafine vs placebo daily for 6 weeks and found that there was no significant benefit between both groups.

In a randomised, double-blind, placebo controlled study Liang et al[191] reported amphotericin B irrigation (40 mg/L) for 4 weeks in CRSsNP showed improvement in symptoms and endoscopic scores but was not superior to saline irrigation group.

In another randomized, double-blind, placebo-controlled study Hashemian et al[192] reported that fluconazole nasal drop 0.2% for a duration of 8 weeks were not superior to placebo in recalcitrant CRS.

Ebbens et al[193] in a large, double-blind, placebo-controlled, multicenter study reported intranasal that 25 mL of amphotericin B lavages (100 μg/mL) twice daily for 3 months did not improve clinical symptoms and polyp size compared to placebo. Subsequently Ebbens et al[194] also demonstrated topical amphotericin B over 13 weeks had no significant effect on inflammatory markers in CRSwNP and CRSsNP consistent with the lack of clinical improvements.

Sacks et al[195] conducted a meta-analysis investigating the role of amphotericin B in the treatment of CRS. In the review which included 5 RCTs, they found that there was no significant difference between treated and placebo groups but there were higher adverse events in the topical antifungal group. This was supported by the meta-analysis conducted by Isaacs et al[196] which included 3 RCTs which showed that there were no significant differences in CT scan, endoscopy and symptom scores between intranasal amphotericin B and saline group. Similarly, meta-analysis by Wang et al[197] which included 5 RCTs showed no difference between both groups in QoLs and endoscopy scores.

However none of the above studies investigated antifungal treatment in AFRS patients alone, and the role of antifungals within this cohort remains unclear. Further high quality research is needed to establish this conclusively.

At present due to the lack of evidence supporting the use of topical and systemic antifungals in CRS the International Consensus Statement on Allergy and Rhinology: Rhinosinusitis[94] has recommended against its use.

Other additives

Carrageenans

Carrageenans are mucoadhesive polysaccharides extracted from red seaweeds from the Rhodophyceae family.

Iota- and Kappa- carrageenans have been known to have antiviral properties[198]. Iota carrageenan sprays were found to improve common cold symptoms especially during the first 4 days by reducing pro-inflammatory cytokines (ie. bFGF, Fractalkine, growth related oncogene, G-CSF, IL-8, IL-1α, IP-10, IL-10, and IFN-α2), reduce duration of symptoms by up to 2 days[199], and reduce viral load in nasal lavages[200]. Koenighofer et al[199] showed significant antiviral efficacy of iota-carrageenans against rhinovirus, coronavirus and influenza A with the greatest benefit observed in coronavirus infected patients. Bennett el al[201] have also recently demonstrated that kappa-carrageenans has antibacterial properties, significantly reducing intracellular *S. aureus* infection in human bronchial epithelial cells *in vitro*. Kappa- carrageenans have been previously reported to improve epithelial barrier function *in vitro*[202] by significantly reducing IL-6 production and increasing transepithelial electrical resistance (TEER) in air-liquid interface cell cultures (ALI) from CRS patients.

Available commercially over the counter as Flo Travel Nasal Spray®, containing both Kappa and Iota-carrageenans, it is encouraged for use during travel due to its mucoadhesive and hydrophilic property which helps maintain nasal tissue hydration and prevents nasal crusting and discomfort associated with long distance flights.

Manuka honey

Manuka honey (*Leptospernum scoparium*) has long been known to have antibacterial properties and more recently anti-biofilm activity against a broad spectrum of gram-positive and gram-negative bacteria. The combined effect of low pH, hyperosmolarity, hydrogen peroxide activity[203] and methylglyoxal (MGO)[204, 205] content confers manuka honey (MH) its unique biofilm-cidal properties. It has also been shown that bacteria

have a low propensity to develop bacterial resistance to MH[206, 207]. In addition, MH has the ability to increase susceptibility of resistant strains to antibiotics[208].

Despite this, its role as a topical antibacterial rinse to treat recalcitrant CRS is still unclear. This is largely due to the body of heterogenous data in the literature on MH, differences in the honey sourced, concentration of MH used, MGO content, dosing intervals and duration and variable treated infections (type of pathogen, single organism vs polymicrobial) has made it difficult to develop a consensus based on data available.

Kilty et al[209] has shown that daily 33% weight/ volume (wt/vol) MH solution of unspecified MGO content showed no evidence of histological epithelial injury when applied to rabbit nasal respiratory mucosa up to 14 days. Paramasivan et al[210] was the first group to suggest that MGO content above 1.8 mg/mL increases risks of ciliary toxicity. In their study they have concluded that twice daily 16.5% (wt/vol) MH with MGO between 0.9 mg/mL and 1.8 mg/mL over 5 days reduces mature *S. aureus* biofilm while showing no toxic effect on the living sinus mucosa and cilia.

Thamboo et al[211] reported a single-blind study investigating daily atomized MH saline solution for 30 days in AFRS and found that there were no significant differences in endoscopy between MH treated and untreated sinuses.

The first randomized controlled, clinician-blinded trial investigating topical MH in recalcitrant CRS was conducted by Lee et al[212]. Patients received 10% (wt/vol) MH of unspecified MGO content or saline sinus irrigations twice daily for 30 days and were offered oral antibiotics and/or oral/topical steroids. Unfortunately, this study was

limited by the difference in baseline disease between treatment and control group which made interpretation of the data difficult.

Due to insufficient data, the International Consensus Statement on Allergy and Rhinology: Rhinosinusitis[94] has not made any recommendations on its use.

Colloidal Silver

Colloidal silver (CS) has significant antibiofilm activity *in vitro* and *in vivo* against *S. aureus*[213, 214], MRSA and *P. aeruginosa*. It has been postulated that colloidal silver exerts its antimicrobial properties via multiple mechanisms. It can act on bacterial cell membranes by disrupting phosphate[215] and sodium channels[216], inhibits mitochondrial ATPase[217] and interacts with bacterial DNA to form dissociable complexes [218, 219].

It has also been reported that colloidal silver has immunomodulatory effects. It has the ability to inhibit matrix metalloproteinases (MMPs) which is pro-inflammatory[19] and metallothionein[19] (MT) which promotes resistance to immune-mediated apoptosis[20]. Both MTs and MMPs have been found at increased levels in patients with CRSwNP [21-23]. CS has also been shown to induce inflammatory cells apoptosis by TNF-α and IL-12 suppression[24].

However, the safety concerns of colloidal silver have been the main obstacle to its clinical use. In 1999, the FDA announced that all over-the-counter CS products for internal and external use were not deemed to be safe and effective[220]. Richter et al[221] was the first to report that the shape and size of colloidal silver nanoparticles affects its cytotoxicity profile. Spherical nanoparticles were shown to be non-toxic in human cell

culture (Tamm-Horsfall Protein 1, Human airway epithelial cell line)[221] and safe in a sheep sinusitis model[214]. However, further clinical studies with close observations of safety and efficacy parameters will be required to ascertain the role of CS in the management of recalcitrant CRS.

To date only one randomised crossover control trial has been conducted, Scott et al[222] reported in recalcitrant CRSsNP patients, over-the-counter CS nasal sprays twice daily over 6 weeks did not improve SNOT-22 or LKS when compared to nasal saline spray. There were no reports of toxicity or adverse events. However this study lacked descriptions of CS concentration and particle properties making the application of this study challenging.

Bacteriophage

Bacteriophage (phage) are viruses that infect bacteria and exist as the most abundant organisms in our biosphere[223]. Phage are highly specific in host range and infect only one host species[224]. This unique profile allow phage to be formulated for (1) diagnostic medicine, using phage particle probes for bacterial detection[225] and identification of tumour targeting agents[226]; (2) therapeutic medicine, using drug-carrying phage[227, 228], gene target and delivery phage[229] and (3) prophylactic medicine, phage as probiotics[230], vaccines[231-233] and in food and agricultural biocontrol[234]. A number of phage have been recognised and approved by the FDA as Generally Recognised as Safe (GRAS) to be sprayed on cattle hide, processed meat and agricultural produce.

History of bacteriophage

In 1896, bacteriologist Ernest Hankin first described antibacterial properties from the waters of Ganges and Jumma against *Vibrio Cholera*. He credited this phenomenon to an unidentifiable, heat labile and filterable subject which he thought contributed to limit the spread of cholera epidemics in India. This discovery was left unexplored for 20 years until Frederick Twort made similar observations while working on micrococci bacteria. He had noticed clear micrococci cultures were able to eradicate white micrococci cultures, but was uncertain if this was due to either a virus or antibacterial enzyme produced by the micrococci itself. It was not until 1917 when a microbiologist Felix d'Herelle had proposed the effect to be caused by a virus capable of infecting bacteria and he later coined the term 'bacteriophage'. He presented his findings of an 'invisible microbe with antagonistic properties' at the Academy Meeting of Sciences but was met with much debate from the scientific committee on whether the effect was truly viral or enzymatic.

However, following his discovery, d'Herelle started using phage to treat patients with dysentery. It was observed that after one dose of phage preparation, patients had started to recover within 24 hours of treatment. Several other people followed using phage to treat a number of suppurative infections, including *Staphylococcal* skin infections and meningitis. However their efforts were eclipsed by the sudden upscale production of penicillin at the onset of World War II.

During World War II, government-sponsored projects spurred pharmaceutical industries to dramatically accelerate the translation of penicillin from laboratory to mass commercial manufacture. Interests shifted away from phage as limited understanding of phage molecular biology made it difficult to reliably mass-produce phage

preparations. Also, the relatively narrow host ranges of phage and inherent difficulties with phage storage and transport during wartime made it less attractive than penicillin.

Although focus on phage has waned in western countries, a number of eastern European countries continued their research on phage.

Re-emerging interest in bacteriophage

Recently interest in phage has re-emerged in the face of escalating antibiotic resistance. In 2017, The World Health Organization (WHO) reported antibiotic resistance to be a serious global threat to public health. With the slowing pipeline of antibiotic production, a priority list of pathogens was released by the WHO to guide research and development for new antimicrobials. Amongst the list, MRSA has been classified as a high priority pathogen and *P. aeruginosa* as critical. Both infections have been observed with increasing prevalence in CRS, especially in recalcitrant CRS.

Pros and Cons of Bacteriophage

Phage confers several advantages over oral antibiotics (Table 7).

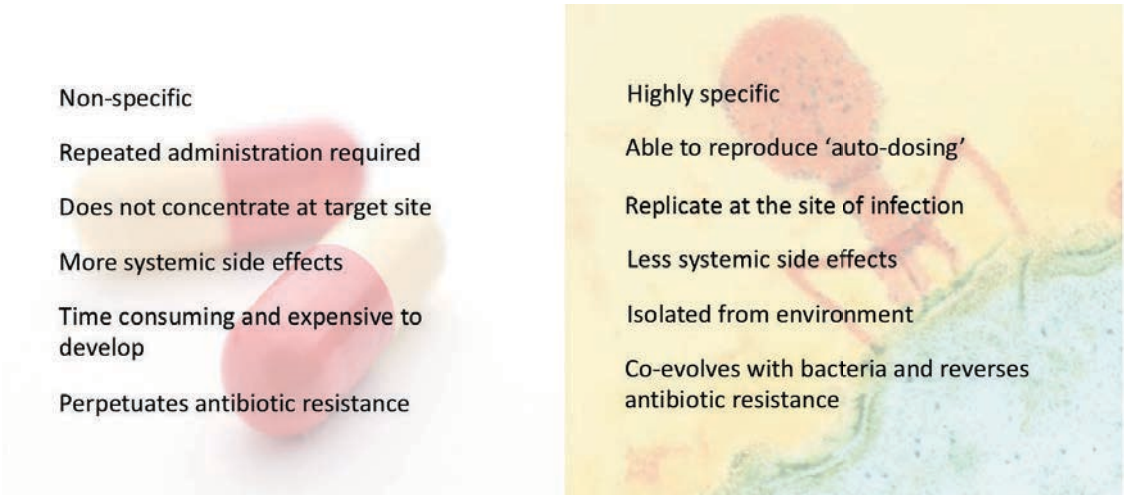

Table 7. Advantages of oral antibiotics (left) vs phage (right).

However, phage are highly specific in nature and have narrow host ranges. Just like antibiotics it is possible for bacteria to develop resistance against phage resulting in bacteriophage insensitive mutants (BIM). Phage mixes/ cocktails have been proposed to increase target host ranges and reduce the risk of BIM development. There are also theoretic concerns that phage therapy could cause large-scale lysis of bacteria and release of endotoxins (Herxheimer effect) although this has yet to be reported in literature. Unlike antibiotics, as phage are living organisms and can be active for as long as there is bacterial persistence, exact pharmacokinetics can be difficult to ascertain and may vary between individuals with differing infection severity. As phage preparations mainly exist in liquid formulations and require stable refrigeration, long term storage and transport can be costly when compared to antibiotics. There are also concerns that by the time of distribution, phage preparations will not remain active for long enough against circulating bacterial strains, however, it has been argued that this is no different from antibiotics and in fact the ability of phage to co-evolve with bacterial is favourable.

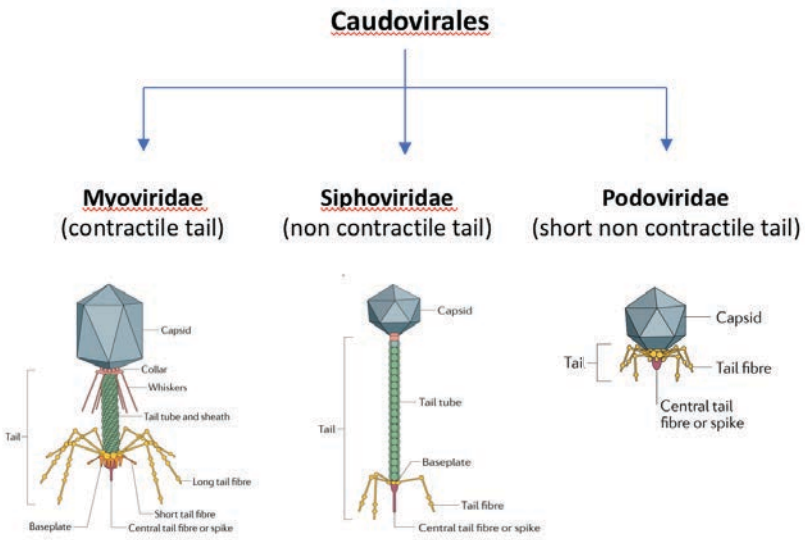

Figure 13. Phage taxonomy as described by Ackermann and Eisenstark as Myoviridae, Siphoviridae, Podoviridae. Adapted from Nobrega et al (2018)[235].

David Bradley first used electron microscopy[236] and fluorescent staining[237] to classify phage into three phenotypes; contractile tail, non-contractile tail and short non-contractile tail. Hans-Wolfgang Ackermann & Abraham Eisenstark later proposed to name them *Myoviridae, Styloviridae,* and *Pedoviridae* respectively. In the early 1980s, the International Committee on Taxonomy of Viruses (ICTV) accepted these three distinct families as Myoviridae, Siphoviridae, and Podoviridae and for the next 30 years this classification system remained unchanged.

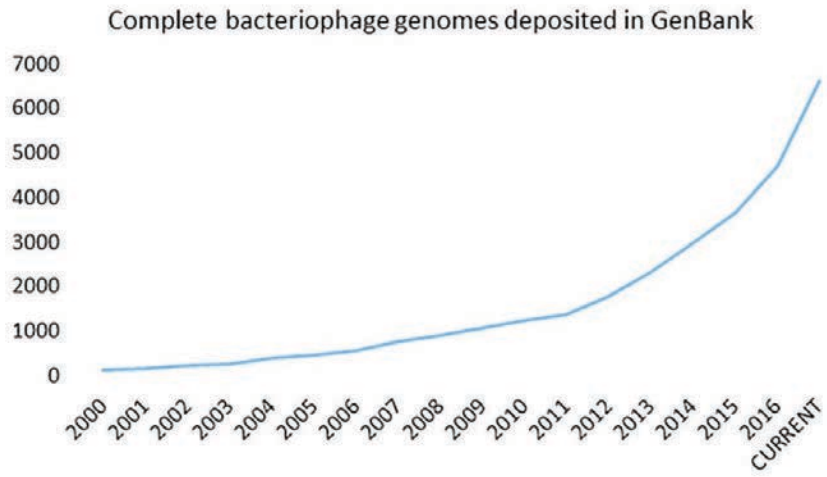

Figure 14. Exponential increase in phage genome discovery over time. Adapted from Tolstoy et al (2018)[238].

The advancement of proteomics and the regeneration of interest in phage prompted a renewed perspective on phage classification. By 2008, there were 349 fully sequenced members of the caudovirales and this number had exponentially increased over the last decade (Figure 14). A decade later, phage taxonomy now classifies caudovirales into 4 families (*Myoviridae, Siphoviridae, Podoviridae and Ackermannviridae*) with more than 22 assigned subfamilies[239] (Figure 15) and this classification is updated annually under the ICTV conventions.

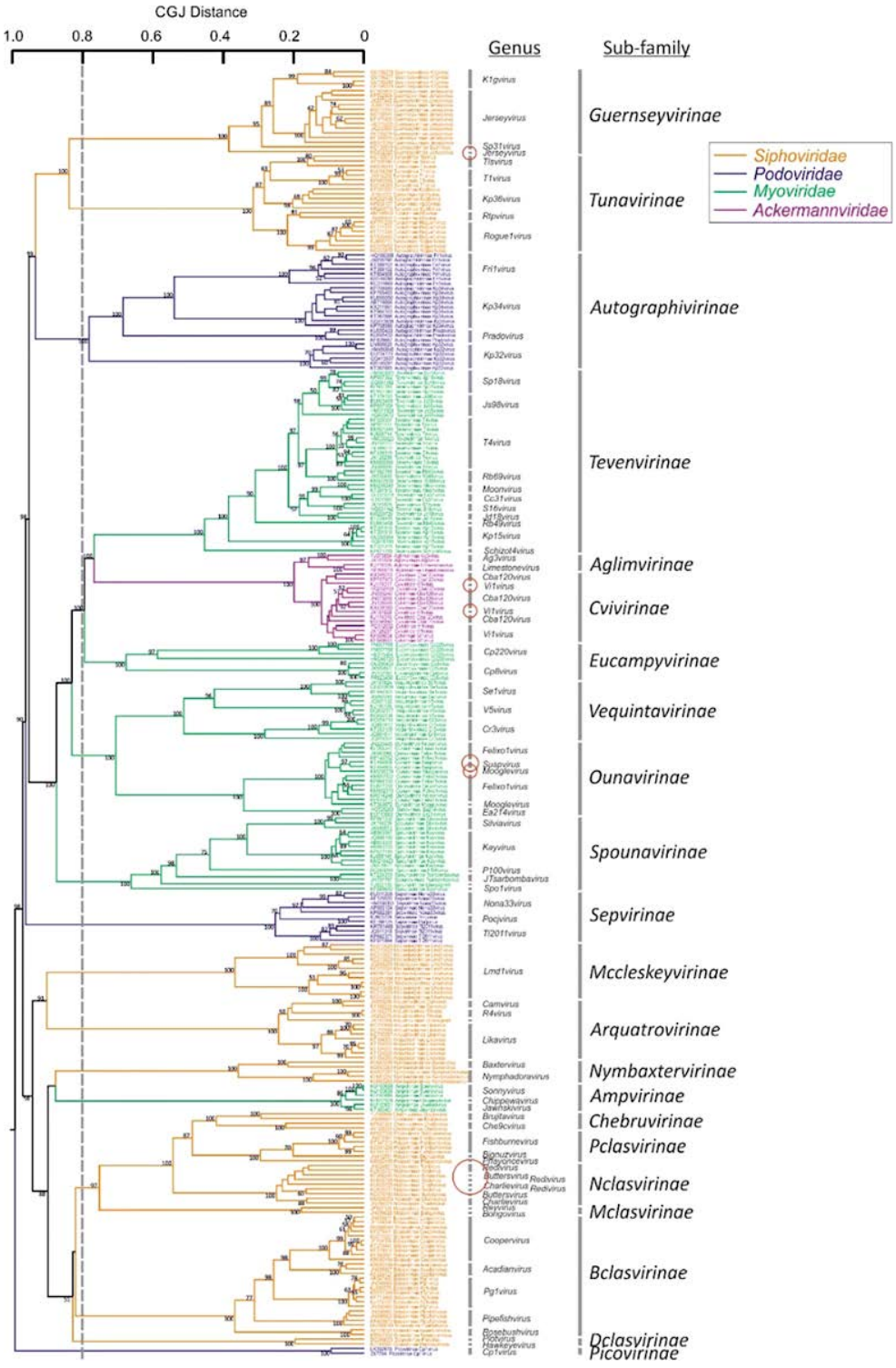

Figure 15. Illustration of phage taxonomy. Adapted from Aiewsakun et al (2018)[239].

Bacteriophage lifecycle

Phage can be divided into three types; lytic, temperate or lysogenic[223, 240-242];

(a) Lytic phage hijack bacterial host cellular machinery to produce progeny phage. Once a critical number of phage particles are formed, the host cell is lysed and progeny phage are released into the surrounding environment to invade new bacterial hosts[223, 240, 241].

(b) Lysogenic phage integrate their DNA into the bacterial host chromosome, and remain latent benignly replicating with the bacteria [223, 240, 241]. When phage DNA is situated within the bacterial genome they are referred to as prophage.

(c) Temperate phage have the characteristics of both these phage types and are able to enter the lytic cycle or integrate into host chromosome[223, 240, 241]. Under appropriate stimulation, temperate phage can exit the host genome and enter the lytic phage cycle.

Lytic phage lifecycle and mechanism of action

Phage therapy primarily utilises lytic phage. This lifecycle/ mechanism of action is described in detail below (Figure 16) using the example of well-studied *Escherichia coli* phage (T4)[243].

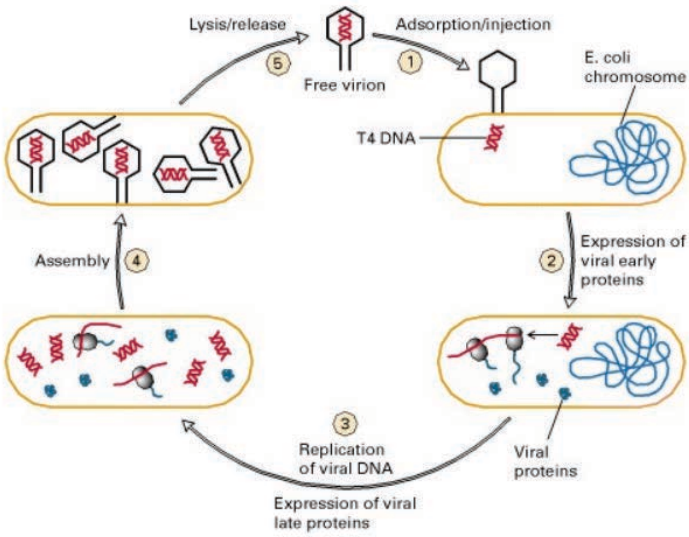

Figure 16. Lytic phage *E. coli* T4 life cycle. Adapted from Lodish et al (2000)[242].

1. Adsorption and injection; Lytic phage infection starts with attachment of phage viral coat tail fibres with specific receptors on the bacterial host cell surface. Then genetic material of phage is injected into the cytoplasm of host bacteria.
2. Transcription; Following that, host cell enzyme begins transcription and translation of phage specific proteins which hijacks host cellular machinery to replicate viral DNA.
3. Replication of DNA; Host cell DNA is degraded to supply nucleotides for the synthesis of viral DNA.
4. Assembly; Progeny phage are assembled within the host cell.
5. Release; Progeny phage produces lysozyme which hydrolyses cell wall peptidoglycan inducing host cell lysis and release of phage.

Bacteriophage therapy

Over the last 60 years, a large volume of work with phage therapy has been from Eastern Europe. Many of these studies were in foreign texts and were not available until Sulakvelidze et al[134] published a review on this body of work (Table 8).

In a Polish institute, Slopek et al[244] conducted several studies involving a total of 550 patients with different suppurative bacterial infections. Successful eradication of bacteria was reported in 92.4% of cases which were primarily antibiotic resistant, including *Klebsiella, Salmonella, Pseudomonas, Staphylococcus, Streptococcus* and *E. coli* infections.

However, the lack of control and blinding groups has stunted the claims of phage therapy and limited its clinical advancement. Furthermore, much work still needs to be done to better understand the complexity of phage biology and phage-host interactions.

Reference(s)	Infection(s)	Target Pathogen(s)	Results
Babalova et al	Bacterial dysentery	*Shigella*	*Shigella* phage were successfully used for prophylaxis of bacterial dysentery.
Bogovazova et al	Infections of skin and nasal mucosa	*K. ozaenae, K. rhinoscleromatis*, and *K. pneumoniae*	Adapted phage were reported to be effective in treating *Klebsiella* infections in all of the 109 patients.
Cislo et al	Suppurative skin infections	*Pseudomonas, Staphylococcus, Klebsiella, Proteus*, and *E. coli*	Thirty-one patients having chronically infected skin ulcers were treated orally and locally with phage. The success rate was 74%.
Ioseliani et al	Lung and pleural infections	*Staphylococcus, Streptococcus, E. coli*, and *Proteus*	Phage were successfully used together with antibiotics to treat lung and pleural infections in 45 patients.
Kochetkova et al	Postoperative wound infections in cancer patients	*Staphylococcus* and *Pseudomonas*	A total of 131 cancer patients having postsurgical wound infections participated in the study. Of these, 65 patients received phage and the rest received antibiotics. Phage treatment was successful in 82% of the cases, and antibiotic treatment was successful in 61% of the cases.
Kucharewicz-Krukowska and Slopek	Various infections	*Staphylococcus, Klebsiella, E. coli, Pseudomonas*, and *Proteus*	Immunogenicity of therapeutic phage was analyzed in 57 patients. The authors concluded that the phage immunogenicity did not impede therapy.
Kwarcinski et al	Recurrent subphrenic abscess	*E. coli*	Recurrent subphrenic abscess (after stomach resection) caused by an antibiotic-resistant strain of *E. coli* was successfully treated with phage.
Litvinova et al	Intestinal dysbacteriosis	*E. coli* and *Proteus*	Phage were successfully used together with bifidobacteria to treat antibiotic-associated dysbacteriosis in 500 low-birth-weight infants.
Meladze et al	Lung and pleural infections	*Staphylococcus*	Phage were used to treat 223 patients having lung and pleural infections, and the results were compared to 117 cases where antibiotics were used. Full recovery was observed in 82% of the patients in the phage-treated group, as opposed to 64% of the patients in the antibiotic-treated group.
Miliutina and Vorotyntseva	Bacterial dysentery and salmonellosis	*Shigella* and *Salmonella*	The effectiveness of treating salmonellosis using phage and a combination of phage and antibiotics was examined. The combination of phage and antibiotics was reported to be effective in treating cases where antibiotics alone were ineffective.

Reference(s)	Infection(s)	Target Pathogen(s)	Results
Perepanova et al	Inflammatory urologic diseases	*Staphylococcus, E. coli*, and *Proteus*	Adapted phage were used to treat acute and chronic urogenital inflammation in 46 patients. The efficacy of phage treatment was 92% (marked clinical improvements) and 84% (bacteriological clearance).
Sakandelidze and Meipariani	Peritonitis, osteomyelitis, lung abscesses, and postsurgical wound infections	*Staphylococcus, Streptococcus*, and *Proteus*	Phage administered subcutaneously or through surgical drains in 236 patients having antibiotic-resistant infections eliminated the infections in 92% of the patients.
Sakandelidze	Infectious allergoses (rhinitis, pharyngitis, dermatitis, and conjunctivitis)	*Staphylococcus, Streptococcus, E. coli, Proteus*, enterococci, and *P. aeruginosa*	A total of 1,380 patients having infectious allergoses were treated with phage (360 patients), antibiotics (404 patients), or a combination of phage and antibiotics (576 patients). Clinical improvement was observed in 86, 48 and 83% of the cases, respectively.
Slopek et al	Gastrointestinal tract, skin, head, and neck infections	*Staphylococcus, Pseudomonas, E. coli, Klebsiella*, and *Salmonella*	A total of 550 patients were treated with phage. The overall success rate of phage treatment was 92%.
Stroj et al	Cerebrospinal meningitis	*K. pneumoniae*	Orally administered phage were used successfully to treat meningitis in a newborn (after antibiotic therapy failed).
Tolkacheva et al	Bacterial dysentery	*E. coli* and *Proteus*	Phage were used together with bifidobacteria to treat bacterial dysentery in 59 immunosuppressed leukemia patients. The superiority of treatment with phage-bifidobacteria over antibiotics was reported.
Weber-Dabrowska et al	Suppurative infections	*Staphylococcus* and various gram-negative bacteria	Orally administered phage were used to successfully treat 56 patients, and the phage were found to reach the patients' blood and urine.
Zhukov-Verezhnikov et al	Suppurative surgical infections	*Staphylococcus, Streptococcus, E. coli*, and *Proteus*	The superiority of adapted phage (phage selected against bacterial strains isolated from individual patients) over commercial phage preparations was reported in treating 60 patients having suppurative infections.

Table 8. Review of phage studies undertaken across Eastern Europe. Adapted from Sulakvelidze et al (2001)[245].

The first controlled clinical trial of a therapeutic phage preparation to evaluate the efficacy of phage was performed in England [246]. The trial dealt with refractory ear infections caused by *P. aeruginosa.* 12 patients were treated with a single dose of phage, 12 received a placebo and both groups were monitored for 42 days. The study reported significant improvement in clinical symptoms for the test group and *P. aeruginosa* counts were lower for the phage treated group.

In Europe, a multicentre, randomised, single-blinded controlled clinical trial is underway to evaluate phage therapy in *Escherichia coli* and *P. aeruginosa* infected burn wounds. This multicentre trial across France, Belgium and Switzerland has been funded by the European Commission (under the 7th Framework Programme for Research and Development), and is expected to lead to significant advancements in the regulatory framework of phage therapy in Europe[247].

The phage presented within this thesis, AB-SA01 (combination of 3 *S. aureus* phage) and AB-PA01 (combination of 4 *P. aeruginosa* phage) had been approved for compassionate use in the USA and Australia. Patients with life-threatening *S. aureus* (n=5) and *P. aeruginosa* (n=3) infections, who did not respond to antibiotics, were given intravenous AB-SA01 (3×10^9 PFU/dose) and nebulised AB-PA01 (4×10^9 PFU/dose) respectively. These were administered concurrently with appropriate antibiotic therapy. 75% of patients had infection successfully treated with no adverse events[248]. This finding is complemented by further safety and efficacy data of topical phage administration into sinuses which will be reviewed below.

Bacteriophage in rhinology

Most of the work in phage therapy has been related to *S. aureus* sinonasal infections, considering it is the most common organism associated with CRS. A few studies have utilized phage cocktails after *in vitro* studies and have shown anti-*S. aureus* phage mixes were superior to single phage as it reduces the risk of developing BIM[249, 250] and provides a wider host range effect[251].

Drilling et al[252] have shown that phage cocktail CTSA is effective against 64/ 66 clinical isolates of CRS with significant reduction in *S. aureus* biofilms. Further studies using phage cocktail CTSA *in vivo* have shown that it is safe over 3 days with significant reduction of *S. aureus* biofilms in sheep frontal sinuses.

Zhang et al[253] assessed the susceptibility of phage cocktail against 65 *S. aureus* clinical isolates from CRS patients and found that there was no significant difference in phage sensitivity against antibiotic resistant and induced antibiotic- tolerant *S. aureus.*

Chibber et al[254] described the efficacy of broad spectrum lytic phage (MR10) *in vitro* on cultured murine nasal cells and *in vivo* murine nasal MRSA colonization model. Intranasal phage treatment was found to be comparable to the efficacy of current treatment mupirocin, with additive effects when used concurrently to achieve a more rapid eradication of MRSA and negating the development of drug resistant mutants.

Apart from using direct phage treatments, several studies have also reported the use of phage proteins to achieve bacteria eradication. Drilling et al[255] used a chimeric

muralytic enzyme (P128) against 3 clinical isolates of *S. aureus* from CRS, including MRSA. P128 combines the specificity of lysostaphin-derived cell wall binding domain with peptidoglycan-hydrolase cell wall degradation domain derived from Phage K (a well-studied *S. aureus* myovirus). There was up to 95.5% reduction of *in vitro S. aureus* biofilms. Rashel et al[256] reported the successful treatment of MRSA nasal infections using intranasal phage lysins (purified and cloned from novel ΦMR11 phage) *in vivo*. Fenton et al[257] utilized intranasal spray of truncated phage lysin (derived from phage K) in a murine nasal MRSA colonization model. It showed rapid elimination of *S. aureus* from nasal mucosa of mice with almost double the activity of native phage lysin.

The study of pseudomonas phage in the field of rhinology is relatively new. A lot of study data had been based on effective phage treatment of lung *P. aeruginosa* clinical isolates from cystic fibrosis patients which are frequently multidrug resistant.

Fong et al[254] evaluated the in vitro efficacy of pseudomonas phage cocktail (CT-PA) against 44 clinical isolates from CRS patients with and without cystic fibrosis and found a 76% reduction in *P. aeruginosa* biofilms after a single dose of phage treatment. Future studies looking at the safety and efficacy of CT-PA in an *in vivo* sinusitis model will be needed to advance its clinical application.

In view of promising preliminary data of phage against *S. aureus* and *P. aeruginosa* (biofilm and multidrug resistant) infections, phage therapy holds potential to be translated into effective clinical treatment for CRS.

Furthermore, with increasing evidence suggesting that commensals in the sinonasal microbiome play a beneficial role in maintaining microbial balance and diversity within sinuses, phage ability to target only the pathogen of interest may also have evident advantages in the management of CRS.

Future potential of bacteriophage in CRS

Recent work on stabilising phage in liquid and dry powder formulations has aimed to improve delivery and penetration of phage treatments to the respiratory tract. To date studies employing phage have mostly been in liquid formulations administered via intranasal instillation or nebulisation. However, freeze drying techniques now enable the formulation of powder phage which allows for long term storage without refrigeration and aids transport[258]. Chang et al[259] recently reported the safety of phage PEV20 dry powder *in vitro* and *in vivo*. This presents an alternative route for phage administration and could potentially be relevant to future otolaryngology medicine.

Deferiprone and Gallium protoporphyrin IX

Richter et al[260-262] first described the potent synergistic antimicrobial properties of Deferiprone and Gallium Protoporphyrin IX (DG). The DG combination is effective in eradicating MRSA, *S. aureus*, *S. epidermidis*, *P. aeruginosa*, and *Acinetobacter johnsonii* biofilms. It is thought to exert its anti-biofilm effects by interfering with the iron metabolism of bacteria, which is involved in membrane bound respiration, bacterial growth, protection against reactive oxygen species, and increases in bacterial virulence factors[263, 264].

Deferiprone is an FDA approved iron chelator used to treat thalassaemia major while Gallium Protoporphyrin IX is a heme analogue with strong antibacterial activity against gram-positive bacteria, gram-negative bacteria and mycobacteria[265, 266].

The hypothesised mechanism of action is that Deferiprone chelates iron from the bacteria's surrounding environment, forcing the bacteria to upregulate their iron transporter proteins, which results in increased uptake of the heme analogue Gallium Protoporphyrin IX into bacterial cells[260, 262] causing cell death. Gallium Protoporphyrin IX although structurally similar to heme, is (a) unable to facilitate electron transfer which is essential for redox reactions in bacterial respiration, DNA synthesis and ATP generation, (b) unable to facilitate efflux of reactive oxygen species and (c) unable to cleave[265, 267, 268].

Richter et al[269] applied DG concurrently and consecutively to *S. aureus* biofilms and was able to demonstrate that when biofilms were first treated with Deferiprone before Gallium Protoporphyrin IX, its antibiofilm activity had doubled the effect of concurrent DG application. This efficacy was further enhanced when biofilms had prolonged exposure to Deferiprone prior to the administration of Gallium Protoporphyrin IX, supporting the mechanism of its synergistic effect.

Recent studies have also shown that DG is effective against planktonic and biofilm-associated *S. aureus* SCV[270]. SCVs with their ability to reside intracellularly within eukaryotic cells aid immune evasion and antibiotic tolerance[62], often contributing to treatment failure and disease recalcitrance[71]. Richter et al[270] demonstrated significant anti-biofilm activity against clinical isolate *S. aureus* parent strain and its gentamicin-

induced SCV, as well as a clinically isolated *S. aureus* SCV. They had also demonstrated DG's ability to potentiate the effect of gentamicin and ciprofloxacin (Cip). The triple drug combination of DG-Cip showed significant antibiofilm effects against all strains when individual compounds had failed, while DG-Gentamicin showed significantly enhanced antibiofilm effects when compared to individual compounds. When applied to an *in vivo* nematode infection model of *Caenorhabditis elegans,* DG treatment significantly prolonged the survival of infected worms and reduced the bacterial burden per worm.

Although the efficacy profile of DG has been observed *in vitro* and *in vivo* (nematodes), its safety and efficacy has not been evaluated in an *in vivo* model of sinusitis.

Xylitol

Xylitol is a five-carbon sugar alcohol sweetener, often used as an artificial sugar in manufactured products to reduce dental caries. It has been found to have indirect antibacterial properties. It exerts its antimicrobial effect by lowering the salt concentrations in sinonasal secretions which enhance the antibacterial activity of salt-sensitive agents in our airway surfaces ie. lysozyme, lactoferrin, secretory leukoproteinase inhibitor, human β-defensins 1 and 2, secretory phospholipase A2, and the cathelicidin LL-37. Innate immune mediators have been shown to be salt-sensitive with increased salt concentrations often inhibiting their activity[271]. Xylitol has also been shown to exert bacteriostatic activity by disrupting glucose cell-wall transport and intracellular glycolysis[272].

Jain et al[273] investigated the anti-biofilm effect of xylitol *in vitro* and demonstrated reduction of *S. epidermidis* biofilm biomass and inhibition of *S. aureus* and *P. aeruginosa* biofilm formation. Weissman et al[274] carried out a prospective, randomized, double-blinded, controlled crossover pilot study to investigate the tolerability and efficacy of sequential xylitol and saline nasal irrigations over 10 days. They found that there was a significant improvement in SNOT-20 scores in the xylitol group compared to saline. There were no changes in visual analogue scale (VAS) scores and no adverse reports except 1 report of minor discomfort during rinse. These studies suggest the potential benefit of xylitol as an additive to regular irrigations and is currently sold under the proprietary name Flo Sinus CRS Kit®.

In a randomized, double-blinded, crossover study, Zabner et al[275] demonstrated that xylitol nasal sprays in normal volunteers for 4 days significantly decreased the number of nasal coagulase-negative *Staphylococcus* compared with saline control. Also in a prospective, randomized, double-blinded crossover study, Weissman et al[274] demonstrated significant improvement in SNOT-20 scores while patients were on xylitol irrigation. Lin et al[276] reported significantly improved symptoms (VAS and SNOT-22) in patients using xylitol nasal irrigations compared to saline. There was also a significant increase in nasal nitric oxide (NO) and inducible nitric oxide synthase mRNA in the maxillary sinuses of patients in the xylitol group, suggesting that patients on xylitol irrigations might benefit from increased NO protective effects which include upregulation of ciliary motility and added antiviral and antibacterial action.

Due to limited evidence on its efficacy, the International Consensus Statement on Allergy and Rhinology: Rhinosinusitis[94] has not made any recommendations on its use.

Surfactants

It is believed that chemical surfactant exerts its anti-biofilm effects via several mechanisms: 1) serves as a biocide and demonstrates synergistic effects with antibiotics[277] 2) serves as a mucolytic agent by reducing water surface tension which improves the penetration of sinus irrigations[278] and 3) disrupts biofilm matrices to enhance the effect of antibiotics[279].

Topical 1% Johnson's baby shampoo, citric acid/zwitterionic surfactant (CAZS) and Sinusurf have been investigated for its role in recalcitrant CRS however safety and efficacy results are mixed.

CAZS has been demonstrated to have significant transient anti-biofilm activity[280] and was noted to cause temporary ciliary denudation *in vivo*[280, 281].

Isaac et al[282] looked at the effects of 1% baby shampoo on mucociliary clearance time of healthy volunteers and found that there was increased mucociliary clearance compared to saline alone. Chiu et al[283] showed that 1% Johnson's baby shampoo twice daily irrigation for 4 weeks in recalcitrant CRS showed 47% overall symptom improvement and 60% improvement in post nasal drip. However, about 11% of patients withdrew from the study due to minor nasal and skin irritation.

Sinusurf solution comprised of proprietary surfactant components of baby shampoo has been discontinued following frequent side effects of nasal burning, epistaxis, headache and temporary anosmia.

Due to the lack of convincing efficacy data on surfactants and in view of the reported ciliotoxic effects, they are currently not widely used in clinical practice.

Mucoactive agents

S-Carboxymethylcysteine (S-CMC) is a mucoregulatory agent used in chronic obstructive pulmonary disease to improve mucociliary clearance. It has anti-inflammatory properties and also an ability to scavenge free radicals[284]. Ohashi et al[285] was first to demonstrate 0.5% S-CMC enhanced ciliary beating frequency *in vitro*. Sugiura et al[286] then followed by demonstrating a dose dependent improvement in ciliary recovery using 10% nebulised S-CMC in an *in vivo* sinusitis model exposed to noxious sulphur dioxide stimuli. In a large randomized controlled trial involving 432 patients, Majima et al[287] studied the use of S-CMC (1500 mg/day) in combination with clarithromycin (200 mg/day) to treat CRS over 12 weeks and found significant improvement in patients' nasal discharge and post nasal drip symptoms when compared to clarithromycin alone. Both groups also showed significant improvement in SNOT-20 and CT scores. However, due to the unspecified CRSwNP and CRSsNP cohorts and based on our current understanding that long term macrolides could improve CRSsNP, it is difficult to interpret the results presented.

Other topical modalities

Chitogel

Chitogel (chitosan and dextran) comprises succinyl-chitosan which is a chitosan polymer produced by the hydrolysis of chitin, found in the exoskeletons of crustaceans. It is a surgical hydrogel FDA approved for use in post endoscopic sinus surgery to

improve patient's outcome[288-291]. It has effective hemostatic[289, 290, 292-298], wound healing[299-301], anti-adhesion[288, 302-310] and antimicrobial[311-313] properties. However, its role as a drug release vehicle in delivering topical treatments to sinus cavities has yet to be explored.

The effective delivery of topical treatments into sinus cavities continues to be one of the main obstacles in modern practice. Our current methods of delivery (sinonasal irrigations and nasal sprays) lack the ideal characteristics of complete sinus distribution and prolonged mucosal contact time to increase local absorption and minimise waste[97]. Patients receive highly variable drug penetration influenced by patient condition, sinus surgery, delivery devices, irrigation volume and pressure and patient positioning[97-100].

Topical application using a hydrogel has the potential to deliver higher concentration of drugs to sinus cavities with enhanced localised action and less systemic side effects. The mucoadhesive properties of hydrogel also increase contact time of topical agents with the sinus mucosa and biofilms[314, 315] augmenting their anti-biofilm effects.

In a prospective, randomised controlled trial, Ha et al[316] investigated the post-operative outcomes of Chitogel combined with Pulmicort Respules® (budesonide, 1 mg/2 mL) on sinus ostia. In this study, Chitogel served as a drug delivery vehicle for budesonide for its anti-inflammatory properties. Using patients as self-controls, Chitodex + budesonide gel was compared with control (nothing) and steroid-only (betamethasone cream). Results have shown that Chitogel with budesonide is superior to both control and steroid-only at improving healing in the early post-operative period. In the frontal sinuses, Chitogel + budesonide gel treated sides maintained 71% of their ostial diameter

at 12 months, compared to 51% in controls (no treatment). Similar results were seen in the sphenoid and maxillary sinuses, although less pronounced.

Rayan et al reported the use of CG as a drug vehicle to deliver Ropivacaine into the sinus cavities of patients post endoscopic sinus surgery for pain management (manuscript in preparation). Although CG-Ropivacaine did not reduce postoperative pain scores on visual analogue scales, a significant reduction in opioid consumption was observed within 24 hours. There was also a statistically significant reduction in adhesions seen in patients treated with CG-Ropivacaine vs CG-Budesonide at 2 weeks post-surgery. This finding could be attributed to the inhibitory effects of local anaesthetics on initial stages of cutaneous wound healing[317] and dampening effects on tensile strength of wounds [318].

The potential role of CG as a topical anti-biofilm agent was alluded to by Richter et al[269]. CG incorporated with novel antibiofilm agents Deferiprone and Gallium Protoporphyrin IX (DG) was shown to be effective *in vitro* against *S. epidermidis, S. aureus,* MRSA, and *P. aeruginosa* biofilms. More importantly, it has also been shown to significantly reduce *S. aureus* small colony variants *in vitro. S. aureus* phenotypes which are generally antibiotic tolerant and difficult to treat.

This finding was unsurprising as the combination of CG-DG had allowed DG to exert its optimum anti-biofilm effect. Enhanced DG efficacy had been previously described when pure compounds were applied consecutively and this effect had been simulated by the release of DG from CG. Richter et al[269] had shown that Deferiprone (hydrophilic) was found to be released from CG completely within the first 48-72 hours whilst

Gallium Protoporphyrin IX (hydrophobic) had a progressive and more sustained release over time.

These studies have consistently demonstrated CG to be an effective drug delivery vehicle into otherwise difficult to access sinus cavities. In some instances it has the potential to enhance the therapeutic effects of treatment agents as it can be utilised to deliver drugs sequentially based on its hydrophilic profile. However, the translational value for its clinical application in recalcitrant CRS still needs to be further evaluated.

Drug eluding biomaterials

In a multicenter, randomized double blinded clinical trial involving 43 patients, bioabsorbable steroid-eluting sinus stent (Propel implants®) following ESS has demonstrated significant reduction in inflammation, polyp formation and adhesions at day 21 to 45 compared to non-drug eluting stents. It was shown to be safe with no adverse events, no quantifiable systemic steroid absorption and no evidence of adrenal axis suppression[319].

In the ADVANCE II multicenter randomized double blinded trial involving 105 patients following ESS, it was shown that bioabsorbable steroid eluting sinus implants (Propel implants®) significantly improve post-surgical outcomes compared with non-drug eluting implants. There was a 45% decrease in frank polyposis, 29% decrease in steroid prescriptions, 52% reduction in adhesion formation and 29% decrease in post-operative interventions. There were also no ocular adverse outcomes observed in this study[320].

This improved post-operative outcome has also been shown to translate into an economic benefit by preventing further costs from post-surgical interventions[321].

Although it has been shown to have beneficial effects on post-surgical outcomes, the International Consensus Statement on Allergy and Rhinology: Rhinosinusitis[94] was not able to come to a consensus on its use due to evidence which is still early and small, despite being of a high level. Regardless, the use of drug eluting biomaterials represents a new forefront in ENT surgery and its potential role as a drug delivery vehicle for anti-biofilm agents has yet to be explored.

Drug carrier nanoparticles

In the field of nanomedicines, biodegradable nanoparticles have been shown to be able to penetrate highly viscoelastic mucus of CRS which advances its potential to deliver localised treatments[322]. However, it is still in the early stages of conception.

Chapter 2: Efficacy of anti-biofilm gel, Chitogel- Mupirocin-Budesonide in a sheep sinusitis model

Statement of Authorship

Title of Paper	Efficacy of anti -biofilm gel, Chitogel -Mupirocin -Budesonide in a sheep sinusitis model
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Principal Author

Name of Principal Author (Candidate)	Mian Li Ooi
Contribution to the Paper	Project design, data collection and analysis and manuscript preparation
Overall percentage (%)	100%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 1/9/2019

Co-Author Contributions

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

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

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

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

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2.1 Abstract

Background: The search for effective topical anti-inflammatory and antibiofilm delivery to manage recalcitrant chronic rhinosinusitis (CRS) continues to be elusive. The ideal topical treatment aims to provide adequate contact time for treatment agents to exert its effect on sinus mucosa. Our study presents the *in vivo* efficacy of Chitogel, combined with anti-inflammatory agent Budesonide and antibiofilm agent Mupirocin (CG-BM) for treatment of *S. aureus* biofilms.

Methods: In established sheep sinusitis model, 15 sheep were randomised into three groups (5 sheep, n=10 sinuses, per treatment); (1) twice daily saline flush (NT), (2) Chitogel (CG) with twice daily saline flush, and (3) CG-BM with twice daily saline flush for 7 days. The anti-inflammatory effect was graded histologically by an independent blinded pathologist. The antibiofilm effect was assessed using LIVE/DEAD BacLight stain and confocal scanning laser microscopy.

Results: No significant differences were observed in histopathology between all groups. A significant reduction in biofilm biomass was shown using COMSTAT2 in CG-BM treated sheep compared to NT controls ($P<0.05$, One-way ANOVA, Kruskal-Wallis test).

Conclusion: CG-BM is effective against *S. aureus* biofilms in a sheep sinusitis model and could represent a viable treatment option in the clinical setting for recalcitrant CRS.

2.2 Introduction

Bacterial biofilms are known to play a role in the pathogenesis of recalcitrant chronic rhinosinusitis[92, 93, 323-325]. Amongst surgically recalcitrant patients about 40-50% of biofilms identified are dominated by *Staphylococcus aureus*[326, 327]. Oral antibiotics are often ineffective against biofilms[328], pressuring a continuous search for topical anti-biofilm agents which allows for increased concentration, localised action and less systemic side effects.

The effective delivery of these topical treatments into sinus cavities remains the main obstacle. Currently topical antibiotics are administered via sinonasal irrigations, nebulisers and nasal sprays. All of these methods lack the ideal characteristics of complete sinus distribution, prolonged mucosal contact time to increase local absorption and minimal waste[97]. Patients receive highly variable drug penetration influenced by patient condition, sinus surgery, delivery devices, irrigation volume and pressure, and patient positioning[97-100].

In this study, we looked at investigating the efficacy of Chitogel (chitosan and dextran), a surgical hydrogel FDA approved for the use after sinus surgery, acting as a drug carrier to deliver the anti-inflammatory effects of budesonide and antibiofilm effects of mupirocin in a previously validated *S. aureus* biofilm sheep sinusitis model[329-331].

Chitogel (CG) comprises succinyl-chitosan which is a chitosan polymer produced by the hydrolysis of chitin, found in the exoskeletons of crustaceans. In the last decade,

Chitogel has been largely used in ENT surgery to improve patient's outcome post endoscopic sinus surgery[288-291] due to its effective hemostatic[289, 290, 292-298], wound healing[299-301], anti-adhesion[288, 302-310] and antimicrobial[311-313] properties. However, its role as a drug release vehicle delivering topical treatments into sinus cavities has yet to be explored.

We hypothesize that by incorporating topical antibiotics into Chitogel we are able to eradicate biofilms by direct application of antibiotic gel into sinuses, increase contact time of topical agents with sinus mucosa and allow use of higher antibiotic concentration at target sites with less systemic side effects. Previous studies have also suggested that chitosan enhances the nasal absorption of topical treatments by the bioadhesivity of the polymer to mucosa and a transient widening of the nasal mucosa tight junctions[314, 315]

The aim of this study was to (i) optimize a sheep sinusitis model for the investigation of a topical gel treatment and (ii) to evaluate the antibiofilm and anti-inflammatory effects of Chitogel-Budesonide-Mupirocin (c) in the treatment of *S. aureus* biofilms in a sheep sinusitis model.

2.3 Materials and Methods

Ethics approval was obtained from the Animal Ethics Committee of The University of Adelaide and the South Australian Health and Medical Research Institute (SAHMRI). All reports on animal experiments have been conducted in accordance with institutional and national guidelines for the care and use of laboratory animals.

Optimisation arm

A total of 5 male merino sheep heads were obtained from the Murray Bridge Abattoir, South Australia. To achieve optimum retention of the gel within the frontal sinuses for 5-7 days, we optimised the method of gel application and determined the appropriate volume of saline flush to be 15mL twice a day commencing 24 hours after gel instillation.

Study arm

Animals

A total of 15 male merino sheep between the dental age of 2 to 4 years were used. All animals were drenched to eradicate the parasite *Oestrus Ovis*.

Bacterial inoculum

Reference strain American Type Culture Collection (ATCC) 25923 Staphylococcus aureus, known to be biofilm forming, was supplied by the Queen Elizabeth Hospital Department of Microbiology, Adelaide. Frozen glycerol stock was defrosted and subcultured for 24 hours in 3mL of nutrient broth (Oxoid, Adelaide, Australia) on a

shaker at 37 ℃ before inoculum was transferred to a 1% nutrient agar plate (Oxoid, Adelaide, Australia). The plate was incubated for 16–18 hours at 37 ℃ and a single colony forming unit (CFU) was diluted to 0.5 McFarland standard in 0.45% sterile saline and transferred on ice for instillation into sheep sinuses.

Chitogel

CG comprised of three components; 5% succinyl-chitosan, 0.3% phosphate buffer and 3% dextran aldehyde (Chitogel®, Wellington). All components were manufactured and sterilised by Chitogel® and cultured for sterility by the Department of Microbiology, Princess Margaret Hospital, Western Australia prior to being used in this study. All stocks were stored at room temperature.

Preparation of Chitogel

Dextran aldehyde was first dissolved in 10 mL of phosphate buffer then mixed with 10 mL of succinyl-chitosan using sterile technique.

Preparation of Chitogel- Budesonide- Mupirocin (CG-BM)

Ten mg of powdered mupirocin (PCCA, Houston) was solubilised under sterile conditions in 6 mL of phosphate buffer 24 hours before application. Mupirocin solution was mixed with 2 mg/4 mL of Pulmicort respules (AstraZeneca Ab, Sodertalje) before being used to dissolve dextran aldehyde and then mixed with 10mL of succinyl-chitosan using sterile technique.

Anaesthetic protocol

All sheep were fasted for 12 hours prior to general anaesthesia. They were induced with intravenous phenobarbitone (19 mg/kg), intubated and placed onto 1.5% to 2% inhalation isoflurane over the course of the procedure. All sheep were placed supine on a wooden cradle and supported with neck slightly flexed on a head ring. Two sprays of Cophenylcaine Forte (ENT Technologies Pty Ltd, Australia) were applied to each nasal cavity 10 minutes prior to any procedures.

Surgical Protocol

As per protocol[329, 330] all sheep had middle turbinectomy and anterior ethmoid complex resection followed by a 3–4 week recovery. Then frontal mini-trephines were placed bilaterally on the sheep's forehead, 1cm lateral from the midline at the level of superior orbital rims. Accurate trephine placements were verified when fluorescein flushed via trephines (0.1 mL diluted in 100 mL of normal saline) can be visualised endoscopically draining from the frontal sinus ostium into nasal cavity.

Efficacy arm

Following frontal trephination, the frontal ostia were packed with petroleum gauze (Vaseline, Kendall, Mansfield, MA). 1 mL of 0.5 McFarland Units *S. aureus* was then instilled into each sinus cavity via mini-trephines and capped. Bacterial biofilms were allowed to form over the next 7 days. On day 8, the petroleum gauze was unpacked and each sheep was randomly assigned into one of three efficacy groups (i) Twice daily saline flush (NT), (ii) Chitogel (CG) and (iii) Chitogel-Budesonide- Mupirocin (CG-BM). For sheep assigned to groups (ii) and (iii), the gels were instilled into each sinus cavity via mini-trephines until gel extrusion from the frontal sinus ostium was visualised under direct endoscopic view. The mini-trephines were then capped. For all

groups, sinuses were irrigated 24 hours later with 15 mL of sterile normal saline twice a day for the remaining 6 days of treatment. On day 8, all sheep were euthanised and sinus mucosa harvested for histopathological analysis and biofilm biomass imaging. Flow diagram describing efficacy arm protocol is represented in Figure 17.

