Host-Virus Interactions in Asthma and Chronic Obstructive Pulmonary Disease

Christopher McCrae

Department of Internal Medicine
and Clinical Nutrition,
Institute of Medicine,
Sahlgrenska Academy,
University of Gothenburg

UNIVERSITY OF
GOTHENBURG

Cover illustration: rhinovirus 2-infected primary bronchial epithelial cells, stained with CellMask (blue), DAPI (red) and an anti-PI4P lipid antibody (green). Image produced by Douglas Ross-Thriepland.

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christopher.mccrae@gu.se

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To Clare, Fraser and Anna

Abstract

Asthma and chronic obstructive pulmonary disease (COPD) are associated with periods of worsened symptoms, known as exacerbations. Severe exacerbations can result in hospitalisation, irreversible decline of the disease and sometimes death. Thus, exacerbations are a major cause of morbidity, mortality and healthcare cost. Treatment or prevention of exacerbations is an area of unmet medical need as the current standard of care has insufficient impact on exacerbation frequency and severity.

Respiratory viral infections are hypothesized to be important triggers of exacerbations. It has been shown that 41-95% of asthma exacerbations and 22-57% of COPD exacerbations are associated with a respiratory virus infection, the most common agent being human rhinovirus (RV). Other viruses frequently associated with exacerbations include respiratory syncytial virus and influenza. Prevention or attenuation of respiratory virus infections could therefore have significant impact on exacerbation frequency and severity.

The mechanisms by which viruses trigger exacerbations are poorly understood, although there is evidence for defective anti-viral interferon (IFN) responses in cells from patients with asthma and COPD. Further investigation of host-virus interactions and their impact on underlying airway disease, may lead to novel therapeutic targets for the prevention of exacerbations.

We investigated host-virus interactions in asthma and COPD through a multi-faceted approach. First, we performed an *in vitro* functional genomics screen using RNA interference (RNAi), to identify targets that are essential for RV replication in primary normal human bronchial epithelial cells. Second, we evaluated the efficacy of inhaled IFNβ-1a for the prevention of severe asthma exacerbations in a Phase 2 trial. Finally, we performed an observational, longitudinal study in COPD patients to investigate the relationship between exacerbations, viral and bacterial infections, air pollution and anti-microbial peptides (AMPs).

In the first study, we identified lanosterol synthase (LSS) as a potential therapeutic target which, when inhibited, blocks RV replication and enhances the RV-induced IFNβ response. We discovered that the mechanism

of this effect was related to the induction of a regulatory sterol, 24(S),25 epoxycholesterol.

In our phase 2 trial (INEXAS), we found that inhaled IFNβ-1a did not prevent the occurrence of severe asthma exacerbations, but improved peak expiratory flow (PEF). In an exploratory analysis, we also identified potential responder subgroups, based on blood eosinophil counts or serum interleukin (IL)-18 levels.

In our COPD cohort, we found that both viral infections and increases in ambient air pollution were associated with exacerbations. Viral exacerbations were strongly associated with upregulation of the IFN response biomarkers, CXCL10, CXCL11 and IFNγ. We went on to discover that the levels of beta-defensin 2 (hBD-2), an AMP expressed by the lung epithelium, is reduced in the sputum of patients who experienced exacerbations, and further found an association between low hBD-2 levels at exacerbation and the presence of a respiratory virus.

The studies presented in this thesis have identified and evaluated key components of host-virus interactions and applied those to the context of asthma and COPD. In all cases, we found the IFN response to be central, not only to the events that occur inside the virus-infected cell, but also to the downstream consequences of infection at the tissue and organ level, likely playing a key role in both anti-bacterial and anti-viral host defense. Despite the extraordinary complexity of the interaction between the virus and its host, this thesis demonstrates that key drivers of this interplay can be identified, manipulated and, hopefully, developed into future medicines for the prevention of asthma and COPD exacerbations.

Keywords

Asthma, COPD, exacerbation, virus, interferon

Sammanfattning på svenska

Hundratals miljoner människor världen över lider av kronisk lungsjukdom så som astma och kronisk obstruktiv lungsjukdom (KOL). Vid både astma och KOL förekommer episoder då man blir försämrad i sin sjukdom, så kallade exacerbationer. Allvarliga exacerbationer leder oftast till sjukvårdsvistelse, försämrad lungfunktion och kan i värsta fall leda till döden. Detta innebär ett lidande för individen och är också mycket kostsamt för sjukvården och samhället. Dessvärre fungerar inte dagens astma och KOL-läkemedel särskilt bra vid just försämrings-episoder. Därför behövs det förbättrad behandling samt förebyggande åtgärder för att minska både frekvensen av exacerbationer och dess svårighetsgrad.
Förkylningsvirus ligger bakom de flesta allvarliga försämringar hos individer med astma och KOL och det kan rent av vara farligt att bli förkyld vid astma och KOL-sjukdom. Studier har visat att 41-95% av astma och 22-57% av KOL exacerbationer är förknippade med luftvägsvirus. Rhinovirus är det vanligaste förekommande förkylningsviruset och det virus som oftast färorsakar försämringar vid astmasjukdom hos både vuxna och barn. Exacerbationer kan även orsakas av influensavirus och respiratoriskt syncytialvirus (RSV). Att förebygga graden av dessa virusinfektioner är viktiga för att minimera antalet exacerbationer samt svårighetsgraden av dessa.
Virus är de minsta biologiska partiklar som kan infektera levande organismer. Virus sprids oftast via kroppsvätskor, direkt kontakt eller via luften. Virus kan inte förflytta sig av egen kraft utan är beroende av en värdcell för att kunna spridas och infektera en levande organism. Hur virus triggar igång immunförsvaret i luftvägarna som leder till astmaförsämring och/eller KOL-försämring är i stort sett outforskat. Dock vet man att astmatiker och KOL patienter har ett bristfälligt försvar mot virus i luftvägsepitelet. Detta beror bland annat på att luftvägsepitelet hos astmatiker och KOL-patienter producerar lägre nivåer av interferoner (IFN), vilket i sin tur leder till ett sämre immunförsvar mot virus och därmed ökad virus mängd.
I denna avhandling har vi studerat virus-värdcell interaktioner för att få en ökad förståelse för vad som sker i luftvägarna hos astmatiker och KOL-patienter vid virusinfektion. Detta är ett viktigt forskningsområde då en ökad förståelse om bakomliggande mekanismer vid virus-triggad astma

och KOL-försämring kan leda till läkemedelsutveckling som förhindrar eller bromsar frekvensen exacerbationer och/eller dess svårighetsgrad orsakade av virus.
I den första studien använde vi oss av en så kallad screening assay för att identifiera potentiella målproteiner som är viktiga för tillväxten av rhinovirus (förkylningsvirus) i epitelceller från luftvägarna. I den andra delstudien använde vi oss av läkemedelsstudien INEXAS där vi undersökte den preventiva effekten av inhalerat IFN-β1a vid astma exacerbationer av svår karaktär. Slutligen genomförde vi en observationsstudie över tid som inkluderade KOL-patienter. Här undersökte vi sambandet mellan exacerbationer, virus och bakterie-infektioner, luftföroreningar samt anti-mikrobiella peptider.
I det första arbetet i avhandlingen (paper I) fann vi att enzymet lanosterolsyntas (LSS) som har betydelse i kolesterolbiosyntesen reglerar tillväxten av rhinovirus (förkylningsvirus) i epitelceller från luftvägarna. När vi använde en substans som inhiberar LSS fann vi att tillväxten av virus minskade i epitelcellerna och att det anti-virala proteinet IFN-β ökade.
I avhandlingens andra arbete (paper II) fann vi att astmatiker som behandlades med IFN-β1a inhalationer förbättrade sitt peak expiratory flow (PEF)-värde. Denna behandling hade dock ingen effekt gällande frekvensen av svåra exacerbationer. När vi gick vidare och gjorde en mer explorativ studie fann vi att de astmatiker som svarade bäst på IFN-β1a hade ett högre antal eosinofiler i blodet, en vit blodcell som är viktig vid både allergier och allergisk astma. Lägre nivåer av proteinet interleukin-18 var också associerat med den astmagrupp som svarade bäst på IFN-β1a behandlingen.
I avhandlingens sista två delarbeten (paper III och paper IV) där vi använde en KOL-kohort fann vi att både virus infektioner och luftföroreningar var förknippade med exacerbationer. Virus-triggade exacerbationer var starkt förknippade med ett ökat uttryck av IFN relaterade biomarkörer så som CXCL10, CXCL11 och IFN-γ. Vi fann också att nivåerna av beta-defensin 2 (hBD-2), en anti-mikrobiella peptid som uttrycks i lungepitelet, var lägre i upphostningsprov från KOL-patienter med tillfällig försämrad sjukdom (exacerbationer). Låga nivåer av hBD-2 vid exacerbation visade sig dessutom vara förknippade med förekomsten av luftvägsvirus hos dessa patienter.
Sammantaget har våra studier identifierat nyckelkomponenter i virus-värdcell interaktioner vid astma och KOL. Vi fann att IFN responser är centrala för virus bekämpning och inte är begränsade till värdcellen utan yttrar sig också på vävnads- och organ-nivå. Våra data föreslår att IFN responser är betydelsefulla även vid ett anti-bakteriellt försvar. Trots den stora komplexiteten av interaktionen mellan virus och dess värdcell demonstrerar

denna avhandling att det är möjligt att identifiera nyckelkomponenter som driver dessa processer. Förhoppningen är att forskning inom detta område leder till utvecklingen av framtida mediciner som förhindrar astma och KOL exacerbationer.

List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Lanosterol Synthase Regulates Human Rhinovirus Replication in Human Bronchial Epithelial Cells.**

McCrae C, Dzgoev A, Ståhlman M, Horndahl J, Svärd R, Große A, Großkopf T, Skujat M-A, Williams N, Schubert S, Echeverri C, Jackson C, Guedán A, Solari R, Vaarala O, Kraan M, Rådinger M.

Accepted for publication in Am J Respir Cell Mol Biol., July 2018

II. **On-Demand Inhaled Interferon-beta 1a (AZD9412) for the Prevention of Severe Asthma Exacerbations: Results of the INEXAS Phase 2a Trial.**

McCrae C, Olsson M, Aurell M, Lundin C, Da Silva CA, Randers F, Paraskos J, Cavallin A, Kjerrulf M, Karlsson K, Wingren C, Marsden M, Monk P, Malmgren A, Gustafson P, Harrison T.

In manuscript.

III. **Study on Risk Factors and Phenotypes of Acute Exacerbations of Chronic Obstructive Pulmonary Disease in Guangzhou, China – Design and Baseline Characteristics.**

Zhou Y, Bruijnzeel PLB, McCrae C, Zheng J, Nihlen U, Zhou R, Van Geest M, Nilsson A, Hadzovic S, Huhn M, Taib Z, Gu Y, Xie J, Ran P, Chen R, Zhong N.

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Abbreviations

ACQ	Asthma control questionnaire
AEC	Airway epithelial cell
ALI	Air-liquid interface
AMP	Anti-microbial peptide
BAL	Bronchoalveolar lavage
BEC	Bronchial epithelial cell
BL	Bronchial lavage
BTS	British Thoracic Society
CDHR3	Cadherin-related family member 3
CH25H	Cholesterol-25-hydroxylase
CXCL	CXC chemokine motif ligand
CXCR	CXC chemokine motif receptor
COPD	Chronic obstructive pulmonary disease
CPE	Cytopathic effect
CRISPR	Clustered regularly spaced short palindromic repeats
DC	Dendritic cells
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EC_{50}	Half maximal effective concentration
24(S),25 EC	Epoxycholesterol
ELISA	Enzyme-linked immunosorbent assay
ePRO	Electronic patient-reported outcomes
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
GINA	Global Initiative for Asthma
GMP	Guanosine monophosphate
GOLD	Global Initiative for Chronic Obstructive Lung Disease
hBD	Human beta defensin
25HC	25-hydroxycholesterol
HMGCoA	3-hydroxy-3methyl-glutaryl-coenzyme A
ICAM-1	Intercellular adhesion molecule 1
ICC	Immunocytochemistry
IgE	Immunoglobulin E
IFN	Interferon

IKKε I kappa B kinase epsilon
IL Interleukin
IRES Internal ribosome entry site
IRF Interferon regulatory factor
ISG Interferon-stimulated gene
ISGF3 Interferon-stimulated gene factor 3
ISH *In situ* hybridisation
JAK Janus kinase
LABA Long-acting B_2 receptor agonist
LDLR Low density lipoprotein receptor
LFA-1 Lymphocyte function-associated antigen 1
LXR Liver X receptor
LSS Lanosterol synthase
mAb Monoclonal antibody
MAP Mitogen activated protein
MDA5 Melanoma differentiation-associated protein 5
miR-132 MicroRNA 132
MOI Multiplicity of infection
mRNA Messenger ribonucleic acid
MSD Mesocale Discovery
Mx1 Myxoma resistance protein 1
NHBE Normal human bronchial epithelial cell
nt Nucleotide
OAS1 Oligoadenylate synthetase 1
OSBP Oxysterol binding protein
PBMC Peripheral blood mononuclear cells
PBS Phosphate-buffered saline
pDC Plasmacytoid dendritic cells
PEF Peak expiratory flow
PI3K Phosphatidyl inositol-3-phosphate kinase
PI4K Phosphatidyl inositol-4-phosphate kinase
PKR Protein kinase R
PLA2G16 Phospholipase A2 Group 16
PFU Plaque-forming units
PM_{10} Particulate matter of $\leq$ 10 μm in diameter
qRT-PCR Quantitative real-time polymerase chain reaction
RIG-I Retinoic acid-inducible gene I
RISC RNA-induced silencing complex
RNAi Ribonucleic acid interference
RNase L Ribonuclease L

RNAseq	Ribonucleic acid sequencing
RSV	Respiratory syncytial virus
RV	Rhinovirus
siRNA	Short interfering ribonucleic acid
SLPI	Secretory leukocyte protease inhibitor
SOCS	Suppressor of cytokine signalling
SREBP	Sterol regulatory element-binding protein
STAT	Signal transducer and activator of transcription
STING	Stimulator of interferon genes
TBK1	Tank-binding kinase 1
$TCID_{50}$	Tissue culture infectious dose 50%
TEER	Transepithelial electrical resistance
TLR	Toll-like receptor
TSLP	Thymic stromal lymphopoietin
TYK	Tyrosine kinase
URTI	Upper respiratory tract infection
UTR	Untranslated region
UV	Ultraviolet

1. Introduction

Asthma and chronic obstructive pulmonary disease (COPD) are chronic lung diseases which are a significant burden to patients, society and healthcare systems. Asthma, which is characterised by reversible airflow limitation and is often caused by allergic inflammation, affects more than 300 million children and adults worldwide (1). COPD differs clinically from asthma in that the airflow obstruction is irreversible and that lung function declines progressively. The hallmark phenotypes of COPD are emphysema (destruction of the lung tissue) and chronic bronchitis (driven by mucus over-production, bacterial colonisation and cough). COPD typically develops later in life and is most commonly caused by tobacco smoking, although other exposures such as indoor air pollution can be important etiological agents, particularly outside of the western world in countries such as China (2). At present, around 65 million people in the world are diagnosed with moderate to severe COPD and by 2030 this disease is predicted to become the 3rd leading cause of death globally (3).

