The role of viruses in chronic rhinosinusitis

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To my long-suffering parents Mary and Michael and to my husband-in-waiting Patrick; we did it! Now onward and upward…

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Thesis declaration

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

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Publications arising from this thesis

Comparative viral sampling in the sinonasal passages; different viruses at different sites

Goggin RK, Bennett CA, Bassiouni A, Bialasiewicz S, Vreugde S, Wormald PJ and Psaltis AJ.

Front. Cell. Infect. Microbiol. 2018 Sep 19;8:334.

The presence of virus significantly associates with chronic rhinosinusitis disease severity

Goggin RK, Bennett CA, Bialasiewicz S, Vediappan RS, Vreugde S, Wormald PJ and Psaltis AJ.

Allergy. 2019 Aug 74(8):1569-1572

Association between viral infection and increased mucosal eosinophils and $CD8^+CD103^+$ T cells in chronic rhinosinusitis

Goggin RK, Bennett CA, Ramezanpour M, Hu H, Fenix K, Javadiyan S, Bialasiewicz S, Bassiouni A, Wormald PJ, Psaltis AJ and Vreugde S.

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Association between viral presence and changes in the bacterial microbiome in chronic rhinosinusitis

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The presence of virus significantly associates with chronic rhinosinusitis disease severity

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Top abstracts section

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Abbreviations

ADSS – Adelaide Disease Severity Score

AdV – Adenovirus

AEC – Airway epithelial cell

AJ – Adherens junction

ALI – Air-liquid interface

AFS – Allergic fungal sinusitis

BoV – Bocavirus

CF – Cystic fibrosis

CMV – Cytomegalovirus

COPD – Chronic obstructive pulmonary disease

CoV – Coronavirus

CPE – Cytopathic effects

CRS – Chronic rhinosinusitis

CRSsNP – Chronic rhinosinusitis without nasal polyps

CRSwNP – Chronic rhinosinusitis with nasal polyps

CSS – Chronic Sinusitis Survey

CT – Computed tomography

Ct – Cycle threshold

DIP – Discharge, Inflammation and Polyp score

DKO – Double knock-out

EBV – Epstein Barr virus

EnV – Enterovirus

ER – Endoplasmic reticulum

ERV3 – Endogenous retrovirus 3

EPS – Exopolysaccharide

FESS – Functional endoscopic sinus surgery

H and E – Haematoxylin and eosin

H. influenzae - *Haemophilus influenzae*

HA – Haemagglutinin

hB/NEC – Human bronchial/nasal epithelial cell

HHV6 – Human herpes virus 6

HPV – Human papilloma virus

HSV – Herpes simplex virus

hTeRT – Human telomerase reverse-transcriptase

ICAM-1 - Intercellular adhesion molecule 1

IFN – Interferon

IFNLR – Interferon lambda receptor

IL – Interleukin

IM/T – Inferior meatus/turbinate

LKS – Lund Kennedy score

LMS – Lund MacKay score

L/URT – Lower/upper respiratory tract

L/URTI – Lower/upper respiratory tract infection

M. catarrhalis – Moraxella catarrhalis

MHC – Major histocompatibility complex

MM/T – Middle meatus/turbinate

MLK – Modified Lund Kennedy score

MPV – Metapneumovirus

N. meningitidis – Neisseria meningitidis

NA – Neuraminidase

NAAT – Nucleic acid amplification tests

OMU – Ostiomeatal unit

P. aeruginosa – Pseudomonas aeruginosa

PAFR – Platelet activating factor receptor

PCR – Polymerase chain reaction

PIV – Parainfluenza virus

PMBC – Peripheral mononuclear blood cell

PND – Post-nasal drip

PPE – Personal protective equipment

PRR – Pattern recognition receptor

POSE – Perioperative Sinonasal Endoscopic score

PVOD – Post-viral olfactory dysfunction

QOD – Questionnaire of Olfactory Disorders

QoL – Quality of life

RSDI – Rhinosinusitis Disability Index

RSOM-31 – 31-Item Rhinosinusitis Outcome Measure

RSV – Respiratory syncytial virus

RT-PCR – Reverse transcription polymerase chain reaction

RV – Rhinovirus

S. aureus – Staphylococcus aureus

S. pneumoniae – Streptococcus pneumoniae

SAg – Superantigen

SCT – Sinusitis Control Test

SLPI – Secretory leucocyte protease inhibitor

SNOT-22 – Sino-Nasal Outcome Test 22

TAP – Transporter associated with antigen processing

Th – T helper type

TJ – Tight junction

TLR – Toll-like receptor

TNF – Tumour necrosis factor

Trm cells – Tissue-resident memory T cells

VAS – Visual Analogue Scale

VP – Virus-positive

VN – Virus-negative

VZV – Varicella zoster virus

WT – Wild type

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Table 5.3 Summary of most abundant bacterial genera. “MRA” indicates mean relative abundance, “P” indicates prevalence (both expressed as percentages).

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Thesis summary

The original research contents of this PhD thesis followed an extensive review of the literature in terms of the viral contribution to chronic rhinosinusitis (CRS); this is described in chapter one. Understanding of the aetiopathogenesis of the disease is of course integral to CRS prevention and treatment. It is also a subject of debate; the roles of bacteria, fungi and disordered innate and adaptive immunity have been investigated. Viruses, however, have received little attention. A commonly encountered clinical paradigm is that of a patient complaining of a viral upper respiratory tract infection (URTI) with the development of CRS symptoms thereafter. This has been investigated in population virome studies, however results regarding any relationship between viruses and CRS these have been inconsistent. Most of these studies have been limited in terms of size, seasonality, viral collection methods and the viral species for which investigators assayed. None have validated their collection methods or investigated for any association between viral presence and more severe disease. In addition to these none have investigated virally-induced changes in the bacterial microbiome in CRS. Microbial disarray is an area of burgeoning interest in many chronic disease processes, CRS included. Respiratory viruses are known to augment local bacterial binding, penetration and persistence. Could virus-induced respiratory epithelial changes be contributing to the disease also?

In order to investigate the above, the first step is to establish and validate a robust sinonasal viral collection method. This is described in the second chapter of this thesis. Sterile cytology brushes under direct endoscopic vision were used for this. 24 patients had two sites sampled immediately prior to endoscopic sinus surgery; the middle and inferior meatuses (MM and IM). Sample DNA and RNA were extracted and underwent PCR for a panel of common

respiratory viruses, including the *Herpesviridae* and endogenous retrovirus 3 (ERV3). The former were chosen for their near-omnipresence in the adult sinuses, and the latter as a marker of sample quality. 18/24 were positive for virus in at least one site, including 8 who were positive at both sites. Only 3 of those 8 demonstrated the same viral species at both sites. 6 showed no virus at either site. All samples demonstrated ERV3 well within published ranges indicating adequate sample quality. From this we concluded that the cytobrushes are an effective method for viral sampling in the nose and sinuses. We also identified a significant discord in viral species between the MM and IM. As such we recommended that both sites are sampled in order to gain truly representative data.

The third chapter of this thesis addresses the shortcomings of published population virome studies. The collection method detailed in the previous paragraph was used to sample from 288 patients over the period of one year. Disease severity data were also collected from these patients (Sino-Nasal Outcome Test 22 scores (SNOT-22), Adelaide Disease Severity Scores (ADSS), Lund MacKay scores (LMS) and Lund Kennedy scores (LKS)). Virus was found to be significantly more prevalent in CRS patients without nasal polyps (CRSsNP) than in controls or CRS patients with nasal polyps (CRSwNP). Viral presence was also found to be associated with significantly worse objective disease (LMS and LKS) but not subjective disease (SNOT-22 and ADSS). This is the first and only CRS virome study to encompass all subsets of the disease, to allow for seasonal variation in viral presence, and to use validated sample collection and processing techniques. We confirmed the long-held suspicion that viruses are more common in patients with CRS. As such we highlighted viruses as important potential targets for CRS prevention and therapy

The fourth chapter investigates the role of eosinophilia and T cell infiltrates in virus-positive versus virus-negative CRS and controls. Sinonasal tissue samples were taken and analysed for presence of eosinophils, $CD8^+$, $CD103^+$ and $CD8^+/CD103^+$ double-positive T cells (Trms). CRS was found to be associated with increased eosinophil and $CD8^+$ $CD103^+$ T cells in excess of that seen in virus-positive controls, implicating viruses in CRS aetiopathogenesis.

The study detailed in the fifth chapter aimed to investigate virus-associated changes in the bacterial CRS sinonasal microbiome again by taking brushings of the sinonasal mucosa. These were analysed for viral presence and the bacterial microbiome was also characterised, using 16S ribosomal RNA gene-targeted amplicon sequencing. Patients were divided into control, non-polyp and polyp groups. Half of each group was virus-positive. No significant differences were seen in relative abundances of the bacterial genera detected, their diversity or stability in any of the groups. A trend towards greater relative abundance of *Haemophilus* spp. was seen in patients reporting a viral illness two to four weeks prior. This early microbiome shift may represent a nidus for superinfection contributing to the development of CRS.

Systematic review of the literature

1.1 Chronic rhinosinusitis

Chronic rhinosinusitis (CRS) is an inflammatory disorder of the nasal and paranasal sinus mucosa. [1, 2] The pathogenesis remains uncertain, but several external and host-related factors are likely at play and a number of theories of have been postulated. [3, 4] Symptoms of CRS may include nasal obstruction, facial pain or pressure, anterior rhinorrhoea or post-nasal drip (PND), and reduction or loss of smell. [2, 3] The presence of two or more of these symptoms for twelve weeks or longer defines the syndrome. Two major disease phenotypes have been observed: without nasal polyps (CRSsNP) and with nasal polyps (CRSwNP). The latter typically causes more severe disease. [5] Published data show CRS is a highly prevalent and costly condition; it affects up to 16% of the population, [6, 7] and this is associated with substantial health care expenditure. [8-10] Despite great strides having been made in the management of CRS in the recent past it has a tendency to persist or recur despite maximal medical and surgical treatment, significantly contributing to the aforementioned costs. [3] Not only are treatment options expensive, but they are often invasive and not without risks to patients. Additionally, rhinosinusitis is the fifth most common diagnosis generating an antibiotic prescription worldwide [11] contributing to the development of resistant bacterial organisms [12, 13] and in turn raiding the armoury of therapies currently available to sufferers.

1.2 Theories of the aetiology and pathogenesis of chronic rhinosinusitis

Despite its prevalence, tendency towards relapse and recalcitrance, and its contribution to global health care costs and the development of antibiotic resistance, the aetiopathogenesis of CRS has been difficult to elucidate. Only a very small proportion of CRS can be attributed at least in part to an existing disorder. These known associations include Wegener's granulomatosis, human immunodeficiency virus, cystic fibrosis (CF) or Kartagener's syndrome. The vast majority of CRS, however, is idiopathic. Much research has been directed into the one factor (should such a factor exist) that unites the clinical and microbiological manifestations of the disease. A number of theories have been described; broadly these are the bacterial hypothesis, the immune hypothesis and the fungal hypothesis, with interest building and evidence emerging for a viral hypothesis.

1.2.1 The bacterial hypothesis

The development of the 16S rRNA bacterial sequencing method has revolutionised research into the bacterial microbiome throughout the body. Previously poorly-characterised due to reliance on more traditional bacterial culture methods, the sinonasal microbiome is now at the forefront of CRS research. Bacterial burden is becoming less of a focus in favour of relative abundance and diversity, [14] and the role of commensal bacteria is under scrutiny. There is a paucity of large-scale investigation into the CRS bacterial microbiome in the published literature, but theories regarding the potentially protective role of commensal bacteria and the disarray caused by overgrowth of more traditionally pathogenic bacteria abound in other organ systems. [15] Of particular interest to this thesis would be the potential for viral pathogens to initiate a disease-causing bacterial microbiome imbalance. Extensive study of

the sinonasal bacterial microbiome in health and disease is currently underway and has exciting implications for treatment of the disease. This is discussed in greater depth in "1.8 The bacterial microbiome of chronic rhinosinusitis".

Pre-dating these new molecular bacterial detection techniques however, *Staphylococcus aureus* (*S. aureus*) overgrowth has long been seen as a major contributor to CRS. [16] These bacteria in particular (along with *Pseudomonas aeruginosa* (*P. aeruginosa*), *Streptococcus pneumoniae* (*S. pneumoniae*), *Haemophilus influenzae* (*H. influenzae*) and *Moraxella catarrhalis* (*M. catarrhalis*)) have been heavily implicated in the formation of biofilms. Biofilms are communities of micro-organisms surrounded by an exopolysaccharide (EPS) matrix (figure 1.1). They are nigh-impossible to detach from the host surface and have the ability to slow their metabolic rate, allowing them to evade host immune responses and antibiotics, and to persist despite environments hostile for bacterial growth. [17] Planktonic *S. aureus* has been observed in up to 50% of CRS patients [18, 19] with *S. aureus* biofilms seen in 25 to 100%. [20] Theoretically biofilm defence-evasion and hostile-proliferation properties would lend them a unique ability to act as a bacterial reservoir. This may allow them to contribute to disease persistence and the downstream effects of simple planktonic bacterial colonisation and infection. However, as disease-inciting entities their role is not clear.

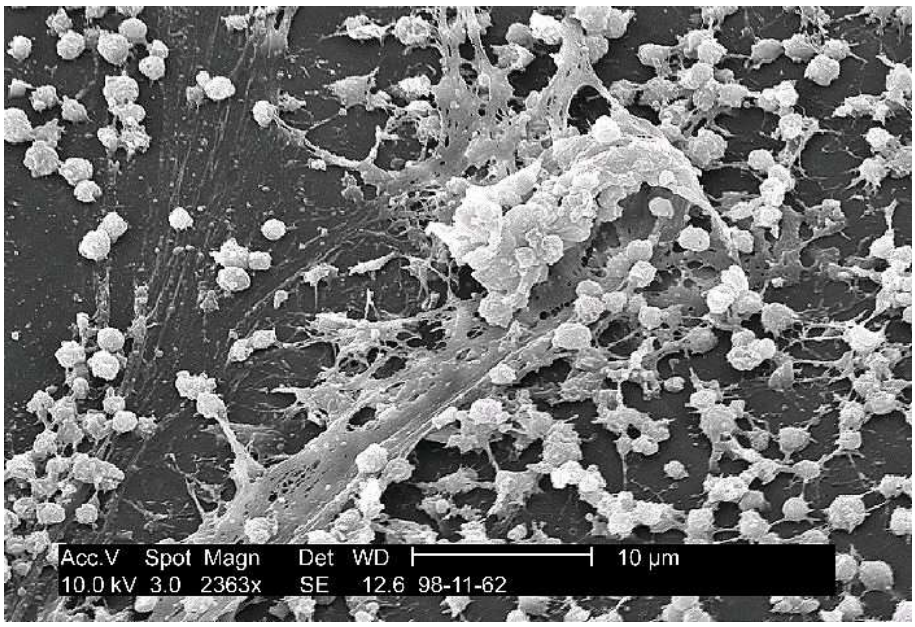


Figure 1.1 Scanning electron micrograph of *S. aureus* biofilms on the inner surface of a needleless connector, showing both cellular material and EPS matrix, reproduced with permission. [21]

S. aureus has an additional potential mechanism by which to cause harm in CRS; that of superantigen (SAg) exotoxin production. SAgs are produced by some bacteria and induce widespread non-specific T-cell activation and subsequent massive cytokine release from multiple immune cell types. *S. aureus* SAgs are associated with approximately 50% of CRSwNP (the more severe subset of the disease) and not with CRSsNP or controls. This is despite *S. aureus* colonisation in CRSsNP and controls. [22] The lack of a demonstrable SAg effect in such a large proportion of CRSwNP patients has led to postulation that these not be causative entities, but rather exacerbators of a pre-existing inflammatory imbalance with a potential role in polyp formation. [23]

1.2.2 The immune hypothesis

Interference with normal host defences by CRS can occur at a number of levels; these include the mechanical and immune barriers to pathogens. More specifically the mechanical barrier encompasses mucus, the muco-ciliary escalator, and tight and adherens junctions (TJs and AJs) between cells. The innate immune barrier includes cytokine-mediated immune cell recruitment, activation of complement, identification and removal of foreign material and debris. The adaptive immune barrier includes antigen presentation and recognition of non-self, generation of pathogen-specific elimination processes, and development of immunological memory. Interference with any of these may be caused by a number of host or environmental factors, but defects in any/all of these elements may result in disruption of the microbiome, increased exposure to pathogens, and/or an exaggerated compensatory innate and/or adaptive response, which can be damaging themselves. [23]

CF patients demonstrate a highly defective muco-ciliary escalator and also an abnormally high incidence of CRS, [24] as do CF gene mutation carriers who do not manifest the disease clinically. [25] In non-CF CRS patients the muco-ciliary escalator has also been shown to be defective. [26] TJs are weaker *in vitro* in CRS cells than in control cells, [27] and permeation of foreign material across this leaky barrier has also been demonstrated. [28]

A wealth of innate antimicrobials and associated molecules are produced in response to sinonasal pathogens, and secretion or activity of many of these are defective in CRS. Examples include lactoferrin (a non-specific antimicrobial), S100s (psoriasin and calprotectin, with roles in wound healing) and PLUNC (an antimicrobial with anti-biofilm properties). Also implicated are the pattern recognition receptor (PRR, responsible for

signalling pathways leading to innate antimicrobial secretion), the toll-like receptors (TLRs, part of the PRR family), bitter taste receptors and interleukin 22 (IL-22). Reports, however, are conflicting as to the magnitude and effect of these changes and their roles in CRSsNP versus CRSwNP. [29-33]

With regards the adaptive immune response in CRSwNP, a T helper type (Th-) 2 profile predominates. This is characterised by excessive eosinophilia, neutrophilia, IL-4, IL-5, IL-10, IL-13, mast cells, B lymphocytes, a reduction in collagen in favour of fibrin, and significant local oedema. [34] Conversely CRSsNP is more classically associated with a Th-1 skewed response. [35] However this is not uniform and can vary in population subtypes. For example, CRSwNP patients with CF exhibit more neutrophilia and a Th-17 cytokine predilection. [36] Multiple attempts have been made to classify CRS based on T-cell and cytokine profiles with no consensus reached. Despite a lack of concrete conclusions these studies do, however, indicate that a defective adaptive immune response is at play in the aetiopathogenesis of CRS.

1.2.3 The fungal hypothesis

In the late 1990s and early 2000s interest in the role of fungal elements and their potential ability to incite and drive CRS was sparked by research in the USA. In a cohort of 210 CRS patients, 96% had fungus cultured from their nasal secretions. Almost all of these went on to meet the diagnostic criteria for allergic fungal sinusitis (AFS) with regards histological and/or culture analysis. [37] This highlighted fungal pathogens as promising factors uniting the hugely diverse group that is idiopathic CRS. This finding was replicated in 2003 and postulated to be associated with the abnormally eosinophilic mucin seen in AFS. [38] This

was reflected in some *in vitro* work carried out on peripheral mononuclear blood cells (PMBCs). Exaggerated proliferative and cytokine responses were seen when PMBCs from CRS patients were exposed to various common or ubiquitous fungi, but the same was not observed in control cells. [39] Unfortunately, however, these results were not replicable. [40] Interest in fungi as the inciting agent for CRS has waned especially since large-scale trials have been unsuccessful in demonstrating any significant benefit with anti-fungal therapies in CRS populations versus healthy individuals. Neither reductions in objective or subjective disease severity scores nor any change in pro-inflammatory cytokine profiles were seen with amphotericin-B nasal lavages. [41, 42] Despite these findings fungi have not been entirely discarded in current CRS research. Abnormally heavy fungal colonisation is still associated with more severe disease and is considered to have an important role in promotion of Th-2 responses typically seen in CRS. [23]

1.2.4 The viral hypothesis and the role of interferons

The role of viruses in the pathogenesis of CRS is uncertain. In some cases patients appear to develop CRS after exposure to viral URTIs. In others with pre-existing disease viral URTIs commonly exacerbate symptoms. Of these various respiratory viruses rhinovirus (RV) is the most prevalent, with coronavirus (CoV) and influenza also making significant contributions. [43] Historically however, it has been difficult even to characterise the exact viruses at play in the sinonasal microbiome. Persistence of respiratory viruses in the mucosa of CRS sufferers higher than that of the general population has been observed to a degree. [44, 45] Preliminary data have shown that this viral infection may hamper a subsequent immune response to bacterial nasal and paranasal sinus infection. [46] Theoretically this may disrupt the microbiome and aggravate, or indeed cause initial development of, the symptoms of the

disease. A reasonable amount is known regarding mechanisms underlying viral-bacterial co-infection in the respiratory tract. It would appear viruses play a role in priming the mucosa for bacterial invasion (discussed further in "1.5 Viral-bacterial co-infection"). Why this may occur more frequently or more vigorously in CRS patients than in the general population is unknown. The culprit may be defective interferon (IFN) responses to viral infection in CRS, leading to prolonged and potentially more frequent and severe bacterial infections in that population. [47]

IFNs are innate immune signalling proteins synthesised and expressed by almost all human cells in response to infection and/or autoimmune disease. There have been three types described in humans; type 1 (consisting largely of IFN-α and IFN-β, but also IFN-ε, IFN-κ and IFN-ω), type 2 (IFN-γ) and type 3 (IFN-λ, of which there are four subtypes; IFN-λs 1-4). IFN-λ1 is also known as IL-29, and IFN-λ2 and IFN-λ3 as IL-28A and IL-28B respectively. [48] They exert their downstream effects by binding to their receptors to induce expression of hundreds of different genes with different methods of modulating the immune response to the original pathogen (figure 1.2). In brief these include:

- Promotion of apoptosis in diseased cells
- Inhibition of viral protein synthesis
- Induction of RNases that digest double-stranded viral DNA in order to limit replication
- Induction of protein kinase, limiting viral replication and inducing apoptosis
- Activation of the tumour suppressor gene p53, which induces apoptosis
- Priming of neighbouring cells for early apoptosis on exposure to virus
- Upregulation of the major histocompatibility complexes (MHC) classes I and II to allow increased recognition and killing of diseased cells

- Activation of macrophages and natural killer cells
- Activation of numerous signalling cascades with ultimate antiviral and anti-proliferative effects, including STAT, CRK and PI3K pathways

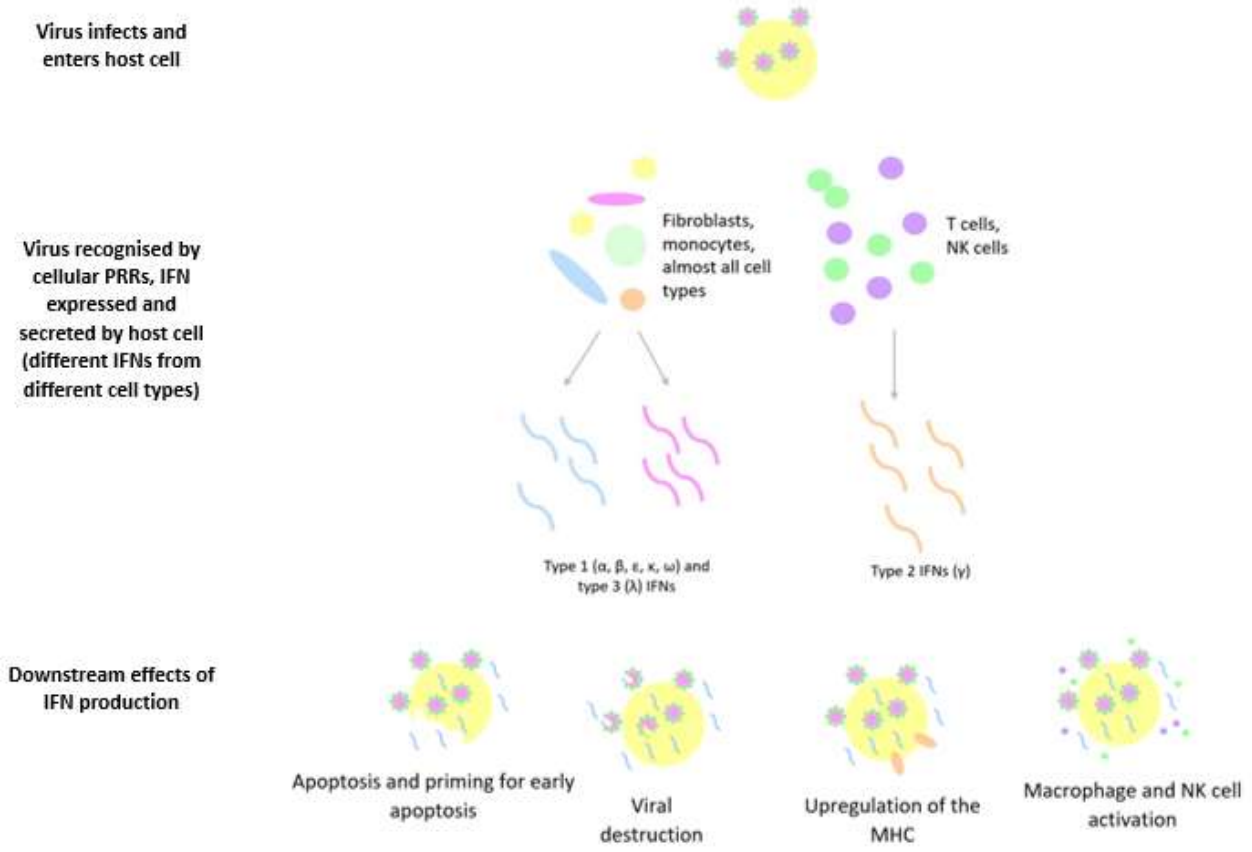


Figure 1.2 Origin and downstream effects of virus-induced IFN expression.

IFN receptors have been found to be IFN type-specific and largely ubiquitous for the type 1 and 2 proteins, ie. the receptors are present in almost all human cell types and can be bound by any IFN molecule of the appropriate type. This is not true of the type 3 IFN-λs; their receptors (IFNLRs) are variably expressed (largely by epithelial immune cells) and bind with differing affinities to differing IFN-λ molecules. [49] All three IFN types have been shown to play a role in the innate immune response to viral infection, but none to a greater degree than IFN-λ. IFN-λ is emerging as more and more important in the early defences and clinical

outcomes of respiratory epithelium to its near-constant onslaught by respiratory viruses. As such it may have a role in the aetiopathogenesis of CRS.