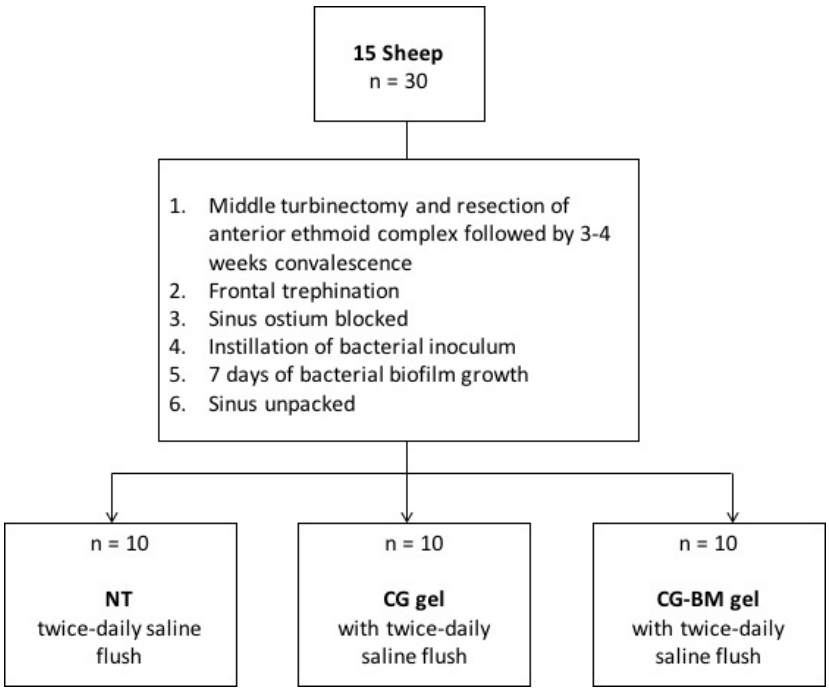

Figure 17. Flow diagram describing efficacy arm protocol.

Biofilm Imaging

From each sinus cavity, two random sections of 1 cm × 1 cm sinus mucosa were sampled and briefly immersed in phosphate buffered solution to wash off planktonic cells. Sampled mucosa was then stained with LIVE/DEAD BacLight stain (Life Technology, Mulgrave, Victoria, Australia) as per manufacturer's instructions. Confocal scanning laser microscope (Zeiss Germany) were used to assess biofilm biomass. Three Z-stack images of highest biofilm presence were taken of each sample

(Image properties: line average 4, 512×512 pixels, Z-stack 80 steps) making a total of 6 Z-stack images per sinus. COMSTAT2 software (Lyngby, Denmark) was utilised to quantify biofilm biomass in each Z-stack.

Histopathology evaluation

One 1 cm x 1 cm mucosal section from each sinus was fixed in 2% formalin solution and sent for histopathology preparation (Adelaide Pathology and Partners). Samples were embedded in paraffin and stained with hematoxylin & eosin. Microscopic evaluation and tissue grading was performed by a pathologist blinded to all clinical data using light microscopy (Eclipse 90i, Nikon instruments Inc, Melville, NY). Acute inflammation (neutrophils), oedema, fibrosis were graded using a likert scale[214, 252, 332]. Acute inflammation was graded from 0-2; 0= none, 1= mild, 2= severe. Oedema and fibrosis were each graded from 0-3; 0=none, 1=mild, 2= moderate, 3=severe.

Statistical analysis

Previous in vivo models have described an average of 60% biofilm biomass reduction with various irrigation treatment[330]. Four sheep were required per arm to obtain a power of 80% to achieve a significance level at $\alpha = 0.05$. Five animals per arm were used in this study as this was our first experience investigating the efficacy of a topical gel treatment.

Comparison of bacterial biofilms between treatment groups were analysed using Kruskal-Wallis 1-way analysis of variance (ANOVA) with Dunn's multiple comparison test. All statistical tests were done using GraphPad Prism 7.0 software (San Diego, CA).

2.4 Results

Anti-inflammatory Effects

There were no significant differences comparing histopathology of sheep sinus mucosa treated with (i) NT, (ii) CG, (iii) CG-BM gel in acute inflammation, oedema, and fibrosis (Figure 18).

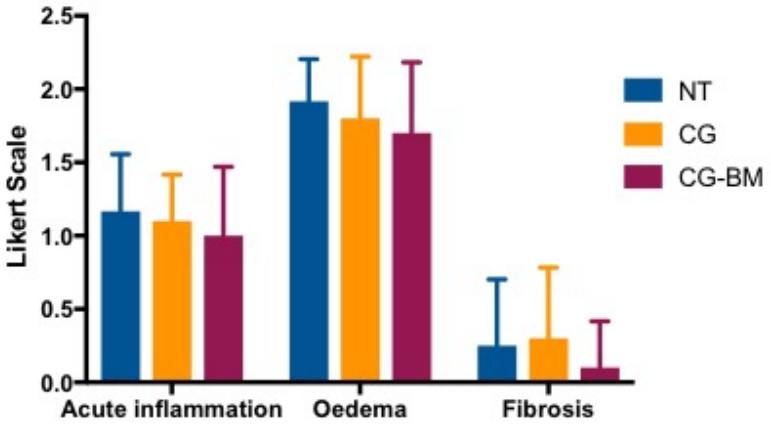

Figure 18. Bar graph showing no difference in histopathology grading of sinus mucosa, specifically there were no anti-inflammatory effects observed between NT and CG-BM treated group.

Antibiofilm Effects

COMSTAT2 assessment of biofilm biomass showed a significant reduction in CG-BM treated sheep compared to NT controls (p= 0.01, One-way ANOVA, Kruskal-Wallis test), but not between NT and CG treated sheep (Figure 19). Compared to no-treatment controls, CG-BM gel and CG gel reduced *S. aureus* biofilms by 90.5% and 20% respectively.

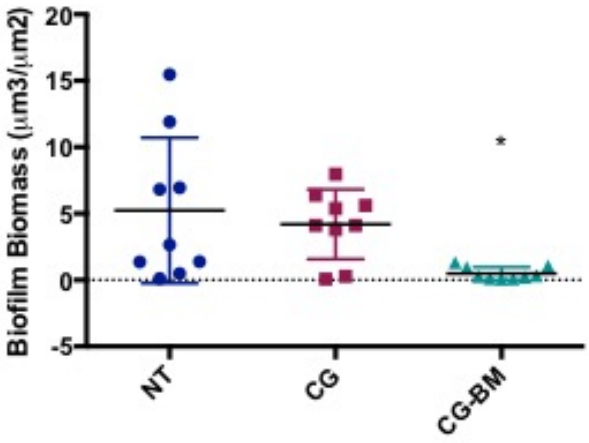

Figure 19. Scatter plots showing COMSTAT computation of *Staphylococcus aureus* biofilm biomass between (1) Twice daily saline flush (NT), (2) CG gel with twice-daily saline flush, and (3) CG-BM gel with twice-daily saline flush. Significant reduction of biofilm biomass seen in CG-BM treated group compared to NT and CG gel.

2.5 Discussion

In this study we demonstrated that CG-BM significantly reduced *S. aureus* biofilms in an in-vivo sinusitis model. Compared to no-treatment controls, CG-BM and CG reduced *S. aureus* biofilms by 90.5% and 20% respectively.

Mupirocin was chosen to be the antimicrobial of choice to be incorporated into Chitogel due to its known clinical efficacy as a nasal irrigation for patients with recalcitrant *S. aureus* CRS[172-176]. Jervis-Bardy et al[175] reported 88.9% of surgically recalcitrant patients had eradication of *S. aureus* using twice daily nasal lavages containing 0.05% mupirocin for 3 weeks. Mupirocin exerts its antimicrobial activity by irreversibly binding to the bacterial enzyme isoleucyl-transfer RNA, thereby preventing isoleucine incorporation during bacterial synthesis[185-187]. It has excellent activity against staphylococci including MRSA, most streptococci, and against certain gram-negative bacteria including *H. influenzae*[186] which are increasingly prevalent in clinical practice.

Budesonide irrigations are now widely recognised to be safe[156, 157, 333] and effective in improving symptoms and endoscopic outcomes post sinus surgery[162, 165, 334]. Luo et al[335] suggested that hydrophobic drugs like steroids are released more slowly from hydrogels, making Chitogel an excellent controlled release vehicle for budesonide. Our department has recently demonstrated that by incorporating budesonide in Chitogel applied post sinus surgery, there was reduced inflammation during the early postoperative period and reduction in the extent of ostial stenosis at 3 and 12 months[336]. Therefore, in this study, we had hypothesized that similar anti-inflammatory effects

would be observed. Although CG-BM treated mucosa showed a trend to improvement in degree of inflammation, acute inflammation, goblet cell hyperplasia, oedema and fibrosis when compared to saline rinses, it was not statistically significant. We postulate that this could be as a result of several different factors. Firstly, only intermediate levels of inflammation were observed in our positive control sheep, indicating that *S. aureus* biofilms might not induce massive inflammation in sheep as would normally be expected. This could be due to the fact that sheep sinus mucosa is naturally colonised by a range of bacteria and might be adapted to the presence of biofilms already. Secondly, the release of budesonide from Chitogel was found to be only up to 18% of the total possible amount over 72 hours (unpublished observations). Optimising the pharmacological formulation of budesonide for improved release from CG might help to improve the anti-inflammatory effects of CG-Bud gel. Also, it is possible that the self-limiting inflammatory process post-surgery is different to inflammation secondary to a bacterial nidus, which might require a higher concentration or longer exposure of budesonide for an increased effect.

CG-BM gel can be applied in the outpatient setting under direct endoscopic view into post-ESS infected sinus cavities via curved and straight suction cannulas. This has the theoretical benefit of effective anti-biofilm delivery specific to infected sinuses regardless of ostium size, patient douching technique and compliance. However further clinical studies are required to explore this treatment viability.

This study has several limitations, one such limitation was our inability to quantify the amount of treatment gel remaining in each sinus over the treatment course due to differing sinus anatomy or drainage. Therefore, it is possible that each sinus was exposed to variable drug concentrations during treatment. Another limitation of this

study remains that in vivo studies cannot fully simulate the conditions of human sinuses and clinical studies will be required to fully characterise the potential of this treatment.

2.6 Conclusion

Our study concludes that CG-BM gel is effective in reducing *S. aureus* biofilms in a sheep sinusitis model. The use of Chitogel as a means to deliver topical therapies like antimicrobial and anti-inflammatory agents offers otolaryngologists an alternative method to treat surgically recalcitrant CRS.

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Chapter 3: Safety and Efficacy of topical Chitogel- Deferiprone- Gallium Protoporphyrin in a sheep model

Statement of Authorship

Title of Paper	Safety and Efficacy of topical Chitogel- Deferiprone- Gallium Protoporphyrin in a sheep model
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Publication Details	Published in Frontiers in Microbiology, section Antimicrobials, Resistance and Chemotherapy. Special research topic edition on Antibiotic Alternatives and Combinational Therapies for Bacterial Infections. On 11 May 2018.

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Name of Principal Author (Candidate)	Mian Li Ooi
Contribution to the Paper	Project design, data collection and analysis and manuscript preparation
Overall percentage (%)	95%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 1/9/2019

Co-Author Contributions

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

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

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

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

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3.1 Abstract

Objectives: Increasing antimicrobial resistance has presented new challenges to the treatment of recalcitrant chronic rhinosinusitis fuelling a continuous search for novel antibiofilm agents. This study aimed to assess the safety and efficacy of Chitogel (Chitogel®, Wellington New Zealand) combined with novel antibiofilm agents Deferiprone and Gallium Protoporphyrin (CG-DG) as a topical treatment against *S. aureus* biofilms *in vivo*.

Methods: To assess safety, 8 sheep were divided into two groups of 7 day treatments (n = 8 sinuses per treatment); (1) Chitogel (CG) with twice daily saline flush, and (2) CG-DG gel with twice daily saline flush. Tissue morphology was analyzed using histology and scanning electron microscopy (SEM). To assess efficacy we used a *S. aureus* sheep sinusitis model. Fifteen sheep were divided into three groups of 7 day treatments (n = 10 sinuses per treatment); (1) twice daily saline flush (NT), (2) Chitogel (CG) with twice daily saline flush, and (3) CG-DG gel with twice daily saline flush. Biofilm biomass across all groups was compared using LIVE/DEAD BacLight stain and confocal scanning laser microscopy.

Results: Safety study showed no cilia denudation on scanning electron microscopy and no change in sinus mucosa histopathology when comparing CG-DG to CG treated sheep. COMSTAT2 assessment of biofilm biomass showed a significant reduction in CG-DG treated sheep compared to NT controls.

Conclusion: Results indicate that CG-DG is safe and effective against *S. aureus* biofilms in a sheep sinusitis model and could represent a viable treatment option in the clinical setting.

3.2 Introduction

Recalcitrant chronic rhinosinusitis is a difficult clinical entity to manage. Bacterial biofilms contribute to disease recalcitrance and have been shown to be associated with more severe disease[92, 93, 323, 324]. Although oral antibiotics are frequently ineffective against biofilms[328], it remains the only option available to achieve symptom control for many recalcitrant patients. However, with the growing prevalence of resistance to first-line antibiotics[337] and the lack of research and development of new antibiotics[338, 339], novel topical anti-biofilm agents are needed to help improve the outcomes in these patients.

Richter et al[260-262] first described the potent synergistic antimicrobial properties of Deferiprone and Gallium Protoporphyrin (DG). This agent targets the iron metabolism that is crucial for bacterial growth and survival[263, 264]. Deferiprone is an iron chelator approved by the U.S. Food and Drug Administration to treat thalassaemia major. Gallium Protoporphyrin IX is a heme analogue with strong antibacterial activity against gram-positive bacteria, gram-negative bacteria and mycobacteria[265, 266]. Gallium Protoporphyrin IX has been shown to kill *S. aureus* and Methicillin-Resistant *S. aureus* (MRSA) in planktonic, biofilm and small colony variant form and has been shown to enhance the antimicrobial properties of commonly used antibiotics[270]. Deferiprone is thought to chelate iron from the bacteria's surrounding environment, forcing the bacteria to upregulate their iron transporter proteins. Deferiprone-dependent increased expression of iron transporter proteins are thought to enhance Gallium Protoporphyrin IX uptake into bacterial cells, thereby augmenting bacterial killing efficacy[260, 262].

Consequently, the synergistic antimicrobial effects are observed mainly when Deferiprone and Gallium Protoporphyrin IX are given consecutively[260].

In this study, DG is incorporated within Chitogel (chitosan and dextran), a surgical hydrogel FDA approved for the use after sinus surgery, which acts as a drug carrier, that can be applied topically to fill the sinus cavities. The gel has been shown to allow the immediate and complete release of Deferiprone whilst Gallium Protoporphyrin IX is released more slowly[262]. This topical application allows higher concentration of drugs to be used for a localised action with less systemic side effects. The mucoadhesive properties of the hydrogel also increases contact time of these topical agents with the sinus mucosa and biofilms[314, 315] augmenting its anti-biofilm effects.

The aims of this study were to assess the safety of CG-DG on healthy sinus mucosa and evaluate its efficacy as an anti-biofilm agent in a previously validated *S. aureus* biofilm-induced sheep sinusitis model.

3.3 Materials and Methods

This study was approved by the Animal Ethics Committee of both The University of Adelaide and the South Australian Health and Medical Research Institute (SAHMRI).

Animals

23 male merino sheep between 2 to 4 dental age (1-2 years of age) were used. All animals were drenched to eradicate the parasite *Oestrus Ovis*. 15 sheep were allocated to the efficacy arm and 8 to the safety arm. For the efficacy arm 5 sheep were randomised to each efficacy group (i) Twice daily saline flush (NT), (ii) Chitogel (CG) and (iii) Chitogel- Deferiprone- Gallium Protoporphyrin (CG-DG). For the safety arm, 4 sheep were randomised to each safety group (i) Chitogel (CG) and (ii) Chitogel-Deferiprone- Gallium Protoporphyrin (CG-DG).

Bacterial inoculum

A known biofilm-forming reference strain of S. *aureus*, ATCC 25923, was supplied by the Department of Microbiology, TQEH. A frozen glycerol stock was defrosted and subcultured overnight in 3mL of nutrient broth (Oxoid, Adelaide, Australia) on a shaker at 37°C for 24 hours before being transferred to a 1% nutrient agar plate (Oxoid). The plate was incubated at 37°C for 16-18 hours, at which point a single colony forming unit (CFU) was diluted in 0.45% sterile saline to 0.5 McFarland standard and transferred on ice for instillation into sheep sinuses.

Chitogel

The Chitogel is made up of a combination of three components; 5% succinyl-chitosan, 0.3% phosphate buffer and 3% dextran aldehyde (Chitogel®, Wellington, NZ). The components are manufactured and sterilized by Chitogel®. All stocks were stored at room temperature.

Deferiprone and Gallium Protoporphyrin

Deferiprone (3-hydroxy-1,2-dimethylpyridin-4(1*H*)-one) (Sigma-Aldrich, St Louis, USA) and Gallium Protoporphyrin IX (Ga-PP IX) (Frontier Scientific, Logan, USA) were stored at room temperature.

Preparation of Chitogel

Dextran aldehyde (0.3 g) was dissolved in 10 mL of phosphate buffer then mixed with succinyl chitosan solution (0.5 g in 10 mL buffer) using sterile technique.

Preparation of Chitogel- Deferiprone- Gallium Protoporphyrin

Deferiprone (20 mM) and Gallium Protoporphyrin (250 μg/mL) were diluted in 10mL of phosphate buffer under sterile conditions the day before use. This prepared solution was then used to dissolve dextran aldehyde prior to mixing with 10 mL of succinyl chitosan using sterile techniques.

Anaesthetic protocol

For every surgical procedure, all sheep underwent general anaesthesia given by an experienced animal handler. Intravenous phenobarbitone was given at induction (19 mg/kg) and sheep were intubated and placed onto 1.5% to 2% inhalation isoflurane to

maintain anaesthesia. Each sheep was placed in a supine position on a wooden cradle and supported on a head ring with neck slightly flexed. Each nasal cavity was sprayed twice with Cophenylcaine Forte (ENT Technologies Pty Ltd, Australia) 10 minutes prior to any procedures.

Surgical Protocol

As per protocol all sheep underwent middle turbinectomy and anterior ethmoid complex resection, which is then followed by a 3-4 week convalescence period. Frontal trephination was later performed by placing mini trephines bilaterally on the sheep's forehead, 1cm lateral from the midline and along a line connecting the superior aspect of the orbital rims. The placement of trephines was confirmed when fluorescein flushed via trephines (0.1 mL diluted in 100 mL of physiological saline) was visualised to be draining from the frontal sinus ostium.

Safety arm

In the safety arm, following frontal trephination, the gels were instilled into each sinus cavity via mini trephines until gel extrusion from the frontal sinus ostium was visualised under direct endoscopic view. The mini trephines were then capped. Gel instilled was left undisturbed within the sinus cavities for 24 hours before beginning sinus irrigation via mini trephines with 15 mL of sterile physiological saline twice a day. On day 8, all safety sheep were euthanized and sinus mucosa harvested for histopathological and SEM analysis.

Efficacy arm

In the efficacy arm, following frontal trephination the frontal ostia were packed with petroleum gauze (Vaseline, Kendall, Mansfield, MA). 1 mL of 0.5 McFarland Units of *S. aureus* was then instilled into each sinus cavity via mini trephines and capped. Bacterial biofilms were allowed to form over the next 7 days. On day 8, the petroleum gauze was removed and each sheep was randomly assigned into one of three efficacy groups (i) Twice daily saline flush (NT), (ii) Chitogel (CG) and (iii) Chitogel-Deferiprone- Gallium Protoporphyrin (CG-DG). For sheep assigned to gel groups (ii) and (iii), the gels were instilled into each sinus cavity via mini trephines until gel extrusion from the frontal sinus ostium was visualized under direct endoscopic view. The mini trephines were then capped. For all groups, sinuses were irrigated 24 hours later with 15 mL of sterile physiological saline twice a day for the remaining 6 days of treatment. On day 8, all sheep were euthanized and sinus mucosa harvested for histopathological analysis and biofilm biomass imaging.

Safety analysis

Histopathology evaluation

One 1 cm x 1 cm mucosal section from each sinus was fixed in 2% formalin solution and sent for histopathology preparation (Adelaide Pathology and Partners, Adelaide, Australia). Samples were embedded in paraffin and stained with hematoxylin & eosin. Microscopic evaluation of tissue damage and inflammation was performed by a pathologist blinded to all clinical data using light microscopy (Eclipse 90i, Nikon instruments Inc, Melville, NY).

Scanning Electron Microscopy Evaluation

From each sinus, a sample of 5 mm x 5 mm tissue was obtained, sonicated in saline, then submerged in SEM fixative (4% paraformaldehyde/1.25% glutaraldehyde in phosphate buffered solution [PBS] + 4 % sucrose, pH 7.2) for at least 24 hours. Tissues were washed in a washing buffer (PBS + 4% sucrose) for 5 minutes then post fixed in 2% OsO_4 in water for 1 hour. All samples underwent a graded dehydration of 70%, 90% and 100% ethanol, then dried using hexamethyldisilazane (HMDS). Following that, all tissues were mounted on stubs and carbon coated. Images were taken using an XL30 Field Emission Gun Scanning Electron Microscope (Phillips, Eindhoven, Netherlands).

Quantification of Plasma Deferiprone and Gallium Protoporphyrin levels

Plasma samples were analysed for Deferiprone and GaPP using high performance liquid chromatography (HPLC) on a Shimadzu UFLC XR (Shimadzu Cooperation, Kyoto, Japan). For the quantification of Deferiprone, 250 µl plasma was mixed with 750 µl methanol (HPLC grade, Merck, Darmstadt, Germany). The samples were vortexed for 1 min and centrifuged for 4 min at 14,800 rpm at room temperature (Eppendorf 5804R, Eppendorf, Hamburg, Germany). The clear supernatant (50 µl) was quantified on a Phenomenex Synergi 4 µm Fusion-RP LC column coupled to a security guard cartridge (Phenomenex, Lane Cove, NSW, Australia) using methanol/0.1 M orthophosphate buffer pH 7.2 (15%: 85%) as mobile phase at a flow rate of 2.0 ml/min. The Deferiprone concentration was detected at 280 nm and calculated against a standard curve ranging from 1.0 to 10.0 µg/ml Deferiprone (R^2>0.992). For the quantification of GaPP, solid phase extraction (SPE) was performed using Oasis PRiME HLB cartridges 1cc/30mg (Waters, Dundas, NSW, Australia). Samples were prepared according to the manufacturer's protocol. Briefly, 250 µl plasma was mixed with 250 µl orthophosphoric acid (4%) and placed in a SPE cartridge. After washing

with 5% methanol in Milli-Q water, 500 µl methanol was used to elute GaPP. The clear eluate (50 µl) was quantified using methanol/0.1 M orthophosphate buffer pH 7.2 (70%: 30%) as mobile phase at a flow rate of 1.0 ml/min. The GaPP concentration was detected at 405 nm and calculated against a standard curve ranging from 0.02 to 10.0 µg/ml GaPP (R^2>0.995).

Efficacy Analysis

Biofilm biomass

Method of biofilm analysis were as described in previous studies[210, 214, 252, 329, 340]. Two random 1cm x 1cm mucosal sections from each sinus were sampled. Each sample was briefly immersed in phosphate buffered solution to wash off planktonic cells and stained with LIVE/DEAD BacLight stain (Life Technology, Mulgrave, VIC, Australia) as per manufacturer's instructions. Biofilm biomass was assessed using confocal scanning laser microscope (LSM 710, Zeiss, Germany). Within each sample 3 of the areas with highest biofilm presence had axial Z stacks recorded to construct a 3D virtual image of the overlying tissue mucosa and biofilm, making a total of 6 Z-stack images per sinus. 80 individual images of each representative area were taken as one Z stack image (Image properties: line average 4, 512x 512 pixels, Z-stack 80 steps). The COMSTAT2 computer software (Lyngby, Denmark) was utilised to quantify biofilm biomass in each Z-stack[341, 342].

Histopathology grading

One 1cm x 1cm mucosal section from each sinus was fixed in 2% formalin solution and sent for histopathology preparation (Adelaide Pathology and Partners). Samples were embedded in paraffin and stained with hematoxylin & eosin. Microscopic evaluation

and tissue grading was performed by a pathologist blinded to all clinical data using light microscopy (Eclipse 90i, Nikon instruments Inc, Melville, NY). Degree of inflammation (lymphocytes, plasma cells, histiocytes and mast cells), acute inflammation (neutrophils), oedema, fibrosis and cilia were graded using an arbitrary scale[214, 252, 332]. Degree of inflammation, oedema and fibrosis were each graded from 0-3; 0=none, 1=mild, 2= moderate, 3=severe. Acute inflammation was graded from 0-2; 0= none, 1= mild, 2= severe. Cilia were graded as minimal loss, focal loss, moderate loss, severe loss.

Statistical analysis

Comparison of mucosal biofilms between treatment groups were analysed using Kruskal-Wallis 1-way analysis of variance (ANOVA) with Dunn's multiple comparison test. Comparison of histopathology grading between treatment groups in the efficacy arm were analysed using Two-way analysis of variance (ANOVA) with Dunnett's multiple comparison test. Statistical significance was considered at $p<0.05$. All statistical tests were done using GraphPad Prism 7.0b software (San Diego, CA).

3.4 Results

Safety arm

Histopathological analysis

Similar mucosal architecture was noted in all sinus samples obtained from CG and CG-DG treated groups, showing a pseudostratified columnar epithelial layer intersected with goblet cells. No squamous metaplasia of epithelium was identified in any samples (Figure 20). These images reflect that the test treatments are safe to apply topically to sinus mucosa.

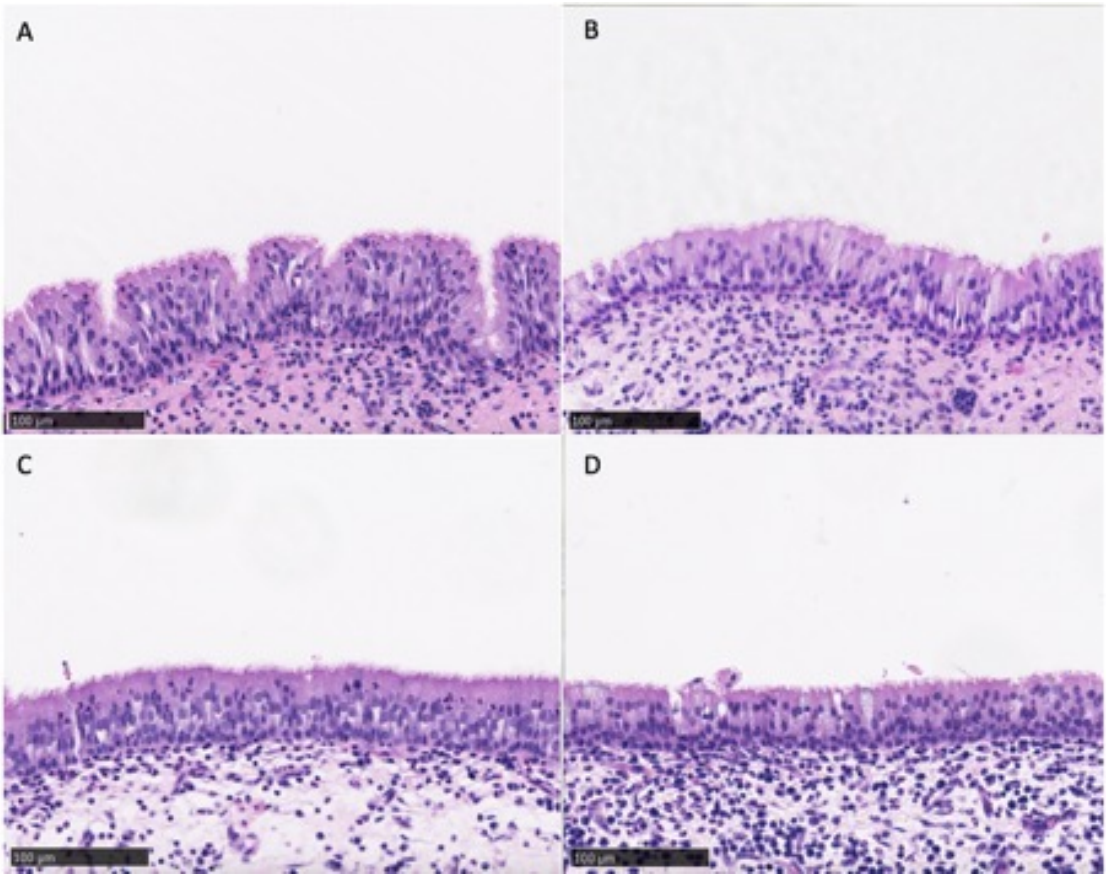

Figure 20. Representative images of sinus mucosa histology harvested from sheep in the safety arm treated with CD (A, B) and CD-DG (C, D). All sinus mucosa showed pseudostratified columnar epithelial layer with no metaplasia, indicating that test treatments were safe for sinus topical application.

SEM tissue analysis

SEM was employed to assess the presence and integrity of cilia present on sinus mucosal samples. In all sinus mucosal samples collected, there were no signs of ciliary denudation in both CG and CG-DG treated groups (Figure 21). These images reflect that the test treatments were not ciliotoxic on ciliated human respiratory cells.

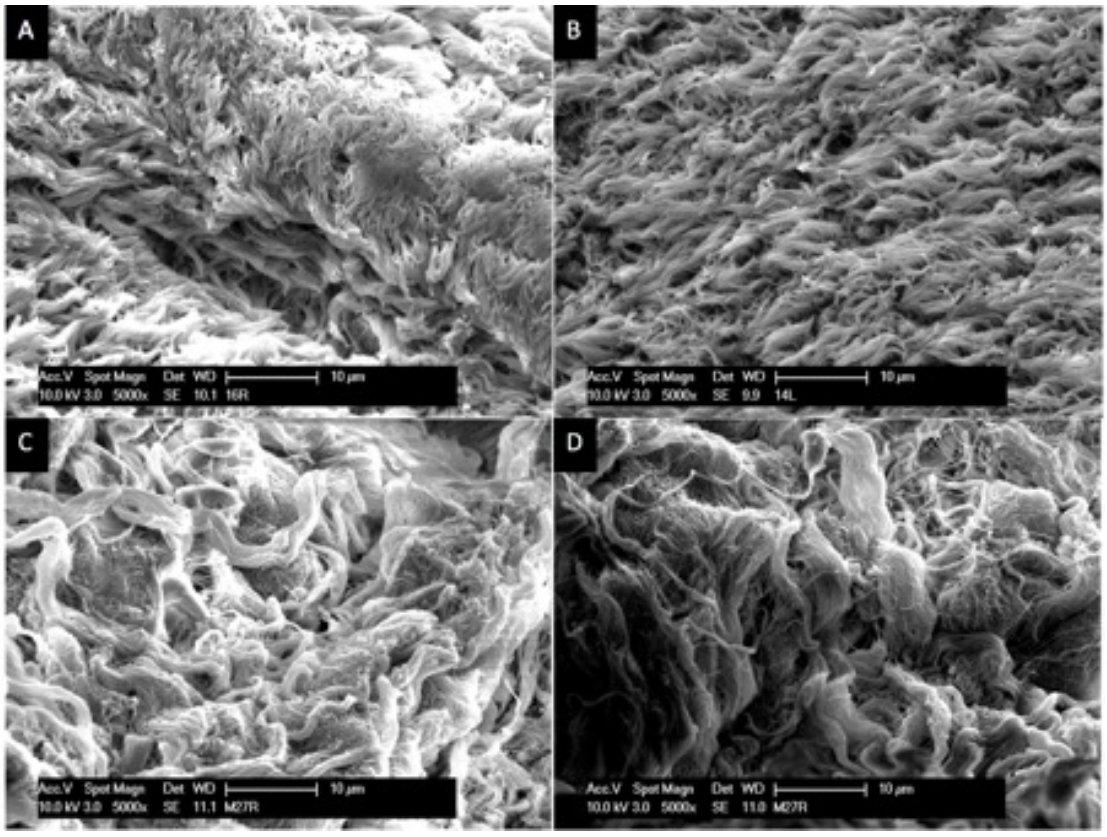

Figure 21. Representative SEM images of sinus mucosa harvested from sheep in the safety arm treated with CG gel (A, B) and CG-DG gel (C, D). SEM allowed assessment for ciliary presence and morphology on sinus mucosa. No ciliary denudation were observed in both treated groups, indicating that test treatments were not ciliotoxic.

Plasma Deferiprone and Gallium Protoporphyrin levels

The maximum Deferiprone concentration was reached after one day (0.18 µg/ml Deferiprone) in the 4 sheep treated with CG-DG (Figure 22). After six days the Deferiprone plasma concentration decreased to 0.03 µg/ml.

GaPP was not detected in the plasma of any of the 4 sheep treated with CG-DG (data not shown). According to the quantification level ranging from 0.02 to 10 µg/ml, this indicates a GaPP plasma concentration was below 0.02 µg/ml.

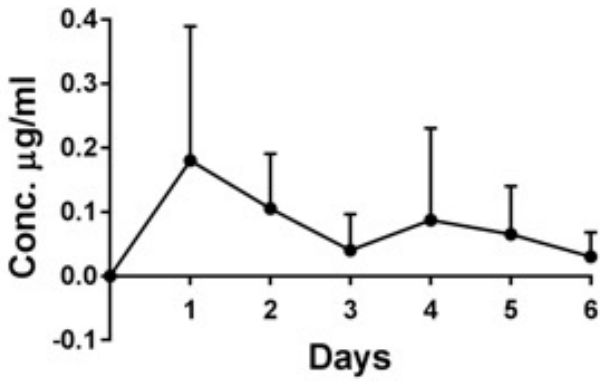

Figure 22. *In vivo* plasma concentration (µg/ml) ± standard deviation of Deferiprone over 6 days, n=4. Maximum Deferiprone plasma level of 0.18 µg/ml was detected at day 1 of topical application to sinuses, which is 110 times less than one oral dose of Deferiprone. No GaPP was detected in the plasma of all 4 sheep treated with CG-DG (data not shown). This indicates that CG-DG has negligible systemic effect from topical sinus application.

Efficacy arm

Biofilm biomass analysis

COMSTAT2 assessment showed a significant reduction of biofilm biomass in CG-DG treated sheep compared to NT controls (p= 0.03, One-way ANOVA, Kruskal-Wallis test), but not between NT and CG treated sheep. Compared to no-treatment controls, CG-DG gel and CG reduced S. aureus biofilms by 82% and 20% respectively (Figure

23). Representative CLSM images showing LIVE/DEAD BacLight staining of *S. aureus* biofilms seen in Figure 24.

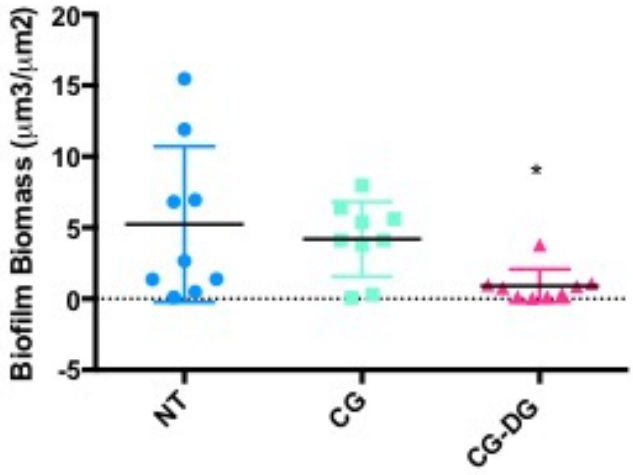

Figure 23. Scatter plots showing COMSTAT computation of *Staphylococcus aureus* biofilm biomass between (A) Twice daily saline flush (NT), (B) CG gel with twice-daily saline flush, and (C) CG-DG gel with twice-daily saline flush. Significant reduction of biofilm biomass seen in CG-DG treated group compared to NT and CG gel. * $P < 0.05$, Kruskal-Wallis 1-way analysis of variance (ANOVA) with Dunn's multiple comparison test.

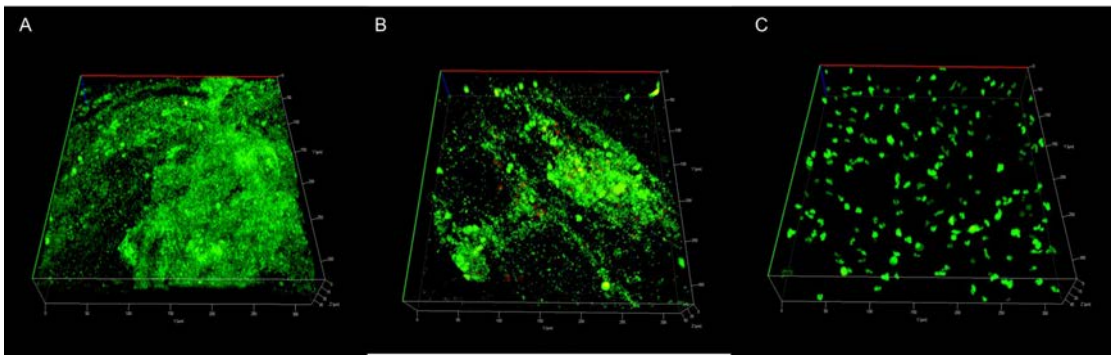

Figure 24. Representative CLSM images of *S. aureus* biofilms stained with LIVE/DEAD Baclight reconstructed into 3D virtual image. Small light green stains represents live bacteria, large dark green stains represents mammalian cells and large red stains represents dead mammalian cells. Sinus mucosa treated with (A) Twice daily saline flush (NT) showing dense population of live bacterial biofilms; (B) CG gel with twice-daily saline flush showing moderate population of live bacterial biofilms; (C) CG-DG gel with twice-daily saline flush showing no bacterial biofilms.

Histopathology analysis of sinus mucosa harvested from sheep in efficacy arm

There was a significant reduction in the degree of inflammation of sheep sinus mucosa between CG-DG treated group and no treatment ($p = 0.0476$, CI 95% 0.004116 to 0.8959). No significant differences were observed in degree of inflammation between CG only group and no treatment controls. Looking at acute inflammation, oedema, fibrosis and cilia, there were no significant differences in sheep sinus mucosa across all groups (Figure 25).

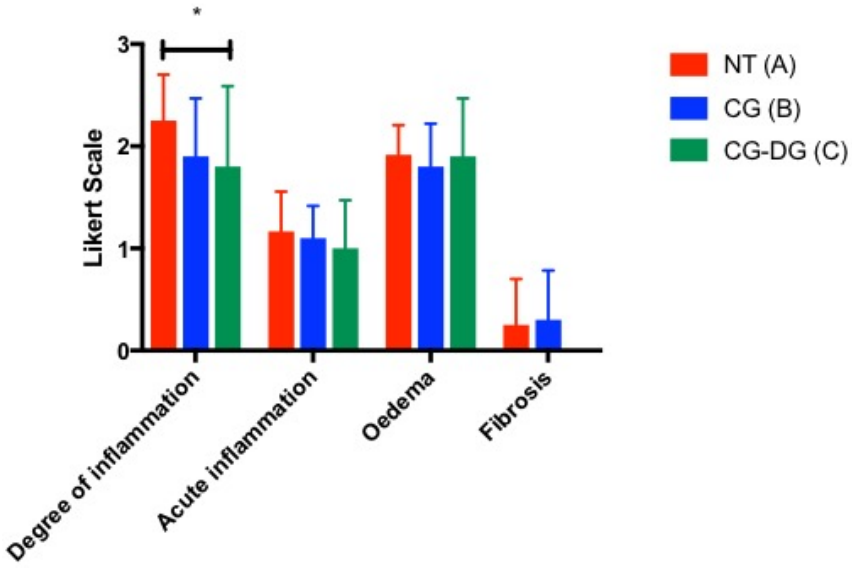

Figure 25. Bar graph showing degree of inflammation, acute inflammation, oedema and fibrosis grading between (A) Twice daily saline flush (NT), (B) CG gel with twice-daily saline flush, and (C) CG-DG gel with twice-daily saline flush. Significant reduction is seen in degree of inflammation of sinus mucosa between CG-DG and NT. * $P < 0.05$, 2-way analysis of variance (ANOVA) with Dunnett's multiple comparison test.

3.5 Discussion

In this study we were able to show that CG-DG is safe and effective in killing *S. aureus* biofilms *in vivo* using a sheep sinusitis model described previously. The anti-inflammatory effects seen in the sinus mucosa of CG-DG group might be due to the effective eradication of biofilms.

The FDA approved oral dose of Deferiprone that is safe to use in humans is up to 75-99 mg/kg/day. Spino et al[343] reported that following an oral dose of 1,500 mg Deferiprone (20 mg/kg) the mean maximum serum Deferiprone concentration (Cmax) of non-iron-loaded healthy subjects was 20 μg/mL. Following one topical CG-DG application the highest plasma Deferiprone concentration measured in this study was 0.18 μg/ml, which is 110 times less than one oral dose of Deferiprone. In addition, GaPP was not detected in the plasma of any of the sheep treated with CG-DG gel. In an in vivo model we have to also account for some accidental oral ingestion of the sinus flushes which may reflect that the true plasma level of Deferiprone might be even lower in human application as patients are instructed to apply sinus rinses head down and allow the rinses to wash out. Therefore, negligible Deferiprone plasma concentrations and the absence of GaPP in plasma, together with no observed adverse effects (e.g. no sinus mucosa damage, no ciliary denudation) indicate safety of CG-DG gel in vivo.

Iron is an essential element for bacterial growth, survival and replication. Deferiprone is an iron-chelator, capable of chelating free iron at the ratio 3:1 and approved by the Food and Drug Administration (FDA) for the treatment of Thalassemia Major [344].

Deferiprone has slight anti-microbial properties by capturing iron from the environment around bacteria, causing a depletion of iron as a nutrient source [345]. Deferiprone also has been shown to accelerate wound healing with enhanced skin closure after topical application *in vivo* [346]. Gallium Protoporphyrin IX belongs to the family of non-iron metalloporphyrins and has antibacterial properties [347]. The compound shows structural similarity to haem, therefore, it can mimic haem as a preferred iron source of bacteria [347]. Once inside the bacterial cell however, non-iron metalloporphyrins such as Gallium Protoporphyrin IX preserve their structure and show antibacterial effects by interfering with essential cellular pathways in the cytoplasm and in the plasma membrane causing bacterial cell death [348]. Combining Deferiprone and Gallium Protoporphyrin IX has potent synergistic antimicrobial properties against a range of bacteria including Multi Drug Resistant bacteria and Methicillin Resistant *S. aureus* (MRSA) [349]. The Deferiprone and Gallium Protoporphyrin IX combination is thought to exert its anti-biofilm effects by interfering with the iron metabolism of *S. aureus* which is involved in membrane bound respiration, bacterial growth, protects against reactive oxygen species, and increases bacterial virulence factors[263, 264].

In this study, CG gel showed the capacity to act as a drug carrier, facilitating the topical delivery of DG to biofilms in the sinonasal cavities. To exert the full anti-biofilm potential of DG it is imperative that Deferiprone is first applied followed by Gallium Protoporphyrin IX[260]. Richter et al[269] described the quick release of hydrophilic Deferiprone from the CG gel within the first 48-72 hours followed by a sustained release of hydrophobic Gallium Protoporphyrin IX reaching 20-25% over 20 days[262] which reinforces the anti-biofilm effects of DG.

In the last decade, Chitogel has been largely used in ENT surgery to improve patient outcomes post endoscopic sinus surgery[288-291] due to its effective hemostatic[289, 290, 292-298], wound healing[299-301], anti-adhesion[288, 302-310] and antimicrobial[311-313] properties and was recently FDA approved for use after sinus surgery. CG gel comprises succinyl-chitosan which is a chitosan polymer produced by the hydrolysis of chitin, found in the exoskeletons of crustaceans. Incorporating DG into CG gel strengthens the gel's anti-biofilm effects which might help improve the outcome of recalcitrant and post endoscopic sinus surgery patients.

CG-DG has been shown to have significant anti-biofilm activity not only against *S. aureus* but also MRSA, *S. epidermidis* and *P. aeruginosa* biofilms[262]. The anti-biofilm activity of DG against multiple pathogens has the added potential of treating polymicrobial infections. This broad activity makes topical CG-DG a valuable treatment alternative that can be applied within the same outpatient setting while waiting for sensitivity result to become available.

In February 2017, the World Health Organisation (WHO) released a global priority list of pathogens to guide research and development of new antibiotics. Amongst the list, MRSA has been classified as a high priority pathogen and *P. aeruginosa* as critical. This also suggests that as a novel antimicrobial agent CG-DG gel has great potential for broader applications in various clinical settings.

3.6 Conclusion

Topically applied CG-DG gel effectively reduced *S. aureus* biofilms with no observed topical and systemic adverse effects in a sheep sinusitis model, indicating safety and efficacy of CG-DG gel in vivo. The use of Chitogel to enhance the delivery of Deferiprone and Gallium Protoporphyrin IX offers otolaryngologists an alternative method to treat surgically recalcitrant CRS.

Clinical trials are currently underway to investigate the safety and efficacy of CG-DG gel in patients with recalcitrant chronic rhinosinusitis and in the post-operative setting.

Chapter 4: Deferiprone has anti-inflammatory properties and reduces fibroblast migration: a new anti-adhesion product

Statement of Authorship

Title of Paper	Deferiprone has anti-inflammatory properties and reduces fibroblast migration: a new anti-adhesion product
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	Published in Nature Scientific Reports, 20 February 2019.

Principal Author

Name of Principal Author (Candidate)	Mahnaz Ramezanpour
Contribution to the Paper	Project design, data collection and analysis and manuscript preparation
Overall percentage (%)	75%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.

Signature Date 6/9/19

Co-Author Contributions

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

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

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

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

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4.1 Abstract

Background: Normal wound healing is a highly regulated and coordinated process. However, tissue injury often results in inflammation with excessive scar tissue formation after 40-70% of operations. Here, we evaluated the effect of the iron chelator Deferiprone on inflammation and the migration of primary nasal fibroblasts and primary human nasal epithelial cells (HNECs) *in vitro*.

Method: The cytotoxicity of Deferiprone was examined by the lactate dehydrogenase assay on primary nasal fibroblasts and air-liquid interface (ALI) cultures of HNECs. Wound closure was observed in scratch assays by using time-lapse confocal scanning laser microscopy. Interleukin-6 (IL-6) and type I collagen protein levels were determined by ELISA. Intracellular Reactive Oxygen Species (ROS) activity was measured by utilizing the fluorescent probe H2DCFDA.

Results: Deferiprone was non-toxic to primary fibroblasts and HNECs. Deferiprone had significant dose-dependent inhibitory effects on the migration, secreted collagen production and ROS release by primary nasal fibroblasts. Deferiprone blocked Poly (I:C)-induced IL-6 production by HNECs but did not alter their migration in scratch assays.

Conclusion: Deferiprone has the potential to limit scar tissue formation and should be considered in future clinical applications.

4.2 Introduction

Scar tissue formation is part of the natural healing process after injury. Tissue repair begins within the first few days of an injury and a variety of cytokines and growth factors are involved in the wound healing processes [350]. In the initial stage of wound healing, thrombin cleaves fibrinogen into fibrin monomers, that act as a scaffold to temporarily seal the bleeding at damaged blood vessels [351]. Neutrophils are then attracted to the wound lesion in response to the degranulation of platelets and the activation of the complement cascade [352]. In the second stage of wound repair (2-7 days), while the number of immune cells and inflammatory cytokines decreases, granulation tissue is formed with keratinocytes and fibroblasts migrating to the injury site. Epithelial cells move in the wound bed and form a thin cell layer to close the wound. At the same time, fibroblasts are attracted from the wound edge or from the bone marrow, proliferate and secrete extracellular matrix (ECM) proteins mainly in the form of collagen to form connective tissue. The last stage of wound repair begins 2-3 weeks after injury and can go for a year or more. In this stage, all of the processes activated after injury decrease, and the temporal ECM (mainly type III collagen) is gradually replaced by the predominant constituent of the normal human dermis (type I collagen). In the normal wound healing process, the scar fades due to reduction of vascularity and shrinks in size from the contraction of the wound under the influence of myofibroblasts [353-355]. However, in an abnormal fibrous wound healing process, the control of tissue repair and regeneration-regulating mechanisms is lost. Clinically, this response is observed as a hypertrophic scar [356, 357]. The distinguishing feature of a hypertrophic scar is the continued proliferation of fibroblasts, with excessive deposition of fibroblast-derived ECM proteins and collagen [358]. Whilst low level inflammation is key to normal

wound healing, the formation of adhesions or hypertrophic scars following injury can be exacerbated by pathological processes resulting in excessive inflammation [359]. These processes include infection and hematoma formation and it has been shown that recruitment of neutrophils and macrophages, producing inflammatory cytokines and reactive oxygen species (ROS) followed by fibroblast migration and proliferation into the wound are critical factors in these processes [352, 360, 361]. Hypertrophic scar formation can negatively impact the outcome of surgery such as abdominal surgery, spinal surgery, vascular surgery and heart surgery. Despite a large number of methods that have been used to reduce surgical scars, the optimal treatment method has not been established. Laser therapy [362], intralesional interferon [363], silicone gel sheeting [364], intralesional corticosteroids [365], pressure therapy [366], bleomycin [367] and onion extract gel [368] are recommended treatments for hypertrophic scar formation which among them, silicone gel, corticosteroids and pressure garments are most common. Silicone gel sheets can be effective in limiting the hypertrophic growth of scars, nevertheless, patients may complain of skin rash, pruritus and excessive sweating [369, 370]. Some studies claimed that pressure therapy may prevent scar formation by suppressing collagen production via limiting the supply of nutrients, oxygen and blood to the scar tissue [371]. Other studies found that there was no significant difference between treatments involving the use of high-pressure garments, lower-pressure garments, or no pressure at all [372]. Intralesional corticosteroid injections are second-line therapies for the treatment of hypertrophic scars. Corticosteroids inhibit the inflammatory process and expression of genes related to collagen and glycosaminoglycan synthesis, decreasing fibroblast proliferation. Intralesional steroid injections are highly responsive (50% to 100%), indicating a profound effect of reducing inflammation on limiting hypertrophic scar formation [368, 373, 374]. However, 63% of the patients experience side effects, especially in the form of

hypopigmentation, skin and subcutaneous fat atrophy and some experience telangiectasia [375].

Deferiprone is an iron chelator with anti-microbial properties[376] that also has properties of free radical scavenging and is known to improve wound healing (skin wounds) [377]. Scavenging ROS after abdominal surgery has been shown to significantly inhibit postoperative adhesion formation [378] .

This study evaluated the wound-healing activities of different concentrations of Deferiprone on primary human fibroblasts and primary human nasal epithelial cells (HNECs) *in vitro*. The goal was to determine the effect of Deferiprone on fibroblast and epithelial cell migration, collagen production, ROS activity and potential for anti-inflammatory effects to evaluate its potential to limit hypertrophic scar tissue formation for future clinical applications.

4.3 Methods

Study population

The study was approved by The Queen Elizabeth Hospital Human Research Ethics Committee, and written informed consent was obtained from all participants for tissue collection and use of clinical information. Patients recruited to the study included those who were undergoing endoscopic sinus surgery for CRS. Exclusion criteria included active smoking, age less than 18 years, pregnancy, and systemic diseases (immunosuppressive disease).

Harvesting and culturing primary Human Nasal Fibroblasts in Vitro.

Sinonasal tissue was biopsied from paranasal sinus mucosa and transferred to a 6-well culture plate with 2 ml Dulbecco's Modified Eagle's medium (DMEM, Invitrogen, UK) supplemented with L-glutamine, 10% Fetal bovine serum (FBS, Sigma, St Louis, USA) and penicillin streptomycin (Gibco, Life Technologies, NY, USA). Every 2-3 days, the tissue was washed gently with 1 ml phosphate-buffered saline (PBS) and medium was replaced with 1.5 ml fresh medium until fibroblasts became confluent after approximately 2 weeks.

Purification of fibroblasts

Once confluent, fibroblasts were washed with 2 ml PBS, trypsinized and collected followed by centrifugation at 400×*g* for 8 minutes. The supernatant was removed and pellet resuspended in 1 ml PBS along with 50 µl Dynabeads Epithelial Enrich (Invitrogen, NY, USA). The tube was wrapped in parafilm and placed on a rotor mixer for 20 minutes at room temperature (RT). Supernatant containing fibroblasts were

transferred to a T25 tissue culture flask (Nunc, Roskilde, Denmark) and the tube containing the remaining beads discarded.

Harvesting and Culturing Human Nasal Epithelial Cells in Vitro.

Primary human nasal epithelial cells (HNECs) were harvested from nasal polyps by gentle brushing in a method described by Ramezanpour et al[379]. Extracted cells were suspended in Bronchial Epithelial Growth Media (BEGM, CC-3170, Lonza, Walkersville, MD, USA), supplemented with 2% Ultroser G (Pall Corporation, Port Washington, NY, USA). The cell suspension was depleted of macrophages using anti-CD68 (Dako, Glostrup, Denmark) coated culture dishes, and HNECs were maintained with B-ALI™ growth medium (Lonza, Walkersville, USA) in collagen coated flasks (Thermo Scientific, Walthman, MA, USA) in a cell incubator at 37°C with 5% CO_2.

Air Liquid Interface Culture

HNECs were grown until 80% confluent then harvested for seeding onto collagen coated 6.5 mm permeable Transwell plates (BD Biosciences, San Jose, California, USA) at a density of 5×10^4 cells per well. Cells were maintained with B-ALI™ growth medium for 2-3 days in a cell incubator at 37°C with 5% CO_2. On day 3 after seeding, the apical media was removed and the basal media replaced with B-ALI™ differentiation media, exposing the apical cell surface to the atmosphere. Human nasal epithelial cultures at air liquid interface (HNEC-ALI) were maintained for a minimum of 21 days for development of tight junctions.

Cytotoxicity Studies

Primary human fibroblasts or HNECs were grown in DMEM and BEGM (Lonza, Walkersville, USA) medium respectively. Cells were maintained in a fully humidified incubator with 5% CO_2 at 37 °C prior to cytotoxicity studies. Cells were exposed to different concentrations of Deferiprone (3-Hydroxy-1,2-dimethyl-4 (1H)-pyridone, Sigma, USA) at different time points, followed by determination of lactate dehydrogenase (LDH) with a cytotoxicity detection kit (Promega, Madison, U.S.). Briefly, 50 µL of the supernatant from each well was mixed with 50 µL of LDH reagent and was incubated for 30 minutes in the dark at RT. The optical density (OD) was measured at 490 nm on a FLUOstar OPTIMA plate reader (BMG Labtech, Ortenberg, Germany). Cell culture studies were performed as three independent experiments.

Wound Healing (Migration) Assay

In the fibroblast wound closure assay, fibroblasts were seeded in 24 well plates, stained with CellTrace™ Violet (Invitrogen/Life Technologies, USA) and allowed to reach 80% confluence in 24 hours. A straight vertical scratch was made down through the fibroblasts and HNEC-ALI cell monolayers by using a 200 µl pipette tip. The media and cell debris was aspirated carefully and culture media with different concentrations of Deferiprone (1 mM, 5 mM, 10 mM, 20 mM) or media only (negative control) added to each well for 72 hours. At time zero, cells were treated with 1 µg/ml mitomycin (Accord Healthcare Inc, NDC 16729-108-11, USA) to inhibit cell proliferation. The wound closure (cell migration) was recorded using time-lapse LSM700 confocal scanning laser microscopy (Zeiss Microscopy, Germany), with an image recorded every 4 hours in a temperature and CO_2 controlled chamber.

Enzyme-Linked Immunosorbent Assay (ELISA)

Supernatants were collected from HNECs and fibroblasts after 24 hours of exposure with different concentrations of Deferiprone in the presence/absence of the pro-inflammatory agent Poly (I:C) (10 μg/ml) [380] or IL-1β (10 ng/ml Sigma, Saint Louis, USA) [381] respectively. Interleukin-6 (IL-6) protein levels were estimated with an ELISA kit using rat anti-human IL-6 antibodies (BD Biosciences, New Jersey, USA), according to the manufacturer's instructions. All measurements were performed in duplicate using a FLUOstar OPTIMA plate reader (BMG Labtech, Ortenberg, Germany). The tissue sample concentration was calculated from a standard curve and corrected for protein concentration.