1.1 Exacerbations of asthma and COPD

Both asthma and COPD are associated with periods of acute disease worsening known as exacerbations (in the case of asthma, these are commonly known as asthma attacks). Exacerbations can be triggered by a number of extrinsic or intrinsic factors, either alone or in combination. For example, asthma exacerbations can be triggered by exposure to the aeroallergen to which the individual is sensitised. Other factors which can cause exacerbations of both asthma and COPD are air pollution, exercise or stress. However, by far the most common triggers of exacerbations are acute respiratory viral infections, as will be described in section 1.2 below. Exacerbations are characterized by a worsening of lung function and an increase in lung inflammation. Severe exacerbations can lead to hospitalisation and in their most severe form can be fatal. In COPD, patients fail to completely recover the loss of lung function which occurs during an exacerbation, thus causing a downward spiral in the progression of the disease (4). In some patients, exacerbations can be frequent events, occurring 2 or more times per year (5). Such patients are often referred to as “frequent exacerbators”.

It is these patients who carry the highest disease and healthcare burden. Unfortunately, despite the combinations of inhaled bronchodilators and anti-inflammatory corticosteroids which are the standard-of-care therapies for asthma and COPD, exacerbations remain a problem. Thus, there is significant unmet need for therapies which prevent exacerbations, and this is currently a major focus of drug discovery and development.

1.2 The role of viruses in asthma and COPD exacerbations

Respiratory viral infections are believed to be the most important and common triggers of asthma and COPD exacerbations. In the case of asthma, this had been contended for decades (6-8), but it was not until the emergence of molecular virology methods in the 1990s that the prevalence of viral infections at exacerbation was fully appreciated (9). Using PCR detection methods, it has been shown that 41-95% of asthma exacerbations and 22-57% of COPD exacerbations are associated with a respiratory virus infection, the most common agent being human rhinovirus (RV) (10, 11). Other viruses frequently associated with exacerbations include respiratory syncytial virus (RSV) and influenza (10, 11). Using sophisticated electronic methods to capture daily patient-reported colds and lower respiratory tract symptoms in COPD patients, coupled with molecular detection of viruses, a recent study has suggested that the prevalence of viral exacerbations may be even greater than suggested by molecular detection methods alone (12).

Another body of evidence supporting the role of viral infections in exacerbations comes from seasonal epidemiology. Seasonal peaks of exacerbations occur contemporaneously with periods of increased risk of infection. For example, the so-called "September peak" of asthma exacerbations in school-age children and younger adults coincides with a peak of RV infections following the post-summer return to school (13-20). In older adults, asthma and COPD exacerbations peak during the Christmas week, a time when families get together, enhancing the spread of viruses such as influenza (14, 21, 22).

Whilst the molecular detection of viruses and the seasonal epidemiology show an association between viral infections and exacerbations, they provide no indication of causality. However, a significant step towards demonstrating that viruses can actually trigger exacerbations was made by the establishment of experimental RV challenge models in asthma and COPD patients. Such models have demonstrated that inoculation of a small dose of RV into the nose gives rise to lower airway changes consistent with the occurrence of an exacerbation (8, 23-27).

Given the above evidence, preventing or attenuating respiratory viral infections poses a substantial opportunity for the prevention of asthma and COPD exacerbations. Moreover, exacerbations associated with viral infections are often more severe and longer in duration than non-viral exacerbations (28). Prevention or attenuation of respiratory virus infections therefore could have significant impact on the burden and progression of asthma and COPD.

1.3 Human rhinovirus (RV)

RV is a respiratory pathogen which circulates year-round and is associated with approximately 50% of episodes of the common cold (29). RV is the virus most commonly detected during exacerbations, typically making up around half to two-thirds of virus positive exacerbations (10, 11). As such, RV deserves particular attention and is consequently one of the most studied viruses in the context of asthma and COPD.

1.3.1 RV structure and virology

RV, a member of the Picornaviridae family, is a non-enveloped virus consisting of a heteromeric protein capsid, containing a single positive strand RNA genome (29). Over 160 genotypes - or serotypes - of RV have been identified and these are grouped based on sequence identity into species A, B or C and also on entry receptor into major (intercellular adhesion molecule-1 (ICAM-1)) and minor (low-density lipoprotein receptor (LDLR) or related proteins) groups (30-34). The RV-C species utilizes a different entry receptor to the above, which has recently been reported to be cadherin-related family member 3 (CDHR3) (35). The RV genome is around 7 kb in length and encodes a single polyprotein which encodes the structural proteins, VP1-4, and the non-structural proteins, 2A-2C and 3A-3D (29) (see **Figure 1**).

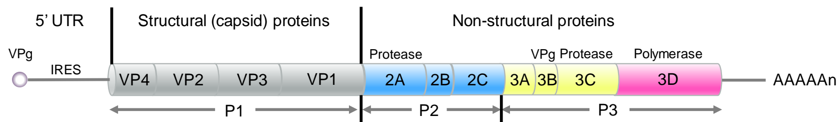

Figure 1. *The RV genome*
The 7 kb RV genome is translated into a single polypeptide which encodes the individual viral proteins. The 5' untranslated region (UTR) contains the VPg priming protein cap (encoded by 3B) and an internal ribosome entry site (IRES). The P1 region encodes the capsid proteins, VP1-VP4. The P2 and P3 regions encode the non-structural proteins, including viral 2A and 3C proteases, and the RNA-dependent RNA polymerase, 3D. The polyprotein is cleaved into the individual proteins by the viral proteases, 2A and 3C. At the 3' end is a poly(A) tail.

The principal natural host cells of RV are the epithelial cells lining the respiratory tract. Initial infection usually occurs in the nasal epithelium and the virus is thought to spread towards to the lower parts of the respiratory tract via the release of progeny virus from infected epithelial cells. Other cell types such as peripheral blood mononuclear cells (PBMC), dendritic cells (DC), macrophages and T cells can also be infected with RV, although there is limited evidence that viral replication occurs in those cells (36-45).

Upon binding to its entry receptor on the cell surface, RV enters the cell via endocytosis. Uncoating of the viral genome occurs by endosomal acidification, and the viral RNA enters the cytoplasm where translation occurs to produce the viral polyprotein (**Figure 2**). A recent study uncovered a novel role for the lipid-modifying enzyme, PLA2G16, in the delivery of RV genome to the cytoplasm (46). Viral proteases cleave the polyprotein into its individual components.

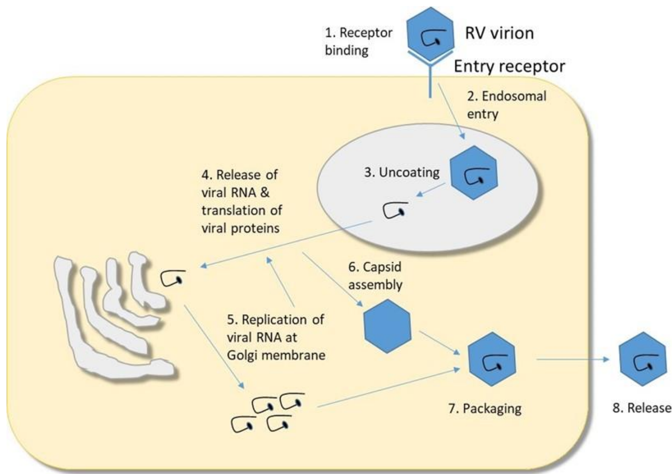

Figure 2. *Simplified diagram of the RV replication cycle*
1. An RV particle enters the airway epithelial cell via its entry receptor (ICAM-1, LDLR or CDHR3). 2. The viral particle then enters the cell via endocytosis. 3. Under low pH, destabilization of the capsid occurs followed by uncoating of the viral +ve stranded RNA genome. 4. The viral genome enters the cytoplasm and begins translation of the viral proteins. 5. Replication organelles are formed from Golgi apparatus membranes, which facilitate the generation of new viral genomes. 6. Structural proteins assemble to form new viral capsids. 7. Viral genomes are packaged into the newly produced capsids, followed by 8. release of progeny virus particles from the cell. Diagram constructed based on data in reference (29).

Certain non-structural proteins such as 2B, 3A and 3AB, interact with host proteins to disrupt internal lipid membranes such as the Golgi apparatus, trans-

Golgi network and endoplasmic reticulum (ER), hijacking those membranes to form viral replication organelles and in so doing, shutting down host protein synthesis and trafficking (47, 48). The RNA-dependent RNA polymerase (3D) assembles on those membranes to facilitate genome replication to produce progeny viral genomes. By a to-date poorly understood process, progeny viral genomes are packaged into assembling capsids to form progeny viral particles, which are then released from the cell by lysis or other means (**Figure 2**). A recent report from Mousnier et al uncovers a previously unknown, essential role for myristoylation of the VP0 precursor in viral capsid assembly (49). Chen et al have shown that RV2 can be released from the cell in a non-lytic manner via phosphatidylserine lipid vesicles (50). A single RV replication cycle takes 8-12 hours, thus supporting rapid spread along the respiratory tract.

1.3.2 RV epidemiology & clinical relevance

Molecular epidemiology studies have shown that multiple RV serotypes circulate at any given time, with no significant over-representation of any particular serotype or species (51-55). In addition to the broad number of circulating serotypes, long-lasting immunity to RV infections is thought to be weak, resulting in life-long susceptibility to RV (56, 57). These factors also make designing vaccine strategies against RV somewhat challenging.

Clinically, all serotypes are believed to be able to cause the common cold and it is not known whether any particular species is more likely to cause colds than another. However, there are apparent differences between species in the severity of the asthma or COPD symptoms that they cause. For example, RV-C is known to be highly pathogenic in younger children and is possibly the most important driver of paediatric viral wheeze in children from 3 years of age (58, 59). When it comes to asthma exacerbations, RV-A and -C have been shown to be more pathogenic than RV-B (60). RV infections in early life are suggested to be major risk factors for the subsequent development of asthma. In a birth cohort study, wheeze associated with RV infection in the first 3 years of life gave a significantly increased odds for the development of asthma by age 6 (odds ratio = 9.8) (61).

Given the large number of RV serotypes which can trigger exacerbations, any therapeutic approach aimed at targeting RV-induced exacerbations needs to be able to target a sufficiently broad spectrum of serotypes.

1.4 Innate anti-viral immunity

1.4.1 The airway epithelium as a barrier to respiratory viruses

The nasal, tracheal and bronchial epithelium provides the first line of innate immune defense against inhaled irritants, particles and pathogens such as respiratory viruses, and is therefore a major determinant of overall host response to infection. The airway epithelium is a pseudostratified barrier, with ciliated cells and mucus-producing goblet cells lining the surface and an underlying pluripotent basal cell population which provides a continuous regenerative supply (see **Figure 3**) (62, 63).

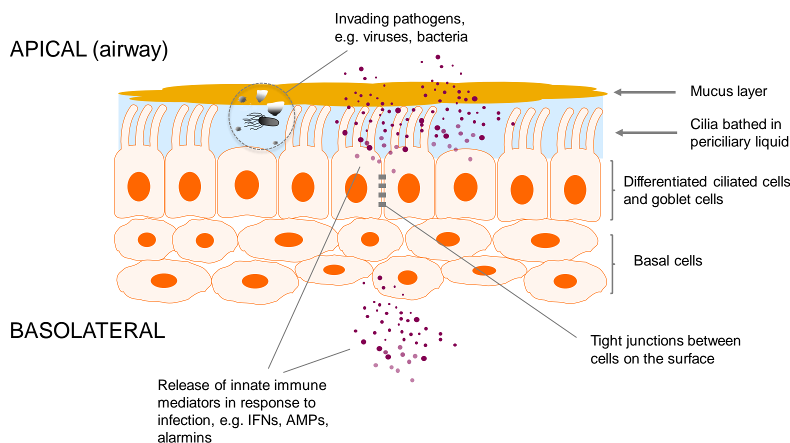

Figure 3. *The airway epithelial barrier.*
The airway epithelium acts as both a physical and a biochemical barrier to infection. Ciliated cells and mucus-producing goblet cells form the airway surface. Tight junctions between these cells prevent the invasion of pathogens and other inhaled particles. The airway surface is lined by cilia bathed in periciliary liquid. An overlying mucus layer, which traps inhaled particles, is transported by the cilia, carrying particles away from the airway. Finally, in response to insult such as an infection, the cells orchestrate a protective response which includes the release of mediators such as IFNs, AMPs and alarmins.

The airway epithelial mucosa acts as both a physical and biochemical barrier to infection, possessing several properties which contribute to host defense (see **Figure 3**). First, the epithelial barrier forms tight junctions between neighbouring cells, preventing the penetration of viruses and other particles through

the barrier (64, 65). Second, the beating cilia of the ciliated cells and the mucus released by the goblet cells act in concert to produce the mucociliary escalator, which traps particles and carries them out of the airways via mucociliary clearance. Third, in response to an insult such as a viral infection, airway epithelial cells rapidly produce and release certain innate response proteins, such as interferons (IFNs), anti-microbial peptides (AMPs e.g. beta defensins) and alarmins (e.g. interleukin (IL)-25, IL-33 and thymic stromal lymphopoietin (TSLP)), which orchestrate immune responses and protect the surrounding cells and tissue from spread of the invading pathogen (66-73). In asthma and COPD, evidence has been provided for dysregulation of all of the above features (62, 64, 65, 72).