In vitro models have identified IFN-λ as a potentially more important contributor to viral respiratory infection than the other IFN types. BEAS-2B (a human bronchial epithelial carcinoma cell line), hBECs (primary human bronchial epithelial cells) and PMBCs have been infected with RV and assayed for IFN-α, IFN-β and IFN-λ. This resulted in a transient, early IFN-α rise, a late IFN-β rise, and a strong and persistent increase in IFN-λ in BEAS-2B. A similar pattern but at very low levels was seen in the hBECSs. All three of the investigated IFNs were strongly and persistently increased in the PBMCs. [50] RSV (respiratory syncytial virus) infected primary paediatric hBECs have shown an exclusive IFN-λ response, with no accompanying rise in type 1 IFNs. [51] This was supported by some work done with hNECs and the A549 cell line; when infected with RSV these mounted purely IFN-λ responses, and when treated with exogenous IFN-λ RSV replication was markedly suppressed. [52] Unfortunately these primary cells were harvested from allergic rhinitis patients, and so are perhaps subject to disordered inflammatory responses to invading pathogens/self already. Regardless these findings do add weight to the concept of IFN-λ being the first line of URT (upper respiratory tract) viral defence. In addition to this RSV has been postulated not only to induce IFN-λ preferentially, but to inhibit type 1 IFNs by the action of its NS1 and NS2 genes. [53] PIV-3 (a virus highly restricted to airway epithelium,) when introduced to the Vero and BEAS-2B cell lines has consistently shown a marked increase in IFN-λ1 expression across human, bovine and dolphin viruses. These cell lines are not known to be particularly representative of normal human airway epithelium, but the results are in keeping with those investigating similar viruses. [54] Influenza is also known to induce IFN-λ expression.

Human and murine airway epithelial cell (AEC) cultures induce both IFN-λ and (to a lesser extent) IFN-β responses. [55]

Animal models have drawn similar conclusions identifying IFN-λ as more active in viral respiratory infection than the other IFN subtypes. Influenza A-infected either wild type (WT), type 1 IFN-deficient or STAT2-deficient mice have displayed greater expression of IFN-λ than IFN-α/IFN-β in both the upper and lower airways. [56] Similarly intranasal IFN-λ administration has been shown to protect type 1 IFN-deficient mice from influenza infection [57] and to reduce influenza viral load and severity of disease in WT mice. [58] Conversely, IFN-α treatment in WT influenza-infected mice does restrict viral replication but worsens symptoms and increases proinflammatory cytokine secretion, innate immune cell recruitment and epithelial cell death. Similar findings have been made in influenza-infected primary human epithelial cells and PMBCs. MPV (metapneumovirus) has also been investigated in this context. This virus was found to induce all four IFN-λ ligands in mice and in the A549 cell line, and to a greater degree than that induced by RSV in those same mice and cells. [59]. SARS-CoV-1 has been investigated in a similar manner using STAT1-deficient mice, type 1 IFN-deficient mice, type 1 and 2 IFN-deficient mice (double knock-out (DKO) mice) and WT mice. STAT1-deficient mice are known to be more susceptible to SARS-CoV-1 and the DKO mice were able to mount only a type 3 IFN response. STAT1-deficient and the DKO mice both had similar peak viral titres and kinetics of viral clearance but developed much higher peak viral titres than their WT counterparts. The DKO mice remained clinically well, but STAT1-deficient mice developed liver pathology and eventually succumbed to neurological disease. From this the investigators concluded the STAT1-deficient mice's inability to control SARS-CoV-1 infection was due to impaired IFN type 1 and 3 signalling, but failure to control systemic viral spread was due to unrelated defects in the STAT1-

deficient mice. [60] Another comparative study used the DKO mice, type 1 IFN-deficient mice and type 3 IFN-deficient mice. These mice were infected with either SARS-CoV-1, influenza A, RSV or MPV. The investigators found that regardless of viral species there was greater pathogenicity, a worse clinical picture and higher replication titres in the lungs of the DKO mice than those lacking one IFN type only. They thus concluded both are important in respiratory tract defence to viral pathogens. [61]

IFN-β has also received specific attention in the literature. This has been largely focused on RV and its role in chronic inflammatory disease of the lower airways, but elements of this work might be hypothesised to reflect the state of play in CRS. 85% of asthma exacerbations are triggered by viruses, RV being the most significant contributor. [62] An excellent paper written by a group of English researchers in 2005 aimed to investigate the mechanism of observed increased RV susceptibility in asthmatics. hBECs were sampled by brushing at bronchoscopy. Cells were taken from 24 asthmatics and 10 controls. Cell cultures were then infected with either RV16, RV1B, medium alone, or UV-inactivated RV16. The authors found a 50 versus 7-fold increase in viral RNA in the supernatants of asthmatic versus control cells. ICAM-1 (intercellular adhesion molecule 1) is the cellular receptor for the major RV group, including RV16. ICAM-1 expression was upregulated in all groups, but much more so in asthmatics. LDLR is the cellular receptor for the minor RV group, including RV1B, but its expression was not investigated in this study. Early impairment of IFN-β mRNA and protein expression in cellular supernatants was found in both groups, but again far more in the asthmatics. Asthmatic cells were then treated with exogenous IFN-β, which induced apoptosis and reduced viral replication. This identifies IFN-β as another potential treatment or preventative target in virus-induced asthmatic exacerbations, and as such may have a role in CRS. [63]

Both IFN-λ and IFN-β appear to be negatively affected by a Th2-skewed inflammatory profile. This is interesting considering many CRS sufferers show a Th2 preponderance with induction of pro-inflammatory IL-4 and IL-13 cytokines. Th2 inflammation has been associated with impaired immunity to RV in *ex vivo* hBECs, [64] and so has been postulated as a potential mechanism behind this increased susceptibility to viral infection. BEAS-2B, hBECs from normal individuals and hNECs (primary human nasal epithelial cells) from patients with allergic rhinitis have been pre-treated with IL-3 and -14 to simulate Th2 inflammation. Cell cultures then exposed to RV produced significantly less IFN-β and IFN-λ and allowed significantly more RV replication. [65] This is notable as it may be part of the mechanism underpinning a defective IFN response to viral infection in CRS.

A study from South Korea attempted to unite this work on IFN in the respiratory tract and CRS. This paper sparked the initial interest demonstrated in this thesis in the IFNs themselves as the potential culprit allowing more frequent or vigorous viral infections in CRS. The investigators took IT hNECs from 10 CRSwNP patients, 3 CRSsNP patients and 14 patients deemed controls (turbinectomy patients suffering from chronic hypertrophic rhinitis). Cells were grown in submerged culture and exposed to RV16 or phosphate-buffered saline as a control. They found a slightly delayed reduction in viral titre and a similarly slight reduction in IFN-β expression in the CRS patients versus control (cytokine profiling was limited to IL-6 and IL-8 and was not significantly different between the groups). From this it was suggested that hNECs display a slightly impaired response to RV infection, but not to the marked degree seen in hBECs, and so perhaps they employ other methods of viral clearance not seen in the lower airways. However, the culture model used (undifferentiated, submerged hNECs rather than the perhaps more relevant ALI model), the small sample size, and the

degree of chronic inflammation likely present even in the control patients do cast some doubt. Additionally, the IFN-λ response was not explored. [47]

Although they are not classically associated with type 3 IFN responses some bacterial infections have also been investigated in this light. Of particular interest to the sinuses *S. aureus* and *P. aeruginosa*-infected type 1 IFN-deficient mice (therefore mounting a heightened type 3 response to the bacteria) have been found to exhibit less pathology without any changes in the composition of their respiratory cellular infiltrates. [66] WT mice also infected with *S. aureus* have been found to mount a type 1 IFN response, at least in part due to protein A expression by the bacteria inducing IFN-β, JAK-STAT signalling and IL-6 production. Type 1 IFN-deficient mice were also infected in the same study and thus were protected from the bacteria, presumably because of their inability to respond to it. [67] Being a largely bacterial disease this finding is intriguing in the case of CRS; might IFN supplementation improve response not just to viral infection, but to bacterial infection also, independent of its antiviral effects?

There exists also the hypothesis that respiratory viruses might persist in the sinuses of CRS patients longer than in healthy individuals, and that this may be part of the mechanism behind the viral hypothesis of CRS. Many DNA viruses of the URT (in particular the *Herpesviridae*) are known to persist in the respiratory mucosa for almost a lifetime. The mechanisms for this vary and many focus on episomal latency or insertion of viral DNA into the host cell genome. As such many of these are irrelevant to the more common RNA viruses of the URT, but a recent study by Oldham *et al.* explored a newer mechanism of respiratory viral latency in some depth. Cellular immunity against viral infections requires antigen presentation by MHC class 1 molecules; antigen peptides enter the host cell, are transported to the endoplasmic

reticulum (ER) by the transporter associated with antigen processing (TAP), are loaded onto the MHC1 by the peptide-loading complex (PLC), and then brought to the cell surface, thus presenting the processed antigen peptides to the immune system. It can then be recognised by cytotoxic CD8+ T cells, which in turn programme the cell for apoptosis. There are many proposed mechanisms of viral evasion of the immune system exerting their effects on any of these steps, but this study investigated the possibility for TAP defects to keep antigens hidden from T cells, resulting in immunodeficiency. Five viral proteins that inhibit TAP were identified from the herpes and cowpox families, the most important being ICP47. This is produced by herpes simplex viruses (HSV) 1 and 2 and blocks TAP activity, reducing MHC1 surface expression, in turn evading recognition by the immune system and resulting in the lifelong infection characteristic of these viruses. [68] There are many human viruses capable of similar latency, if by different mechanisms (for example hepatitis C, HIV and some of the other *Herpesviridae*) and so it is not unreasonable to suspect that upper respiratory viruses may be capable of similar evasion of the immune system. This would be of significant interest to the viral hypothesis of CRS; are the common cold viruses not just more prevalent in CRS populations, but are they (and thus their deleterious effects on the immune response to further infection) more persistent also?

1.3 Disease severity in chronic rhinosinusitis

CRS severity can be gauged subjectively using various symptom-based patient questionnaires. These include the SNOT-22 (Sino-Nasal Outcome Test 22) questionnaire, the ADSS (Adelaide Disease Severity Score) and the Visual Analogue Score (VAS). Objectively, severity can be measured using computed tomography (CT) and the Lund MacKay score (LMS), or endoscopically using the Lund Kennedy score (LKS).

1.3.1 Subjective measurement

Multiple quality of life (QoL) questionnaires have been established for assessment of symptom severity and to measure efficacy of interventions in CRS. Most address rhinological symptoms, their effect on the patient's activities of daily living (ADLs), the emotional impact of the disease, or a combination of such. Most also attempt to correlate with objective disease measurement tools and disease outcomes. Questionnaires include the SNOT -16, -20 or -22, ADSS, 31-Item Rhinosinusitis Outcome Measure (RSOM-31), VAS, Rhinosinusitis Disability Scale (RSDI), Sinusitis Control Test (SCT), Questionnaire of Olfactory Disorders (QOD) and Chronic Sinusitis Survey (CSS). The SNOT-22 (figure 1.3, modified from the SNOT-20) has been the most widely accepted of these, and was validated in 2009 by Hopkins *et al*. [69] The SNOT-22 has faced criticism for its length and lack of specificity in terms of rhinological symptoms. This led to the development of the shorter and more directed ADSS in 2013 (figure 1.4). [70] Multiple versions of the VAS (figure 1.5) exist; it has also received criticism for its difficulty to complete; overwhelmed with choice, patients are often confused as to which point best suits them. It also requires precise post-questionnaire distance measurement, and interpretation of the resulting wide range of raw scores has been

problematic. [71] A recent systematic review deemed the SNOT-22, QOD and SCT as the most valid available tools. [72] However, these can all be quite lengthy and thus difficult for patients to complete. This is largely due to inclusion of details of emotional well-being, generalised systemic symptoms and patient expectations and preferences, and so the choice of questionnaire must take this into account.

Below you will find a list of symptoms and social / emotional consequences of your rhinosinusitis. We would like to know more about these problems and would appreciate your answering the following questions to the best of your ability. There are no right or wrong answers, and only you can provide us with this information.
Please rate your problems as they have been over the past **two weeks.**

Considering how severe the problem is when you experience it and how frequently it happens, please rate each item below on how 'bad' it is by *circling the number* that corresponds with how you feel using this scale →	No Problem	Very Mild Problem	Mild or slight Problem	Moderate Problem	Severe Problem	Problem as bad as it can be
1. Need to blow nose	0	**1**	2	3	4	5
2. Sneezing	0	**1**	2	3	4	5
3. Runny nose	0	**1**	2	3	4	5
4. Cough	0	**1**	2	3	4	5
5. Nasal obstruction	0	**1**	2	3	4	5
6. Loss of smell or taste	0	**1**	2	3	4	5
7. Post-nasal discharge	0	**1**	2	3	4	5
8. Thick nasal discharge	0	**1**	2	3	4	5
9. Ear fullness	0	**1**	2	3	4	5
10. Dizziness	0	1	2	3	4	5
11. Ear pain	0	1	2	3	4	5
12. Facial pain/pressure	0	1	2	3	4	5
13. Difficulty falling asleep	0	1	2	3	4	5
14. Wake up at night	0	1	2	3	4	5
15. Lack of good night's sleep	0	1	2	3	4	5
16. Wake up tired	0	1	2	3	4	5
17. Fatigue	0	1	2	3	4	5
18. Reduced productivity	0	1	2	3	4	5
19. Reduced concentration	0	1	2	3	4	5
20. Frustrated/restless/irritable	0	1	2	3	4	5
21. Sad	0	1	2	3	4	5
22. Embarrassed	0	1	2	3	4	5

Figure 1.3 Sino-Nasal Outcome Test 22 (adapted from Hopkins *et al.*). [69]

Nasal obstruction	1 2 3 4 5
Rhinorrhoea	1 2 3 4 5
Post-nasal drip	1 2 3 4 5
Headache or facial pain	1 2 3 4 5
Sense of smell	1 2 3 4 5
Quality of life†	
How do your symptoms affect your quality of life?	0———7

*1 = no symptoms, 2 = mild, 3 = moderate, 4 = severe, 5 = extreme. †0 = no effect; 7 = maximal effect.

Figure 1.4 Adelaide Disease Severity Score (adapted from Naidoo *et al.*). [70]

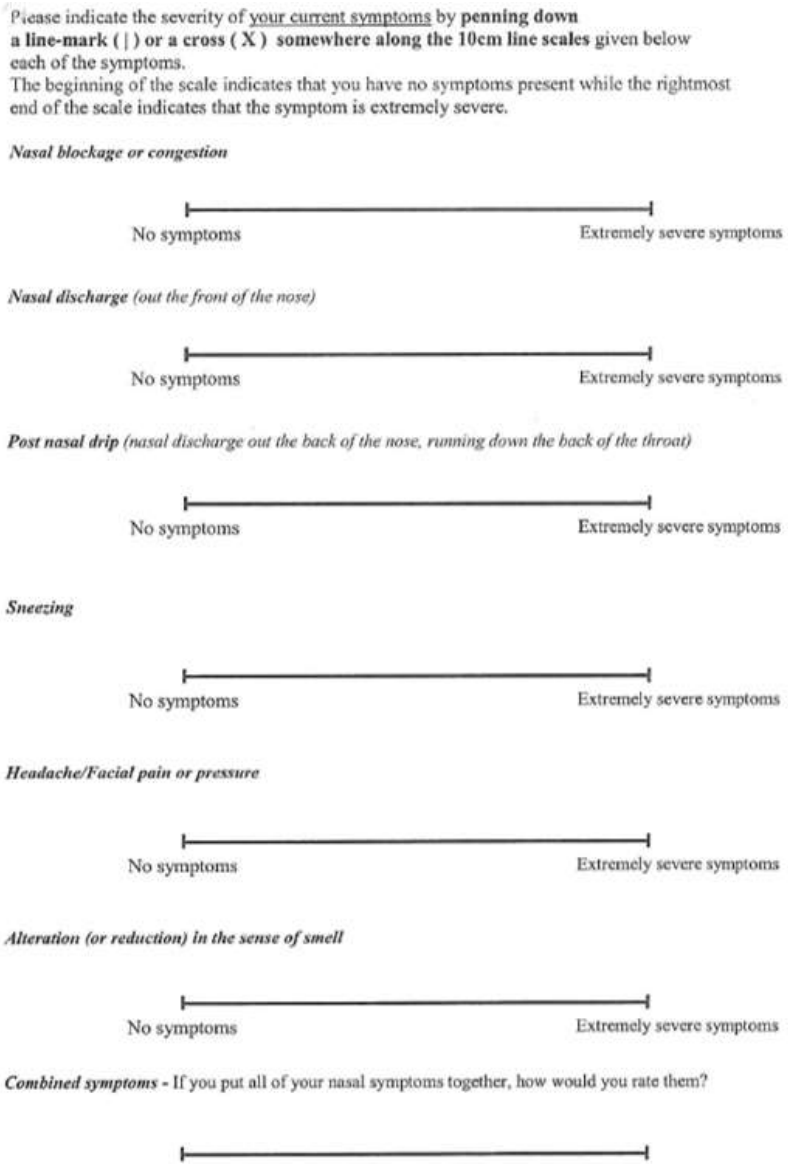


Figure 1.5 Visual Analogue Scale (adapted from Doulaptisi *et al.*). [73]

1.3.2 Objective measurement

Multiple scoring tools have been developed for objectively assessing CRS severity and again no consensus exists as to their optimal use. The most popular are likely the LMS and LKS.

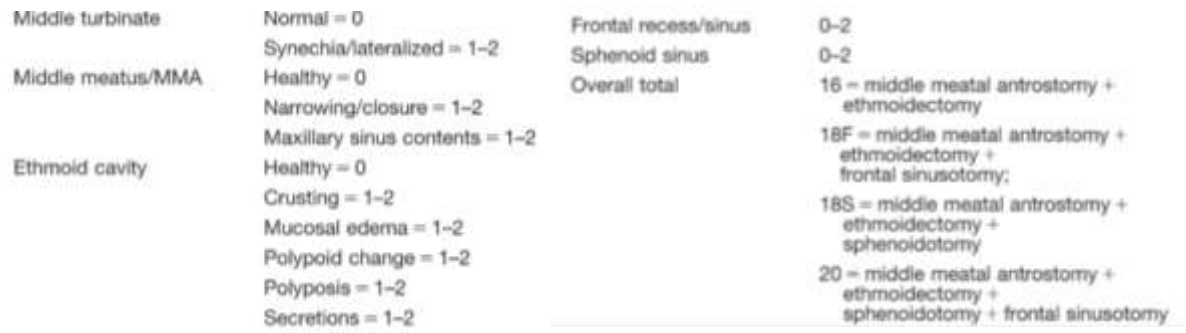
There do exist other radiological scoring systems for sinus disease besides the LMS. These include the systems suggested by Jorgensen *et al*, [74] Weber *et al.* [75] and Newman *et al.* [76]) Historically however, the LMS (figure 1.6) [77] has been the most widely employed. It uses CT imaging of the sinuses and scores the maxillary, anterior ethmoid, posterior ethmoid, sphenoid and frontal sinuses 0-2 on each side. 0 indicates no disease, 1 indicates partial opacification and 2 indicates complete opacification. The ostiomeatal unit (OMU) is scored either 0 or 2 on each side (0 indicating no obstruction, 2 indicating obstruction). This gives a total score out of 24. Its popularity is likely due to a combination of its simplicity, [78] reproducibility, [79] and high intra- and inter-observer agreement. [80] More recent work has exhibited its reliability when derived from radiologists' reports in the absence of the images themselves. [81] The LMS, however, correlates poorly with symptom scores [78] and has received criticism for the simplicity of its scoring. There exists a significant difference between mild mucosal disease and severe disease that has not yet reached complete CT opacification, but in both of these instances the sinus in question will be scored 1.

	No abnormality	Partial opacification	Total opacification
Anterior ethmoid			
R	0	1	2
L	0	1	2
Posterior ethmoid			
R	0	1	2
L	0	1	2
Maxillary			
R	0	1	2
L	0	1	2
Frontal			
R	0	1	2
L	0	1	2
Sphenoid			
R	0	1	2
L	0	1	2
Ostiomeatal complex	Non-obstructed		Obstructed
R	0		2
L	0		2

Figure 1.6 Lund MacKay scoring system (adapted from Lund and MacKay). [77]

Existing endoscopic scores include the Perioperative Sinonasal Endoscopic (POSE) score (figure 1.7), the Discharge, Inflammation, Polyp (DIP) score (figure 1.8) and the LKS (figure 1.9). The LKS, perhaps the most often used of these, is simple to undertake during a standard three-pass rigid nasal endoscopy. It scores polyps, oedema, discharge, crusting and scarring each from 0 to 2 on each side for a total score out of 20. It has not, however, been validated and has been reported to correspond minimally with subjective severity scoring systems. [82] The modified LKS (MLK, figure 1.10) excludes the scoring of scarring and crusting. Both elements are present largely in populations having already undergone functional endoscopic sinus surgery (FESS, the LKS being originally designed for post-operative use). Crusting is mitigated by the common use of sinus rinses both before and after surgery. As such a large proportion of patients who have not yet undergone surgery will have severe disease but score nothing for scarring and crusting. The POSE also includes scarring and requires scoring of specific sinuses. Correct scoring of many areas mentioned in the POSE requires complete visualisation which cannot be undertaken without FESS ie. despite being named the

Perioperative Sinonasal Endoscopic score it cannot be undertaken preoperatively. Psaltis *et al*. described the MLK score and compared it to other existing scoring systems. Patients undertook SNOT-22 and VAS pre- and post-operatively, while surgeons undertook the LKS, POSE, DIP score and MLK score intra-operatively. For analysis the SNOT-22 scores were subdivided into symptom-specific and generalised responses, and the VAS scores were subdivided into major and minor symptom groups. The DIP was found to correlate only weakly with total post-operative SNOT-22 scores. The LKS was found to correlate only weakly with post-operative symptom-specific SNOT-22 scores, with a modest correlation with total VAS scores. The POSE correlated only with post-operative symptom-specific SNOT-22 scores and major symptom VAS scores. The authors were unable to correlate the POSE with pre-operative questionnaires given the nature of this post-operative scoring system. The MLK was found to correlate positively both pre- and post-operatively with symptom-specific SNOT-22 and major symptom VAS scores. [83] As such the MLK may represent the most useful endoscopic scoring tool currently available.



Middle turbinate	Normal = 0 Synechia/lateralized = 1–2
Middle meatus/MMA	Healthy = 0 Narrowing/closure = 1–2 Maxillary sinus contents = 1–2
Ethmoid cavity	Healthy = 0 Crusting = 1–2 Mucosal edema = 1–2 Polypoid change = 1–2 Polyposis = 1–2 Secretions = 1–2
Frontal recess/sinus	0–2
Sphenoid sinus	0–2
Overall total	16 = middle meatal antrostomy + ethmoidectomy 18F = middle meatal antrostomy + ethmoidectomy + frontal sinusotomy; 18S = middle meatal antrostomy + ethmoidectomy + sphenoidotomy 20 = middle meatal antrostomy + ethmoidectomy + sphenoidotomy + frontal sinusotomy

Figure 1.7 Perioperative Sinonasal Endoscopic scoring system (adapted from Wright *et al*.). [84]

Discharge	0 = absent discharge
	5 = thick mucus
	10 = purulent discharge.
Inflammation	0 = no inflammation
	5 = moderate inflammation
	10 = severe inflammation
Polyps/edema	0 = normal mucosa
	5 = marked edema/no polyps
	10 = polyps filling nasal cavity

Figure 1.8 Discharge, Inflammation and Polyp scoring system (adapted from Durr *et al*.).

[85]

Polyps	0 = no polyps
	1 = polyps in middle meatus only
	2 = beyond middle meatus
Edema	0 = absent
	1 = mild
	2 = severe
Discharge	0 = no discharge
	1 = clear, thin discharge
	2 = thick, purulent discharge
Scarring	0 = absent
	1 = mild
	2 = severe
Crusting	0 = absent
	1 = mild
	2 = severe

Figure 1.9. Lund Kennedy scoring system (adapted from Lund *et al*.). [86]

Polyps	0 = no polyps
	1 = polyps in middle meatus only
	2 = beyond middle meatus
Edema	0 = absent
	1 = mild
	2 = severe
Discharge	0 = no discharge
	1 = clear, thin discharge
	2 = thick, purulent discharge

Figure 1.10 Modified Lund Kennedy scoring system (adapted from Psaltis *et al*.). [83]

1.4 Virology of the upper aerodigestive tract

The human upper aerodigestive tract is a highly colonised area playing host to a myriad of viruses, bacteria, bacteriophage and other micro-organisms. Here discussed is a brief introduction to some of the more common viruses found in the sinonasal passages; adenovirus (AdV), influenza virus, coronavirus (CoV), parainfluenza virus (PIV), rhinovirus (RV), respiratory syncytial virus (RSV), metapneumovirus (MPV), bocavirus (BoV), enterovirus (EnV) and the *Herpesviridae.*

1.4.1 Adenovirus

AdVs (figure 1.11) are non-enveloped double-stranded DNA viruses largely causing febrile illnesses in children, URT syndromes and pneumonia. Less commonly the AdVs cause otitis media, conjunctivitis, gastroenteritis and cystitis. Transmission is via aerosol droplets, faecal-oral, contaminated fomites or vertical. There are over 50 subtypes classified into groups A to F based on haemagglutination pattern. Groups B and C are associated with URT disease and are most prevalent. [87] Viral shedding usually occurs from the URT for three to five days, and from the eye and stool for more than two weeks. However this can persist for months in some cases. Diagnosis is largely by polymerase chain reaction (PCR). Paired serology taken during acute illness and in the following days to weeks can indicate recent infection, but circulating anti-adenoviral antibodies are seen in most individuals by the age of 10. [88] Thus serology has limited clinical application. The antiviral agent cidofovir can be used to combat AdV but is usually reserved for the immunocompromised and very severe disease. Infection control includes standard measures of patient cohorting, personal protective equipment (PPE) and decontamination of instruments and the patient environment.