Collagen ELISA Assay

Primary human nasal fibroblasts were seeded in 24-well tissue-culture plates at a density of 5×10^5 grown in DMEM until confluent. Duplicate wells were stimulated with Deferiprone at 1 mM, 5 mM, 10 mM and 20 mM in DMEM in the presence/absence of L-Ascorbic acid-2 phosphate (100 mM) (113170-55-1, Sigma-Aldrich) for 48 hours. Following treatment, the supernatant was collected and the protein level of type I collagen was measured with a procollagen type I C-peptide ELISA kit (Takara Bio Inc, Otsu, Japan). Experimental procedures followed the manufacturer's instructions. Briefly, 20 μl of culture medium and 100 μl of the antibody-POD conjugate solution were sequentially added into microtiter plates and reacted for 3 hours at 37°C. After 4 × washing with washing buffer solution (1× PBST), 100 μl of the substrate solution was added and incubated for 15 minutes at RT. Finally, the stop solution (100 μl) was added and corresponding absorbance was recorded at 450 nm using a FLUOstar OPTIMA plate reader (BMG Labtech, Ortenberg, Germany).

Evaluation of oxidative stress

Primary nasal fibroblast cells were cultured in DMEM with 10% FBS and seeded into black wall 96-well plates (Life Technologies, Australia) and incubated for 24 h in a humidified incubator with 5% CO_2 at 37 °C. Cells were washed with PBS and 10 μM of 2′, 7′-dichlorodihydrofluorescein diacetate (H2DCFDA; Invitrogen Life Technologies, Carlsbad, CA, USA) was added for 1 h, at 37°C in the dark. Cells were then washed twice with PBS and exposed to scratching injury by dragging a 100 μL pipette tip linearly on the confluent monolayers in the presence of different Deferiprone concentrations (1 mM, 5 mM, 10 mM and 20 mM). The fluorescence intensity was then measured using a microplate reader at filter range Ex/Em: 492/525 nm at 1h intervals over 5 h.

Statistical analysis

A repeated-measures ANOVA was used for statistical analysis of the wound closure. Data is presented as the mean ± SEM. Statistical analyses of LDH assay and ELISA assays were carried out using ANOVA, followed by Tukey HSD post hoc test. These tests were performed using SPSS software (version 22). The Pearson product-moment correlation coefficient was determined by using R software to find correlations of Deferiprone dosages with OD values in the oxidative stress assay. Statistical significance was defined as a P value of less than 0.05.

4.4 Results

In vitro cytotoxicity of Deferiprone

The cytotoxic effect of different concentrations of Deferiprone (1 mM, 5 mM, 10 mM, 20 mM) was determined by the LDH assay, evaluating the survival of HNECs (Figure 26A) and fibroblasts (Figure 26B) relative to the negative control over time. Different exposure times (1 h, 2 h, 3 h, 4 h, 5 h and 6 h) showed no significant increase in LDH release with any concentration of Deferiprone in HNECs or fibroblasts ($p > 0.05$). The positive control (0.5% Triton X-100) and negative control (medium) demonstrated expected toxicity values.

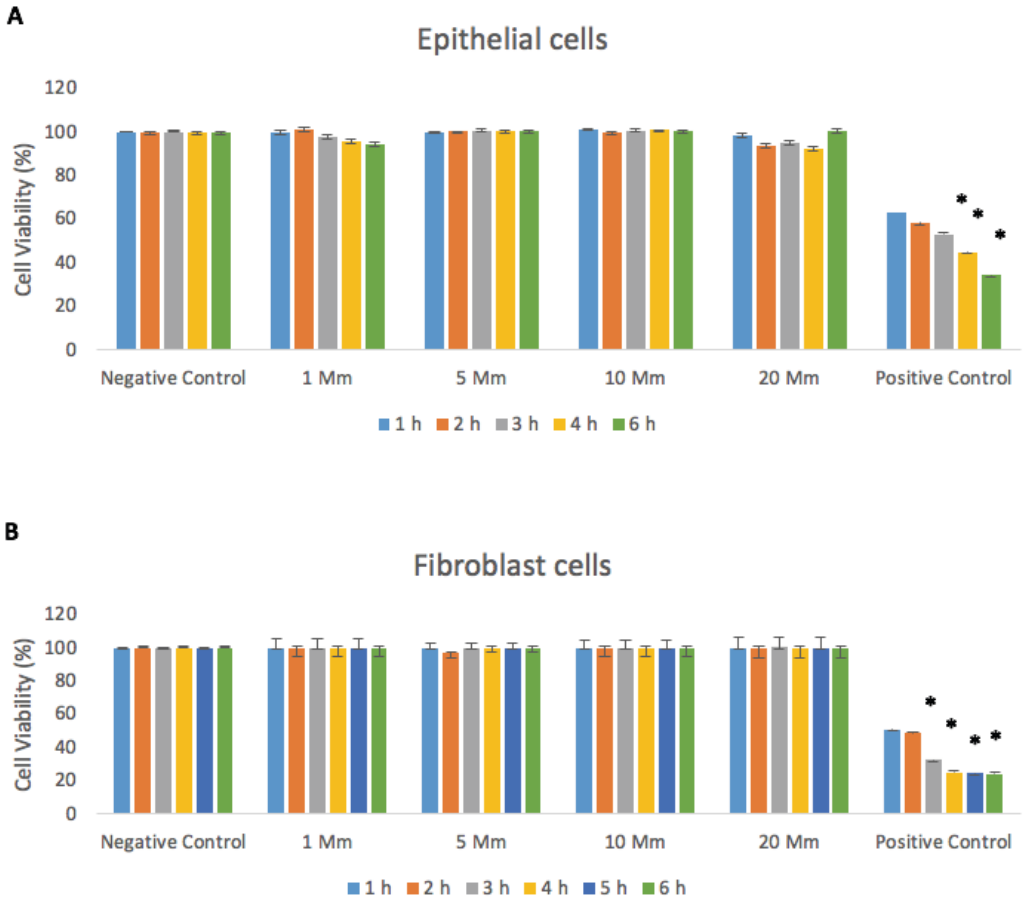


Figure 26. Cell viability of HNECs and human nasal fibroblast monolayers derived from CRS patients. Viability relative to no treatment control cells as determined by the LDH assay, 1 hr, 2 hr, 3 hr, 4 hr, 5 hr and 6 hours after application of Deferiprone (1 mM, 5 mM, 10 mM, 20 mM), negative control (medium), and positive control (0.5% Triton X-100) in HNECs (A) and primary human nasal fibroblasts (B) derived from CRS patients. Cell viability was calculated relative to the untreated cells as negative control. The values are shown as means ± SEM, n = 3. ANOVA, followed by Tukey HSD post hoc test. (* $p < 0.05$).

Effect of Deferiprone on human nasal epithelial cell and primary fibroblast cell migration in vitro.

To examine the influence of Deferiprone on sinonasal wound resealing *in vitro*, time course studies were performed during active wound closure. HNEC-ALI cultures and primary fibroblasts were treated with different concentrations of Deferiprone or negative control in scratch assays. In HNEC-ALI cultures, untreated (control) wounds healed with full re-epithelialization by 68 hours. Incubation with four different concentrations of Deferiprone for up to 68 hours did not show any significant delay in wound healing (Figure 27A). Untreated primary fibroblasts closed the wound after 44 hours. Incubation with 20 mM Deferiprone caused a significant delay in wound closure at 24 h and at all-time points measured thereafter. In addition, lower 10 mM and 5 mM Deferiprone concentrations significantly delayed fibroblast migration after 44 hours (Figure 27B).

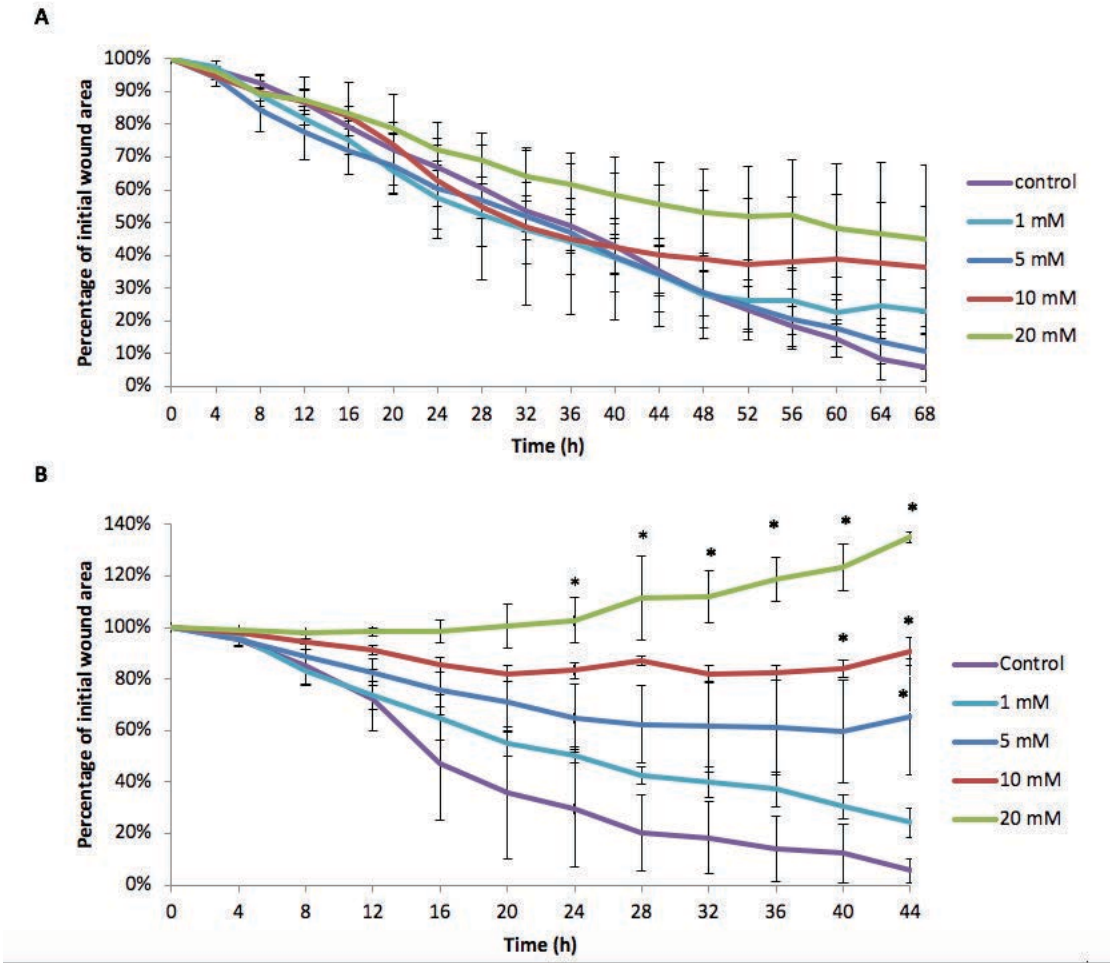
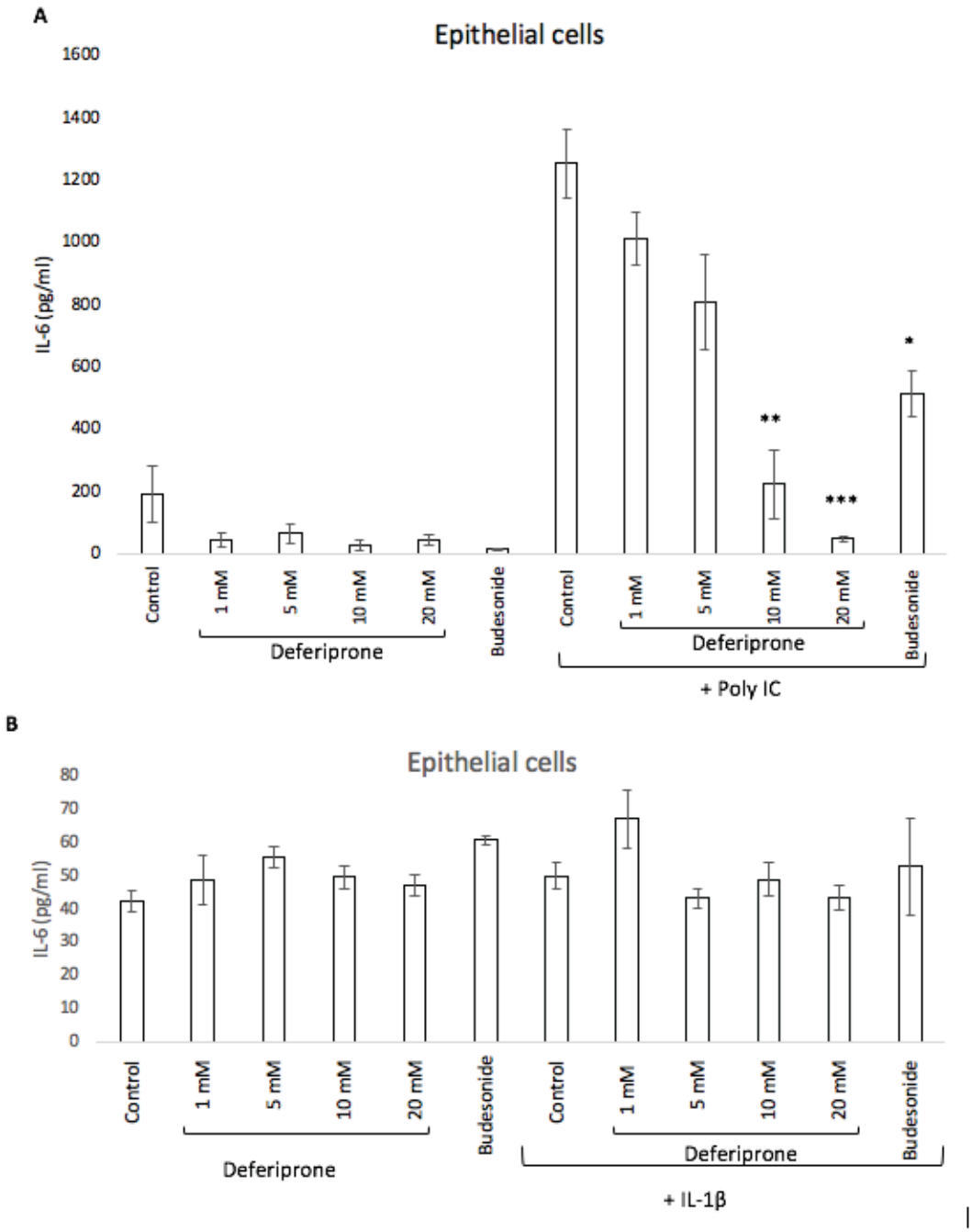


Figure 27. Scratch assays of primary human nasal epithelial cells and primary fibroblasts in the presence of different Deferiprone concentrations over time. The mean percentage of wound area in scratch assays of primary human nasal epithelial cells (A) and sinonasal fibroblasts (B) in the presence of different concentrations of Deferiprone (1 mM, 5 mM, 10 mM, 20 mM) or negative (medium) control over time. The values are shown as mean ± SEM, n = 3. ANOVA, followed by Tukey HSD post hoc test. * $p < 0.05$.

Effect of Deferiprone on the inflammatory response in human nasal epithelial cells and human sinonasal fibroblasts

To determine the potential of Deferiprone to dampen a pro-inflammatory response, Deferiprone at different concentrations was applied to HNECs or fibroblasts in the presence or absence of the pro-inflammatory agent Poly (I:C) or IL-1β. Budesonide was used as an anti-inflammatory standard of care control and significantly reduced IL-6 production in both HNECs ($p = 0.03$) and fibroblasts ($p = 0.001$) in the presence of

pro-inflammatory agents. In HNECs, application of 10 mM and 20 mM of Deferiprone for 24 hours significantly reduced IL-6 protein concentrations (80% reduction, p=0.001 and 96% reduction, p=0.0001 respectively) in the presence of Poly (I:C) (Figure 28A) compared with negative control. In contrast, Deferiprone did not alter the secretion of IL-6 in nasal fibroblasts in the presence or absence of IL-1β after 24 hours (Figure 28D).



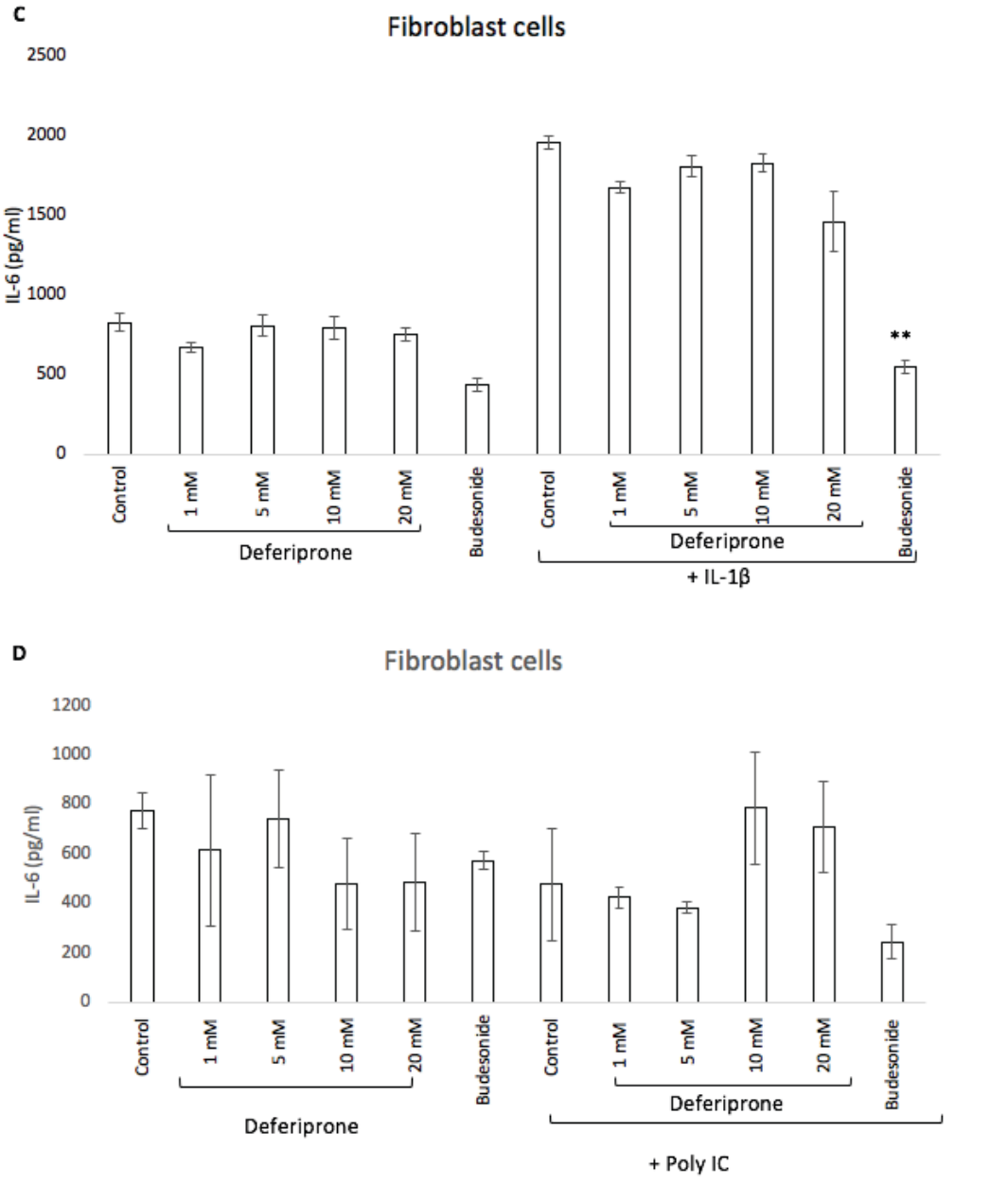


Figure 28. IL-6 production was measured using ELISA on human nasal epithelial cells (A &B) or nasal fibroblast cells (C&D) in the presence or absence of the pro-inflammatory agent Poly (I:C) or IL-1β. Budesonide (1 mg/ 2 ml) was used as anti-inflammatory standard of care control and medium was used as negative control. ANOVA, followed by Tukey HSD post hoc test. (*=p<0.05, **= p < 0.001, ***=p< 0.0001); values are shown as means ± SEM.

Effect of Deferiprone on the release of collagen by primary nasal fibroblasts

Application of different concentrations of Deferiprone for 24 hours significantly reduced collagen protein concentrations in supernatants of fibroblast monolayers

derived from CRS patients (p<0.0001) (Figure 29A). In addition, Deferiprone at different concentrations was applied to fibroblasts in the presence of L-Ascorbic acid-2 phosphate (ASC), known to induce collagen production by fibroblasts [382]. Deferiprone significantly inhibited collagen secretion in the presence ASC (Figure 29B).

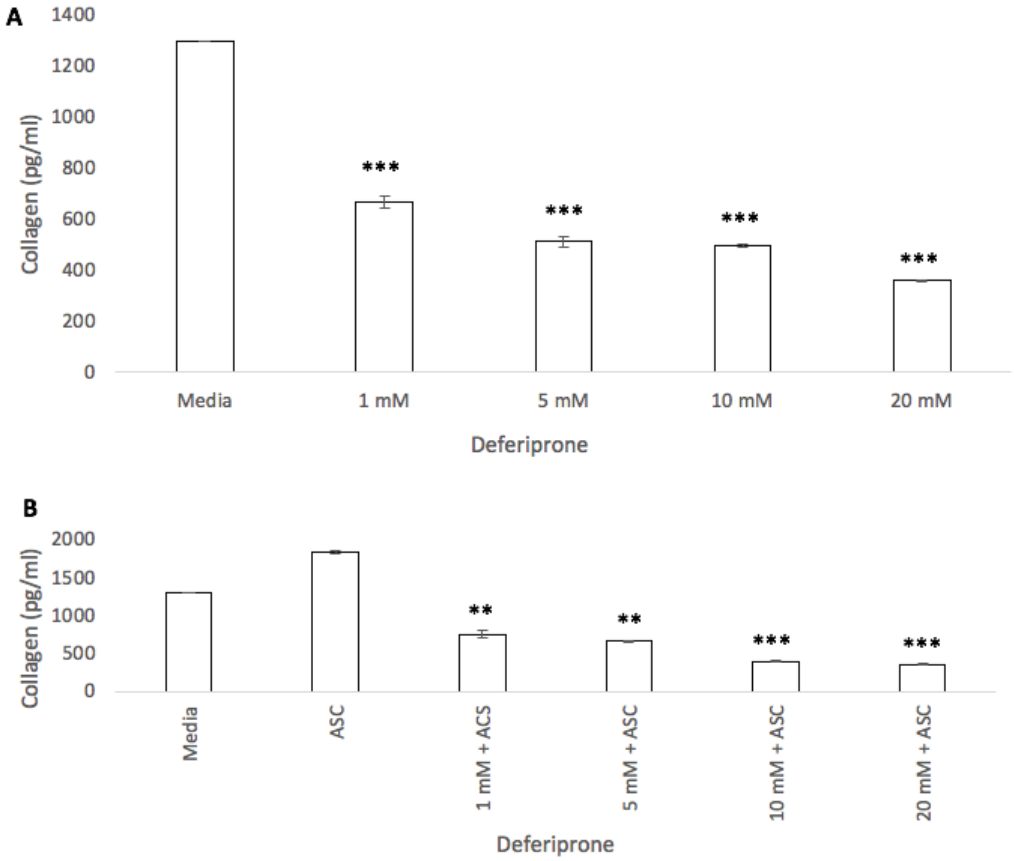


Figure 29. Collagen release was measured by ELISA in primary nasal fibroblasts treated with Deferiprone in the absence (A) or presence (B) of L-Ascorbic acid-2 phosphate (ASC). Primary human nasal fibroblasts were treated with 1 mM, 5 mM, 10 mM and 20 mM Deferiprone for 24 hours. Media only and L-Ascorbic acid-2 phosphate (ASC) acted as a negative and positive control respectively. Bars stand as means ± standard deviation (n=4). (** p < 0.001(*** p < 0.0001). ANOVA, followed by Tukey HSD post hoc test.

Effect of Deferiprone on the release of reactive oxygen species in primary nasal fibroblasts

We then measured reactive oxygen species (ROS) production by utilizing the fluorescent probe H2DCFDA. This compound accumulates inside the cells and is oxidized by ROS to the corresponding fluorescent chromophore. The fluorescence intensity was measured using a microplate reader at filter range Ex/Em: 492/525 nm at 1h intervals over 5 h. There was a significant negative correlation between Deferiprone dose and ROS release in scratch assay in fibroblast cells at all-time points measured (Pearson product-moment correlation) (Figure 30).

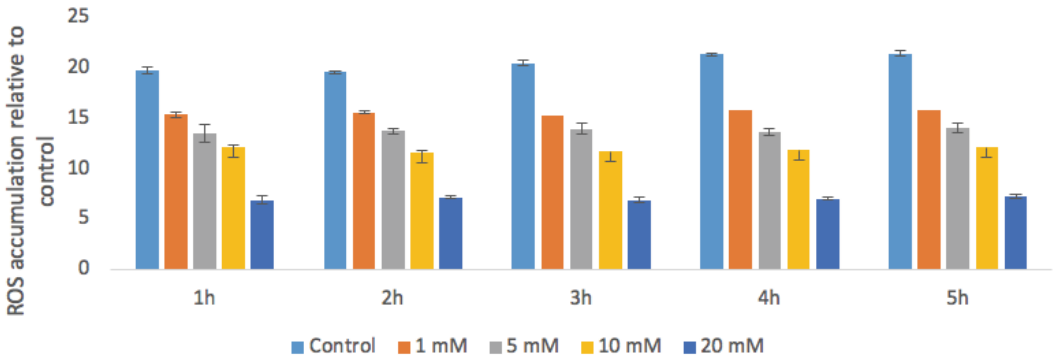


Figure 30. ROS quantification by measuring the dichlorofuorescein diacetate (H2DCFDA) probe activation through ROS generation in primary nasal fibroblast monolayers. Data is expressed as ROS accumulation relative to untreated cells (control). Statistical analysis was performed by Pearson product-moment correlation.

4.5 Discussion

In this study on the effect of Deferiprone on wound healing activities, we made five fundamental observations. First, Deferiprone was non-toxic when applied onto primary fibroblasts and HNECs *in vitro*. Second, Deferiprone significantly delayed fibroblast migration in a dose-dependent way when compared with untreated samples. In contrast, HNECs did not show any significant change in their migration rate after Deferiprone treatment. Third, Deferiprone blocked IL-6 production in pro-inflammatory conditions in HNECs but not in fibroblasts. Fourth, Deferiprone reduced collagen protein concentrations in the supernatants of fibroblast monolayers in the presence or absence of L-Ascorbic acid-2 phosphate (ASC). Finally, Deferiprone inhibited ROS release in a dose dependant manner in primary nasal fibroblasts compared with untreated samples. The absence of toxicity is in line with our previous reports where treatment with Deferiprone (alone or in combination with other compounds) showed no significant toxic effects *in vitro* when applied to L929 and NuLi-1 cell lines [376] and *in vivo* when applied to the sinus mucosa of sheep [383]. It has been demonstrated that the critical time interval to block adhesions is primarily in the first few days after the initial injury and that the extent of adhesion formation is largely dependent on the level of inflammation, ROS production, collagen production and fibroblast migration during that time [359, 384]. Here, the 44 h exposure to 5 mM, 10 mM and 20 mM Deferiprone caused a significant delay in fibroblast migration, whereas HNECs re-epithelialized at the same rate as the untreated control. Our data implies that Deferiprone might be helpful in treating post-surgical adhesions as it limits fibroblast cell-adhesions without negatively affecting the re-epithelialization. Fibroblasts and smooth muscle cells are responsible for collagen synthesis and any factor that decreases collagen synthesis results in a longer-lasting

wound healing. Our data indicate that stimulation of the fibroblasts with Deferiprone decreased collagen production in a dose-dependent manner. For further scrutiny, we treated the fibroblasts in the presence of ASC which is known to stimulate collagen accumulation and cell proliferation [382]. Interestingly, we found Deferiprone significantly inhibited collagen secretion even in the presence of ASC. Consistent with our finding, Deferiprone has been shown to inhibit the proliferation of skin fibroblasts and frataxin- depleted neuroblastoma-derived cells *in vitro* [385]. The study showed that Deferiprone impaired aconitase activity through reduced synthesis of the iron-sulfur cluster machinery which makes it a potent chelator of mitochondrial matrix iron [385].

Balanced inflammation is critical to the normal healing process after tissue injury. However, excessive inflammation can result in hypertrophic scar formation and chronic inflammation is a critical factor in delaying the wound healing of chronic wounds [359, 386]. Critical inflammatory cells involved in this process are neutrophils and macrophages [359] and their recruitment is associated with the induction of ROS and inflammatory cytokine production [387]. In an *in vitro* wound model of hypertrophic scar fibroblasts, a microarray analysis indicated the interleukin 6 (IL-6) signaling pathway to be the main pathway involved in the early response to injury in those cells [388]. Moreover, IL-6, a key chemoattractant for monocytes and a macrophage activator, along with other proinflammatory factors, such as interleukin (IL)-1α, IL-1β and tumor necrosis factor-α are upregulated in hypertrophic scar tissues [386]. Moreover, decreased levels of IL-6 characterize foetal wounds, known to heal without scarring, and the addition of IL-6 to foetal wounds leads to scarring [389].

Together these findings indicate that limiting inflammation and specifically proinflammatory cytokines such as IL-6 and ROS production, as well as inhibiting the

migration of fibroblasts and limiting their collagen production might be key to limiting hypertrophic scar formation. Our findings indicate that Deferiprone has the potential to do just that as, in addition to reducing the migration of fibroblasts into the wound and decreasing their collagen production, Deferiprone manifestly reduced IL-6 protein production by HNECs in pro-inflammatory conditions and decreased ROS in a dose-dependent manner in fibroblasts.

4.6 Conclusion

In conclusion, the results of this study indicate that Deferiprone was not toxic to primary fibroblasts or HNECs. Deferiprone, in a dose and time-dependent way, delayed primary nasal fibroblast migration in scratch assays, decreased their collagen and ROS production and reduced immune cytokine IL-6 production by HNECs. Together, our observations indicate that Deferiprone may have the potential to limit scar tissue formation in future clinical applications.

Chapter 5: Long-term safety of topical bacteriophage application to the frontal sinus region

Statement of Authorship

Title of Paper	Long-term safety of topical bacteriophage application to the frontal sinus region
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
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Principal Author

Name of Principal Author (Candidate)	Amanda Drilling
Contribution to the Paper	Project design, data collection and analysis and manuscript preparation
Overall percentage (%)	85%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 31/1/20

Co-Author Contributions

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

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

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

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

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5.1 Abstract

Background: Staphylococcus aureus biofilms contribute negatively to a number of chronic conditions, including chronic rhinosinusitis (CRS). With the inherent tolerance of biofilm-bound bacteria to antibiotics and the global problem of bacterial antibiotic resistance, the need to develop novel therapeutics is paramount. Phage therapy has previously shown promise in treating sinonasal S. aureus biofilms.

Methods: This study investigates the long term (20 days) safety of topical sinonasal flushes with bacteriophage suspensions. The bacteriophage cocktail NOV012 against S. aureus selected for this work contains two highly characterized and different phage, P68 and K710. Host range was assessed against S. aureus strains isolated from CRS patients using agar spot tests. NOV012 was applied topically to the frontal sinus region of sheep, twice daily for 20 days. General sheep wellbeing, mucosal structural changes and inflammatory load were assessed to determine safety of NOV012 application.

Results: NOV012 could lyse 52/61 (85%) of a panel of locally derived CRS clinical isolates. Application of NOV012 to the frontal sinuses of sheep for 20 days was found to be safe, with no observed inflammatory infiltration or tissue damage within the sinus mucosa.

Conclusion: NOV012 cocktail appears safe to apply for extended periods to sheep sinuses and it could infect and lyse a wide range of S. aureus CRS clinical isolates. This indicates that phage therapy has strong potential as a treatment for chronic bacterial rhinosinusitis.

5.2 Introduction

Staphylococcus aureus is an opportunistic bacterial pathogen forming biofilms, which are known to be involved in a number of infective chronic diseases[174, 323, 390-393]. These include osteomyelitis[391], endocarditis[390], as well as infections of indwelling devices[392]. In addition, sinonasal bound biofilms of S. aureus are known to impact negatively in chronic rhinosinusitis (CRS). The presence of such infections in CRS reportedly leads to more frequent out-patient visits[323], increased risk of recurrent infections and antibiotic use[174] as well as poorer postoperative progression[394, 395]. Such biofilms are up to 1000-fold more tolerant of current antibiotic therapies than their planktonic counterparts[396]. Further, increased levels of antibiotic resistance observed in pathogenic bacteria around the globe[397] also limit the success of antibiotic therapies. It is important that new therapies which effectively treat such infections are identified. One alternative to antibiotics, originally described in the early 1900s and coming back into focus, is bacteriophage "phage" therapy[398]. Beneficially, phage are not only effective against planktonic infections, but can also infect and lyse biofilm bound cells[166, 399-406]. Recently, we have shown that topical phage therapy has potential against pathogenic *S. aureus* bacterial biofilms, using an animal model of rhinosinusitis[252]. Our previous work demonstrated the safety of once daily phage application into the frontal sinuses of sheep for 3 days[252]. The first aim of the current work was to examine the safety of phage administration for a substantially longer period of time. In the current study we investigated the effects of a longer-term (20 day) application of twice-daily frontal sinus bacteriophage flushes in sheep. A cocktail of two phage against S. aureus, designated NOV012, was used. NOV012 contains two highly-characterized phage, K710 and P68.

Both the parental version of phage K710, phage K[407], and phage P68[408] have had their genomes completely sequenced, and they have been shown to lack any known genes that could increase the virulence of *S. aureus* or confer resistance to antibiotics[407, 408]. Phage K710 and P68 have been shown to be active against a wide range of S. aureus isolates from the United Kingdom and Europe[409].The second aim of this work was to extend these observations by examining the susceptibility of local (Australian) S. aureus isolates to the phage in NOV012.

5.3 Methods and Materials

Bacteriophage

A phage cocktail (NOV012) comprised of preparations of highly purified phage, K710 and P68, was obtained from Novolytics Pty. Ltd. (Warrington, United Kingdom). This cocktail of phage is functionally similar to the CTSA cocktail (Special Phage Services, Brookvale, NSW, Australia) which we used previously[252]. For commercial reasons CTSA was unavailable. The concentration of phage in NOV012 stock was maintained at 1 x 10^8 PFU/mL for in vivo and in vitro work. To produce a heat-inactivated version of the cocktail, the phage stock was treated at 121◦C for 15 min and tested by plaque assay to confirm complete inactivation prior to use.

Bacteria

Investigation of S. aureus strains isolated from CRS patients was approved by The Queen Elizabeth Hospital Human Ethics Committee. Written informed consent was obtained for all study participants. To isolate S. aureus from sinonasal swabs, Columbian blood agar plates, colistin nalidixic acid plates or cystine lactose electrolyte deficient plates (all from Thermofisher Scientific, Australia) were employed. Latex agglutination tests and antibiotic sensitivity testing were performed by the commercial laboratory Adelaide Pathology Partners and used to confirm identification of S. aureus and MRSA strains (data not shown). Antibiotic resistance was determined using disc diffusion methods as according to Clinical and Laboratory Standards Institute (CLSI) recommendations (CLSI, 2012). Isolates were then subcultured in nutrient broth

overnight (Thermo Fisher, Scoresby, Victoria, Australia) and stored in nutrient broth with 20% glycerol at −80◦C.

CRS Bacterial Isolate Susceptibility to Phage Infection

Bacterial sensitivity to phage infection was assessed by spotting phage onto bacterial lawns in an agar overlay system. Briefly, S. aureus isolates were cultured overnight at 37◦C with shaking for 16–18 h in nutrient broth. Overnight cultures were diluted 1:30 in liquid 0.4% nutrient agar which was overlayed onto 1% nutrient agar. Dilutions of P68, K710, and the cocktail of these (NOV012) were spotted onto soft agar plates and allowed to dry. Plates were inverted and incubated overnight at 37◦C. Spots were assessed the next day and phage titres and plaque morphologies recorded.

Plaques were ranked "+++" (highly sensitive, clear plaque) through to "+" (slightly sensitive, plaque barely discernible), and plaques of status intermediate between these were ranked "++" (moderately sensitive). Results were termed "lysis from without" (LWO) when zones of inhibition were observed however discreet plaques were not evident when the assay used diluted phage stocks. Bacterial isolates were considered susceptible to phage if a plaque was discernible. To determine the strain type of the isolates, pulsed-field gel electrophoresis was used as described by O'Brien et al[410]. Isolates with ≥80% similarity or <6 band differences were considered the same strain. Efficiency of plating was determined using the concentration of phage determined from infection of the S. aureus reference strain ATCC 25923[252].

Frontal Sinus Access and Treatment

Animal work performed in this study was approved by the South Australian Health and Medical Research Institute and the University of Adelaide Animal Ethics Committees. Access to the ovine frontal sinus was achieved through the placement of mini-trephines, as described[329]. Once accessed, sinuses where flushed twice daily with 50 mL of treatment using an extension cannula for 20 days. Sheep were treated with one of three different treatments: 0.9% saline, 0.9% saline containing 2x 10^6 PFU/mL heat-inactivated phage. NOV012 (HIp) or 0.9% saline containing 2 x 10^6 PFU/mL active NOV012 (NOV012). Each treatment group consisted of 4 sheep. One trephine from each treatment group became blocked during the treatment, resulting in n = 7 sinuses per group. Sheep were monitored for general wellbeing during treatment. At the completion of treatment, sheep were euthanized and sinus tissue harvested for analysis. Microbiology swabs were taken to determine the bacterial composition of the sinus.

Histology and Scanning Electron Microscopy

Mucosal sections were dissected and placed in either 10% formalin for histological analysis, or scanning electron microscopy (SEM) buffer [4% paraformaldehyde/1.25% glutaraldehyde in phosphate-buffered solution (PBS) with 4% sucrose] for SEM. For histological analysis, tissue was embedded into paraffin blocks, sectioned, mounted on slides, and stained using haematoxylin and eosin (H&E). Sections were examined by an experienced tissue pathologist (author CJ). Sections were identified only by a code to ensure the examining pathologist was unaware of which treatment had been provided to the animals. The examining pathologist graded the tissue for levels of inflammation, oedema, fibrosis, and presence or absence of goblet cell hyperplasia. For SEM, tissue was counterstained using 2% osmium tetroxide and dehydrated using a graded series of 70–100% ethanol washes. The tissue was chemically dried using hexamethyldisilazane

(Sigma Aldrich) and mounted on SEM stub specimen mounts (Ted Pella, Redding, CA). The stubs were then coated in carbon using a standard carbon coater (Ted Pella) and viewed using an XL30 field emission Gun scanning electron microscope (Philips, Eindhoven, Netherlands). Five images of each tissue section were captured at magnification 2500x where at least 50% of the image allowed visualization of the tissue surface. Each image was broken down into 2 cm^2 grid sections and scored either: 1, full cilia coverage; 0.5, some cilia coverage; 0, no cilia present. Not counted, mucus covering cilia or epithelial layer, so tissue could not be visualized.

Isolation of Phage from Serum Samples

Serum samples were collected from all sheep prior to the first treatment flush. Further, sheep in the inactivated phage group and in the control group had serum samples taken on days 7, 14 and 19 after the first flush. Phage-treated sheep had serum samples taken 10 min, and 1, 2, and 4 h after flush 1, and 18 h after the second flush. Serum samples were not found to contain phage at any of the tested time points. Therefore on days 7, 14, and 19, serum samples were harvested directly after phage flush 1 as well as 1 and 2 h post-flush 1 and 18 h post-flush 2. A mucosal sample (1 g) harvested from each phage-treated sheep on day 2L was processed and filtered as previously described by Drilling et al[252]. All harvested serum and mucosal samples were tested for infectious phage using the agar overlay plaque assay method. Serum (1 ml) or processed mucosal sample was mixed with cultures of S. aureus strain ATCC 25923 (cultured as above) and incubated at room temperature for 15 min. Samples were then mixed with 2 mL of 0.7% nutrient agar and overlayed onto 1% nutrient agar plates. Plates were examined every 24 h for 3 days for the presence of plaques. Agar overlays were performed in triplicate.

Statistics

Statistical analyses were performed using SPSS version 23 software (IBM R SPSS R Statistics, New York, USA). Fisher's exact tests were used to compare the range of S. aureus stains that the phage preparations (P68 vs. K710 vs. cocktail) could infect and kill. All other statistical comparisons were performed using Kruskal-Wallis analysis and post-hoc with Bonferroni correction[411].

5.4 Results

Infection Range of Phage P68 and K710 Against CRS-Derived S. aureus Clinical Isolates

S. aureus isolates from 61 patients diagnosed with CRS were examined (Table 9). When tested for strain type by pulse-field gel electrophoresis, 25 different strains were identified. Each strain contained 1–6 different clonal types. Clonal types R1, R3, and T3 were observed in the isolate population more twice.

When tested for susceptibility to K710, P68, and NOV012, 36/61 isolates (59%) were found sensitive to phage K710, 45 (74%) were sensitive to P68, and 52 (85%) were sensitive to NOV012 (Table 9). The cocktail was able to infect significantly more *S. aureus* strains compared to single K710 application (p = 0.0022).

Pulsotype	K710	P68	NOV012	Pulsotype	K710	P68	NOV012
A1	LWO	LWO	LWO	N2	+++	++	+++
A2	+++	++	+++	N3	+++	+++	+++
A3	+	+	+	O1	+++	+++	+++
B1	+++	+	+++	02	+++	+	+++
B2	LWO	+++	+++	O3	+++	R	+++
C1	LWO	++	+	P	LWO	LWO	LWO
C2	++	R	++	Q	LWO	+++	+++
C3	+	R	+	R1	R	+++	+++
C4	R	+	+	R1	R	++	++
C5	+	LWO	+	R2	+++	+++	+++
C6	R	+	+	R3	R	+++	+++
D1	LWO	R	LWO	R3	R	+++	+++
D2	R	R	R	R4	R	+++	+++
D3	LWO	+	+	R5	+++	+++	+++
D4	LWO	R	LWO	R6	+++	+++	+++
E	+++	+++	+++	S1	++	+	++
F	+++	+++	+++	S2	+++	++	+++
G	+	R	+	T1	LWO	R	LWO
H1	LWO	LWO	LWO	T2	LWO	+++	+++
H2	R	+++	+++	T2	+++	+++	+++
H3	R	R	R	T3	+++	+++	+++

H4	+++	+++	+++	T4	+++	R	+++
H5	+++	+++	+++	T5	LWO	+++	+++
H6	+++	+++	+++	U	+++	++	+++
I1	+++	+++	+++	V1	+++	+++	+++
I2	+++	+++	+++	V1	+++	+++	+++
J	+++	+++	+++	W	LWO	R	LWO
K	+++	+++	+++	X	+++	+++	+++
L	+++	+++	+++	Y1	+++	+++	+++
M	+++	LWO	+++	Y2	LWO	+++	+++
N1	LWO	++	++				

Table 9. Sensitivity of *Staphylococcus aureus* isolates of CRS origin to bacteriophage lysis

The cocktail lysed more strains compared to P68, however this was not statistically significant. Five CRS isolates were identified to be methicillin-resistant *S. aureus* (MRSA) isolates. Three of the five MRSA isolates were susceptible to K710 and all five MRSA isolates were susceptible to P68. Efficiency of plating for each isolate ranged from 0.002 to 4-fold for K710, 2.7E-06 to 6.7-fold for P68 and 4E-06 to 8-fold for NOV012.

Animal Studies

Observations of Sheep General Health

During phage administration to sheep, there was no change in the general well-being of three out of four sheep in each of the control group and the phage group. One sheep in the control group and one in the phage group experienced some loss of appetite during the treatment period. Both sheep were found to have infections at the site of catheter insertion, which was thought to be the cause of appetite disruption. Antibiotic treatment during the treatment period, in accordance with the ethics committee approved experimentation protocol, improved the appetite of both sheep. The general health of all four sheep treated with Hip was as expected and no change in sheep well-being was observed in this group.

Histology of Sheep Sinus Mucosa

H&E stained tissue sections taken from sheep euthanized at the end of the treatment period (Figure 31) were examined for inflammation, oedema, fibrosis and the presence or absence of goblet cell hyperplasia. There were no significant differences between the groups in regard to each of these parameters (data not shown).

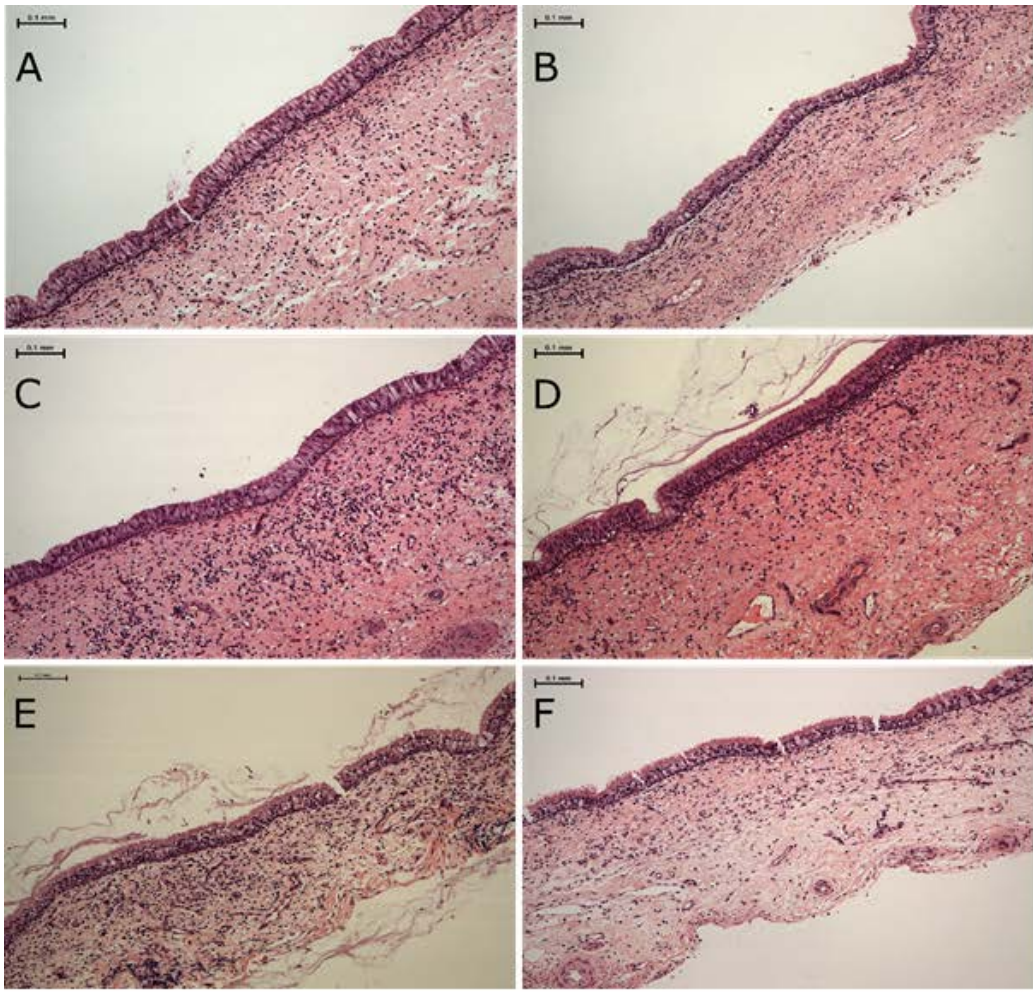

Figure 31. Haematoxylin and eosin stained sheep nasal mucosa sections. Sections are of tissues taken from animals euthanized after 20 days of treatment as below. No significant differences in tissue inflammation, oedema, fibrosis and the presence or absence of goblet cell hyperplasia was observed between treatment groups (A,B) control, (C,D) heat-inactivated NOV012 (H*ip*) treatment and (E,F) active NOV012 treatment.

Presence and Appearance of Cilia of Mucosa Tissue

SEM was performed to allow closer inspection of sinus mucosa cilia. Cilia of all seven sinus samples harvested from Hip treatment were able to be visualized. The cilia of one control treated sheep sample and one phage treated sample were obscured from view by mucus, hence these samples were excluded from analysis. The appearance and coverage of the cilia was similar across the groups (Figure 32). Further, scoring results indicated that there was no difference across the groups in regard to cilia coverage (Figure 33).

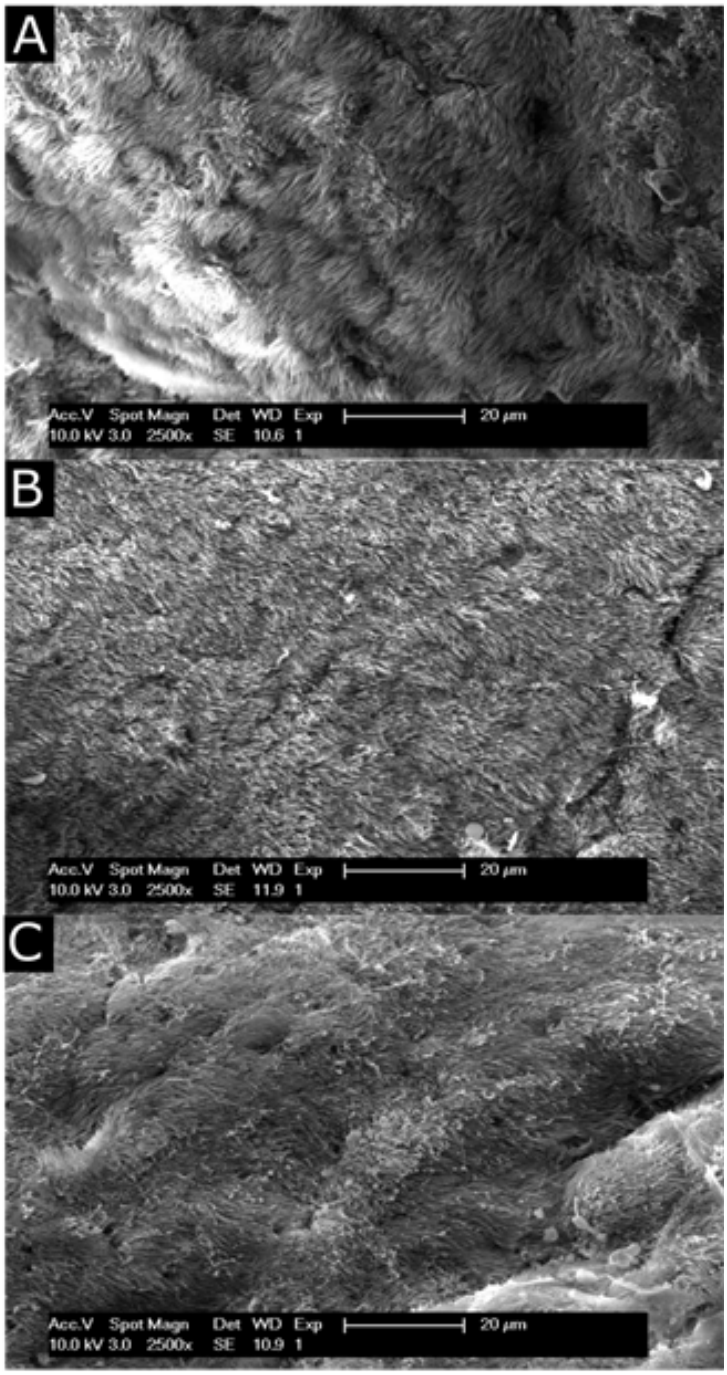

Figure 32. Representative images of sheep nasal mucosa viewed using scanning electron microscopy. Cilia coverage and morphology were similar between all treatment groups including (A) control, (B) heat-inactivated NOV012 (Hi*p*) treatment and (C) active NOV012 treatment.

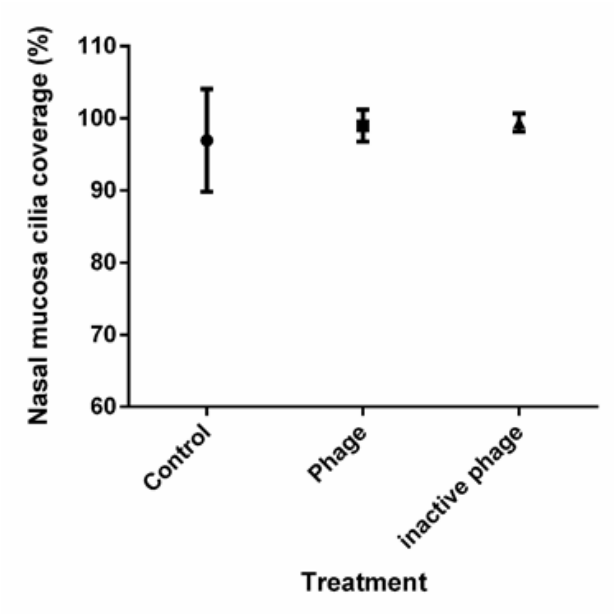

Figure 33. Percentage of nasal mucosal area covered in cilia observed using scanning electron microscopy. Five images of each tissue section were captured at 2500x magnification, divided into 2cm^2 grid sections and each grid scored according to 1=full cilia coverage, 0.5=some cilia coverage, 0=no cilia present. This graph shows the average percentage coverage across the three treatment groups, no statistical difference was observed between the groups.

Phage Detection in Sheep Serum

Using plaque assays, no infectious phage were detected in serum of the CONT, Hip, or NOV012-treated sheep taken at any timepoint during the experiment.

5.5 Discussion

Phage therapy has almost a 100 year history of human application for treating bacterial infections[398]. Accompanying this long history is an outstanding record of safety. There are numerous reports of the safety of phage therapy in animal[412-416] and in human trials[417-422]. Our previous work showed that short term bacteriophage application is safe when applied topically to the sheep sinonasal region[252]. This study aimed to extend this work by assessing longer-term (20 days) sinonasal phage application. This safety data is needed to support the further preclinical development of phage therapy in general and NOVO12 specifically as it is likely that phage therapy would be used in patients for an extended period of time, at least 2 weeks.

It is recognized that phage may interact with some aspects of the host immune system, and it is important to ensure they do not elicit adverse immune responses[423]. Supporting our previous work[252], this study shows that 20 days of topical phage therapy did not modify or damage the architecture of the sinus mucosal lining. Phage application for this extended period did not appear to increase or alter the profile of immune cells in the sinus mucosa. A limitation of this study is that only the presence of the cells was examined, whereas stimulation of the cells in relation to immune effector molecules such as cytokines was not investigated. Previous work has investigated this parameter, applying phage T4 or purified phage T4 proteins to mice and humans[424]. It was shown that such products did not stimulate the production of inflammatory related cytokines and reactive oxygen species[424]. In contrast, it has been suggested that through complex bacterial-phagehost interaction, phage can reduce ROS production[425, 426]. Recent

research has also investigated the effect on phage infectivity when exposed to cell lines that mounted an inflammatory response to the phage.

This study found that the phage still retained infectivity against bacterial cells[427]. It is not known whether phage can traverse the sinonasal mucosal barrier to gain access to the bloodstream. During the 20 days of treatment, no phage were detected in the bloodstream.

A limitation of this study, which relied on plaque assays, is that it could not determine whether inactivated phage or phage genomes entered the bloodstream. A further limitation is that this study did not address the question of whether the sheep developed antibodies against phage. It has been shown that neutralizing antibodies may be produced following phage treatment[428], and our future work will examine this possibility. This is of interest because it is not yet confirmed whether development of anti-phage antibodies will have a negative impact on phage therapy[245]. Recent research however has shown positive results, showing that development of antibodies may not necessarily strongly impact the clinical success of phage therapy[429, 430].

In addition to examining the safety profile of phage cocktail NOV012, this study also investigated the NOV012 host range in the context of CRS S. aureus infections. The findings of this study build on our previous work[252] that suggests using a cocktail of phage assisted in overcoming issues of matching phage to bacteria. We found that phage K710 was effective against 59% of the S. aureus strains in our panel of isolates, which was increased to 85% with addition of phage P68.

Using cocktails rather than a single phage has the additional benefit of reducing the rate of generation of bacteria resistant to phage infection[431]. This work has implications beyond the treatment of CRS. For example, nasal colonization with *S. aureus* increases the risk of surgical wound infection[432]. S. aureus decolonization reduces such risks[433, 434], and phage have potential to achieve this. Other potential applications include topical treatment of burn wounds[435] and treatment of indwelling catheter infections[436]. Importantly, this therapy can be broadened to target other bacterial pathogens.