1.4.2 The type I and III interferon response

One of the major components of cellular innate anti-viral immunity is the IFN response. Upon viral infection with respiratory viruses such as RV, RSV and influenza, pattern recognition receptors such as toll-like receptor (TLR) 3/7/8, retinoic acid-inducible gene (RIG)-I and melanoma differentiation-associated protein (MDA) 5, recognise the viral RNA genome and trigger, via interferon response factor (IRF) 3 or IRF7 signalling, the transcription and release of type I (IFNα and IFNβ) and type III IFNs (IFNλ1,2,3) (**Figure 4**) (74-76). The type I IFNs, which are produced by the majority of cell types in response to viral infection, bind to their receptor, IFNAR, on surrounding cells. The type III IFNs, which are primarily released by virus-infected epithelial cells, bind to a complex of the IL-10 receptor β chain and the IFNλ1 receptor α chain. In the case of both type I and III IFN receptor engagement, a signaling cascade is activated which results in the formation of a signal transducer and activator of transcription (STAT) 1-STAT2-IRF9 signalling complex (termed the interferon-stimulated gene factor (ISGF) 3 complex) (see **Figure 4**).

Signalling in response to type I and III IFNs results in a broad transcriptional response involving several hundred so-called IFN-stimulated genes (ISG) (**Figure 4**) (77). These genes have diverse functions such as prevention of viral genome replication (e.g. myxoma resistance protein 1 (Mx1)) and cleaving host and viral RNA (oligoadenylate synthetase 1 (OAS1), ribonuclease L (RNase L)), inducing a so-called anti-viral state in the responding cell, which serves to limit viral spread (74, 78). Other ISGs are released from the cell and serve to activate cells of the adaptive immune system, such as CXC motif chemokine ligand (CXCL) 10 and CXCL11, which induce migration of T cells via CXC motif

chemokine receptor (CXCR) 3 (79). Thus, the type I and III IFN response orchestrates a broad innate and adaptive immune response to viral infection.

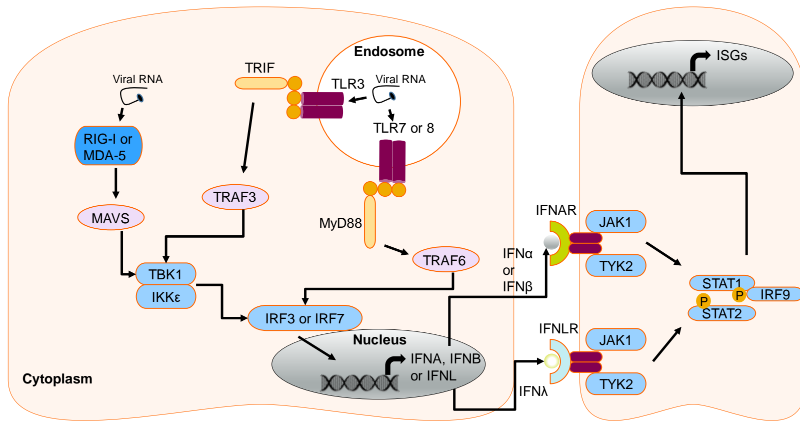

Figure 4. *Type I and III IFN signalling.*
Upon infection with an RNA virus, the viral RNA genome is sensed either in the endosome by TLR3, 7 or 8, or in the cytoplasm by the helicases RIG-I or MDA5. This leads to the activation of a signalling pathway via tank-binding kinase (TBK)1 and I-kappa B kinase (IKK) ε, leading to the translocation of the transcription factors IRF3 or IRF7 to the nucleus and expression of the IFNA, IFNB or IFNL genes. IFNα, β, and λ are then released from the cell and bind to their respective receptors on neighbouring cells. Receptor activation leads to signalling via Janus kinase (JAK) 1 and tyrosine kinase (TYK) 2 to form the ISGF3 signalling complex, which consists of phosphorylated STAT1, STAT2 and IRF9. The ISGF3 complex drives the transcription of several hundred ISGs.

1.4.3 Evidence for defective innate anti-viral immunity in asthma and COPD

The immune mechanisms by which viral infections can trigger exacerbations remain poorly understood. Several studies have demonstrated an impaired type I and III IFN response in bronchial epithelial cells (BEC), bronchoavleolar lavage cells or PBMC from patients with asthma or COPD (25, 39, 66, 80). However, not all studies have confirmed this IFN deficiency (81). In the case of asthma, it has recently been suggested that IFN impairment is observed in a subgroup of patients with severe, therapy-resistant atopic or neutrophilic asthma, but not in patients with well-controlled asthma (82-84). In one study, Spann et al showed that IFN impairment in cells from asthmatic patients compared to controls was virus-specific, suggesting that disease-related perturbations may occur in host pathways that are utilised by some viruses but not others (85).

The precise signaling mechanisms leading to IFN deficiency are not well understood. In asthma, there is growing evidence for an inverse relationship between IFN responses and allergic type 2 inflammation, including immunoglobulin (Ig)E signaling, eosinophilic inflammation and group 2 innate lymphoid cells (36, 81, 86-92). The expression of SOCS1 (suppressor of cytokine signaling 1) protein, which is an inhibitor of cytokine production including interferons, has been shown to be increased in the asthmatic epithelium (93). Another recent study identified altered expression of certain microRNA which regulate TLR7 expression in alveolar macrophages, thus giving rise to deficient type I IFN production (41). Less is known about how IFN responses may be suppressed in COPD, although *in vitro* studies suggest that cigarette smoke exposure suppresses IFN responses to RV (79). Furthermore, decreased influenza-induced IFN responses in BEC from COPD patients was shown to be linked to increased levels of miR-132 and decreased formation of protein kinase R (PKR)-mediated anti-viral stress granules (94).

1.5 Therapeutic interventions for viral exacerbations

1.5.1 Direct anti-virals

Since the 1970s, extensive efforts have gone into discovering and developing anti-viral drugs against RV, initially as a treatment for the common cold but more recently for the prevention of asthma exacerbations (29, 95). The most well-studied anti-RV mechanisms have been 3C protease inhibitors and viral capsid binders, which were demonstrated to have broad coverage across the rhinovirus serotypes (although notably, capsid inhibitors do not inhibit RV-C) (96, 97). The most advanced RV 3C protease inhibitor, rupintrivir, showed a modest reduction in cold symptoms in a phase 2 clinical trial (98, 99). No further development was reported thereafter.

RV capsid inhibitors have reached a more advanced stage of clinical development. Pleconaril, initially discovered by Sterling Winthrop and subsequently developed by ViroPharma, was tested as an oral drug in two phase 3 trials for the common cold (100). Although the primary endpoint was met, the drug was not approved by the Food and Drug Administration because the level of clinical benefit was deemed to be insufficient, there was a drug-drug interaction risk with the contraceptive pill, and evidence was found for a rapid emergence of drug-resistant mutations (101, 102). Although intranasal pleconaril was subsequently

evaluated by Merck and showed a modest reduction in asthma exacerbation rates, no further development has been reported (103). Most recently, vapendavir, a capsid inhibitor discovered by Biota, was tested in a phase 2 trial in asthmatics reporting cold symptoms (SPIRITUS), but no improvement in asthma control was observed (104, 105).

Although direct anti-viral drugs are available for RSV (Synagis) and influenza (Tamiflu), these are licensed specifically to treat severe infections in at risk populations (106, 107). Due to the relatively small proportion of exacerbations associated with RSV or influenza infections, they are unlikely to be viable as stand-alone therapies for the prevention of exacerbations (10, 11). Annual influenza vaccination programs are available in many countries to prevent complications in at-risk populations, including asthma and COPD patients, but there is no evidence that flu vaccination reduces susceptibility to exacerbations caused by viruses other than influenza (108, 109).

1.5.2 Host-targeted therapies

Targeting host proteins essential for virus infection has two major benefits over targeting viral proteins: first, it is easier to identify common pathways essential for a broad(er) spectrum of virus species; second, the barrier to development of drug-resistant mutant strains is – at least in principal – higher.

1.5.2.1 Targeting the major group RV entry receptor, ICAM-1

ICAM-1 is the entry receptor for the major group of RV, which represents approximately 90% of RV-A and RV-B serotypes (30). Due to the large number of serotypes that utilize this receptor, blocking ICAM-1 is an approach that has received some attention for the prevention of RV infections or RV-induced exacerbations.

One approach to this has been to deliver recombinant soluble ICAM-1 (sICAM-1) to the airway. In four trials of inhaled tremacamra in healthy subjects experimentally challenged with RV, cold severity was reduced (110). However, pharmacokinetics of the drug were poor and the drug required dosing 6 times daily. To-date, no further development has been reported.

Efforts have also been made to develop ICAM-1 blocking monoclonal antibodies (mAbs). Traub et al. reported the discovery of an anti-human ICAM-1 mAb, 14C11, which blocked RV infection but spared ICAM-1/lymphocyte func-

tion-associated antigen 1 (LFA-1) interactions, thus potentially leaving host defense intact (111). However, there are no reports of clinical development of this molecule.

1.5.2.2 Interferon-related therapies

Perhaps one of the most attractive ways of targeting the host to prevent viral exacerbations is to boost the body's own innate anti-viral defense mechanism, i.e. IFN. Type I IFNs have been explored as therapeutic proteins for several decades, first with IFNα and more recently with IFNβ. A number of small clinical trials have been performed in asthmatics with recombinant inhaled or intranasal human IFNα, with mixed success and some evidence of poor tolerability (112-118). More recently, Synairgen performed a phase 2 trial of inhaled recombinant IFNβ-1a for the preventions of asthma worsening following symptomatic colds (119). Although the primary endpoint of asthma control was not met in the overall study cohort, in the subgroup of patients with difficult-to-treat asthma, IFNβ-1a significantly improved asthma control and lung function. Interestingly, an epidemiological study of multiple sclerosis patients showed that the use of systemic IFNβ-1a therapy reduced the odds ratio for hospitalisations due to respiratory illnesses, although the specifics of those illnesses (e.g. viral etiology) were not reported (120).

Approaches that would boost endogenous IFN production, for example via stimulation of TLR3, 7/8 or 9, may also be of interest for the prevention of exacerbations. Such agonists have reached early clinical development, although to-date there are no reports from clinical trials specifically evaluating them for the prevention of viral exacerbations (121-126).

1.5.2.3 Targeting regulators of intracellular lipids hijacked by viruses

Phospatidylinositol-4-phosphate (PI4P) lipids are a major component of the Golgi and ER membranes. PI4 kinases (PI4K) are essential for the formation of these lipids. Recent work has shown the PI4KIIIβ isoform to be essential for the replication of a wide range of Picornaviruses, including RV (127-129). In fact, PI4KIIIβ was shown to be the molecular target of enviroxime, a picornavirus inhibitor compound first discovered in the 1970s (130). Selective inhibitors of PI4KIIIβ have been shown to prevent the virus-induced disruption of the Golgi apparatus and the formation of viral replication organelles which occur early

during infection, indicating that PI4KIIIβ plays a non-redundant role in this process (47, 131). Importantly, PI4KIIIβ inhibitors have been shown to inhibit representatives of the RV-A, -B and -C species (132). Although some PI4KIIIβ inhibitors have shown toxicity in preclinical models, this pathway remains interesting as a route to target RV-triggered exacerbations (131, 133).

1.5.2.4 Anti-inflammatory mechanisms

One hypothesis is that inhibition of virus-induced inflammation may prevent viral exacerbations. p38 mitogen-activated protein (MAP) kinase and extracellular signal-regulated kinase (ERK) inhibitors have shown inhibitory effects on viral replication *in vitro*, as well as inhibitors of phospatidylinositol-3 kinases (PI3K) (134-136). In the case of both p38 MAK kinase and PI3K inhibitors, recent, unpublished clinical trials have reported beneficial effects on COPD exacerbations (137, 138). One recent trial addressed the hypothesis that inflammation triggered directly by innate viral RNA recognition may be an important driver of exacerbations. In this trial, a monoclonal TLR3 blocking antibody was tested in an experimental RV challenge model but failed to prevent RV-induced worsening of asthma symptoms and lung function (139).

1.5.2.5 Evidence for anti-viral effects of existing therapies

As mentioned previously, exacerbations remain a problem for many asthma and COPD patients, despite current standard-of-care therapies. The mainstay therapy for asthma is the combination of an inhaled corticosteroid (ICS) to dampen inflammation and an inhaled long-acting B_2 receptor agonist (LABA) as a bronchodilator (140). For COPD, long-acting muscarinic receptor antagonists are also included in the therapy guidelines (141). Whilst these therapies do reduce exacerbation frequency, there are limited data on whether they specifically prevent virus-triggered exacerbations (142, 143). However, Reddel et al showed that budesonide (ICS) and formoterol (LABA) therapy reduced the rate of common cold-related asthma exacerbations (144). Several *in vitro* studies have investigated whether corticosteroids and/or LABA directly impair RV replication or RV-induced IFN responses. Whilst some studies report no effect on either parameter, others show impairment of IFN responses with corticosteroids (145-149). The differences between these studies may reflect differences in protocols and/or the specific drug(s) used.

The macrolide antibiotic, azithromycin, has been reported to induce IFN responses to *in vitro* and *in vivo* RV infection (150-152). Other macrolide analogues were subsequently shown to possess increased anti-viral effects compared to azithromycin (153). In a 48-week randomized, placebo-controlled trial, oral azithromycin significantly reduced the rate of exacerbations in patients with persistent, uncontrolled asthma (154).

Omalizumab is an anti-IgE mAb which is licensed for the treatment of patients with severe asthma (155). The ICATA and PROSE trials demonstrated a significant effect of omalizumab in preventing exacerbations in asthmatic children during the September peak, and *ex vivo* experiments in PBMC and plasmacytoid (p)DC showed that the clinical benefit in PROSE correlated with the level of RV-triggered IFNα release (40, 87, 156).

Interestingly, lebrikizumab, an anti-IL-13 mAb currently under development by Genentech, was trialed in severe asthmatics and although it did not show promise in terms of reducing overall exacerbation frequency, seasonal exacerbation peaks were suppressed (90, 157).

The results with omalizumab and lebrikizumab support the pre-clinical findings showing an inverse relationship between type-2 allergic inflammation and type I IFN responses. It will be intriguing to determine whether other biological therapeutics targeting type-2 allergic inflammation (e.g. anti-IL-4, anti-IL-5 and anti-IL-5 receptor mAbs) or the type-2-inducing alarmins that are upstream (e.g. anti-TSLP), will show evidence of selectively preventing seasonal or viral exacerbations. Pertinent to this, benralizumab, an anti-IL-5 receptor blocking mAb, has been shown to consistently reduce exacerbations across all seasons (158).