Some work done by a group of researchers in Germany in 1987 investigated the potential for AdV to persist long-term in human lymphoid tissue. Samples of tonsillar and adenoid tissue were taken and assessed for presence of AdV2 using *in situ* hybridisation and cell culture over a period of eight weeks. AdV2 is one of the most commonly isolated sub-species from the C-group of AdVs, along with types 1, 5 and 6, and *in situ* hybridisation was quite a novel technique for the time. During the period of culture the isolated primary cells were intermittently assessed for cytopathic effect (CPE), and AdV2 was successfully identified both in samples displaying and not displaying such. There was unfortunately no discussion of numbers of samples used, statistical analyses employed, and there was very little explanation of control samples. Despite this the results remain intriguing as to the possibility of truly latent (ie. insertion of viral genomic material into the native cellular genome, with only periodic expression of viral RNA) adenoviral infection in human tissues. [89] This may be relevant to CRS given its relapsing nature but has not been specifically investigated in the sinonasal tracts, or in CRS.

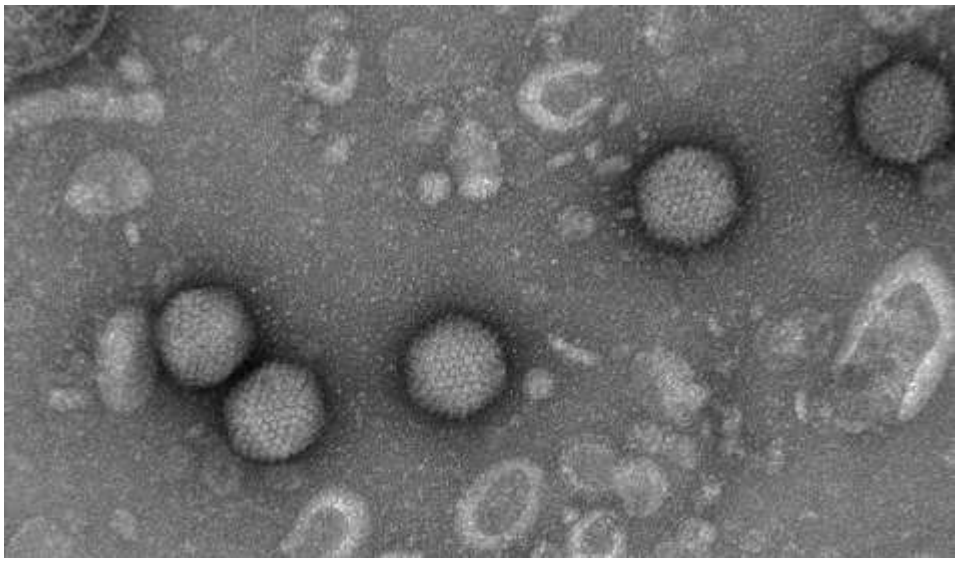


Figure 1.11 Electron microscope image of adenovirus, reproduced with permission. [90]

1.4.2 Influenza virus

Influenza viruses (figure 1.12), more commonly known as the flu, are enveloped RNA viruses of the family *Orthomyxoviridae*. Epidemics occur nearly every year, mainly in winter. Influenza is classified into groups A, B and C (with a recently proposed fourth D group) all characterised by the presence of two major surface glycoproteins. These are haemagglutinin (HA) and neuraminidase (NA). HA is involved in virus-target binding and viral entry into the cell, while NA mediates release of progeny virions after intracellular replication. The segmented nature of the influenza genome allows high reassortment rates in different virus particles infecting the same cell. This causes both major and minor changes in the envelope glycoproteins (termed antigenic shift and antigenic drift, respectively). These result in regular influenza outbreaks of varying sizes. Influenza classically causes both upper and lower respiratory tract (LRT) symptoms, but can be complicated by pneumonia, myositis and rhabdomyolysis, central nervous system disease (encephalopathy, meningitis, transverse myelitis and Guillain-Barre syndrome) and cardiovascular disease (ischaemic heart disease, myocarditis and pericarditis). Complications can also arise secondary to bacterial superinfection, such as bacterial pneumonia, acute sinusitis and otitis media. Complicated influenza can be especially problematic at extremes of age and in the immunocompromised. Viral shedding occurs 24 to 48 hours prior to symptom onset and can continue for up to 10 days. NA inhibitors (zanamivir, oseltamivir and peramivir) reduce symptom duration by one to three days if initiated within 48 hours of onset. [91] These, however, are largely reserved for high risk populations. High risk populations include infants and the elderly, pregnant and post-partum women, the morbidly obese, patients with significant comorbidities, the immunocompromised, and any patients requiring hospitalisation for the sequelae of their

influenza infection. Again, preventative measures include patient cohorting or isolation, PPE and vaccination (recommended for all individuals older than six months). [92]

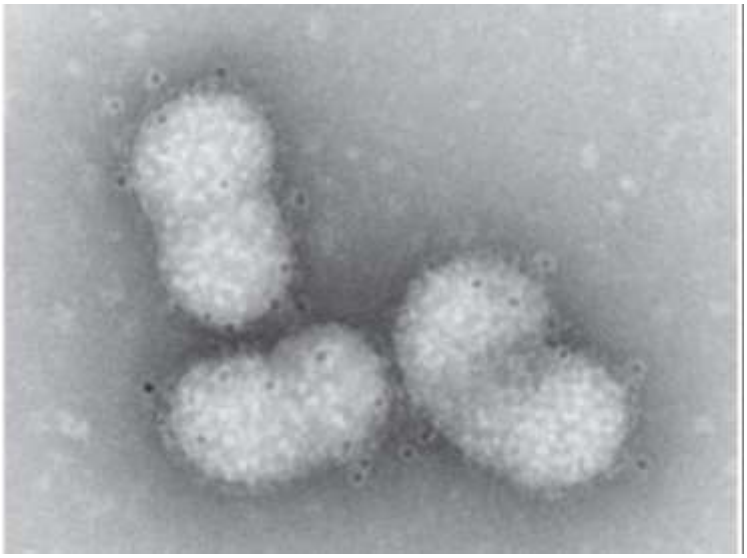


Figure 1.12 Electron microscope image of influenza virus, reproduced with permission. [93]

1.4.3 Coronavirus

CoVs are enveloped RNA viruses classified into four genera (α, β, γ, and δ) of which only two (α and β) contain the human CoVs. The human CoVs are 229E, NL63, HKU1, OC43 and the more recently discovered MERS, SARS-CoV-1 and SARS-CoV-2 (CoVID-19) strains. Many of the coronaviruses, like AdV, are almost ubiquitous, but like influenza cause respiratory illness largely in winter in temperate climates. Transmission is via contact with infected secretions or aerosolised droplets. CoVs cause URT symptoms and have been implicated in otitis media, pneumonia and inflammation of the lower airways. Given there is currently no treatment available diagnosis of CoV infection has limited clinical application beyond patient cohorting and PPE.

1.4.4 Parainfluenza virus

Only distantly related to influenza, PIVs are enveloped RNA viruses of the family *Paramyxoviridae* (which also includes RSV and MPV). The virus is transmitted via aerosolised droplets or direct contact with infected secretions, and serotypes 1 to 4 have been identified. Prevalence is PIV3, 1, 2 and 4 in descending order. [94] PIV causes URT symptoms and is associated with pneumonia, exacerbations of chronic airways disease, otitis media, acute sinusitis, and meningitis, myocarditis and pericarditis. The latter three are rare occurrences. Again, the more severe sequelae are seen largely in extremes of age or in the immunocompromised. Diagnosis is also largely by PCR; serology is clinically impractical for similar reasons to those detailed for AdV. However without any available treatment modalities besides supportive care, laboratory-confirmed PIV infection is of little immediate clinical consequence.

Of interest in the sinuses PIV has been associated with post-viral olfactory dysfunction (PVOD) after the common cold. Little investigation has been done into this particular association, but one study did show significant PIV3 presence in inferior turbinate (IT) scrapings in patients complaining of PVOD up to 12 months following reports of initial URTI. 88% of PVOD sufferers demonstrated PIV3 versus only 9% of control patients undergoing isolated septoplasty. Said initial cold, however, was not investigated to confirm viral presence preceding the onset of PVOD. [95]

1.4.5 Rhinovirus

A member of the picornavirus family, RV is a small, non-enveloped RNA virus that proliferates in the relatively cold environment of the nasopharynx (33 to 35°C). There are over a hundred human RVs and these are further classified into A, B or (the newest) C groups. Within the A and B groups there also exist major and minor RV types; these are based on the cellular receptors used by the virus when attaching and initiating infection in the host cell. ICAM-1 is used by the 89 major types, and LDLR is used by the 10 minor types. The receptor for the RVC group may be CDHR3, but this is unconfirmed. [96] Transmission is via aerosolised droplets, direct contact with infected secretions, or fomites. Of particular interest is RV's ability to remain viable outside of the host for up to three hours. The incubation period is 24 to 72 hours, as is the case for most URT viruses. Infected cells exist in the host in patches, and nasopharyngeal RV shedding usually occurs for five to seven days. This corresponds to average duration of symptoms but can continue for up to three weeks. RV is a major culprit of symptoms associated with the common cold but is also responsible for exacerbations of chronic obstructive pulmonary disease (COPD) and asthma. It is also associated with a more severe clinical picture in infants, the elderly and the immunocompromised. There are currently no available treatments or vaccines, and there is little to no cross-protection amongst the many serotypes.

RV has a known association with acute sinusitis; this has been well documented for decades, including by a group of American researchers. They recruited healthy volunteers and infected them with RV39. They then compared pre- and post-infection symptom scores, MRI evidence of development of sinus disease, nasal secretion volume and viral shedding in nasal lavages. Over half (18) of their volunteers were successfully infected with RV, but only four

of these developed any radiologically visible acute sinus changes. There were no significant differences in symptom scores between these four patients and those with persistently normal MRIs. Interestingly however the volume of the nasal secretions in these presumed acutely sinusitic participants increased five-fold. In this study the researchers did not, however, use any control patients with sham infections. Neither did they follow up the four patients displaying RV-induced sinus disease long-term to investigate the possibility of transformation into a chronic disease process. [97]

1.4.6 Respiratory syncytial virus

Another RNA virus of the *Paramyxoviridae* family is RSV. First isolated as the cause of a nosocomial outbreak of colds in chimpanzees in the mid-1950s, [98] RSV has both A and B subtypes. Both are usually present in outbreaks but the A subtypes generally cause more severe disease. RSV is almost ubiquitous by the age of two, and transmission is via direct contact with infected secretions, fomites or (less commonly) aerosol droplets. RSV, like RV, can survive for hours outside of the host. Incubation is somewhat longer than most URT viruses at four to six days. RSV causes both URT and LRT disease largely in infants and children. It is associated with significant apnoea in infants, conjunctivitis, and occasionally bacterial superinfection in the respiratory tract, urinary tract and central nervous system. Its tendency to induce asthma and recurrent wheeze in children has been particularly problematic. [99] Laboratory diagnosis, when required, is largely by PCR of nasopharyngeal secretions. Care is supportive, but there is some emerging evidence for ribavirin (a nucleoside analogue) in adults with RSV after haematopoietic cell transplant. [100] Palivizumab (a monoclonal antibody against the F glycoprotein of RSV) can be used for prevention in high-

risk infants. Although multiple vaccines are in development none are currently commercially available.

Some investigation into RSV and its action in chronically inflamed lower airways has been undertaken. This included two very similar studies comparing RSV infection in CF and healthy well-differentiated nasal, tracheobronchial and bronchiolar epithelial cells grown at air-liquid interface (ALI). As do many of the articles mentioned in this review of the literature, the investigators chose these cells as they can be grown into an organ culture closely mimicking the *in vivo* state of their parent cells. The ALI model allows the development of multilayered, pseudostratified, mucociliary tissue including basal support structures. The first of these studies investigated preferential infection sites. Despite direct inoculation of apical, basal and intermediate cell types, there was a clear predilection for the apical, ciliated cells across all three airway collection sites. The later of these two studies found that RSV caused no visible CPE in this ALI model up to three months after infection. This was in the absence of an adaptive immune response, as is the case in the ALI environment. [101] This suggests the pathogenicity of RSV may depend largely on said adaptive immunity, rather than direct CPE. Conversely, the earlier study found demonstrable CPE on light microscopy as early as two hours post-infection. [102] The author of this review of the literature cannot discern any obvious differences in the two articles that would account for this, but would suggest that a mucosal explant model, which would retain at least some of its immune capabilities and be vastly simpler to generate, would be another acceptable choice for investigation of the above.

1.4.7 Metapneumovirus

The third member of the *Paramyxoviridae* family pertinent to this thesis (along with PIV and RSV) is MPV. Genetically similar to RSV, MPV is an enveloped RNA virus which, also like RSV, has A and B subtypes with two clades in each. These are termed A1, A2, B1 and B2. The virus was first isolated in 2001 so formal transmission and shedding kinetics studies have not yet been completed. It would appear most children have been infected by the age of five. [103] MPV causes URT disease, bronchiolitis and exacerbations of asthma and pneumonia largely in the very young or very old. It also has a possible association with encephalitis [104] and exacerbations of COPD. [105] RT-PCR (reverse transcription polymerase chain reaction) is well-developed for MPV detection, but again, current treatment is supportive only and so laboratory diagnosis is useful largely for infection control. Vaccines are under development, but none are near licensure.

A group of researchers in Brisbane in 2014 highlighted the lack of investigation into chronic airways diseases and viruses other than RV. As such they investigated the innate immune response of children not yet diagnosed but at risk of asthma to RSV and MPV. Primary nasal and tracheal epithelial cells were taken to compare the upper and lower airways. These cells were cultured, transfected, and assessed for viral replication and inflammatory cytokine production. Of particular interest, again, were IFN-β and IFN-λ. As stated previously, deficiency in these has been identified in severely asthmatic adults. The researchers sought to determine if this deficiency precedes and therefore may have a role in development of asthma, or if it is acquired, perhaps by viral infection itself in susceptible individuals. Their main finding was that RSV-infected tracheal epithelial cells produced less IFN-β versus control. Interestingly none of the other three groups (RSV-infected hNECs, MPV-infected

tracheal cells, and MPV-infected nasal cells) showed any change in inflammatory cytokine production when compared with controls. All four groups, however, displayed significantly greater viral replication. This would suggest that higher viral loads are seen in atopy and wheeze (the study recruitment criteria) but the impaired anti-viral response is not IFN-mediated, and an IFN deficiency does not precede the development of asthma in wheezy/atopic children. Participants for this work were recruited at time of elective otolaryngologic surgery, largely for adenotonsillectomy. This group are themselves subject to chronic airways inflammation, and so even in the absence of asthmatic risk factors are perhaps not as "healthy", (and therefore perhaps not as capable of mounting a normal innate immune response) as would be ideal for this study. [106]

1.4.8 Bocavirus

Human BoV is a parvovirus first identified in Sweden in 2005 [107] and causes largely LRT illness in children, especially under the age of two. It is most prevalent during winter and is commonly found in the presence of additional respiratory viruses. It has been associated loosely with human gastrointestinal disease and has been detected in serum. [108, 109] Being a relatively new respiratory pathogen understanding of its role in causality of clinically apparent disease is limited. As yet there are no treatment strategies available bar supportive measures, PPE and patient cohorting.

1.4.9 Enterovirus

EnV is a small RNA virus of the picornavirus family closely related to RV. EnV causes disease throughout the year in patients of all ages, but most often in small children.

Designations A to J of EnV have been identified and transmission is faecal-oral. Apart from both URT and LRT disease EnVs are classically associated with poliomyelitis, as well as exanthemas, meningitis, encephalitis, conjunctivitis, pleurodynia and peri/myocarditis. Most EnV infections are self-limiting and do not require specific treatment outside of supportive care. In severe cases intravenous immunoglobulin has been used to anecdotal benefit in EnV-encephalomyelitis. [110] The capsid inhibitor pleconaril has shown some favourable results in neonatal populations. [111] Administration of the poliovirus vaccine is currently undertaken worldwide as part of the WHO plan for eradiation, which has been largely successful in its aims.

1.4.10 *Herpesviridae*

The *Herpesviridae* are a large family of DNA viruses, nine of which are known to infect humans. Among these are HSV (herpes simplex virus), CMV (cytomegalovirus), EBV (Epstein Barr virus), VZV (varicella zoster virus), HHV6 (human herpes virus 6) and HPV (human papilloma virus). *Herpesviridae* are rampant in humans; up to 90% of adults have been infected with at least one of HSV, CMV, EBV and VZV, as evidenced by persistent latent infection in many. [109] There have been multiple mechanisms postulated to result in the lifelong infection seen in these viruses. The most commonly held theories include the encoding of a homologue to IL-10 [112] and downregulation of the MHC class 1 on the surface of infected cells. [113] The former inhibits synthesis of pro-inflammatory cytokines and the latter prevents T cell recognition of non-self. The clinical manifestations of *Herpesviridae* infection are varied and are summarised in table 1.1. The majority of these are self-limiting and require no treatment in immunocompetent individuals, but for severe cases, in extremes of age and in the immunocompromised there are a number of antiviral options.

These are also summarised in table 1.1, but evidence for most of these is minimal. Vaccination schedules are well established for VZV and HPV primary prevention, and EBV vaccination is in the animal trial stage. Currently no vaccines available for any of the *Herpesviridae* have a mitigating effect on latent infection.

Viral species	**Possible clinical manifestations**	**Systemic antivirals available**
HSV	Cutaneous lesions, encephalitis, meningitis, hepatitis, pneumonitis, oesophagitis, epiglottitis/laryngitis in children	Acyclovir, famciclovir, valacyclovir
EBV	Infectious mononucleosis, lymphoma, stomach/nasopharyngeal carcinoma, associated with multiple sclerosis	Acyclovir
CMV	Mononucleosis, gastrointestinal upset, encephalitis, Guillain-Barré syndrome, peri/myocarditis, pulmonary and ocular manifestations	Ganciclovir, valganciclovir, foscarnet, cidofovir
HHV6	Mononucleosis, encephalitis, pneumonitis	Ganciclovir, foscarnet, cidofovir
HPV	Cutaneous lesions, anogenital malignancies	Immunotherapy (imiquimod, sinecatechins, IFNs)
VZV	Chicken pox, shingles, encephalitis, pneumonitis and bronchitis	Acyclovir, famciclovir, valacyclovir

Table 1.1 Known *Herpesviridae* clinical manifestations and possible systemic antiviral treatments available.

1.5 Viral-bacterial coinfection

It is historically very well documented that viruses increase susceptibility to secondary bacterial infection. Given the extensive implication of bacteria in the aetiopathogenesis of CRS (see section 1.2.2), any event that might allow greater bacterial colonisation and infection (an URTI for example) would be of significant interest. Research into viral-bacterial co-infection, however, has largely focused on the LRT. This is largely due to the significant mortality and morbidity associated with diseases such as asthma and COPD. Research is also plagued with difficulty when attempting to identify the exact viral pathogens involved in this association. Investigation into the mechanisms behind viral-bacterial co-infection in the LRT has elucidated a number of biological changes likely responsible for the clinically observed phenomenon of increased secondary bacterial infection susceptibility. These may be somewhat applicable to the URT. These changes can be grouped into factors affecting epithelial barrier function, those affecting bacterial binding, those influencing innate and adaptive immunity, changes in the lower respiratory microenvironment and direct interactions between the viruses and bacteria themselves. [114]

1.5.1 Interactions in the lower respiratory tract

Influenza in particular has been repeatedly linked to bacterial infection in the LRT, often *S. pneumoniae*. Significantly increased rates of bacterial pneumonia during influenza outbreaks have been reported for decades, but isolation of the preceding virus in human populations is difficult given the generally rapid clearance of such pathogens from the airways. [115, 116] Perhaps the most infamous example of this synergistic relationship is the Spanish flu outbreak of 1918 and 1919. This was the largest and most devastating influenza pandemic of the 20th century. The morbidity and mortality it caused are believed to have resulted largely

from complications of bacterial superinfection (mainly with *S. pneumoniae* and *S. aureus*). [115] Influenza has also been linked with bacterial infections outside of the respiratory tract. Parallel outbreaks of influenza and meningococcal infection in the UK in 1989 were investigated with matched peri- and post-morbid virus serology and compared with healthy age-matched controls. Significant correlation was found between influenza A and *Neisseria meningitidis* (*N. meningitidis*) infection. [117] The authors of this study postulated this to represent either early virus-driven nasopharyngeal mucosal injury allowing greater bacterial adherence and penetrance, or a more central mechanism involving generalised virus-driven immunosuppression.

1.5.2 Effects on epithelial barrier function

Respiratory viruses cause increased mucus production in order to eliminate said virus, as indeed do any pathogens of the respiratory tract. This, however, can be counterproductive. Excessive mucus production is associated with obstruction and reduced mucociliary clearance, allowing further colonisation and infection by additional potential pathogens. [118] Viruses can also reduce ciliary beat frequency, [119] cause ciliary dyskinesia [120] and reduce numbers of ciliated cells. [102] They can cause epithelial cell death resulting in large-scale physical disruption to the epithelial barrier. [121] On a smaller scale they can induce changes in TJ proteins and cell polarisation allowing pathogen spread through gaps in said barrier. [122-124]

1.5.3 Effects on bacterial binding

Particular host receptors are upregulated by particular viral infections, and some of these host cell surface molecules can also act as binding sites for bacteria. ICAM-1 and fibronectin, both native cell surface receptors, are upregulated in influenza infection. Both have been shown to allow increased binding of *H. influenzae*, *S. aureus* and *S. pneumoniae*. [125] Influenza, RV, RSV and PIV have all been shown to induce platelet activating factor receptor (PAFR) expression in infected cells. This allows greater pneumococcal binding *in vitro*. [126-128] Unfortunately this result has not been replicated *in vivo*. In a mouse model of influenza PAFR blockage has effected no change in mortality or disease severity after bacterial superinfection. [129, 130] This does not necessarily suggest that PAFR upregulation has no role in this co-infection, but perhaps that the mechanisms behind said co-infection are multifactorial in nature.

Viral proteins expressed on the surface of host cells may also act as bacterial binding sites. The RSV attachment glycoprotein (G protein) can be bound by *H. influenzae*, [131] *S. pneumoniae*, [131, 132] and *P. aeruginosa*. [133] Influenza HA has been shown to bind to group A streptococci in a mouse model. [134]

1.5.4 Effects on innate and adaptive immunity

Common respiratory viruses can severely deplete local macrophage stores. This has been seen in a mouse model of influenza, in whom restoration of alveolar macrophage populations remedied an observed increased susceptibility to pneumococci. [135] This may also hamper neutrophil recruitment as this relies to some degree upon chemokine expression by local

immune cells. Indeed influenza and pneumococcal co-infection has been shown to induce direct neutrophil apoptosis. [136] Viruses can also impair the actions of natural killer cells, [137] they can interfere with antigen presenting cells, [138, 139] and impair T cell function. The lower airways of mice co-infected with influenza and *S. pneumoniae* produce less CD8+ and CD4+ T cells, less of their associated cytokines, and their Th-17 cells have been shown to produce less IL-17. [140-142]

These viruses can also interfere with PRRs, including the TLRs. If cell PRRs encounter multiple pathogens over time, feedback mechanisms designed to prevent over-activation of adaptive immunity start to cause delayed or absent responses to further infection. This has been observed both in RV-infected pulmonary epithelial cells [143] and in RSV-infected mice [144] both faced with bacterial challenge. In the case of the mice, this poor response to further pathogens persisted for months after viral clearance.

Suppression of innate immunity has been induced by the RSV G protein, [145] and non-structural proteins of influenza and RSV. [146-148] The effects of this suppression on subsequent bacterial infection have not been elucidated, but it would be reasonable to hypothesise this would augment bacterial spread. [114]

There may also be an element of virus-induced local antimicrobial peptide suppression at play. RV-infected COPD patients show much higher rates of secondary bacterial infections than non-infected COPD patients, and their sputum shows vastly reduced levels of the antimicrobial peptides secretory leucocyte protease inhibitor (SLPI) and elafin (another protease inhibitor). [149]

1.5.5 Effects on the respiratory microenvironment

Viruses can change the availability of nutrients in the airways; for example influenza NA can cleave sialic acid from airway mucins. This can be metabolised by some pneumococci, and so it follows these bacteria may grow and divide more readily. [150] *H. influenzae* can also increase respiratory cell expression of ICAM-1, which influenza can then use as an attachment site. This leads to increased bacterial susceptibility and a heightened inflammatory response. [151] ICAM-1 over-expression also seen in RV infection has been postulated to be responsible for increased *S. aureus* internalisation by pneumocytes in the lower airways. [152] Virus-induced temperature change and availability of other intracellular micronutrients released on cell lysis can variably encourage biofilm formation, induce release of planktonic bacteria from biofilms, and accommodate their further spread in the respiratory tract. [153, 154]

1.5.6 Direct viral-bacterial interactions

There is some emerging evidence that apart from the indirect viral-bacterial interactions detailed previously, viruses and bacteria may directly interact in the lower airways. It is established that the RSV G protein can bind to pneumococci, which has been shown to change the bacterial transcriptome and render the bacteria more virulent. [155] There is some evidence that RSV may act as a direct coupling agent between epithelial cells and *P. aeruginosa*, however this work was undertaken in AEC lines only. [133] Influenza NA can alter the structure of some meningococcal capsules, leaving them with an enhanced ability to adhere to epithelium. [156]

1.5.7 Interactions in the upper respiratory tract

Extensive investigation into viral-bacterial interactions in the LRT has been undertaken. There is a wealth of evidence that such an interaction is also likely in the URT; influenza has been consistently linked to acute bacterial sinusitis [157] and to otitis media in up to 40% of children infected with the virus. [158] Despite this the URT (and in particular the sinonasal passages) has not received attention equal to its lower counterpart.