5.6 Conclusion

This work confirms that the NOV012 phage cocktail infects a broad range of *S. aureus* isolates, including a number of MRSA isolates, from CRS patients. Further, we find that longer term (20 day) topical application of the cocktail is safe for sheep sinonasal application. This safety data supports the potential for the use of phage as a topical antimicrobial treatment in CRS and will help build the profile of the product to lead to the ability to use the product in clinical trials and eventually commercially.

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Chapter 6: Safety and efficacy of a bacteriophage cocktail in an in vivo model of pseudomonas aeruginosa sinusitis

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Overall percentage (%)	75%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 6/9/19

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

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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6.1 Abstract

Background: *Pseudomonas aeruginosa* (PA) is a bacterial pathogen that frequently displays antibiotic resistance. Its presence within the sinuses of chronic rhinosinusitis sufferers is associated with poorer quality of life. Lytic bacteriophages (phage) are viruses that infect, replicate within, and lyse bacteria, causing bacterial death.

Aim: To assess the safety and efficacy of a PA phage cocktail (CT-PA) in a sheep model of rhinosinusitis.

Methods: The sheep rhinosinusitis model was adapted to simulate PA infection in sheep frontal sinuses. To assess efficacy, after a 7-day biofilm formation period, sheep received twice-daily frontal trephine flushes of CT-PA or saline for one week. Biofilm quantitation on the frontal sinus mucosa was performed using LIVE/DEAD BacLight staining. To assess safety, sheep received twice-daily frontal trephine flushes of CT-PA or vehicle control for 3 weeks. Blood and faecal samples were collected throughout treatment. Histopathology of frontal sinus, lung, heart, liver, spleen, and kidney tissue was performed. Sinus cilia were visualised using scanning electron microscopy (SEM). Results: The Efficacy arm showed statistically significant reduction in biofilm biomass with all concentrations of CT-PA tested ($p<0.05$). Phage titration of sinuses showed phage presence was maintained for at least 16 hours after the final flush. All Safety arm sheep completed 3 weeks of treatment. Phage was detected consistently in faeces and sporadically in blood and organ samples. Histology and SEM of tissues revealed no treatment-related damage.

Conclusion: CT-PA was able to decrease frontal sinus PA biofilm at concentrations of 10^8 to 10^{10} PFU/mL. No safety concerns were noted.

6.2 Introduction

Chronic rhinosinusitis (CRS) is an inflammatory condition of the nose and paranasal sinuses, persisting for 12 weeks or longer[437]. The prevalence of CRS amongst the general population ranges from 8% to 12% between different continents, and it carries a significant economic burden[438-442]. From a public health perspective, CRS also accounts for a significant burden of antibiotic use, with antibiotics being prescribed during 69% of 47.9 million outpatient visits for CRS, over a 5-year period in the United States[443].

One of the foremost concerns regarding the overuse of antibiotics is the emergence of antimicrobial resistance in bacteria[444]. Since the discovery of the antibacterial properties of penicillin in the 1940s, the introduction of new antibiotics to widespread clinical use has been followed by the appearance of bacteria that are resistant to those antibiotics[445]. The bacterial species commonly found in the sinuses of CRS sufferers are no exception. Antibiotic resistance has been noted in up to 46% of isolates from the sinuses of CRS patients[446].

In addition to antibiotic resistance, the formation of bacterial biofilms on the sinonasal mucosa of CRS patients plays a role in disease recalcitrance[92, 447, 448]. Bacterial biofilms consist of a community of bacteria living within a self-produced extracellular polymeric matrix, and attached to a biotic or abiotic surface[449]. Biofilm formation confers increased tolerance to antibiotics through various mechanisms, including impeded antibiotic penetration through the biofilm matrix, expression of resistance genes, accumulation of antibiotic degrading enzymes, and changes in cellular metabolism[450].

Pseudomonas aeruginosa is a biofilm-forming gram-negative bacteria that displays significant rates of both intrinsic and acquired antibiotic resistance [451, 452]. It is present in the sinuses of 9% of patients with CRS, and up to 49% of patients with both cystic fibrosis (CF) and CRS[453, 454].

Bacteriophages (phage) have been proposed as a novel treatment for bacterial infections, especially those caused by multidrug resistant bacteria[455, 456]. Lytic phage are bacterial viruses that infect, replicate within, and then lyse bacteria to release copies of the phage, resulting in bacterial death[457]. Phage offer several advantages over conventional antibiotics in targeting bacterial infection in CRS. They are able to penetrate bacterial biofilms, have activity against multidrug resistant isolates, and are species specific, preventing deleterious effects on commensal bacterial flora[254, 458, 459]. We have recently reported on the *in vitro* activity of an anti-*P. aeruginosa* phage cocktail (CT-PA) against *P. aeruginosa* CRS clinical isolates, including several multidrug resistant isolates[254].

We have previously developed the sheep sinusitis model, which simulates *S. aureus* bacterial biofilm infection in the frontal sinuses[460]. We adapted this model to simulate *P. aeruginosa* biofilm infection in CRS. The aims of this study were to assess the *in vivo* efficacy and safety of the anti-*P. aeruginosa* phage cocktail using this animal model, as well as to assess the local and systemic distribution of the cocktail when applied topically to the paranasal sinuses.

6.3 Methods

This study was approved by the Animal Ethics Committees of the University of Adelaide and the South Australian Health and Medical Research Institute, Adelaide, South Australia. The use of previously collected *P. aeruginosa* clinical isolates in this study was approved the Human Research Ethics Committee of The Queen Elizabeth Hospital, Adelaide, South Australia.

Bacterial strains and growth conditions

A clinical CRS *P. aeruginosa* strain was isolated from an endoscopically-guided sinus swab and MLST sequence typed, as previously described[254]. The chosen isolate was MLST sequence type 1399, and displays significant biofilm formation as well as sensitivity to the phage cocktail *in vitro*[254]. The isolate was stored in 25% glycerol in nutrient broth at -80°C. Isolates were plated from frozen glycerol stocks onto 1.5% nutrient agar, and broth cultures were grown in nutrient broth. Agar plates and broth cultures were incubated at 37°C.

Bacteriophage cocktail

1 mL aliquots of a bacteriophage cocktail (CT-PA) containing equal concentrations of each of 4 *P. aeruginosa* bacteriophages (Pa 193, Pa 204, Pa 222, Pa 223) in phosphate buffered saline supplemented with magnesium (PBS+Mg) were supplied by AmpliPhi Biosciences (Brookvale, New South Wales, Australia). Pa 193 and Pa 204 are of the Myoviridae family, and Pa 222 and Pa 223 are of the Podoviridae family. All 4 phage have been characterised as strictly lytic by genome sequencing (unpublished data).

Adaptation of the Sheep Rhinosinusitis model

The sheep rhinosinusitis model as described by Ha et al[460] was adapted for use in this study.

A total of 32 Merino cross wethers sheep were used. 12 sheep were used in the model Optimisation arm, 14 sheep in the treatment Efficacy arm, and 6 sheep in the treatment Safety arm. Power calculations based on the results of a similar study by Drilling et al[461] indicated that 5 sinuses per group in the Efficacy arm would be sufficient to detect the estimated minimum effect size, at an alpha-level of 0.05 with 80% statistical power. Two sheep in the Efficacy arm did not complete the study protocol, necessitating the use of 2 additional sheep. One sheep died from aspiration pneumonitis secondary to regurgitation during induction of anaesthesia, and another sheep had aberrant sinus anatomy that prevented effective occlusion of the frontal sinus ostia by nasal packing.

Optimisation arm protocol

The aims of the Optimisation arm were to assess the amount of *P. aeruginosa* bacterial culture required to achieve consistent biofilm formation in the frontal sinuses, and to assess any negative impact on animal welfare in the adapted model. 3 groups of 4 sheep each were used. Each group consisted of 2 sheep with *P. aeruginosa* culture inoculated into the frontal sinuses bilaterally, and 2 contemporaneous control sheep that had only sterile 0.9% saline inoculated into their frontal sinuses. Infection sheep in the 1st, 2nd and 3rd groups were inoculated with 1 mL x 10^7 CFU/mL, 1 mL x 10^8 CFU/mL, and 2 mL x 10^8 CFU/mL respectively into each frontal sinus. Control sheep received equivalent volumes of sterile saline. Animal welfare was monitored a minimum of twice daily through assessment for signs of pain, anorexia, dehydration, respiratory distress, and systemic infection. After a 7 day biofilm formation period, sheep were

humanely killed, and the frontal sinuses harvested for biofilm quantification and fluorescence in situ hybridisation (FISH) biofilm detection.

Efficacy arm protocol

The treatment Efficacy arm protocol was adapted from the protocol previously described by Drilling et al[461]. Following the insertion of pre-sterilised frontal trephines and nasal packing, a gentle lavage of 2 mL sterile saline into the frontal sinuses was performed, and microbiology swabs (Σ-Transwab Amies, Medical Wire & Equipment, Corsham, England) of the lavage fluid were sent to a clinical laboratory (Clinpath Laboratories, Adelaide, South Australia) in order to assess the commensal flora of the sheep's sinuses. Each frontal sinus was then inoculated with 2 mL of 10^8 CFU/mL *P. aeruginosa* culture, based on the results of the Optimisation arm. Each treatment group consisted of 3 sheep, who each received a twice daily flush of treatment agent diluted into 50 mL sterile 0.9% saline, into each frontal trephine. Sheep received the same treatment to their left and right frontal sinuses. The four different treatments were 1 mL of: 1) 0.9% saline, 2) 10^8 PFU/mL CT-PA, 3) 10^9 PFU/mL CT-PA, and 4) 10^{10} PFU/mL CT-PA. Following 7 days' treatment, sheep were humanely killed and frontal sinuses harvested for biofilm quantification, histopathology, and phage enumeration. Microbiology swabs were also taken from the inside of each harvested sinus, and sent to the clinical laboratory for culture as described above.

Faecal samples were collected from all phage-treated sheep prior to the administration of the first flush, and on Day 7 of treatment. 9mL blood samples were collected into Vacuette lithium heparin tubes without gel (Greiner Bio-One, Kremsmünster, Austria) from sheep via a jugular vein catheter at 1, 2 and 4 hours following the first trephine

flush, and again at 1, 2, 4 and 8 hours following the morning trephine flush on Day 7 of treatment. Phage enumeration was carried out on faecal and blood samples as described below.

Safety arm protocol

The treatment Safety arm protocol was adapted from the protocol described by Drilling et al[461]. Two treatment groups of 3 sheep each were used. Sheep had twice-daily flushes of treatment agent diluted in 50mL sterile 0.9% saline into each frontal trephine, for 21 days. The two treatments were 1 mL 10^{10} PFU/mL CT-PA and 1mL PBS+Mg (vehicle control). Following the treatment period, sheep were humanely killed, and frontal sinuses as well as brain, heart, lungs, liver, spleen and kidneys were harvested for histopathology and phage enumeration. Sinuses were also assessed for ciliary integrity, as described below.

Faecal samples were collected from sheep on Days 8 and 15 of treatment, and following humane killing. Blood samples were collected via jugular vein catheters into Vacuette lithium heparin tubes without gel from sheep on Days 1, 8, and 15 of treatment, and just prior to humane killing. Phage enumeration was carried out on faecal and blood samples as described below.

<u>Biofilm quantification on sinus mucosa</u>

A previously defined protocol was used to quantify biofilm present on the frontal sinus mucosa[462, 463]. Frontal sinuses were placed into Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies, Carlsbad CA, USA), and transported to the laboratory on ice. Under the laminar flow hood, two 1 cm^2 pieces of mucosa were dissected at random

from each frontal sinus, and stained with LIVE/DEAD BacLight Bacterial Viability Kit (Life Technologies Australia, Mulgrave VIC, Australia). The entire piece of mucosa was then examined at 20x magnification using a confocal microscope (Zeiss LSM700, Carl Zeiss AG, Oberkochen, Germany). Z-stack images (80 slices, interval 0.80) were taken of the three largest biofilms from each piece of mucosa. COMSTAT version 2.1 was used to measure the biomass of biofilms, with threshold set manually to minimise background staining[160, 341, 464].

Fluorescence in situ hybridisation (FISH) biofilm detection

A 1 cm^2 piece of mucosa from each sinus in the Optimisation arm was collected after humane killing, and transported to the laboratory in DMEM, on ice. Samples were stored at -80°C prior to FISH analysis (protocol previously validated by Foreman et al[465]). Samples were defrosted and processed following the protocol described by Foreman et al[465], using an *E. coli*/*P. aeruginosa* PNA FISH probe (AdvanDx, Woburn MA, USA) that specifically detects *P. aeruginosa* and *E. coli* with Texas Red and fluorescein-labelled probes respectively. Following the FISH protocol, epithelial cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, Sydney, Australia). The mucosa was then examined at 20x, 40x, and 80x magnification using a confocal microscope (Zeiss LSM700, Carl Zeiss AG, Oberkochen, Germany) to identify *P. aeruginosa* biofilms.

Histopathology

A 1 cm^2 piece of sinus mucosa was collected from each frontal sinus after humane killing, and placed directly into 10% neutral buffered formalin (Sigma-Aldrich). For all samples from Efficacy group sheep, following 72 hours fixation, samples were placed

into 70% ethanol and processed using an Excelsior AS tissue processor (Thermo Scientific), and embedded in paraffin using the HistoStar Embedding Workstation (Thermo Scientific). Samples from Safety groups were processed and embedded by RDDT VivoPharm (Bundoora, VIC, Australia). Haematoxylin and eosin slides were prepared and examined by RDDT VivoPharm. Based on a previously published protocol, the parameters in Table 10 were graded by a veterinary pathologist (J.F.) who was blinded to the treatment groups[466].

Degree of inflammation • Grade 0 - no inflammation • Grade 1 - mild: <30% of superficial half of stroma occupied by inflammatory cells at a density of 50+ cells/high powered field • Grade 2 - moderate: 30% - 60% of superficial half of stroma occupied by inflammatory cells at a density of 50+ cells/high powered field • Grade 3 - severe: >60% of superficial half of stroma occupied by inflammatory cells at a density of 50+ cells/high powered field
Percentage of acute inflammatory cells (neutrophils)
Epithelial hyperplasia • Grade 0: absent / minimal • Grade 1: mild • Grade 2: mild-moderate • Grade 3: moderate • Grade 4: moderate-severe • Grade 5: severe
Fibrosis • Grade 0: no fibrosis • Grade 1: mild fibrosis • Grade 2: moderate fibrosis • Grade 3: severe fibrosis

Table 10. Histopathology grading of sinus mucosa.

Phage sensitivity testing of post-treatment isolates

P. aeruginosa isolates cultured from the sinuses of phage-treated sheep in the Efficacy arm post-treatment were tested for sensitivity to CT-PA using the spot test assay described by Mazzocco et al[467].

Assessment of sinus ciliary integrity

Assessment of mucosal ciliary integrity was performed using scanning electron microscopy, as described by Ha et al[460]. Following humane killing sheep in the Safety groups, a 1 cm^2 piece of mucosa was dissected from each frontal sinus and placed directly into sterile PBS. Samples were transported to the laboratory on ice, and sonicated (Soniclean 80T, Soniclean, Adelaide, Australia) in an ice bath for 30 minutes to remove the adherent mucus layer. Samples were then transferred into 1 mL of electron microscopy fixative (4% paraformaldehyde/1.25% glutaraldehyde in PBS with 4% sucrose). After 72 hours, samples were washed in buffer, post-fixed in osmium tetroxide, and dehydrated by placing in sequentially increasing concentrations of ethanol (70%, 90%, and 100%), and then hexamethyldisilazine. Samples were then mounted onto stubs, coated with platinum, and viewed using a Philips XL30 field emission scanning electron microscope (Philips, Amsterdam, Netherlands).

Haematology and blood biochemistry

Blood samples were collected from Safety sheep via a central venous catheter inserted into the jugular vein, prior to the first phage flush (Day 1) and prior to humane killing (Day 22). Vacuette K3EDTA and serum separator clot activator tubes (Greiner Bio-One, Kremsmünster, Austria) were used to collect blood for haematology and serum

biochemistry panels respectively. Testing was performed by a clinical laboratory (SA Pathology, Adelaide, South Australia).

Phage enumeration in tissues and faeces

Following humane killing and harvesting of frontal sinus mucosa, a 1 cm^2 piece of mucosa from each sinus was dissected aseptically and placed directly into 5 mL sterile phosphate buffered saline (PBS). 1 cm^3 organ samples were collected from the brain, lungs, heart, liver, kidney, and spleen of all Safety arm sheep, as well as 4 Efficacy arm sheep (# 19, 21, 22 and 23). All samples were transported to the laboratory in separate 5mL aliquots of sterile PBS, on ice. Phage titre in faecal and organ samples was measured using previously published methodology[461]. 1 g of sheep faeces in 5 mL sterile PBS spiked with 20 µL of 10^8 PFU/mL CT-PA was used as a positive control for faecal samples. 5 mL sterile PBS spiked with 20 µL 10^8 PFU/mL CT-PA was used as a positive control for organ and sinus samples. Phage enrichment of samples was performed as described by Drilling et al[461], with the use of the *P. aeruginosa* isolate in place of *S. aureus*.

Phage enumeration in blood

Blood tubes were transported to the laboratory on ice, and centrifuged at 1500 rpm (Eppendorf 5810R centrifuge, Eppendorf AG, Hamburg, Germany) for 20 minutes at 4°C. 3 mL of sheep blood spiked with 200 µL of 10^8 PFU/mL CT-PA was used as a positive control. The plasma was then filtered through a 0.22 µm syringe filter. The small drop plaque assay was used to titrate the filtered plasma for phage. Sample phage enrichment was performed as described above.

Statistics

Biofilm biomass data were analysed using a linear effects model with piece nested in side and sheep. A log transformation was applied to the data prior to analysis due to violations of the distributional assumptions of a linear regression model (normally distributed with constant variance). The data were transformed back to the original scale prior to reporting. As a result, post hoc comparisons represent the ratio of two geometric means. All analyses were completed using SAS v9.4 (SAS Institute Inc., Cary, NC, USA).

Kruskal-Wallis H test (SPSS version 24, IBM, Armonk NY, USA) was performed to compare histopathological parameters between groups.

6.4 Results

Optimisation arm

Sheep across all groups displayed mild to moderate appetite loss in the first 24 to 48 hours following insertion of trephines and nasal packing, which resolved after 48 hours. One Control sheep ("inoculated" with saline only) was excluded from analysis due to a large amount of purulent discharge that developed around its frontal trephines, with swabs growing coliforms. No other adverse events occurred.

Macroscopic examination of the frontal sinus mucosa revealed inflamed mucosa in all sinuses inoculated with *P. aeruginosa*, with frank purulent secretions within sinuses inoculated with 1mL or 2mL x 10^8 CFU/mL. Only 2 Control sinuses from one sheep displayed mucosal erythema, of which one sinus contained frank pus, which swabbed positive for coliforms. The remainder of Control sinuses had macroscopically normal mucosa.

BacLight staining of infected sinuses showed the presence of biofilm structures consisting of green-fluorescing rod-shaped organisms (Figure 34). FISH biofilm detection using the *E. coli*/*P. aeruginosa* probe confirmed the presence of biofilm structures comprised of red-fluorescing rods, consistent with *P. aeruginosa* biofilms (Figure 35). Quantification of the biomass of BacLight-stained live biofilm structures by COMSTAT analysis of z-stack images showed that sinuses inoculated with *P. aeruginosa* grew significantly more biofilm compared to Control sinuses (mixed model analysis, p = 0.001, 0.02, and 0.004 for 1 mL x 10^7, 1 mL x 10^8, and 2 mL x 10^8 CFU/mL

respectively). There were no significant differences in biomass between the different amounts of *P. aeruginosa* inoculated. However, the sinuses inoculated with 2mL x 10^8 CFU/mL *P. aeruginosa* had biofilms distributed more evenly within the pieces of mucosa examined. Figure 36 shows the biomass of BacLight-stained biofilms in the different groups.

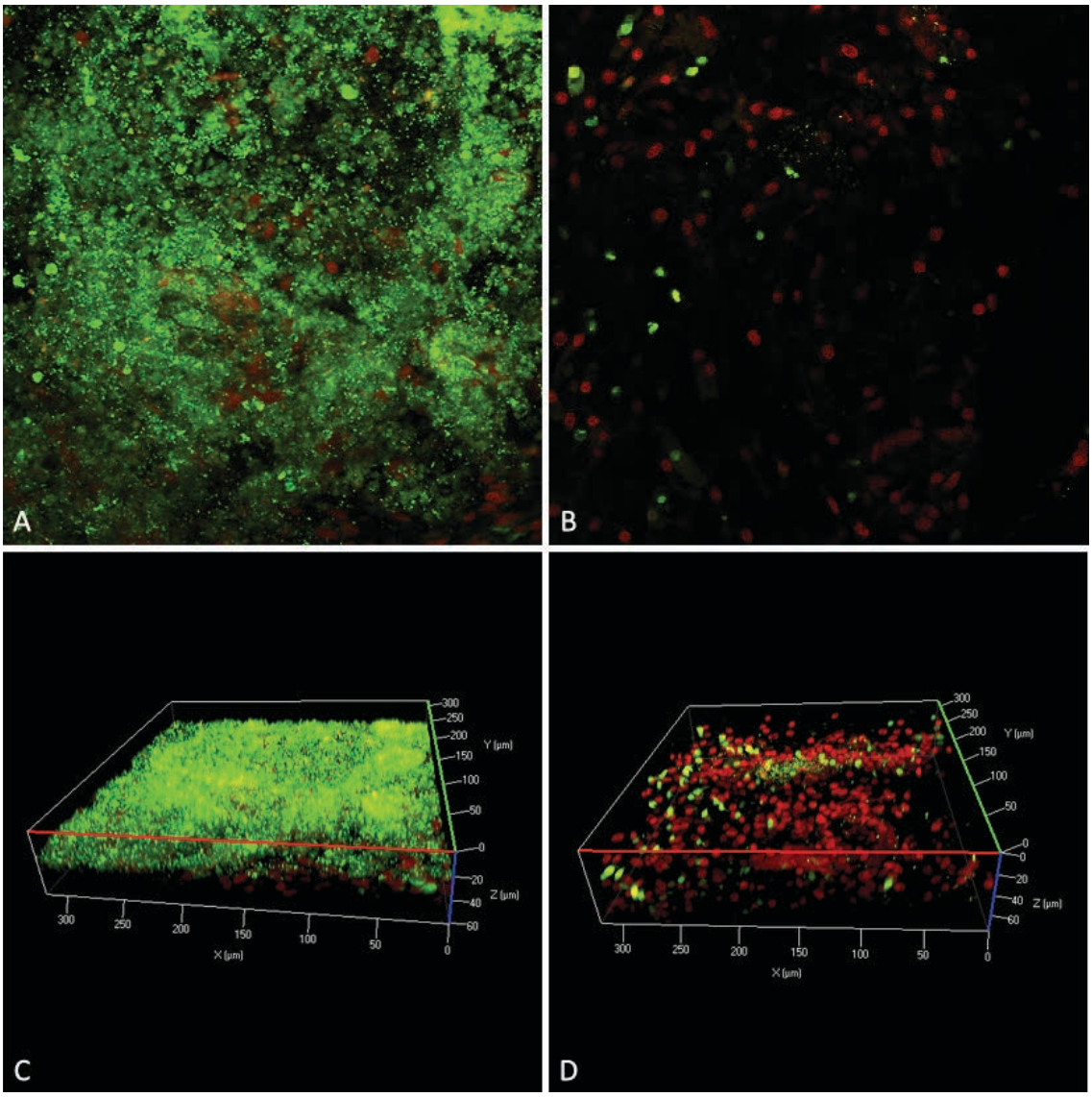


Figure 34. 2D cross-sectional images (A & B) and 3D reconstructions of z-stack images (C & D) (taken at 20x magnification) of BacLight-stained biofilm in a sinus inoculated with 2 mL x 10^8 CFU/mL P. aeruginosa (A & C), and in a matched control sinus inoculated with 2 mL sterile 0.9% saline (B & D).

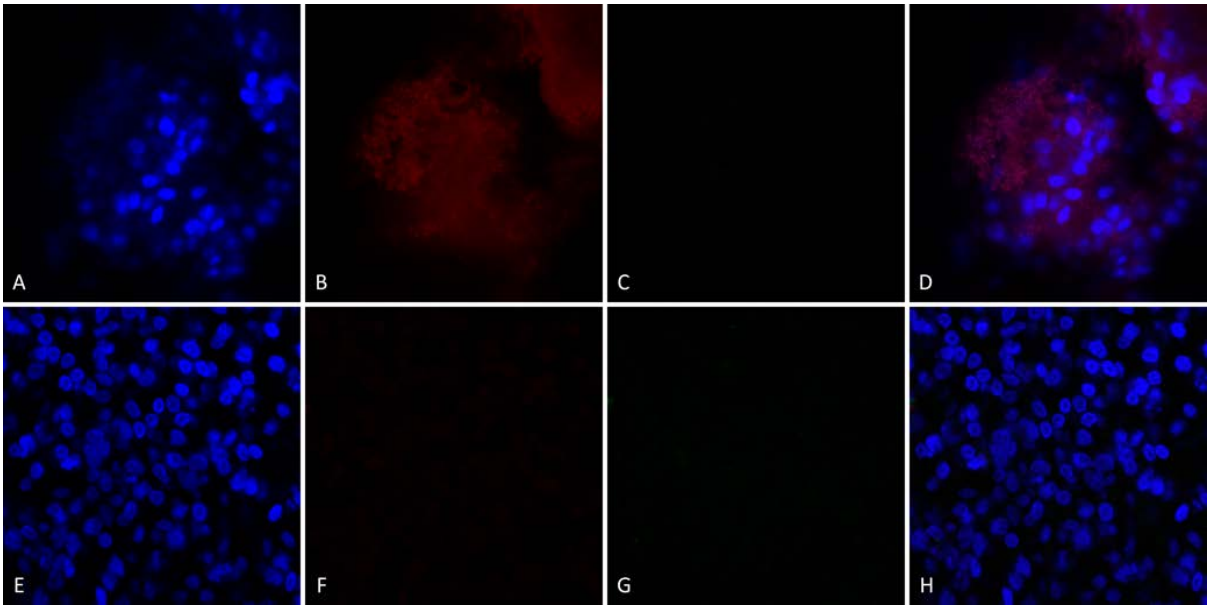


Figure 35. Split channel images showing: epithelial cell nuclei counterstained blue with DAPI (A & E), Texas Red-tagged PNA FISH probe for P. aeruginosa (B & F), fluorescein-tagged PNA FISH probe for E. coli (C & G), and merged images (D & H) from a sinus inoculated with 2 mL x 10^8 CFU/mL P. aeruginosa (A, B, C & D) and a matched control sinus inoculated with 2 mL sterile 0.9% saline (E, F, G & H).

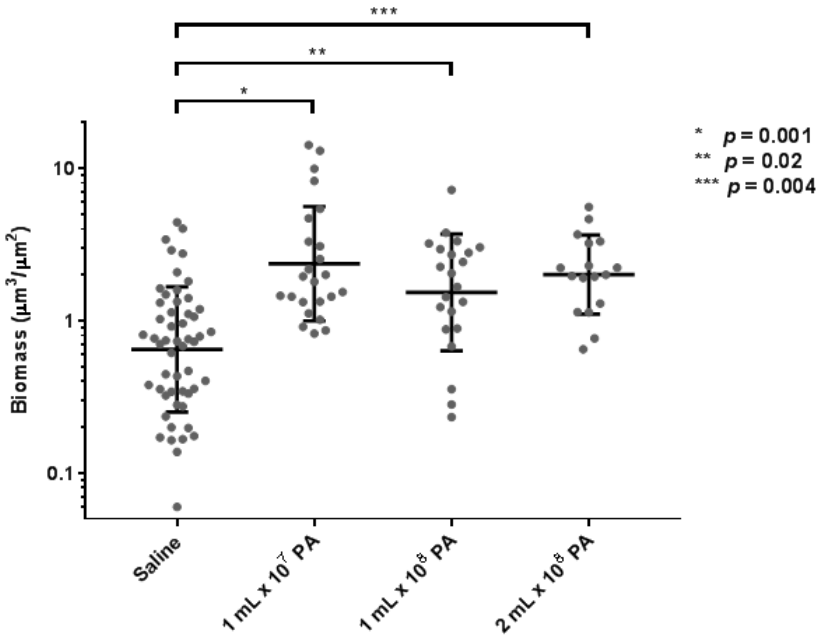


Figure 36. Biomass of sinus biofilms by dose of P. aeruginosa inoculated into frontal sinus. The complete area of two randomly selected 1 cm^2 pieces of sinus mucosa from each frontal sinus was assessed for biofilms. Each data point represents the biomass of each of the three largest biofilms (as measured by Comstat quantification of biofilm biomass) from each piece of sinus mucosa. Log-transformed biofilm

biomass data were analysed using a mixed effects model with piece nested in side and sheep. Error bars represent geometric mean ± geometric standard deviation. PA: Pseudomonas aeruginosa

Efficacy arm

Pre-inoculation sinus microbiology

The swab of pre-inoculation frontal sinus lavage was obtained bilaterally from all sheep in the 10^8 and 10^{10} PFU/mL CT-PA and Saline treatment groups, and in 5 out of 6 sinuses in the 10^9 PFU/mL CT-PA treatment group (23 sinus cavities in total). Overall, 15 of 23 sinuses had growth on the pre-inoculation swab; of these, 9 sinuses grew *P. aeruginosa*. Four swabs grew *Pseudomonas fluorescens*, 1 grew coliforms, and 1 grew skin flora. The outcome of pre-inoculation swabs is shown in Table 11.

Pre-inoculation swab growth	**Treatment group**				**Total**
	Saline	**10^8 CT-PA**	**10^9 CT-PA**	**10^{10} CT-PA**	
P. aeruginosa	1	1	3	4	9
P. fluorescens	2	2	0	0	4
Coliforms	0	0	0	1	1
Skin flora	0	1	0	0	1
No growth	3	2	2	1	8
Total no. of swabs taken	6	6	5	6	23

Table 11. Pre-innoculation sinus microbiology randomized to different treatment groups.

Biofilm biomass

Mean biomass was significantly lower in the three CT-PA treatment groups when compared to saline (10^{10} PFU/mL: $p = 0.0007$; 10^9 PFU/mL: $p = 0.049$; 10^8 PFU/mL: $p = 0.003$). The three treatment groups did not differ significantly from each other. The biomass of frontal sinus biofilms following treatment for each group is displayed in Figure 37.

Regarding the effect of commensal sinus bacteria cultured from pre-inoculation lavage on post-treatment biofilm biomass, type III tests did not show a significant interaction between the presence of commensal *P. aeruginosa* prior to inoculation and treatment effect (p = 0.195). However, a significant interaction was present between the presence of non-*P. aeruginosa* bacteria prior to inoculation and treatment effect (p = 0.004). Post hoc comparisons demonstrate significant reductions in biofilm with all CT-PA treatments compared to saline when bacterial species other than *P. aeruginosa* were absent, but a lack of significant reduction when they were present (Table 12).

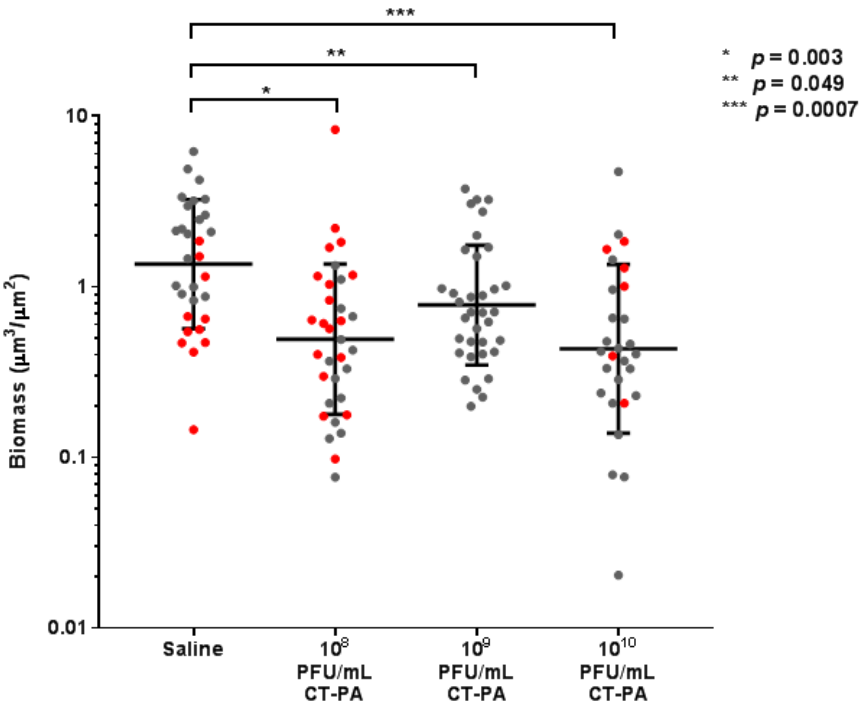


Figure 37. Efficacy arm: biomass of frontal sinus biofilms following treatment. The complete area of two randomly located 1 cm^2 pieces of sinus mucosa from each frontal sinus was assessed for bacterial biofilms using LIVE/DEAD BacLight staining. Each data point represents the biomass of each of the three largest biofilms (as measured by COMSTAT quantification of biofilm biomass) from each piece of sinus mucosa. Red data points represent data from sinuses that contained non-Pseudomonas aeruginosa bacterial species prior to inoculation with P. aeruginosa, whilst grey data points are from sinuses that did not contain non-P. aeruginosa bacterial species prior to inoculation. Log-transformed biofilm biomass data from all sinuses were analysed using a linear effects model with piece nested in side and sheep. Error bars represent geometric mean ± geometric SD.

Other bacterial species present pre- inoculation	Comparison between groups		Ratio of geometric means (95% CI)	p value
No	10^8 CT-PA	Saline	0.15 (0.07-0.35)	**<0.0001**
	10^9 CT-PA	Saline	0.36 (0.18-0.73)	**0.006**
	10^{10} CT-PA	Saline	0.17 (0.08-0.35)	**<0.0001**
	10^{10} CT-PA	10^8 CT-PA	1.08 (0.49-2.38)	0.846
	10^{10} CT-PA	10^9 CT-PA	0.46 (0.24-0.89)	**0.022**
	10^8 CT-PA	10^9 CT-PA	0.42 (0.20-0.90)	**0.027**
Yes*	10^8 CT-PA	Saline	1.11 (0.45-2.75)	0.813
	10^{10} CT-PA	Saline	1.22 (0.36-4.06)	0.744
	10^{10} CT-PA	10^8 CT-PA	1.09 (0.36-3.36)	0.873

Table 12. Post hoc comparisons between groups with and without bacterial species other than *P. aeruginosa* present prior to inoculation.

* The 10^9 CT-PA treatment group did not have any bacterial species other than *P. aeruginosa* grown on swabs of pre-inoculation lavage samples, thus no comparisons with this group could be performed for this outcome.

Biomass of frontal sinus biofilms following treatment

The complete area of two randomly located 1 cm^2 pieces of sinus mucosa from each frontal sinus was assessed for bacterial biofilms using LIVE/DEAD BacLight staining. Each data point represents the biomass of each of the three largest biofilms (as measured by Comstat quantification of biofilm biomass) from each piece of sinus mucosa. Red data points represent data from sinuses that contained non-Pseudomonas aeruginosa bacterial species prior to inoculation with P. aeruginosa, whilst grey data points are from sinuses that did not contain non-P. aeruginosa bacterial species prior to inoculation. Log-transformed biofilm biomass data from all sinuses were analysed using a linear effects model with piece nested in side and sheep. Error bars represent geometric mean ± geometric SD.

Post-treatment sinus microbiology

Post-treatment sinus swabs showed growth of *P. aeruginosa* in 4, 5, and 2 out of 6 sinuses in the 10^8, 10^9, and 10^{10} PFU/mL CT-PA treatment groups respectively, compared to 5 out of 6 sinuses treated with saline only. The outcome of post-treatment swabs by treatment groups is shown in Table 13.

Post-treatment swab growth	**Treatment group**			
	Saline	**10^8 CT-PA**	**10^9 CT-PA**	**10^{10} CT-PA**
P. aeruginosa	5	4	5	2*
P. fluorescens	0	0	0	0
Coliforms	0	2	0	4*
Skin flora	0	0	1	0
No growth	1	0	0	1
Total no. of swabs taken	6	6	6	6*

Table 13. Results of post-treatment sinus microbiology.

* One swab grew both *P. aeruginosa* as well as coliforms. All post-treatment *P. aeruginosa* isolates from CT-PA-treated sheep were sensitive to CT-PA on the spot test assays (results not shown).

Sinus histopathology

There were no statistically significant differences in degree of inflammation, fibrosis, epithelial hyperplasia, or percentage of acute inflammatory cells between treatment groups. Degree of inflammation ranged from mild to severe in all groups (Figure 38), whilst the inflammatory infiltrate was predominantly (≥ 95%) lymphoplasmacytic in 18 of 24 (75%) sinuses. Of the sinuses that displayed a more neutrophilic infiltrate, 3 were treated with saline, 2 with 10^8 PFU/mL CT-PA, and 1 with 10^{10} PFU/mL CT-PA (Figure 39). None of the sinuses were found to have fibrosis.

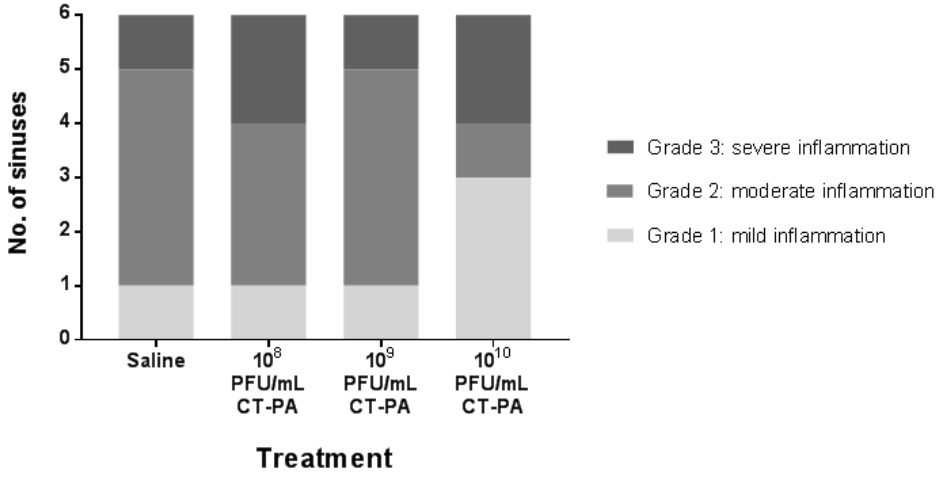


Figure 38. Degree of histopathological sinus inflammation by treatment group.

Grade 1: <30% of superficial half of stroma occupied by inflammatory cells at a density of 50+ cells/high powered field
Grade 2: 30% - 60% of superficial half of stroma occupied by inflammatory cells at a density of 50+ cells/high powered field
Grade 3: >60% of superficial half of stroma occupied by inflammatory cells at a density of 50+ cells/high powered field

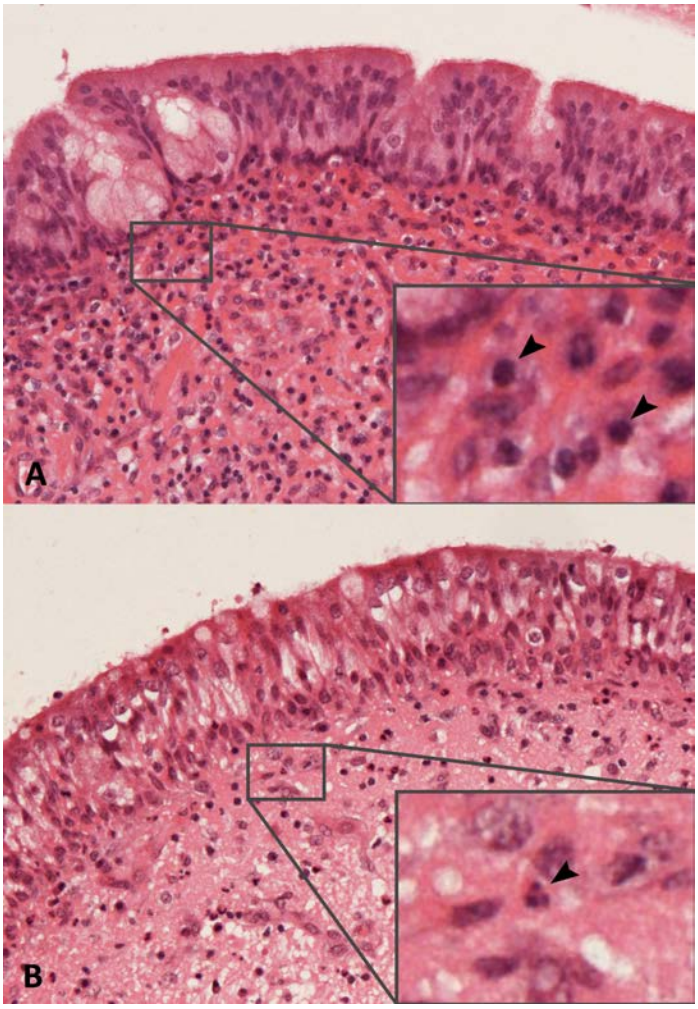


Figure 39. Sinus histology showing (A) predominantly lymphoplasmacytic infiltrate (arrowheads point to lymphocytes) vs. (B) more neutrophilic infiltrate (arrowhead points to neutrophil) in two sinuses treated with saline in the Efficacy arm.

Local and systemic distribution of phage

Sheep were humanely killed between 16 and 18 hours following administration of the last sinus flush. Measurable phage titres were detected in the sinus tissue of 13 out of 18 CT-PA-treated sinuses, with an additional 4 sinuses showing the presence of phage after enrichment. Post-treatment phage titres are shown in Table 14.

Sheep	Side	Treatment (PFU/mL CT-PA)	Sinus mucosa phage titre (PFU/cm^2)
21	R	10^8	4.00E+03
21	L	10^8	1.95E+05
22	R	10^8	1.34E+06
22	L	10^8	*
23	R	10^8	*
23	L	10^8	4.00E+03
13	R	10^9	3.00E+03
13	L	10^9	1.85E+04
14	R	10^9	6.00E+03
14	L	10^9	4.00E+03
15	R	10^9	8.25E+04
15	L	10^9	5.85E+06
10	R	10^{10}	*
10	L	10^{10}	-
11	R	10^{10}	7.63E+04
11	L	10^{10}	7.27E+06
12	R	10^{10}	*
12	L	10^{10}	4.00E+03

Table 14. Sinus phage titres 16 to 18 hours following final flush.

R: right; L: left
- : phage not detected
*: phage detected after enrichment

Phage was detected in blood samples from 1 out of 9 CT-PA-treated sheep on Day 7 of treatment, at all time points (titres of $8.8x10^3$, $1.24x10^4$, $3.8x10^3$, and $3.0x10^3$ PFU/mL at 1, 2, 4, and 8 h post-dose respectively). Phage was detected after sample enrichment in blood samples from 2 sheep on Day 1 of treatment. No sheep had detectable phage in faecal samples taken prior to initiation of sinus flushes, and 6 sheep had phage detected in Day 7 faecal samples.

Safety arm

All Safety arm sheep completed 21 days of treatment. One sheep (#30) was found deceased on the morning of Day 22; pathological findings were consistent with exacerbation of a background interstitial pneumonia by aspiration of the sinus flush (see *Histopathology* findings for further detail). Two sheep experienced minor infection around the trephine sites, without associated loss of appetite, fever, or other signs of systemic illness. No other adverse events occurred.

Haematology and biochemistry

No clinically significant changes in complete blood count, white blood cell differential count, serum biochemistry, liver function tests, cholesterol, calcium, phosphate, albumin, or globulins were noted between blood samples taken prior to the first flush (Day 1) and following the completion of flushes (Day 22).

Histopathology

There was no significant difference in degree of inflammation, fibrosis, or percentage of acute inflammatory cells between the CT-PA and vehicle control-treated groups. The degree of epithelial hyperplasia was significantly lower in the CT-PA group compared to the vehicle control group ($p = 0.043$). Photomicrographs of sinus histology are shown in Figure 40.

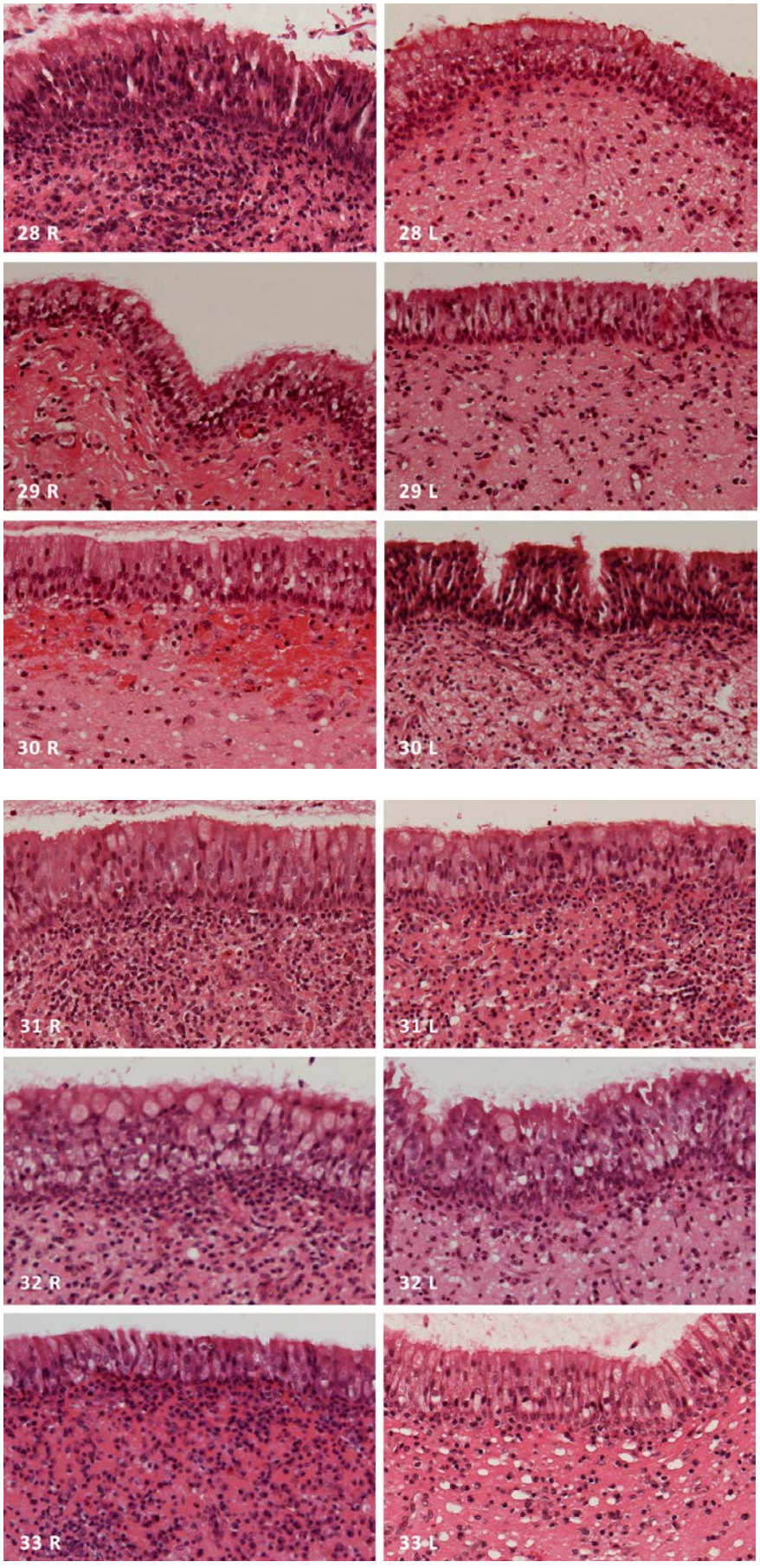

28 R
28 L
29 R
29 L
30 R
30 L
31 R
31 L
32 R
32 L
33 R
33 L

Figure 40. Photomicrographs of haematoxylin and eosin stained sinus mucosa slides (10x magnification) from each sheep in the Safety arm (# 28, 29 & 30: CT-PA-treated sheep; # 31, 32 & 33: vehicle control-treated sheep; R: right frontal sinus; L: left frontal sinus).

No clinically significant histopathological findings were noted in any of the brain, heart, kidney, liver, or spleen samples. All lung samples from both CT-PA and vehicle control-treated sheep were noted to have changes consistent with mild alveolar interstitial pneumonia, likely due to endemic lungworm. Lung samples from sheep #30 additionally showed abundant alveolar macrophages and occasional multinucleated giant cells (Figure 41).

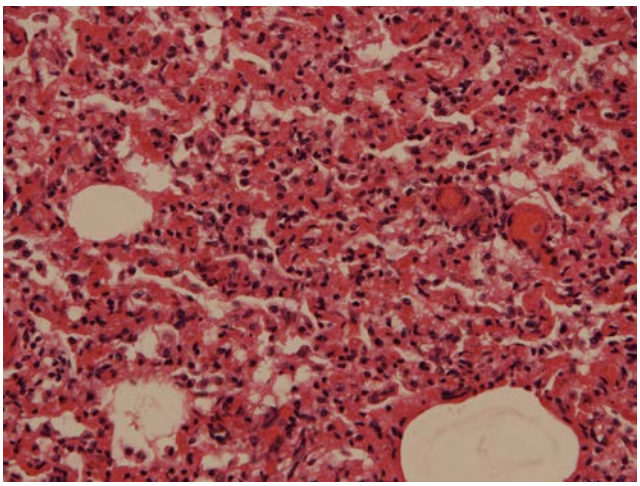
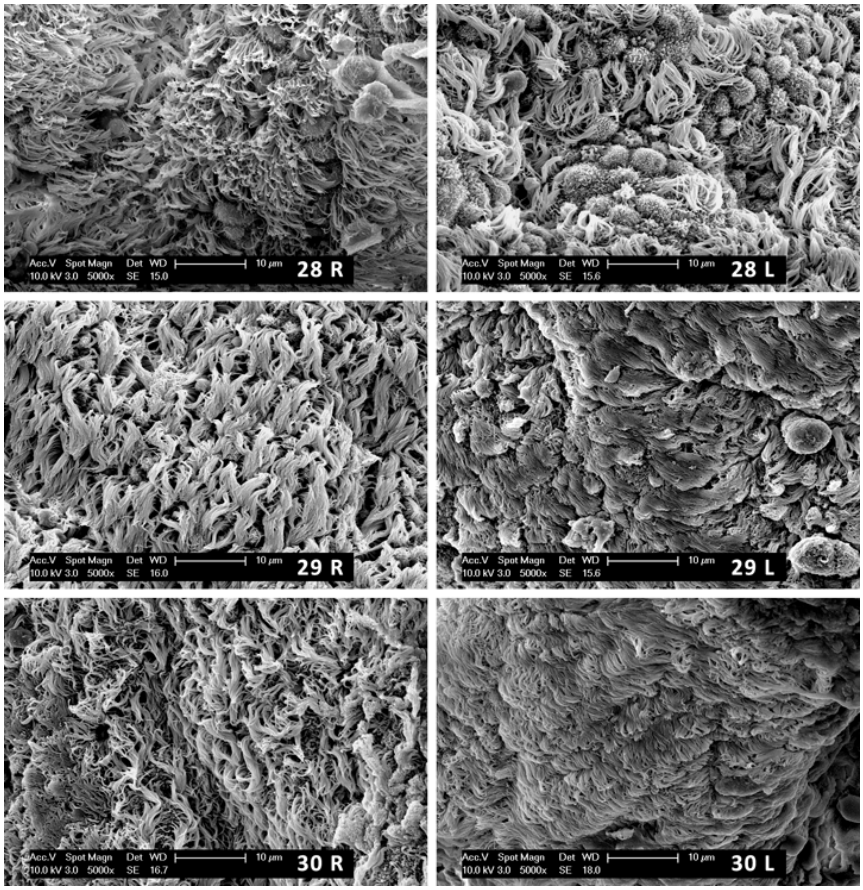


Figure 41. Photomicrograph of haematoxylin and eosin stained lung tissue (20x magnification) from sheep #30.

Sinus ciliary integrity

One sinus treated with the phage cocktail (#28 left) and one sinus treated with the vehicle control (#33 left) showed some cells possessing microvilli, as well as cells with normal cilia. Another sinus treated with vehicle control (#32 left) had an obscuring overlying mucus layer covering the majority of the sample, showing mostly cells with microvilli or short cilia in the areas not covered by the mucus layer. Cilia were

preserved in all other sinuses. Representative images of each sinus are shown in Figure 42.



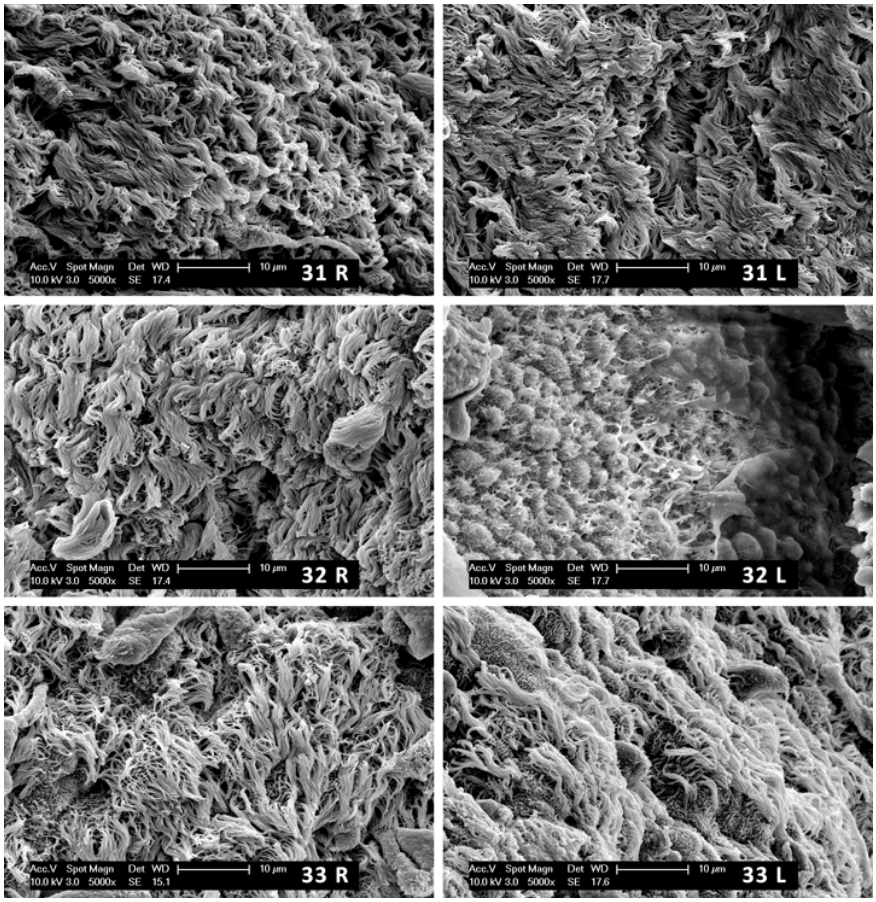


Figure 42. SEM images of frontal sinus mucosal samples from each sheep in the Safety arm (#28, 29 & 30: CT-PA-treated sheep; #31, 32 & 33: vehicle control-treated sheep; R: right frontal sinus; L: left frontal sinus). 5 of 6 sinuses in the CT-PA-treated group show intact cilia, with 1 sinus (#28 left) showing a mix of ciliated cells and cells possessing microvilli. 4 of 6 sinuses in the vehicle control-treated group show intact cilia, with 1 sinus (#33 left) showing a mix of ciliated cells and cells possessing microvilli, and another sinus (#32 left) having an obscuring overlying mucus layer and cells possessing microvilli or short cilia.

Local and systemic distribution of phage

Phage was detected in the faeces of all CT-PA-treated sheep at all time points throughout treatment, with average titres of 6.87×10^3, 5.64×10^4, and 2.03×10^4 PFU/g on Days 8, 15, and 22 respectively. Phage was not detected in any of the faecal samples from vehicle control-treated sheep.