Statins, which inhibit 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase, the key rate-limiting enzyme in the cholesterol biosynthetic pathway, are the mainstay therapy for the control of hypercholesterolemia. Since many respiratory viruses hijack certain components of lipid biosynthesis and metabolism, it seems reasonable to hypothesise that statins may reduce the risk of viral infections. Indeed, statins have been shown to inhibit RSV replication *in vitro* (159-161). Interestingly, epidemiological studies have explored statin use in relation to asthma and COPD and shown a reduction in asthma hospitalization or COPD exacerbations (162, 163). It is unknown whether these findings are due to an effect of statins on respiratory viral infections. However, a randomized controlled trial of simvastatin in COPD patients failed to show any benefit on exacerbation rates (164).

1.6 The relationship between viral and bacterial infections in the lung

1.6.1 Viral infections and susceptibility to secondary bacterial infections

Colonisation of the airways with bacterial pathogens such as non-typeable Haemophilus influenzae, Streptococcus pneumoniae and Moraxella catarrhalis, is common in COPD patients (165, 166). During COPD exacerbations, bacterial load in the lung has been shown to increase and recent studies investigating the airway microbiome have demonstrated changes in bacterial composition (167). It has been suggested that viral infection may down-regulate host defence leading to enhanced bacterial proliferation and inflammation. Mallia et al. have recently demonstrated that experimental rhinovirus infection is associated with a very high rate of bacterial exacerbation in COPD patients (26), whilst George et al. showed similar findings in COPD exacerbations associated with naturally occurring RV infections (168).

1.6.2 Type I interferons and bacterial infections

A mechanistic explanation for bacterial outgrowth during a viral COPD exacerbation could be the induction of the type I IFN response, which could compromise the anti-bacterial immune response (169). Several investigators have reported improved survival and improved clearance of secondary bacterial infection following influenza infection in mice lacking IFNAR or treated with a neutralising antibody to IFNAR1 (170-174). Similar results were observed in mice treated with the synthetic TLR3 and RIG-I agonist, poly(I:C), followed by Streptococcus pneumoniae infection (175). Furthermore, influenza-induced type I IFN signalling in mice suppresses Th17-mediated protection from secondary Staphylococcous aureus infection (176). However, other studies have shown protective effects of type I IFN signalling on bacterial infection and invasion (177, 178). The above studies were all performed in mice and evidence in humans is lacking. An ongoing clinical trial of inhaled IFNβ-1a therapy in COPD patients may shed some light on this (179).

1.6.3 Anti-microbial peptides

A key component of cellular anti-microbial defence is the production and release of so-called anti-microbial peptides (AMPs). These form a family of small proteins which act to destroy invading pathogens, typically by disrupting the lipid membrane or envelope. The main families of AMPs include the defensins (alpha and beta), cathelicidins (e.g. LL-37) and protegrins (180, 181). Secretory leukoprotease inhibitor (SLPI), elafin and pentraxin 3 have also been shown to act as AMPs (26).

Human beta defensins (hBD) are cationic AMPs produced primarily by epithelial cells throughout the body (180). They are known to exert anti-bacterial, anti-viral and anti-fungal effects. There are four well-characterised beta defensins (hBD-1 -4), although several others have been identified in the human genome. hBD-1 is constitutively expressed whilst hBD-2, 3 and 4 are inducible. hBD-2 is the most highly expressed in the lung epithelium and is thus thought to be the most important beta defensin in terms of host defense in the airways (182). In addition to its anti-microbial activity, some reports have demonstrated that hBD-2 has a direct role in modulating cells of the innate and adaptive immune systems (183-188). As such, hBD-2 plays an important role in lung host defense and acts on the interface between the innate and adaptive immune systems.

Some evidence exists that the production of AMPs is altered in the COPD lung. For example, levels of hBD-2 are reduced in the central airways of COPD patients (189). There are also reports of a genetic association between hBD-2 copy number and COPD, although this is controversial (190-192). A recent *in vitro* study demonstrated a synergistic induction of hBD-2 in BEC co-infected with RV and Pseudomonas aeruginosa, but the level of induction was suppressed in cells from COPD patients (67). In accordance with this, when experimentally inoculated with RV, COPD patients expressed lower levels of several AMPs in sputum, compared to healthy smoker controls (26). The lungs of COPD patients are known to be rich in proteases such as cathepsins and neutrophil elastase (NE) which can degrade AMPs, which is one mechanism by which levels of AMPs may be reduced in the COPD lung (193).

The interaction between viral infections, AMP production and disease suggest that AMPs may be a key component of the susceptibility of COPD patients to colonization and outgrowth of pathogenic bacteria.

2. Aims

The overall aim of this thesis is to identify mechanisms of host-virus interactions which may ultimately lead to novel therapeutic targets for the prevention of virus-triggered exacerbations in asthma and COPD. The thesis contains both a pre-clinical part involving a search for proteins essential for RV replication *in vitro*, and a clinical part involving both a clinical trial in asthmatics and a non-interventional biomarker study in COPD patients. The specific aims of these studies are presented below.

Paper I

- Identify novel targets for inhibition of RV infection in airway epithelial cells, by conducting a high throughput, RNA interference (RNAi)-based phenotypic screen of RV infection of primary bronchial epithelial cells.
- Study the most promising hit from the screen further, to confirm specificity and investigate molecular mechanisms.

Paper II

- Investigate the efficacy of inhaled IFNβ-1a for the prevention of severe asthma exacerbations following cold symptoms.
- Evaluate patient subgroups based on exploratory biomarkers to identify potential responder sub-populations.

Paper III

- Conduct a 2-year longitudinal observational study in COPD patients to investigate the importance of different trigger factors on the occurrence and phenotype of exacerbations.
- Determine the relationship of exacerbations with infection status and blood and airway biomarkers.

Paper IV

- Investigate the relationship between the AMP, hBD-2, and risk of exacerbations in COPD patients.
- Study how hBD-2 levels associate with viral and bacterial pathogen status during stable disease and at the time of exacerbation.

3. Methods

3.1 *In vitro* models of RV infection

Cell lines such as HeLa OHIO and HeLa H1 are highly susceptible to RV infection. A rapid, lytic infection occurs in these lines, with profound cytopathic effect (CPE) observable after a single or very few infection cycles. For this reason, HeLa OHIO or H1 cells are the lines of choice for virus propagation and titration. Ease of culture and a relatively simple readout (CPE) also make HeLa infection assays a useful tool in drug discovery and they are highly amenable to performing high throughput screening (194). However, the major limitation of performing studies in these cell lines is that the nature of the infection is very different to what is observed when infecting airway epithelial cells (AEC), i.e. the natural host cell, with RV. Instead of a profoundly lytic infection, the majority of AEC appear to survive multiple rounds of infection, unless they are inoculated with very high multiplicities of infection (MOI). HeLa cells are known to produce a weak IFN response compared to AECs, which may in part explain their high susceptibility to infection. To study virus-host interactions in HeLa cells could therefore potentially lead to findings of limited physiological relevance, unless subsequently confirmed in AEC. For this reason, all work in **Paper I** was performed in primary normal human bronchial epithelial cells (NHBE) cells. Whilst an immortalised AEC cell line such as A549 or H292 would have been more convenient to use for a high throughput screen, we used the primary cells as our starting point because these are more physiologically relevant and are closer to the *in vivo* phenotype. We found that we could establish a high throughput RV infection assay in NHBE cells with sufficient robustness and reproducibility with which to perform an RNAi screen.

The choice of readout from an RV infection assay depends largely on the main purpose of the study. However, irrespective of the main goal, it is essential to perform some measure of viral replication. This is because all other readouts are dependent on the viral load in the culture. Viral load can be measured in several different ways, in either supernatants (shed virus) or in cell lysates (intracellular virus). In NHBE cells, it is usually easier to detect intracellular virus than shed virus. RT-PCR is a common method and if one wants to obtain a nominal quantification, a viral standard curve can be included and there are even commercially available qRT-PCR-based kits that enable the calculation of plaque-

forming units (PFU)-equivalents. If it is important to measure the true concentration of live shed virus (i.e. PFU/ml or tissue culture infectious dose $(TCID)_{50}$), supernatants can be transferred to HeLa cells for a CPE assay and there are also commercially available kits for this purpose. Imaging is a useful tool which permits an assessment of what proportion of cells are infected. This can either be done by *in situ* hybridisation (ISH), using probes to detect RV genomic RNA (as utilised in **Paper I**) or replicative strand RNA, or by immunocytochemistry (ICC) using antibodies specific to viral double-stranded DNA or certain viral proteins (e.g. 2C, 3A (48) or VP2). Quantification of the signal can then be performed using image analysis software such as Definiens, as described in **Paper I**.

In terms of measuring the host response to infection, detailed transcriptional analysis can be performed using qRT-PCR or RNAseq. Due to the large number of ISGs of different functions expressed during the IFN response, whole transcriptome analysis is advantageous if one wants to capture a holistic view of the nature and kinetics of the IFN response under different infection conditions. Released proteins can be measured in culture supernatants using immunoassays such as ELISA, AlphaLisa or Mesoscale Discovery (MSD) assays. It should be noted that measuring type I and III IFN proteins directly can be challenging, since they are produced in relatively small quantities. It is common that the levels of IFN in supernatants post-RV infection can be below the limit of quantification of the available immunoassays. A contributory factor is that RV has been shown effectively evade IFN responses (195). One solution to this problem is to measure one of the IFN response proteins which are released from the cell, e,g, CXCL10 or ISG15 (196). An alternative or complementary readout is to measure IFN gene transcription by qRT-PCR, as done in **Paper I**. On a technical note, when performing immunoassays on supernatants, the samples still contain live virus and appropriate biosafety precautions must be taken throughout the procedure. Inactivation of the virus by UV treatment can be performed prior to analysis, but this is not advisable since certain proteins can also be affected by UV treatment.

Although NHBE cells are more physiologically relevant and closer to the *in vivo* phenotype than any cell line, when grown in submerged culture they are more similar to the basal cells which underlie the differentiated ciliated and goblet cells which line the airway surface. It is those terminally differentiated cells which usually become exposed to virus *in vivo*. This is a limitation of the submerged cell NHBE cell model such as that used in the RNAi screen in **Paper I**. To reach the next level of complexity and physiological relevance, it is of importance to study infection of ciliated and goblet cells. This can be done *in vitro* by differentiating NHBE cells at air-liquid interface (ALI; **Figure 5**, see **Paper I**,

supplemental methods, for further details on how this is performed) (197). ALI cultures form all the different epithelial cell types that are observed in the airway, have beating cilia, produce mucus and form tight junctions. ALI cultures can be inoculated on the apical (air) side and virus replication and cellular responses measured over several days post-infection. Similar readouts to those described above can be utilized. Moreover, the ALI model permits a much more in-depth study of the epithelial response to infection with readouts of barrier function (e.g. transepithelial electrical resistance, TEER) and permeability (e.g. using fluorescent particles such as FITC-dextran) becoming possible (198, 199). The ability to sample both the apical (air) and basolateral (medium) sides of the culture allows an assessment of the directionality of released proteins such as IFNs, AMPs and alarmins. Furthermore, a time course of samples can be taken from the apical side without destroying the culture, permitting longer duration studies with fewer individual cultures. As with submerged NHBE, ALI cultures can be infected readily with RV, and often a more robust infection occurs in ALI. Of particular note, the only available *in vitro* model of RV-C infection is the ALI culture, since the entry receptor, CDHR3, is selectively expressed by ciliated cells (96, 200).

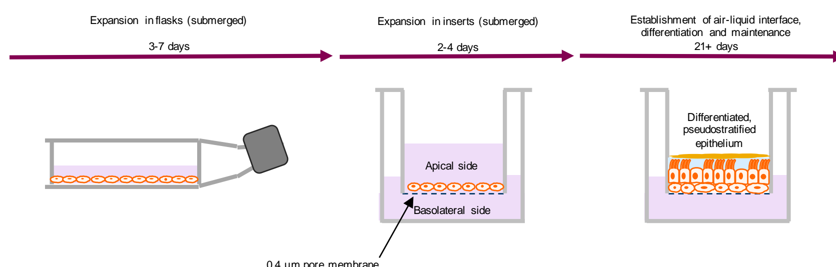

Figure 5. Schematic diagram illustrating the generation of air-liquid interface (ALI) cultures of airway epithelial cells.

One limitation of the ALI culture model is that it consists only of AEC and does not capture the crosstalk which occurs in tissue with other cell types such as endothelial cells, fibroblasts and immune cells. Development of co-culture or chip-based microfluidics models has been reported (201-203). Furthermore, *ex vivo* lung explant or precision-cut lung slice models of viral infection have been described (204-206). Although this would be an attractive next step, these more complex models are yet to be explored in the context of the findings of **Paper I**.

3.2 RNAi as a platform for phenotypic screening

RNAi is a powerful molecular gene silencing tool, first described in the late 1990s and subsequently established in mammalian cells in 2001 (207, 208). Whilst siRNA rapidly became a widely used research tool, efforts also spawned in the pharmaceutical and biotech industry to develop RNAi therapeutics. At the time of writing this thesis, the very first RNAi therapeutic was approved by the FDA (209). RNAi utilizes the RNA-induced silencing complex (RISC) to destroy specific short RNA sequences. Short interfering (si)RNA molecules are double-stranded RNAs which are 21-23 nucleotides (nt) in length containing 2 nt overhangs at the 3' end. One strand is complementary to the target mRNA and thus the siRNA molecule can be designed to have specificity for any given gene. Delivery to the cell can be performed using specific lipid-based transfection methods, as described in **Paper I**, although other methods can be used. Once in the cell, the complementary (targeting) strand is incorporated into RISC, which uses the targeting strand as a sequence-specific probe for targeting mRNA for cleavage by argonaute 2, resulting in the silencing of that specific gene. The efficiency and kinetics of the gene silencing depend on multiple parameters including protein half-life, siRNA sequence, transfection efficiency, knock-down efficiency and cell type.