A South Korean group took IT hNECs grown at ALI and investigated components of the cells' TJs and AJs after infection with RV. Expression of both was reduced compared to control non-infected cells. [159] This was supported by some work in Japan in 2011 using what appeared to be a similar protocol but with RSV in place of RV, however this was a very small study with very little explanation of methodology in the publication. [160] This has been extrapolated to some investigation into subsequent bacterial adherence in virus-infected tissues. A group in Japan infected primary tracheal epithelial cells with RV or RSV and super-infected these with *S. pneumoniae*. They found significantly increased bacterial adherence in the presence of both viruses. [127] The same South Korean group grew hNECs at ALI, infected these with RV, super-infected with either *S. aureus, S. pneumoniae* or *H. influenzae*. They then assessed expression of relevant host-cell adhesion molecules. They found there was significantly increased adhesion of all bacteria to the virus-infected cells. [46] The previously discussed link between influenza and meningococci (see section 1.5.1) has also been investigated in the upper airways. 19 nasopharyngeal mucosal explants were taken from the ITs of patients with nonallergic nasal obstruction, infected with influenza B or PBS as a control, and then with group B *N. meningitidis*. Pre-innoculation with influenza had no influence on bacterial infection when compared with control explants. [161] However,

influenza B is classically less virulent than the A subtype. Additionally only one strain of influenza and *N. meningitidis* each were investigated, and the influenza B was not strictly WT, being initially isolated in 1979 well before this study in 1999.

Animal models of viral rhinosinusitis have been difficult to establish, and as such results have been mixed. Some animal work has been done to elucidate the effects of different influenza subtypes on subsequent bacterial infection. In a ferret model 90% of subjects pre-inoculated with H3N2 (a classically more virulent strain of influenza) developed clinical and microbiological *S. pneumoniae* infection in the form of sinusitis or otitis media. In contrast only 10% of subjects pre-inoculated with the classically less-virulent H1N1 or influenza B developed such infections. [162] Sendai virus is a highly pathogenic murine respiratory virus that can be used to mimic many aspects of RV infection seen in humans. These aspects include clearance of acute infection within ten days but persistence of T-suppressor and T-regulatory cells and airway hyperresponsiveness long after said clearance. The mouse model of Sendai virus does not, however, display the eosinophilia so characteristic of most virally-exacerbated CRS seen in humans. [163]

Much like many other respiratory viruses, AdV has long been suspected to correlate with increased respiratory bacterial infections. A Swedish group in 1994 investigated the possibility of increased bacterial adherence to respiratory epithelial cells pre-infected with AdV. They found increased adherence of multiple *S. pneumoniae* strains in both the A549 cell line and hNECs. Conversely, no change in bacterial adherence was found when the streptococci were substituted for *H. influenzae.* This is perhaps not unexpected in the A549 cells; these originate from alveolar basal epithelial cells, and *H. influenzae* is known to infect largely the URT. [164]

RSV has been identified as a primer of both the upper and lower airways to secondary bacterial infection and allergic responses. As such, RSV may be of particular note when investigating the response of a chronically inflamed sinonasal tract to viral infection. Hament *et al.* compared adherence of various strains of *S. pneumoniae* to respiratory epithelial cell monolayers infected with RSVB. The investigators found a two- to ten-fold increase in bacterial adherence to the cells pre-infected with virus versus control, even though this varied somewhat amongst the bacterial serotypes. The host cells used were from two human carcinomatous cell lines (HEp-2 and A549). These two, along with most other respiratory epithelial cell lines, are widely accepted as poor mimickers of true *in vivo* cell behaviour. [165] In perhaps a more representative model, Das *et al*. harvested well-differentiated healthy hNECs from turbinectomy patients. These were transfected with RSV or control media in an attempt to induce this "priming" of the airways. Subsequently the pro-inflammatory cytokine tumour necrosis factor α (TNF-α) or a control compound were added, and the resulting cytokine profile was analysed. They found significantly elevated levels of IL-6, IL-8 and RANTES in RSV infection alone, and an even greater elevation of these with the addition of TNF-α to RSV-infected cells. In order to aid analysis the researchers used a green fluorescent protein-expressing RSV from RSV strain A2. There was little discussion of the similarity of this recombinant virus to its WT parent, so there could perhaps be some question regarding the *in vivo* relevance of these results. However, the over-arching implication of RSV in the induction of innate immune hyper-responsiveness remains compelling, and further experiments are needed to validate this in a diseased (eg. CRS) model. [166]

Supportive work in the same vein has been done in Japan, investigating the cytokine profile and mechanistic interactions between RSV and *in vitro* upper AECs. Human telomerase

reverse-transcriptase-infected hNECs (hTeRT, giving the cells an extended lifespan) from hypertrophic rhinitis or CRS patients were infected with RSV. This induced pro-inflammatory cytokines IL-8 and TNF-α itself. During this same study analysis of TJ protein expression was undertaken. Interestingly this was markedly increased in the infected cells. This increase in TJ expression also appeared to correlate with increased RSV budding from the apical cell surfaces. This is an intriguing finding as it does not correlate with current theories of viral disturbance of the epithelial barrier (ie. TJ disruption). The use of hTeRT-treated hNECs has been somewhat validated in this and previous studies, [167, 168] but substantiation of this observation in untreated hNECs would add significant weight. [169]

1.6 Viral collection and processing methods

Traditional viral sampling is taken from the inferior nasal septum and anterior nasal floor. These are easily accessible in the primary care setting (ie. endoscopic equipment and expertise are not required) and cause patients little discomfort. The posterior nasopharyngeal wall has been recommended historically, but endoscopic confirmation of access would be required. No evidence exists however, for any of these three sites over each other or indeed any other location in the sinonasal passages. Additionally the inferior nasal septum, anterior nasal floor and posterior nasopharyngeal wall are relatively close to airborne pathogens and therefore risk of contamination. They are relatively distant from the sinuses and may be subject to pooling of potentially contaminating secretions. Secretions often containing non-replicating, extra-cellular, non-pathogenic virus. This is particularly pertinent to the anterior nasal floor.

Viral collection techniques are another point of interest. Methods investigated in the literature include nasal washes, aspirates, brushings and traditional viral swabs [44, 45, 170-180]. Heikkinen *et al*. looked at viral swabs and nasal aspirates in the detection of influenza in children and found no statistically significant difference between the two. [181] Spiridakou *et al*. compared viral swabs, nasal aspirates, washes and brushings and their ability to sample a range of common URT viruses. The only significant difference found was in detection of RV with brushes and washes. RV detection was slightly higher in the washes, however the brushing was taken after all three other samples had already been harvested. As such much of the epithelial, virus-containing layer may have already been removed. In addition to this the washes could well be contaminated by ambient, non-replicating, non-pathogenic virus either in the air of the sinonasal tracts or in mucosal secretions. [182]

An additional concern when sampling respiratory viruses is ensuring adequate cellular collection. Clinically relevant, actively replicating URT viruses are intra-cellular and have been shown largely to reside in the upper epithelial layers of respiratory mucosa. [118] Traditional viral sampling brushes are smaller, softer and more flexible than cell sampling brushes. This is more comfortable for the conscious patient and less traumatic to the mucosa, but again risks sampling largely mucosal secretions and not the cells in which the viruses reside. [182] Cytology brushes are larger and more rigid. It follows that these cause more discomfort and trauma, and if sampling from specific, more posterior areas in the sinonasal passages is required their size means these samples may be contaminated with cells from areas more anterior during insertion and removal. However, they have the significant advantage of increased cellular sampling yield. [183]

Viruses, being obligate intracellular pathogens, are considerably more difficult to isolate and identify than bacteria. As such the arrival of nucleic acid amplification tests (NAATs) in the late 1980s has revolutionised viral detection methods. Prior to this respiratory viral pathogens were found either by cell culture in amenable cell lines or by antigen detection. Cell culture is a lengthy process and so is largely irrelevant in a clinical sense; by the time virus is grown and identified, the patient will have either recovered or succumbed. It also requires viable viral samples and so the processing laboratory must be nearby, and requires experienced interpretation of CPE. Many viruses will cause specific CPE in specific cell types, but this is not universal. Additionally, some common viruses do not grow well in cell culture, or do not do so at all. Antigen detection is a much faster, more clinically relevant method. Unfortunately it is also poorly sensitive and requires the existence of said antigens, which is unfortunately not the case for some very common viruses, RV and CoV for example. NAATs

are not without their drawbacks; they can detect virus in asymptomatic patients which can complicate interpretation. They can also be more expensive than the methods detailed above, and the presence of sequence variants may give false negatives. Sequence variants are especially prevalent in largely-RNA URT viruses. However, NAATs amplify even tiny amounts of viral nucleic acid to a readily detectable level, they are highly sensitive even when used on a non-viable sample, and they are rapid. This allows prompt patient treatment and cohorting. For these reasons most of this literature review will focus on studies employing NAATs (largely PCR) in their viral detection, as indeed will the work undertaken in our own studies. [184]

1.7 Viruses and chronic rhinosinusitis

Most research into the microbial influences on CRS has focused on the role of bacteria rather than viruses. This is partly due to the more common isolation of bacteria from CRS patients, as well as their ease of study. With this said, most patients with CRS do recollect a viral illness preceding the development of sinus symptoms, suggesting that viral infection may play an important role in the initiation of the inflammatory process. Although most virome studies of the upper airway to date have been population-based rather than disease-specific, there have been a few studies examining the prevalence of certain viruses in CRS cohorts.

1.7.1 Conventional URTI-causing viruses and CRS

Table 1.2 summarises the viral CRS studies that have been performed, including their methodology and salient findings.

In 2006, Jang *et al.* isolated RV in IT scrapings of 21% of CRS patients and found RV to be completely absent in control patients. This prompted them to hypothesise that RV may be important in the aetiopathogenesis of CRS. This study did suffer from numerous limitations however, including small sample size, the absence of a truly healthy control group, seasonal sampling bias and the absence of polyps in the CRS cohort. Furthermore the possibility that the IT may not truly represent the paranasal sinuses in terms of viral burden must also be mentioned. [44] These limitations may explain why a follow-up mucosal explant model by Wang *et al.* failed to demonstrate any increase in RV susceptibility between CRS polyp tissue and control tissue [185]

A later, larger study by Cho *et al.* sought to address many of the above deficiencies. In their study they examined nasal lavage and IT scrapings from CRS patients with and without polyps, compared to a more appropriate control group of patients undergoing tonsillectomy or thyroidectomy. Unlike the Jang *et al.* study they did not limit their assay to RV alone and instead screened for a large number of common viruses using PCR. They found 50.5% of CRS and 28% of control nasal lavages positive for one or more viruses, and 71% of CRS and 30% of control scrapings positive for one or more viruses. However, given their collection methods, again it could be questioned how representative their samples were of the actual sinuses of these patients. [45] A similar study was done in China during winter comparing viral presence in MM scrapings of CRS patients with and without polyps as well as controls. They found similarly high rates of viral detection in all groups. Unfortunately the set of viruses for which they searched was comparatively small, only encompassing RV, RSV, influenza, PIV and CoV. [170] Similar work was done in Pittsburgh in the USA over the course of a year. This again used MM scrapings but with the addition of anterior ethmoid scrapings. All controls were negative for viruses while 20% of CRSsNP and 80% of CRSwNP were positive. Unfortunately the sample size was so small (32 patients) that the results are not compelling. [171] A group in New Zealand took ethmoid or sphenoid mucosal samples (no further details were given regarding the nature of these) from only 13 CRS patients and 2 healthy controls during summer and autumn. These were assessed for a wide array of viruses, including some not uniformly addressed in the aforementioned studies (BoV, CMV, HHV6 and EBV). They did not find any conventional viruses in any of the samples but did identify low titre HHV6 and EBV in the CRS patients only. The authors themselves identified their sample size and timing of sample collection as significant pitfalls in their work. This is especially true of their controls, numbering only two. [172] Many conventional URTI-causing viruses reside largely in the epithelial layer. As such it could be postulated that

the whole mucosal samples used in this study might not give the high virus titres seen in some of the other population studies that used epithelial sampling methods. This also applies to a Brazilian study conducted cross-seasonally over two years. The aim of this study was to identify any seasonality in virus detection in CRS patients. The authors hypothesised that there may be a continual viral presence either in excess/of a different nature/both in CRS patients at times when virus circulation is generally low in healthy populations. A discovery such as this might add weight to the theory that a continual viral presence contributes to chronic inflammatory symptoms in CRS. The investigators took samples of nasal and maxillary mucosa as well as nasal lavages from a reported 100 CRS patients at time of surgery. Patients were recruited both with and without polyps, none reported any current respiratory illness, but on closer inspection the cohort appears to be only 93 patients. They found a 54% overall viral detection rate. This was comprised largely of MPV and RV and was concentrated in winter/spring of 2010 and spring of 2011. The authors thus concluded that these viruses are unlikely to be contributing to CRS symptoms given these are times of high viral circulation in the general population, however they investigated no specific control subjects in their work. [173] Another group lacking a control group is that of Abshirini *et al.* who collected mucus specimens during FESS from 53 CRSwNP and 23 CRSsNP patients over the course of nearly a year in Iran. No specific cellular collection was undertaken and the mucus collection method was not detailed. The mucus specimens underwent RT-PCR specifically for RV and RSV (it is unclear why the authors chose only these pathogens) and found a 22% and 9% presence of these respectively, with a 6% dual presence. From these results the authors concluded these viruses have a role in CRS pathogenesis, but without a healthy control group and any intra-cellular collection method such a conclusion cannot be validated. [174] Some earlier work (1992 to 1994) done in West Virginia also investigated viral presence in CRS patients only, and did not use a control group for comparison.

However, this study was undertaken during the beginnings of commercial PCR availability and so was focused more on the detection method rather than the identification of viruses for its own merit. 20 patients undergoing sinus surgery (no season was identified) had tissue samples extracted. These were analysed for RSV and AdV presence using both PCR and more traditional methods, as well as simultaneous bacterial culture. Four patients (20%) were positive for RSV using PCR, and none were positive for AdV using PCR. One sample each was positive for the aforementioned viruses using immunofluorescence and viral culture. 40% of the samples were positive on bacterial culture (anaerobes > *H. influenzae* > *S. pneumoniae* > *P. aeruginosa*), but this was not discussed in great depth. Of note would have been any association between bacterial and viral presence. However this is unlikely to have displayed any significance either way given the experimental conditions and sample size. [175]

Despite some areas of concern, common to all of these studies are the viruses most prevalent in various populations. Exact numbers vary, but these include RV, influenza, PIV, RSV, MPV, CoV and AdV.

Study	Sample size	Viruses screened	Season	Collection method	Results and author's conclusion
Abshirini *et al*, 2015 [174]	0 controls, 23 CRSsNP, 53 CRSwNP	RSV, RV	All (one year)	Sinonasal mucus	High prevalence of RV and RSV; 28.94% RV-positive, 11.84% RSV-positive, 7.89% both
Cho *et al*, 2013 [45]	50 controls (thyroid/tonsil surgery), 49 CRSsNP, 62 CRSwNP	AdV, BoV, CoV, EnV, influenza, MPV, PIV, RSV, RV	Late summer, autumn, early spring	Nasal lavage, IT scrapings	Viruses and CRS may be associated; 50.5% CRS lavage positive vs. 26% control, 64% CRS scrapings positive vs. 15% control. RV most prevalent
Jang *et al*, 2006 [44]	27 controls (OSA surgery), 39 CRSsNP, 0 CRSwNP	RV	Late winter, spring	Nasal lavage, IT scrapings	RV may be associated with CRS; 21% CRS scrapings positive vs. 0% controls, all lavages negative
Liao *et al*, 2014 [170]	53 controls (septoplasties), 61 CRSsNP, 67 CRSwNP	CoV, influenza, PIV, RSV	All (two years)	MM scrapings	CRS and virus likely not linked, no correlation with disease severity; 75.47% controls positive vs. 73.77% CRSsNP vs. 68.66% CRSwNP
Lima *et al*, 2015 [173]	0 controls, 32 CRSsNP, 60 CRSwNP	AdV, BoV, CoV, EnV, influenza, MPV, RSV, RV	All (two years)	Nasal lavage, mucosal biopsies (MT/polyp/maxillary sinus)	Viruses in CRS seasonal; 54% positive, MPV most prevalent, peaks in winter and spring
Ramadan *et al*, 1997 [175]	No controls, 20 CRS (polyp status unspecified)	AdV, RSV	All (two years)	Mucosal biopsies (ethmoid/maxillary sinus)	PCR more sensitive for viral detection than culture; 20% samples positive on PCR vs. 5% on culture
Rowan *et al*, 2015 [171]	14 controls (skull base tumours), 8 CRSsNP, 13 CRSwNP	AdV, CoV, influenza, PIV, MPV, RSV	Late winter, spring, summer, early autumn	Viral swabs (MM/ anterior ethmoid)	Viruses and CRS may be associated; controls all negative, CRSsNP 80% positive, CRSwNP 20% positive. Virus associated with severity in CRSsNP
Wood *et al*, 2011 [172]	2 controls (skull base tumours), 8 CRSsNP, 5 CRSwNP	AdV, BoV, CoV, influenza, PIV, MPV, RSV, RV	Summer, early autumn	Mucosal biopsies (location not specified)	Viruses and CRS unlikely related; no viral detection in any sample

Table 1.2 Summary of published virome studies

1.7.2 The *Herpesviridae* and CRS

Viruses known to cause long-term latent infection often without significant upper respiratory symptoms include EBV, HHV6, HSV, VZV, HPV and CMV. These pathogens have a wide tissue distribution, and so lend themselves to investigation as potential initiating or perpetuating factors in CRS. Since the advent of large-scale PCR testing little research has been done into the prevalence of these in the sinonasal tracts. What has been done has focused largely on EBV and HHV6 and their potential role in nasal polyposis. A group in Hong Kong investigated the role of EBV in the pathogenesis of a wide range of tumours. They took healthy nasopharyngeal tissue samples during autopsy from patients without apparent EBV-related disease (ie. with presumably healthy nasopharyngeal mucosa). They found 80% (8/10 total) of these were positive for EBV. [176] The same group also investigated EBV presence in PCR-tested nasal polyp tissue. They found the virus to be present in 69% (9/13) of their samples. [177] Researchers in Greece took this further in 2009, comparing nasal polyp and adjacent turbinate mucosa samples from 23 CRSwNP patients to turbinate samples from 13 patients undergoing nasal corrective surgery. The specimens underwent PCR testing for HPV, CMV, HSV, VZV, HHV6 and EBV. EBV was detected in 35% of the polyp samples (8/23), HPV in 13% (5/23), HSV in 8% (2/23) and CMV in 4% (1/23). Only the adjacent turbinates of patients positive for HPV in their polyps were positive for viruses (HPV in 2/46, 4%). All other disease and control samples were negative for the viruses tested. Based on their findings the authors concluded that EBV presence influences the pathogenesis of nasal polyps, and that HPV, HSV and CMV are likely to play less important roles. This would be more compelling if the control population were composed of CRSsNP patients, if the CRS and control groups were composed of similar numbers, and if the one sample positive for CMV had shown statistical significance. [179] Additional work

was undertaken in Italy in 2014 again comparing nasal polyp and adjacent turbinate mucosa samples from 35 CRSwNP patients taken at time of FESS. Pre- and post-operative IT or MT (middle turbinate) scrapings were also taken in 29 of the 35 patients. HHV6 was found in 12.1% of samples (3/35 polyps, 11/31 adjacent turbinates, 1/29 preoperative scraping). EBV was found in 10.5% (8/35 polyps, 3/31 turbinates, 1/29 preoperative and 1/29 postoperative scraping). CMV was found in 1.6% (1/35 polyps and 1/29 postoperative scraping) and HSV in 1.6% also (2/35 polyps). These samples were also investigated for more common URT viruses (influenza, RSV, AdV, MPV, CoV, PIV, RV, EnV and BoV). Only one preoperative scraping was positive (PIV). The authors drew similar conclusions to the aforementioned study; that EBV may have an initiating or perpetuating role in polyp development in CRS, and that the other viruses likely do not. [180] There was, however, no appropriate control group in this study with polyp samples only compared to adjacent tissue from the same patients. This tissue adjacent to polyps in CRSwNP patients is undoubtedly still affected by the disease. Although not explicitly stated in either study, sampling of all mucosal layers rather than pure epithelial sampling was likely chosen here as EBV has previously been found in deeper lymphoid tissue. [177, 178] A summary of the investigation into the *Herpesviridae* and CRS can be seen in table 1.3.

Reference	Demographics	Viruses	Season	Collection	Results and author's conclusion
Costa *et al*, 2014 [180]	0 separate control patients, used adjacent turbinate tissue 0 CRSsNP 35 CRSwNP	CMV, EBV, HHV6, HSV, VZV	Autumn, winter, early spring	Biopsies of polyps and adjacent mucosal turbinate tissue, turbinate scrapings	No association between polyps and herpesviruses; polyps 40.1% positive, adjacent turbinates 45.2% positive, scrapings 6.8% positive, HHV6 most prevalent
Tao *et al*, 1996 [177]	0 controls 0 CRSsNP 13 CRSwNP	EBV	Not specified	Mucosal biopsies (location not specified	Polyps may be associated with EBV; 69% positive
Zaravinos *et al*, 2009 [179]	13 separate control patients (?septoplasties) plus adjacent turbinate tissue from polyp patients 0 CRSsNP 23 CRSwNP	CMV, EBV, HHV6, HSV, VZV	Not specified	CRS: biopsies of polyp and adjacent mucosal turbinate tissue, control: IT/MT mucosal biopsies	EBV presence may be associated with polyposis in CRS; 14 polyp samples positive, 2 adjacent turbinates positive, controls all negative. EBV most prevalent

Table 1.3 Summary of investigation of CRS and the *Herpesviridae.*

1.8 The bacterial microbiome of chronic rhinosinusitis

The bacterial microbiome of the sinonasal passages in health and disease has received significant attention in the recent past. Work is underway to characterise it and devise methods of manipulating it for therapeutic ends. In-depth discussion of the CRS microbiome is beyond the scope of this thesis, however some findings are generally agreed upon. There appears to be a dysbiosis within CRS with a similar bacterial burden to that seen in healthy individuals, but CRS sinuses lack biodiversity. [186] Typically this lack of biodiversity manifests as increased prevalence of species seen as more traditionally pathogenic, with a reduction in prevalence of bacteria more traditionally seen as commensal. [187] Various studies have been undertaken on varying scales using varying collection methods, laboratory-based analysis and bioinformatic techniques. As such the resulting data have been difficult to compare. An excellent meta-analysis by Wagner Mackenzie *et al*. attempted to standardise and re-analyse many of the existing studies. They concluded the bacteria most prevalent both in the healthy microbiome and in that of CRS are *Staphylococcus*, *Propionibacterium*, *Corynebacterium* and *Streptococcus* species, as well as an unclassified lineage of Actinobacteria. The CRS microbiome was confirmed as dysbiotic, with reduced relative abundance of Actinobacteria and *Propionibacterium* species and an increased abundance of *Corynebacterium* species. When the dataset from normal individuals was analysed without *Burkholderia* or *Propionibacterium* species network fragmentation was increased. This suggests these may be "gatekeeper" genera with roles in maintaining the healthy microbiome. [188]

Little investigation has been undertaken into the changes induced in the respiratory microbiome in the presence of viral infection. What has been studied has focused largely either on the lower airways or on animal models; it is important to note that replication of the

human microbiome in an animal model is exceedingly complex. Wang *et al*. showed that pathogen-free mice (lacking *S. aureus* colonisation as well as other relevant inflammatory cytokines) were more susceptible to influenza-mediated death. [189] Leung *et al*. recruited bacterial pneumonia patients both with and without H1N1 influenza infection and compared their oropharyngeal microbiota. They found a significant increase in *Pseudomonas*, *Bacillus* and *Ralstonia* species in the influenza group. This was accompanied by significant reductions in *Prevotella*, *Veillonella* and *Neisseria* species (all normal oral commensals). The authors did not investigate the microbiome of the lower airways in these patients, despite their disease being focused there. [190] de Steenhuijsen *et al*. focused on children with RSV infection of the lower airway and found clusters of patients with significantly more *H. influenzae*, *Streptococcus*, *Corynebacterium*, *Moraxella* or *S. aureus*. The presence of any of these also correlated with more severe disease. Again, the microbiome samples were taken from the nasopharynx despite the virus under investigation existing in the lower airways. [191] One study did focus purely on the upper airways; nasopharyngeal swabs were taken from 177 patients with clinically suspected viral URTI. 47 of these were virus negative on PCR with the remainder positive for one of either influenza, RV, MPV or RSV. These swabs were compared with 48 swabs from healthy controls. This showed significant reduction in bacterial diversity and loss of bacteria known to form part of the core healthy bacterial microbiome. The authors also found significant increase in particular URT bacteria known to colonise the healthy airway, but also often implicated in respiratory disease (including *S. aureus, H. influenzae, S. pneumoniae* and *M. catarrhalis*). No difference was found in microbiota profiling between the different virus groups, however with only 23 to 28 patients in each statistical significance was unlikely given the enormous diversity seen in these profiles between individuals. During recruitment of controls one patient identified was asymptomatic but positive for influenza A. This individual displayed a bacterial microbial

profile similar to those of non-virus-infected controls. Overall these observations suggest viral susceptibility and the manifestation of viral disease may be influenced by virally-induced changes in the sinonasal microbial profile. [192]

1.9 Cellular infiltrates and chronic rhinosinusitis

As previously mentioned CRS has traditionally been classified into two phenotypes; CRSsNP and CRSwNP. A significant degree of heterogeneity exists amongst patients within each of these phenotypic classifications however, and so their validity has been debated in recent years. Clear definition of CRS endotypes is valuable as it may predict disease progression, disease recurrence and response to treatment, and may identify therapeutic targets. Studies have investigated and attempted to cluster CRS endotypes based on downstream molecular and cellular markers, and eosinophilia and different T cell populations have been identified as important clusters. [193-195]

1.9.1 Eosinophilia and CRS

Eosinophils normally make up about 1-3% or peripheral white cells and play important roles in fighting viral and parasitic infections. They have been implicated in T cell antigen presentation, and mediate allergic responses. Their degranulation has been postulated to be partially responsible for the inflammation and tissue damage seen in CRS. Tissue eosinophilia has been classically associated with CRSwNP and elevated levels of Th-2 cytokines, whereas CRSsNP is associated with tissue neutrophilia and elevated Th-1 cytokines. [195] Again there are many individuals whose disease does not follow these inflammatory and phenotypic patterns, but when present excessive eosinophilia is associated with more extensive disease, worse symptom scores and higher recurrence rates. [196-198] Several chemokines and cytokines have been implicated in augmenting eosinophil survival and facilitating their differentiation in CRS mucosa, [199, 200] but the factors inducing their presence in the first instance remain uncertain. Given the known association of eosinophils

and viral infection, it is not unreasonable to hypothesise that viruses may be an eosinophilia-inciting factor in CRS.