Phage was only detected after sample enrichment in the blood of one CT-PA-treated sheep (# 30) on Day 22. Phage was not detected in blood samples from any of the other 5 sheep.

On phage titration of organ samples, phage was not detected in any samples from vehicle control-treated sheep. For CT-PA-treated sheep, phage was not detected in any brain samples. Measurable phage titres were found in only one lung sample (# 28) at a titre of 3.06×10^4 PFU/g. Phage was not detected in any other lung samples, even after sample enrichment. Phage was detected only after enrichment in one liver, one spleen, one kidney, and 2 heart samples (# 28 and 30). Phage was detected after enrichment in 3 of 6 sinus samples from CT-PA-treated sheep, but not at detectable levels prior to enrichment.

6.5 Discussion

Bacterial biofilms have been identified on the sinonasal mucosa of 44% to 55% of patients undergoing endoscopic sinus surgery (ESS) for CRS, compared to 0% to 8% of healthy controls[447, 448, 468]. The presence of mucosal biofilms is associated with poorer outcomes following ESS[395, 469]. More specifically, the presence of *P. aeruginosa* within a polymicrobial biofilm has been associated with increased symptomatic and radiographic severity of CRS, and *in vitro* biofilm formation by *P. aeruginosa* CRS isolates is associated with unfavourable disease progression following ESS[92, 470]. Thus *P. aeruginosa* biofilm formation appears to be a significant factor impacting on CRS disease severity and treatment outcomes.

To our knowledge, this is the first study examining safety and efficacy of a novel treatment for biofilm-associated *P. aeruginosa* sinus infection in a large animal model. Whilst models of *P. aeruginosa* biofilm on airway epithelial cells *in vitro*, and in rabbit maxillary sinuses *in vivo*, have been developed, the sheep rhinosinusitis model enables simulation of treatment application via a sinus flush in frontal sinuses similar in size to the human paranasal sinuses[471, 472].

The ability of the phage cocktail to reduce the biomass of *P. aeruginosa* biofilms *in vivo* is in concordance with numerous studies demonstrating *P. aeruginosa* biofilm removal by bacteriophages *in vitro*[403, 473-480]. Phage have also shown efficacy in treating *P. aeruginosa* biofilm-associated infections *in vivo*, such as canine chronic otitis and a murine model of keratitis[416, 481]. Of particular relevance to CF-associated CRS, phage

have also demonstrated *in vivo* efficacy against *P. aeruginosa* CF isolates in murine models of lung infection[482-484].

Clinical trials of phage cocktails for treatment of *P. aeruginosa* infections in humans have had mixed results. Wright et al[420] found a statistically significant improvement in symptom severity and clinician-assessed disease severity, as well as a drop in *P. aeruginosa* counts, in a double-blind randomised placebo-controlled trial in patients with antibiotic-resistant chronic *P. aeruginosa* otitis. Conversely, a clinical trial of a phage cocktail designed to target both *P. aeruginosa* and *S. aureus*, applied to colonised burn wounds, did not find any advantage over standard of care[485]. It is worth noting that the authors describe several pitfalls of the study design, which may explain their results.

One aspect of phage therapy for CRS that we have attempted to address is whether targeting *P. aeruginosa* alone, in the context of co-infection with other bacterial species, is likely to have any beneficial effects. Our statistical analysis suggests that the presence of another bacterial species on pre-treatment sinus cultures predicts a poor outcome in terms of biofilm removal by CT-PA. The LIVE/DEAD BacLight stain does not differentiate between bacterial species with similar morphology (eg. *P. aeruginosa* and *E. coli*), so the presence of biofilms consisting partially or completely of non-*P. aeruginosa* species in these sinuses cannot be excluded. Given the species specificity of the phage cocktail, non-*P. aeruginosa* species are unlikely to be sensitive to the phage cocktail, which may explain the lack of biofilm removal in these sinuses. *In vitro* studies of mixed species biofilms (*P. aeruginosa* with *K. pneumoniae* or *Escherichia coli*) have demonstrated a protective effect against phage targeted at a single species alone[486, 487]. The aforementioned studies suggest that simultaneous treatment with other

anti-bacterial or anti-biofilm matrix agents such as xylitol or Tween 20 may overcome this effect[486, 487].

We were not able to detect any significant differences in histopathological severity of sinus inflammation between treatment groups in the Efficacy arm. Whilst correlations between tissue eosinophilia and radiological or endoscopic severity of CRS have been previously reported, the relationship between the severity of microscopic tissue inflammation, biofilms, and CRS treatment outcomes is unclear[488-490]. As such, the sheep *P. aeruginosa* rhinosinusitis model was developed primarily to assess efficacy of biofilm removal, rather than any effect on tissue inflammation. Potential confounding factors for analysis of tissue inflammation include the proximity of mucosal samples to the site of trephination, and the amount and species of commensal bacteria present in the sinuses. In addition, whilst the presence of *P. aeruginosa* is very uncommon in cultures from the healthy human sinonasal tract, we routinely found *P. aeruginosa* within the sinuses of sheep prior to experimental inoculation without clinical signs of infection [491-493]. *P. aeruginosa* may form part of the commensal flora of the paranasal sinuses in sheep and thus may not induce overt signs of infection and inflammation, even when artificially instilled.

Persisting high phage titres in the sinuses of Efficacy arm sheep up to 18 hours following the final flush, and the ongoing sensitivity of post-treatment *P. aeruginosa* isolates to CT-PA, indicates that the twice-daily dosing regime was appropriate. We did not assess whether regrowth of biofilms occurs upon cessation of phage flushes, however the continued presence of and bacterial sensitivity to the phage suggests that there is potential for an ongoing treatment effect. The finding of detectable phage in

faeces during the treatment period was not unexpected, as many sheep were observed to swallow some of the sinus flushes. A human clinical trial of oral anti-*E. coli* phage T4 also detected faecal phage when subjects were administered 10^5 PFU/mL phage in drinking water[494].

The sporadic detection of phage in sheep blood and organ samples, in most cases only after sample enrichment, suggests that phage may reach the systemic circulation at low levels after topical application into the paranasal sinuses. There are few other studies of the penetration of topically-administered phage into the systemic circulation. Bogovazova et al[495] identified the presence of phage in the blood and internal organs of mice within 24 hours following the intranasal administration of *K. pneumoniae* phage. Conversely, Drilling et al[461] did not find measurable phage titres in the blood or internal organs of sheep administered *S. aureus* phage in the sheep rhinosinusitis model. Studies of intravenous administration of phage suggest that neutralising antibody formation and the reticuloendothelial system may play a role in inactivation and clearance of phage from the circulation, which could explain the variation in findings between studies[496, 497].

The SEM appearance of some cells with microvilli or short cilia in 3 of 12 sinuses in the Safety arm is consistent with changes seen in regenerating human sinus mucosa, up to 6 months following sinus surgery[498]. Given the 3 week time frame between trephine insertion and the collection of samples for SEM, it is possible that the observed changes are due to healing of mucosal trauma incited during trephine insertion. Similar findings have been noted in other studies utilising the sheep rhinosinusitis model, where a small

number of sinuses in both treatment and control arms demonstrated some areas of loss of cilia[461, 466].

6.6 Conclusion

In conclusion, the CT-PA phage cocktail has demonstrated efficacy in decreasing biofilm biomass in sinuses infected with *P. aeruginosa* biofilms, with no treatment-related adverse effects noted within the limitations of the sheep model. Given increasing concerns regarding antibiotic resistance and overuse, further investigation of the therapeutic potential of phage therapy in CRS would be valuable.

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Disclosures

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All authors have read the journal's authorship agreement and policy on disclosure of potential conflicts of interest.

Chapter 7: Safety and Tolerability of Bacteriophage Therapy for Chronic Rhinosinusitis Due to *Staphylococcus aureus*

Statement of Authorship

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Principal Author

Name of Principal Author (Candidate)	Mian Li Ooi
Contribution to the Paper	Project design, data collection and analysis, manuscript preparation
Overall percentage (%)	100%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 1/9/2019

Co-Author Contributions

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

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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7.1 Abstract

Objective: To investigate the safety, tolerability, and preliminary efficacy of ascending multiple intranasal doses of investigational phage cocktail AB-SA01 in patients with recalcitrant CRS due to *S. aureus*.

Design, Setting, And Participants: This phase 1, first-in-humans, open-label clinical trial of multiple ascending doses was conducted at a single tertiary referral center from December 1, 2015, through September 30, 2016, with follow-up completed on December 31, 2016. Patients with recalcitrant CRS (aged 18-70 years) in whom surgical and medical treatment had failed and who had positive *S. aureus* cultures sensitive to AB-SA01 were recruited. Findings were analyzed from February 2 through August 31, 2017.

Interventions: Three patient cohorts (3 patients/cohort) received serial doses of twice-daily intranasal irrigations with AB-SA01 at a concentration of 3×10^8 PFU for 7 days (cohort 1); 3×10^8 PFU for 14 days (cohort 2); 3×10^9 PFU for 14 days (cohort 3).

Main Outcomes and Measures: The primary study outcome was the safety and tolerability of intranasal AB-SA01. Safety observations included vital signs, physical examinations. Clinical laboratory test results, and adverse events. The secondary outcome was preliminary efficacy assessed by comparing pretreatment and posttreatment microbiology results, disease-relevant endoscopic Lund-Kennedy Scores, and symptom scores using a visual analog scale and Sino-Nasal Outcome Test–22.

Results: All 9 participants (4 men and 5 women; median age, 45 years [interquartile range, 41.0-71.5 years]) completed the trial. Intranasal phage treatment was well tolerated, with no serious adverse events or deaths reported in any of the 3 cohorts. No change in vital signs occurred before and 0.5 and 2.0 hours after administration of AB-SA01 and at the exit visit. No changes in biochemistry were found except for 1 participant in cohort 3 who showed a decrease in blood bicarbonate levels on exit visit, with normal results of physical examination and vital signs. All biochemistry values were normalized 8 days later. No changes in temperature were recorded before, during, or after treatment. Six adverse effects were reported in 6 participants; all were classified as mild treatment-emergent adverse effects and resolved by the end of the study. Preliminary efficacy results indicated favorable outcomes across all cohorts, with 2 of 9 patients showing clinical and microbiological evidence of eradication of infection.

Conclusions And Relevance: Intranasal irrigation with AB-SA01 of doses to 3×10^9 PFU for 14 days was safe and well tolerated, with promising preliminary efficacy observations. Phage therapy could be an alternative to antibiotics for patients with CRS.

Trial Registration: ACTRN12616000002482

7.2 Introduction

The management of recalcitrant chronic rhinosinusitis (CRS) is increasingly challenged by infections with difficult-to-treat biofilms and multidrug resistant bacteria. Antibiotics can alleviate symptoms in acute exacerbations of recalcitrant CRS but fail to eradicate the biofilm nidus resulting in a relapsing course of disease.[85] Amongst surgically recalcitrant patients, up to 50% of biofilms identified are dominated by *Staphylococcus aureus* (*S. aureus*).[326] With a growing prevalence of resistance to first-line antibiotics and lack of research and development of new antibiotics, novel anti-biofilm agents are needed to help control disease in these patients.

Bacteriophage (phage) therapy was proposed as an antibacterial treatment as early as the 1910s. Early clinical results as well as the controversy surrounding the field due to poor understanding of the nature of phage are reviewed elsewhere.[499,245] Increasing interest for its potential to treat bacterial infections has been recently driven by the exponential increase of antibiotic resistant strains.[253] Phage are viruses that infect only one or a few closely related bacterial species with no pathogenic effect on mammalian cells. Phage can be divided into two broad types; obligately lytic or temperate phage (also called lysogenic).[223, 241] Lytic phage hijack the bacterial host cellular machinery to produce progeny phage, kill the cell to re-enter the surrounding environment, and proceed to invade new bacterial hosts. Temperate phage integrate their genome into the host genome and remain dormant, benignly replicating with the bacteria until triggered to enter the lytic cycle. Phage therapy employs obligately lytic phage to achieve maximal bacterial elimination and minimize the risks for horizontal gene transfer.[245, 398]

Phage therapy offers several potential advantages over oral antibiotics.[500] For example, biofilms are more effectively removed by phage[501] but are up to 1000 folds more resistant to antibiotics[87]. Phage offer a highly specific, targeted treatment that is expected to cause less disruption of the normal microbiota than broad-spectrum antibiotics, resulting in fewer systemic side effects. Their mechanism of action means that phage self-replicate at the site of infection with enhanced therapeutic effect at the intended target site, theoretically reducing the need for frequent administration. Phage can be effective against antibiotic resistant strains like methicillin resistant *Staphylococcus aureus* (MRSA) and have the potential to alter the resistance profile of antibiotic-resistant strains.[502, 503]

In vitro studies with anti-*S. aureus* phage determined that the tested phage mixes were superior to single phage for reducing the risk of developing bacteriophage insensitive mutants (BIM)[249, 250] and providing a wider host range effect.[251] The phage cocktail used in the present study, AB-SA01, is an equipotent mixture of 3 lytic phage that belong to the *Myoviridae* family. The AB-SA01 component phage are obligately lytic, incapable of specialized transduction and contain no known antibiotic resistance or bacterial virulence genes (S. Morales, Personal Communication). Related phage demonstrated short term[252] and long term[504] safety and efficacy in an established sheep *S. aureus* biofilm sinusitis model.

Designing and executing robust clinical trials is key to building a greater understanding of the short and long-term clinical impact of phage therapy and required for licensure.

The purpose of this first-in-human, open label study was to determine the safety and tolerability of intranasal application of the phage cocktail AB-SA01 in patients with recalcitrant *S. aureus* CRS. In addition, we determined the feasibility of our trial protocol, including preliminary efficacy assessments.

7.3 Methods and Materials

Participants and Study Design

Ethics approval was granted by the Central Northern Adelaide Health Service Human Research Ethics Committee (CALHN HREC) to conduct the trial within its network of teaching hospitals in Adelaide, Australia. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol inclusion and exclusion criteria are outlined in Table 15. Patients aged between 19-70 who gave informed consent and were able to comply with the trial protocol and who previously had endoscopic sinus surgery were included into the study if they presented with *S. aureus* sinus infection sensitive to AB-SA01.

Inclusion Criteria	**Exclusion criteria**
Between 18 – 70 years of age AND able to give written informed consent.	Participants with diagnosis of cystic fibrosis or ciliary dyskinesia.
Must have two or more ongoing symptoms of CRS (nasal discharge, postnasal drip, nasal obstruction, facial pain or pressure, reduced sense of smell) AND Endoscopic evidence of CRS	Participants who are immunocompromised
ESS >6 weeks prior to enrolment	Participants who are pregnant or breastfeeding
Positive *S. aureus* sinonasal swab sensitive to AB-SA01 within 14 days of enrolment	Participants taking oral corticosteroids or oral antibiotics
Adequate use of birth control for participants and partners of childbearing potential during the study and up to 1 month following the last dose of AB-SA01. Adequate birth control is defined as use double barrier contraception (condom AND one other form of contraception ie. oral contraceptive pills, intrauterine device, injectable birth control/ depot, birth control patch, nuva ring, documented surgical sterilization at least 6 months prior to enrolment)	Used oral antibiotics within 1 month prior to screening

For females, menopausal is defined by cessation of menstrual period for > 12 months or documented FSH > 40mIU/mL	
Female participants of childbearing potential must have a negative serum pregnancy test at screening AND negative urine pregnancy test on Day 1.	Participants with positive *P. aeruginosa* sinonasal swab
Able to comply with the requirements of the protocol and willing to return to this centre for postoperative follow-up care	Any clinically significant laboratory abnormality

Table 15. Inclusion and exclusion criteria.

This was a prospective, open-label, phase 1 clinical trial conducted between December 2015 to September 2016. Each cohort was serially dosed with AB-SA01 intranasal irrigations in the following ascending dosage regimens: twice daily intranasal irrigations of AB-SA01 at a concentration of 3×10^8 PFU for 7 days (cohort 1); or 3×10^8 PFU for 14 days (cohort 2); or 3×10^9 PFU for 14 days (cohort 3).

In Cohort 1, all three participants were dosed serially, and only after successful completion of a safety and tolerability assessment by a safety medical committee (SMC), which consisted of the principal investigator, a virologist, medical monitor and project manager. Once Cohort 1 was completed, the safety data was reviewed by the SMC before Cohort 2 was commenced. After the sentinel subject from Cohort 2 proceeded without safety concerns, the remaining two participants in Cohort 2 were dosed in parallel. The use of a sentinel subject was then repeated for Cohort 3 (Figure 43).

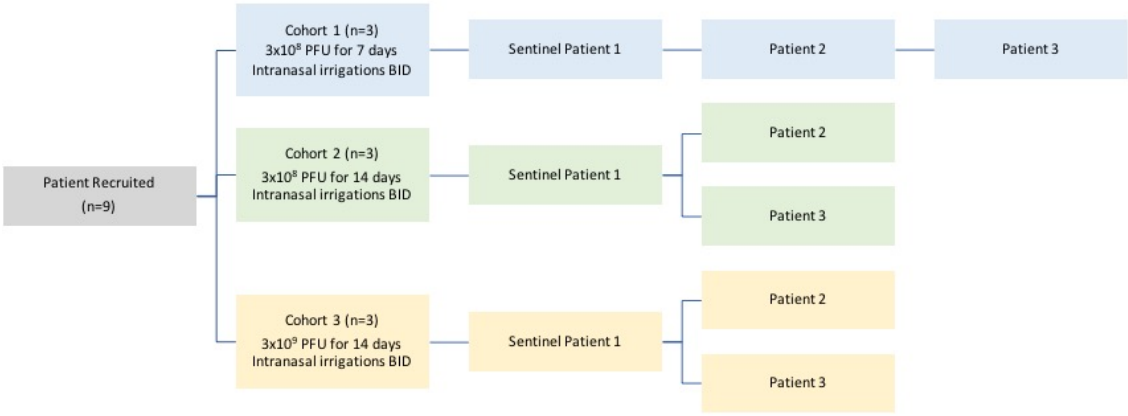

Figure 43. Flow diagram describing participant flow and specific administered treatments.

Bacteriophage Investigational Product

Investigational bacteriophage product AB-SA01 was produced under phase-appropriate Good Manufacturing Practices and supplied by AmpliPhi Biosciences Corporation (San Diego, USA).

Sensitivity of *S. aureus* Clinical Isolates to AB-SA01

Patient *S. aureus* cultures from nasal swabs were collected from Clinpath Laboratories (Adelaide, Australia) and transferred to the Basil Hetzel Institute Laboratories (Adelaide, Australia) for testing. The bacteria were streaked on a 1% nutrient agar plate and grown overnight at 37°C. Colonies were picked using a sterile 1 µL loop and transferred to 3 mL nutrient broth followed by incubation with shaking (180 rpm) at 37°C for 16-18 hrs. Phage sensitivity, defined as productive bacteriophage infection as demonstrated by the presence of individual phage plaques, was determined in triplicates using the soft agar overlay technique as described previously.[251, 253] ATCC 25923 was

obtained from the ATCC and used as a positive control in the assay.[251] Only patients carrying a clinical isolate that was sensitive to AB-SA01 were eligible to be in the study.

Instructions to prepare the intranasal sinus lavage

Supplies of trial products given to participants are detailed in Table 16.

	Supplies of trial products
Cohort 1	14 x 1 mL phage vials + 2 excess vials 16 x sterile pipettes 16 x 350 mL Mount Franklin spring water 16x Neilmed sinus rinse bottles Temperature Log Omron digital thermometer (underarm)
Cohort 2 & 3	28 x 1 mL phage vials + 2 excess vials 30 x sterile pipettes 30 x 350 mL Mount Franklin spring water 30 x Neilmed sinus rinse bottles Temperature Log Omron digital thermometer (underarm)

Table 16. Supplies of trial products.

Participants were instructed to store the AB-SA01 vials away from light and in the refrigerator. Prior to use, participants were asked to fill a rinse bottle (NeilMed Pharmaceuticals, Santa Rosa, CA, USA) with 240 mL of Mount Franklin Spring Water (Coca-Cola Amatil Pty Ltd, Australia), add the proprietary buffered salts sachets (pharmaceutical grade sodium chloride and sodium bicarbonate), followed by adding 1 mL of AB-SA01. Participants then performed the rinses by squeezing the bottle gently to deliver 120 mL of the mixed solution into each nasal cavity. Participants performed nasal irrigations twice daily using a new bottle to deliver each dose.

Data collection

Study protocol detailing screening visit, dosing visit, exit visit and follow up (via telephone 7 days after exit visit) is described in Figure 44.

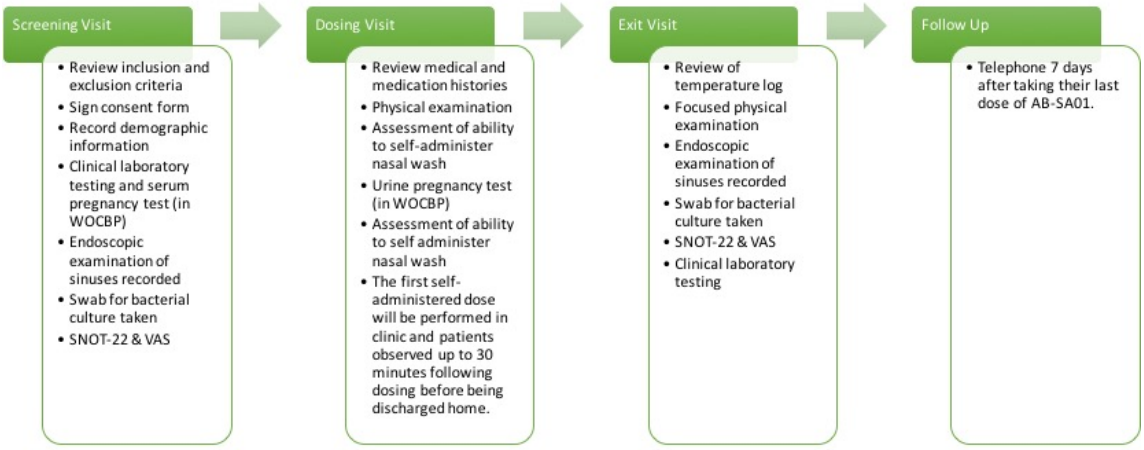

Figure 44. Flow diagram describing detailed trial protocol at all time points of the study.

Outcome measurements - Safety

Clinical biochemistry tests were conducted on screening visit and on exit visit. The panel of biochemistry tests assessed were as follows:

Haematology. Haemoglobin, haematocrit, erythrocytes, platelets, leukocytes (including eosinophils, neutrophils, basophils, lymphocytes and reticulocytes).

Serum chemistry. Blood urea nitrogen, creatinine, total bilirubin, direct bilirubin, urate, albumin, alkaline phosphatase, creatine kinase, aspartate aminotransferase, alanine aminotransferase, glucose, bicarbonate.

Temperature log. Participants were asked to self-monitor temperature twice daily at home and complete a temperature log throughout the duration of the trial to be handed in at exit visit.

Concomitant medications. All medications taken during 30 days prior to screening were recorded and reviewed by the principal investigator to determine whether the participant was suitable for inclusion. Participants were asked to report all medications taken during the course of the trial. Participants on routine intranasal corticosteroids (INC) on enrolment were instructed to continue throughout the duration of the study, with at least two hours between application of the investigational drug and INC.

Clinical examination. A general physical examination including vital signs (body temperature, heart rate, respiratory rate, blood pressure) was conducted on dosing visit (prior to dosing, 0.5 hrs and two hrs after dosing) and on exit visit.

Adverse events. Participants were assessed for adverse events (AEs) coded using the Medical Dictionary for Regulatory Activities (MedDRA®, Version 2.1) for the duration of the study during clinic visits and at follow-up.

Outcome measurements- Efficacy

Preliminary efficacy was evaluated by the semi-quantitative assessment of pre- and post-treatment bacterial cultures. All patients completed symptoms score questionnaire at every visit, using Sino-Nasal Outcome Test-22 (SNOT-22)[505] (22 items, each scored from 0–5; total score range 0-110) and Visual Analogue Scale (VAS)[506] (average of six items; each scored from 0-100, total score range 0–100). All patients also had entry and

exit endoscopic videos recorded and scored by an independent blinded surgeon using the Lund Kennedy Score (LKS)[505, 507] (score range, 0–20).

7.4 Results

Baseline demographic and clinical characteristics are shown in Table 17.

	Cohort 1	Cohort 2	Cohort 3
Age, year	54·57 (52-58)	50 (39-69)	58 (37-69)
Gender, male	1 (33%)	0 (0%)	3 (100%)
History of polyposis	1 (33%)	1 (33%)	2 (67%)
Frontal drillouts	1 (33%)	2 (67%)	2 (67%)
Visual analogue scale	60·14 (36·57-78·14)	33·76 (20·14-49)	29·62 (18·14-46·71)
SNOT-22 score	72 (45-95)	28·67 (26-34)	31·67 (8-70)
Lund-Kennedy score	11 (8-13)	5·67 (4-7)	7 (6-8)

Table 17. Baseline patient demographics and clinical characteristics. Data are medians (interquartile range) or numbers (%).

A total of 28 patients gave written informed consent of which 19 patients were excluded because of negative bacterial cultures (n=4), positive bacterial cultures but no *S. aureus* growth (n=9), *S. aureus* positive cultures but insensitive to AB-SA01 (n=3), study withdrawal prior to first treatment (n=3). In this study, the sensitivity of *S. aureus* CRS isolates to AB-SA01 was 80% (n=12/15).

Tolerability, adverse effects, compliance. All nine participants were compliant and completed the trial indicating the irrigations were well tolerated, validating the feasibility of administration route and trial design. There were no serious AEs (SAEs), and no AE led to withdrawal of study drug or discontinuation from the study. A total of six AEs were reported throughout the study in six of the nine participants, all of which were classified as treatment-emergent AEs (TEAEs). All TEAEs were of mild severity and resolved by the end of the study. One TEAE was reported in one of the

three participants in Cohort 1 (diarrhoea). Three TEAEs were reported in the three participants in Cohort 2 (epistaxis, oropharyngeal pain and cough). Two TEAEs were reported in two of the three participants in Cohort 3 (rhinalgia and blood bicarbonate decreased). Details of AEs reported are listed in Table 18.

Adverse event	Relevant medical history	Cohort	Time of onset	Severity	Causality/ relation to study treatment	Adverse event description	Action taken	Outcome
Loose bowels	History of Crohn's disease	1	Day 5 of phage treatment	Mild	Unlikely	Exacerbation of a chronic pre-existing condition	Dose not changed	Recovered
Self-resolved epistaxis	History of intermittent epistaxis	2	Day 10 of phage treatment	Mild	Unlikely	Exacerbation of an intermittent pre-existing condition	Dose not changed	Recovered
Symptoms of upper respiratory tract infection	N/A	2	Day 11 of phage treatment	Mild	Unlikely	New condition detected after phage treatment even though it may have been present prior to the start of the study	Dose not changed	Recovered
Oropharyngeal pain	N/A	2	Day 11 of phage treatment	Mild	Unlikely	New condition detected after phage treatment even though it may have been present prior to the start of the study	Dose not changed	Recovered
Rhinalgia	N/A	3	Day 1 of phage treatment	Mild	Likely	New condition detected after phage treatment	Dose not changed	Recovered
Low serum bicarbonate level	N/A	3	Day 14	No awareness of symptom	Unlikely	New condition detected after phage treatment	Dose not changed	Recovered

Table 18. Adverse events report.

Vital signs. There has been no change in vital signs before administration of AB-SA01 and post administration at 0.5 hrs, 2 hrs and on exit visit.

Biochemistry. No changes in biochemistry were found pre- and post-treatment, except for one participant in cohort 3 who showed a decrease in blood bicarbonate levels on exit visit. The patient attended an unscheduled visit and had normal physical examination and vital signs. Blood test was repeated eight days later and all biochemistry parameters were within normal values.

Temperature. No change in temperature records pre- , during and post-treatment.

Preliminary Efficacy Results

Efficacy data describes observed trends and no statistical analysis has been performed due to the small sample size.

Microbiology outcome. All patients had reduction in *S. aureus* growth and 2/9 patients had negative cultures post treatment. Data is summarized in Table 19.

	Pre Treatment	**Post Treatment**
Cohort 1	Heavy	Moderate
	Moderate	Light
	Heavy MRSA	Moderate MRSA
Cohort 2	Moderate	Light
	Light	Negative
	Heavy	Negative
Cohort 3	Moderate S. aureus	Light MRSA*
	Heavy	Light
	Heavy	Light

Table 19. Bacterial load reported as scant, light, moderate or heavy (equivalent to 1+, 2+, 3+ or 4+) by laboratory with associated phage sensitivity.

Visual Analogue Scores (VAS) were reduced (Cohorts 1 and 3) pre-treatment and post-treatment (Figure 45A, Table 20).

SNOT-22 scores were reduced (Cohorts 1 and 3) pre-treatment and post-treatment (Figure 45B, Table 20).

Lund Kennedy scores (LKS). There was a consistent trend showing improvement in LK endoscopic scores across all cohorts with greatest improvement noted in Cohort 3 (Figure 45C, Table 20).

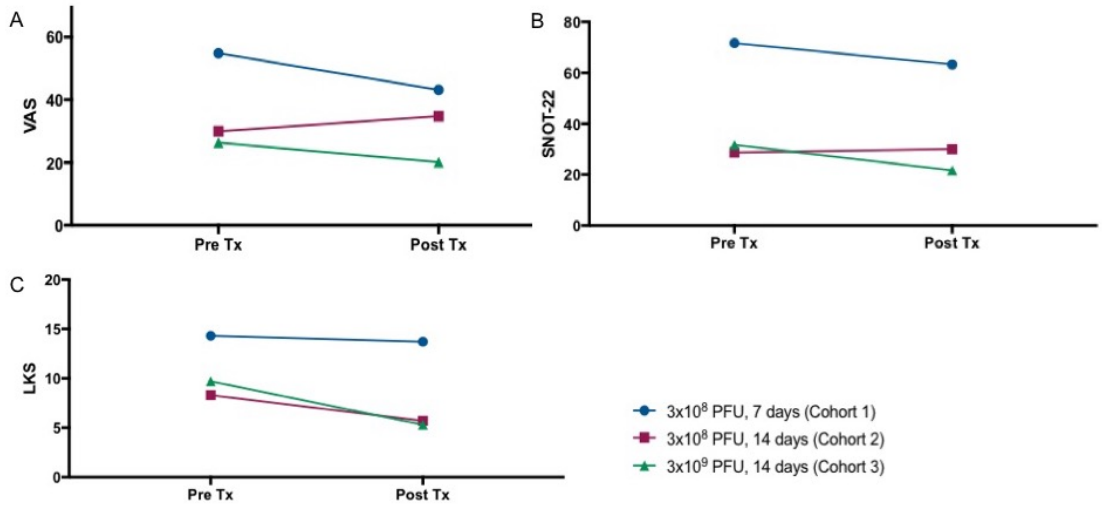

Figure 45. Line graph showing median values pre-AB-SA01 treatment (Pre Tx) and post-AB-SA01 treatment (Post Tx) for: (A) VAS scores, (B) SNOT-22, (C) LKS scores across all cohorts.

	VAS	SNOT-22	LKS
Cohort 1	-11.71	-8.4	-0.6
Cohort 2	4.81	1.3	-2.6
Cohort 3	-6.25	-10	-4.4

Table 20. Mean difference of (A) VAS scores, (B) SNOT-22, (C) LKS scores across individual cohorts. Above 0 (positive values) means deterioration from pre-treatment, below 0 (negative values) means improvement from pre-treatment.

3 months follow up. Five patients received further antibacterial treatment after cessation of the study and four patients did not receive any further treatment post phage therapy. These four patients (one patient from cohort 1, two patients with negative cultures from cohort 2, one patient from cohort 3) were followed up at three months with VAS, SNOT-22 and LKS assessment. There was a continuing trend towards further improvement in all outcome measures. Results are shown in Figure 46.

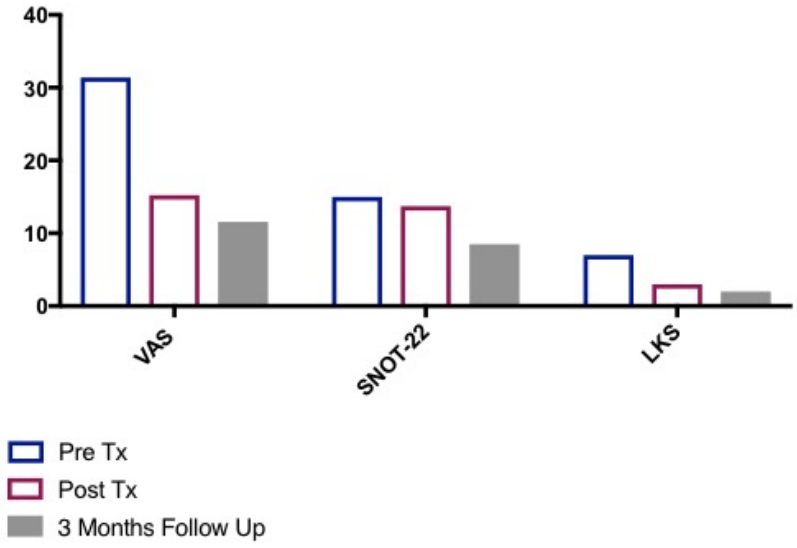

Figure 46. 3 month follow-up data

7.5 Discussion

This study indicated that twice daily intranasal irrigations up to 3×10^9 PFU for 14 days was safe and well-tolerated with no serious adverse events. Our safety result is consistent with the body of literature that is currently available with regards to the safety of phage use. A number of phase 1 human clinical trials have been conducted after application of phage topically (to the skin) or orally, with none of those studies reporting any serious adverse events. In a placebo-controlled phase 1 study, 42 patients were recruited testing the safety of phage mixes against *S. aureus*, *P. aeruginosa,* and *E. coli* for the treatment of chronic venous leg ulcers. Patients were treated for 12 weeks with post-treatment follow-up up to 24 weeks with no reported significant side effects.[508] Another phase 1 study was conducted where a single spray phage cocktail active against *P. aeruginosa,* and *S. aureus* was applied on colonized burn wounds in nine patients. No adverse events, clinical abnormalities or changes in laboratory results were observed.[509] The first modern double-blinded controlled clinical trial using phage to treat refractory *P. aeruginosa* ear infections was conducted in England in 2007.[246] 24 patients were randomised to receive a single dose of phage or placebo and both groups were monitored for 42 days. *P. aeruginosa* counts were lower for the phage-treated group with no reported adverse events.

The safety of phage treatment has also been recorded in healthy adults after oral administration. A pilot study tested the safety of a coliphage in fifteen healthy adult volunteers.[510] Two different doses of the T4 coliphage (10^3 and 10^5 PFU/mL) were mixed with drinking water. The counts of normal *E. coli* flora did not decrease and no adverse events were reported. In a follow-up study, 15 healthy adults from Bangladesh

received a phage cocktail composed of nine *E. coli* phage at two different concentrations up to 3×10^9 PFU.[511] The results showed no adverse events by self-report or clinical examination. The laboratory tests for liver, kidney, and haematology function were also reported as normal. Importantly, oral phage treatment had no impact on the faecal microbiota composition.

Phage preparations administered to humans with CRS has been reported in the past with favorable outcomes with 76-78% efficacy in infection control and no significant side effects.[512, 513] However, these studies were inconsistent, lacked controls and did not have comprehensive safety outcome measures. Mills et al[514] administered alpha with occasionally beta lysate staphylococcus bacteriophages via a nebulizer over individualized durations followed by monthly maintenance doses. While Weber-Dabrowska et al[513] administered phage orally and topically from repeated antral punctures for duration ranging from 4-12 weeks.

Acknowledging the statistical limitations of our safety trial, we are only able to comment on the trends observed in this study on efficacy. The preliminary efficacy observations seen in this study are promising. Although there were no significant changes in the validated symptom scores relevant to sinus disease (VAS and SNOT-22 scores), the clinical improvements seen endoscopically may be explained by a reduction in bacterial load and suspected anti-inflammatory effects of phage. In the context of infection, phage have been reported to show anti-inflammatory effects by reducing neutrophils and pro-inflammatory cytokines such as IL-6, IL-1β[515, 516] and by reducing reactive oxygen species production.[426]

Interestingly, continued clinical observations of certain patients beyond the formal duration of the trial showed that there might be a sustained clinical effect of up to three months post-phage treatment. This may be due to phage persistence and prophylactic potential of phage, although this was not assayed. Future clinical studies may benefit from longer follow up periods. Previous *in vivo* studies conducted by our department have identified low levels of active phage still present within sinuses 24 hours post-administration.[252] This is consistent with various other studies which have shown serum phage persistence from 48 hours up to 38 days post inoculation[517, 518] with clearance of phage by the reticuloendothelial system (RES).[519] The intranasal administration of phage in the sinuses may prolong phage persistence bypassing the RES, especially in the presence of remaining bacterial cells which would enable self-replication. Wright et al[246] reported 200-fold amplification of *P. aeruginosa* phage over 42 days after just a single dose of treatment. Multiple studies have also suggested the role of phage in conferring a protective role and assisting bacterial clearance when subsequent infection is encountered.[516-518]

7.6 Conclusion

This study concludes that AB-SA01 phage cocktail intranasal irrigations are safe and well tolerated up to 3×10^9 PFU for 14 days with no dose-limiting side effects. The preliminary efficacy data is promising and suggests that prolonged antimicrobial effects are possible allowing for a more targeted approach in treating recalcitrant *S. aureus* sinus infections and associated inflammation. Further work must be performed to determine the optimal dose regimen and demonstrate the efficacy of AB-SA01 in a statistically powered randomised controlled trial.

Acknowledgements

Dr Peter Speck is acknowledged for his introduction of phage therapy to our department and for his expertise on previous phage studies. We would also like to thank Professor Eric Gowans for his participation as our virology expert on our safety monitoring committee.

Chapter 8: Topical colloidal silver for the treatment of recalcitrant chronic rhinosinusitis: A pilot study

Statement of Authorship

Title of Paper	Topical colloidal silver for the treatment of recalcitrant chronic rhinosinusitis: A pilot study
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
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Principal Author

Name of Principal Author (Candidate)	Mian Li Ooi
Contribution to the Paper	Project design, data collection and analysis, manuscript preparation
Overall percentage (%)	100%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 1/9/2019

Co-Author Contributions

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

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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8.1 Abstract

Background: The management of recalcitrant chronic rhinosinusitis (CRS) is challenged by difficult-to- treat polymicrobial biofilms and multidrug resistant bacteria. This has led to the search for broad-spectrum non-antibiotic antimicrobial therapies. Colloidal silver (CS) has significant antibiofilm activity in vitro and in vivo against S. aureus, MRSA and P. aeruginosa. However, due to the lack of scientific efficacy, it is only currently used as an alternative medicine. This is the first study looking at the safety and efficacy of colloidal silver in recalcitrant CRS.

Methods: Patients were included when they had previously undergone endoscopic sinus surgery and presented with signs and symptoms of sinus infection with positive bacterial cultures. 22 patients completed the study. Patients were allocated to 10-14 days of culture- directed oral antibiotics with twice daily saline rinses (n=11) or 10 days of twice daily 0.015mg/mL colloidal silver rinses (n=11). Safety observations included pre- and post- treatment serum silver levels, University of Pennsylvania Smell Identification Test (UPSIT) and adverse event (AE) reporting. Efficacy was assessed comparing microbiology results, Lund Kennedy Scores (LKS) and symptom scores using Visual Analogue Scale (VAS) and Sino-Nasal Outcome Test (SNOT-22).

Results: CS demonstrated good safety profile with no major adverse events, no changes in UPSIT and transient serum silver level changes in 4 patients. CS patients had 1/11 (9.09%) negative cultures, compared to 2/11 (18.18%) in the control group upon

completion of the study. Whilst not statistically significant, both groups showed similar improvement in symptoms and endoscopic scores.

Conclusion: This study concludes that twice daily CS (0.015mg/mL) sinonasal rinses for 10 days is safe but not superior to culture-directed oral antibiotics. Further studies including more patients and looking at longer treatment or improving the tonicity of the solution for better tolerability should be explored.

8.2 Introduction

The management of recalcitrant chronic rhinosinusitis (CRS) is increasingly challenged by difficult-to-treat polymicrobial biofilms and multidrug resistant bacteria which antibiotics often cannot effectively eradicate. For recalcitrant patients, antibiotics often alleviate symptoms in acute exacerbations but fail to eradicate the biofilm nidus which periodically sheds planktonic organisms resulting in a relapsing and remitting course of disease[85]. This has fuelled a continuous search for broad-spectrum topical non-antibiotic anti-biofilm therapies. Topical agents allow increased concentration, localised action, less systemic side effects and lessen the risk of antibiotic resistance.

To date, numerous topical agents have been tested and although some have shown anti-biofilm activity [175, 210, 260, 261, 269, 283, 330, 520, 521], none have been widely accepted as a treatment option in recalcitrant CRS. Recent evidence suggests that colloidal silver may be effective against bacterial biofilms. We have previously shown that colloidal silver showed significant anti-biofilm activity *in vitro* and *in vivo* against *S. aureus*[213, 214], and against methicillin-resistant *S. aureus* (MRSA) and *P. aeruginosa* biofilms. Spherical nanoparticles were also shown to be non-toxic in human cell culture (THP-1, Nuli-1)[221] and safe in a sheep sinusitis model[214]. Moreover, they were physically stable for over 6 months in storage with no observed loss in anti-biofilm activity.[221]

However, due to the lack of evidence for their efficacy, it is only currently used as an alternative medicine. This is the first study investigating the safety and efficacy of colloidal silver in recalcitrant CRS patients.

8.3 Methods and Materials

Participants and study design

This was a prospective, open-label, single-blinded, pilot study looking at the safety and efficacy of colloidal silver sinonasal rinses in patients with recalcitrant chronic rhinosinusitis between December 2016 to July 2017. Ethics approval was granted by the Central Northern Adelaide Health Service, Ethics of Human Research Committee (TQEH/LMH/MH HREC) to conduct the trial within its network of teaching hospitals in Adelaide, Australia. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

A total of 22 patients were enrolled in the study (8 females, 14 males, aged 27-86). Patients were allocated to either the colloidal silver arm (CS) (n = 11) or control arm (CON) (n = 11) depending on availability of silver stock and patient's adverse reaction to culture-sensitive oral antibiotics (Figure 47). Full inclusion and exclusion criterias are outlined in Table 21. Baseline demographic and clinical characteristic are demonstrated in Table 22.

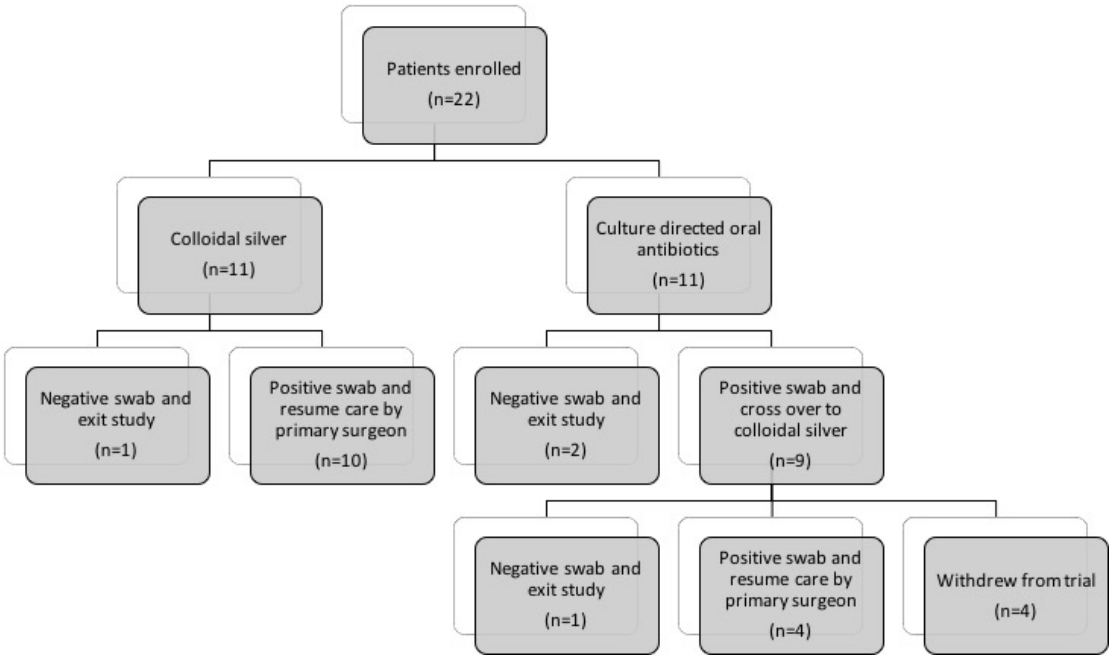

Figure 47. Flow diagram describing patients allocated to (1) Culture-directed oral antibiotics with twice daily saline flush (CON) and (2) Colloidal silver (CS) with twice-daily saline flush.

Inclusion Criteria	Exclusion criteria
Ongoing symptoms of CRS despite at least one trial of oral antibiotics	Allergy to silver
ESS >12 weeks prior to enrolment	Antibiotics in previous 2 weeks
Positive bacterial microbiology swab	Taking oral corticosteroids
Over 18 years of age AND able to give written informed consent	Pregnant or breastfeeding
Willing to return to this centre for postoperative follow-up care	Immunocompromised

Table 21. Inclusion and exclusion criteria.

	CON (n=11)	CS (n=11)
Age, year	61 (52-72)	60 (47-73)
Gender, male	7 (63.6%)	7 (63.6%)
History of polyposis	9 (81.82%)	8 (72.73%)
Frontal Drillouts	7 (63.64%)	9 (81.82%)
Visual analogue scale	38.29 (22.14-51.86)	49.72 (28.75-65)
SNOT-22 score	38.55 (23-59)	58.01 (43-75)
Lund-Kennedy score	6.82 (4-10)	8.57 (6-10)

Table 22. Baseline patient demographics and clinical characteristics.

CS patients were provided with 20 sealed bottles of pre-filled 120 mL colloidal silver solution in standard nasal irrigation squeeze bottles. Patients were instructed to store these bottles away from light and in the refrigerator. Prior to use, patients were asked to warm the solution to room temperature, fill the rinse bottle to 240 mL with cooled boiled water, then perform the rinses twice daily for 10 days. Patients are to apply gentle pressure onto squeeze bottles which delivers the solution through the inner tube and out of the tip of the bottle into the nostril. CS patients were specifically instructed not to add the usual proprietary buffered salts sachets to avoid chemical interaction with the colloidal silver nanoparticles. All squeeze bottles were provided by NeilMed Pharmaceuticals (Santa Rosa, CA). If there were signs of persistent infection on endoscopic examination and a positive culture swab post- treatment, CS patients exited the study and resumed treatment based on clinical grounds.

CON patients received a 10 to 14-day course of culture-directed oral antibiotics and were instructed to perform twice daily saline rinses similar to the delivery of colloidal silver. If the patient had persistent infection on endoscopic examination and a positive culture swab at the end of treatment, they received CS.

Those taking INCs on enrolment were instructed to continue throughout the duration of the study.

Synthesis of silver nanoparticles

Spherical silver nanoparticles were prepared as previously described[221]. Briefly, a mixture of 6.25 mL water, 1.25 mL sodium citrate (1% wt.), 1.25 mL silver nitrate (1% wt.) and 50 µl potassium iodide (300 µM) was prepared under stirring at room temperature and incubated for 5 min. This mixture was added to 237.5 mL of boiling water that included 250 µl ascorbic acid (0.1M). The colourless solution changed to yellow and finally slightly orange, indicating particle formation. The silver nanoparticles were further boiled for 1 hour under reflux and stirring at 1500 rpm. After cooling, the silver nanoparticles were characterised by UV-Vis spectrometry and transmission electron microscopy (quality control). This confirmed a spherical particle shape and size of approximately 40 nm. Silver nanoparticles were stored in amber glass flasks under dark condition at 4ºC prior to utilisation as a nasal rinse.

Efficacy Assessment. Endoscopic guided sinonasal swabs were taken at every scheduled visit for microbiological evaluation. All patients completed symptoms score questionnaire at every visit, using Sino-Nasal Outcome Test-22 (SNOT-22)[505] (22 items, each scored from 0–5; total score range 0-110) and Visual Analogue Scale (VAS)[506] (average of 6 items and an overall symptom score; each scored from 0-100, total score range 0–100). At each visit, all patients had entry and exit endoscopic videos recorded and scored by a blinded surgeon using the Lund Kennedy Score (LKS)[505, 507] (score range, 0–20).

Safety Assessment. All patients on CS treatment were required to have pre- and post-treatment serum silver levels and completed the University of Pennsylvania Smell Identification Test (UPSIT). If serum silver level post-treatment was above normal

limits, a repeat serum silver level was performed 7 days later to confirm return to baseline. Patients were advised to report any adverse outcomes while on the study.

Data Analysis

Statistical power was calculated for the primary end-point of culture negativity post-treatment. Power analysis estimates determined a sample size of 11 patients per group would be required to achieve statistical significance (80%, $p<0.05$) based on response rates of 25% and 90% in the control and silver groups, respectively.

All results were statistically analysed at the completion of the study using 2-way analysis of variance (ANOVA) and student's t-test, with a significance value set at $p<0.05$.

8.4 Results

Efficacy

Microbiology Result. 2/11 (18.18%) patients in CON group had negative swabs while 1/11 (9.09%) CS patients had negative swabs upon completion of treatment. List of pathogens treated in both cohorts are described in Table 23.

Before colloidal silver	After colloidal silver
Heavy *MRSA* + Light *P. aeruginosa*	Heavy *MRSA* + Scant *P. aeroginosa*
Heavy *S. aureus*	Moderate *S. aureus*
Heavy *S. aureus* + Heavy *P. aeruginosa*	Moderate *S. aureus* + Moderate *P. aeroginosa*
Moderate *S. aureus*	Light *S. aureus* + Light *S. Pneumoniae*
Scant *K. oxytoca*, Scant *H. influenza*	No growth
Heavy *H. influenza*	Heavy *S. aureus* + Light *E. cloacae* + Light *H. influenzae*
Moderate *K. oxytoca* + Moderate *P. aeruginosa*	Heavy *K. oxytoca* + Moderate *P. aeruginosa*
Light *P. aeruginosa*	Moderate *S. aureus*
Heavy *S. aureus*	Moderate *S. aureus*
Heavy *S. aureus* + Heavy *P. aeruginosa*	Heavy *S. aureus*
Heavy *S. aureus*	Heavy *S. aureus* + Moderate *M. Morganii*

Before oral antibiotics	Antibiotics	After oral antibiotics	After colloidal silver
Heavy S. aureus	Augmentin DF	Moderate *P. aeruginosa* + Heavy *S. aureus*	Moderate *S. pneumoniae* + Moderate *S. aureus*
Moderate P. aeruginosa	Ciprofloxacin	Light P. stutzeri	Withdrew due to other commitments
Heavy H. influenzae	Bactrim DS	No growth	
Heavy *S. aureus*	Augmentin DF	Heavy *S. Maltophilia*	No growth
Moderate *S. aureus*	Augmentin DF	No growth	
Heavy *E. Coli*	Augmentin DF	Moderate *E. coli*	Withdrew due to flush discomfort
Heavy *S. aureus*	Cephalexin	Moderate *S. aureus* + Light *H. influenzae*	Withdrew due to lack of efficacy
Moderate *S. aureus*	Augmentin DF	Moderate *S. aureus* + Light *H. influenzae*	Heavy *S. aureus* + Light *E. Coli*

Moderate *E. aerogenes*	Ciprofloxacin	Moderate *E. aerogenes* + Scant *S. aureus*	Withdrew due to due to external injury
Moderate *S. pneumoniae* + Scant *S. aureus*	Augmentin DF	Light *S. aureus*	Light *S. aureus*
Moderate *S. pneumoniae* + Scant *S. aureus*	Bactrim DS	Moderate *S. aureus* + Scant *Alternaria sp*	Light *S. aureus*

Table 23. Standard semi-quantitative analysis of bacterial load reported as scant, light, moderate or heavy (equivalent to 1+, 2+, 3+ or 4+) by laboratory.

Visual Analogue Scale (VAS). VAS scores in both CON and CS groups showed a similar trend of improvement post-treatment, but both were not statistically significant (CON 1.728 [95% CI -7.785 to 11.24] vs CS 3.536 [95% CI -5.977 to 13.05]) (Figure 48).

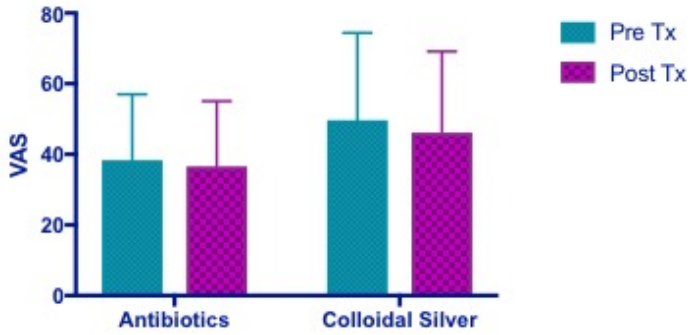

Figure 48. Bar graph showing no significant difference in VAS scores between CON and CS treated groups.

***Sino-Nasal Outcome Test -22 (SNOT-22*).** Patients in the CON group showed no change in SNOT-22 scores post- treatment while CS group showed a trend towards an improvement in SNOT-22 scores, but it was not statistically significant (CON -0.6364 [95% CI -6.673 to 5.4] vs CS 5.818 [95% CI -0.2183 to 11.85]) (Figure 49).

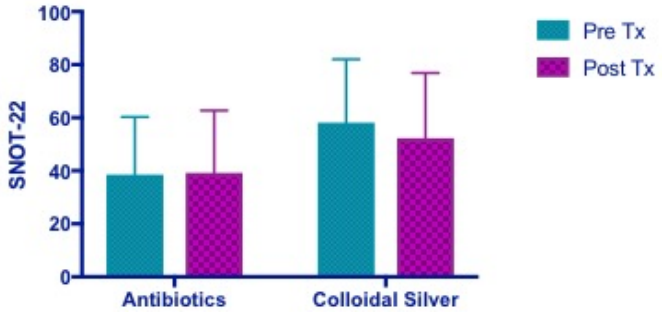

Figure 49. Bar graph showing no change in SNOT-22 scores in CON group, while CS group showed a trend of improved SNOT-22 scores, but not statistically significant.

Lund Kennedy Score (LKS). Both CON and CS group showed trends of similar improvements in Lund Kennedy Scores but this was not statistically significant (CON 1.818 [95% CI -1.373 to 5.009] vs CS 2.167 [95% CI -2.154 to 6.488]) (Figure 50).

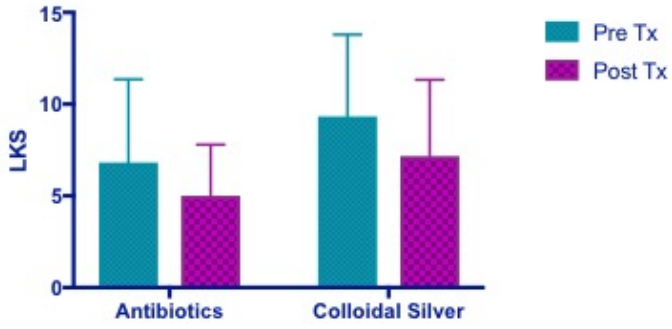

Figure 50. Bar graph showing similar improvements in LKS in both CON and CS groups, but not statistically significant.

Subgroup analyses: Crossover silver arm

5 patients completed the crossover colloidal silver arm after failing oral antibiotics. Subgroup analyses were performed comparing VAS, SNOT-22 and LKS scores of

patients while on either treatment. The mean score difference post antibiotic treatment vs post CS treatment were compared using Wilcoxon matched-pairs signed rank tests. However, due to the small sample size of our subgroup analyses, data presented is focused on describing observed trends.

Microbiology Result of Crossover arm. 1/5 patient had successful infection eradication from colloidal silver treatment after failing culture-sensitive oral antibiotics.