Whole-genome and druggable-genome siRNA libraries have been produced by several commercial suppliers, making this tool highly amenable to phenotypic drug target screening. The fact that primary AEC can be transfected efficiently using siRNA led us to choose this method for performing a phenotypic screen to identify host targets essential for RV infection. An alternative was to screen one of AstraZeneca's small molecule compound libraries. However, many compounds possess either unknown pharmacology or mixed pharmacology against several proteins. Whilst compound screens have the advantage that they yield chemical starting points for the design of a drug-like molecule, any output of a compound screen would require deconvolution to identify the molecular target(s) responsible for the phenotypic effect. The advantage of siRNA is that the target is already known from the start, hence choosing this methodology for the screen described in **Paper I**. In order to increase the chances of identifying drugable targets, we chose to use a druggable genome library representing approximately 10,500 genes.

There are several issues that need to be considered when performing an siRNA functional genomics screen, since hit-calling from such screens is known to be susceptible to false negatives and false positives (210, 211). First, although the individual siRNA molecules in a library have been optimized by the supplier,

it should be borne in mind that knock-down efficiency will vary, and low efficiency knock-down may result in a lack of phenotypic effect. Second, sequence related off-target effects can occur, leading to potentially spurious findings. Third, siRNA delivery can give rise to cytotoxicity, again leading potentially to false negative or positive results, depending on the phenotypic response being measured. Finally, one common off-target effect comes from the fact that siRNA can induce the anti-viral IFN response in a non-sequence specific manner, which is a particular concern when performing a viral infection screen. In **Paper I**, these issues were controlled for as much as possible. For example, 3 independent siRNA molecules per gene were utilized, the IFN response was assessed using control siRNA during the assay development stage, multiple positive and negative control siRNA were included on each screening plate, and we included a stringent cytotoxicity threshold at the data analysis stage. Furthermore, we confirmed the effect of the most interesting screening hit, lanosterol synthase (LSS), was not a false positive, using pharmacological inhibitors. The details of this can be found in **Paper I**.

Since the emergence of RNAi screening, CRISPR-Cas9 technology has emerged as a more elegant and precise method for performing functional genomics screens (212-214). The advantages of CRISPR/-Cas9 over RNAi are that it provides the possibility to functionally knock out, knock in or fine-tune the expression of a given gene permanently. One limitation is that this is currently not feasible in primary cells with a short lifespan, such as NHBE cells.

3.3 Human studies

In paper II-IV, written informed consent was obtained from all subjects and ethical approval was obtained from the respective ethics committees in the different countries and study centres.

3.3.1 Asthmatic subjects (**Paper II**)

Subjects with severe asthma (GINA step 4-5) and a documented history of frequent severe exacerbations were enrolled for the clinical trial described in **Paper II** . The choice of this population was based on previous experience in a phase 2 trial of inhaled IFNβ-1a (119). To ensure the enrollment of patients susceptible to virus-triggered exacerbations, an additional requirement was that the exacerbations should be reported by the subject to have been triggered by the common

cold or flu. A clear limitation of the latter is recall bias, although the exacerbations themselves had to be documented. Of note, using an epidemiological method, a subgroup of asthmatics in the West Sweden Asthma Study cohort reported only having exacerbations following colds or flu, whilst another, separate group of frequent exacerbators only reported other triggers such as allergen, stress and exercise (215-217). This suggests that a specific "virus susceptible" subgroup may exist among asthma exacerbators.

3.3.2 Subjects with COPD (**Papers III-IV**)

In **Papers III-IV**, all subjects were recruited at the Guangzhou Institute for Respiratory disease and were residents of a large metropolitan area surrounding Guangzhou, one of China's largest cities. A broad cohort of COPD patients was recruited across all disease severities (GOLD 0-4). In addition, a cohort of control subjects without COPD was recruited. GOLD 0 is a classification of "at risk" subjects without a diagnosis of COPD but with certain symptoms and risk factors, such as a history of heavy smoking. In the more recent reviews of the GOLD criteria, GOLD 0 was not included (218). However, this remains an interesting group to study, particularly in light of recent findings in the group of subjects from the SPIROMICS cohort with COPD-like symptoms but preserved lung function (219).

Glossary

GINA (Global Initiative for Asthma) is a medical guidelines organisation which works with public health officials and healthcare professionals globally, to reduce asthma prevalence, morbidity and mortality. The GINA global strategy for asthma management guidelines are updated on an annual basis and are divided into 4 levels (GINA 1-4) based on level of asthma severity and disease control. More information on these levels can be found in ref 138 and www.ginaasthma.org.

GOLD (Global Initiative for Chronic Obstructive lung Disease) is an organization with works with public health officials and healthcare professionals around the world to raise awareness of chronic obstructive pulmonary disease and to improve its prevention and treatment. The GOLD committee provides COPD management guidelines which are reviewed on a regular basis. The current treatment guidelines are divided into GOLD levels A-D based on a combination of symptoms scores and exacerbation frequency. More information on the guidelines can be found in ref 139.

3.3.3 Clinical trial to assess an "on-demand" therapeutic intervention for viral exacerbations of asthma (**Paper II**)

Most exacerbation trials have tested the effect of chronic therapy on rates of exacerbations. Typically, these studies are performed over a 6 to 12-month period in order to obtain sufficient numbers of events for statistical power (142, 156, 157). In **Paper II**, we utilized a less conventional trial design in order to assess the efficacy of therapeutic dosing of the study drug, modelling an "on-demand" treatment approach. Briefly, patients were recruited to the clinic and randomized to a 14-day treatment of IFNβ-1a or placebo within 48 hours of reporting cold symptoms. Further details on the study design can be found in **Paper II**.

There are several advantages of this "on-demand" trial design. One advantage is that since the patient was recruited to the clinic at the time of an event, detailed assessments could be made throughout and after the event, and frequent sampling could be done for biomarker analysis. Long term exacerbation trials rarely involve such a detailed assessment of the exacerbation events. Another advantage is that the design closely mimics how the drug would be anticipated to be used in real-life, with patients starting to take a course of treatment when they recognize symptoms of a cold.

There are also a number of challenges when it comes to performing such a trial design. For example, there are significant operational and logistical issues in ensuring a participant gets the first dose of study drug within 48 hours of reporting cold symptoms. Randomisation was performed at this time and it was therefore not feasible for the patient to have the course of treatment stored at home. Another method to assess a therapeutic intervention would be to use an experimental RV challenge model. Although the design used in **Paper II** has the advantage that all events were naturally occurring, the experimental challenge model allows greater flexibility in time of dosing, for example allowing a comparison of prophylactic and therapeutic dosing. However, there are ethical considerations in performing experimental RV challenge in severe asthmatics.

Although there are data on how many exacerbations are linked to viral infections (10, 11), it is less clear what proportion of colds leads to asthma exacerbations. This had a significant impact on the ability to accurately predict exacerbation event rates. Since only one course of treatment was given per subject, we relied on reasonable odds that a single cold would lead to an exacerbation.

As described in the Introduction, seasonal differences in exacerbations can occur, and it has been shown that the seasonality of asthma exacerbations in the Northern Hemisphere mirrors that of the Southern Hemisphere (14). This trial was performed in multiple centres across several continents in both hemispheres, in order to capture these geographical differences.

In general, exacerbation trials in both asthma and COPD tend to have lower-than-predicted exacerbation rates based on 12-month history of exacerbations (142, 156, 157). This may be in part due to the beneficial effect to the patient of being on a trial, e.g. better compliance to standard-of-care medication, better sense of wellbeing and ready access to study physicians. Severe exacerbation event rates were also low in our on-demand trial, and the above factors may have contributed to this.

3.3.4 Longitudinal exacerbation study in COPD patients (**Papers III-IV**)

Longitudinal, observational studies have the advantage that the natural history of disease can be assessed over time without significant patient interference or intervention. In the case of the study described in Papers III and IV, subjects were followed for two years. All subjects visited the clinical annually for assessments and sampling (baseline, 12 months and 24 months). A subgroup of the cohort was selected for more in depth assessment of daily symptoms using an electronic patient-reported outcomes (ePRO) device. Using a Blackberry smartphone, EXACT-PRO, a COPD symptom questionnaire which can sensitively detect the early onset of exacerbation, was administered to subjects in this subgroup on a daily basis (220). Previous studies have demonstrated close to 100% compliance and exacerbation detection using this ePRO method (22). When signs of exacerbation were detected, the subject was recruited to the clinic for assessments and sampling. Thus, subjects were assessed on an annual basis during stable disease, and at the time of exacerbation, enabling a within-subject comparison of clinical parameters and biomarkers at stable disease and exacerbation. One limitation, however, is that only 1 clinic visit was made at the time of exacerbation, so it was not possible to study the time course of events during exacerbation and at recovery.

The 2-year observation period with annual assessments and sampling afforded the ability to assess the stability of different parameters over time. However, it is likely that 2 years is not a sufficient length of time to assess disease progression. Typically, studies of disease progression, e.g. lung function decline, follow patients for 3-5 years (4, 221).

3.3.5 Sputum sampling

Sampling of sputum is a relatively non-invasive method to obtain fluid from the lining of the upper airways, permitting an assessment of different cell-associated and soluble biomarkers. The most abundant cell types in sputum are macrophages, lymphocytes, neutrophils and eosinophils (222, 223). There are two procedures normally used for the sampling of sputum. Induced sputum involves the inhalation of sterile saline (e.g. 4% PBS) followed by the subject coughing up and spitting out the sputum sample. This procedure is uncomfortable for the subject and is done with measurement of lung function before and after the saline inhalation. The second procedure, expectorate sputum, is non-invasive and involves the subject coughing up and spitting out sputum without any prior saline inhalation. For **Paper II**, only expectorate sputum was obtained to avoid risk to patients and any interference with the trial's efficacy endpoints. For **Papers III-IV**, either induced and expectorate sputum was collected, depending on whether it was safe to perform the induced sputum procedure.

Sputum is expectorated into a petri dish which is then placed on ice for rapid processing. Processing involves separating the sputum plugs from saliva, weighing them followed by addition of PBS and centrifugation to separate the cell fraction from the sputum supernatant. At this point, a fraction of the supernatant ("PBS supernatant") was stored at -80°C for further use. Afterwards, a fraction of the remaining sputum was treated with the reducing agent dithiothreitol (DTT) (e.g. Sputasol or Sputolysin), in order to break down the sputum plug. A small aliquot of this sample was removed for differential cell count and assessment of cell viability using trypan blue. A fraction of the sputum sample was also centrifuged onto a cytospin slide for future cell analysis. The remaining sample was centrifuged and DTT supernatants collected and stored at -80°C for further use. DTT may affect the ability to measure certain proteins in sputum supernatant, hence the advantage of collection both untreated ("PBS") sputum and DTT sputum supernatants. For **Paper II**, the remaining sputum cells were stored in RNA lysis buffer for further analysis. Any sample with more than 10% squamous cell contamination and/or less than 75% cell viability, was deemed unsuitable for further analysis.

In **Paper II**, subjects were recruited at multiple clinical trial centres, few fo which were either experienced or equipped to perform sputum processing. In order to obtain sputum samples from those centres, expectorate sputum was collected directly into a tube and frozen immediately at -80°C, providing so-called "spit and freeze" sputum samples. Although these samples could not be used for soluble protein biomarker analysis, they could be put into RNA lysis buffer for RNA isolation and RT-PCR for gene expression or pathogen detection.

A limitation of sputum collection is that measurements made in sputum do not necessarily reflect ongoing biological of pathological processes in the lower airway. A further limitation is the potential for contamination from the oropharynx. Sampling of the lower airways, by collection of bronchial lavage or bronchoalveolar lavage, can be performed via bronchoscopy. However, this is more invasive and for ethical reasons is not always feasible, especially in more severe disease or during exacerbations. For this reason, bronchial sampling was not performed in the studies described in **Papers II-IV**.

3.4 Biomarker analysis methods

3.4.1 Pathogen detection

In **Papers II-IV**, detection of a panel of respiratory viruses was performed in nasal lavage or sputum samples using RT-PCR (see individual papers for the details). In **Papers III-IV**, a small number of pathogenic bacterial species were included in the RT-PCR panel. Furthermore, traditional bacterial culture methods were used for the detection of a larger range of pathogenic bacterial species in sputum.

It was noted in the analysis for **Paper II** that virus detection rates were higher in sputum than in nasal lavage (see section 4.2). Although it is not clear whether this is due to the infection having cleared from the nose and spread to the lung at the time of sampling or, alternatively, due to differences in sample quality between nasal lavage and sputum, this phenomenon has been noted previously ((22) and Synairgen, personal communication).

3.4.2 Host response and inflammatory biomarkers

In **Papers II-IV**, a range of soluble markers were measured in sputum and/or serum in order to assess lung and systemic inflammatory cytokines, IFN-related markers and AMPs. These assays were performed using either ELISA or MSD immunoassays. As noted in section 3.1, many IFNs and also inflammatory biomarkers are expressed at levels which are too low for most immunoassays to quantify. This is especially the case for the type I IFNs. For example, of >500 sputum samples measured in the COPD study used in **Paper III-IV**, IFNβ was detected by MSD assay in only 38 samples (not shown). Several of the samples

with failed IFNβ detection were exacerbations in which a respiratory virus had been detected. Even in a previous trial of inhaled IFNβ-1a, IFNβ could not be detected in serum by ELISA at any time point post-treatment (Synairgen, personal communication).

For the above reason, assessment of the IFN response in clinical samples such as sputum and serum are best performed using IFN response biomarkers such as CXCL10, CXCL11 and ISG15. This was the reason for choosing such biomarkers in **Paper II** and **Paper IV**.

mRNA biomarkers can be measured by qRT-PCR or RNAseq in the sputum cell fraction. In **Paper II**, a panel of 5 ISGs was measured by qRT-PCR in either sputum cells or "spit and freeze" sputum, to assess the activation of the IFN response by inhaled IFNβ-1a.

3.5 Statistical methods

All statistical analyses reported in **Papers I-IV** were performed using either GraphPad Prism, R or Spotfire software. Non-parametric or parametric analysis was used as appropriate depending on whether or not the parameter was normally distributed. A p-value of less than 0.05 was regarded as statistically significant. Where multiple comparisons were involved, adjustment for multiple testing was performed. Full details of the individual statistical analyses performed are described in **Papers I-IV**.

4. Results and Discussion

The main findings of **Papers I-IV** are summarized in this chapter. Unpublished results of relevance to the findings are also included.