1.9.2 T lymphocytes and CRS

T lymphocytes are another cellular population of significant interest in CRS that form important parts of the immune response to respiratory viral infection. [201, 202] $CD4^+$ T cells and their associated cytokines have long been implicated in CRS pathogenesis. [203] Cytotoxic $CD8^+$ T cells have been investigated in less depth in CRS, but do appear to have roles in modulation of autoimmune and allergic pathologies outside of their more classical cytotoxic activity. [204] $CD8^+$ T cells have been found in greater numbers in CRS mucosa and have been associated with their own set of pro-inflammatory cytokines which may be contributing to disease pathogenesis. [204-206] As is the case for other T cell types $CD8^+$ T cells have regulatory subtypes (tissue-resident memory T cells, Trm); these express CD103. $CD103^+$ T cells (also known to be expressed on $CD4^+$ regulatory T cells) have roles in controlling the potentially damaging effect of cytotoxic T cells when present in high numbers. [207] As yet the $CD8^+$ and $CD103^+$ subsets of Trms have not been investigated in CRS patients, or in virus-positive CRS.

Summary of the systematic review of the literature

CRS is an extremely prevalent and costly disease of the nasal and paranasal sinus mucosa that is difficult to treat. Symptoms include nasal obstruction, anterior rhinorrhoea, PND, headaches or facial pain/pressure and anosmia as well as other non-specific systemic complaints. Disease severity and response to treatment can be evaluated using various tools, including validated patient reported outcome measures such as the SNOT-22 or ADSS, and the objective LMS, LKS or MLK.

There are a number of theories as to the aetiopathogenesis of CRS. These include the bacterial, immune and fungal hypotheses. The bacterial hypothesis encompasses microbiota dysbiosis, *S. aureus* overgrowth, SAg production and biofilm formation. The immune hypothesis includes disruption of the epithelial mechanical barrier, innate and adaptive immune systems. The fungal hypothesis was initially seen as a very promising element that might unite all cases of idiopathic CRS. It has lost momentum due to lack of consensus in results and the inability of large-scale antifungal trials to demonstrate significant clinical benefit. A more recent school of thought highlights viruses as possible contributors as well. Anecdotally it is extremely common to encounter CRS patients who report having contracted the common cold with the development of CRS thereafter. Viruses are associated with other chronic inflammatory diseases (such as asthma, COPD and CF) and bacterial infections of the respiratory tract. They are also associated with eosinophilia and T cell infiltrates, which are in turn associated with more severe CRS that is more difficult to treat and more likely to relapse. The combination of these two observations has ignited interest in the role that viruses may play in the development of CRS. Also driving this interest are known mechanisms of viral-bacterial co-infection in the respiratory tracts. These include effects on epithelial barrier

function, bacterial binding, innate and adaptive immunity, the respiratory microenvironment, direct viral-bacterial interactions and a defective IFN response. These may be pertinent to CRS given the role of bacteria in its aetiopathogenesis; the bacterial microbiome is under intense scrutiny in the current literature. It is generally accepted that CRS sufferers exhibit a dysbiosis with reduced biodiversity, reduced commensal bacteria and increased pathogenic bacteria in their sinuses. However, minimal investigation has been undertaken with regards the effect of viruses on the respiratory microbiota.

Common URT viruses include RV, influenza, CoV, AdV, PIV, RSV, MPV and less commonly BoV and EnV. The *Herpesviridae* (EBV, HHV6, HPV, CMV, VZV and HSV) are also overwhelmingly prevalent, but their near-ubiquity and capability of remaining latent has made interpretation of their presence difficult. Methods for collection of these viruses in the sinonasal passages have not yet been investigated in any depth. Population virome studies have formed the bulk of the literature on the possible role of viruses in CRS. However, differences in patient cohorts, collection methods, season of collection and the viruses for which investigators assayed make comparison of these data difficult. Overall it would seem the two disease entities are correlated, but more comprehensive, standardised investigation encompassing both the viral and bacterial microbiome of CRS is required.

Aims

1. To establish and validate a standardised method for viral sampling in the sinonasal passages
2. To characterise the sinonasal virome in CRS and to investigate correlation of viral presence with disease severity
3. To investigate association of eosinophilia and T lymphocytes with viral presence in CRS
4. To investigate virally-induced changes in the CRS microbiome

Comparative viral sampling in the sinonasal passages; different viruses at different sites

Statement of authorship

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This paper reports on original research 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.

As the principal author RKG contributed to study design, sample collection and processing, data analysis, and writing of the manuscript (70%). CAB contributed to sample collection (5%). AB contributed to statistical analysis (2.5%). SB contributed to sample processing and writing of the manuscript (7.5%). SV contributed to study design and review of the manuscript (5%). PJW and AJP contributed to study design, sample collection and review of the manuscript (5% each).

All authors certify the author contribution statement detailed above is accurate, and that the sum of co-author contributions is equal to 100% less the candidate's stated contribution. All authors give permission for this manuscript to be reproduced in this thesis.

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2.1 Abstract

Background: With the emergence of the microbiome as an important factor in health and disease in the respiratory tract standardised, validated techniques are required for its accurate characterisation. No standardised technique has been reported specifically for viral sampling in the sinonasal passages.

Aim: To optimise viral sampling techniques from the sinonasal cavity.

Methods: Sterile cytology brushes were used under endoscopic guidance to sample the sinonasal mucosa at time of endoscopic sinus surgery at both the MM and IM. DNA and RNA were extracted from the samples and underwent PCR or RT-PCR testing respectively for a panel of fifteen common URT viruses.

Results: 24 adult patients were recruited for this study. 18/24 (75%) patients were positive for virus in at least one site, while 8/24 (33%) were positive for virus at both sites. The mean number of viruses identified at the two sites were similar (0.875 +/- 0.899 at the MM versus 0.750 +/- 1.032 at the IM). 6/24 (25%) of patients showed no virus at either site, while 3/24 (12.5%) demonstrated the same viral species at both sites.

Conclusion: Although the number of viruses present at different sites with the nasal cavity are similar, discord exists in the viral species between sites. It is therefore recommended that both sites are sampled in the clinical and research setting better to characterise the viral species within the nasal cavity.

2.2 Introduction

The role of the healthy human microbiome in prevention and eradication of disease is an area of burgeoning interest in recent years. The interplay between various colonising organisms, their relative abundance and the importance of a fine microbial balance has been shown to be essential for normal functioning of multiple organ systems, not least respiratory. [208, 209] Conversely, disruption of this balance between viruses, bacteria and single-celled eukaryotes has been implicated in numerous disease processes, including acute infective processes as well as many chronic inflammatory diseases. [208]

Microbial dysbiosis (specifically bacterial) has been implicated in several respiratory diseases, including asthma [210] and CRS. [211] Persistent nasal and paranasal sinus inflammation characteristic of CRS affects up to 16% of the western population [212] and manifests as nasal congestion, facial pain or pressure, anterior or post-nasal drainage, and reduction or loss of smell. [2] Although the exact aetiopathogenesis of this condition remains elusive, it is considered multifactorial in origin. Current theories include the fungal hypothesis, the bacterial hypothesis (implicating dysbiosis with *S. aureus* overgrowth, SAg production and biofilm formation) and an overactive immune response (resulting in chronic inflammation and defective mechanical and innate immune barriers to infection in the CRS population). [23] An area that is anecdotally suggested to play a role in CRS pathogenesis is a viral dysbiosis. [44, 45] This is due to self-reports by many CRS patients that their symptoms almost invariably developed after an initial viral URTI. Research into the ideal method to sample the sinonasal bacterial microbiome is ongoing, [213] however similar efforts to investigate and standardise sampling of the virome have not been made.

Studies attempting to investigate the upper respiratory virome are limited. The lack of standardisation in sampling has led to conflicting results regarding the presence of virus and the composition of the virome. Collection techniques employed thus far include nasal washes, aspirates, brushings and traditional viral swabs, with viral analysis performed by PCR. [44, 45, 170-180] Few studies have compared sampling methods; Heikkinen *et al*. found no difference in the detection of childhood influenza comparing nasal swabs and aspirates. [181] Spyridaki *et al*. found a higher detection of RV in nasal lavages compared with nasal brushings but found no difference in any other viruses tested when comparing these to nasal aspirates and swabs. [182] To date there have been no studies that have compared different sites within the sinuses and nasopharynx in terms of viral detection.

The aim of the study here presented was to establish differences in viral detection and species sampled from different sinonasal sites, in an effort to validate and standardise viral collection techniques and facilitate further investigation of the sinonasal virome.

2.3 Materials and methods

2.3.1 Study participants

Patients for this study were recruited from the tertiary rhinologic practices of the two senior authors (PJW and AJP). This study was carried out in accordance with the recommendations of the Central Adelaide Local Health Network Ethics Committee (HREC/15/TQEH/132). The protocol was approved by the same. All subjects gave written informed consent in accordance with the Declaration of Helsinki. Patients were included in this study if they were older than eighteen years of age and were undergoing endoscopic surgery. Control patients consisted of patients with an absence of clinical or radiologic evidence of CRS. CRS patients fulfilled the diagnostic criteria for CRS as outlined in the American guidelines. [214] The radiological severity of disease was scored for all patients using the LMS. [77]

2.3.2 Sampling and processing

Using an aseptic technique, endoscan cytology brushes (McFarlane Medical, Melbourne, Australia) were used to sample the sinonasal mucosa (figure 2.1) of the left and right MM and IM of each patient. This was done under endoscopic visualisation with caution to avoid cross-contamination from neighbouring tissue. The samples were then placed in a viral transport medium (89% Roswell Park Memorial Institute medium supplemented with 9% foetal bovine serum, 1% amphotericin B and 1% penicillin streptomycin (all Gibco by ThermoFisher, Waltham, USA)) and immediately transported on ice to the laboratory for processing. Sample material was removed from the brushes and centrifuged at 4°C and 1700rpm for seven minutes in order to isolate cellular material. The supernatant was discarded, after which samples were stabilised with 35µL RPE Buffer (Qiagen, Hilden,

Germany) and 3.5µL beta-mercaptoethanol (Gibco by ThermoFisher, Waltham, USA) and stored at -80°C.

Samples were thawed in batches to undergo RNA and DNA extraction using an AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany). This yielded DNA samples of 200µL (average concentration 96.37ng/µL, range 10.3 to 383.3ng/µL) and RNA samples of 60µL (average concentration 58.58ng/µL, range 4 to 247.3ng/µL).

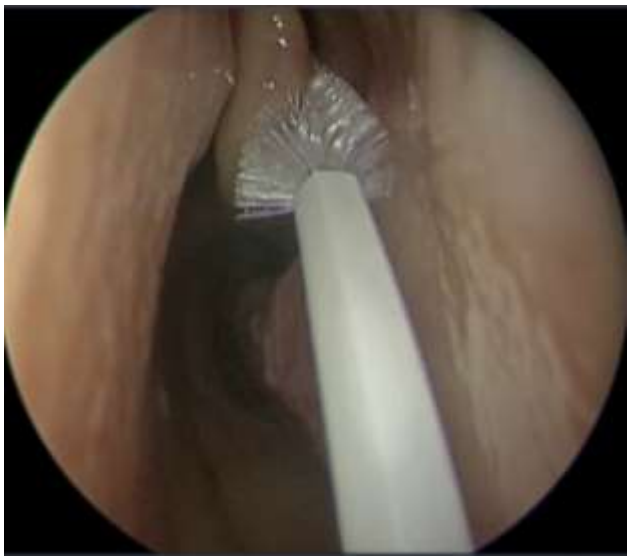

Figure 2.1 Cytology brushing of sinonasal mucosa.

2.3.3 PCR/RT-PCR

Extracted DNA and RNA were stored at -80°C until batch testing for a range of URT viruses using real-time PCR. The panel included RV, influenza A-C, PIV 1-4, RSV A and B, CoV HKU-1, OC43, NL63 and 229E, EnV, MPV, AdV, BoV, polyomaviruses WUPyV and KIPyV, EBV, CMV, HHV6, HSV 1 and 2, and VZV. All DNA extracts first underwent an ERV3 assay (present as two copies per human diploid cell) in order to confirm respiratory sample collection quality.

Briefly, DNA extracts were screened for ERV3, AdV, BoV, WUPyV, KIPyV, CMV, EBV, VZV, HSV 1 and 2 and HHV6 using an identical set of conditions previously optimised so as not to compromise sensitivity (table 2.1). Said conditions were 8 pmole of each primer, 3.2pmol of the respective probe(s) and 2µL of template, made up to a final reaction volume of 20 µL using the Bioline Sensi Mix II Probe PCR mix kit (Bioline Australia). Details of the target genes, primer and probe sequences are summarised in tables 4 and 5. Samples then underwent the following cycling conditions: 94°C for two minutes, followed by 45 cycles of 95°C for fifteen seconds and 60°C for sixty seconds. The RNA extracts were tested for RV, influenza A to C, PIV 1 to 4, RSV A and B, CoVs HKU-1, NL63, OC43 and 229E, EnV and MPV (table 2.2) using identical quantities of primer, probe and template to the DNA reactions but with the Bioline SensiFAST Probe One-Step RT-PCR kit (Bioline, Sydney, Australia). There were two exceptions to these quantities; the IV A/B duplex where asymmetric probe amounts were used (6.4pmol and 3.2pmol respectively) and the RV assay where 16pmol of probe was used. Samples then underwent the following cycling conditions: 45°C for twenty minutes, and 45 cycles of 95°C for fifteen seconds and 60°C for sixty seconds. All samples were run with both positive and negative controls; the positive controls were either previously established clinically positive samples, or synthetic controls specific for each assay. All cycling was conducted on Viia7 instruments (ThermoFisher, Scoresby, Australia). Viral detection was defined as a cycle threshold (Ct) of forty or less.

Reaction mix	Virus	Target gene	Primer, probe sequences (5'-3')	Source
11	Adenovirus	Hexon	GCCACGGTGGGGTTTCTAAACTT GCCCCAGTGGTCTTACATGCACATC FAM-TGCACCAGACCCGGGCTCAGGTACTCCGA-BHQ1	[215]
12	Polyomavirus WU	NCCR	GCCGACAGCCGTTGGATATA TTTCAGGCACAGCAAGCAAT FAM-AGGGTCACCATTTTTATTTCAGATGGGCA-BHQ1	[216]
	Polyomavirus KI	NCCR	GAACTTCTACTGTCCTTGACACAGGTA GGATTAGAACTTACAGTCTTAGCATTTCAG Q670-ACCCTTTGTAGGCCAAAGGAGAGTGAAGG-BHQ2	
	Polyomavirus KI	STAg	CACAGGTGGTTTTCTATAAATTTTGTACTT GAAGCAGTGGGATGTATGCATTC YAK-TGCATTGGCATTCGTGATTGTAGCCA-BBQ	
13	Bocavirus	VP1	GGCAGAATTCAGCCATACTCAAA TCTGGGTTAGTGCAAACCATGA FAM-AGAGTAGGACCACAGTCATCAGACACTGCTCC-BHQ1	[217]
14	Cytomegalo-virus	MIE	AACTCAGCCTTCCCTAAGACCA GGGAGCACTGAGGCAAGTTC FAM-CAATGGCTGCAGTCAGGCCATGG-BHQ1	[218]
15	Epstein Barr virus	BALF5	CGGAAGCCCTCTGGACTTC CCCTGTTTATCCGATGGAATG FAM-TGTACACGCACGAGAAATGCGCC-BHQ1	[219]
16	Varicella zoster virus	ORF38	AAGTTCCCCCCGTTCGC TGGACTTGAAGATGAACTTAATGAAGC FAM-CCGCAACAACTGCAGTATATATCGTCTCA-BHQ1	[218]
17	Herpes simplex 1	gD	CGGCCGTGTGACACTATCG CTCGTAAAATGGCCCCTCC FAM-CCATACCGACCACACCGACGAACC-BHQ1	[220]
	Herpes simplex 2	gD	CGCCAAATACGCCTTAGCA GAAGGTTCTTCCCGCGAAAT VIC-CTCGCTTAAGATGGCCGATCCCAATC-BHQ1	[218]
18	Herpes virus 6	DNA Pol	TGCTCGGACTGCATCTTGGA TTATTGCCGTGTGTTGCGATT FAM-TTAACATAATCCACCGTGGAACAAAGCATCT-BHQ1	[221]
19	Endogenous retrovirus 3	ENV	CATGGGAAGCAAGGGAACTAATG CCCAGCGAGCAATACAGAATTT FAM-TCTTCCCTCGAACCTGCACCATCAAGTCA-BHQ1	[222]

Table 2.1 Target gene, probe and primer details for DNA viruses.

Reaction mix	Virus	Target gene	Primer, probe sequences (5'-3')	Source
1	Rhinovirus	5’ UTR	CY+AGCC+TGCGTGGY GAAACACGGACACCCAAAGTA FAM-TCCTCCGGCCCCTGAATGYGGC-BHQ1	[223]
2	Influenza A	Matrix	CTTCTAACCGAGGTCGAAACGTA GGTGACAGGATTGGTCTTGTCTTTA Q670-TCAGGCCCCCTCAAAGCCGAG-BHQ2	[224]
3	Influenza B	Matrix	GCATCTTTTGTTTTTTATCCATTCC CACAATTGCCTACCTGCTTTCA FAM-TGCTAGTTCTGCTTTGCCTTCTCCATCTTCT-BHQ1	[225]
	Influenza C	Matrix	CATAATTGAACTTGTCAATGGTTTTGT TTCAGGCATAATTGTGGTCTTTATATCT FAM-CTCGGCAGATGGGAGAGATGGTGTG-BHQ1	Personal communic-ation
4	RSV A	Nucleocapsid	AGATCAACTTCTGTCATCCAGCAA TTCTGCACATCATAATTAGGAGTATCAAT FAM-CACCATCCAACGGAGCACAGGAGAT –BHQ1	[226]
	RSV B	Nucleocapsid	AAGATGCAAATCATAAATTCACAGGA TGATATCCAGCATCTTTAAGTATCTTTATAGTG YAK-TATGTCC+AGG+TTAGGAAG+G+G+AA-BBQ	
5	Parainfluenza 1	Hemagglutinin-neuraminidase	TTTAAACCCGGTAATTTCTCATACCT CCCCTTGTTCCTGCAGCTATT FAM-TGACATCAACGACAACAGGAAATCATGTTCTG-BHQ1	[225]
	Parainfluenza 2	Nucleocapsid	AGAGTTCCAACATTCAATGAATCAGT CTCAAGAGAAATGTCATTCCCATCT YAK-CCTCTGTATTGCTCATGCATAGCACGGA-BBQ	
6	Parainfluenza 3	Nucleocapsid	CGGTGACACAGTGGATCAGATT AGGTCATTTCTGCTAGTATTCATTGTTATT Q670-TCAATCATGCGGTCTCAACAGAGCTTG-BHQ2	

	Parainfluenza 4A	Phosphoprotein	GCAATTAAGGCAYTAGAAGTRA AATTGTGGCAAGTGAACC FAM-TTTGTCAACTTTCCCYTCAATCCTG-BHQ1	[227]
	Parainfluenza 4B	Phosphoprotein	TCCHATAATCGTCACTGGYA TATTTTAAGTGCATCTATACGAAC Q670-ACAAAATGGGTCTTGCTARCGG-BHQ2	
7	Coronavirus HKU1	Polymerase	CCTTGCGAATGAATGTGCT TTGCATCACCACTGCTAGTACCAC FAM-TGTGTGGCGGTTGCTATTATGTTAAGCCTG-BHQ1	[228]
8	Coronavirus OC43	Nucleocapsid	CGATGAGGCTATTCCGACTAGGT CCTTCCTGAGCCTTCAATATAGTAACC Q670-TCCGCCTGGCACGGTACTCCCT-BHQ2	[229]
	Coronavirus NL63	Polyprotein 1a	ACGTACTTCTATTATGAAGCATGATATTAA AGCAGATCTAATGTTATACTTAAAACTACG YAK-ATTGCCAAGGCTCCTAAACGTACAGGTGTT-BBQ	[230]
	Coronavirus 229E	Nucleocapsid	CAGTCAAATGGGCTGATGCA AAAGGGCTATAAAGAGAATAAGGTATTCT FAM-CCCTGACGACCACGTTGTGGTTCA-BHQ1	[229]
9	Metapneumo-virus	Nucleocapsid	CATATAAGCATGCTATATTAAAAGAGTCTC CCTATTTCTGCAGCATATTTGTAATCAG FAM-TGYAATGATGAGGGTGTCACTGCGGTTG-BHQ1	[231]
10	Enterovirus	5’ UTR	CCTGAATGCGGCTAATCC TTGTCACCATWAGCAGYCA FAM-CCGACTACTTTGGGWGTCCGTGT-BHQ1	[232]

Table 2.2 Target gene, probe and primer details for RNA viruses. “+” indicates a locked nucleic acid (eg. +A is a locked nucleic adenine analogue.

2.3.4 Statistical analysis

Statistics were performed using software from Scientific Python, namely SciPy and pandas through the Jupyter Notebook interface. [233] McNemar's test was used to test for significantly different proportions of viral positivity between sites. Paired Student's t test was used to compare the mean number of viruses detected between sites. Percentage agreement was calculated for viral detection between both sites for both number and species of viruses detected. Chi square test was used to investigate any correlation between viral presence and control/disease status. Statistical significance was defined as a p-value of <0.05.

2.4 Results

2.4.1 Patient characteristics

24 patients were recruited at time of endoscopic surgery; this included fourteen men and ten women, with an age range of 19 to 79 years, and a mean age of 51 years. Seven patients had CRSsNP, eight had CRSwNP and nine were controls. Demographics and patient characteristics are summarised in table 2.3. All patients in the CRS groups underwent FESS, while those in the control group underwent trans-sphenoidal resections of pituitary masses.

Mean age (years)	46.5	45.6	61.1
Sex	3 M, 6 F	4 M, 3 F	7 M, 1 F
Diagnosis	9 controls	7 CRSsNP	8 CRSwNP

Table 2.3 Summary of patient demographics and characteristics.

2.4.2 Viral detection and analysis

ERV3 was detected in all patient samples, with a median Ct of 22.5 (range 19.3-28.0), showing adequate cellular material was captured throughout the collection and DNA extraction phases. 18 patients were positive for at least one virus in at least one site (18/24, 75%), while six (6/24, 25%) were negative for any of the viruses for which the samples were screened (table 2.4). Similar rates of viral detection were seen between the MM and IM overall (52% positivity at the MM versus 48% at the IM; p= 0.55, McNemar's test). The mean number of viruses detected at the MM was 0.875 +/- 0.899, versus 0.750 +/- 1.032 at the IM. The mean number of viruses detected did not differ significantly between both sites

(p=0.57, paired t test). Interestingly the majority of patients (63%) did not show an intranasal correlation between sites. Of the nine patients demonstrating similar findings at both sites, only three demonstrated viral presence with six showing an absence of virus at all sites. Fifteen patients were inconsistent between the two sites; this included 4 patients who exhibited virus or viruses at both sites but of different species at each (table 2.4) These findings correspond to a percentage agreement of only 31 between the MM and IM in terms of number of viruses detected (i.e. not accounting for viral species). When analysing for viral species there was only a percentage agreement of 27 between the sites. No correlation was found between control/disease phenotype and viral presence (p=0.68, Chi-square test).

Patient number	Diagnosis	Site of sampling	Viruses identified	Patient number	Diagnosis	Site of sampling	Viruses identified
1	Control	MM	None	13	Control	MM	EBV, HHV6
		IM	None			IM	EBV
2	CRSwNP	MM	None	14	CRSsNP	MM	None
		IM	None			IM	Influenza A, HHV6
3	CRSsNP	MM	None	15	CRSsNP	MM	None
		IM	None			IM	EBV
4	Control	MM	None	16	CRSwNP	MM	EBV
		IM	None			IM	None
5	CRSwNP	MM	None	17	CRSsNP	MM	HHV6
		IM	None			IM	None
6	Control	MM	None	18	Control	MM	HHV6
		IM	None			IM	None
7	CRSwNP	MM	Influenza A, HHV6	19	CRSwNP	MM	None
		IM	Influenza A, HHV6			IM	EBV
8	Control	MM	HHV6	20	CRSwNP	MM	EBV, HHV6
		IM	HHV6			IM	None
9	Control	MM	EBV, HHV6	21	CRSsNP	MM	HHV6
		IM	EBV, HHV6			IM	None
10	CRSsNP	MM	HHV6	22	CRSwNP	MM	EBV, HHV6
		IM	EBV			IM	None
11	CRSsNP	MM	Influenza A	23	CRSwNP	MM	PIV2
		IM	Influenza A, EBV			IM	None
12	Control	MM	HHV6	24	Control	MM	None
		IM	EBV			IM	EBV

Table 2.4 Viral species identified at middle and inferior meatuses.

2.5 Discussion

A standardised, validated technique for viral sampling in the sinonasal passages has not yet been described. This study shows a significant discrepancy in viral presence and species between just two of the sites commonly sampled, highlighting the need for such a standardisation. This indicates that viral sampling needs to be conducted with a cytobrush in both the IM and the MM.