Visual Analogue Scale (VAS) of Crossover Arm. There were slight improvements in VAS scores after culture sensitive oral antibiotics and CS treatment. There was a trend of greater improvement in VAS while on CS compared to when patients were treated with culture sensitive oral antibiotics. It is also observed that patients' VAS scores appeared to return to baseline after completing course of oral antibiotics and before commencing colloidal silver which is consistent with what is observed in clinical practice (Figure 51). Mean difference in VAS scores when patients were on culture sensitive oral antibiotics 4.546 [95% CI -8.156 to 17.25] vs CS treatment 5.94 [95% CI -3.347 to 15.23], p = 0.4750.

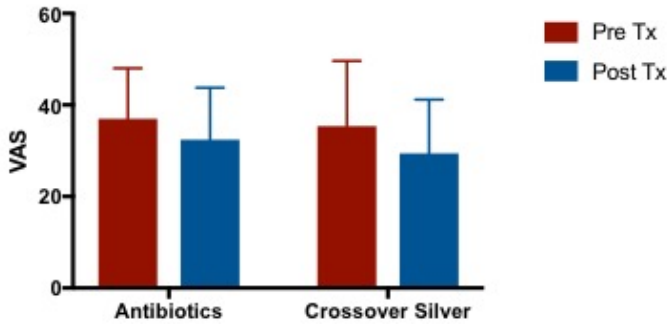

Figure 51. Bar graph comparing VAS scores of patients following failed culture sensitive oral antibiotics and crossed-over to CS treatment

Sino-Nasal Outcome Test -22 (SNOT-22) of Crossover Arm. There were no changes in SNOT-22 scores after culture sensitive oral antibiotics treatment but showed trends of improvement when patients were crossed over to CS treatment. (Figure 52). Mean difference in SNOT-22 scores when patients were on culture sensitive oral antibiotics 0.2 [95% CI -2.021 to 2.421] vs CS treatment -13 [95% CI -22.42 to -3.585], p = 0.06.

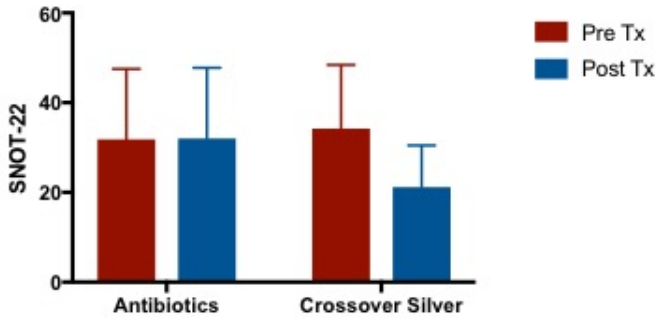

Figure 52. Bar graph comparing SNOT-22 scores of patients following failed culture sensitive oral antibiotics and crossed-over to CS treatment.

Lund Kennedy Score (LKS) of Crossover Arm. Patients demonstrated an improvement in LKS post antibiotic treatment and further improvements were observed after completion of CS treatment. (Figure 53). Mean difference in LKS scores when patients were on culture sensitive oral antibiotics -2.8 [95% CI -7.311 to 1.711] vs CS treatment -1.4 [95% CI -4.259 to 1.459], p = 0.50.

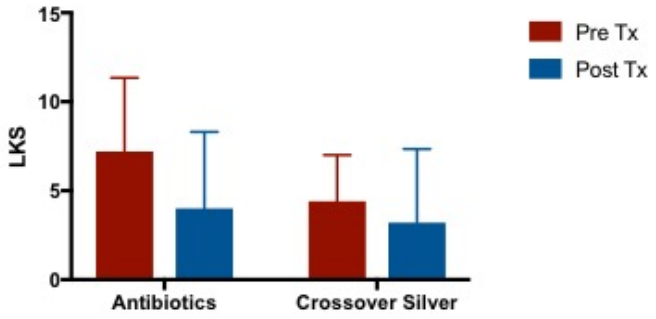

Figure 53. Bar graph comparing LKS scores of patients following failed culture sensitive oral antibiotics and crossed-over to CS treatment

Safety

Serum Silver Levels. 4 patients who had received CS had serum silver levels that were above normal limits measured within 24 hours after receiving final silver dose. 3 patients had a repeat test 10 days after study exit which saw serum silver levels had returned to normal parameters. 1 patient had serum silver levels which were above normal ranges pre-treatment and on repeat test had returned to baseline. Our laboratory reference indicates that argyria can be present at serum silver levels of approximately 100 nmol/L, the highest level of serum silver level recorded in our study was 57.3 nmol/L.

Smell Test. There were no significant changes in smell pre- and post- treatment between both groups measured using the University of Pennsylvania Smell Identification Test (UPSIT).

Adverse Events. There were no serious adverse events reported.

8.5 Discussion

In this study, looking at the primary end-point of culture negativity post-treatment, CS has not been shown to be superior to culture-directed oral antibiotics. Although interesting to note, CS patients had more severe baseline disease when compared to CON, but demonstrated comparable improvement in subjective symptoms and objective endoscopic scores suggesting it may be more than just a placebo effect. It is possible that CS treatment over 10 days is sufficient to demonstrate symptomatic and endoscopic improvement but insufficient time to achieve bacterial eradication. Indeed, when compared with topical mupirocin rinses which have been one of the more successful topical treatments for recalcitrant patients[172-176], mupirocin has been used as a twice-daily rinse over 3-4 weeks. We believe that this reflects the duration of CS treatment needs to be further optimised. A longer study period including a larger number of study participants would be needed to assess the safety and efficacy of CS topical application in these patients.

The spherical colloidal silver nanoparticles used in this study has been shown to have substantial anti-biofilm activity *in vitro* with 96%, 97%, and 98% biofilm reduction of *S. aureus,* MRSA, and *P. aeruginosa* respectively[221]. It has been postulated that colloidal silver exerts its antimicrobial properties via multiple mechanisms. It can act on bacterial cell membranes by disrupting phosphate[215] and sodium channels[216], inhibits mitochondrial ATPase[217] and interacts with bacterial DNA to form dissociable complexes [218, 219].

Some immunomodulatory functions of colloidal silver have also been observed in the literature. It has the ability to inhibit matrix metalloproteinases (MMPs) which is pro-inflammatory[522] and metallothionein[522] (MT) which promotes resistance to immune-mediated apoptosis[523]. Both MTs and MMPs have been found at increased levels in patient with chronic rhinosinusitis with nasal polyps (CRSwNP) [524-526]. Colloidal silver has also been shown to induce inflammatory cells apoptosis by TNF-α and IL-12 suppression[527]. An improved host response might be able to account for the efficacy observed in the colloidal silver cohort even though there was no eradication of bacteria.

However, one of the limitations of this study is the time-consuming process of manufacturing CS rinses using small scale equipment. Currently, to prepare sufficient CS for a 10-day treatment course a full-time laboratory personnel requires over 10-15 hours. If production cannot be upscaled, CS could be evaluated as an adjunct to oral antibiotics.

In the literature, silver has been described to exhibit low toxicity with minimal risks expected from clinical exposure. Silver is absorbed into the systemic circulation as a protein complex and eliminated by the liver and kidneys.[528] Prolonged silver exposure commonly associated with occupational and/or systemic administration can lead to deposition of silver particles in skin (argyria), eye (argyrosis) and other organs[529]. Argyria is a cosmetic concern with irreversible blue-grey skin discoloration in sun-exposed areas, but not life-threatening.

Reported cases of silver toxicity are limited. In the literature, very little data exists correlating serum silver levels with symptomatic presentation of argyria and at present

there are no medical guidelines available regarding its use. The World Health Organisation reported that a person can have a total lifetime oral intake of approximately 10 g of silver with no observed adverse effects[530]. The United States Environmental Protection Agency's has reported that a maximum acceptable oral *dose* of silver to be 0.005 mg/kg/day or about 0.35mg for a 70 kg person a day, every day during their lifetime[531]. In this study patients will be exposed to a total of 72 mg of topical CS rinses, which is well under the total lifetime amount of 10 g and to an equivalent of 7.2 mg/day of topical silver treatment for 10 days. Our laboratory reference of serum silver levels indicates argyria could be present when serum silver levels exceed 100 nmol/L. The serum silver levels were well below this concentration and no symptoms of argyria were observed in any patient of this study.

Although this study has shown that colloidal silver is safe based on serum silver levels and smell tests, the discomfort of using CS rinses have been noted. This discomfort is likely due to the tonicity and temperature of the rinses and possible stinging properties from silver. To improve the tonicity of the rinse solution for better tolerability, we are currently looking at mixing colloidal silver with 5% dextrose isotonic solution.

8.6 Conclusion

This study concludes that twice daily CS (0.015 mg/mL) sinonasal rinses for 10 days is safe but not superior to culture-directed oral antibiotics. Future studies looking at optimizing the tolerability, duration of treatment and investigating the role of CS as an adjunct treatment to oral antibiotics should be explored and evaluated in a randomised, double-blinded, placebo-controlled trial.

Chapter 9: Manuka honey sinus irrigations in recalcitrant chronic rhinosinusitis: Phase 1 randomized single-blinded placebo-controlled trial

Statement of Authorship

Title of Paper	Manuka honey sinus irrigations in recalcitrant chronic rhinosinusitis: Phase 1 randomized, single-blinded, placebo-controlled trial
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	Published in International Forum of Allergy and Rhinology, 28 August 2019

Principal Author

Name of Principal Author (Candidate)	Mian Li Ooi
Contribution to the Paper	Project design, data collection and analysis, manuscript preparation
Overall percentage (%)	100%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 1/9/2019

Co-Author Contributions

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

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

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

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

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9.1 Abstract

Background: Manuka honey (MH) has significant anti-biofilm activity *in vitro* and *in vivo* against *S. aureus*, *MRSA* and *P. aeruginosa*. This is the first randomised, single-blinded, placebo-controlled Phase 1 clinical trial investigating the safety and preliminary efficacy of MH with augmented methylglyoxal (MGO) rinses in recalcitrant chronic rhinosinusitis (CRS).

Methods: Patients were included after previously undergoing endoscopic sinus surgery and presenting with signs and symptoms of sinus infection with positive bacterial cultures on sinus swabs. Patients were randomised to receive 14 days of twice daily 16.5% MH + 1.3 mg/mL MGO sinonasal rinses and concurrent 10 days of placebo tablets (MH), or 14 days of twice daily saline sinonasal rinses and concurrent 10 days of culture directed antibiotic therapy (CON). Safety observations included University of Pennsylvania Smell Identification Test (UPSIT) and adverse event (AE) reporting. Efficacy was assessed comparing microbiology results, Lund Kennedy Scores (LKS) and symptom scores using Visual Analogue Scale (VAS) and Sino-Nasal Outcome Test (SNOT-22).

Results: 25 patients completed the study. MH demonstrated good safety profile with no major adverse events and no changes in UPSIT. 6/10 (60%) MH patients had a reduction in bacterial culture rate with 1/10 of those having negative cultures, compared to 12/15 (80%) in the control group with 7/15 having negative cultures upon completion of the study.

Conclusion: This study concludes that twice daily 16.5% MH augmented with 1.3 mg/mL MGO sinonasal rinses alone for 14 days is safe but not superior to culture-directed oral antibiotics and twice daily saline rinses.

9.2 Introduction

Recalcitrant chronic rhinosinusitis (CRS) associated with bacterial biofilms continues to be a challenging clinical entity. For recalcitrant patients, antibiotics often alleviate symptoms in acute exacerbations. However, they fail to eradicate the biofilm nidus which periodically sheds planktonic organisms resulting in a relapsing and remitting course of disease[85]. This repeated use of antibiotics raises increasing concern of propagating antibiotic resistance. Therefore, the search for novel topical anti-biofilm therapies continues to be an area of interest. Topical agents allow increased concentration, localised action, confer less systemic side effects and lessen the risk of antibiotic resistance.

Manuka honey (MH) (*Leptospernum scoparium*) has antibiofilm effects against a broad spectrum of gram-positive and gram-negative bacteria, including *Streptococcus* and *Staphylococcus* species, *Pseudomonas aeruginosa, Escherichia coli, Proteus mirabilis, Enterobacter cloacae* and *K. pneumoniae*[532-535]. The combined effect of low pH, hyperosmolarity, hydrogen peroxide activity[203] and methylglyoxal (MGO) content [204, 205] confers MH its unique biofilm-cidal properties. In order to effectively deliver MH to the sinuses it has to be diluted to 16.5%[536]. A previous optimization pilot study conducted by our department indicated that 16.5% MH is the upper limit of tolerability (unpublished data). At this dilution our previous studies have shown that the antibiofilm property of MH can be significantly enhanced by increasing the natural MGO content of MH up to 1.8mg/mL and still remained safe to sinonasal mucosa cilia and mucosa [537, 538]. Its low propensity to develop bacterial resistance[206, 207], ability to increase

susceptibility of resistant strains to antibiotics[208], anti-oxidant and anti-inflammatory[539] properties have further fuelled interest in MH being an alternative to oral antibiotics.

Despite this, its potential as a topical antibacterial rinse to treat recalcitrant CRS exacerbations is still unclear due to the lack of consistent data on its safety and efficacy. Differences in the honey sourced, concentration of MH used, MGO content, dosing intervals and duration, the type of infection treated (types of pathogen, single organism or multispecies), has made it difficult to develop a consensus on its use.

This is the first randomized, single-blinded placebo-controlled Phase 1 clinical trial evaluating the safety and preliminary efficacy of MH rinses in recalcitrant CRS.

9.3 Methods and Materials

Manuka Honey, saline rinses and medications

Augmented manuka honey (MH), consisting of 16.5% MH augmented with 1.3 mg/mL Methylglyoxal (MGO), was obtained from Watson & Son LP (Masterton, New Zealand). NeilMed Pharmaceuticals (Santa Rosa, CA, USA) packaged the augmented MH and isotonic saline rinse solutions. Placebo tablets were obtained from Pharmaceutical Packaging Professionals (Melbourne, Victoria, Australia). Oral antibiotics were supplied by the Department of Pharmacy Clinical Trials Unit, The Queen Elizabeth Hospital, Woodville, Australia.

Participants and study design

This was a prospective, randomised, single-blinded, placebo-controlled Phase 1 human clinical trial evaluating the safety and preliminary efficacy of MGO-augmented MH sinonasal rinses in patients with recalcitrant CRS, after endoscopic sinus surgery, between February 2016 and January 2018. Ethics approval was granted by the Central Northern Adelaide Health Service, Ethics of Human Research Committee (Protocol Number HREC/14/TQEHLMH/165) to conduct the trial within its network of teaching hospitals in Adelaide, Australia. All subjects gave written informed consent in accordance with the Declaration of Helsinki. Full inclusion and exclusion criteria are outlined in Table 24.

Inclusion Criteria	Exclusion criteria
Must have two or more ongoing symptoms of CRS (nasal discharge, postnasal drip, nasal obstruction, facial pain or pressure, reduced sense of smell) AND Endoscopic evidence of CRS	Allergy to honey
Ongoing symptoms of CRS despite at least one trial of oral antibiotics	Antibiotics in previous 2 weeks
ESS >12 weeks prior to enrolment	Taking oral corticosteroids
Patent sinuses deemed suitable for trial on investigator's discretion	Pregnant or breastfeeding
Positive bacterial microbiology swab	Immunocompromised or systemic disease (ie. Granulomatosis polyangiitis, cystic fibrosis)
Over 18 years of age AND able to give written informed consent	
Willing to return to this centre for postoperative follow-up care	

Table 24. Inclusion and exclusion criteria.

On the screening visit, all participants who were eligible and consented to trial had endoscopic videos recorded and endoscopically guided sinonasal swabs taken for microbiological evaluation. Culture swabs were taken under direct endoscopic guidance of frank mucopus visualized draining from sinus ostia or within sinuses. Microscopy, culture and sensitivity tests were performed with standard semi-quantitative analysis of bacterial culture rate reported as scant, light, moderate or heavy (equivalent to 1+, 2+, 3+ or 4+) by the laboratory, with no bacteria cultured reported as no growth or negative swab.

Patients completed symptom score questionnaires using Sino-Nasal Outcome Test-22 (SNOT-22)[505] (22 items, each scored from 0–5; total score range 0-110) and a Visual Analogue Scale (VAS)[506] (an overall score and 6 individual scores on nasal obstruction,

anterior rhinorrhea, postnasal drainage, sneezing, headache/ facial pressure, alteration of sense of smell; each scored from 0-100, total score range 0–100). To assess safety, all patients completed the University of Pennsylvania Smell Identification Test (Sensonics International, NJ, USA), a validated assessment tool to measure olfaction[540] and were advised to report any adverse outcomes during and after the study. Endoscopic videos were scored by a blinded independent surgeon using the Lund Kennedy Score (LKS)[505, 507] (score range, 0–20).

After culture and antimicrobial sensitivity result became available within 7-10 days, a blinded physician prescribed an appropriate oral antibiotic for the patient to be handed to the Department of Pharmacy Clinical Trials Unit, The Queen Elizabeth Hospital, Woodville, Australia. Patients were randomised to either the manuka honey arm (MH) (n = 10) or control arm (CON) (n = 15) based on a computer-generated list. Based on patients' randomised cohort, the appropriate treatment agents were dispensed by the hospital pharmacist directly to participants.

MH group patients were given 28 NeilMed rinse bottles each containing 240 mL of 16.5% MH augmented with 1.3 mg/mL MGO and 20 placebo tablets. CON group patients were given 28 NeilMed rinse bottles containing isotonic saline solution and 10 days of culture directed oral antibiotic tablets. Both the MH and isotonic saline solutions were premixed in bottles. The MH solution is the same consistency as saline, except the MH solution had an orange tinge and tastes sweet. However, patients were informed at the start of the trial that both solutions have been made to appear and taste the same. We elected not to add sweeteners to the placebo saline arm so as not to add possible confounding ingredients to the solution that may affect efficacy.

They were also instructed to store all irrigation bottles in the refrigerator (4-8°C) for the duration of the trial (as requested by the Ethics committee). Prior to each use, patients were asked to place irrigation bottles in the microwave for no longer than 20 seconds, invert the bottle 2-3 times, then perform the rinse twice daily for 2 weeks. In this study, all patients were on topical budesonide irrigations prior to enrolment and were given specific instructions to continue on the frequency they had been on prior to recruitment to ensure that the factor of topical INCS has been controlled for.

Patients were reviewed 2 weeks post- treatment and a repeat endoscopic bacterial swab was obtained with post- treatment UPSIT, SNOT-22, VAS and LKS scores recorded.

Data Analysis

All results were statistically analysed at the completion of the study. The statistical software used was SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Baseline demographic characteristics between both treatments cohorts were statistically analysed (Table 25). Linear regression and mixed effects models were used to investigate the association between 6 individual symptoms (VAS difference) and both treatment groups (Table 26). Linear mixed-effects models were used to investigate the association between the outcomes VAS (overall score), SNOT-22 and LKS over time; within groups post–treatment vs pre- treatment (Post Tx vs Pre Tx) and between groups (CON vs MH group). A compound symmetry covariance structure was used to adjust for repeated measurements over time (pre and post treatment measurements were taken). A linear regression was performed to investigate the association between differences in UPSIT over time between treatment groups. Assumptions of a linear model were found to be

upheld throughout by inspection of histograms and scatter plots of residuals and predicted values. Results are given in Table 27.

9.4 Results

Patient characteristics

A total of 25 patients completed the study (11 females, 14 males, aged 27-86). All patients had complete ESS (total frontosphenoethmoidectomy and maxillary antrostomy) with a number of patients who have had frontal drillouts for recalcitrant disease. Patients needed to be at least 3 months after surgery with patent sinus ostia prior to being considered for recruitment. This was based on previously published literature which suggests that it takes at least 3 months post-surgery for the sinuses to stabilize. In this study, patients had their last surgery on average 5 years ago. 10/15 patients in CON were CRSwNP while 10/10 patients in MH were CRSwNP. Baseline demographic and further clinical characteristics are demonstrated in Table 25 and there were no significant differences in baseline demographic characteristics between both treatment groups.

	CON (n=15)	**MH (n=10)**	**P value**
Age, year	63.5 (47.3-69)	58 (48-68)	0.3968
Gender, female	7/15	4/10	1.0000
Visual analogue scale	35.6 (23-54.7)	40.5 (27.4-55.2)	0.8958
SNOT-22 score	35(22-57)	38.5 (22.3-56)	0.9748
Lund-Kennedy score	7.5 (6-10)	9 (6-12)	0.5206
CRSwNP	10/15	10/10	0.0613
Frontal Drillout	10/15	7/10	1.0000
Asthma	5/15	5/10	0.4422
ASA sensitivity	1/15	1/10	1.0000
Allergic rhinitis	6/15	6/10	0.4283
Current smoker	0	0	1.0000
Average no of previous sinus surgeries per patient	2 (1.5-7)	3 (1-3)	0.4699

Table 25. Baseline patient demographics and clinical characteristics. Data are medians (interquartile range) or numbers.

Efficacy

Microbiology Result. 7/15 (47%) patients in CON group had negative swabs and a further 5/15 (33%) patients had a reduction in standard semi-quantitative bacterial culture rate when comparing pre-treatment with post-treatment. 1/10 (10%) MH patient had negative swabs and a further 5/10 (50%) had a reduction in standard semi-quantitative bacterial culture rate. Microbiology results for both groups are described in Table 26.

Oral antibiotics		**Manuka Honey**	
Pre Treatment	**Post Treatment**	**Pre Treatment**	**Post Treatment**
Heavy *S. aureus*	No growth	Heavy *S. aureus*	No growth
Heavy *S. aureus*/ Heavy *Citrobacter*	Moderate *S. aureus*	Heavy *S. aureus*	Moderate *S. aureus*
Heavy *S. aureus*	No growth	Moderate *S. aureus*	Light *S. aureus*
Moderate *S. aureus*	No growth	Heavy *S. aureus*/ Heavy *H. Influenzae*	Light *S. aureus*/ Moderate *H. Influenzae*
Moderate *S. aureus*	No growth	Scanty *S. aureus*	Light *S. aureus*
Heavy *S. aureus*	No growth	Heavy *S. aureus*	Scant *S. aureus*
Moderate *H. Influenzae*	No growth	Heavy *K. Pneumoniae*	Heavy *K. Pneumoniae*/ Heavy *MRSA**
Moderate *S. Pneumoniae*/ Moderate *Pseudomonas*	No growth	Moderate *S. aureus,* Light *Pseudomonas*	Moderate *P. aeruginosa*
Scanty *S. aureus*	Light *S. aureus*	Moderate *S. aureus*	Moderate *E coli*
Moderate *S. aureus*	Scanty *S. aureus*	Moderate *Pseudomonas*	Moderate *Pseudomonas*/ Moderate *S. aureus*
Moderate *S. aureus*	Moderate *S. aureus*		
Heavy *S. aureus*	Scanty *S. aureus*		
Moderate *MRSA*/ Moderate *Pseudomonas*	Moderate *Pseudomonas*		
Moderate *S. aureus*	Moderate *S. aureus*		
Heavy *S. aureus*	Light *S. aureus*		

Table 26. List of pathogens Pre- and post- treatment culture results in both cohorts. Standard semi-quantitative analysis of bacterial load reported as scant, light, moderate or heavy (equivalent to 1+, 2+, 3+ or 4+) by laboratory. *P. aeruginosa = Pseudomonas aeruginosa*, *MRSA = Methicllin resistant staphylococcus aureus*, *S. aureus = Staphylococcus aureus*, *H. influenza = Haemophilus Influenzae, E. cloaca = Enteroboacter cloacae, S. pneumonia = Streptococcus pneumonia*, *E. coli = Escherichia coli, K. pneumoniae = Klebsiellla pneumoniae, Citrobacter koseri = Citrobacter*

* Patient had previously cultured MRSA

Visual Analogue Scale (VAS)

Patients in both CON and MH groups showed no significant difference in Post Tx vs Pre Tx overall symptom measured with VAS (CON -9.93 [95% CI -20.51, 0.65] vs MH -2.1 [95% CI -15.06, 10.86]) (Figure 54). There was no statistically significant interaction between groups (CON vs MH) and time for the outcome of overall symptoms VAS (interaction P value=0.3427). There was also no statistically significant difference in individual symptom scores for nasal obstruction, anterior rhinorrhea, postnasal drainage, sneezing, headache/ facial pressure, alteration of sense of smell between the CON and MH groups over time (Table 27) and no significant difference in VAS scores between symptoms (global P value=0.9259).

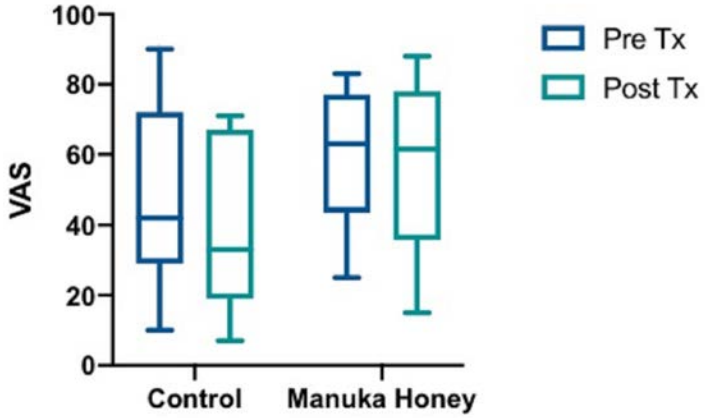

Figure 54. Box plot graph showing Visual Analogue Scale (VAS) scores pre- and post- treatment in CON group and MH treated group.

Outcome	*Predictor*	*Comparison*	*Estimate (95% CI)*	*Global P value*
Nasal blockage	Group	Control vs Manuka	-8.35 (-24.56, 7.86)	0.3127
Nasal discharge	Group	Control vs Manuka	2.95 (-13.18, 19.08)	0.7200
Postnasal drip	Group	Control vs Manuka	-13.28 (-30.38, 3.83)	0.1282

Outcome	*Predictor*	*Comparison*	*Estimate (95% CI)*	*Global P value*
Sneezing	Group	Control vs Manuka	3.88 (-9.02, 16.79)	0.5554
Headache facial pain or pressure	Group	Control vs Manuka	-2.79 (-14.96, 9.39)	0.6537
Reduction in smell	Group	Control vs Manuka	-7.88 (-23.04, 7.29)	0.3087

Table 27. Linear regressions of individual symptoms (difference in VAS score) versus treatment.group.

Sino-Nasal Outcome Test -22 (SNOT-22)

Patients in both CON and MH groups showed no significant difference in total SNOT-22 scores Post Tx vs Pre Tx (CON -6.3 [95% CI -13.5, 0.8] vs MH -4.4 [95% CI -13.1, 4.4]) (Figure 2). There was no statistically significant interaction between groups (CON vs MH) and time for the outcome SNOT 22 (interaction P value=0.7197). (Table 28 and Figure 55)

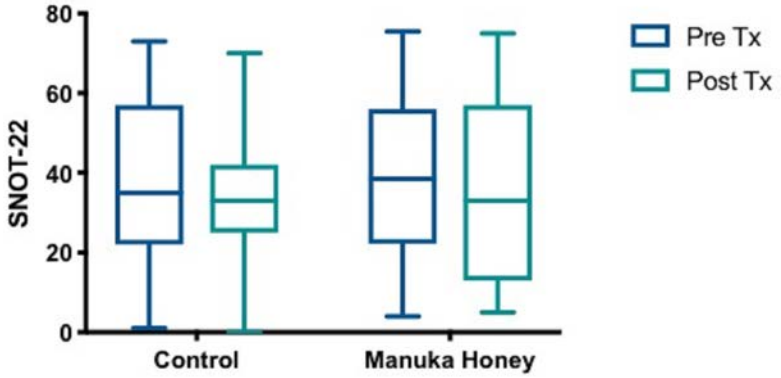

Figure 55. Box plot graph showing SNOT-22 pre- and post- treatment in CON group and MH treated group.

Outcome	Mean (SD) – Control Pre Tx	Mean (SD) – Control Post Tx	Mean (SD) – Manuka Pre Tx	Mean (SD) – Manuka Post Tx	Constant	Comparison	Estimate (95% CI)	Comparison P value	Interaction P value
VAS (Overall symptoms)	48.3 (24.4)	38.4 (21.4)	58.9 (20.5)	56.8 (24.1)	Pre Tx	Control vs Manuka	-10.57 (-29.74, 8.61)	0.2660	0.3427
					Post Tx	Control vs Manuka	-18.40 (-37.57, 0.77)	0.0592	
					Control	Post Tx vs Pre Tx	-9.93 (-20.51, 0.65)	0.0644	
					Manuka	Post Tx vs Pre Tx	-2.10 (-15.06, 10.86)	0.7404	
SNOT 22	39.3 (22.4)	33.0 (18.1)	39.1 (23.1)	34.7 (24.0)	Pre Tx	Control vs Manuka	0.3 (-18.0, 18.6)	0.9747	0.7197
					Post Tx	Control vs Manuka	-1.7 (-20.0, 16.6)	0.8492	
					Control	Post Tx vs Pre Tx	-6.3 (-13.5, 0.8)	0.0796	
					Manuka	Post Tx vs Pre Tx	-4.4 (-13.1, 4.4)	0.3144	
LKS	7.9 (2.3)	5.7 (3.5)	8.9 (3.1)	6.8 (2.1)	Pre Tx	Control vs Manuka	-1.0 (-3.5, 1.5)	0.4298	0.9136
					Post Tx	Control vs Manuka	-1.1 (-3.6, 1.4)	0.3783	
					Control	Post Tx vs Pre Tx	-2.2 (-3.6, -0.8)	0.0033*	
					Manuka	Post Tx vs Pre Tx	-2.1 (-3.8, -0.5)	0.0150*	
	Mean (SD) – difference for Control		**Mean (SD) - difference for Manuka**						
UPSIT	0.4 (4.3)		-2.4 (6.4)			Control vs Manuka	2.8 (-1.6, 7.2)	0.2141	-

Table 28. Regression results and descriptive statistics for four outcome variables

Lund Kennedy Score (LKS)

For the CON group, there was a statistically significant difference in LKS outcome Post Tx vs Pre Tx (comparison P value=0.0033). Patients Post Tx had a mean LKS score 2.2 units less than patients Pre Tx (estimate=-2.2, 95% CI: -3.6,-0.8). For the MH group, there was a statistically significant difference in LKS outcome Post Tx vs Pre Tx (comparison P value=0.0150). Patients Post Tx had a mean LKS score 2.1 units less than patients Pre Tx (estimate=-2.1, 95% CI: -3.8,-0.5). There was no statistically significant interaction between groups (CON vs MH) and time for the outcome LKS (interaction P value=0.9136), which indicates that although both cohorts had significant improvement Post Tx there is no evidence that either treatment were more superior. (Table 28 and Figure 56)

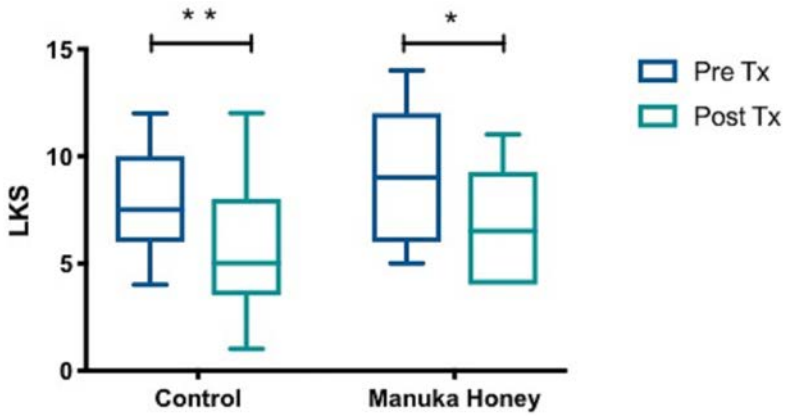

Figure 56. Box plot graph showing Lund Kennedy Scores (LKS) pre- and post- treatment in CON group and MH treated group.

Safety

Smell Test. There were no significant differences in smell pre- and post- treatment between both groups measured using the University of Pennsylvania Smell

Identification Test (UPSIT), (p=0.21, 2.8 [95% CI -1.6, 7.2]. Above 0 (positive values) means improvement in smell, below 0 (negative values) means deterioration in smell. MH group showed some improvement in smell but this was not statistically significant. (Table 28 and Figure 57)

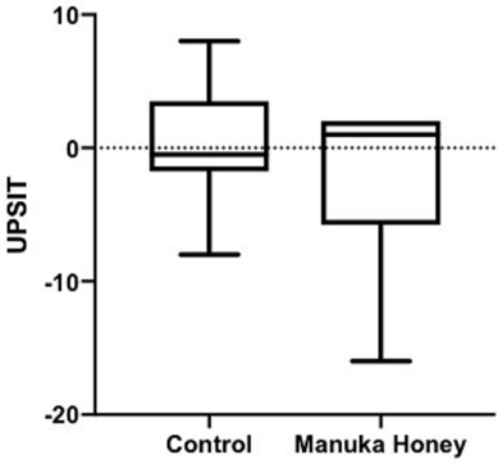

Figure 57. Box plot graph demonstrating University of Pennsylvania Smell Identification Test (UPSIT) scores pre- and post- treatment in CON group and MH treated group.

Adverse Events. There were no serious adverse events (SAEs). Tolerance was not specifically measured but all patients tolerated the MH rinses well and no patient withdrew from the study due to tolerability issues. 4 patients from MH group were withdrawn from the trial; 1 had spontaneous epistaxis following the first application of MH rinse which self- resolved; 1 required antibiotic treatment for community acquired pneumonia during the trial; 2 had contaminated MH rinses. Thereafter 0.01% of Benzylkonium Chloride was added to all MH and control rinses as a preservative.

9.5 Discussion

This Phase 1 safety study confirms the tolerability and safety profile of twice daily 16.5% MH + 1.3 mg/mL MGO sinonasal rinses over 14 days. In terms of secondary outcomes, culture-directed oral antibiotics with saline rinses appear to be superior to MH only in eradication of infection on culture. Culture negativity observed in CON was not reflected in symptoms improvement measured using VAS and SNOT-22. Both CON and MH groups did not show statistically significant improvement in VAS and SNOT-22 outcomes. This appears to be consistent with clinical observations of patients having variable symptoms improvement post oral antibiotics.

Interestingly, both CON and MH groups demonstrated significant endoscopic improvements in LKS suggesting a level of efficacy was demonstrated with the MH cohort. Although not comparable to outcomes observed in CON group in microbiological eradication, the MH group did demonstrate a reduction in bacterial growth for the majority of patients with corresponding significant improvement in LKS, suggesting that MH irrigations may have therapeutic value, perhaps with modification to the dosing or regimen duration.

Considering this cohort of patients all had multiple revision surgeries for recalcitrant disease and are often on frequent courses of oral antibiotics, the option of MH irrigations offering demonstrable improvement in just 2 weeks without the potential adverse effects known to oral antibiotics warrants further investigation and optimisation of this treatment option.

There has been mixed data in the literature about the role of MH as a topical treatment for recalcitrant CRS. Thamboo et al[211] reported daily 2mL atomized MH- saline spray to single nostrils for 30 days in allergic fungal rhinosinusitis (AFRS) using the contralateral nostril as control. The study found significant improvement in SNOT-22 scores but no change in culture result or difference in endoscopy scores between MH treated and untreated sinuses. However, the study's findings suggested that patients with high serum IgE levels were more likely to respond favorably.

The first randomized controlled, single-blinded trial investigating topical MH in recalcitrant CRS was conducted by Lee et al[212]. Patients received 10% (wt/vol) MH with unspecified MGO content or saline sinus rinses twice daily for 30 days. Both cohorts were offered oral antibiotics and/or oral/topical steroids. In that study, it was reported that 42% of patients on MH rinses had eradication of infection compared to 19% of patients on saline rinses. Unfortunately, this study was limited by the difference in baseline disease between treatment and control group. The control group had more CRSwNP patients with higher Lund Mackay scores, and greater proportion of patients with asthma and aspirin sensitivity. However, it is interesting to note that the treatment duration in this study was over 30 days.

It is possible that in our study the duration of MH treatment (14 days) was insufficient to achieve bacterial eradication, particularly since the majority of patients showed a reduction in microbial culture load which was reflected clinically with improvement in endoscopic scores. The study had been designed as a 14 day treatment as we considered 1) matching the duration of both treatment arms whilst we also set out to ascertain MH's

minimum effective dose, 2) the feasibility of patients transporting and storing more than 2 weeks of MH treatment (> 6.7 Litres). We believe that the duration of MH treatment may need to be further optimised, especially taking into account the outcome of other studies using MH rinses for up to 4 weeks with more favourable microbiology results[212]. Previous cohort studies with topical treatments for recalcitrant CRS, such as mupirocin rinses, are commonly used as a twice-daily rinse over 3-4 weeks[172-176].

MH has been shown *in vitro* to augment the effects of antibiotics[208, 541, 542]. Synergistic effects of MH have been demonstrated with rifampicin, tetracycline, imipenem and mupirocin against *MRSA*; and rifampicin, tetracycline and colistin against *P. aeruginosa*. Interestingly, studies have also shown that in the presence of low dose MH, resistance of MRSA to oxacillin can be reversed[543] and confers improved bacteriostatic efficacy at below minimum inhibitory concentrations (MIC)[541]. This raises the potential role of MH rinses being used as an adjuvant to antibiotics.

MH rinses used in this study incorporated MGO at 1.3 mg/mL, which was well below the reported threshold for ciliary toxicity while retaining sufficient anti-biofilm potency. Indeed, Paramasivan et al[210] were the first to highlight that different MGO concentrations in MH affected MH's anti-biofilm efficacy and safety profile to the sinus mucosa. In their study they have concluded that twice daily 16.5% (wt/vol) MH augmented with MGO between 0.9 mg/mL and 1.8 mg/mL over 5 days reduced mature *S. aureus* biofilm while showing no toxic effect on ovine sinus mucosa and cilia.[210] In line with these findings, there were no serious adverse effects or changes in smell observed in patients post MH treatment.

There was however 2 incidents of contaminated MH rinses which developed a visible suspended discoloured substance within the wash. These washes were discarded by the patients. The addition of benzylkonium chloride did not alter the taste, colour, viscosity or smell of the MH solution and were unlikely to cause unblinding of the subjects. Although we made best possible attempts to preserve blinding. It is possible that patients could perceive a difference in the taste or smell of the MH.

As this was primarily a phase 1 safety study, patient recruitment was deliberately curtailed. As a result it was underpowered for assessing efficacy. Whilst a positive trend in subjective and objective symptom scores was observed in patients treated with MH rinses, statistical significance was reached only for Lund-Kennedy scores. In contrast to our study, where the control group was treated with oral antibiotics and saline nasal rinses, previous studies testing efficacy of MH rinses compared outcomes with saline rinses alone. It is hypothesised that MH could confer some therapeutic advantages when used over a longer period as our study showed outcome measures for the MH group trended towards positive outcomes similar to outcome measures seen in the control group. An adequately powered study testing MGO augmented MH rinses over a prolonged period of up to 30 days is needed to confirm this hypothesis.

9.6 Conclusion

This study has shown that MH with augmented MGO is safe following application for 2 weeks but not superior to culture-directed antibiotic therapy and saline rinses in eradicating bacterial cultures. However, a significant improvement in endoscopic sinus scores can be seen in both groups, which suggests some potential benefit from the MH rinses in the management of recalcitrant CRS. Future studies looking at optimizing the duration of treatment or the role of MH as an adjunct to antibiotics should be assessed in a larger sample size.

Acknowledgement

We acknowledge Suzanne Edwards from the University of Adelaide for her statistical support services.

Thesis Summary

In the past decade we have witnessed an exciting time in rhinology that has evolved our understanding of the pathomechanics of Chronic Rhinosinusitis (CRS). Our historical classification of CRS based on clinical phenotype is now considered insufficient to adequately define and manage this disease. With new understanding of the different CRS subtypes now termed as endotypes we are now able to better appreciate the nature of the chemical processes driving this disease and it has provided valuable insights into the incongruent responses observed in our patients.

This new understanding has resulted in a complete shift in the paradigm of our clinical management, with a gradual shift away from the one-size-fits-all approach, towards personalized treatments specific to the pathomechanics driving each patient's disease. It has also reflected an increased shift towards intensifying medical management to complement surgical management in CRS. This has become particularly relevant in managing the subset of patients with recalcitrant CRS.

These are patients who have previously undergone endoscopic sinus surgery who present with recurrent symptoms of sinus infection with frank mucopus visualized draining from sinus ostia or within sinuses. With increasing polymicrobial and multidrug resistant infection, compounded by the lack of development of new antibiotics, the management of recalcitrant CRS has been increasingly challenging as we try to gradually shift away from overprescribing oral antibiotics. As we know oral antibiotics are ineffective against biofilms, biofilms have demonstrated up to 1000x resistance against oral antibiotics than in its planktonic form. Repeated use of oral

antibiotics further drives antimicrobial resistance and depletes sinonasal microbiome diversity.

Topical antimicrobial therapies have been proposed for their antibiofilm properties at higher concentrations at a locally targeted site, without the many adverse effects known to oral antibiotics. Although the concept of topical therapies in recalcitrant CRS is appealing it has been met with sluggish reception. We hope that this thesis adds to this existing body of work and offers exciting directions for further research in this area of rhinology study. This thesis presents several novel topical antimicrobial agents in the treatment of recalcitrant CRS in different phases of preclinical and clinical development.

Our first challenge was to reassess our current methods of topical drug delivery into sinus cavities. Current options lack the ideal characteristics of thorough sinus distribution, sufficient mucosal contact time for local drug absorption and minimal waste. We attempted to reconsider a novel method of drug delivery, using Chitogel® which comprises succinyl-chitosan produced by the hydrolysis of chitin, found in the exoskeletons of crustaceans. Chitogel has been FDA approved for use in rhinology to improve patient's outcome post endoscopic sinus surgery due to its effective hemostatic, wound healing, anti-adhesion and antimicrobial[311-313] effects. However its potential as a slow release drug delivery vehicle to distribute anti-biofilm therapies into sinus cavities to manage recalcitrant CRS has yet to be explored. Chitosan has been postulated to increase nasal mucosal absorption of drugs through tight junctions due to the bioadhesivity properties of the chitin polymer.

Our first study incorporated Chitogel with known antibiofilm agent Mupirocin and intranasal corticosteroid Budesonide previously found to have positive outcomes in recalcitrant CRS and optimised the application protocol of the gel. Chitogel-Budesonide-Mupirocin (CG-BM) was found to significantly reduce *S. aureus* biofilms *in vivo* and is the first study to suggest an alternative method of topical drug delivery to sinuses. Although histopathology analysis showed CG-BM reduced the degree of inflammation, acute inflammation, goblet cell hyperplasia, oedema, and fibrosis of infected sinonasal mucosa when compared to no treatment, it did not reach statistical significance. We postulate that this combination gel treatment could be further optimised by increasing the dose of incorporated budesonide as efficacy observed towards self-limiting inflammatory process post-surgery could be different to inflammation secondary to a bacterial nidus. Also taking into account the limited and slow release of budesonide from Chitogel (18% from the total 2mg incorporated), which is still far below the safety dose of long-term intranasal budesonide use of 2mg/day.

Our next study investigated a novel combination of antimicrobial agents Deferiprone and Gallium Protoporphyrin IX. It represents the first *in vivo* study of this novel antimicrobial agent targeting the iron metabolism pathways crucial for bacterial survival[263, 264]. We incorporated Deferiprone and Gallium Protoporphyrin IX into Chitogel. Chitogel enhances the therapeutic effect of this antimicrobial combination due to its unique drug release sequence profile. Deferiprone (hydrophilic) is released first from the hydrogel and is thought to chelate iron from the bacteria's surrounding environment, forcing the bacteria to upregulate their iron transporter proteins. Gallium Protoporphyrin IX (hydrophobic), which is released at a more sustained rate from the

hydrogel, acts as a heme analogue to encourage uptake into bacterial cells, thereby augmenting its antimicrobial efficacy. CG-DG has known anti-biofilm activity not only against *S. aureus* but also MRSA, *S. epidermidis* and *P. aeruginosa* biofilms[262]. It has been reported that CG-DG surpasses the efficacy of ciprofloxacin against pseudomonas infections without the adverse effects of ciprofloxacin and has potent synergistic properties against MRSA. In our *in vivo* sheep sinusitis model, CG-DG was found to be safe in sinonasal application and effective in killing *S. aureus* biofilms. Histopathology analysis also showed a significant reduction in the degree of inflammation in sheep sinus mucosa between CG-DG treated group and no treatment.

Although the anti-inflammatory effects observed in our *in vivo* model could be due to effective eradication of biofilms, it prompted us to further characterise the therapeutic properties of Deferiprone on wound healing. In our third paper, we were able to demonstrate that Deferiprone significantly delayed fibroblast migration in a dose-dependent way without affecting the re-epithelisation rate of human nasal epithelial cells (HNECs). Deferiprone also reduced pro-inflammatory cytokine IL-6, collagen secretion and reactive oxygen species release from HNECs and sinonasal fibroblasts. Deferiprone's inherent wound healing properties has potential in preventing adhesions post endoscopic sinus surgery which commonly leads to recalcitrant CRS.

In our subsequent studies we present our translational research on bacteriophage therapy. Bacteriophage (phage) therapy was first proposed as an antibacterial treatment as early as the 1910s and subsequently fell out of favour during the war that paved the way for penicillins. However, with the exponential increase of antibiotic resistant infections in the last decade, interest in phage has been revived. Phage are viruses that

infect only one or a few closely related bacterial species with no pathogenic effect on mammalian cells. This selective antimicrobial therapy with the ability to preserve sinonasal microbiota is an exciting new alternative. Its value in the field of rhinology has increased relevance now in light of our current understanding of microbial diversity and sinus health.

Our fourth paper looked at the long-term safety of a *S. aureus* phage cocktail to the frontal sinuses *in vivo*. NOV012 comprises 2 bacteriophage strains K710 and P68 and has an 85% efficacy against local CRS clinical isolates including MRSA. 2×10^6 pfu/mL of active NOV012 bacteriophage cocktail was found to be safe in sheep sinonasal application over 20 days with no phage detected in the blood stream. This safety data supports the potential use of bacteriophage as a topical antimicrobial treatment in CRS.

In the fifth paper we presented the safety and efficacy of *Pseudomonas aeruginosa* bacteriophage cocktail (CT-PA) over 3 weeks *in vivo*. *Pseudomonas aeruginosa* has been known as a phenomenon of bacterial resistance and declared by the World Health Organisation as critical priority for new antibiotic development. CT-PA was found to be safe in sinonasal application and effective in reducing pseudomonas biofilms in all 3 CT-PA concentrations of 1 $x10^8$-10^{10} PFU/mL. In this study, phage were found sporadically in blood and organs but were found consistently in faeces.

Our sixth paper presents a phase-1 human clinical trial investigating the safety and preliminary efficacy of *S. aureus* bacteriophage in recalcitrant CRS. This was a single-

center, open-label study of ascending dose intranasal phage cocktail AB-SA01 consisting of 3 phage targeting *S. aureus*. As the minimum effective dose for AB-SA01 for the treatment of *S. aureus* CRS has not yet been determined, we used published literature and preclinical studies with AB-SA01 phage for guidance to determine a dose that was likely to be safe and effective. This data suggests that in an acute model, doses higher than 1×10^7 PFU per phage within the cocktail are required for efficacy. In our previous *S. aureus* frontal sinusitis sheep study, a 2 x 10^6 PFU/mL daily dose was administered for 3 consecutive days (efficacy) and twice daily for 20 days (safety). While the efficacy data showed a significant reduction in biofilm biomass, there was no complete eradication, suggesting that a higher dose regimen could be necessary. In this study, each cohort was serially dosed with AB-SA01 intranasal irrigations in ascending dosage regimens of twice daily 3×10^8 PFU for 7 days (cohort 1); or 3×10^8 PFU for 14 days (cohort 2); or 3×10^9 PFU for 14 days (cohort 3). This study concluded that intranasal irrigations of AB-SA01 were safe and well tolerated up to 3×10^9 PFU for 14 days with no dose-limiting side effects. Preliminary efficacy data was promising and suggests that prolonged antimicrobial effects are possible allowing for a more targeted approach in treating recalcitrant *S. aureus* sinus infections.

In our seventh paper, we presented a pilot study investigating the safety and efficacy of colloidal silver (CS) sinus irrigations in recalcitrant CRS. CS is available over the counter to the wide public as an alternative medicine and has remained largely unregulated. CS has long been used in wound dressings with known antimicrobial activity against *S. aureus*[213, 214], including methicillin-resistant *S. aureus* (MRSA) and *P. aeruginosa* biofilms. Spherical nanoparticles were shown to be non-toxic in human

cell culture (THP-1, Nuli-1) and safe and effective in a sheep sinusitis model. Some immunomodulatory functions of CS have also been observed in the literature. It has the ability to inhibit matrix metalloproteinases, which are pro-inflammatory, and metallothioneins, which promote resistance to immune-mediated apoptosis, found increasingly in patients with chronic rhinosinusitis with nasal polyps. Colloidal silver has also been shown to induce inflammatory cell apoptosis by suppression of TNF-α and IL-12. This study demonstrated that 0.015 mg/mL spherical CS sinus irrigations are safe over 10 days but not superior to oral antibiotics in recalcitrant CRS. Both oral antibiotic and CS group showed improvement in VAS, SNOT-22 and LKS but did not achieve statistical significance. Although this study has shown that colloidal silver is safe based on serum silver levels and smell tests, the discomfort of using CS rinses has been noted and work is currently under way to optimise this treatment irrigation.

In our final paper we investigated the role of manuka honey (MH) sinus irrigations augmented with methylgloxal (MGO) in recalcitrant CRS in a phase 1 randomized single-blinded placebo-controlled trial. MH (*Leptospernum scoparium*) has broad spectrum antibiofilm effects against *Streptococcus* and *Staphylococcus* species, *Pseudomonas aeruginosa, Escherichia coli, Proteus mirabilis, Enterobacter cloacae* and *K. pneumoniae*. Its low pH, hyperosmolarity, hydrogen peroxide activity and methylglyoxal (MGO) content confers MH its unique anti-biofilm properties. Its low propensity to develop bacterial resistance, ability to increase susceptibility of resistant strains to antibiotics, anti-oxidant and anti-inflammatory properties have further fuelled interest in MH being an alternative to oral antibiotics. This study has shown that MH with augmented MGO is safe following application for 2 weeks but not superior to culture-directed antibiotic therapy and saline rinses in eradicating bacterial infection.

However, a significant improvement in LKS can be seen in both groups, which suggests some potential benefit from the MH rinses without the adverse effects of oral antibiotics. Future studies looking at optimizing the duration of treatment should be assessed in a larger sample size.

With a gradual shift away from overprescribing oral antibiotics, we expect topical antimicrobial therapies will gain traction in the coming years. We hope that this body of work will offer new perspectives in the management of recalcitrant CRS and will encourage further research in novel topical treatment alternatives.

The future

We have arrived at a cornerstone of change in the management of CRS. With shifting understanding of CRS pathomechanics and the discovery of phenotypes, endotypes and microbiotypes we anticipate that the future of clinical research will also evolve with it. Clinical trials in the future will incorporate identification of disease subclasses to better assess patients' response to more personalised treatment with a greater emphasis in immunomodulatory effects of therapeutics.

Bibliography

1. Rosenfeld RM, Andes D, Neil B, et al. Clinical practice guideline: Adult sinusitis. *Otolaryngology–Head and Neck Surgery.* 2007;137:S1-S31.

2. Lethbridge-Cejku M, Schiller JS, Bernadel L. Summary health statistics for U.S. adults: National Health Interview Survey, 2002. *Vital and health statistics. Series 10, Data from the National Health Survey.* 2004:1-151.

3. Bhattacharyya N. Incremental Health Care Utilization and Expenditures for Chronic Rhinosinusitis in the United States. *Annals of Otology, Rhinology & Laryngology.* 2011;120:423-427.

4. Bhattacharyya N. Functional limitations and workdays lost associated with chronic rhinosinusitis and allergic rhinitis. *American journal of rhinology & allergy.* 2012;26:120-122.

5. Australian Health Survey: First results, 2011–12. *Australian Bureau of Statistics.*Cat. no. 4364.0.55.001. Canberra: ABS, 2012. .

6. Senior BA, Glaze C, Benninger MS. Use of the Rhinosinusitis Disability Index (RSDI) in rhinologic disease. *Am J Rhinol.* 2001;15:15-20.

7. Gliklich RE, Metson R. The health impact of chronic sinusitis in patients seeking otolaryngologic care. *Otolaryngol Head Neck Surg.* 1995;113:104-109.

8. Payne SC, Borish L, Steinke JW. Genetics and phenotyping in chronic sinusitis. *Journal of Allergy and Clinical Immunology.* 2011;128:710-720.

9. Van Bruaene N, Perez-Novo CA, Basinski TM, et al. T-cell regulation in chronic paranasal sinus disease. *J Allergy Clin Immunol.* 2008;121:1435-1441, 1441.e1431-1433.

10. Van Bruaene N, Derycke L, Perez-Novo CA, et al. TGF-β signaling and collagen deposition in chronic rhinosinusitis. *Journal of Allergy and Clinical Immunology.* 2009;124:253-259.e252.

11. Bachert C, Gevaert P, Holtappels G, Cuvelier C, Van Cauwenberge P. Nasal Polyposis: From Cytokines to Growth. *American Journal of Rhinology.* 2000;14:279-290.

12. Bachert C, Wagenmann M, Hauser U, Hauser U, Rudack C. IL-5 synthesis is upregulated in human nasal polyp tissues. *J Allergy Clin Immunol.* 1997;99.

13. White AA, Stevenson DD. Aspirin-Exacerbated Respiratory Disease. *New England Journal of Medicine.* 2018;379:1060-1070.

14. Kennedy JL, Borish L. Chronic sinusitis pathophysiology: the role of allergy. *American journal of rhinology & allergy.* 2013;27:367-371.

15. Tomassen P, Vandeplas G, Van Zele T, et al. Inflammatory endotypes of chronic rhinosinusitis based on cluster analysis of biomarkers. *J Allergy Clin Immunol.* 2016;137:1449-1456.e1444.

16. Bachert C, Akdis CA. Phenotypes and Emerging Endotypes of Chronic Rhinosinusitis. *The journal of allergy and clinical immunology. In practice.* 2016;4:621-628.

17. Lou H, Meng Y, Piao Y, et al. Cellular phenotyping of chronic rhinosinusitis with nasal polyps. *Rhinology.* 2016;54:150-159.

18. Liao B, Liu JX, Li ZY, et al. Multidimensional endotypes of chronic rhinosinusitis and their association with treatment outcomes. *Allergy.* 2018;73:1459-1469.
19. Cao PP, Li HB, Wang BF, et al. Distinct immunopathologic characteristics of various types of chronic rhinosinusitis in adult Chinese. *J Allergy Clin Immunol.* 2009;124:478-484, 484.e471-472.
20. Wang X, Zhang N, Bo M, et al. Diversity of TH cytokine profiles in patients with chronic rhinosinusitis: A multicenter study in Europe, Asia, and Oceania. *J Allergy Clin Immunol.* 2016;138:1344-1353.
21. Stammberger H, Posawetz W. Functional endoscopic sinus surgery. Concept, indications and results of the Messerklinger technique. *European archives of oto-rhino-laryngology : official journal of the European Federation of Oto-Rhino-Laryngological Societies (EUFOS) : affiliated with the German Society for Oto-Rhino-Laryngology - Head and Neck Surgery.* 1990;247:63-76.
22. Krause HF. Allergy and chronic rhinosinusitis. *Otolaryngol Head Neck Surg.* 2003;128:14-16.
23. Antunes MB, Gudis DA, Cohen NA. Epithelium, cilia, and mucus: their importance in chronic rhinosinusitis. *Immunology and allergy clinics of North America.* 2009;29:631-643.
24. Belafsky P, Kissinger P, Davidowitz SB, Amedee RG. HIV sinusitis: rationale for a treatment algorithm. *The Journal of the Louisiana State Medical Society : official organ of the Louisiana State Medical Society.* 1999;151:11-18.
25. Borgo CD, Forno AD, Ottaviani F, Fantoni M. Sinusitis in HIV-Infected Patients. *Journal of Chemotherapy.* 1997;9:83-88.
26. Oksenhendler E, Gerard L, Fieschi C, et al. Infections in 252 patients with common variable immunodeficiency. *Clin Infect Dis.* 2008;46:1547-1554.
27. Carr TF, Koterba AP, Chandra R, et al. Characterization of specific antibody deficiency in adults with medically refractory chronic rhinosinusitis. *Am J Rhinol Allergy.* 2011;25:241-244.
28. Vanlerberghe L, Joniau S, Jorissen M. The prevalence of humoral immunodeficiency in refractory rhinosinusitis: a retrospective analysis. *B-ent.* 2006;2:161-166.
29. Miljkovic D, Psaltis A, Wormald PJ, Vreugde S. T regulatory and Th17 cells in chronic rhinosinusitis with polyps. *Int Forum Allergy Rhinol.* 2016;6:826-834.
30. Miljkovic D, Psaltis A, Wormald PJ, Vreugde S. Naive and effector B-cell subtypes are increased in chronic rhinosinusitis with polyps. *Am J Rhinol Allergy.* 2018;32:3-6.
31. Miljkovic D, Psaltis AJ, Wormald PJ, Vreugde S. Chronic Rhinosinusitis with Polyps Is Characterized by Increased Mucosal and Blood Th17 Effector Cytokine Producing Cells. *Frontiers in physiology.* 2017;8:898-898.
32. Miljkovic D, Ou J, Kirana C, et al. Discordant frequencies of tissue-resident and circulating CD180-negative B cells in chronic rhinosinusitis. *Int Forum Allergy Rhinol.* 2017;7:609-614.
33. Shipman WD, Dasoveanu DC, Lu TT. Tertiary lymphoid organs in systemic autoimmune diseases: pathogenic or protective? *F1000Research.* 2017;6:196-196.