4.1 Lanosterol synthase is a regulator of rhinovirus infection in primary NHBE cells (**Paper I**)

The initial aim of the research which led to **Paper I** was to perform a phenotypic screen using RNAi, identify novel targets that inhibit viral replication. The ambition was that confirmed hits could be taken forward for the development of new therapeutics for the prevention of virus-triggered exacerbations. The original screening cascade included both RV and RSV. Given the fact that RV and RSV are highly unrelated, our rationale was that if we can identify targets that apply to both RV and RSV, they are likely to be relevant to a broad spectrum of respiratory viruses.

For the primary screen of the entire drugable-genome siRNA library, we chose RV since it is the most common virus type associated with exacerbations. However, the choice of RV2, a representative of the minor group of RV, was made for pragmatic reasons. Minor group serotypes are members of the RV-A species and are defined based on the entry receptor, LDLR and related proteins. The minor group makes up less than 10% of RV serotypes. The preference would have been to use a representative of the major group since this represents around 90% of RV-A and B serotypes (note that RV-C would not have been feasible for this screen since it only infects differentiated AEC grown at ALI). However, during the assay development and optimization stage, of the 6 serotypes evaluated (RV2, 10, 14, 16, 22 and 41), RV2 provided by far the best and most robust high-throughput assay.

The original siRNA screening cascade is illustrated in **Figure 6**. At Pass 3/4 (technical and biological validation), we intended to include infection assays for two additional viruses, RV16 (major group) and RSV. However, the results were disappointing. First, we were unable to establish a sufficiently robust medium-throughput RV16 infection assay in NHBE cells which would permit the testing

of the 25 genes which were selected after Pass 2. We therefore decided not to include the RV16 assay. Although we did succeed in developing a suitable RSV infection assay, only three of the “high-confidence” hits, DNAJC3, EEF1G and ZNF614, inhibited RSV. We chose not to follow up on these because at the time there were limited or no tools for further target validation and, in the case of ZNF614, very little knowledge of its function. However, DNAJC3 is of particular interest due to the fact that there are reports of a role in coxsackievirus 3B infection (another Picornavirus), suggesting that it is involved in the infection of several viral species (224).

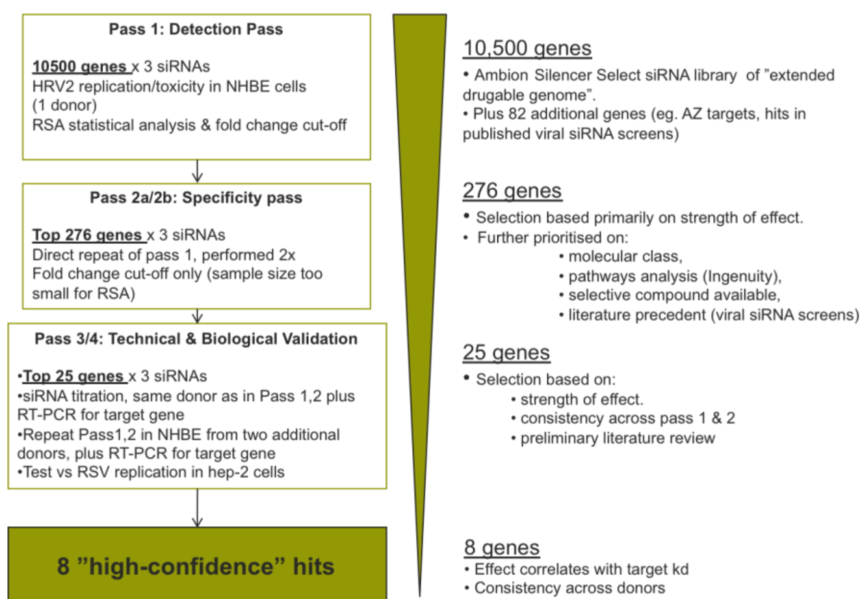

Figure 6. The siRNA screening cascade.

Several RNAi-based phenotypic screens for virus replication have been reported in the literature ((210, 211, 225). In general, very few common genes have emerged from those screens, even in cases where the same virus was used. This highlights the limitations described above around false positives and negatives, and also perhaps indicates the impact of different cell types, model systems and protocol on the eventual outcome. In spite of these potential concerns, we identified several hits that have previously been shown to be involved in viral replication (e.g. oxysterol binding protein (OSBP) genes, DNAJC3, GTF3C2, NR1H3), making us confident in the validity of our screening output (47, 224, 226-230).

Following the conclusion of the siRNA screening cascade, we chose to focus our efforts on LSS due to the availability of selective small molecule inhibitors which would allow us to rapidly confirm the specify of the siRNA results. We proceeded to confirm that pharmacological inhibition of LSS inhibits RV replication. The results of those experiments were promising but left us with a conundrum: the anti-viral potencies of all three LSS inhibitors tested were 5-10-fold higher than their potencies in an assay of LSS enzymatic activity. From such a finding, one would normally conclude this would be the result of some off-target effect of the compound. However, two things led us to believe that something else might be the reason. First, the same phenomenon occurred with all three compounds, which are from different chemical series. Second, the compounds were all clinical candidates and had thus gone through rigorous testing for selectivity (231, 232).

Rowe et al had shown previously that partial inhibition of LSS in murine macrophages, gives rise to an increase in 24(S),25 epoxycholesterol (EC) (233). This gave a potential biological explanation for the high anti-viral potency of the LSS inhibitors. Accordingly, when we measured 24(S),25 EC in our RV2 infection assay, we confirmed that low concentrations of LSS inhibitor were associated with an increase in 24(S),25 EC levels. Intriguingly, the EC_{50} for 24(S),25 EC induction was remarkably close to the antiviral $EC_{50.}$ As described in **Paper I**, we subsequently confirmed that exogenous 24(S),25 EC had an anti-viral effect in its own right. We first thought that the mechanism for this anti-viral effect might be due to activation of the liver X receptor (LXR), since 24(S),25 EC is a potent agonist of this receptor (234). Indeed, we confirmed that LSS inhibitors induced the LXR target gene, ABCA1. However, we were unable to reproduce the anti-viral effect with the selective small molecule LXR agonist, GW3965. Furthermore, the LXR antagonist, GSK2033, failed to block that anti-viral effect of either LSS inhibitors or 24(S),25 EC. This led us to believe that the other known function of 24(S),25 EC, i.e. blocking the activation of the transcription factor sterol regulatory element-binding protein (SREBP), might be responsible for anti-viral effect. Further studies are required, for example using siRNA targeting SREBP, to confirm this.

Given that the siRNA screen and the majority of our follow-up experiments were performed using the minor group serotype, RV2, it was necessary to gain confidence that LSS inhibitors would possess anti-viral activity against a broader range of RV serotypes. Accordingly, we confirmed anti-viral efficacy against the major group serotype, RV16. We chose to conduct those experiments in ALI cultures, for two main reasons. First, we wanted to demonstrate the anti-viral activity of this mechanism in a more physiologically relevant model of infection.

Second, our previous experience told us that RV16 establishes a more robust infection in ALI than it does in submerged NHBE cells. It would have been valuable to have tested an RV-C strain in the ALI infection model but, at the time, we did not have the possibility do so. Confirming the findings of the siRNA screen, we failed to show any effect of the small molecule inhibitors on RSV replication in hep-2 cells (unpublished, data not shown).

Recent reports have suggested an interplay between type I IFN responses and the cholesterol biosynthetic pathway (235). It was found that cholesterol-25-hydroxylase (CH25H) is an ISG. CH25H catalyses the production of 25-hydroycholesterol (25HC), which was shown to inhibit the growth of several viruses (235-237). Subsequently, York et al showed more broadly that IFN signaling counteracts cholesterol biosynthesis and vice versa and went on to suggest a potential mechanism for this, via STING (stimulator of interferon genes) signaling in response to cyclic GMP (238). This led us to hypothesise that perturbing cholesterol synthesis via LSS could lead to an increase in the IFN response to RV. We confirmed this to be the case, with a 4-fold increase in IFNB mRNA in the presence of the LSS inhibitor BIBB-515. Although this might imply that enhancement of the IFN response could explain the anti-viral effects that we have observed, we do not believe this to be the case. This is because the observed antiviral effect precedes the production of significant levels of IFNβ. Instead, we believe that LSS inhibition may in fact have a direct impact on viral replication as well as an ability to increase virus-induced IFN responses.

Our discovery of the role of LSS adds to the increasing number of lipid regulation and lipid metabolism components that are found to be essential for viral replication, such as PI4KIIIb, OSBP and LXR, which have been discussed earlier. Clearly, the synthesis and localization of lipids and lipid membranes are likely crucial for virus propagation, throughout the replication cycle from entry to exit. Another important cellular process involving regulation of lipid membranes is autophagy, and there is growing evidence that viruses can control this process to facilitate their own replication. For example, autophagy regulation has been shown to play a role in RV replication and also IFN responses (46, 145, 239, 240). Further research is warranted to further investigate the precise role of autophagy throughout the viral infection cycle.

Beyond LSS, we also interrogated further the output of the primary siRNA screen. For funding reasons, we were limited to advancing around 250 hit mRNA to Pass 2. However, this was only a proportion of the mRNA which passed the pre-defined hit criteria, based on either redundant siRNA activity (RSA) or a fold-change cut-off. We wanted to identify pathways and molecular functions which may be over-represented in the hits, and to have sufficient statistical power, we wanted to maximise the number of hits included. To do this, we used

a third statistical analysis method, by performing a t-test based on the mean value of the three siRNA molecules for each gene, adjusting for multiple testing using Benjamini-Hochberg. A total of 1129 mRNA passed at least one of the 3 criteria, and we used this as the basis of the pathway enrichment analysis using Ingenuity Pathways Analysis (241). At the canonical pathway level, the most highly over-represented pathways included IL-1, nuclear factor (NF) κB and IFN signaling. A central role of NFκB was revealed, supporting a hijack hypothesis which has been reported in the literature (**Figure 7**) (242). At the molecular function level, the most highly over-represented were those involved in viral replication and apoptosis/survival (**Figure 8**). The latter finding gave further confidence that the screen was successful and identified genes of known importance to viral infection.

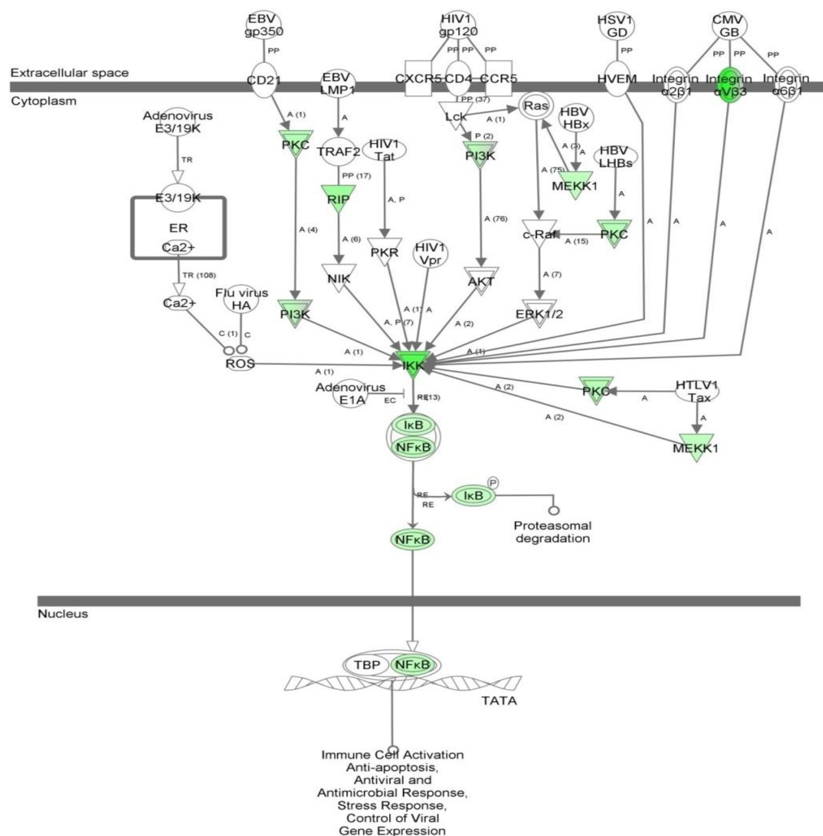

Figure 7. Pathway enrichment analysis of the siRNA primary screen output.
"NFkB activation by viruses" canonical pathway overlaid with mRNA present in the 1129 "hit" list (green). Analysis was performed using Ingenuity Pathways Analysis.

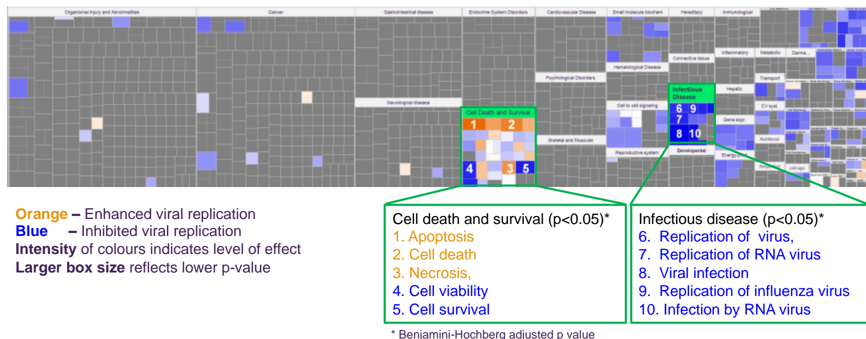

Figure 8. Diseases and molecular functions represented by the 1129 hit mRNA from the siRNA primary screen. *Analysis was performed using Ingenuity Pathways Analysis.*

4.2 The efficacy of on-demand inhaled IFNβ-1a for the prevention of severe asthma exacerbations (**Paper II**)

Since Wark et al at the University of Southampton, UK, first reported evidence for deficient type I IFN responses in BEC from asthmatic patients in 2005, efforts began to develop inhaled recombinant IFNβ-1a (the same drug substance that had been licensed in the 1990s as a systemic therapy for the treatment of relapsing/remitting multiple sclerosis), for the prevention of virus-triggered asthma exacerbations (66, 120). This led to the formation of Synairgen Research Ltd., a biotech company which span out of the University of Southampton. Synairgen advanced inhaled IFNβ-1a to a phase 2 trial in asthmatics with a broad range of disease severity (British Thoracic Society (BTS) guidelines 1-5) (243). In that trial, asthmatics were given a 14-day course of nebulized IFNβ-1a or placebo following cold or flu symptoms. The primary endpoint was the 6-item asthma control questionnaire (ACQ-6). Although the primary endpoint was not met in the overall cohort, a planned subgroup analysis showed a significant benefit of IFNβ-1a in the patients with "difficult-to-treat" asthma, i.e. BTS 4-5 (119).