Collection variability has the potential to impact respiratory viral detection significantly. The sample volume and location, as well as the documented uneven distribution of viruses within the nasal cavity, can all contribute to false negatives. [234] Given that clinically relevant, actively replicating viruses of the URT are intra-cellular and largely reside in the upper epithelial layers of the mucosa, [118] adequate cell sampling is an important consideration when searching for viruses. Traditional viral sampling brushes have the advantages of causing less trauma to the delicate mucosa and thus less discomfort to a conscious patient, but risk sampling largely secreted elements rather than the cells themselves. [182] Viruses do certainly reside in these secretions, but this may not necessarily represent actively replicating virus causing disease. For these reasons we elected to use cytology brushes for this study. Cytology brushes are designed specifically for cell sampling due to their larger and more rigid design than traditional viral sampling brushes. Although this may potentially increase the risk of trauma or discomfort to the awake patient, when used in the anaesthetised patient, as was the case in this study, they have the significant advantage of increased cellular sampling yield. [183]

As mentioned viral yields are also difficult to compare in respiratory samples, as sample volume can vary widely. The samples here averaged a DNA concentration of 96.37ng/µL and an RNA concentration of 58.58ng/µL, but with ranges of 10.3 to 383.3ng/µL and 4 to 247.3ng/µL respectively. To minimise the impact of this variability on results all samples underwent an ERV3 assay prior to PCR. This has been identified previously as a positive indicator of respiratory sample quality, and all samples were well within previously published target ranges. [235, 236]

Viral sampling is traditionally performed from the inferior nasal septum and anterior nasal floor as they are easily accessible and cause minimal patient discomfort. The posterior nasopharyngeal wall is also traditionally endorsed, but confirmation of access to this site is difficult without endoscopic equipment. There is no evidence however that these three sites are any more or less appropriate. These areas may indeed be less than ideal due to their relative proximity to airborne pathogens (and therefore risk of contamination), their distance from areas of particular interest (such as the paranasal sinuses), and the tendency for pooling of potentially contaminating secretions in these areas. The MM (sampled in our study) remains relatively simple to access but is further away from potential sources of contamination, and receives drainage from a much wider area including the maxillary, frontal and anterior ethmoid sinuses. There are indeed a number of other sites in the nasopharynx not investigated here, for example the superior meatus, the sphenoethmoidal recess and the post-nasal space, however these are difficult to reach without endoscopic equipment that is not readily available in the primary care setting, and can be subject to contamination from other more anterior sites during insertion and removal of sampling instruments. Should these areas demonstrate greater viral presence than the MM and IM the specialist input required to access the sinuses themselves would likely delay or miss altogether the diagnosis and window for

anti-viral treatment. Large-scale economic viability of the collection method here proposed also warrants mention; pooling of viral samples from the same patient prior to analysis and limitation of viral testing to a smaller panel of more prevalent, clinically relevant pathogens would be prudent, however selection of such a panel requires further investigation.

Common, clinically relevant upper respiratory viruses are largely of the RNA subtype, and include RV, influenza, RSV and MPV, and to a lesser extent CoV, PIV, and EnV. Of the DNA viruses here investigated AdV is certainly a notable URT pathogen. BoV has been linked largely with lower respiratory illness. [237] The other DNA viruses here investigated were chosen not primarily for their clinical relevance in viral respiratory disease, but instead for either their near-ubiquity, their ability to remain latent in the respiratory tract, or both. EBV and HHV6 have here shown themselves to be particularly useful in testing viral sampling methods as they are almost omnipresent in the adult sinonasal passages, and are rarely entirely cleared after first infection.

Effort was made in this study to identify any correlation between control/CRSsNP/CRSwNP status and viral presence. Patient reports of recent viral infection, SNOT-22 scores, LMS, LMK and RT-PCR Ct values were collected for all patient and samples, but the sample size here was too small to demonstrate any significant differences. The inclusion of the extremely common herpesviruses (seen, as expected, in many of our controls) also skewed any such results. This is an area that requires significant further investigation.

Neither of the sites from whence samples were taken were more or less likely to be positive than the other. Our observation that the MM and IM only completely agree in terms of viral presence or absence as well as viral species in 27% of cases indicates a significant proportion

of viruses present would not be identified were only one site to be sampled. Our findings suggest viral sampling from the sinonasal passages should be taken from both sites in both nasal cavities. The sampling method here described has significant implications for further research into a field of emerging importance in both rhinologic and also respiratory disease on a larger scale.

The presence of virus significantly associates with chronic rhinosinusitis disease severity

Statement of authorship

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This paper reports on original research 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.

As the principal author RKG contributed to study design, sample collection and processing, data analysis, statistical analysis and writing of the manuscript (70%). CAB and RSV contributed to sample collection (2.5% each). SB contributed to sample processing and writing of the manuscript (10%). SV contributed to study design and review of the manuscript (5%). PJW and AJP contributed to study design, sample collection and review of the manuscript (5% each).

All authors certify the author contribution statement detailed above is accurate, and that the sum of co-author contributions is equal to 100% less the candidate's stated contribution. All authors give permission for this manuscript to be reproduced in this thesis.

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3.1 Abstract

Background: Patients with CRS often implicate a viral URT infection as a disease-inciting event. However, the presence and identity of these viruses and their relationship to disease phenotype and severity in CRS patients is unknown. The aim of this study was to investigate the CRS virome in relation to disease characteristics.

Methods: Endoscopically-guided sterile cytology brushes were used to sample the mucosa immediately prior to sinus surgery. DNA/RNA extracts underwent PCR/RT-PCR testing for a panel of common respiratory viruses. Disease severity data was collected from each patient prior to sampling: SNOT-22, ADSS, LMS and LKS.

Results: 288 patients were included in the study; 71 controls, 133 CRSsNP) and 84 CRSwNP patients. Virus was significantly more prevalent in CRSsNP patients compared to controls; 20.30% of CRSsNP versus 15.48% of CRSwNP and 7.04% of controls. LMS and LKS were worse in the CRSsNP group with virus than the CRSsNP group without virus (LMS 9.56 ± 1.07 vs. 6.5 ± 0.43, LKS 6.07 ± 0.71 vs. 4.21 ± 0.32). SNOT-22 scores and ADSS were not significantly different for patients with virus versus patients without.

Conclusions: Virus is more prevalent in CRSsNP patients and is associated with worse objective disease. This potentially implicates viruses in the pathophysiology of CRS, and as such presents a new preventative and therapeutic target.

3.2 Introduction

CRS is an inflammatory disorder of the nasal and paranasal sinuses occurring with and without nasal polyps (CRSsNP and CRSwNP). Although not objectively demonstrated, an initial viral insult is commonly described by patients prior to development of CRS. If viruses were demonstrated to play a role in CRS, novel prophylactic and/or therapeutic targets might be uncovered.

Findings in previous studies investigating CRS and viruses are variable. [44, 45, 171, 172] Possible reasons include small sample sizes, unvalidated collection methods, seasonal limitation, heterogenous CRS cohorts and limited viral species screening. No studies to date have investigated disease severity in relation to viral presence.

We aimed to investigate the sinonasal virome of CRS patients in relation to disease phenotype, to compare it to healthy controls, and to explore any association between more severe disease and viral presence. Cytobrush samples were taken from the sinonasal passages and DNA/RNA extracts underwent PCR for a number of viral species and strains. The *Herpesviridae* were excluded due to their near-ubiquity in adult sinuses.

3.3 Methods

3.3.1 Study participants and data collection

Patient recruitment was undertaken from the tertiary rhinologic practices of two of the senior authors (AJP and PJW). The protocol was approved and the study was undertaken as per the Central Adelaide Local Health Network Ethics Committee (HREC/15/TQEH/132). Specifically, all participants gave verbal and written informed consent in accordance with the Declaration of Helsinki. Patients over the age of 18 years undergoing endoscopic surgery were included in this study. Control patients had no clinical, radiologic or endoscopic evidence of CRS. CRS patients met the American diagnostic criteria for such. [214]. All patients in the CRS groups were undergoing FESS, while those in the control group were undergoing trans-sphenoidal resections of pituitary masses or septoturbinoplasties. The symptomatic severity of disease was scored for all patients using the validated SNOT-22 and the ADSS. The radiological severity of disease was calculated using the LMS, and the endoscopic severity of disease was calculated using the LKS.

3.3.2 Viral sampling and processing

Viral sampling was undertaken using a previously published protocol from the authors' department. [238] Briefly, endoscan cytology brushes (McFarlane Medical, Melbourne, Australia) were used to sample the left and right middle meatus (MM) and inferior meatus (IM) mucosa with an aseptic technique and under endoscopic visualisation. Samples were transported on ice and stored at -80°C in accordance with the aforementioned protocol. Prior to processing samples were thawed and underwent RNA and DNA extraction using an

AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany). DNA samples of 200µL were obtained (average concentration 96.37ng/µL, range 10.3 to 383.3ng/µL), as were RNA samples of 60µL (average concentration 58.58ng/µL, range 4 to 247.3ng/µL).

3.3.3 Viral PCR/RT-PCR

Specifics regarding PCR/RT-PCR methods appear in a previously published protocol. [238] In brief, DNA and RNA extracts were stored at -80°C until real-time PCR batch testing for a panel of respiratory viruses. These were AdV, BoV, CoV, EnV, influenza, MPV, PIV 1-4, RSV and RV. An initial ERV3 assay was undertaken in the DNA extract fractions prior to assays for the aforementioned viruses to ensure adequate sample quality.

DNA extracts were tested for ERV3, AdV and BoV using the Bioline Sensi Mix II Probe PCR mix kit (Bioline, Sydney, Australia) under conditions previously described. [238] Said conditions had been optimised prior to testing so as not to compromise sensitivity. Details of the target genes, primer and probe sequences and cycling conditions have also been detailed previously. [238] RNA extracts were screened for CoVs HKU-1, NL63, OC43 and 229E, EnV, influenza A, B and C, MPV, PIV 1 to 4, RSV A and B and RV using the Bioline SensiFAST Probe One-Step RT-PCR kit (Bioline, Sydney, Australia). Again, target genes, primer and probe sequences and cycling conditions have been detailed previously. Cycling was conducted on Viia7 instruments (ThermoFisher, Scoresby, Australia). Viral detection was defined as a Ct of forty or less.

3.3.4 Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.03 (San Diego, USA). Chi-square test was used to compare viral detection in control, CRSsNP and CRSwNP patients, and to compare seasonal viral detection across those three groups. Kruskal-Wallis tests with post-hoc pairwise comparisons were used to compare SNOT-22 scores, ADSS, LMS and LKS in control, CRSsNP and CRSwNP patients, and to investigate correlation of disease severity with viral species. Benjamini-Hochberg false discovery rate was used to correct p-values for multiple pairwise comparisons. Statistical significance was defined at a p-value of <0.05.

3.4 Results

3.4.1 Patient characteristics and viral detection

288 patients were recruited: 71 controls, 133 CRSsNP and 84 CRSwNP (table 3.1). 45/288 patients were virus-positive: 5 control, 27 CRSsNP and 13 CRSwNP (figure 3.1). The rate of viral positivity was significantly higher in the CRSsNP group ($p<0.05$).

Diagnosis	71 controls	133 CRSsNP	84 CRSwNP
Mean age (years)	53.82	48.79	51.05
Number of female patients	35	70	29
Season (spring:summer:autumn:winter)	15:13:16:27	28:33:51:21	19:15:30:19

Table 3.1 Summary of patient demographics and characteristics for viral analysis.

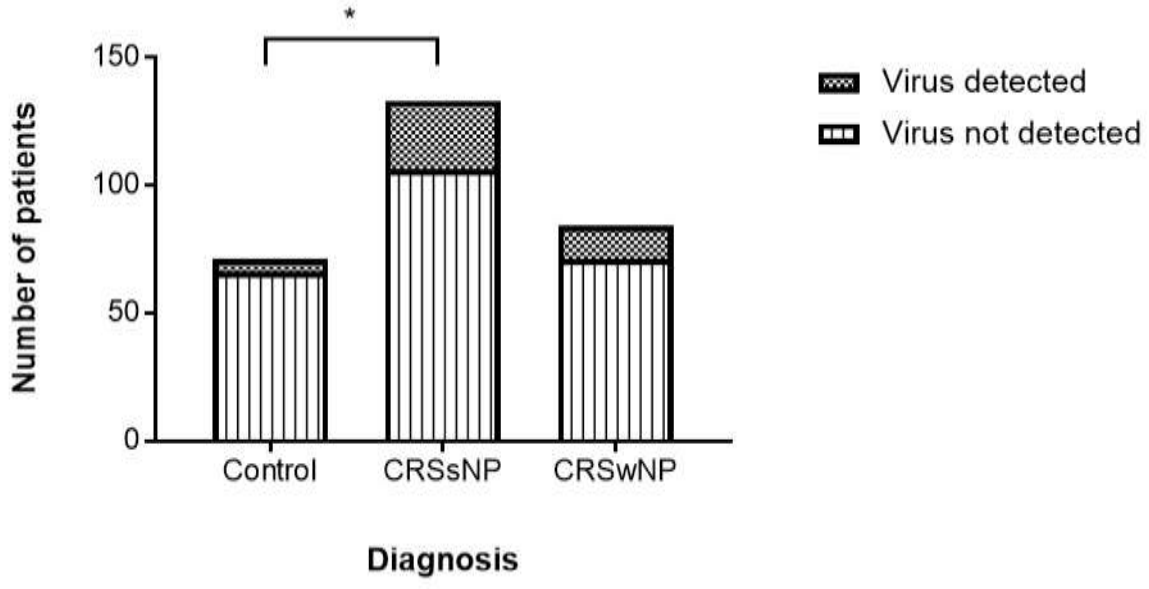

Figure 3.1 Detection of virus by PCR in controls and CRS patients. Number of patients

where virus was detected and not detected in controls, CRSsNP and CRSwNP. Comparison of positivity of virus detected, * $p<0.05$, Chi-square test.

3.4.2 Correlation with disease severity

Objective disease severity scores LMS and LKS revealed significantly worse disease in the CRSsNP virus positive cohort compared with the CRSsNP virus negative cohort ($p<0.05$, figure 3.2). No significant differences were observed in the control or CRSwNP cohorts. Subjective scores (SNOT-22 and ADSS) revealed no difference between patients with or without virus in any of the groups (figure 3.2). PCR Cts also revealed no difference between virus-positive or negative individuals (table 3.2).

Viral species	**Number of positive patients**	**Mean SNOT-22**	**Mean ADSS**	**Mean LMS**	**Mean LKS**	**Mean Ct**
Adenovirus*	1*	67*	21*	4*	4*	33.57*
Bocavirus	4	46.75	13.75	6.75	9.25	36.21
Coronavirus	16	41.06	13.94	6.56	5.44	33.85
Enterovirus	3	38	14	9.33	6.67	34.26
Influenza	9	46	17	10.22	7	35.44
Parainfluenza	3	31	16	14	10.33	32.25
Rhinovirus	16	38.19	14.88	9.88	6.5	29.02
RSV*	1*	47*	19*	7*	12*	38.09*

Table 3.2 Details of virus-positive patients, including viral species, mean severity scores and cycle threshold values. *Indicates viral species for which only one patient was positive, so mean score not able to be calculated. Instead that one patient's scores have been recorded.

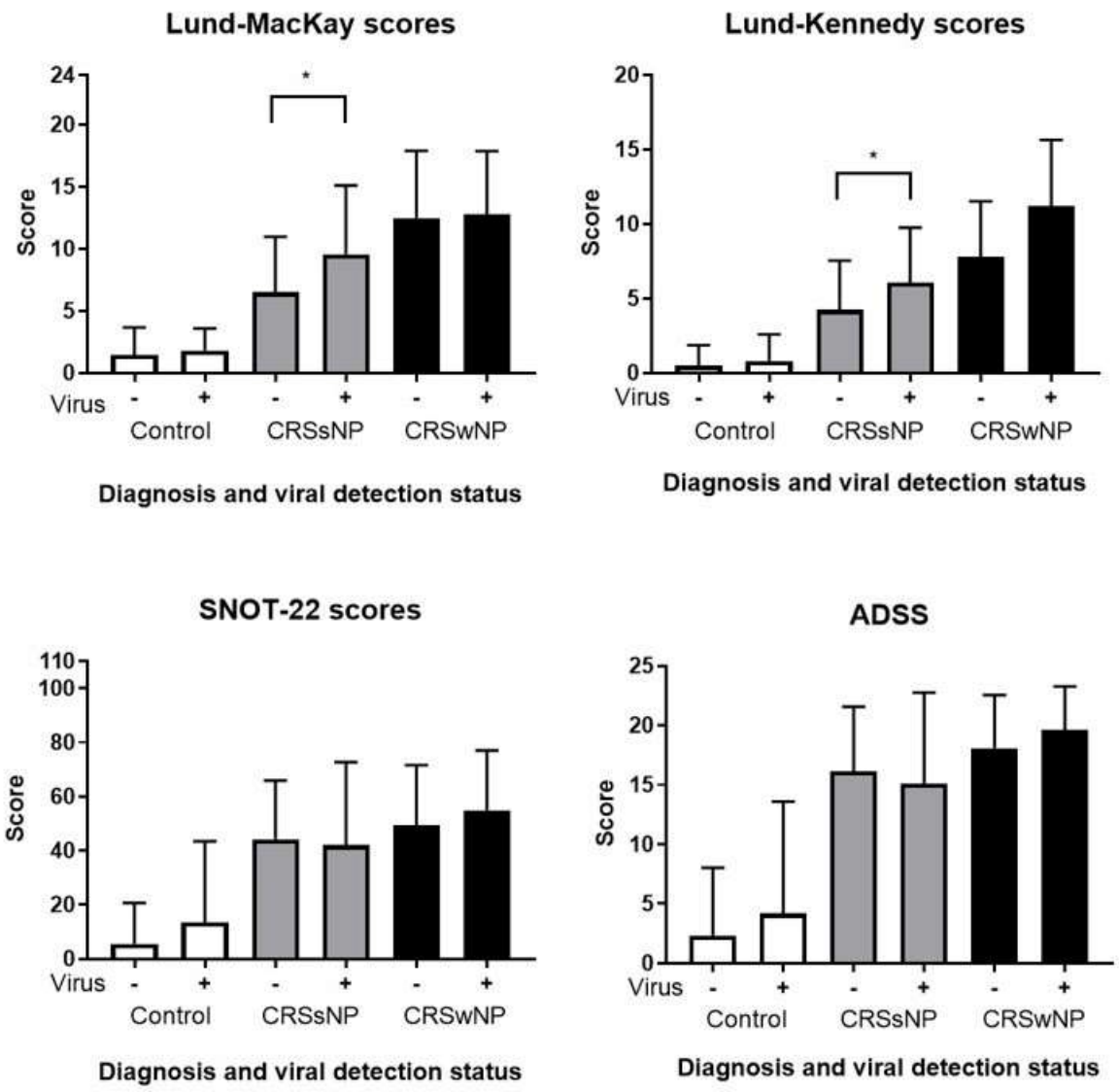

Figure 3.2 Objective (top) and subjective (bottom) disease scores in virus negative and virus positive patients. Grouped and compared by diagnosis (control, CRSsNP or CRSwNP), * $p<0.05$, Kruskal-Wallis tests.

3.4.3 Viral species detection and seasonal spread

Viral species detected did not vary significantly from previously published studies; these were largely RV and CoV (tables 3.2 and 3.3 and figure 3.3). Peak viral detection occurred in

spring and winter; there was no significant difference in detection when analysed by season (figure 3.4).

Viral species +/- strain	**Number of positive control patients**	**Number of positive CRSsNP patients**	**Number of positive CRSwNP patients**
Adenovirus	1	0	0
Bocavirus	1	2	1
Coronavirus HKU	1	0	1
Coronavirus NL63	2	3	1
Coronavirus OC43	1	2	3
Coronavirus 229E	0	1	1
Enterovirus	1	1	1
Influenza A	0	7	1
Influenza B	0	1	0
Influenza C	0	0	0
Parainfluenza 1	0	0	0
Parainfluenza 2	0	0	1
Parainfluenza 3	0	1	1
Parainfluenza 4	0	0	0
Rhinovirus	1	13	2
RSV A	0	0	1
RSV B	0	0	0

Table 3.3 Details of viral species, strains and patient diagnoses.

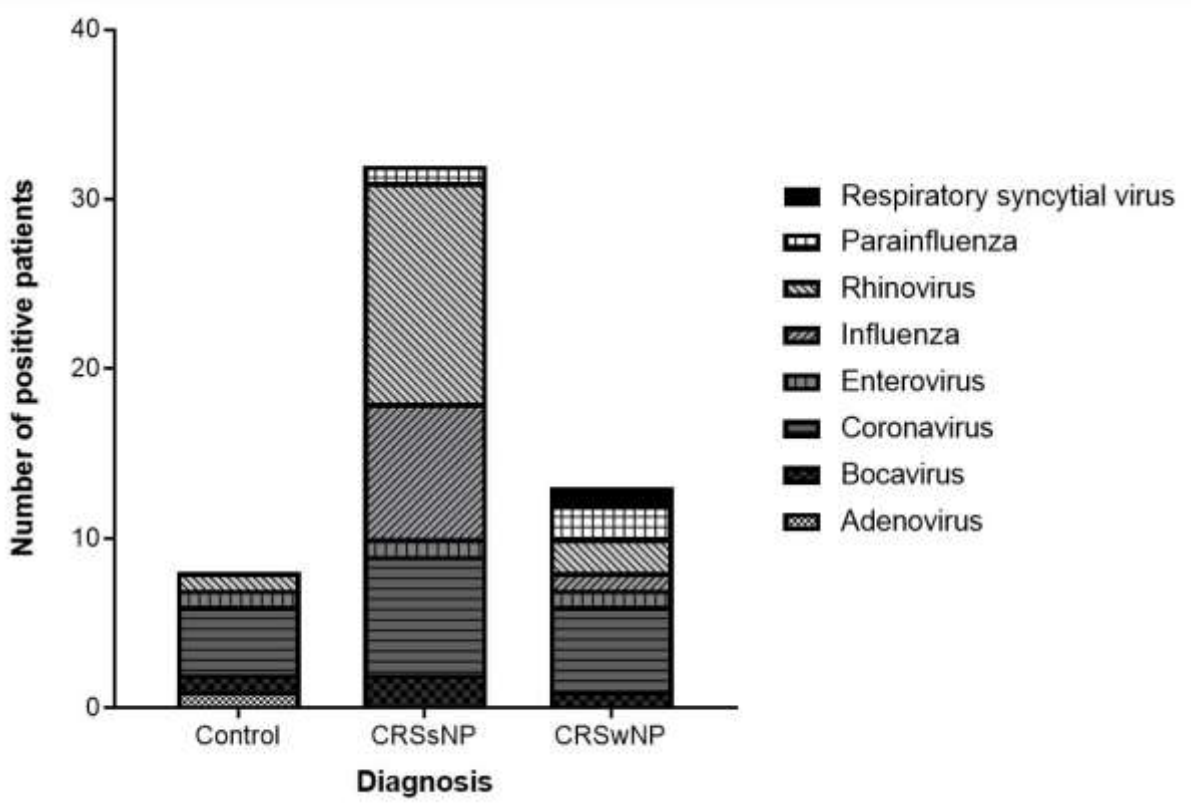

Figure 3.3 Viral species detected by PCR in virus positive patients. Control compared with CRS.

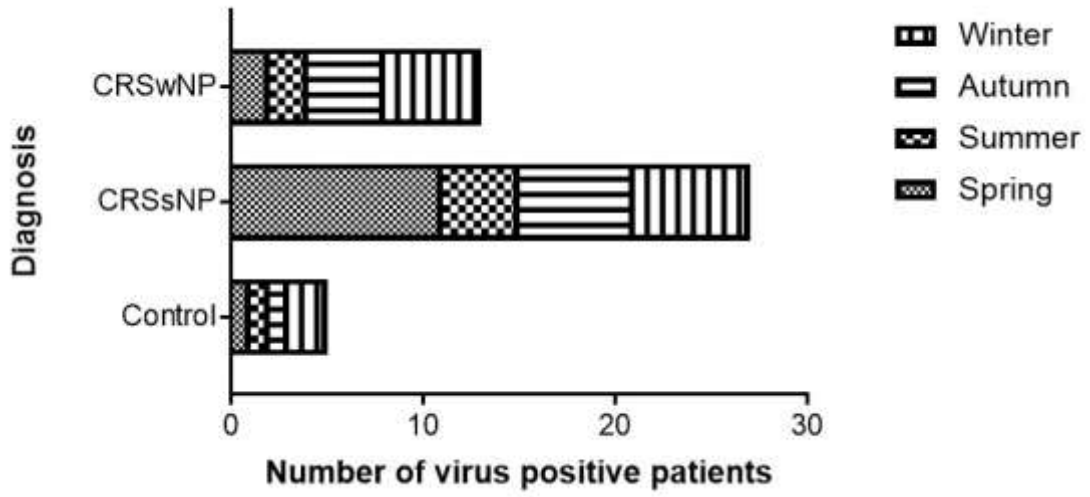

Figure 3.4 Seasonal spread of viral detection by PCR. Grouped by diagnosis (control, CRSsNP, CRSwNP).

3.5 Discussion

This study identified common respiratory viruses as more prevalent in CRSsNP patients than in controls. It is the first study to demonstrate their significant association with more severe radiological and endoscopic disease in CRSsNP patients but not CRSwNP patients.

The lack of any significant difference in subjective symptom scores in any of the groups is not unexpected. Absence of correlation between subjective and objective measures of disease severity has been well documented. [82] Although the inclusion of non-rhinologic questions in the SNOT-22 score is a possible explanation, no difference was observed when using the more specific ADSS. Another possible explanation may the timing of sampling. As most viruses tested in the assay are shed from the nasopharynx up to three weeks after symptom resolution it is possible that sampling occurred either during this time, or early in the infection prior to symptom development.

The viruses identified largely consistent with those seen in previous CRS studies, with the exception that this study did not identify MPV. The main viruses observed across all cohorts were RV and CoV, with influenza featuring strongly in the CRS group. However, it seems likely there is no one virus with a particular contribution to CRS not also seen in the general population. The size of the virus-positive cohort limited sub-analysis of viral species/strains with regard to disease severity. As such we were unable to determine whether one particular viral species is associated with worse disease.

Seasonality is an important concern when sampling for respiratory viruses. These are known to be most prevalent in winter and the early part of spring, an observation supported by this

study. Importantly we also showed strong viral positivity in summer and autumn. This highlights a clear short fall of previous CRS virome studies that limited sampling to winter and spring.

The mechanisms underlying the viral contribution to CRS are unknown. Similarly unknown is whether the higher rates of viral infection here observed are a cause or a consequence of CRS. CRS has been well established as a bacterial disease, encompassing bacterial overgrowth, SAg and biofilm formation, and disruption of the microbiome. [16] The link between the bacterial and viral hypotheses of CRS aetiopathogenesis may lie in the ability of viruses to prime the airways for bacterial infection. Viruses damage the epithelial barrier by increasing mucus production, reducing ciliary presence and reducing tight junction expression. [118-120, 122, 159, 160] Viruses bind directly to bacteria and upregulate host cell surface molecules to facilitate bacterial-host adherence. [126, 131, 132] Viruses hamper the innate immune system with effects on neutrophil and macrophage recruitment and impairment of natural killer, antigen presenting and T-cell activity, leaving the mucosa at risk for bacterial invasion. [135-137] Viruses alter temperature and variably exhaust or increase the availability of micronutrients, which can allow bacteria in planktonic and biofilm form to proliferate. [150, 153] The general population, however, is subject to a near-constant onslaught from respiratory viruses but only 16% of individuals develop CRS. The link between these two entities may be related to the IFNs and their signalling pathways. These cytokines are expressed by almost all cells in response to pathogen invasion and play key roles in innate antiviral immunity. [239] Deficient IFN responses to viral infection have been consistently demonstrated in asthma, a similar disease process to CRS, as well as in CRS itself. [47, 63, 240] We hypothesise this lack of early antiviral activity in CRS patients may result in more frequent, severe or persistent viral infections, paving the way for the bacterial

invasion so critical for disease development. Further research, however, is required to clarify this hypothesis.