34. Lau A, Lester S, Moraitis S, et al. Tertiary lymphoid organs in recalcitrant chronic rhinosinusitis. *Journal of Allergy and Clinical Immunology.* 2017;139:1371-1373.e1376.
35. Paramasivan S, Lester S, Lau A, et al. Tertiary lymphoid organs: A novel target in patients with chronic rhinosinusitis. *Journal of Allergy and Clinical Immunology.* 2018;142:1673-1676.
36. Song J, Wang H, Zhang Y-N, et al. Ectopic lymphoid tissues support local immunoglobulin production in patients with chronic rhinosinusitis with nasal polyps. *Journal of Allergy and Clinical Immunology.* 2018;141:927-937.
37. Grogan JL, Ouyang W. A role for Th17 cells in the regulation of tertiary lymphoid follicles. *European journal of immunology.* 2012;42:2255-2262.
38. Mahdavinia M, Suh LA, Carter RG, et al. Increased noneosinophilic nasal polyps in chronic rhinosinusitis in US second-generation Asians suggest genetic regulation of eosinophilia. *J Allergy Clin Immunol.* 2015;135:576-579.
39. Wu D, Wang J, Zhang M. Altered Th17/Treg Ratio in Nasal Polyps With Distinct Cytokine Profile: Association With Patterns of Inflammation and Mucosal Remodeling. *Medicine.* 2016;95:e2998.
40. Videler WJ, Georgalas C, Menger DJ, Freling NJ, van Drunen CM, Fokkens WJ. Osteitic bone in recalcitrant chronic rhinosinusitis. *Rhinology.* 2011;49:139-147.
41. Georgalas C. Osteitis and paranasal sinus inflammation: what we know and what we do not. *Curr Opin Otolaryngol Head Neck Surg.* 2013;21:45-49.
42. Oue S, Ramezanpour M, Paramasivan S, et al. Increased IL-13 expression is independently associated with neo-osteogenesis in patients with chronic rhinosinusitis. *Journal of Allergy and Clinical Immunology.* 2017;140:1444-1448.e1411.
43. Ramadan HH, Fornelli R, Ortiz AO, Rodman S. Correlation of allergy and severity of sinus disease. *Am J Rhinol.* 1999;13:345-347.
44. Yacoub M-R, Trimarchi M, Cremona G, et al. Are atopy and eosinophilic bronchial inflammation associated with relapsing forms of chronic rhinosinusitis with nasal polyps? *Clinical and molecular allergy : CMA.* 2015;13:23-23.
45. Robinson S, Douglas R, Wormald PJ. The relationship between atopy and chronic rhinosinusitis. *Am J Rhinol.* 2006;20:625-628.
46. Kim S-D, Cho K-S. Samter's Triad: State of the Art. *Clinical and experimental otorhinolaryngology.* 2018;11:71-80.
47. Hastan D, Fokkens WJ, Bachert C, et al. Chronic rhinosinusitis in Europe – an underestimated disease. A GA2LEN study. *Allergy.* 2011;66:1216-1223.
48. Eriksson J, Ekerljung L, Sundblad BM, et al. Cigarette smoking is associated with high prevalence of chronic rhinitis and low prevalence of allergic rhinitis in men. *Allergy.* 2013;68:347-354.
49. Gwaltney JM, Jr., Phillips CD, Miller RD, Riker DK. Computed tomographic study of the common cold. *The New England journal of medicine.* 1994;330:25-30.
50. Cho GS, Moon BJ, Lee BJ, et al. High rates of detection of respiratory viruses in the nasal washes and mucosae of patients with chronic rhinosinusitis. *Journal of clinical microbiology.* 2013;51:979-984.
51. Jang YJ, Kwon HJ, Park HW, Lee BJ. Detection of rhinovirus in turbinate epithelial cells of chronic sinusitis. *Am J Rhinol.* 2006;20:634-636.

52. Goggin RK, Bennett CA, Bialasiewicz S, et al. The presence of virus significantly associates with chronic rhinosinusitis disease severity. *Allergy.* 2019.
53. Goggin RK, Bennett CA, Bassiouni A, et al. Comparative Viral Sampling in the Sinonasal Passages; Different Viruses at Different Sites. *Frontiers in Cellular and Infection Microbiology.* 2018;8.
54. Meltzer EO, Hamilos DL, Hadley JA, et al. Rhinosinusitis: establishing definitions for clinical research and patient care. *J Allergy Clin Immunol.* 2004;114:155-212.
55. Bent JP, 3rd, Kuhn FA. Diagnosis of allergic fungal sinusitis. *Otolaryngol Head Neck Surg.* 1994;111:580-588.
56. Manning SC, Holman M. Further evidence for allergic pathophysiology in allergic fungal sinusitis. *The Laryngoscope.* 1998;108:1485-1496.
57. Ponikau JU, Sherris DA, Kern EB, et al. The diagnosis and incidence of allergic fungal sinusitis. *Mayo Clinic proceedings.* 1999;74:877-884.
58. Van Crombruggen K, Zhang N, Gevaert P, Tomassen P, Bachert C. Pathogenesis of chronic rhinosinusitis: inflammation. *J Allergy Clin Immunol.* 2011;128:728-732.
59. Brook I. Microbiology and antimicrobial management of sinusitis. *The Journal of laryngology and otology.* 2005;119:251-258.
60. E. Bolger W. *Gram Negative Sinusitis: An Emerging Clinical Entity?*1994.
61. Foreman A, Psaltis AJ, Tan LW, Wormald P-J. Characterization of Bacterial and Fungal Biofilms in Chronic Rhinosinusitis. *American Journal of Rhinology & Allergy.* 2009;23:556-561.
62. Proctor RA, van Langevelde P, Kristjansson M, Maslow JN, Arbeit RD. Persistent and relapsing infections associated with small-colony variants of Staphylococcus aureus. *Clin Infect Dis.* 1995;20:95-102.
63. Proctor RA, von Eiff C, Kahl BC, et al. Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. *Nature reviews. Microbiology.* 2006;4:295-305.
64. Brouillette E, Martinez A, Boyll BJ, Allen NE, Malouin F. Persistence of a Staphylococcus aureus small-colony variant under antibiotic pressure in vivo. *FEMS Immunol Med Microbiol.* 2004;41:35-41.
65. von Eiff C. Staphylococcus aureus small colony variants: a challenge to microbiologists and clinicians. *International journal of antimicrobial agents.* 2008;31:507-510.
66. Ou JJJ, Drilling AJ, Cooksley C, et al. Reduced Innate Immune Response to a Staphylococcus aureus Small Colony Variant Compared to Its Wild-Type Parent Strain. *Frontiers in cellular and infection microbiology.* 2016;6:187-187.
67. Massey RC, Buckling A, Peacock SJ. Phenotypic switching of antibiotic resistance circumvents permanent costs in Staphylococcus aureus. *Current biology : CB.* 2001;11:1810-1814.
68. Bayston R, Ashraf W, Smith T. Triclosan resistance in methicillin-resistant Staphylococcus aureus expressed as small colony variants: a novel mode of evasion of susceptibility to antiseptics. *J Antimicrob Chemother.* 2007;59:848-853.
69. Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM. Microbial biofilms. *Annu Rev Microbiol.* 1995;49:711-745.

70. Massey RC, Peacock SJ. Antibiotic-resistant sub-populations of the pathogenic bacterium Staphylococcus aureus confer population-wide resistance. *Current Biology.* 2002;12:R686-R687.
71. Garcia LG, Lemaire S, Kahl BC, et al. Antibiotic activity against small-colony variants of Staphylococcus aureus: review of in vitro, animal and clinical data. *J Antimicrob Chemother.* 2013;68:1455-1464.
72. Abreu NA, Nagalingam NA, Song Y, et al. Sinus microbiome diversity depletion and Corynebacterium tuberculostearicum enrichment mediates rhinosinusitis. *Science translational medicine.* 2012;4:151ra124-151ra124.
73. Jervis Bardy J, Psaltis AJ. Next Generation Sequencing and the Microbiome of Chronic Rhinosinusitis: A Primer for Clinicians and Review of Current Research, Its Limitations, and Future Directions. *The Annals of otology, rhinology, and laryngology.* 2016;125:613-621.
74. Bassiouni A, Paramasivan S, Shiffer A, et al. Microbiotyping the sinonasal microbiome. *bioRxiv.* 2019:549311.
75. Paramasivan S, Bassiouni A, Shiffer A, et al. The international sinonasal microbiome study (ISMS): a multi-centre, multi-national collaboration characterising the microbial ecology of the sinonasal cavity. *bioRxiv.* 2019:548743.
76. Cope EK, Goldberg AN, Pletcher SD, Lynch SV. Compositionally and functionally distinct sinus microbiota in chronic rhinosinusitis patients have immunological and clinically divergent consequences. *Microbiome.* 2017;5:53.
77. Cleland EJ, Drilling A, Bassiouni A, James C, Vreugde S, Wormald PJ. Probiotic manipulation of the chronic rhinosinusitis microbiome. *Int Forum Allergy Rhinol.* 2014;4:309-314.
78. Ramsey MM, Freire MO, Gabrilska RA, Rumbaugh KP, Lemon KP. Staphylococcus aureus Shifts toward Commensalism in Response to Corynebacterium Species. *Front Microbiol.* 2016;7:1230.
79. De La Cochetiere MF, Durand T, Lepage P, Bourreille A, Galmiche JP, Dore J. Resilience of the dominant human fecal microbiota upon short-course antibiotic challenge. *Journal of clinical microbiology.* 2005;43:5588-5592.
80. Raymond F, Ouameur AA, Deraspe M, et al. The initial state of the human gut microbiome determines its reshaping by antibiotics. *The ISME journal.* 2016;10:707-720.
81. Dethlefsen L, Relman DA. Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. *Proceedings of the National Academy of Sciences of the United States of America.* 2011;108 Suppl 1:4554-4561.
82. Zaura E, Brandt BW, Teixeira de Mattos MJ, et al. Same Exposure but Two Radically Different Responses to Antibiotics: Resilience of the Salivary Microbiome versus Long-Term Microbial Shifts in Feces. *mBio.* 2015;6:e01693-01615.
83. Jakobsson HE, Jernberg C, Andersson AF, Sjolund-Karlsson M, Jansson JK, Engstrand L. Short-term antibiotic treatment has differing long-term impacts on the human throat and gut microbiome. *PLoS One.* 2010;5:e9836.
84. Cryer J, Schipor I, Perloff JR, Palmer JN. Evidence of Bacterial Biofilms in Human Chronic Sinusitis. *ORL.* 2004;66:155-158.

85. Foreman A, Jervis-Bardy J, Wormald P-J. Do biofilms contribute to the initiation and recalcitrance of chronic rhinosinusitis? *The Laryngoscope.* 2011;121:1085-1091.
86. Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms: from the Natural environment to infectious diseases. *Nature Reviews Microbiology.* 2004;2:95.
87. Romling U, Balsalobre C. Biofilm infections, their resilience to therapy and innovative treatment strategies. *Journal of internal medicine.* 2012;272:541-561.
88. Stewart PS. Theoretical aspects of antibiotic diffusion into microbial biofilms. *Antimicrobial agents and chemotherapy.* 1996;40:2517-2522.
89. Brown MR, Allison DG, Gilbert P. Resistance of bacterial biofilms to antibiotics: a growth-rate related effect? *J Antimicrob Chemother.* 1988;22:777-780.
90. Solano C, Echeverz M, Lasa I. Biofilm dispersion and quorum sensing. *Current Opinion in Microbiology.* 2014;18:96-104.
91. Burmolle M, Webb JS, Rao D, Hansen LH, Sorensen SJ, Kjelleberg S. Enhanced biofilm formation and increased resistance to antimicrobial agents and bacterial invasion are caused by synergistic interactions in multispecies biofilms. *Appl Environ Microbiol.* 2006;72:3916-3923.
92. Bendouah Z, Barbeau J, Hamad WA, Desrosiers M. Biofilm formation by Staphylococcus aureus and Pseudomonas aeruginosa is associated with an unfavorable evolution after surgery for chronic sinusitis and nasal polyposis. *Otolaryngol Head Neck Surg.* 2006;134:991-996.
93. Psaltis AJ, Weitzel EK, Ha KR, Wormald PJ. The effect of bacterial biofilms on post-sinus surgical outcomes. *Am J Rhinol.* 2008;22:1-6.
94. Orlandi RR, Kingdom TT, Hwang PH, et al. International Consensus Statement on Allergy and Rhinology: Rhinosinusitis. *Int Forum Allergy Rhinol.* 2016;6 Suppl 1:S22-209.
95. Iro H, Mayr S, Wallisch C, Schick B, Wigand ME. Endoscopic sinus surgery: its subjective medium-term outcome in chronic rhinosinusitis. *Rhinology.* 2004;42:200-206.
96. Dursun E, Korkmaz H, Eryilmaz A, Bayiz U, Sertkaya D, Samim E. Clinical predictors of long-term success after endoscopic sinus surgery. *Otolaryngol Head Neck Surg.* 2003;129:526-531.
97. Harvey RJ, Schlosser RJ. Local drug delivery. *Otolaryngol Clin North Am.* 2009;42:829-845, ix.
98. Liang J, Lane AP. Topical Drug Delivery for Chronic Rhinosinusitis. *Current Otorhinolaryngology Reports.* 2013;1:51-60.
99. Beule A, Athanasiadis T, Athanasiadis E, Field J, Wormald PJ. Efficacy of different techniques of sinonasal irrigation after modified Lothrop procedure. *Am J Rhinol Allergy.* 2009;23:85-90.
100. Grobler A, Weitzel EK, Buele A, et al. Pre- and postoperative sinus penetration of nasal irrigation. *Laryngoscope.* 2008;118:2078-2081.
101. Möller W, Schuschnig U, Khadem Saba G, et al. Pulsating aerosols for drug delivery to the sinuses in healthy volunteers. *Otolaryngology–Head and Neck Surgery.* 2010;142:382-388.
102. Kimbell JS, Segal RA, Asgharian B, et al. Characterization of deposition from nasal spray devices using a computational fluid dynamics model of the human

nasal passages. *Journal of aerosol medicine : the official journal of the International Society for Aerosols in Medicine.* 2007;20:59-74.
103. Suman JD, Laube BL, Dalby R. Comparison of nasal deposition and clearance of aerosol generated by nebulizer and an aqueous spray pump. *Pharm Res.* 1999;16:1648-1652.
104. Laube BL. Devices for aerosol delivery to treat sinusitis. *Journal of aerosol medicine : the official journal of the International Society for Aerosols in Medicine.* 2007;20 Suppl 1:S5-17; discussion S17-18.
105. Hyo N, Takano H, Hyo Y. Particle deposition efficiency of therapeutic aerosols in the human maxillary sinus. *Rhinology.* 1989;27:17-26.
106. Saijo R, Majima Y, Hyo N, Takano H. Particle deposition of therapeutic aerosols in the nose and paranasal sinuses after transnasal sinus surgery: a cast model study. *Am J Rhinol.* 2004;18:1-7.
107. Weber R, Keerl R, Radziwill R, et al. Videoendoscopic analysis of nasal steroid distribution. *Rhinology.* 1999;37:69-73.
108. Itoh H, Smaldone GC, Swift DL, Wagner HN. Mechanisms of aerosol deposition in a nasal model. *Journal of Aerosol Science.* 1985;16:529-534.
109. Penttila M, Poulsen P, Hollingworth K, Holmstrom M. Dose-related efficacy and tolerability of fluticasone propionate nasal drops 400 microg once daily and twice daily in the treatment of bilateral nasal polyposis: a placebo-controlled randomized study in adult patients. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology.* 2000;30:94-102.
110. Keith P, Nieminen J, Hollingworth K, Dolovich J. Efficacy and tolerability of fluticasone propionate nasal drops 400 microgram once daily compared with placebo for the treatment of bilateral polyposis in adults. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology.* 2000;30:1460-1468.
111. Tos M, Svendstrup F, Arndal H, et al. Efficacy of an aqueous and a powder formulation of nasal budesonide compared in patients with nasal polyps. *Am J Rhinol.* 1998;12:183-189.
112. Andersson M, Berglund R, Greiff L, et al. A comparison of budesonide nasal dry powder with fluticasone propionate aqueous nasal spray in patients with perennial allergic rhinitis. *Rhinology.* 1995;33:18-21.
113. Djupesland PG, Skretting A, Winderen M, Holand T. Breath actuated device improves delivery to target sites beyond the nasal valve. *Laryngoscope.* 2006;116:466-472.
114. Hansen FS, Djupesland PG, Fokkens WJ. Preliminary efficacy of fluticasone delivered by a novel device in recalcitrant chronic rhinosinusitis. *Rhinology.* 2010;48:292-299.
115. Djupesland PG, Vlckova I, Hewson G. Impact of Baseline Nasal Polyp Size and Previous Surgery on Efficacy of Fluticasone Delivered with a Novel Device: A Subgroup Analysis. *American Journal of Rhinology & Allergy.* 2010;24:291-295.
116. Miller TR, Muntz HR, Gilbert ME, Orlandi RR. Comparison of Topical Medication Delivery Systems after Sinus Surgery. *The Laryngoscope.* 2004;114:201-204.
117. Negley JE, Krause H, Pawar S, Reeves-Hoche MK. RinoFlow nasal wash and sinus system as a mechanism to deliver medications to the paranasal sinuses:

results of a radiolabeled pilot study. *Ear, nose, & throat journal.* 1999;78:550-552, 553-554.
118. Giroux M, Hwang, P., Prasad, A,. Controlled particle disperson: Applying vertical flow to optimise nasal drug deposition. *Drug Delivery Tech.* 2005:44-49.
119. Hwang PH, Woo RJ, Fong KJ. Intranasal deposition of nebulized saline: a radionuclide distribution study. *Am J Rhinol.* 2006;20:255-261.
120. Moller W, Schuschnig U, Khadem Saba G, et al. Pulsating aerosols for drug delivery to the sinuses in healthy volunteers. *Otolaryngol Head Neck Surg.* 2010;142:382-388.
121. Moller W, Schuschnig U, Celik G, et al. Topical drug delivery in chronic rhinosinusitis patients before and after sinus surgery using pulsating aerosols. *PLoS One.* 2013;8:e74991.
122. Valentine R, Athanasiadis T, Thwin M, Singhal D, Weitzel EK, Wormald PJ. A prospective controlled trial of pulsed nasal nebulizer in maximally dissected cadavers. *Am J Rhinol.* 2008;22:390-394.
123. Abadie WM, McMains KC, Weitzel EK. Irrigation penetration of nasal delivery systems: a cadaver study. *Int Forum Allergy Rhinol.* 2011;1:46-49.
124. Wormald PJ, Cain T, Oates L, Hawke L, Wong I. A comparative study of three methods of nasal irrigation. *Laryngoscope.* 2004;114:2224-2227.
125. Harvey RJ, Debnath N, Srubiski A, Bleier B, Schlosser RJ. Fluid residuals and drug exposure in nasal irrigation. *Otolaryngol Head Neck Surg.* 2009;141:757-761.
126. Olson DE, Rasgon BM, Hilsinger RL, Jr. Radiographic comparison of three methods for nasal saline irrigation. *Laryngoscope.* 2002;112:1394-1398.
127. Pynnonen MA, Mukerji SS, Kim HM, Adams ME, Terrell JE. Nasal saline for chronic sinonasal symptoms: a randomized controlled trial. *Arch Otolaryngol Head Neck Surg.* 2007;133:1115-1120.
128. Thomas WW, 3rd, Harvey RJ, Rudmik L, Hwang PH, Schlosser RJ. Distribution of topical agents to the paranasal sinuses: an evidence-based review with recommendations. *Int Forum Allergy Rhinol.* 2013;3:691-703.
129. Snidvongs K, Chaowanapanja P, Aeumjaturapat S, Chusakul S, Praweswararat P. Does nasal irrigation enter paranasal sinuses in chronic rhinosinusitis? *Am J Rhinol.* 2008;22:483-486.
130. Harvey RJ, Goddard JC, Wise SK, Schlosser RJ. Effects of endoscopic sinus surgery and delivery device on cadaver sinus irrigation. *Otolaryngol Head Neck Surg.* 2008;139:137-142.
131. Singhal D, Weitzel EK, Lin E, et al. Effect of head position and surgical dissection on sinus irrigant penetration in cadavers. *Laryngoscope.* 2010;120:2528-2531.
132. Raghavan U, Logan BM. New method for the effective instillation of nasal drops. *The Journal of laryngology and otology.* 2000;114:456-459.
133. Morén F, Björnek K, Klint T, Wagner ZG. A comparative distribution study of two procedures for administration of nose drops. *Acta Oto-Laryngologica.* 1988;106:286-290.
134. Kayarkar R, Clifton NJ, Woolford TJ. An evaluation of the best head position for instillation of steroid nose drops. *Clinical Otolaryngology & Allied Sciences.* 2002;27:18-21.

135. Wilson R. Effect of head position on the efficacy oftopical treatment of chronic mucopurulent rhinosinusitis. *Thorax.* 1987:631-632.
136. Beule A, Athanasiadis T, Athanasiadis E, Field J, Wormald P-J. Efficacy of Different Techniques of Sinonasal Irrigation after Modified Lothrop Procedure. *American Journal of Rhinology & Allergy.* 2009;23:85-90.
137. Harvey R, Hannan SA, Badia L, Scadding G. Nasal saline irrigations for the symptoms of chronic rhinosinusitis. *The Cochrane database of systematic reviews.* 2007:Cd006394.
138. Chong LY, Head K, Hopkins C, et al. Saline irrigation for chronic rhinosinusitis. *The Cochrane database of systematic reviews.* 2016;4:Cd011995.
139. Luk CK, Dulfano MJ. Effect of pH, viscosity and ionic-strength changes on ciliary beating frequency of human bronchial explants. *Clinical science (London, England : 1979).* 1983;64:449-451.
140. Lansley AB, Sanderson MJ, Dirksen ER. Control of the beat cycle of respiratory tract cilia by Ca2+ and cAMP. *The American journal of physiology.* 1992;263:L232-242.
141. Eveloff JL, Warnock DG. Activation of ion transport systems during cell volume regulation. *The American journal of physiology.* 1987;252:F1-10.
142. Bachmann G, Hommel G, Michel O. Effect of irrigation of the nose with isotonic salt solution on adult patients with chronic paranasal sinus disease. *European archives of oto-rhino-laryngology : official journal of the European Federation of Oto-Rhino-Laryngological Societies (EUFOS) : affiliated with the German Society for Oto-Rhino-Laryngology - Head and Neck Surgery.* 2000;257:537-541.
143. Hauptman G, Ryan MW. The effect of saline solutions on nasal patency and mucociliary clearance in rhinosinusitis patients. *Otolaryngol Head Neck Surg.* 2007;137:815-821.
144. Talbot AR, Herr TM, Parsons DS. Mucociliary clearance and buffered hypertonic saline solution. *Laryngoscope.* 1997;107:500-503.
145. Homer JJ, Dowley AC, Condon L, El-Jassar P, Sood S. The effect of hypertonicity on nasal mucociliary clearance. *Clin Otolaryngol Allied Sci.* 2000;25:558-560.
146. Ural A, Oktemer TK, Kizil Y, Ileri F, Uslu S. Impact of isotonic and hypertonic saline solutions on mucociliary activity in various nasal pathologies: clinical study. *The Journal of laryngology and otology.* 2009;123:517-521.
147. Kanjanawasee D, Seresirikachorn K, Chitsuthipakorn W, Snidvongs K. Hypertonic Saline Versus Isotonic Saline Nasal Irrigation: Systematic Review and Meta-analysis. *Am J Rhinol Allergy.* 2018;32:269-279.
148. Rudmik L, Soler ZM. Medical Therapies for Adult Chronic Sinusitis: A Systematic ReviewMedical Therapies for Adult Chronic SinusitisMedical Therapies for Adult Chronic Sinusitis. *JAMA.* 2015;314:926-939.
149. Joe SA, Thambi R, Huang J. A systematic review of the use of intranasal steroids in the treatment of chronic rhinosinusitis. *Otolaryngol Head Neck Surg.* 2008;139:340-347.
150. Rudmik L, Schlosser RJ, Smith TL, Soler ZM. Impact of topical nasal steroid therapy on symptoms of nasal polyposis: a meta-analysis. *Laryngoscope.* 2012;122:1431-1437.

151. Kalish L, Snidvongs K, Sivasubramaniam R, Cope D, Harvey RJ. Topical steroids for nasal polyps. *The Cochrane database of systematic reviews.* 2012;12:Cd006549.
152. Chong LY, Head K, Hopkins C, Philpott C, Schilder AG, Burton MJ. Intranasal steroids versus placebo or no intervention for chronic rhinosinusitis. *The Cochrane database of systematic reviews.* 2016;4:Cd011996.
153. Chong LY, Head K, Hopkins C, Philpott C, Burton MJ, Schilder AG. Different types of intranasal steroids for chronic rhinosinusitis. *The Cochrane database of systematic reviews.* 2016;4:Cd011993.
154. Snidvongs K, Kalish L, Sacks R, Craig JC, Harvey RJ. Topical steroid for chronic rhinosinusitis without polyps. *The Cochrane database of systematic reviews.* 2011:Cd009274.
155. Rudmik. High Volume Sinonasal Budesonide Irrigations for Chronic Rhinosinusitis: An Update on the Safety and Effectiveness. *Adv Pharmacoepidemiol Drug Safety.* 2014;Vol 3:1-5.
156. Bhalla RK, Payton K, Wright ED. Safety of budesonide in saline sinonasal irrigations in the management of chronic rhinosinusitis with polyposis: lack of significant adrenal suppression. *J Otolaryngol Head Neck Surg.* 2008;37:821-825.
157. Welch KC, Thaler ER, Doghramji LL, Palmer JN, Chiu AG. The effects of serum and urinary cortisol levels of topical intranasal irrigations with budesonide added to saline in patients with recurrent polyposis after endoscopic sinus surgery. *Am J Rhinol Allergy.* 2010;24:26-28.
158. Seiberling KA, Chang DF, Nyirady J, Park F, Church CA. Effect of intranasal budesonide irrigations on intraocular pressure. *Int Forum Allergy Rhinol.* 2013;3:704-707.
159. Smith KA, French G, Mechor B, Rudmik L. Safety of long-term high-volume sinonasal budesonide irrigations for chronic rhinosinusitis. *Int Forum Allergy Rhinol.* 2016;6:228-232.
160. Soudry E, Wang J, Vaezeafshar R, Katznelson L, Hwang PH. Safety analysis of long-term budesonide nasal irrigations in patients with chronic rhinosinusitis post endoscopic sinus surgery. *International Forum of Allergy & Rhinology.* 2016;6:568-572.
161. Rotenberg BW, Zhang I, Arra I, Payton KB. Postoperative care for Samter's triad patients undergoing endoscopic sinus surgery: a double-blinded, randomized controlled trial. *Laryngoscope.* 2011;121:2702-2705.
162. Snidvongs K, Pratt E, Chin D, Sacks R, Earls P, Harvey RJ. Corticosteroid nasal irrigations after endoscopic sinus surgery in the management of chronic rhinosinusitis. *Int Forum Allergy Rhinol.* 2012;2:415-421.
163. Rawal RB, Deal AM, Ebert CS, Jr., et al. Post-operative budesonide irrigations for patients with polyposis: a blinded, randomized controlled trial. *Rhinology.* 2015;53:227-234.
164. Kosugi EM, Moussalem GF, Simoes JC, et al. Topical therapy with high-volume budesonide nasal irrigations in difficult-to-treat chronic rhinosinusitis. *Brazilian journal of otorhinolaryngology.* 2016;82:191-197.
165. Kang TW, Chung JH, Cho SH, Lee SH, Kim KR, Jeong JH. The Effectiveness of Budesonide Nasal Irrigation After Endoscopic Sinus Surgery in Chronic Rhinosinusitis With Asthma. *Clinical and Experimental Otorhinolaryngology.* 2017;10:91-96.

166. Tait S, Kallogjeri D, Suko J, Kukuljan S, Schneider J, Piccirillo JF. Effect of Budesonide Added to Large-Volume, Low-pressure Saline Sinus Irrigation for Chronic Rhinosinusitis: A Randomized Clinical Trial. *JAMA otolaryngology--head & neck surgery.* 2018;144:605-612.
167. Yoon HY, Lee HS, Kim IH, Hwang SH. Post-operative corticosteroid irrigation for chronic rhinosinusitis after endoscopic sinus surgery: A meta-analysis. *Clinical Otolaryngology.* 2018;43:525-532.
168. Cherian LM, Cooksley C, Richter K, et al. Effect of commercial nasal steroid preparation on bacterial growth. *Int Forum Allergy Rhinol.* 2019.
169. Rudmik L, Hoy M, Schlosser RJ, et al. Topical therapies in the management of chronic rhinosinusitis: an evidence-based review with recommendations. *Int Forum Allergy Rhinol.* 2013;3:281-298.
170. Lee VS, Davis GE. Culture-directed topical antibiotic treatment for chronic rhinosinusitis. *American Journal of Rhinology & Allergy.* 2016;30:414-417.
171. Videler WJ, van Drunen CM, Reitsma JB, Fokkens WJ. Nebulized bacitracin/colimycin: a treatment option in recalcitrant chronic rhinosinusitis with Staphylococcus aureus? A double-blind, randomized, placebo-controlled, cross-over pilot study. *Rhinology.* 2008;46:92-98.
172. Uren B, Psaltis A, Wormald P-J. Nasal Lavage With Mupirocin for the Treatment of Surgically Recalcitrant Chronic Rhinosinusitis. *The Laryngoscope.* 2008;118:1677-1680.
173. Solares CA, Batra PS, Hall GS, Citardi MJ. Treatment of chronic rhinosinusitis exacerbations due to methicillin-resistant Staphylococcus aureus with mupirocin irrigations. *Am J Otolaryngol.* 2006;27:161-165.
174. Jervis-Bardy J, Wormald PJ. Microbiological outcomes following mupirocin nasal washes for symptomatic, Staphylococcus aureus-positive chronic rhinosinusitis following endoscopic sinus surgery. *Int Forum Allergy Rhinol.* 2012;2:111-115.
175. Jervis-Bardy J, Boase S, Psaltis A, Foreman A, Wormald PJ. A randomized trial of mupirocin sinonasal rinses versus saline in surgically recalcitrant staphylococcal chronic rhinosinusitis. *Laryngoscope.* 2012;122:2148-2153.
176. Seiberling KA, Aruni W, Kim S, Scapa VI, Fletcher H, Church CA. The effect of intraoperative mupirocin irrigation on Staphylococcus aureus within the maxillary sinus. *Int Forum Allergy Rhinol.* 2013;3:94-98.
177. Desrosiers MY, Salas-Prato M. Treatment of chronic rhinosinusitis refractory to other treatments with topical antibiotic therapy delivered by means of a large-particle nebulizer: results of a controlled trial. *Otolaryngol Head Neck Surg.* 2001;125:265-269.
178. Di Cicco M, Alicandro G, Claut L, et al. Efficacy and tolerability of a new nasal spray formulation containing hyaluronate and tobramycin in cystic fibrosis patients with bacterial rhinosinusitis. *Journal of Cystic Fibrosis.* 2014;13:455-460.
179. Ezzat WF, Fawaz SA, Rabie H, Hamdy TA, Shokry YA. Effect of topical ofloxacin on bacterial biofilms in refractory post-sinus surgery rhino-sinusitis. *European archives of oto-rhino-laryngology : official journal of the European Federation of Oto-Rhino-Laryngological Societies (EUFOS) : affiliated with the German Society for Oto-Rhino-Laryngology - Head and Neck Surgery.* 2015;272:2355-2361.

180. Antunes MB, Feldman MD, Cohen NA, Chiu AG. Dose-dependent effects of topical tobramycin in an animal model of Pseudomonas sinusitis. *Am J Rhinol.* 2007;21:423-427.
181. Moss RB, King VV. Management of Sinusitis in Cystic Fibrosis by Endoscopic Surgery and Serial Antimicrobial Lavage: Reduction in Recurrence Requiring Surgery. *JAMA Otolaryngology–Head & Neck Surgery.* 1995;121:566-572.
182. Mainz JG, Schadlich K, Schien C, et al. Sinonasal inhalation of tobramycin vibrating aerosol in cystic fibrosis patients with upper airway Pseudomonas aeruginosa colonization: results of a randomized, double-blind, placebo-controlled pilot study. *Drug Des Devel Ther.* 2014;8:209-217.
183. Berkhout MC, van Velzen AJ, Touw DJ, de Kok BM, Fokkens WJ, Heijerman HG. Systemic absorption of nasally administered tobramycin and colistin in patients with cystic fibrosis. *J Antimicrob Chemother.* 2014;69:3112-3115.
184. Strateva T, Yordanov D. Pseudomonas aeruginosa - a phenomenon of bacterial resistance. *J Med Microbiol.* 2009;58:1133-1148.
185. Boon RJ, Beale AS, Sutherland R. Efficacy of topical mupirocin against an experimental Staphylococcus aureus surgical wound infection. *J Antimicrob Chemother.* 1985;16:519-526.
186. Sutherland R, Boon RJ, Griffin KE, Masters PJ, Slocombe B, White AR. Antibacterial activity of mupirocin (pseudomonic acid), a new antibiotic for topical use. *Antimicrob Agents Chemother.* 1985;27:495-498.
187. Parenti MA, Hatfield SM, Leyden JJ. Mupirocin: a topical antibiotic with a unique structure and mechanism of action. *Clin Pharm.* 1987;6:761-770.
188. Kim JS, Kwon SH. Mupirocin in the Treatment of Staphylococcal Infections in Chronic Rhinosinusitis: A Meta-Analysis. *PloS one.* 2016;11:e0167369-e0167369.
189. Carr TF, Hill JL, Chiu A, Chang EH. Alteration in Bacterial Culture After Treatment With Topical Mupirocin for Recalcitrant Chronic Rhinosinusitis. *JAMA otolaryngology-- head & neck surgery.* 2016;142:138-142.
190. Kennedy DW, Kuhn FA, Hamilos DL, et al. Treatment of chronic rhinosinusitis with high-dose oral terbinafine: a double blind, placebo-controlled study. *Laryngoscope.* 2005;115:1793-1799.
191. Liang K-L, Su M-C, Shiao J-Y, et al. Amphotericin B Irrigation for the Treatment of Chronic Rhinosinusitis without Nasal Polyps: A Randomized, Placebo-controlled, Double-blind Study. *American Journal of Rhinology.* 2008;22:52-58.
192. Hashemian F, Hashemian F, Molaali N, Rouini M, Roohi E, Torabian S. Clinical effects of topical antifungal therapy in chronic rhinosinusitis: a randomized, double-blind, placebo-controlled trial of intranasal fluconazole. *EXCLI J.* 2016;15:95-102.
193. Ebbens FA, Scadding GK, Badia L, et al. Amphotericin B nasal lavages: Not a solution for patients with chronic rhinosinusitis. *Journal of Allergy and Clinical Immunology.* 2006;118:1149-1156.
194. Ebbens FA, Georgalas C, Luiten S, et al. The effect of topical amphotericin B on inflammatory markers in patients with chronic rhinosinusitis: A multicenter randomized controlled study. *The Laryngoscope.* 2009;119:401-408.

195. Sacks P-Lt, Harvey RJ, Rimmer J, Gallagher RM, Sacks R. Antifungal therapy in the treatment of chronic rhinosinusitis: a meta-analysis. *American journal of rhinology & allergy.* 2012;26:141-147.
196. Isaacs S, Fakhri S, Luong A, Citardi MJ. A meta-analysis of topical amphotericin B for the treatment of chronic rhinosinusitis. *International Forum of Allergy & Rhinology.* 2011;1:250-254.
197. Wang T, Su J, Feng Y. The effectiveness topical amphotericin B in the management of chronic rhinosinusitis: a meta-analysis. *European archives of oto-rhino-laryngology : official journal of the European Federation of Oto-Rhino-Laryngological Societies (EUFOS) : affiliated with the German Society for Oto-Rhino-Laryngology - Head and Neck Surgery.* 2015;272:1923-1929.
198. Wang W, Zhang P, Yu G-L, et al. Preparation and anti-influenza A virus activity of κ-carrageenan oligosaccharide and its sulphated derivatives. *Food Chemistry.* 2012;133:880-888.
199. Koenighofer M, Lion T, Bodenteich A, et al. Carrageenan nasal spray in virus confirmed common cold: individual patient data analysis of two randomized controlled trials. *Multidisciplinary Respiratory Medicine.* 2014;9:57.
200. Eccles R, Meier C, Jawad M, Weinmüllner R, Grassauer A, Prieschl-Grassauer E. Efficacy and safety of an antiviral Iota-Carrageenan nasal spray: a randomized, double-blind, placebo-controlled exploratory study in volunteers with early symptoms of the common cold. *Respiratory Research.* 2010;11:108.
201. Bennett C, Ramezanpour M, Cooksley C, Vreugde S, Psaltis AJ. Kappa-carrageenan sinus rinses reduce inflammation and intracellular Staphylococcus aureus infection in airway epithelial cells. *Int Forum Allergy Rhinol.* 2019;9:918-925.
202. Ramezanpour M, Murphy J, Smith JLP, Vreugde S, Psaltis AJ. In vitro safety evaluation of human nasal epithelial cell monolayers exposed to carrageenan sinus wash. *International Forum of Allergy & Rhinology.* 2017;7:1170-1177.
203. Allen KL, Molan PC, Reid GM. A survey of the antibacterial activity of some New Zealand honeys. *The Journal of pharmacy and pharmacology.* 1991;43:817-822.
204. Adams CJ, Manley-Harris M, Molan PC. The origin of methylglyoxal in New Zealand manuka (Leptospermum scoparium) honey. *Carbohydrate Research.* 2009;344:1050-1053.
205. Mavric E, Wittmann S, Barth G, Henle T. Identification and quantification of methylglyoxal as the dominant antibacterial constituent of Manuka (Leptospermum scoparium) honeys from New Zealand. *Molecular nutrition & food research.* 2008;52:483-489.
206. Blair SE, Cokcetin NN, Harry EJ, Carter DA. The unusual antibacterial activity of medical-grade Leptospermum honey: antibacterial spectrum, resistance and transcriptome analysis. *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology.* 2009;28:1199-1208.
207. Cooper RA, Jenkins L, Henriques AF, Duggan RS, Burton NF. Absence of bacterial resistance to medical-grade manuka honey. *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology.* 2010;29:1237-1241.

208. Liu M, Lu J, Müller P, et al. Antibiotic-specific differences in the response of Staphylococcus aureus to treatment with antimicrobials combined with manuka honey. *Frontiers in Microbiology.* 2014;5:779.
209. Kilty SJ, AlMutairi D, Duval M, Groleau MA, De Nanassy J, Gomes MM. Manuka honey: histological effect on respiratory mucosa. *Am J Rhinol Allergy.* 2010;24:e63-66.
210. Paramasivan S, Drilling AJ, Jardeleza C, Jervis-Bardy J, Vreugde S, Wormald PJ. Methylglyoxal-augmented manuka honey as a topical anti-Staphylococcus aureus biofilm agent: safety and efficacy in an in vivo model. *Int Forum Allergy Rhinol.* 2014;4:187-195.
211. Thamboo A, Thamboo A, Philpott C, Javer A, Clark A. Single-blind study of manuka honey in allergic fungal rhinosinusitis. *J Otolaryngol Head Neck Surg.* 2011;40:238-243.
212. Lee Victoria S, Humphreys Ian M, Purcell Patricia L, Davis Greg E. Manuka honey sinus irrigation for the treatment of chronic rhinosinusitis: a randomized controlled trial. *International Forum of Allergy & Rhinology.* 2016;7:365-372.
213. Goggin R, Jardeleza C, Wormald PJ, Vreugde S. Colloidal silver: a novel treatment for Staphylococcus aureus biofilms? *Int Forum Allergy Rhinol.* 2014;4:171-175.
214. Rajiv S, Drilling A, Bassiouni A, James C, Vreugde S, Wormald PJ. Topical colloidal silver as an anti-biofilm agent in a Staphylococcus aureus chronic rhinosinusitis sheep model. *Int Forum Allergy Rhinol.* 2015;5:283-288.
215. Schreurs WJ, Rosenberg H. Effect of silver ions on transport and retention of phosphate by Escherichia coli. *J Bacteriol.* 1982;152:7-13.
216. Semeykina AL, Skulachev VP. Submicromolar Ag+ increases passive Na+ permeability and inhibits the respiration-supported formation of Na+ gradient in Bacillus FTU vesicles. *FEBS Lett.* 1990;269:69-72.
217. Chappell JB, Greville GD. Effect of silver ions on mitochondrial adenosine triphosphatase. *Nature.* 1954;174:930-931.
218. Modak SM, Fox CL, Jr. Binding of silver sulfadiazine to the cellular components of Pseudomonas aeruginosa. *Biochem Pharmacol.* 1973;22:2391-2404.
219. Rosenkranz HS, Rosenkranz S. Silver sulfadiazine: interaction with isolated deoxyribonucleic acid. *Antimicrob Agents Chemother.* 1972;2:373-383.
220. Over-the-counter drug products containing colloidal silver ingredients or silver salts. Department of Health and Human Services (HHS), Public Health Service (PHS), Food and Drug Administration (FDA). Final rule. *Federal register.* 1999;64:44653-44658.
221. Richter K, Facal P, Thomas N, et al. Taking the Silver Bullet Colloidal Silver Particles for the Topical Treatment of Biofilm-Related Infections. *ACS Appl Mater Interfaces.* 2017;9:21631-21638.
222. Scott JR, Krishnan R, Rotenberg BW, Sowerby LJ. The effectiveness of topical colloidal silver in recalcitrant chronic rhinosinusitis: a randomized crossover control trial. *Journal of Otolaryngology - Head & Neck Surgery.* 2017;46:64.
223. Clokie MR, Millard AD, Letarov AV, Heaphy S. Phages in nature. *Bacteriophage.* 2011;1:31-45.
224. Holmfeldt K, Middelboe M, Nybroe O, Riemann L. Large Variabilities in host strain susceptibility and phage host range govern interactions between lytic

marine phages and their Flavobactetium hosts. *Applied and environmental microbiology.* 2007;73:6730-6739.
225. Singh A, Arutyunov D, Szymanski CM, Evoy S. Bacteriophage based probes for pathogen detection. *The Analyst.* 2012;137:3405-3421.
226. Nilsson F, Tarli L, Viti F, Neri D. The use of phage display for the development of tumour targeting agents. *Adv Drug Deliver Rev.* 2000;43:165-196.
227. Bar H, Yacoby I, Benhar I. Killing cancer cells by targeted drug-carrying phage nanomedicines. *BMC Biotechnology.* 2008;8:37.
228. Yacoby I, Shamis M, Bar H, Shabat D, Benhar I. Targeting Antibacterial Agents by Using Drug-Carrying Filamentous Bacteriophages. *Antimicrobial Agents and Chemotherapy.* 2006;50:2087-2097.
229. Reardon S. Modified viruses deliver death to antibiotic-resistant bacteria. *Nature.* 2017;546:586-587.
230. Aleshkin AV, Rubalskii EO, Volozhantsev NV, et al. A small-scale experiment of using phage-based probiotic dietary supplement for prevention of E. coli traveler's diarrhea. *Bacteriophage.* 2015;5:e1074329-e1074329.
231. Charles-Nino C, Pedroza-Roldan C, Viveros M, Gevorkian G, Manoutcharian K. Variable epitope libraries: New vaccine immunogens capable of inducing broad human immunodeficiency virus type 1-neutralizing antibody response. *Vaccine.* 2011;29:5313-5321.
232. Prezzi C, Nuzzo M, Meola A, et al. Selection of antigenic and immunogenic mimics of hepatitis C virus using sera from patients. *Journal of immunology (Baltimore, Md. : 1950).* 1996;156:4504-4513.
233. Meola A, Delmastro P, Monaci P, et al. Derivation of vaccines from mimotopes. Immunologic properties of human hepatitis B virus surface antigen mimotopes displayed on filamentous phage. *Journal of immunology (Baltimore, Md. : 1950).* 1995;154:3162-3172.
234. Coffey B, Rivas L, Duffy G, Coffey A, Ross RP, McAuliffe O. Assessment of Escherichia coli O157:H7-specific bacteriophages e11/2 and e4/1c in model broth and hide environments. *Int J Food Microbiol.* 2011;147:188-194.
235. Nobrega FL, Vlot M, de Jonge PA, et al. Targeting mechanisms of tailed bacteriophages. *Nature Reviews Microbiology.* 2018;16:760-773.
236. Bradley DE. The structure of coliphages. *Journal of general microbiology.* 1963;31:435-445.
237. Bradley DE. The fluorescent staining of bacteriophage nucleic acids. *Journal of general microbiology.* 1966;44:383-391.
238. Tolstoy I, Kropinski AM, Brister JR. Bacteriophage Taxonomy: An Evolving Discipline. *Methods in molecular biology (Clifton, N.J.).* 2018;1693:57-71.
239. Aiewsakun P, Adriaenssens EM, Lavigne R, Kropinski AM, Simmonds P. Evaluation of the genomic diversity of viruses infecting bacteria, archaea and eukaryotes using a common bioinformatic platform: steps towards a unified taxonomy. *The Journal of general virology.* 2018;99:1331-1343.
240. O'Flaherty S, Ross RP, Coffey A. Bacteriophage and their lysins for elimination of infectious bacteria. *FEMS Microbiol Rev.* 2009;33:801-819.
241. Guttman B, Raya R, Kutter E. Basic Phage Biology. In: Elizabeth Kutter, Sulakvelidze A, eds. *Bacteriophages, Biology and Applications*. Boca Raton: CRC Press; 2004:29-66.

242. Lodish H, Berk A, Zipursky SL, Matsudaira P, Baltimore D, Darnell J. *Molecular Cell Biology.* 4 ed. New York: W. H. Freeman; 2000.
243. Lodish H BA, Zipursky SL, et al. Molecular Cell Biology. 4th Edition ed: New York: W. H. Freeman; 2000.
244. Slopek S, Weber-Dabrowska B, Dabrowski M, Kucharewicz-Krukowska A. Results of bacteriophage treatment of suppurative bacterial infections in the years 1981-1986. *Archivum immunologiae et therapiae experimentalis.* 1987;35:569-583.
245. Sulakvelidze A, Alavidze Z, Morris JG, Jr. Bacteriophage therapy. *Antimicrob Agents Chemother.* 2001;45:649-659.
246. Wright A, Hawkins CH, Anggard EE, Harper DR. A controlled clinical trial of a therapeutic bacteriophage preparation in chronic otitis due to antibiotic-resistant Pseudomonas aeruginosa; a preliminary report of efficacy. *Clinical Otolaryngology* 2009;34: 349–357.
247. Verbeken G, De Vos D, Vaneechoutte M, Merabishvili M, Zizi M, Pirnay JP. European regulatory conundrum of phage therapy. *Future microbiology.* 2007;2:485-491.
248. Aslam S, Gilbey T, Maddocks S, et al. 1642. Safety and Efficacy of Bacteriophage Therapy: Analysis of Clinical Case Series Data. *Open Forum Infectious Diseases.* 2018;5:S47-S47.
249. Mizoguchi K, Morita M, Fischer CR, Yoichi M, Tanji Y, Unno H. Coevolution of Bacteriophage PP01 and Escherichia coli O157:H7 in Continuous Culture. *Applied and Environmental Microbiology.* 2003;69:170-176.
250. Nale JY, Spencer J, Hargreaves KR, et al. Bacteriophage Combinations Significantly Reduce Clostridium difficile Growth In Vitro and Proliferation In Vivo. *Antimicrobial Agents and Chemotherapy.* 2016;60:968-981.
251. Drilling A, Morales S, Jardeleza C, Vreugde S, Speck P, Wormald PJ. Bacteriophage reduces biofilm of Staphylococcus aureus ex vivo isolates from chronic rhinosinusitis patients. *Am J Rhinol Allergy.* 2014;28:3-11.
252. Drilling A, Morales S, Boase S, et al. Safety and efficacy of topical bacteriophage and ethylenediaminetetraacetic acid treatment of Staphylococcus aureus infection in a sheep model of sinusitis. *Int Forum Allergy Rhinol.* 2014;4:176-186.
253. Zhang G, Zhao Y, Paramasivan S, et al. Bacteriophage effectively kills multidrug resistant Staphylococcus aureus clinical isolates from chronic rhinosinusitis patients. *International Forum of Allergy & Rhinology.* 2018;8:406-414.
254. Fong SA, Drilling A, Morales S, et al. Activity of Bacteriophages in Removing Biofilms of Pseudomonas aeruginosa Isolates from Chronic Rhinosinusitis Patients. *Frontiers in Cellular and Infection Microbiology.* 2017;7.
255. Drilling AJ, Cooksley C, Chan C, Wormald PJ, Vreugde S. Fighting sinus-derived Staphylococcus aureus biofilms in vitro with a bacteriophage-derived muralytic enzyme. *Int Forum Allergy Rhinol.* 2016;6:349-355.
256. Rashel M, Uchiyama J, Ujihara T, et al. Efficient elimination of multidrug-resistant Staphylococcus aureus by cloned lysin derived from bacteriophage phi MR11. *The Journal of infectious diseases.* 2007;196:1237-1247.

257. Fenton M, Casey PG, Hill C, et al. The truncated phage lysin CHAP(k) eliminates Staphylococcus aureus in the nares of mice. *Bioengineered bugs.* 2010;1:404-407.
258. Leung SS, Parumasivam T, Gao FG, et al. Production of Inhalation Phage Powders Using Spray Freeze Drying and Spray Drying Techniques for Treatment of Respiratory Infections. *Pharm Res.* 2016;33:1486-1496.
259. Chang RYK, Wallin M, Lin Y, et al. Phage therapy for respiratory infections. *Advanced drug delivery reviews.* 2018;133:76-86.
260. Richter K, Ramezanpour M, Thomas N, Prestidge CA, Wormald PJ, Vreugde S. Mind "De GaPP": in vitro efficacy of deferiprone and gallium-protoporphyrin against Staphylococcus aureus biofilms. *Int Forum Allergy Rhinol.* 2016;6:737-743.
261. Richter K, Van den Driessche F, Coenye T. Innovative approaches to treat Staphylococcus aureus biofilm-related infections. *Essays In Biochemistry.* 2017;61:61-70.
262. Richter K, Thomas N, Claeys J, et al. A Topical Hydrogel with Deferiprone and Gallium-Protoporphyrin Targets Bacterial Iron Metabolism and Has Antibiofilm Activity. *Antimicrob Agents Chemother.* 2017;61.
263. Weinberg ED. Iron availability and infection. *Biochim Biophys Acta.* 2009;1790:600-605.
264. Braun V. Iron uptake mechanisms and their regulation in pathogenic bacteria. *Int J Med Microbiol.* 2001;291:67-79.
265. Stojiljkovic I, Kumar V, Srinivasan N. Non-iron metalloporphyrins: potent antibacterial compounds that exploit haem/Hb uptake systems of pathogenic bacteria. *Mol Microbiol.* 1999;31:429-442.
266. Hijazi S, Visca P, Frangipani E. Gallium-Protoporphyrin IX Inhibits Pseudomonas aeruginosa Growth by Targeting Cytochromes. *Front Cell Infect Microbiol.* 2017;7:12.
267. Stojiljkovic I, Evavold BD, Kumar V. Antimicrobial properties of porphyrins. *Expert opinion on investigational drugs.* 2001;10:309-320.
268. Reniere ML, Torres VJ, Skaar EP. Intracellular metalloporphyrin metabolism in Staphylococcus aureus. *Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine.* 2007;20:333-345.
269. Richter K, Thomas N, Claeys J, et al. A Topical Hydrogel With Deferiprone and Gallium-Protoporphyrin Targets Bacterial Iron Metabolism and Has Antibiofilm Activity. *Antimicrob Agents Chemother.* 2017.
270. Richter K, Thomas N, Zhang G, et al. Deferiprone and Gallium-Protoporphyrin Have the Capacity to Potentiate the Activity of Antibiotics in Staphylococcus aureus Small Colony Variants. *Frontiers in Cellular and Infection Microbiology.* 2017;7.
271. Goldman MJ, Anderson GM, Stolzenberg ED, Kari UP, Zasloff M, Wilson JM. Human beta-defensin-1 is a salt-sensitive antibiotic in lung that is inactivated in cystic fibrosis. *Cell.* 1997;88:553-560.
272. Miyasawa-Hori H, Aizawa S, Takahashi N. Difference in the xylitol sensitivity of acid production among Streptococcus mutans strains and the biochemical mechanism. *Oral microbiology and immunology.* 2006;21:201-205.
273. Jain R, Lee T, Hardcastle T, Biswas K, Radcliff F, Douglas R. *The in vitro effect of xylitol on chronic rhinosinusitis biofilms*2016.

274. Weissman JD, Fernandez F, Hwang PH. Xylitol nasal irrigation in the management of chronic rhinosinusitis: a pilot study. *Laryngoscope.* 2011;121:2468-2472.
275. Zabner J, Seiler MP, Launspach JL, et al. The osmolyte xylitol reduces the salt concentration of airway surface liquid and may enhance bacterial killing. *Proceedings of the National Academy of Sciences of the United States of America.* 2000;97:11614-11619.
276. Lin L, Tang X, Wei J, Dai F, Sun G. Xylitol nasal irrigation in the treatment of chronic rhinosinusitis. *Am J Otolaryngol.* 2017;38:383-389.
277. Kofonow JM, Adappa ND. In vitro Antimicrobial Activity of SinuSurf. *ORL; journal for oto-rhino-laryngology and its related specialties.* 2012;74:179-184.
278. Rohrer JW, Dion GR, Brenner PS, et al. Surfactant improves irrigant penetration into unoperated sinuses. *Am J Rhinol Allergy.* 2012;26:197-200.
279. Cohen M, Kofonow J, Nayak JV, et al. Biofilms in chronic rhinosinusitis: A review. *American Journal of Rhinology and Allergy.* 2009;23:255-260.
280. Valentine R, Jervis-Bardy J, Psaltis A, Tan LW, Wormald P-J. Efficacy of Using a Hydrodebrider and of Citric Acid/Zwitterionic Surfactant on a Staphylococcus Aureus Bacterial Biofilm in the Sheep Model of Rhinosinusitis. *American Journal of Rhinology & Allergy.* 2011;25:323-326.
281. Tamashiro E, Banks CA, Chen B, et al. In vivo effects of citric acid/zwitterionic surfactant cleansing solution on rabbit sinus mucosa. *Am J Rhinol Allergy.* 2009;23:597-601.
282. Isaacs S, Fakhri S, Luong A, Whited C, Citardi MJ. The effect of dilute baby shampoo on nasal mucociliary clearance in healthy subjects. *Am J Rhinol Allergy.* 2011;25:e27-29.
283. Chiu AG, Palmer JN, Woodworth BA, et al. Baby shampoo nasal irrigations for the symptomatic post-functional endoscopic sinus surgery patient. *Am J Rhinol.* 2008;22:34-37.
284. Hooper C, Calvert J. The role for S-carboxymethylcysteine (carbocisteine) in the management of chronic obstructive pulmonary disease. *International journal of chronic obstructive pulmonary disease.* 2008;3:659-669.
285. Ohashi Y, Nakai Y, Sugiura Y, Ohno Y, Okamoto H, Hayashi M. Effect of S-carboxymethylcysteine on ciliary activity in chronic sinusitis. *Rhinology.* 1993;31:107-111.
286. Sugiura Y, Ohashi Y, Nakai Y. Improvement of mucosal pathology of the sinuses after exposure to sulfur dioxide by nebulization of S-carboxymethylcysteine. *Acta oto-laryngologica. Supplementum.* 1997;531:10-16.
287. Majima Y, Kurono Y, Hirakawa K, et al. Efficacy of combined treatment with S-carboxymethylcysteine (carbocisteine) and clarithromycin in chronic rhinosinusitis patients without nasal polyp or with small nasal polyp. *Auris, nasus, larynx.* 2012;39:38-47.
288. Athanasiadis T, Beule AG, Robinson BH, Robinson SR, Shi Z, Wormald PJ. Effects of a novel chitosan gel on mucosal wound healing following endoscopic sinus surgery in a sheep model of chronic rhinosinusitis. *Laryngoscope.* 2008;118:1088-1094.