Following the results of Synairgen's trial, inhaled IFNβ-1a was in-licensed by AstraZeneca for further development. Given that only a small subgroup showed efficacy in the previous trial, we set out to design a trial which would evaluate that patient subgroup with sufficient statistical power. The Interferon for Exacerbations of Asthma (INEXAS) trial therefore studied severe asthma pa-

tients (GINA 4-5, similar to BTS 4-5), using a similar study design to the previous trial (243, 244). Due to medical need and commercial value, we chose severe exacerbations, instead of ACQ-6, to be the primary endpoint, and powered the study accordingly.

The outcome of the primary endpoint was negative, although there was a beneficial effect of IFNβ-1a on lung function. A detailed account and discussion of the trial and its results can be found in **Paper II**. Here, I will discuss further some of the findings related to the virus detection and biomarker analysis that were not covered in **Paper II**.

Virus detection was performed retrospectively in nasal lavage and sputum samples collected during the first week of treatment. As outlined in **Paper II**, randomization to study drug was determined based on confirmed cold or flu symptoms. We elected not to utilize virus positivity as a randomization criterion because we believed based on previous findings that cold symptoms may be more sensitive that the virus test itself (12). Furthermore, waiting for a virus test result would have further delayed treatment start. We utilized a commercially available RT-PCR kit for the detection of 21 respiratory pathogens (FTD respiratory pathogens 21 kit). Overall, the percent of patients positive for any virus or RV was 54% or 17%, respectively. This is somewhat lower than expected when comparing to previous studies (10, 43, 119). Although RV was, as expected, the most common virus species detected, the proportion of all virus detections (19 of 59, 32%) was particularly low (**Figure 9**). It is not known why this was the case although, as discussed below, it may relate to differences between participating countries in the viruses circulating at the time of conducting the trial.

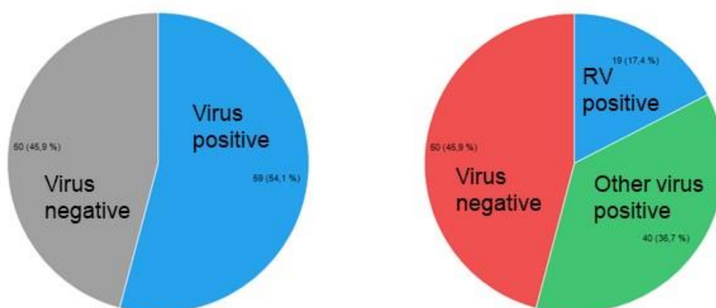

Figure 9. Subjects positive or negative for respiratory viruses in the INEXAS trial.
A panel of 21 respiratory pathogens were detected by RT-PCR in nasal lavage and sputum samples, during the first week of the treatment period. A subject was regarded as virus or RV positive if there was a positive detection in any sample at any visit.

We also analysed virus positivity by visit and sample type. At randomization, virus positivity was lower in nasal lavage (30%) than in sputum (38%). The same trend was observed for RV positivity (6% in nasal lavage and 13% in sputum) (**Figure 10**). As mentioned in Section 3.4.1, lower detection rates in nasal samples compared to sputum have been observed previously. Further virus detection was performed in sputum at days 3 and 7 of the treatment period. As expected, virus positivity declined over time, with 51% at day 3 and 45% at day 4. The same was the case for RV positivity (17% at day 3 and 15% at day 4). There was no evidence for a difference between active and placebo at any time point, although numbers were low.

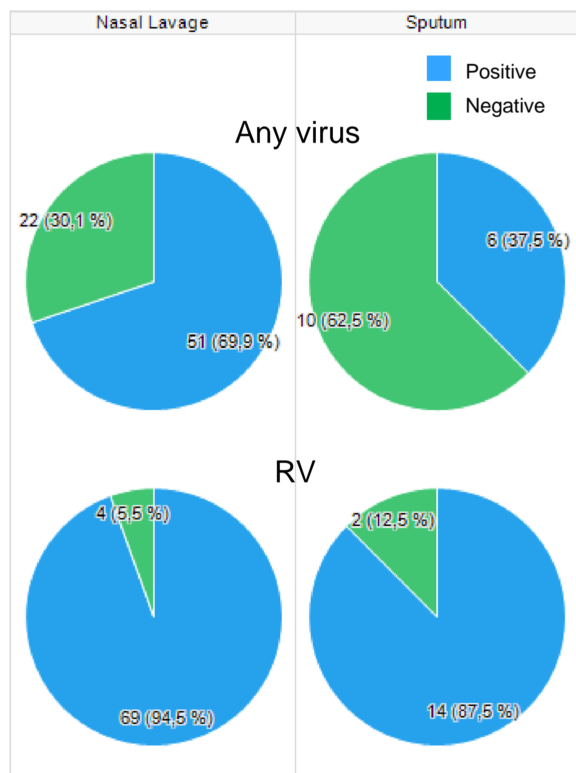

Figure 10. Subjects positive or negative for respiratory viruses at randomisation by sample type in the INEXAS trial.
A panel of 21 respiratory pathogens were detected by RT-PCR in nasal lavage (left) and sputum (right) samples. Upper panel: any virus. Lower panel: RV.

We then compared virus positivity in patients with or without clinically confirmed colds based on the Jackson Cold Score (see ref (245, 246) and methods in **Paper II**). We hypothesized that patients with confirmed colds would be enriched for virus positivity. Indeed, when comparing patients who fulfilled both criteria for clinically confirmed colds with those who met neither, overall virus positivity was higher (59% vs 40%). However, no such trend was observed for RV (18% vs 20%) (**Figure 11**).

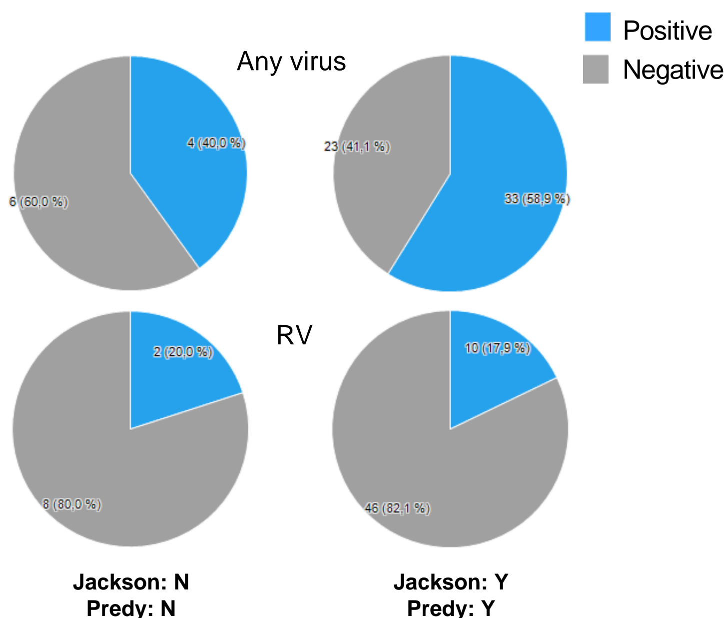

Figure 11. Subjects positive or negative for respiratory viruses by clinical cold status in the INEXAS trial.
A panel of 21 respiratory pathogens were detected by RT-PCR in nasal lavage and sputum samples. A subject was regarded as virus or RV positive if there was a positive detection in any sample at any visit. Upper panel: any virus. Lower panel: RV. Left panel: cold not confirmed by either Jackson or Predy methods (245, 246). Right panel: clinically confirmed cold by both Jackson and Predy methods.

Further analysis of the virus detection results was performed by country (**Figure 12**). Of the 7 countries included in the trial, the 3 with highest virus detection rates were South Korea, Spain and Columbia (71%, 71% and 67%, respectively). The picture was somewhat different for RV, with France, Australia and Columbia showing the highest RV detection rates (50%, 33% and 21%, respectively). It should be noted however that for France and Columbia, data were available for only 2 and 3 samples, respectively, with one sample in each country

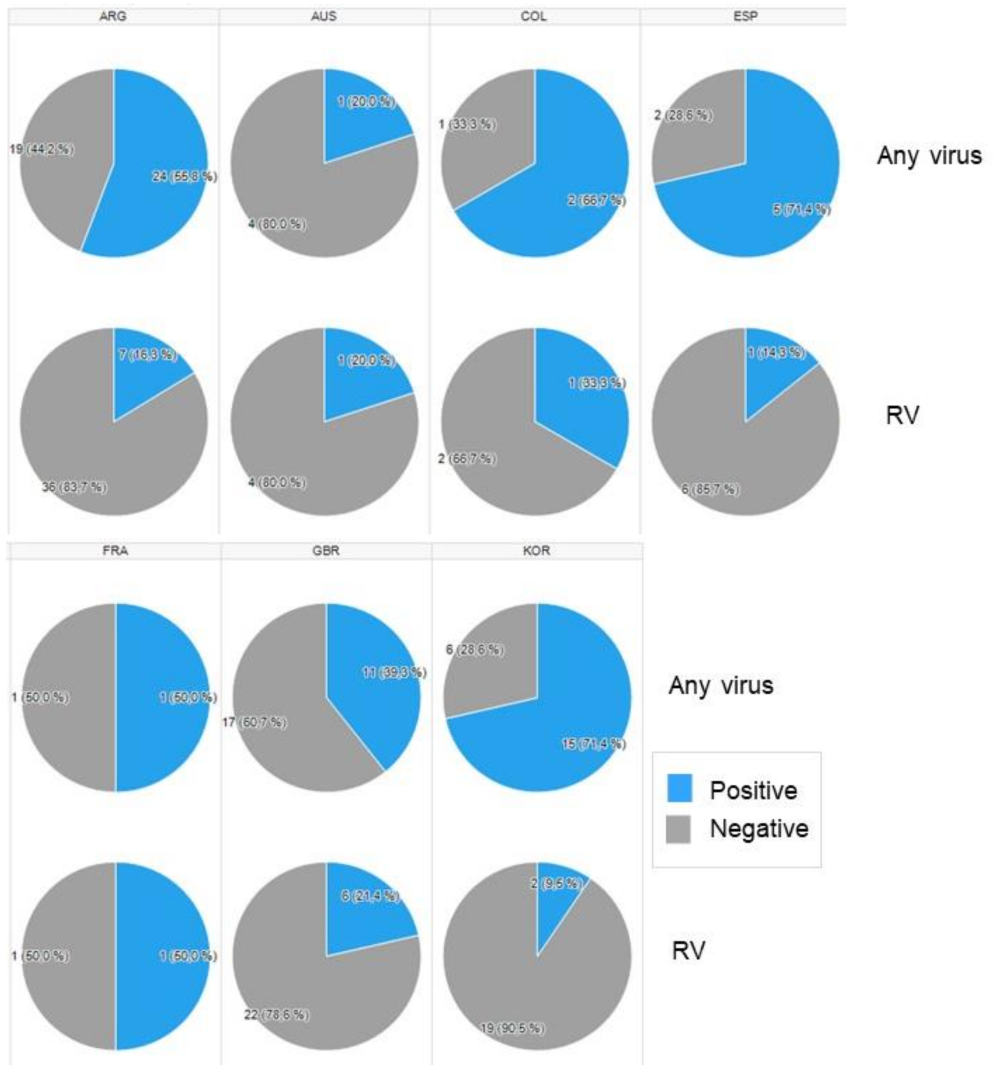

Figure 12. Subjects positive or negative for respiratory viruses by country in the INEXAS trial.
A panel of 21 respiratory pathogens were detected by RT-PCR in nasal lavage and sputum samples. A subject was regarded as virus or RV positive if there was a positive detection in any sample at any visit. Upper panel: any virus. Lower panel: RV. ARG: Argentina. AUS: Australia. COL: Columbia. ESP: Spain. FRA: France. GBR: United Kingdom. KOR: South Korea.

being positive for RV. As a proportion of all viruses detected, RV differed substantially between countries, ranging from 13% in South Korea to 100% in France, Australia and Columbia. Interestingly, a large proportion of subjects were recruited in Argentina at a time when there was an ongoing flu epidemic.

Twenty-four (56%) subjects recruited in Argentina were virus positive, of which 7 (29%) were RV positive. When it comes to flu, 4 (17%) were positive for either H1N1, influenza A or influenza B. This was by far the highest flu detection rate of any country, with only 3 other positive detections (Columbia, Spain and United Kingdom).

In terms of virus species, the 3 most commonly detected viruses were RV (14% of samples), RSV (7%) and metapneumovirus (6%). Influenza was less commonly detected (3% for H1N1 and 1% for FluA and FluB, respectively).

CXCL10 is a well-described marker of the IFN response and has been shown to be a robust biomarker of viral exacerbations (247-250). Hence, we utilized serum CXCL10 as a biomarker to measure both the endogenous IFN response and the response to the drug. As described in **Paper II**, this enabled us to confirm pharmacodynamic activity of the drug as well as to compare subgroups based on the level of endogenous IFN response. Given the close relationship between CXCL10 and viral load and that in RV challenge studies, viral load correlates with symptoms (24, 248), we hypothesized that CXCL10 levels would have a relationship to infection status, cold symptoms and asthma symptoms. We therefore subdivided subjects based on their CXCL10 level at the time of reporting symptoms, before the first dose of study drug. Surprisingly, we saw no relationship between CXCL10 levels and any of the above parameters. We also evaluated CXCL10 levels in those subjects with severe exacerbations and again, saw no difference compared to the overall study cohort. However, we did find one interesting trend, in that the robustness of the CXCL10 response at randomisation differed markedly between countries. In Argentina, CXCL10 levels increased to the greatest extent, whilst in UK and South Korea, almost no increase in CXCL10 was observed (**See Figure 13**). We could not find any other clinical correlate with this inter-country CXCL10 difference, although it is intriguing to speculate that this may relate to the higher rate of influenza infections in Argentina. Indeed, although there were only 7 influenza positive subjects, the CXCL10 level at randomization tended to be higher in those than in subjects positive for other viruses (not shown).