Our results confirm a long-held suspicion that viruses are more common in CRS than in the general population. Viral presence is associated with more severe sinus disease measured by LMS and LKS. This has the potential to lead to exciting new developments in viral prophylaxis and anti-viral therapy in the prevention and possible treatment of CRS.

Association between viral infection and increased mucosal eosinophils and $CD8^+CD103^+$ T cells in chronic rhinosinusitis.

Statement of authorship

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Published as a letter to the editor; format modified for the purposes of this thesis.

This paper reports on original research 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.

As the principal author RKG contributed to study design, sample collection and processing, data analysis, statistical analysis and writing of the manuscript (70%). CAB contributed to sample collection (2.5%). MR and KF contributed to sample processing (2.5% each). SB contributed to sample processing and review of the manuscript (7.5%). PJW and AJP contributed to study design, sample collection and review of the manuscript (5% each). SV contributed to study design and review of the manuscript (5%).

All authors certify the author contribution statement detailed above is accurate, and that the sum of co-author contributions is equal to 100% less the candidate's stated contribution. All authors give permission for this manuscript to be reproduced in this thesis.

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4.1 Introduction

CRSwNP patients frequently present with extensive eosinophilic inflammation, severe symptom scores and disease recalcitrance. Different immune cell types and their secreted products are associated with tissue eosinophilia [199], however the initial insult causing the recruitment of these cells in CRS remains unclear. $CD8^+$ T cells have been found to be enriched in CRS patients in association with eosinophilic disease. [205] In response to alloantigen encounter at mucosal surfaces, $CD8^+$ T cells can be induced to express CD103. [207] CD103 expression is required for eosinophil recruitment in the context of allergic airway inflammation, and is associated with airway hyper-responsiveness. [205] Eosinophils are also recruited into the airways in response to viral infection to support innate host defence, while also contributing to the pathophysiology of disease [201]. $CD8^+$ $CD103^+$ T cells, also known as tissue-resident memory T cells (Trm cells) are thought to have a major role in viral immunity at mucosal surfaces. [241-243]

As yet $CD103^+$ cell subsets, including $CD8^+$ $CD103^+$ Trm cells, and their relationship to tissue eosinophilia and the presence of virus has not been investigated in CRS. In a recent study, we analysed the sinonasal virome in 288 CRS and control patients showing that viruses were more prevalent in CRS and were associated with significantly worse disease severity scores. [244] The present study utilised tissue samples from virus positive and virus negative CRS and control patients to investigate the number of eosinophils, $CD8^+$, $CD103^+$ and $CD8^+$ $CD103^+$ Trm cells in relation to viral infection. We hypothesised that these cell types would be more abundant in virus positive CRS.

4.2 Methods

Viral sampling was undertaken from consented patients using mucosal cytology brushings of the left and right middle and inferior meatuses under endoscopic visualisation at the time of endoscopic sinus surgery. DNA/RNA extracts underwent PCR/RT-PCR testing for a panel of common respiratory viruses (AdV, BoV, CoV, EnV, influenza, MPV, PIV, RSV and RV). Mucosal samples were taken at the same time as the aforementioned swabs and processed to undergo Haematoxylin and Eosin (H and E) or immunofluorescence staining for eosinophils and $CD8^+$ and $CD103^+$ cells respectively (the differing methodology resulting in the two study arms).

4.2.1 Study participants

Patients were recruited from the tertiary rhinologic practices of two of the senior authors (PJW and AJP). This study was carried out in accordance with the recommendations of the Central Adelaide Local Health Network Ethics Committee, and the protocol was approved by the same (HREC/15/TQEH/132). Subjects gave written informed consent in accordance with the Declaration of Helsinki. Patients were included in this study if they were older than eighteen years of age and were undergoing endoscopic surgery. No patients in this study suffered from asthma or aspirin-exacerbated respiratory disease, none were smokers, none were on oral or topical steroids in the two weeks before the sample collection day, and none reported symptoms of viral illness in the two weeks before the sample collection day. Control patients did not have any clinical or radiologic evidence of CRS. CRS patients fulfilled the diagnostic criteria for CRS as outlined in the American guidelines. [245]

4.2.2 Viral sampling, processing and analysis

Viral sampling, processing and analysis was undertaken using a previously published departmental protocol [238]. Briefly, endoscan cytology brushes (McFarlane Medical, Melbourne, Australia) were used to sample the left and right MM and IM mucosa with an aseptic technique and under endoscopic visualisation. Samples were transported on ice and stored at -80°C. Samples were thawed for processing and underwent RNA and DNA extraction using an AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany). These were again stored until real-time PCR batch testing for AdV, BoV, CoV, EnV, influenza, MPV, PIV, RSV and RV. An initial ERV3 assay was undertaken in the DNA extract fractions prior to assays for the disease-causing viruses to ensure adequate sample quality, and to ensure detection of active, intra-cellular viral DNA/RNA. Specifics regarding PCR target genes, primer and probe sequences and cycling conditions have also been detailed previously. [238] Viral detection was defined as a Ct of forty or less.

4.2.3 Mucosal sampling, processing and analysis

Mucosal tissue was harvested by sharp dissection with an aseptic technique as a routine part of patients' endoscopic sinus surgery. Bony elements were removed from the tissue after which it was placed in transport medium (Dulbecco's Modified Eagle Medium, Gibco by ThermoFisher, Waltham, USA) on ice. Once in the laboratory it was divided into small pieces and placed in 10% neutral buffered formalin for at least 24 hours, after which it was embedded in paraffin. These samples were cut into 5μm-thick slices, mounted on histological slides, stained with H and E and scanned using a NanoZoomer 2.0HT digital whole-slide imager (Hamamatsu, Japan). The images were then assessed by two blinded observers for

eosinophilia using previously described techniques. [246] In brief, images were scanned for areas of high cellular infiltration. Six areas of 0.035mm^2 were selected from the subepithelial layer from each slide, eosinophils were counted and a mean eosinophil count was determined for each patient.

A separate set of 5µm-thick slices were taken from a subset of the aforementioned paraffin blocks and mounted on histological slides for immunofluorescence microscopy. Some patients had no further tissue available and so were not able to be included in this arm of the study. The slides were de-paraffinised by sequential immersion in solutions of xylene, 100% ethanol, 90% ethanol and PBS. Antigen retrieval was performed using a pressure-cooker method with the slides immersed in sodium citrate. Slides were washed with PBS and then blocked with serum-free blocker (Dako, Glostrup, Denmark) for 60 minutes at room temperature. CD8 and CD103 antibodies (clones CD/144B and EPR4166(2) respectively, both Abcam, Cambridge, UK) were diluted in 10% FCS TBST (1:5 dilution for the former and 1:250 for the latter). 50-100µL was added to each slide and incubated overnight at 4°C. Slides were washed twice in PBST in preparation for secondary antibody addition. These were anti-mouse IgG 647 and anti-rabbit IgG CY3, both diluted in 10% FCS TBST at a ratio of 1:200, and 50-100µL per slide. The slides were incubated for one hour at room temperature in the dark, and then washed twice with PBST. 200µg/mL of 4′,6-diamidino-2-phenylindole (DAPI; Sigma, Aldrich) was added to resolve nuclei, and allowed to incubate for ten minutes at room temperature in the dark. The slides were rinsed twice in PBS, after which 50-100µL of TrueBlack dye (Biotium, Fremont, USA, diluted 1:20 in 70% ethanol) was added to reduce autofluorescence. The slides were washed a final three times in PBS after which a drop of anti-fade mounting medium (Dako, Glostrup, Denmark) was added before cover-slipping. Samples were visualised by using a LSM700 confocal laser scanning

microscope (Zeiss Microscopy, Jena, Germany). Images were assessed for T cell infiltration using the aforementioned eosinophil methodology.

4.2.4 Statistical analysis

Statistics were performed using R statistical software (R Foundation for Statistical Computing, Vienna, Austria) and the scientific Python package (scipy). Mann Whitney tests were used to compare means of inflammatory cell counts between various study groups and subgroups, with False Discovery Rate corrections for multiple comparisons. Multivariate permutation-based ANOVA models (as implemented in the R package 'lmPerm') were used to investigate the effect of the clinical covariates (disease phenotype, viral status) as well as their interaction terms, on the various mean inflammatory cell counts. Spearman tests were used to investigate any correlation between the eosinophils and the two T cell types. Statistical significance was defined as a p-value of < 0.05.

4.3 Results

4.3.1 Patient characteristics

58 patients were recruited to the study: 6 controls, 33 CRSsNP and 19 CRSwNP. 30 were negative for any of the viruses tested (VN), and 28 were positive (VP). Patients in the different groups were matched for age, gender, disease phenotype and the season in which samples were taken (table 4.1). Eosinophils, $CD8^+$ and $CD103^+$ cell numbers were counted by two blinded observers; these are summarised in table 4.2. Viral species identified are summarised in table 4.3.

Study arm	Eosinophils		T lymphocytes	
Viral detection status	Virus-negative	Virus-positive	Virus-negative	Virus-positive
Diagnosis (control:CRSsNP:CRSwNP)	3:17:10	3:16:9	3:16:9	3:15:8
Mean age (years)	50.6	49.9	50.9	49.8
Sex (M:F)	19:11	19:9	17:11	17:9
Season (spring:summer:autumn:winter)	10:6:9:5	8:6:9:5	8:6:9:5	8:5:9:4

Table 4.1 Summary of patient demographics and characteristics for eosinophil and T lymphocyte analysis.

	Control		CRSsNP		CRSwNP	
	VN	**VP**	**VN**	**VP**	**VN**	**VP**
Mean eosinophil counts	0.28	1.94	0.20	1.76	1.02	4.65
Mean $CD8^+$ T cell counts	7.72	9.56	5.86	28.29	15.48	36.29
Mean $CD103^+$ T cell counts	5.69	11.69	4.96	24.80	12.94	31.70
Mean $CD103^+CD8^+$ Trm cell counts	4.25	6.89	3.08	20.47	9.86	23.13

Table 4.2 Mean cell counts. "VP" indicates virus-positive, "VN" indicates virus-negative.

Study arm	Eosinophils	T lymphocytes
Control	BoVx1, CoV x2	As for eosinophil arm
CRSsNP	BoV x2, CoV x4, influenza A x5, RV x5	BoV x2, CoV x4, influenza A x5, RV x4
CRSwNP	BoV x1, CoV x3, EnV x1, influenza A x1, RSV x1, RV x2	BoV x1, CoV x3, influenza A x1, RSV x1, RV x2

Table 4.3 Viral species identified.

4.3.2 Viral detection and analysis

Mean mucosal eosinophil, $CD8^+$ T cells, $CD103^+$ cells and $CD8^+CD103^+$ Trm cell counts were significantly greater in VP CRSsNP and VP CRSwNP than in their VN counterparts.

Eosinophils were significantly more abundant in VP CRSwNP than in VP controls. $CD8^+$ T cells, $CD103^+$ cells and $CD8^+CD103^+$ Trm cells were also significantly more abundant in VP CRSsNP and VP CRSwNP than in VP controls (figures 4.1 and 4.2).

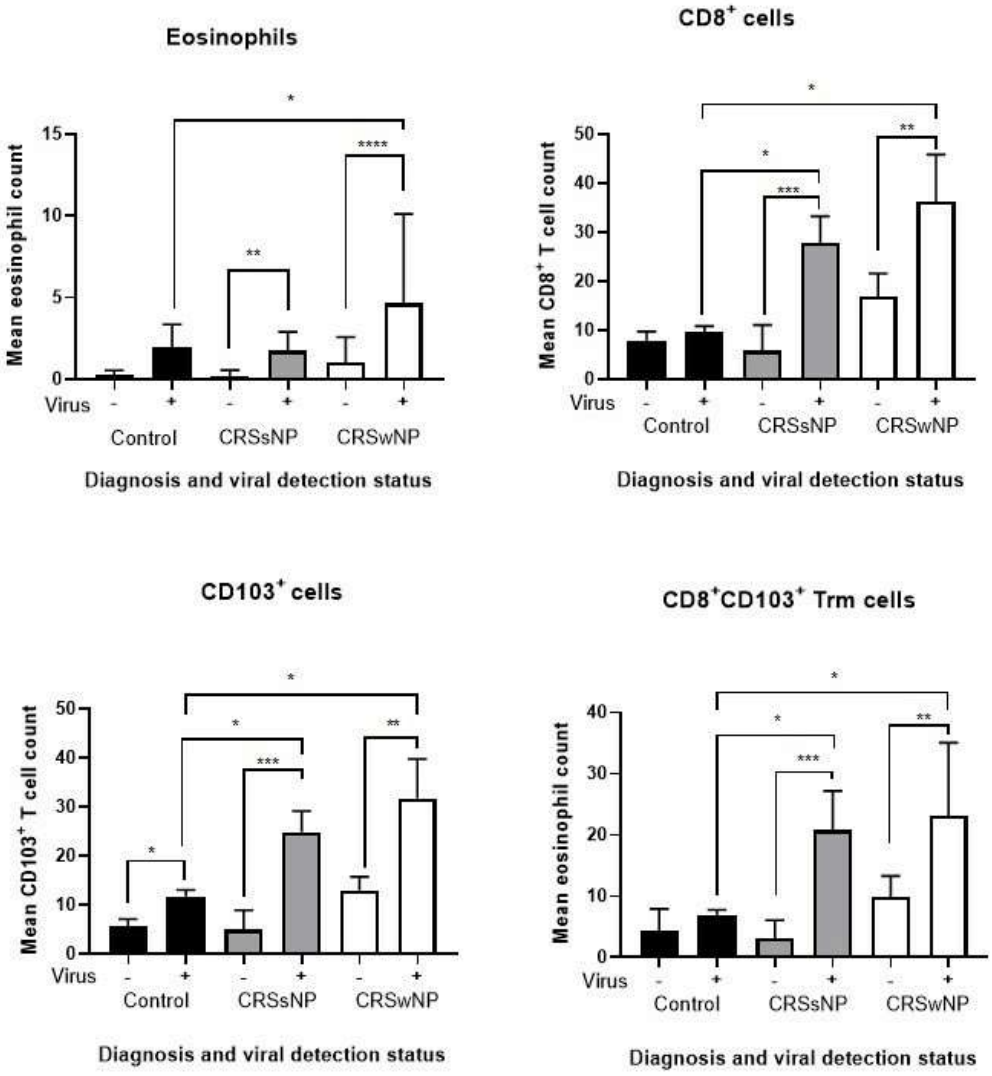

Figure 4.1 Mean cell counts of eosinophils (A), CD8+ cells (B), CD103+ cells (C) and CD8+ CD103+ Trm cells (D) in virus negative (-) and virus positive (+) controls (n = 6), CRSsNP (n = 17 top left, 16 elsewhere) and CRSwNP (n = 10 top left, 9 elsewhere) patients. * $p<0.05$, ** $p<0.005$, *** $p<0.0005$, **** $p<0.0001$

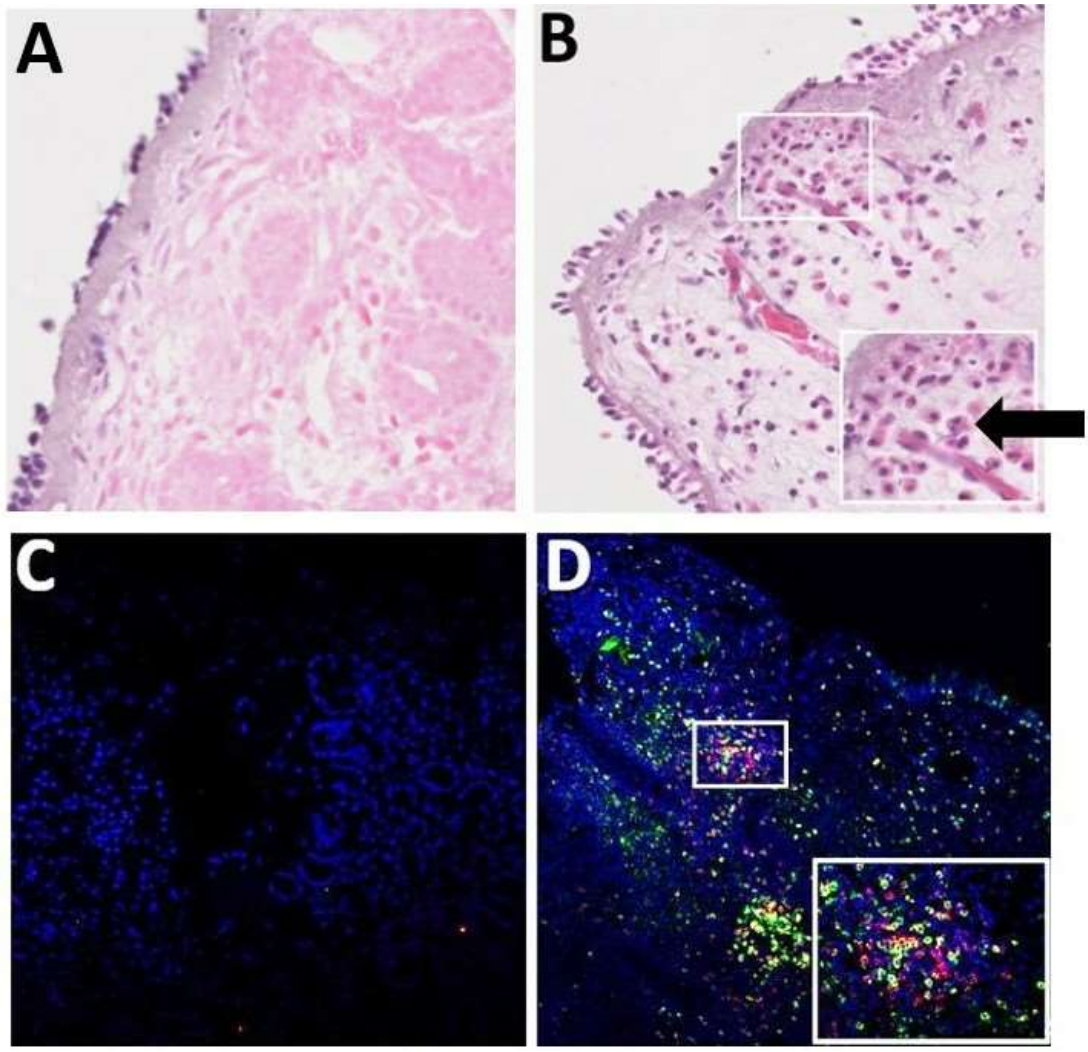

Figure 4.2 Sinonasal mucosa of representative virus negative control (A, C) and virus positive CRSwNP patient (B, D). (A, B) Haematoxylin and Eosin stained tissue with eosinophils evident in inset image B (arrow indicates eosinophil). (C, D) immunofluorescence staining $CD8^+$ T cells red, $CD103^+$ cells green and $CD8^+$ $CD103^+$ Trm cells yellow/orange. DAPI stains nuclei blue.

Bivariate permutation ANOVA models for each of the four cell types confirmed the significant association of disease phenotype and viral detection status with the outcome variables (inflammatory cell counts) in all four models. These models also showed a

significant interaction between disease phenotype and viral status in determining $CD8^+$, $CD103^+$ and $CD8^+CD103^+$ Trm cell counts (tables 4.4 to 4.7).

Poisson model: Eosinophil average ~ factor (phenotype) + factor (virus)			
Term	Coefficient (β)	Standard Error	P value
Intercept	-1.1125	0.4643	0.0166
Factor (phenotype) CRSsNP	-0.1320	0.4288	0.7583
Factor (phenotype) CRSwNP	0.9396	0.4142	0.0233
Factor (virus) 1	1.7509	0.3006	< 0.0001
• Null deviance: 141.491 on 53 degrees of freedom • Residual deviance: 72.907 on 50 degrees of freedom			

Table 4.4 Poisson model of eosinophil average.

Poisson model: $CD8^+$ T cell average ~ factor (phenotype) + factor (virus)			
Term	Coefficient (β)	Standard Error	P value
Intercept	1.23775	0.15030	< 0.0001
Factor (phenotype) CRSsNP	0.87423	0.14521	< 0.0001
Factor (phenotype) CRSwNP	1.34350	0.14609	< 0.0001
Factor (virus) 1	1.37696	0.06983	< 0.0001
• Null deviance: 826.09 on 53 degrees of freedom • Residual deviance: 216.02 on 50 degrees of freedom			

Table 4.5 Poisson model of $CD8^+$ T cell average.

Poisson model: $CD103^+$ T cell average ~ factor (phenotype) + factor (virus)			
Term	Coefficient (β)	Standard Error	P value
Intercept	1.14435	0.15383	< 0.0001
Factor (phenotype) CRSsNP	0.73168	0.14677	< 0.0001
Factor (phenotype) CRSwNP	1.19785	0.14782	< 0.0001
Factor (virus) 1	1.48061	0.07755	< 0.0001
• Null deviance: 725.42 on 53 degrees of freedom • Residual deviance: 162.89 on 50 degrees of freedom			

Table 4.6 Poisson model of $CD103^+$ T cell average.

Poisson model: DP Trm cell average ~ factor (phenotype) + factor (virus)			
Term	Coefficient (β)	Standard Error	P value
Intercept	0.4693	0.2029	0.0207
Factor (phenotype) CRSsNP	1.0018	0.1934	< 0.0001
Factor (phenotype) CRSwNP	1.4286	0.1948	< 0.0001
Factor (virus) 1	1.6048	0.0952	< 0.0001
• Null deviance: 705.98 on 53 degrees of freedom • Residual deviance: 248.92 on 50 degrees of freedom			

Table 4.7 Poisson model of DP Trm cell average.

4.4 Discussion

This is the first study linking viral infection with tissue eosinophilia and accumulation of $CD8^+$ T cells, $CD103^+$ cells and $CD8^+CD103^+$ Trm cells in the context of CRS. Strikingly, all four cell-types were increased in VP CRS patients compared to VP controls and our ANOVA multivariate analysis indicated that both viral status and CRS diagnosis positively affected inflammatory cell counts. The significant interaction terms in these models indicate that CRS patients might react to viral infection by inciting a more exacerbated immune response compared to controls and that viral infection might contribute to inflammatory cell infiltration in a subset of CRS patients. Both allergens and viruses are known to induce tissue eosinophilia and recent studies indicate CD103 expression plays a critical role in these processes [244, 247]. In line with these findings, this study also shows significantly increased $CD103^+$ cell numbers in the presence of virus in CRS patients and controls. $CD8^+CD103^+$ Trm cells were also increased in VP CRS patients compared to VP controls and represented the majority of the total $CD103^+$ cell population. Unlike Trm cells in the lung, Trm cells in nasal mucosa have been shown to develop independently of local antigen recognition and can persist for extended periods potentially explaining their presence also in VN patients. [248] Apart from expression on $CD8^+$ T cell subpopulations, CD103 is also expressed on a subset of $CD4^+$ T cells, natural killer cells and dendritic cells. [249] These cells have been reported to play host-protective roles in the context of infection and detrimental roles in the context of chronic inflammation. [249] Our study has some limitations. The exact role of $CD103^+$ expressing cells including $CD8^+CD103^+$ Trm cells and their role in protection against viral infection and/or contribution to chronic inflammation in CRS is unknown and warrants further investigation. Also, the potential for causal relationships between viral infection of the sinuses and influx of eosinophils and $CD8^+$ $CD103^+$ Trm cells is not known. Thirdly, the

control group size in this study was small relative to the CRSsNP and CRSwNP groups; this is due to the rarity of viral positivity in control patients.

In conclusion, this study links viral infection in CRS with mucosal eosinophilia and accumulation of $CD8^+$ T cells, $CD103^+$ T cells and $CD8^+$ $CD103^+$ Trm cells, implicating viruses as possible inciting factors in the dysregulated immune response in CRS. More investigation is required to elucidate a causative relationship, should one exist.

Association between viral presence and changes in the bacterial microbiome in chronic rhinosinusitis

Statement of authorship

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This paper reports on original research 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.

As the principal author RKG contributed to study design, sample collection and processing, data analysis, statistical analysis and writing of the manuscript (70%). CAB contributed to sample collection (2.5%). CC contributed to sample processing (2.5%). AB contributed to statistical analysis (2.5%). SB contributed to sample processing and review of the manuscript (7.5%). SV contributed to study design and review of the manuscript (5%). PJW and AJP contributed to study design, sample collection and review of the manuscript (5% each).

All authors certify the author contribution statement detailed above is accurate, and that the sum of co-author contributions is equal to 100% less the candidate's stated contribution. All authors give permission for this manuscript to be reproduced in this thesis.

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5.1 Abstract

Background: Factors at play in the aetiopathogenesis of CRS have been postulated to include a bacterial dysbiosis and a recently established greater susceptibility to viral infection. There is potential for the presence of virus to influence the resident bacterial microbiome, thereby altering the course of the disease. The aim was to investigate virus-associated changes in the bacterial CRS sinonasal microbiome.

Methods: Brushings of the sinonasal mucosa were taken at time of endoscopic sinus surgery. Viral detection was undertaken using PCR or RT-PCR on extracted DNA and RNA. Bacterial characterisation was undertaken using 16S ribosomal RNA gene-targeted amplicon sequencing. Analysis of bacterial abundance, diversity and stability with regard to viral presence was undertaken using the Quantitative Insights Into Microbial Ecology (QIIME 2) platform.

Results: 82 adult patients were recruited for this study: 10 controls, 49 CRSsNP and 23 polyp CRSwNP patients. Half of each group was virus-positive. No significant differences were seen in relative abundances of the bacterial genera detected, their diversity or stability in any of the groups. A trend towards greater relative abundance of *Haemophilus* spp. was seen in patients reporting a viral illness two to four weeks prior, which was not apparent in patients reporting such between one and two months prior.