289. Chung Y-J, An S-Y, Yeon J-Y, Shim WS, Mo J-H. Effect of a Chitosan Gel on Hemostasis and Prevention of Adhesion After Endoscopic Sinus Surgery. *Clinical and Experimental Otorhinolaryngology.* 2016;9:143-149.
290. Valentine R, Athanasiadis T, Moratti S, Hanton L, Robinson S, Wormald PJ. The efficacy of a novel chitosan gel on hemostasis and wound healing after endoscopic sinus surgery. *Am J Rhinol Allergy.* 2010;24:70-75.
291. Ngoc Ha T, Valentine R, Moratti S, Robinson S, Hanton L, Wormald PJ. A blinded randomized controlled trial evaluating the efficacy of chitosan gel on ostial stenosis following endoscopic sinus surgery. *Int Forum Allergy Rhinol.* 2013;3:573-580.
292. Chou TC, Fu E, Wu CJ, Yeh JH. Chitosan enhances platelet adhesion and aggregation. *Biochem Biophys Res Commun.* 2003;302:480-483.
293. Klokkevold PR, Lew DS, Ellis DG, Bertolami CN. Effect of chitosan on lingual hemostasis in rabbits. *J Oral Maxillofac Surg.* 1991;49:858-863.
294. Klokkevold PR, Subar P, Fukayama H, Bertolami CN. Effect of chitosan on lingual hemostasis in rabbits with platelet dysfunction induced by epoprostenol. *J Oral Maxillofac Surg.* 1992;50:41-45.
295. Pusateri AE, McCarthy SJ, Gregory KW, et al. Effect of a chitosan-based hemostatic dressing on blood loss and survival in a model of severe venous hemorrhage and hepatic injury in swine. *J Trauma.* 2003;54:177-182.
296. Rao SB, Sharma CP. Use of chitosan as a biomaterial: studies on its safety and hemostatic potential. *J Biomed Mater Res.* 1997;34:21-28.
297. Valentine R, Athanasiadis T, Moratti S, Robinson S, Wormald PJ. The efficacy of a novel chitosan gel on hemostasis after endoscopic sinus surgery in a sheep model of chronic rhinosinusitis. *Am J Rhinol Allergy.* 2009;23:71-75.
298. Valentine R, Boase S, Jervis-Bardy J, Dones Cabral JD, Robinson S, Wormald PJ. The efficacy of hemostatic techniques in the sheep model of carotid artery injury. *Int Forum Allergy Rhinol.* 2011;1:118-122.
299. Biagini G, Bertani A, Muzzarelli R, et al. Wound management with N-carboxybutyl chitosan. *Biomaterials.* 1991;12:281-286.
300. Stone CA, Wright H, Clarke T, Powell R, Devaraj VS. Healing at skin graft donor sites dressed with chitosan. *Br J Plast Surg.* 2000;53:601-606.
301. Azad AK, Sermsintham N, Chandrkrachang S, Stevens WF. Chitosan membrane as a wound-healing dressing: characterization and clinical application. *J Biomed Mater Res B Appl Biomater.* 2004;69:216-222.
302. Costain DJ, Kennedy R, Ciona C, McAlister VC, Lee TD. Prevention of postsurgical adhesions with N,O-carboxymethyl chitosan: examination of the most efficacious preparation and the effect of N,O-carboxymethyl chitosan on postsurgical healing. *Surgery.* 1997;121:314-319.
303. Diamond MP, Luciano A, Johns DA, Dunn R, Young P, Bieber E. Reduction of postoperative adhesions by N,O-carboxymethylchitosan: a pilot study. *Fertil Steril.* 2003;80:631-636.
304. Kennedy R, Costain DJ, McAlister VC, Lee TD. Prevention of experimental postoperative peritoneal adhesions by N,O-carboxymethyl chitosan. *Surgery.* 1996;120:866-870.
305. Cabral JD, McConnell MA, Fitzpatrick C, et al. Characterization of the in vivo host response to a bi-labeled chitosan-dextran based hydrogel for postsurgical adhesion prevention. *J Biomed Mater Res A.* 2015;103:2611-2620.

306. Medina JG, Das S. Sprayable chitosan/starch-based sealant reduces adhesion formation in a sheep model for chronic sinusitis. *Laryngoscope.* 2013;123:42-47.
307. Medina JG, Steinke JW, Das S. A chitosan-based sinus sealant for reduction of adhesion formation in rabbit and sheep models. *Otolaryngol Head Neck Surg.* 2012;147:357-363.
308. Vlahos A, Yu P, Lucas CE, Ledgerwood AM. Effect of a composite membrane of chitosan and poloxamer gel on postoperative adhesive interactions. *Am Surg.* 2001;67:15-21.
309. Zhou J, Elson C, Lee TD. Reduction in postoperative adhesion formation and re-formation after an abdominal operation with the use of N, O - carboxymethyl chitosan. *Surgery.* 2004;135:307-312.
310. Zhou J, Lee JM, Jiang P, Henderson S, Lee TD. Reduction in postsurgical adhesion formation after cardiac surgery by application of N,O-carboxymethyl chitosan. *J Thorac Cardiovasc Surg.* 2010;140:801-806.
311. Paramasivan S, Jones D, Baker L, et al. The use of chitosan-dextran gel shows anti-inflammatory, antibiofilm, and antiproliferative properties in fibroblast cell culture. *Am J Rhinol Allergy.* 2014;28:361-365.
312. No HK, Park NY, Lee SH, Meyers SP. Antibacterial activity of chitosans and chitosan oligomers with different molecular weights. *Int J Food Microbiol.* 2002;74:65-72.
313. Rhoades J, Roller S. Antimicrobial actions of degraded and native chitosan against spoilage organisms in laboratory media and foods. *Appl Environ Microbiol.* 2000;66:80-86.
314. Illum L, Farraj NF, Davis SS. Chitosan as a novel nasal delivery system for peptide drugs. *Pharm Res.* 1994;11:1186-1189.
315. Nakamura K, Maitani Y, Lowman AM, Takayama K, Peppas NA, Nagai T. Uptake and release of budesonide from mucoadhesive, pH-sensitive copolymers and their application to nasal delivery. *Journal of Controlled Release.* 1999;61:329-335.
316. Ha T, Valentine R, Moratti S, Hanton L, Robinson S, Wormald PJ. The efficacy of a novel budesonide chitosan gel on wound healing following endoscopic sinus surgery. *Int Forum Allergy Rhinol.* 2018;8:435-443.
317. Brower MC, Johnson ME. Adverse effects of local anesthetic infiltration on wound healing. *Regional anesthesia and pain medicine.* 2003;28:233-240.
318. Kesici S, Kesici U, Ulusoy H, Erturkuner P, Turkmen A, Arda O. [Effects of local anesthetics on wound healing]. *Revista brasileira de anestesiologia.* 2018;68:375-382.
319. Murr AH, Smith TL, Hwang PH, et al. Safety and efficacy of a novel bioabsorbable, steroid-eluting sinus stent. *Int Forum Allergy Rhinol.* 2011;1:23-32.
320. Marple BF, Smith TL, Han JK, et al. Advance II: A Prospective, Randomized Study Assessing Safety and Efficacy of Bioabsorbable Steroid-Releasing Sinus Implants. *Otolaryngology–Head and Neck Surgery.* 2012;146:1004-1011.
321. Rudmik L, Smith TL. Economic Evaluation of a Steroid-Eluting Sinus Implant following Endoscopic Sinus Surgery for Chronic Rhinosinusitis. *Otolaryngol Head Neck Surg.* 2014;151:359-366.

322. Lai SK, Suk JS, Pace A, et al. Drug carrier nanoparticles that penetrate human chronic rhinosinusitis mucus. *Biomaterials.* 2011;32:6285-6290.
323. Singhal D, Foreman A, Jervis-Bardy J, Wormald PJ. Staphylococcus aureus biofilms: Nemesis of endoscopic sinus surgery. *Laryngoscope.* 2011;121:1578-1583.
324. Singhal D, Psaltis AJ, Foreman A, Wormald PJ. The impact of biofilms on outcomes after endoscopic sinus surgery. *Am J Rhinol Allergy.* 2010;24:169-174.
325. Prince AA, Steiger JD, Khalid AN, et al. Prevalence of biofilm-forming bacteria in chronic rhinosinusitis. *American Journal of Rhinology.* 2008;22:239-245.
326. Foreman A, Psaltis AJ, Tan LW, Wormald PJ. Characterization of bacterial and fungal biofilms in chronic rhinosinusitis. *Am J Rhinol Allergy.* 2009;23:556-561.
327. Stephenson MF, Mfuna L, Dowd SE, et al. Molecular characterization of the polymicrobial flora in chronic rhinosinusitis. *J Otolaryngol Head Neck Surg.* 2010;39:182-187.
328. Costerton JW. Overview of microbial biofilms. *J Ind Microbiol.* 1995;15:137-140.
329. Ha KR, Psaltis AJ, Tan L, Wormald PJ. A sheep model for the study of biofilms in rhinosinusitis. *Am J Rhinol.* 2007;21:339-345.
330. Le T, Psaltis A, Tan LW, Wormald PJ. The efficacy of topical antibiofilm agents in a sheep model of rhinosinusitis. *Am J Rhinol.* 2008;22:560-567.
331. Ooi ML, Drilling AJ, Craig J, et al. Efficacy of topical anti- Staphylococcus aureus biofilm agent Chitodex Mupirocin Budesonide gel in an in vivo sheep sinusitis model. *Australian Journal of Otolaryngology.* 2019.
332. Boase S, Jervis-Bardy J, Cleland E, Pant H, Tan L, Wormald PJ. Bacterial-induced epithelial damage promotes fungal biofilm formation in a sheep model of sinusitis. *Int Forum Allergy Rhinol.* 2013;3:341-348.
333. Sachanandani NS, Piccirillo JF, Kramper MA, Thawley SE, Vlahiotis A. The effect of nasally administered budesonide respules on adrenal cortex function in patients with chronic rhinosinusitis. *Arch Otolaryngol Head Neck Surg.* 2009;135:303-307.
334. Jang DW, Lachanas VA, Segel J, Kountakis SE. Budesonide nasal irrigations in the postoperative management of chronic rhinosinusitis. *Int Forum Allergy Rhinol.* 2013;3:708-711.
335. Luo Y, Kirker KR, Prestwich GD. Cross-linked hyaluronic acid hydrogel films: new biomaterials for drug delivery. *Journal of Controlled Release.* 2000;69:169-184.
336. Ha T VR, Moratti S, Hanton L, Robinson S, Wormald PJ. The efficacy of a novel budesonide chitosan gel on wound healing following endoscopic sinus surgery. *International Forum of Allergy and Rhinology.* 2017:In press.
337. Organization TWH. Antibiotic resistance. *Media Centre, Fact Sheets, The World Health Organization.* 2016.
338. Conly JM, Johnston BL. Where are all the new antibiotics? The new antibiotic paradox. *The Canadian Journal of Infectious Diseases & Medical Microbiology.* 2005;16:159-160.
339. Organization TWH. Antibacterial Agents in Clinical Development. An analysis of the antibacterial clinical development pipeline, including

tuberculosis. In: Medicines and health products Aow, Rational use of medicines, The World Health Organization, ed2017:48.
340. Singhal D, Jekle A, Debabov D, et al. Efficacy of NVC-422 against Staphylococcus aureus biofilms in a sheep biofilm model of sinusitis. *Int Forum Allergy Rhinol.* 2012;2:309-315.
341. Heydorn A, Nielsen AT, Hentzer M, et al. Quantification of biofilm structures by the novel computer program comstat. *Microbiology.* 2000;146:2395-2407.
342. Klinger-Strobel M, Suesse H, Fischer D, Pletz MW, Makarewicz O. A Novel Computerized Cell Count Algorithm for Biofilm Analysis. *PLoS One.* 2016;11:e0154937.
343. Spino M, Connelly J, Tsang Y-C, Fradette C, Tricta F. Deferiprone Pharmacokinetics with and without Iron Overload and in Special Patient Populations. *Blood.* 2015;126:3365-3365.
344. Olivieri NF, Brittenham GM, McLaren CE, et al. Long-term safety and effectiveness of iron-chelation therapy with deferiprone for thalassemia major. *N. Engl. J. Med.* 1998;339:417-423.
345. de Léséleuc L, Harris G, KuoLee R, Chen W. In vitro and in vivo biological activities of iron chelators and gallium nitrate against Acinetobacter baumannii. *Antimicrob. Agents Chemother.* 2012;56:5397-5400.
346. Mohammadpour M, Behjati M, Sadeghi A, Fassihi A. Wound healing by topical application of antioxidant iron chelators: kojic acid and deferiprone. *Int Wound J.* 2013;10:260-264.
347. Stojiljkovic I, Kumar V, Srinivasan N. Non-iron metalloporphyrins: potent antibacterial compounds that exploit haem/Hb uptake systems of pathogenic bacteria. *Mol. Microbiol.* 1999;31:429-442.
348. Reniere ML, Torres VJ, Skaar EP. Intracellular metalloporphyrin metabolism in Staphylococcus aureus. *Biometals.* 2007;20:333-345.
349. Richter K, Ramezanpour M, Thomas N, Prestidge CA, Wormald PJ, Vreugde S. Mind "De GaPP": in vitro efficacy of deferiprone and gallium-protoporphyrin against Staphylococcus aureus biofilms. *Int Forum Allergy Rhinol.* 2016.
350. Enoch S, Leaper DJ. Basic science of wound healing. *Surgery (Oxford).* 2008;26:31-37.
351. Singer AJ, Clark RA. Cutaneous wound healing. *New England journal of medicine.* 1999;341:738-746.
352. Martin P, D'Souza D, Martin J, et al. Wound healing in the PU. 1 null mouse—tissue repair is not dependent on inflammatory cells. *Current Biology.* 2003;13:1122-1128.
353. Werner S, Krieg T, Smola H. Keratinocyte–fibroblast interactions in wound healing. *Journal of Investigative Dermatology.* 2007;127:998-1008.
354. Gurtner GC, Werner S, Barrandon Y, Longaker MT. Wound repair and regeneration. *Nature.* 2008;453:314-321.
355. Lovvorn HN, Cheung DT, Nimni ME, Perelman N, Estes JM, Adzick NS. Relative distribution and crosslinking of collagen distinguish fetal from adult sheep wound repair. *Journal of pediatric surgery.* 1999;34:218-223.
356. English RS, Shenefelt PD. Keloids and hypertrophic scars. *Dermatologic Surgery.* 1999;25:631-638.
357. Linares HA, Larson DL. Elastic tissue and hypertrophic scars. *Burns.* 1976;3:4-7.

358. Atiyeh BS. Nonsurgical management of hypertrophic scars: evidence-based therapies, standard practices, and emerging methods. *Aesthetic plastic surgery.* 2007;31:468-492.
359. Rajan V, Murray R. The duplicitous nature of inflammation in wound repair. *Wound Practice & Research: Journal of the Australian Wound Management Association.* 2008;16:122.
360. Martin P, Leibovich SJ. Inflammatory cells during wound repair: the good, the bad and the ugly. *Trends in cell biology.* 2005;15:599-607.
361. Dovi JV, Szpaderska AM, DiPietro LA. Neutrophil function in the healing wound: adding insult to injury? *Thrombosis and haemostasis.* 2004;92:275-280.
362. Goldman MP, Fitzpatrick RE. Laser treatment of scars. *Dermatologic Surgery.* 1995;21:685-687.
363. Lee JP, Jalili RB, Tredget EE, Demare JR, Ghahary A. Antifibrogenic Effects of Liposome-Encapsulated IFN-α2b Cream on Skin Wounds in a Fibrotic Rabbit Ear Model. *Journal of interferon & cytokine research.* 2005;25:627-631.
364. O'Brien L, Jones DJ. Silicone gel sheeting for preventing and treating hypertrophic and keloid scars. *The Cochrane Library.* 2013.
365. Golladay ES. Treatment of keloids by single intraoperative perilesional injection of repository steroid. *Southern medical journal.* 1988;81:736-738.
366. Anzarut A, Olson J, Singh P, Rowe BH, Tredget EE. The effectiveness of pressure garment therapy for the prevention of abnormal scarring after burn injury: a meta-analysis. *Journal of Plastic, Reconstructive & Aesthetic Surgery.* 2009;62:77-84.
367. España A, Solano T, Quintanilla E. Bleomycin in the treatment of keloids and hypertrophic scars by multiple needle punctures. *Dermatologic Surgery.* 2001;27:23-27.
368. Koc E, Arca E, Surucu B, Kurumlu Z. An open, randomized, controlled, comparative study of the combined effect of intralesional triamcinolone acetonide and onion extract gel and intralesional triamcinolone acetonide alone in the treatment of hypertrophic scars and keloids. *Dermatologic Surgery.* 2008;34:1507-1514.
369. Karagoz H, Yuksel F, Ulkur E, Evinc R. Comparison of efficacy of silicone gel, silicone gel sheeting, and topical onion extract including heparin and allantoin for the treatment of postburn hypertrophic scars. *Burns.* 2009;35:1097-1103.
370. So K, Umraw N, Scott J, Campbell K, Musgrave M, Cartotto R. Effects of enhanced patient education on compliance with silicone gel sheeting and burn scar outcome: a randomized prospective study. *Journal of Burn Care & Research.* 2003;24:411-417.
371. Puzey G. The use, of pressure garments on hypertrophic scars. *Journal of tissue viability.* 2002;12:11-15.
372. Esselman PC, Thombs BD, Magyar-Russell G, Fauerbach JA. Burn rehabilitation: state of the science. *American journal of physical medicine & rehabilitation.* 2006;85:383-413.
373. Manuskiatti W, Fitzpatrick RE. Treatment response of keloidal and hypertrophic sternotomy scars: comparison among intralesional corticosteroid,

5-fluorouracil, and 585-nm flashlamp-pumped pulsed-dye laser treatments. *Archives of dermatology.* 2002;138:1149-1155.

374. Tang Y-W. Intra-and postoperative steroid injections for keloids and hypertrophic scars. *British journal of plastic surgery.* 1992;45:371-373.

375. George W. Linear lymphatic hypopigmentation after intralesional corticosteroid injection: report of two cases. *Cutis.* 1999;64:61-64.

376. Richter K, Ramezanpour M, Thomas N, Prestidge CA, Wormald PJ, Vreugde S. Mind "De GaPP": in vitro efficacy of deferiprone and gallium-protoporphyrin against Staphylococcus aureus biofilms. *International forum of allergy & rhinology.* Vol 6: Wiley Online Library; 2016:737-743.

377. Mohammadpour M, Behjati M, Sadeghi A, Fassihi A. Wound healing by topical application of antioxidant iron chelators: kojic acid and deferiprone. *International wound journal.* 2013;10:260-264.

378. ten Raa S, Van den Tol MP, Sluiter W, Hofland LJ, van Eijck CH, Jeekel H. The role of neutrophils and oxygen free radicals in post-operative adhesions. *Journal of Surgical Research.* 2006;136:45-52.

379. Ramezanpour M, Moraitis S, Smith JL, Wormald P, Vreugde S. Th17 cytokines disrupt the airway mucosal barrier in chronic rhinosinusitis. *Mediators of inflammation.* 2016;2016.

380. Ramezanpour M, Murphy J, Smith JL, Vreugde S, Psaltis AJ. In vitro safety evaluation of human nasal epithelial cell monolayers exposed to carrageenan sinus wash. *International forum of allergy & rhinology.* Vol 7: Wiley Online Library; 2017:1170-1177.

381. Kim KS, Jung H, Shin IK, Choi BR, Kim DH. Induction of interleukin-1 beta (IL-1β) is a critical component of lung inflammation during influenza A (H1N1) virus infection. *Journal of medical virology.* 2015;87:1104-1112.

382. Hata RI, Senoo H. L-ascorbic acid 2-phosphate stimulates collagen accumulation, cell proliferation, and formation of a three-dimensional tissuelike substance by skin fibroblasts. *Journal of cellular physiology.* 1989;138:8-16.

383. Ooi ML, Richter K, Drilling AJ, et al. Safety and Efficacy of topical Chitogel-Deferiprone-Gallium Protoporphyrin in a sheep sinusitis model. *Frontiers in Microbiology.* 2018;9:917.

384. Munireddy S, Kavalukas SL, Barbul A. Intra-abdominal healing: gastrointestinal tract and adhesions. *Surgical Clinics.* 2010;90:1227-1236.

385. Goncalves S, Paupe V, Dassa EP, Rustin P. Deferiprone targets aconitase: implication for Friedreich's ataxia treatment. *BMC neurology.* 2008;8:20.

386. Dong X, Mao S, Wen H. Upregulation of proinflammatory genes in skin lesions may be the cause of keloid formation. *Biomedical reports.* 2013;1:833-836.

387. Wlaschek M, Scharffetter-Kochanek K. Oxidative stress in chronic venous leg ulcers. *Wound Repair and Regeneration.* 2005;13:452-461.

388. Tosa M, Ghazizadeh M, Shimizu H, Hirai T, Hyakusoku H, Kawanami O. Global gene expression analysis of keloid fibroblasts in response to electron beam irradiation reveals the involvement of interleukin-6 pathway. *Journal of investigative dermatology.* 2005;124:704-713.

389. Liechty KW, Adzick NS, Crombleholme TM. Diminished interleukin 6 (IL-6) production during scarless human fetal wound repair. *Cytokine.* 2000;12:671-676.

390. Petti CA, Fowler VG, Jr. Staphylococcus aureus bacteremia and endocarditis. *Cardiology clinics.* 2003;21:219-233, vii.
391. Ziran BH. Osteomyelitis. *J Trauma.* 2007;62:S59-60.
392. Baldoni D, Steinhuber A, Zimmerli W, Trampuz A. In vitro activity of gallium maltolate against Staphylococci in logarithmic, stationary, and biofilm growth phases: comparison of conventional and calorimetric susceptibility testing methods. *Antimicrob Agents Chemother.* 2010;54:157-163.
393. Foreman A, Boase S, Psaltis A, Wormald PJ. Role of bacterial and fungal biofilms in chronic rhinosinusitis. *Curr Allergy Asthma Rep.* 2012;12:127-135.
394. Foreman A, Psaltis AJ, Tan LW, Wormald PJ. Characterization of bacterial and fungal biofilms in chronic rhinosinusitis. *Allergy Rhinol (Providence).* 2010;1:10.
395. Singhal D, Psaltis AJ, Foreman A, Peter-John W. The impact of biofilms on outcomes after endoscopic sinus surgery. *Am J Rhinol Allergy.* 2010;24:169-174.
396. Anwar H, Dasgupta MK, Costerton JW. Testing the susceptibility of bacteria in biofilms to antibacterial agents. *Antimicrobial agents and chemotherapy.* 1990;34:2043-2046.
397. Roca I, Akova M, Baquero F, et al. The global threat of antimicrobial resistance: science for intervention. *New microbes and new infections.* 2015;6:22-29.
398. Carlton RM. Phage therapy: past history and future prospects. *Archivum immunologiae et therapiae experimentalis.* 1999;47:267-274.
399. Doolittle MM, Cooney JJ, Caldwell DE. Lytic infection of Escherichia coli biofilms by bacteriophage T4. *Canadian journal of microbiology.* 1995;41:12-18.
400. Corbin BD, McLean RJ, Aron GM. Bacteriophage T4 multiplication in a glucose-limited Escherichia coli biofilm. *Canadian journal of microbiology.* 2001;47:680-684.
401. Sillankorva S, Oliveira R, Vieira MJ, Sutherland IW, Azeredo J. Bacteriophage Phi S1 infection of Pseudomonas fluorescens planktonic cells versus biofilms. *Biofouling.* 2004;20:133-138.
402. Curtin JJ, Donlan RM. Using bacteriophages to reduce formation of catheter-associated biofilms by Staphylococcus epidermidis. *Antimicrob Agents Chemother.* 2006;50:1268-1275.
403. Fu W, Forster T, Mayer O, Curtin JJ, Lehman SM, Donlan RM. Bacteriophage cocktail for the prevention of biofilm formation by Pseudomonas aeruginosa on catheters in an in vitro model system. *Antimicrob Agents Chemother.* 2010;54:397-404.
404. Cerca N, Oliveira R, Azeredo J. Susceptibility of Staphylococcus epidermidis planktonic cells and biofilms to the lytic action of staphylococcus bacteriophage K. *Letters in applied microbiology.* 2007;45:313-317.
405. Lu TK, Collins JJ. Dispersing biofilms with engineered enzymatic bacteriophage. *Proceedings of the National Academy of Sciences of the United States of America.* 2007;104:11197-11202.
406. Carson L, Gorman SP, Gilmore BF. The use of lytic bacteriophages in the prevention and eradication of biofilms of Proteus mirabilis and Escherichia coli. *FEMS Immunology & Medical Microbiology.* 2010;59:447-455.

407. O'Flaherty S, Coffey A, Edwards R, Meaney W, Fitzgerald GF, Ross RP. Genome of staphylococcal phage K: a new lineage of Myoviridae infecting gram-positive bacteria with a low G+C content. *J Bacteriol.* 2004;186:2862-2871.
408. Vybiral D, Takac M, Loessner M, Witte A, von Ahsen U, Blasi U. Complete nucleotide sequence and molecular characterization of two lytic Staphylococcus aureus phages: 44AHJD and P68. *FEMS microbiology letters.* 2003;219:275-283.
409. Takac M, Blasi U. Phage P68 virion-associated protein 17 displays activity against clinical isolates of Staphylococcus aureus. *Antimicrob Agents Chemother.* 2005;49:2934-2940.
410. O'Brien FG, Udo EE, Grubb WB. Contour-clamped homogeneous electric field electrophoresis of Staphylococcus aureus. *Nature protocols.* 2006;1:3028-3033.
411. Theodorsson-Norheim E. Kruskal-Wallis test: BASIC computer program to perform nonparametric one-way analysis of variance and multiple comparisons on ranks of several independent samples. *Computer methods and programs in biomedicine.* 1986;23:57-62.
412. Soothill J, Hawkins C, Anggard E, Harper D. Therapeutic use of bacteriophages. *The Lancet. Infectious diseases.* 2004;4:544-545.
413. Biswas B, Adhya S, Washart P, et al. Bacteriophage therapy rescues mice bacteremic from a clinical isolate of vancomycin-resistant Enterococcus faecium. *Infection and immunity.* 2002;70:204-210.
414. Wills QF, Kerrigan C, Soothill JS. Experimental bacteriophage protection against Staphylococcus aureus abscesses in a rabbit model. *Antimicrob Agents Chemother.* 2005;49:1220-1221.
415. McVay CS, Velásquez M, Fralick JA. Phage therapy of Pseudomonas aeruginosa infection in a mouse burn wound model. *Antimicrobial agents and chemotherapy.* 2007;51:1934-1938.
416. Hawkins C, Harper D, Burch D, Anggard E, Soothill J. Topical treatment of Pseudomonas aeruginosa otitis of dogs with a bacteriophage mixture: a before/after clinical trial. *Veterinary microbiology.* 2010;146:309-313.
417. Markoishvili K, Tsitlanadze G, Katsarava R, Morris JG, Jr., Sulakvelidze A. A novel sustained-release matrix based on biodegradable poly(ester amide)s and impregnated with bacteriophages and an antibiotic shows promise in management of infected venous stasis ulcers and other poorly healing wounds. *International journal of dermatology.* 2002;41:453-458.
418. Bruttin A, Brussow H. Human volunteers receiving Escherichia coli phage T4 orally: a safety test of phage therapy. *Antimicrob Agents Chemother.* 2005;49:2874-2878.
419. Rhoads DD, Wolcott RD, Kuskowski MA, Wolcott BM, Ward LS, Sulakvelidze A. Bacteriophage therapy of venous leg ulcers in humans: results of a phase I safety trial. *J Wound Care.* 2009;18:237-238, 240-233.
420. Wright A, Hawkins CH, Anggard EE, Harper DR. A controlled clinical trial of a therapeutic bacteriophage preparation in chronic otitis due to antibiotic-resistant Pseudomonas aeruginosa; a preliminary report of efficacy. *Clinical otolaryngology : official journal of ENT-UK ; official journal of Netherlands Society for Oto-Rhino-Laryngology & Cervico-Facial Surgery.* 2009;34:349-357.

421. Sarker SA, Sultana S, Reuteler G, et al. Oral Phage Therapy of Acute Bacterial Diarrhea With Two Coliphage Preparations: A Randomized Trial in Children From Bangladesh. *EBioMedicine.* 2016;4:124-137.
422. McCallin S, Alam Sarker S, Barretto C, et al. Safety analysis of a Russian phage cocktail: from metagenomic analysis to oral application in healthy human subjects. *Virology.* 2013;443:187-196.
423. Kaur T, Nafissi N, Wasfi O, Sheldon K, Wettig S, Slavcev R. Immunocompatibility of Bacteriophages as Nanomedicines. *Journal of Nanotechnology.* 2012;2012:13.
424. Miernikiewicz P, Dąbrowska K, Piotrowicz A, et al. T4 phage and its head surface proteins do not stimulate inflammatory mediator production. *PloS one.* 2013;8:e71036-e71036.
425. Przerwa A, Zimecki M, Switala-Jelen K, et al. Effects of bacteriophages on free radical production and phagocytic functions. *Medical microbiology and immunology.* 2006;195:143-150.
426. Miedzybrodzki R, Switala-Jelen K, Fortuna W, et al. Bacteriophage preparation inhibition of reactive oxygen species generation by endotoxin-stimulated polymorphonuclear leukocytes. *Virus Research.* 2008;131:233-242.
427. Khan Mirzaei M, Haileselassie Y, Navis M, Cooper C, Sverremark-Ekström E, Nilsson AS. Morphologically Distinct Escherichia coli Bacteriophages Differ in Their Efficacy and Ability to Stimulate Cytokine Release In Vitro. *Frontiers in microbiology.* 2016;7:437-437.
428. Kucharewicz-Krukowska A, Slopek S. Immunogenic effect of bacteriophage in patients subjected to phage therapy. *Archivum immunologiae et therapiae experimentalis.* 1987;35:553-561.
429. Żaczek M, Łusiak-Szelachowska M, Jończyk-Matysiak E, et al. Antibody Production in Response to Staphylococcal MS-1 Phage Cocktail in Patients Undergoing Phage Therapy. *Frontiers in Microbiology.* 2016;7.
430. Łusiak-Szelachowska M, Weber-Dąbrowska B, Jończyk-Matysiak E, Wojciechowska R, Górski A. Bacteriophages in the gastrointestinal tract and their implications. *Gut Pathogens.* 2017;9:44.
431. Chan BK, Abedon ST, Loc-Carrillo C. Phage cocktails and the future of phage therapy. *Future microbiology.* 2013;8:769-783.
432. Perl TM, Roy MC. Postoperative wound infections: risk factors and role of Staphylococcus aureus nasal carriage. *J Chemother.* 1995;7 Suppl 3:29-35.
433. Chen AF, Wessel CB, Rao N. Staphylococcus aureus screening and decolonization in orthopaedic surgery and reduction of surgical site infections. *Clinical orthopaedics and related research.* 2013;471:2383-2399.
434. Bode LG, Kluytmans JA, Wertheim HF, et al. Preventing surgical-site infections in nasal carriers of Staphylococcus aureus. *The New England journal of medicine.* 2010;362:9-17.
435. Mousa HA. Burn and scald injuries. *Eastern Mediterranean health journal = La revue de sante de la Mediterranee orientale = al-Majallah al-sihhiyah li-sharq al-mutawassit.* 2005;11:1099-1109.
436. Piraino B. Staphylococcus aureus infections in dialysis patients: focus on prevention. *ASAIO journal (American Society for Artificial Internal Organs : 1992).* 2000;46:S13-17.

437. Fokkens WJ, Lund VJ, Mullol J, et al. EPOS 2012: European position paper on rhinosinusitis and nasal polyps 2012. A summary for otorhinolaryngologists. *Rhinology.* 2012;50:1-12.
438. Bhattacharyya N. The economic burden and symptom manifestations of chronic rhinosinusitis. *Am J Rhinol.* 2003;17:27-32.
439. Gliklich RE, Metson R. Economic implications of chronic sinusitis. *Otolaryngology--head and neck surgery : official journal of American Academy of Otolaryngology-Head and Neck Surgery.* 1998;118:344-349.
440. Hastan D, Fokkens WJ, Bachert C, et al. Chronic rhinosinusitis in Europe--an underestimated disease. A GA(2)LEN study. *Allergy.* 2011;66:1216-1223.
441. Hirsch AG, Stewart WF, Sundaresan AS, et al. Nasal and sinus symptoms and chronic rhinosinusitis in a population-based sample. *Allergy.* 2017;72:274-281.
442. Shi JB, Fu QL, Zhang H, et al. Epidemiology of chronic rhinosinusitis: results from a cross-sectional survey in seven Chinese cities. *Allergy.* 2015;70:533-539.
443. Smith SS, Evans CT, Tan BK, Chandra RK, Smith SB, Kern RC. National burden of antibiotic use for adult rhinosinusitis. *Journal of Allergy and Clinical Immunology.* 2013;132:1230-1232.
444. Antibiotic Resistance Threats in the United States, 2013: Centers for Disease Control and Prevention; 2013.
445. Sengupta S, Chattopadhyay MK, Grossart H-P. The multifaceted roles of antibiotics and antibiotic resistance in nature. *Frontiers in Microbiology.* 2013;4:47.
446. Kingdom TT, Swain RE, Jr. The microbiology and antimicrobial resistance patterns in chronic rhinosinusitis. *Am J Otolaryngol.* 2004;25:323-328.
447. Chen H-H, Liu X, Ni C, et al. Bacterial biofilms in chronic rhinosinusitis and their relationship with inflammation severity. *Auris Nasus Larynx.* 2012;39:169-174.
448. Psaltis AJ, Ha KR, Beule AG, Tan LW, Wormald PJ. Confocal scanning laser microscopy evidence of biofilms in patients with chronic rhinosinusitis. *Laryngoscope.* 2007;117:1302-1306.
449. Flemming HC, Wingender J. The biofilm matrix. *Nat Rev Microbiol.* 2010;8.
450. de la Fuente-Núñez C, Reffuveille F, Fernández L, Hancock REW. Bacterial biofilm development as a multicellular adaptation: antibiotic resistance and new therapeutic strategies. *Current Opinion in Microbiology.* 2013;16:580-589.
451. D'Agata E. Pseudomonas aeruginosa and Other Pseudomonas species. In: Bennett JE, Dolin R, Blaser MJ, eds. *Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases*. 8th ed. New York: Saunders; 2015.
452. Sader HS, Huband MD, Castanheira M, Flamm RK. Pseudomonas aeruginosa Antimicrobial Susceptibility Results from Four Years (2012 to 2015) of the International Network for Optimal Resistance Monitoring Program in the United States. *Antimicrobial agents and chemotherapy.* 2017;61.
453. Cleland EJ, Bassiouni A, Wormald PJ. The bacteriology of chronic rhinosinusitis and the pre-eminence of Staphylococcus aureus in revision patients. *Int Forum Allergy Rhinol.* 2013;3:642-646.

454. Rasmussen J, Aanaes K, Norling R, Nielsen KG, Johansen HK, von Buchwald C. CT of the paranasal sinuses is not a valid indicator for sinus surgery in CF patients. *J Cyst Fibros.* 2012;11:93-99.
455. Pires DP, Vilas Boas D, Sillankorva S, Azeredo J. Phage Therapy: a Step Forward in the Treatment of Pseudomonas aeruginosa Infections. *Journal of virology.* 2015;89:7449-7456.
456. Sulakvelidze A, Alavidze Z, Morris JG. Bacteriophage Therapy. *Antimicrobial agents and chemotherapy.* 2001;45:649-659.
457. Guttman B, Raya R, Kutter E. Basic Phage Biology. In: Kutter E, Sulakvelidze A, eds. *Bacteriophages: Biology and Applications*. Boca Raton: CRC Press; 2004.
458. Hanlon GW. Bacteriophages: an appraisal of their role in the treatment of bacterial infections. *International Journal of Antimicrobial Agents.* 2007;30:118-128.
459. Vilas Boas D, Almeida C, Sillankorva S, Nicolau A, Azeredo J, Azevedo NF. Discrimination of bacteriophage infected cells using locked nucleic acid fluorescent in situ hybridization (LNA-FISH). *Biofouling.* 2016;32:179-190.
460. Ha KR, Psaltis AJ, Tan L, Wormald P-J. A sheep model for the study of biofilms in rhinosinusitis. *Am J Rhinol.* 2007;21:339-345.
461. Drilling A, Morales S, Boase S, et al. Safety and efficacy of topical bacteriophage and ethylenediaminetetraacetic acid treatment of Staphylococcus aureus infection in a sheep model of sinusitis. *Int Forum Allergy Rhinol.* 2014;4:176-186.
462. Singhal D, Jekle A, Debabov D, et al. Efficacy of NVC-422 against Staphylococcus aureus biofilms in a sheep biofilm model of sinusitis. *Int Forum Allergy Rhinol.* 2012;2:309-315.
463. Singhal D, Boase S, Field J, Jardeleza C, Foreman A, Wormald P-J. Quantitative analysis of in vivo mucosal bacterial biofilms. *Int Forum Allergy Rhinol.* 2012;2:57-62.
464. Vorregaard M. Comstat2 - a modern 3D image analysis environment for biofilms. *Informatics and Mathematical Modelling*. Kongens Lyngby, Denmark: Technical University of Denmark; 2008.
465. Foreman A, Psaltis AJ, Tan LW, Wormald PJ. Characterization of bacterial and fungal biofilms in chronic rhinosinusitis. *Am J Rhinol Allergy.* 2009;23:556-561.
466. Jardeleza C, Thierry B, Rao S, et al. An in vivo safety and efficacy demonstration of a topical liposomal nitric oxide donor treatment for Staphylococcus aureus biofilm–associated rhinosinusitis. *Translational Research.* 2015;166:683-692.
467. Mazzocco A, Waddell TE, Lingohr E, Johnson RP. Enumeration of Bacteriophages Using the Small Drop Plaque Assay System. In: Clokie MRJ, Kropinski AM, eds. *Bacteriophages: Methods and Protocols, Volume 1: Isolation, Characterization, and Interactions*. Totowa, NJ: Humana Press; 2009:81-85.
468. Pinto Bezerra TF, de Melo Pádua FG, Ogawa AI, Santiago Gebrim EMM, Nascimento Saldiva PH, Voegels RL. Biofilm in Chronic Sinusitis with Nasal Polyps: Pilot study. *Brazilian Journal of Otorhinolaryngology (English Edition).* 2009:788-793.

469. Psaltis AJ, Weitzel EK, Ha KR, Wormald P-J. The effect of bacterial biofilms on post-sinus surgical outcomes. *Am J Rhinol.* 2008;22:1-6.
470. Foreman A, Wormald PJ. Different biofilms, different disease? A clinical outcomes study. *Laryngoscope.* 2010;120:1701-1706.
471. Woodworth BA, Tamashiro E, Bhargave G, Cohen NA, Palmer JN. An in vitro model of Pseudomonas aeruginosa biofilms on viable airway epithelial cell monolayers. *Am J Rhinol.* 2008;22:235-238.
472. Perloff JR, Palmer JN. Evidence of bacterial biofilms in a rabbit model of sinusitis. *Am J Rhinol.* 2005;19:1-6.
473. Ahiwale S, Tamboli N, Thorat K, Kulkarni R, Ackermann H, Kapadnis B. In vitro management of hospital Pseudomonas aeruginosa biofilm using indigenous T7-like lytic phage. *Current Microbiology.* 2011;62:335-340.
474. Alves DR, Perez-Esteban P, Kot W, et al. A novel bacteriophage cocktail reduces and disperses Pseudomonas aeruginosa biofilms under static and flow conditions. *Microbial biotechnology.* 2015.
475. Danis-Wlodarczyk K, Olszak T, Arabski M, et al. Characterization of the newly isolated lytic bacteriophages KTN6 and KT28 and their efficacy against pseudomonas aeruginosa biofilm. *PLoS ONE.* 2015;10.
476. Holguin AV, Rangel G, Clavijo V, et al. Phage PhiPan70, a Putative Temperate Phage, Controls Pseudomonas aeruginosa in Planktonic, Biofilm and Burn Mouse Model Assays. *Viruses.* 2015;7:4602-4623.
477. Kim S, Rahman M, Seol SY, Yoon SS, Kim J. Pseudomonas aeruginosa bacteriophage PA1Ø requires type IV pili for infection and shows broad bactericidal and biofilm removal activities. *Applied and environmental microbiology.* 2012;78:6380-6385.
478. Li LY, Yang HJ, Yue H. Isolation and classification of the bacteriophages of Pseudomonas aeruginosa and their application on biofilm control. *Chinese Journal of Microbiology and Immunology (China).* 2011;31:330-334.
479. Morales S, Mearns G, Cole R, Smithyman A. Bacteriophage treatment inhibits and reduces biofilm formation by Pseudomonas aeruginosa strains from cystic fibrosis patients. *Clinical Microbiology and Infection.* 2012;18:387-388.
480. Pires D, Sillankorva S, Faustino A, Azeredo J. Use of newly isolated phages for control of Pseudomonas aeruginosa PAO1 and ATCC 10145 biofilms. *Research in Microbiology.* 2011;162:798-806.
481. Fukuda K, Ishida W, Uchiyama J, et al. Pseudomonas aeruginosa keratitis in mice: effects of topical bacteriophage KPP12 administration. *PLoS One.* 2012;7:e47742.
482. Alemayehu D, Casey PG, McAuliffe O, et al. Bacteriophages phiMR299-2 and phiNH-4 can eliminate Pseudomonas aeruginosa in the murine lung and on cystic fibrosis lung airway cells. *MBio.* 2012;3:e00029-00012.
483. Morello E, Saussereau E, Maura D, Huerre M, Touqui L, Debarbieux L. Pulmonary bacteriophage therapy on Pseudomonas aeruginosa cystic fibrosis strains: first steps towards treatment and prevention. *PLoS One.* 2011;6:e16963.
484. Pabary R, Singh C, Morales S, et al. S103 Anti-Pseudomonal Bacteriophage Cocktail Reduces Inflammatory Responses in the Murine Lung. *Thorax.* 2012;67:A50-A51.

485. Rose T, Verbeken G, de Vos D, et al. Experimental phage therapy of burn wound infection: Difficult first steps. *International Journal of Burns and Trauma.* 2014;4:66-73.
486. Chhibber S, Bansal S, Kaur S. Disrupting the mixed-species biofilm of klebsiella pneumoniae B5055 and pseudomonas aeruginosa PAO using bacteriophages alone or in combination with xylitol. *Microbiology (United Kingdom).* 2015;161:1369-1377.
487. Kay MK, Erwin TC, McLean RJC, Aron GM. Bacteriophage ecology in Escherichia coli and pseudomonas aeruginosa mixed-biofilm communities. *Applied and environmental microbiology.* 2011;77:821-829.
488. Bhattacharyya N, Vyas DK, Fechner FP, Gliklich RE, Metson R. Tissue eosinophilia in chronic sinusitis: quantification techniques. *Arch Otolaryngol Head Neck Surg.* 2001;127:1102-1105.
489. Soler ZM, Sauer DA, Mace J, Smith TL. Relationship Between Clinical Measures and Histopathologic Findings in Chronic Rhinosinusitis. *Otolaryngology-Head and Neck Surgery.* 2009;141:454-461.
490. Harvey RJ, Lund VJ. Biofilms and chronic rhinosinusitis: systematic review of evidence, current concepts and directions for research. *Rhinology.* 2007;45:3-13.
491. Köck R, Werner P, Friedrich AW, et al. Persistence of nasal colonization with human pathogenic bacteria and associated antimicrobial resistance in the German general population. *New Microbes and New Infections.* 2016;9:24-34.
492. Kwashie ANA, Muibat AF, Modupe AI, Adejumoke BJM, Kehinde AO. A survey of bacterial isolates cultured from apparently healthy individuals in South-Western Nigeria. *International Journal of Tropical Medicine.* 2012;7:130-137.
493. Morrison AJ, Jr., Wenzel RP. Epidemiology of infections due to Pseudomonas aeruginosa. *Rev Infect Dis.* 1984;6 Suppl 3:S627-642.
494. Bruttin A, Brüssow H. Human volunteers receiving Escherichia coli phage T4 orally: A safety test of phage therapy. *Antimicrobial agents and chemotherapy.* 2005;49:2874-2878.
495. Bogovazova GG, Voroshilova NN, Bondarenko VM. [The efficacy of Klebsiella pneumoniae bacteriophage in the therapy of experimental Klebsiella infection]. *Zhurnal mikrobiologii, epidemiologii, i immunobiologii.* 1991:5-8.
496. Nungester WJ, Watrous RM. Accumulation of Bacteriophage in Spleen and Liver Following Its Intravenous Inoculation. *Proceedings of the Society for Experimental Biology and Medicine.* 1934;31:901-905.
497. Łusiak-Szelachowska M, Zaczek M, Weber-Dabrowska B, et al. Phage neutralization by sera of patients receiving phage therapy. *Viral Immunology.* 2014;27:295-304.
498. Toskala E, Rautiainen M. Electron Microscopy Assessment of the Recovery of Sinus Mucosa after Sinus Surgery. *Acta Oto-Laryngologica.* 2003;123:954-959.
499. Eaton MD, Bayne-Jones S. Bacteriophage therapy: Review of the principles and results of the use of bacteriophage in the treatment of infections. *Journal of the American Medical Association.* 1934;103:1769-1776.
500. Loc-Carrillo C, Abedon ST. Pros and cons of phage therapy. *Bacteriophage.* 2011;1:111-114.

501. Harper DR, Parracho HMRT, Walker J, et al. Bacteriophages and Biofilms. *Antibiotics.* 2014;3:270-284.
502. Chan BK, Sistrom M, Wertz JE, Kortright KE, Narayan D, Turner PE. Phage selection restores antibiotic sensitivity in MDR Pseudomonas aeruginosa. *Scientific Reports.* 2016;6:26717.
503. Asavarut P, Hajitou A. The phage revolution against antibiotic resistance. *The Lancet Infectious Diseases.* 2014;14:686.
504. Drilling AJ, Ooi ML, Miljkovic D, et al. Long-Term Safety of Topical Bacteriophage Application to the Frontal Sinus Region. *Front Cell Infect Microbiol.* 2017;7:49.
505. Kennedy JL, Hubbard MA, Huyett P, Patrie JT, Borish L, Payne SC. Sino-nasal Outcome Test (SNOT-22): A predictor of post-surgical improvement in patients with chronic sinusitis. *Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology.* 2013;111:246-251.e242.
506. Walker FD, White PS. Sinus symptom scores: what is the range in healthy individuals? *Clin Otolaryngol Allied Sci.* 2000;25:482-484.
507. Lund VJ, Kennedy DW. Quantification for staging sinusitis. The Staging and Therapy Group. *Ann Otol Rhinol Laryngol Suppl.* 1995;167:17-21.
508. Rhoads D, Wolcott R, Kuskowski M, Wolcott B, Ward L, Sulakvelidze A. Bacteriophage therapy of venous leg ulcers in humans: results of a phase I safety trial. *Journal of wound care.* 2009;18:237-238, 240-233.
509. Rose T, Verbeken G, Vos DD, et al. Experimental phage therapy of burn wound infection: difficult first steps. *International Journal of Burns and Trauma.* 2014;4:66-73.
510. Bruttin A, Brussow H. Human volunteers receiving *Escherichia coli* phage T4 Orally: a safety test of phage therapy. *Antimicrobial agents and chemotherapy.* 2005;49:2874-2878.
511. Sarkar S, McCallin S, Baretto C, et al. Oral T4-like phage cocktail application to healthy adult volunteers from Bangladesh. *Virology* 2012;434:222-232.
512. Ernest MA. Staphylococcus bacteriophage lysate aerosol therapy of sinusitis. *The Laryngoscope.* 1956;66:846-858.
513. Weber-Dabrowska B, Mulczyk M, Gorski A. Bacteriophage therapy of bacterial infections: an update of our institute's experience. *Archivum immunologiae et therapiae experimentalis.* 2000;48:547-551.
514. Mills AE. Staphylococcus bacteriophage lysate aerosol therapy of sinusitis. *Laryngoscope.* 1956;66:846-858.
515. Wang Z, Zheng P, Ji W, et al. SLPW: A Virulent Bacteriophage Targeting Methicillin-Resistant Staphylococcus aureus In vitro and In vivo. *Frontiers in Microbiology.* 2016;7.
516. Pabary R, Singh C, Morales S, et al. Antipseudomonal Bacteriophage Reduces Infective Burden and Inflammatory Response in Murine Lung. *Antimicrob Agents Chemother.* 2016;60:744-751.
517. Capparelli R, Parlato M, Borriello G, Salvatore P, Iannelli D. Experimental phage therapy against Staphylococcus aureus in mice. *Antimicrob Agents Chemother.* 2007;51:2765-2773.
518. Chhibber S, Kaur S, Kumari S. Therapeutic potential of bacteriophage in treating Klebsiella pneumoniae B5055-mediated lobar pneumonia in mice. *Journal of Medical Microbiology.* 2008;57:1508-1513.

519. Westwater C, Kasman LM, Schofield DA, et al. Use of genetically engineered phage to deliver antimicrobial agents to bacteria: an alternative therapy for treatment of bacterial infections. *Antimicrob Agents Chemother.* 2003;47:1301-1307.
520. Alandejani T, Marsan J, Ferris W, Slinger R, Chan F. Effectiveness of honey on Staphylococcus aureus and Pseudomonas aeruginosa biofilms. *Otolaryngology--head and neck surgery : official journal of American Academy of Otolaryngology-Head and Neck Surgery.* 2009;141:114-118.
521. Jardeleza C, Foreman A, Baker L, et al. The effects of nitric oxide on Staphylococcus aureus biofilm growth and its implications in chronic rhinosinusitis. *Int Forum Allergy Rhinol.* 2011;1:438-444.
522. Wright JB, Lam K, Buret AG, Olson ME, Burrell RE. Early healing events in a porcine model of contaminated wounds: effects of nanocrystalline silver on matrix metalloproteinases, cell apoptosis, and healing. *Wound Repair Regen.* 2002;10:141-151.
523. Dutsch-Wicherek M, Tomaszewska R, Strek P, Wicherek L, Skladzien J. The analysis of RCAS1 and DFF-45 expression in nasal polyps with respect to immune cells infiltration. *BMC Immunol.* 2006;21.
524. Eisenberg G, Pradillo J, Plaza G, Lizasoain I, Moro MA. [Increased expression and activity of MMP-9 in chronic rhinosinusitis with nasal polyposis]. *Acta Otorrinolaringol Esp.* 2008;59:444-447.
525. Sauter A, Stern-Straeter J, Sodha S, Hormann K, Naim R. Regulation of matrix metalloproteinases (MMP)-2/-9 expression in eosinophilic chronic rhinosinusitis--cell culture by interleukin-5 and -13? *In Vivo.* 2008;22:415-421.
526. Wicherek L, Galazka K, Lazar A. Analysis of Metallothionein, RCAS1 Immunoreactivity Regarding Immune Cell Concentration in the Endometrium and Tubal Mucosa in Ectopic Pregnancy during the Course of Tubal Rupture. *Gynecol Obstet Invest.* 2007;65.
527. Bhol KC, Schechter PJ. Topical nanocrystalline silver cream suppresses inflammatory cytokines and induces apoptosis of inflammatory cells in a murine model of allergic contact dermatitis. *Br J Dermatol.* 2005;152:1235-1242.
528. Lansdown AB. Silver in health care: antimicrobial effects and safety in use. *Curr Probl Dermatol.* 2006;33:17-34.
529. Tomi NS, Kranke B, Aberer W. A silver man. *Lancet.* 2004;363:532.
530. Organisation TWH. Silver in drinking water: Background document for the development of WHO Guidelines for Drinking Water Quality. *WHO, Geneva, Switzerland.* 1996.
531. Fung MC, Bowen DL. Silver products for medical indications: risk-benefit assessment. *J Toxicol Clin Toxicol.* 1996;34:119-126.
532. Cooper RA, Halas E, Molan PC. The efficacy of honey in inhibiting strains of Pseudomonas aeruginosa from infected burns. *The Journal of burn care & rehabilitation.* 2002;23:366-370.
533. Lu J, Turnbull L, Burke CM, et al. Manuka-type honeys can eradicate biofilms produced by Staphylococcus aureus strains with different biofilm-forming abilities. *PeerJ.* 2014;2:e326.
534. Maddocks SE, Lopez MS, Rowlands RS, Cooper RA. Manuka honey inhibits the development of Streptococcus pyogenes biofilms and causes reduced

expression of two fibronectin binding proteins. *Microbiology.* 2012;158:781-790.

535. Majtan J, Bohova J, Horniackova M, Klaudiny J, Majtan V. Anti-biofilm effects of honey against wound pathogens Proteus mirabilis and Enterobacter cloacae. *Phytotherapy research : PTR.* 2014;28:69-75.

536. Jervis-Bardy J, Foreman A, Bray S, Tan L, Wormald P-J. Methylglyoxal-infused honey mimics the anti-Staphylococcus aureus biofilm activity of manuka honey: Potential Implication in Chronic Rhinosinusitis. *The Laryngoscope.* 2011;121:1104-1107.

537. Jervis-Bardy J, Foreman A, Bray S, Tan L, Wormald PJ. Methylglyoxal-infused honey mimics the anti-Staphylococcus aureus biofilm activity of manuka honey: potential implication in chronic rhinosinusitis. *Laryngoscope.* 2011;121:1104-1107.

538. Paramasivan S, Drilling AJ, Jardeleza C, Jervis-Bardy J, Vreugde S, Wormald PJ. Methylglyoxal-augmented manuka honey as a topical anti-Staphylococcus aureus biofilm agent: safety and efficacy in an in vivo model. *Int Forum Allergy Rhinol.* 2014;4:187-195.

539. Stephens JM, Schlothauer RC, Morris BD, et al. Phenolic compounds and methylglyoxal in some New Zealand manuka and kanuka honeys. *Food Chemistry.* 2010;120:78-86.

540. Doty RL, Shaman P, Kimmelman CP, Dann MS. University of Pennsylvania Smell Identification Test: a rapid quantitative olfactory function test for the clinic. *Laryngoscope.* 1984;94:176-178.

541. Jenkins R, Cooper R. Improving antibiotic activity against wound pathogens with manuka honey in vitro. *PloS one.* 2012;7:e45600-e45600.

542. Liu MY, Cokcetin NN, Lu J, et al. Rifampicin-Manuka Honey Combinations Are Superior to Other Antibiotic-Manuka Honey Combinations in Eradicating Staphylococcus aureus Biofilms. *Front Microbiol.* 2017;8:2653.

543. Cooper R, Jenkins RE. Synergy between oxacillin and manuka honey sensitizes methicillin-resistant Staphylococcus aureus to oxacillin. *Journal of Antimicrobial Chemotherapy.* 2012;67:1405-1407.