Conclusion: No significant differences in the composition of the bacterial microbiome in virus-negative or positive patients was seen. A trend was seen towards early microbiome shift in the context of recent viral infection, which may represent a nidus for superinfection in these patients.

5.2 Introduction

The bacterial microbiome of the aerodigestive tract and specifically the sinonasal passages is an area receiving increasing attention in current literature. This is of significant interest to research into CRS. This inflammation of the nose and paranasal sinuses of greater than twelve weeks duration is characterised by symptoms of anterior rhinorrhoea, PND, headaches, facial pain or pressure, nasal obstruction and hyposmia. Aetiopathogenesis theories are many but a significant focus remains on microbial imbalance, and methods of manipulating this for therapeutic ends.

A bacterial dysbiosis has been shown to exist within CRS with patients showing a lack of biodiversity compared to healthy controls. [186] Bacterial species historically seen as more pathogenic are found to be relatively more prevalent in CRS than bacterial species that have been considered commensal. [187] Studies investigating the CRS microbiome have varied in scale, methodology and analysis and so data have been difficult to compare. Efforts have been made to standardise these, concluding that the healthy sinonasal microbiome consists mainly of *Staphylococcus*, *Propionibacterium*, *Corynebacterium* and *Streptococcus* species while the microbiome in CRS shows a reduction in the relative abundance of Actinobacteria and *Propionibacterium* species, with significantly more prevalent *Corynebacterium* species. [188]

URT viruses have recently been shown to be more common in CRS than in healthy individuals. The presence of these viruses is also associated with more severe subjective and objective disease. [244] These findings potentially implicate viruses as an inciting and/or exacerbating factor in the immune dysregulation of CRS. Viral-bacterial co-infection is

known to have deleterious effects on epithelial barrier function, bacterial binding and innate and adaptive immunity. [114] It has been suggested that viruses may induce changes in the bacterial microbiome potentially causing more severe disease in the LRT. [191, 192] However, little is known about the specific changes in bacterial aerodigestive populations seen in the presence of viral infection in CRS.

The aim of this study was thus to investigate virus-associated changes in the bacterial CRS sinonasal microbiome, , hypothesising that viral infection would indeed alter the composition of resident bacteria.

5.3 Materials and methods

5.3.1 Study participants

Study participants were recruited from the tertiary rhinologic practices of two of the senior authors (PJW and AJP). This study was carried out in accordance with the recommendations of the Central Adelaide Local Health Network Ethics Committee, with their approval of the protocol (HREC/15/TQEH/132). In accordance with the Declaration of Helsinki study participants gave written informed consent. Patients were included in this study if they were older than eighteen years of age and were undergoing endoscopic nasal surgery. Control patients did not have any clinical or radiologic evidence of CRS, and were undergoing trans-sphenoidal resections of pituitary masses or surgery to the septum or ITs. CRS patients fulfilled the diagnostic criteria for CRS as outlined in the American guidelines, and were undergoing FESS. [245] Patients who had used antibiotics or steroids in the two months prior to the study day were excluded. No patients in this study suffered from asthma or aspirin-exacerbated respiratory disease and none were smokers, On the study day patients were asked to report the timing of their last URTI with the following time points; current, within the last 1-2 weeks, within the last 2-4 weeks, within the last 1-2 months, or more than 2 months prior.

5.3.2 Viral sampling, processing and analysis

Viral sampling, processing and analysis was undertaken using a previously published departmental protocol [238]. Briefly, the left and right MM and IM mucosa was sampled using endoscan cytology brushes (McFarlane Medical, Melbourne, Australia). This was conducted with endoscopic visualisation and aseptic technique. Samples were transported on

ice and stored at -80°C. At time of processing samples were thawed for RNA and DNA extraction using an AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The products of these were stored and subsequently real-time PCR batch tested. An initial assay for ERV3 was undertaken in the DNA extract fractions to ensure adequate sample collection quality. Assays were then undertaken for AdV, BoV, CoV, EnV, influenza, MPV, PIV, RSV and RV. PCR target genes, primer and probe sequences, the nature of positive and negative controls and cycling conditions have been published previously. [238] A Ct of forty or less indicated viral detection.

5.3.3 Bacterial sampling and processing

Bacterial sampling was also undertaken intra-operatively with an aseptic technique and endoscopic visualisation. Guarded, flocked swabs (Copan Italia S.p.A, Brescia, Italy) were inserted into the MMs of all patients on both sides and rotated seven times before removal. Swabs were stored at -80°C until batch thawing for DNA extraction and analysis as follows. Swab heads were cut into small pieces and 180µL of enzymatic lysis buffer (Qiagen, Hilden, Germany) was added and left overnight at room temperature. 5mm steel beads agitated for 20 seconds at 15Hz in a Qiagen Tissue Lyser were used to homogenise the pieces, followed by 5 minutes of further homogenisation with 0.1mm glass beads at 30Hz. DNA extraction was then undertaken in accordance with the Qiagen DNeasy Blood and Tissue Kit protocol (Qiagen, Hilden, Germany), and stored at -80°C until sequencing.

5.3.4 Bacterial 16S-sequencing

PCR amplification and sequencing were performed by the Australian Genomics Research Facility. Gene libraries were generated by amplifying the V3 to V4 (341F–806R) hypervariable region of the 16S rRNA. AmpliTaq Gold 360 Master Mix (Life Technologies, Mulgrave, Australia) with primers CCTAYGGGRBGCASCAG in the forward sequence and GGACTACNNGGGTATCTAAT in the reverse sequence were used to generate PCR amplicons. These underwent fluorometric measurement (Invitrogen Picogreen; Thermo Fisher Scientific, Waltham, MA, USA) and normalised. Quantitative PCR (KAPA Biosystems, Cape Town, South Africa) was used to quantify the equimolar pool. This was arranged for sequencing on the Illumina MiSeq (Illumina Inc., San Diego, CA, USA) with 300 base paired end chemistry. All samples in this study were sequenced in one run.

5.3.5 Bioinformatic pipeline

QIIME 2 (version 2018.11) [250] was employed for the bioinformatic pipeline in this study. PEAR was used to combine forward and reverse reads [251] through the QIIME 2 plugin q2-pear (https://github.com/bassio/q2-pear). The QIIME 2 plugin q2-quality-filter [252] was used to quality-filter the combined sequences. Minimum quality parameter was 20 [253]. Deblur (q2-deblur plugin with setting “trim-size” = 435 with default parameters otherwise) was used for denoising and to form Amplicon Sequence Variant (ASV). [253] Greengenes 16S database (version 13.8 August 2013, the 99% clustered similarity sequences) was used as reference. [254] and the QIIME 2 BLAST-based q2-feature-classifier was used as taxonomy classifier [255].

Prior to analysis, n=400 reads were chosen as the rarefaction depth cut-off. Rarefaction plots (for total number of ASVs and for Shannon's alpha diversity index) were performed (figure 5.1). Relative abundance comparisons were done at the genus level. The taxonomic assignment of the one DNA-negative control sample containing extraction reagents only was explored. The bacterial genus *Flavobacterium* was present in high relative abundance in this sample and in relatively low abundance in many samples, so this genus was excluded before downstream statistical analyses. Mean relative abundance and genera prevalence were calculated. Alpha diversity was measured using Shannon's diversity and Faith's phylogenetic diversity index, [256] calculated using Sci-kit bio (version 0.5.3).

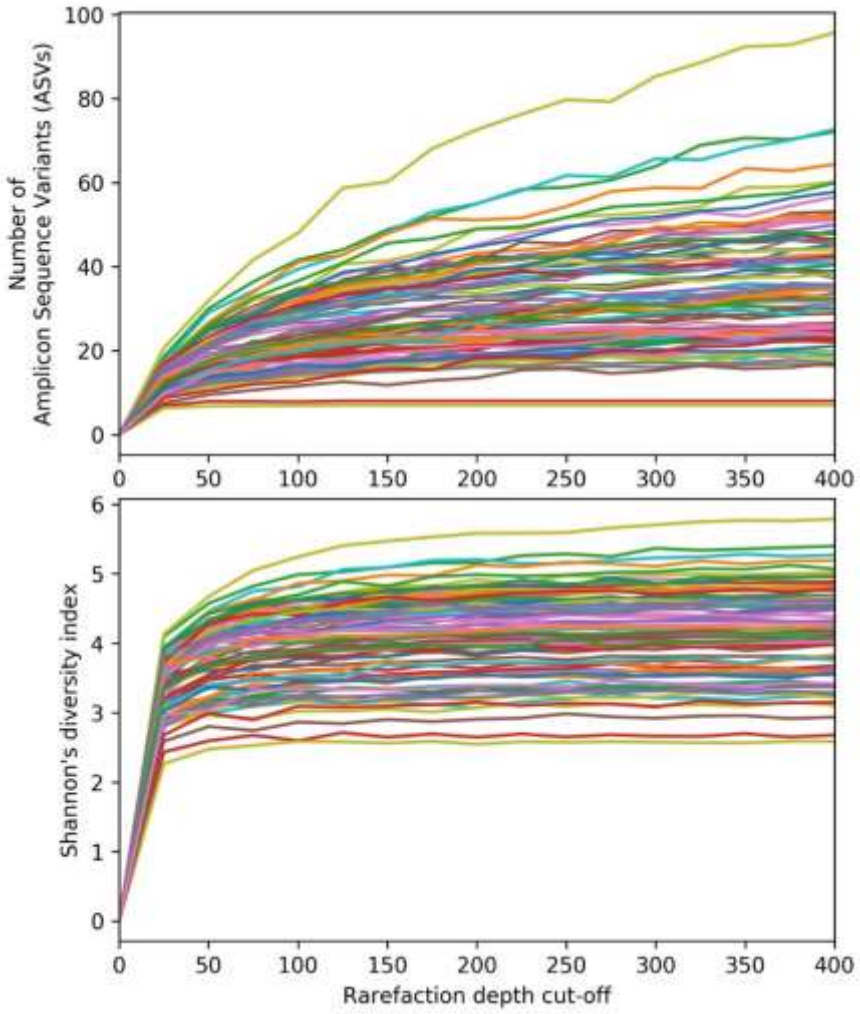

Figure 5.1 Rarefaction plots

Rank variability is a per-sample index, and a surrogate for microbiome stability. It is defined by Martí et al. as “the absolute difference between each taxon rank and the overall rank”. [257] Rank variability was calculated using a Python implementation of the equations previously described. [257]

5.4 Results

5.4.1 Patient characteristics

82 patients were recruited: 10 controls, 49 CRSsNP and 23 CRSwNP. 41/82 patients were virus-positive: 5 control, 24 CRSsNP and 12 CRSwNP. Virus-positive and virus-negative patients were age and season-matched within the three groups (control, CRSsNP and CRSwNP). Demographics and patient characteristics are summarised in table 5.1.

	Control	**CRSsNP**	**CRSwNP**
Number with each diagnosis	10	49	23
Number of virus-positive patients	5	24	12
Mean age (years)	35.5	50.0	45.0
Male: female	4:6	27:22	22:1
Season sample obtained (spring:summer:autumn:winter)	2:2:2:4	20:8:12:9	4:4:8:7

Table 5.1 Summary of patient demographics and characteristics.

5.4.2 Viral detection

ERV3 was detected in all samples. Mean ERV3 Ct was 22.9, indicating that adequate cellular material was obtained in all cases. 41 patients were positive for one or more of the disease-causing viruses assayed, while 41 were virus-negative. RV was the most prevalent of the species assayed; MPV was not detected in any of the samples. Fourteen patients were

positive for more than one viral species. Details of viral species detected are summarised in table 5.2.

Viral species	**Number of positive control patients**	**Number of positive CRSsNP patients**	**Number of positive CRSwNP patients**
Adenovirus	1	0	0
Bocavirus	1	2	1
Coronavirus	4	6	5
Enterovirus	1	1	1
Influenza	0	6	1
Parainfluenza	0	1	2
Rhinovirus	1	13	1
RSV	0	0	1

Table 5.2 Details of viral species and patient diagnoses.

5.4.3 Bacterial microbiome outcomes: taxonomy

The most abundant taxa are found in table 5.3. The genera *Corynebacterium*, *Staphylococcus*, *Streptococcus*, *Haemophilus*, and *Moraxella* were the most abundant. We found no significant difference in differential abundance of the five most abundant genera by diagnosis (i.e. control versus CRSsNP versus CRSwNP; Kruskal-Wallis $p > 0.05$, figure 5.2).

	Control				CRSsNP				CRSwNP			
	Virus-pos		Virus-neg		Virus-pos		Virus-neg		Virus-pos		Virus-neg	
	MRA	**P**	**MRA**	**P**	**MRA**	**P**	**MRA**	**P**	**MRA**	**P**	**MRA**	**P**
Corynebacterium	26.00	80.0	44.85	100.0	33.39	58.33	31.71	60.87	23.75	66.67	48.05	58.33
Staphylococcus	47.06	80.0	33.05	100.0	22.45	58.33	24.49	69.57	26.85	66.67	23.55	50.00
Streptococcus	9.12	20.0	0.00	0.0	4.86	12.50	9.94	26.09	0.00	0.00	3.62	16.67
Haemophilus	0.00	0.0	0.00	0.0	10.55	16.67	3.50	8.70	5.42	8.33	0.00	0.00
Moraxella	0.94	20.0	4.35	40.0	7.18	16.67	0.00	0.00	0.00	0.00	3.68	8.33
Proteus	0.00	0.0	0.00	0.0	0.00	0.00	0.00	0.00	8.38	8.33	10.00	8.33
Porphyromonas	0.00	0.0	0.00	0.0	6.11	8.33	0.00	0.00	3.60	8.33	0.00	0.00
Genus unidentified (Enterobacteriaceae)	1.25	20.0	0.00	0.0	3.11	12.50	4.84	4.35	0.00	0.00	0.00	0.00
Alloiococcus	0.00	0.0	0.90	40.0	0.00	0.00	2.96	4.35	4.20	8.33	0.95	8.33
Genus unidentified (Cytophagaceae)	0.00	0.0	5.45	40.0	1.68	25.00	1.87	17.39	0.00	0.00	0.00	0.00

Table 5.3 Summary of most abundant bacterial genera. “MRA” indicates mean relative abundance, “P” indicates prevalence (both expressed as percentages).

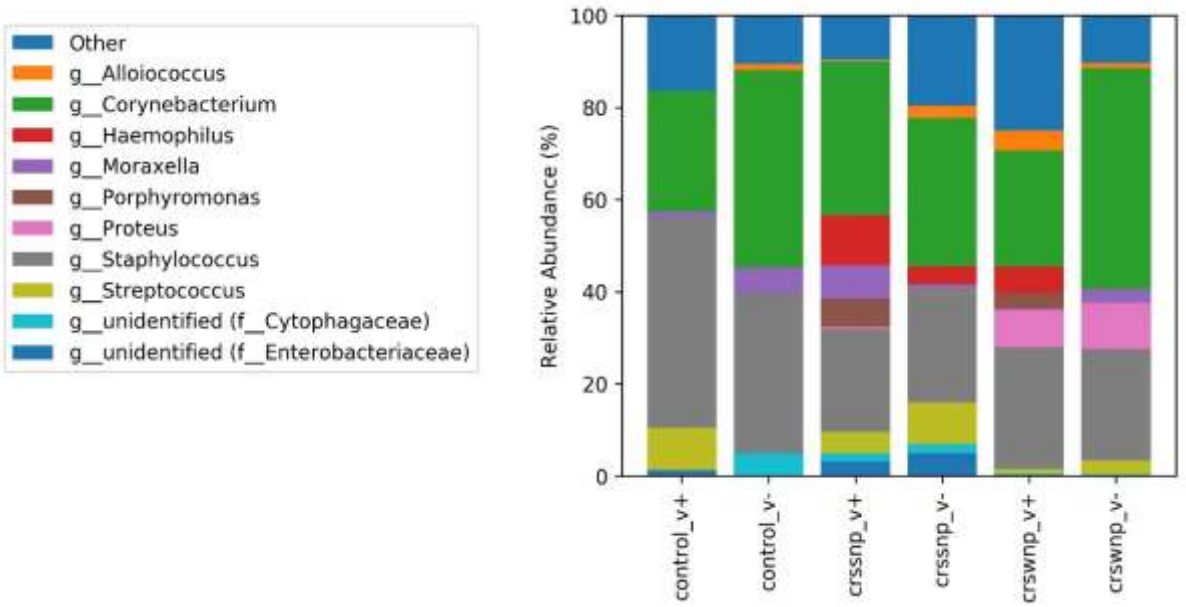

Figure 5.2 Relative abundances of the most abundant bacterial genera. "v+" indicates viral positivity, "v-" indicates viral negativity.

5.4.4 Viral covariates as predictors of bacterial taxa abundances

We found no significant association between viral presence or number of viruses detected and the abundances of the aforementioned most abundant bacterial genera (Kruskal-Wallis, $p > 0.05$). Moreover there was no statistically significant association detected between the presence of CoV, influenza or RV and bacterial relative abundances.

The above associations were again tested in a further subgroup analysis, with separate analyses for control, CRSsNP, and CRSwNP groups. This yielded no significant associations in any of the three patient groups ($p > 0.05$). However, a trend towards a shift in bacterial microbiome composition manifest as a greater relative abundance of *Haemophilus* spp. was seen in patients reporting a viral URTI two to four weeks prior to the study day (figure 5.3).

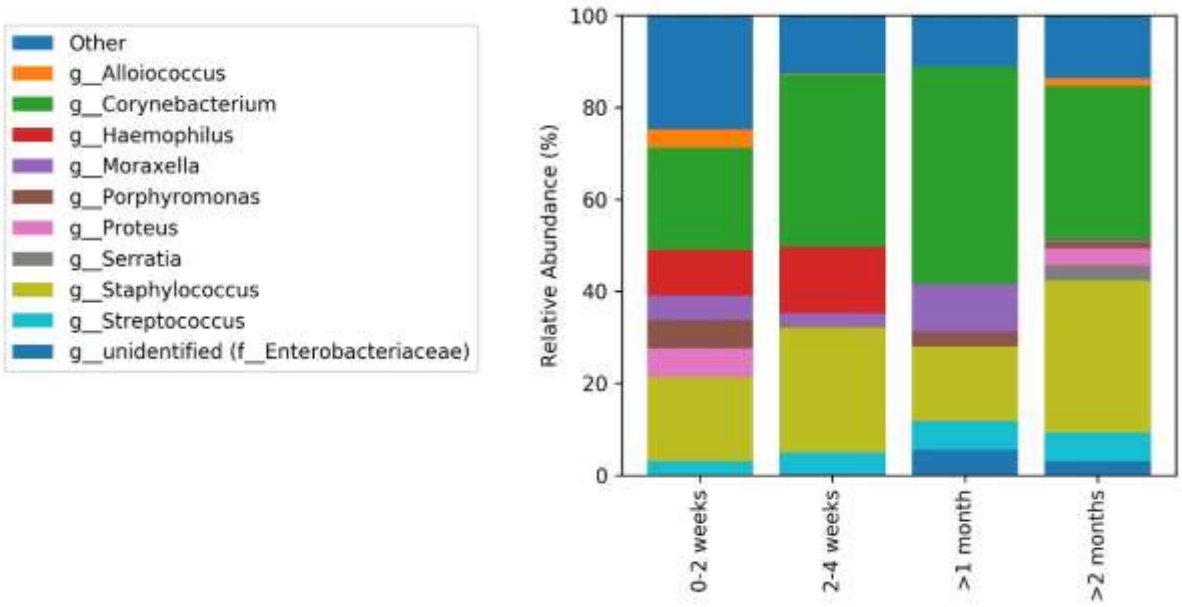

Figure 5.3 Relative abundances of the most abundant bacteria grouped by time since last patient-reported viral infection.

5.4.5 Viral covariates as predictors of bacterial diversity and stability

We calculated Faith's and Shannon's indices as markers of both phylogenetic and non-phylogenetic alpha diversity. We also calculated rank variability; a per-sample surrogate for microbiome stability as mentioned earlier. We found no significant association between bacterial genera abundances and viral status covariates such as viral presence, number of viruses, and presence of specific viruses (influenza, CoV or RV). ($p > 0.05$)

5.5 Discussion

This study seeks to compare the bacterial microbiome seen in virus-positive and virus-negative individuals both with and without CRS. The most prevalent viruses seen in all groups were CoV, influenza and RV. The most abundant bacterial genera seen were *Corynebacterium, Staphylococcus, Streptococcus, Haemophilus,* and *Moraxella*. We observed a trend towards changes in the microbiome in patients reporting a viral infection less than a month prior to the study date. This trend was not seen in patients reporting their last viral infection more than a month prior. This may indicate an early shift in the microbiome associated with viral infection, which may then normalise after a month. Despite a lack of significance in this small sample size, this may indicate a tendency towards superinfection and worsening of disease in the CRS population. Greater investigation with a larger sample size and repeat sampling over time is required to elucidate this further. A longitudinal, prospective model would be required in order to confirm initial viral infection; our findings were based purely on patient-suspected pathology.

No significant differences were seen in bacterial abundance, diversity or stability between virus-negative or virus-positive individuals within the control, CRSsNP or CRSwNP groups with regard to the other viral covariates tested (presence or absence of virus, number of viruses or presence of specific viruses). This is in contrast to previously reported effects of viral presence on the microbiome in non-CRS populations [258-260]. Our study focuses on CRS patients, is smaller and geographically different to these studies, but its strengths lie in a robust and previously validated viral collection method, [238] an undertaking of more in-depth analysis beyond viral presence or absence alone, and the use of age and season-matched virus-negative control groups. Ding *et al.* compared swabs from control and

influenza-infected individuals (sample size of 40 versus 215). They found *Corynebacterium* and *Streptococcus* to be more abundant in controls, with virus-positive swabs dominated by *Moraxella* and *Dolosigranulum*. [260] This result is interesting not least due to the rarity of identification of *Dolosigranulum* in adult CRS URT bacterial profiles; the low biomass of airway samples carries risk of contamination if not carefully screened prior to and following analysis. Borges *et al*. compared swabs from twelve patients with severe acute respiratory infections: six with influenza, and six with undisclosed non-influenza causative viral organisms. Despite their small sample size they found significant differences in abundance of fifteen different bacterial genera, however no control group was presented. [259] Rosas-Salazar *et al*. compared the infantile microbiome in the presence of either RV or RSV, and found significant differences in eleven genera. Again, no control group was presented, and in all three of the aforementioned studies viral collection methods were prone to contamination. No method to ensure cellular collection was employed, and as such even viral presence cannot be confidently asserted in these studies.

The negative effects of a viral/bacterial co-infection have long been established in many body systems, but of significant interest in any such co-infection model is the nature of the original inciting pathogen. This is a cross-sectional study investigating the characteristics of sinonasal microbiota at a solitary time point (that of endoscopic sinonasal surgery). Should differences in these microbiota have been observed between control and CRS patients, we would be unable to determine whether it is the composition of the bacterial microbiome that predisposes to viral infection, or vice versa. To elicit such information a longitudinal study model would be required. To our knowledge this has not yet been undertaken in these cohorts. An additional limitation is a lack of gender balance in the CRSwNP patient sample, and the known differing immune response profile of CRSsNP and CRSwNP patients.

CRSsNP is associated with a Th-1 skewed response, while CRSwNP is associated with a Th-2 skewed, eosinophilic response. [34, 35] Viral infections themselves are also associated with eosinophilia. [247] These factors have the potential to impact the detection of virus and/or the microbiome balance observed in this study. [261]

Of additional interest but requiring further research would be whether there is indeed correlation between patient reporting of a current viral infection, and the presence of virus in the sinonasal passages at that time. Only three patients in this study reported such, limiting any analyses thereof. An additional limitation is that the symptoms of CRS and of a viral URTI are indeed similar but with a marked difference in duration.

In conclusion this study compares the bacterial microbiome in virus negative and virus positive controls, CRSsNP and CRSwNP, and has uncovered no significant differences in its composition. However, a trend towards early microbiome shift in patients who report a recent viral infection has been uncovered. Larger, longitudinal investigation is required to investigate this further.

Thesis synopsis and future directions

This thesis provides novel insights into the role of viruses in CRS. We have first established a robust, reproducible and validated collection method for sinonasal viruses. We then finally established that viruses are indeed more common in CRS than in the general population, and that their presence is associated with more severe disease. We showed that virus-positive CRS is associated with eosinophil and $CD8^+$, $CD103^+$ and double-positive T cell presence in excess of that seen in virus-positive controls. This implicates viruses as a possible inciting factor in the dysregulated innate and adaptive immune responses seen in CRS. Our final study uncovers a trend towards early bacterial microbiome shift in the context of recent viral infection, which may represent a nidus for superinfection contributing to the development of CRS.

The research presented in this work is but a gateway to potential further investigation. We live in an era of increasing antibiotic resistance with emergence of novel pathogens (both viral and bacterial) and widely accessible long-distance travel enabling pandemics. This work was undertaken, and indeed the last sentence written, prior to the CoVID-19 outbreak; never has this been so clearly demonstrated. As such, intimate knowledge of both healthy and disease-inducing microbes, and the interplay amongst these, is essential to rationalise targets and develop new prevention and therapeutic strategies. Not least of these are vaccines and early antiviral therapy for those at risk. An exciting area in need of more research is the role of interferons in the post-viral immune cascade. The development of small-molecule modulators is still in its infancy but shows promise in malignancy, and as such these could be promising as anti-viral pharmacological agents in CRS. Information is also lacking with

regards the response of a previously bacterially infected or colonised airway to secondary viral infection, and the contribution of fungus to this and airway immune functions. It is also very difficult to simulate the constantly-barraged, highly unsterile human respiratory environment; no viral or bacterial infection ever takes hold without the simultaneous presence of multiple other colonisers and/or pathogens. Greater knowledge of the healthy and diseased human respiratory microbiomes will allow more accurate investigation of the interplay amongst viruses, bacteria, fungi, bacteriophage and protozoa.

More research is needed to elucidate the cause-consequence relationship of CRS and viruses. The mechanism behind increased rate of viral infections in CRS also remains to be seen. In this thesis we postulate this may be related to defective IFN responses to viral infection in CRS populations. Characterisation of these responses is needed; this may lead to manipulation of IFN in CRS as a therapeutic strategy in a disease that remains so very unpleasant, highly prevalent and costly.

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