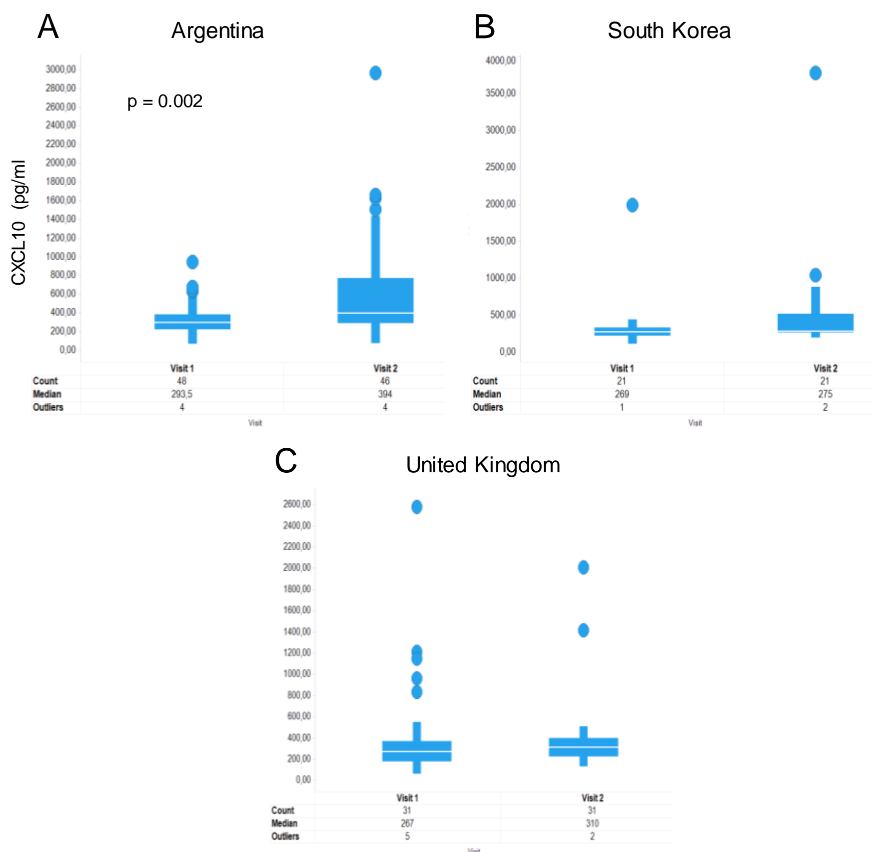

Figure 13. Serum CXCL10 levels from screening to randomisation by country in the INEXAS trial. *CXCL10 was measured in serum samples using an MSD V-plex immunoassay. Levels are compared between Visit 1 (screening) and Visit 2 (randomisation visit, at time of cold/flu symptoms but before first dose of study drug) in subjects enrolled in Argentina (A), South Korea (B) or United Kingdom (C). p-value is determined using Kruskal-Wallis.*

We measured a panel of inflammatory biomarkers in serum at baseline, throughout the treatment period and at follow-up (3 days and 2 weeks after end-of-treatment), and the majority of this analysis is described in **Paper II**. We included the type-2 allergic inflammatory cytokines IL-4, IL-5 and IL-13 based on the evidence that there is a counteractivity between IFN signaling and type-2 inflammation, as discussed in Section 1.4.3. We hypothesized that inhaled IFNβ-1a would reduce IL-4, IL-5 and IL-13 levels in serum. Although we were unable to measure IL-4 and IL-5 in all but a few samples, we obtained sufficient IL-13 data

to perform a comparison of the active and placebo groups. At baseline and throughout the treatment, there were no differences in IL-13 levels between active and placebo. However, at the 2-week follow-up visit, IL-13 levels were lower in the IFNβ-1a group compared to placebo (**Figure 14**). It is tempting to speculate that activation of the IFN response may have resulted in a switch of the immune response away from allergic type-2 inflammation, akin to what has been shown for agonists of the type I IFN-inducing receptors, TLR7 and TLR9 (124, 251, 252). To test this hypothesis in allergic asthmatic patients, an inhaled TLR9 agonist is currently being evaluated in the phase 2 trial (253). If the hypothesis is supported by this trial, inhaled IFNβ-1a could be another interesting approach to achieve the same goal.

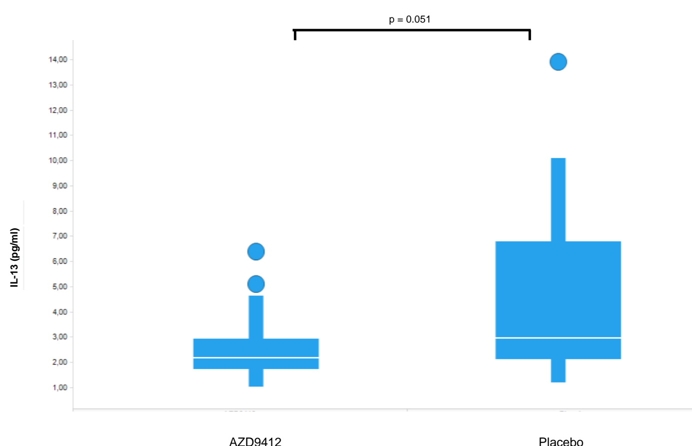

Figure 14. Serum IL-13 levels at 2 weeks post end of inhaled IFNβ-1a or placebo treatment. *IL-13 was measured in serum samples using an MSD U-plex immunoassay. AZD9412: IFNβ-1a. p-value is determined using Kruskal-Wallis.*

One possible explanation for the lack of efficacy in the INEXAS trial is that starting treatment within 48 hours of cold symptoms is too late to prevent a worsening of asthma. This is supported by a recent trial of the RV capsid binder vapendavir and also the time courses of upper and lower respiratory tract symptom scores in asthmatics following experimental RV challenge (27, 105). To get around this, other dosing regimens such as post-exposure or seasonal prophylaxis are worthy of exploration. However, when it comes to prophylaxis, further safety studies would be required to show tolerability to inhaled IFNβ-1a dosing over longer periods of time. Should the results of future tolerability studies impose limits on dosing frequency, less frequent dosing (e.g. once per week) could be evaluated. However, the kinetics of the IFN response has not been explored

extensively enough to know what dosing intervals could be therapeutically viable. We have recently shown that overnight treatment of macrophages with IFNβ is sufficient to induce ISGs, and protect cells from influenza infection, for up to 1 week (unpublished observations). This provides evidence that once per week, prophylactic dosing could be a viable approach to evaluate the efficacy of inhaled IFNβ-1a for the prevention of virus-triggered exacerbations.

4.3 The role of viruses, bacteria and human beta defensin-2 in exacerbations of COPD (**Papers III-IV**)

Infections are believed to be important drivers of COPD both in stable disease and at exacerbation. The importance of respiratory viral infections as exacerbation triggers is well established. Different endotypes of exacerbation have been characterized based on the presence or absence of viral or bacterial pathogens, although the prevailing opinion in the field is that the viral infection is the initial trigger, which then precipitates an outgrowth of pathogenic bacteria in the lung. As discussed in Section 1.6.1, evidence from both experimental RV challenge and naturally occurring RV infections supports this (26, 168).

To address to some depth the role of different trigger factors in COPD exacerbations, we chose to perform a 2-year longitudinal observational study in a cohort of COPD patients in Guangzhou, China. The motivation for performing the study in China was based on several considerations. First, since air pollution is known to be an important driver of exacerbations in China, and Guangzhou is a large metropolitan area with high pollution levels (254), we had the possibility to study air pollution, and its interaction with viruses and bacteria, as a driver of exacerbations. Second, China is a nation of strategic importance to AstraZeneca, both as a research base and as a major market for respiratory medicines. Third, similar exacerbation studies had been performed in European and North American cohorts, and the cohort at Guangzhou gave us the possibility to compare and contrast findings from those studies (249). Finally, Professors Nanshan Zhong and Rongchang Chen at the Guangzhou Institute for Respiratory Diseases are leading experts in COPD, with access to patients across the severity spectrum (many previous western cohort studies were enriched for frequent exacerbators and at Guangzhou, the cohort would be more representative of a real-world population).

The Guangzhou COPD study was completed over a 2.5-year period, with the first subject enrolled in mid-2010 and last subject, last visit in early 2013. The

overall exacerbation rate was 0.4 per year, with very little seasonal variation. This contrasts with previous studies which have shown a peak around Christmas time (21, 22). However, since the Christmas peak is believed to be precipitated by families gathering to celebrate, and Christmas is not a major celebration in China, it is not entirely surprising that such a peak was not observed in Guangzhou. The month of March showed a higher exacerbation rate in two of the three years. Following the completion of all patient visits, a biomarker plan was devised to permit the investigation of biological endotypes associated with the clinical, microbiological and environmental data that had already been gathered. A wide range of biomarkers were measured in sputum, serum and/or plasma and multivariate modelling of all data is ongoing.

One such biomarker, hBD-2, became the central topic of **Paper IV**, due to its relevance to host defense against respiratory pathogens. Our hypothesis was that COPD patients have deficient immunity against pathogens, and previous literature on hBD-2 in COPD made this AMP an intriguing candidate (26, 67, 189, 191, 192, 255, 256). We initially found that serum and sputum hBD-2 levels were not significantly different between COPD patients and control subjects. However, it was clear that some patients had lower levels of hBD-2 in sputum. We went on to show that the patients with lower sputum hBD-2 tended to be those who had at least 1 exacerbation during the 2-year observation period. Further analysis showed that low hBD-2 levels were associated with risk of exacerbation in following 12 months. We next hypothesized that low hBD-2 levels might associate with the presence of sputum pathogenic bacteria during stable disease. However, no such association could be found, and neither was there any association with respiratory viruses in the nose or sputum. Subsequently, we turned our attention to hBD-2 levels at exacerbation. We expected hBD-2 levels to increase at exacerbation, given that is it inducible following inflammatory signaling (257). However, although some patients showed an increase of hBD-2 from stable disease to exacerbation, others did not. When we compared pathogen status at exacerbation in patients with low or high hBD-2 levels at exacerbation, we found that patients with low hBD-2 were more often positive for a respiratory virus. This might suggest that respiratory viral infection somehow suppresses the hBD-2 response, which may in turn render the airways more susceptible to outgrowth of pathogenic bacteria. For a summary of the viral and bacterial detection rates at stable disease and exacerbation, please refer to **Paper IV**.

In agreement with the work of others, we showed that virus positive exacerbations were characterized by high levels of serum and sputum CXCL10, CXCL11 and IFN-γ (248-250). As discussed in Section 1.6.2, there is evidence for a relationship between IFN signaling and promotion of bacterial infections, leading us to speculate that the virus-induced IFN response could be one mechanism

by which hBD-2 levels are suppressed in the lungs of COPD patients at the time of exacerbation. This a topic worthy of further investigation.

There are clear limitations to our findings. First, we only investigated a single AMP in **Paper IV**. We chose to study hBD-2 due to the fact that it is inducible (and therefore arguably more susceptible to dysregulation), it is exclusively expressed by epithelial cells, and there are reports of both genetic association with, and altered expression in, COPD. However, as discussed in Section 1.6.3, there are several families of AMPs which are expressed in the lung, and it would be of interest to investigate the association of those with exacerbations. For example, Mallia et al demonstrated reduced levels of SLPI and elafin following experimental RV challenge in COPD patients (26). Another limitation is that our findings are based on a single cohort. It would be important to replicate these findings in additional cohorts, in both China and other parts of the world.

Additional analyses have been performed on the Guangzhou study which were beyond the scope of **Paper IV**. Daily air pollution levels (sulphur dioxide (SO_2) and particulate matter of ≤ 10 µm in diameter (PM_{10})) were collected throughout the study. First, we investigated whether the time course of either pollutant was correlated with exacerbation rates. Although there was no overall correlation, we found that the highest monthly average level of PM_{10} occurred contemporaneously with the highest peak of exacerbation frequency, in March 2012 (**Figure 15**). Furthermore, in a discrimator analysis for the prediction of exacerbations, PM_{10} emerged as one of the strongest predictors (**Figure 16**) (250). A more detailed analysis of the 6-week period covering these peaks revealed 2 weeks with increased exacerbation frequency, which were preceded by sharp increases in PM_{10}. A 1-week lag time between the PM_{10} increase and disease events is similar to what has been described previously (258, 259). There may be an interaction between air pollution and other exacerbation risk factors such as viral infections (260-262). To this end, we investigated the correlation between PM_{10} or SO_2 and virus positive and virus-negative exacerbation rates separately. Intriguingly, we found a significant correlation between SO_2 levels and virus-positive exacerbations (**Figure 17**). There are some reports which support a link between SO_2 and innate immunity, but further mechanistic studies are warranted (263-269).

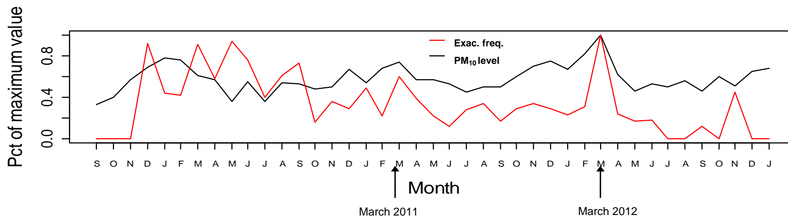

Figure 15. The relationship between air pollution (PM_{10}) and exacerbation frequency in the Guangzhou COPD cohort.
Monthly maximum PM_{10} values (black line) and exacerbation frequencies (red line) expressed as proportion of the maximum observed value.

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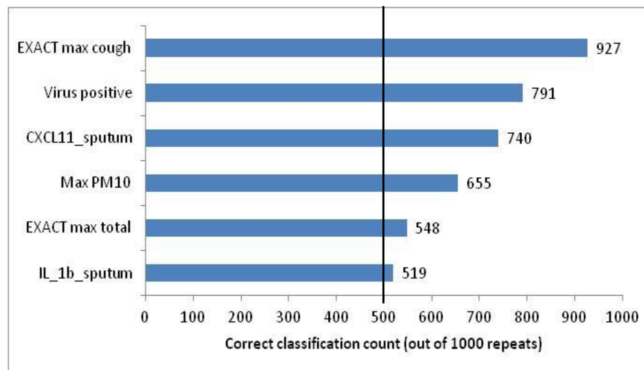
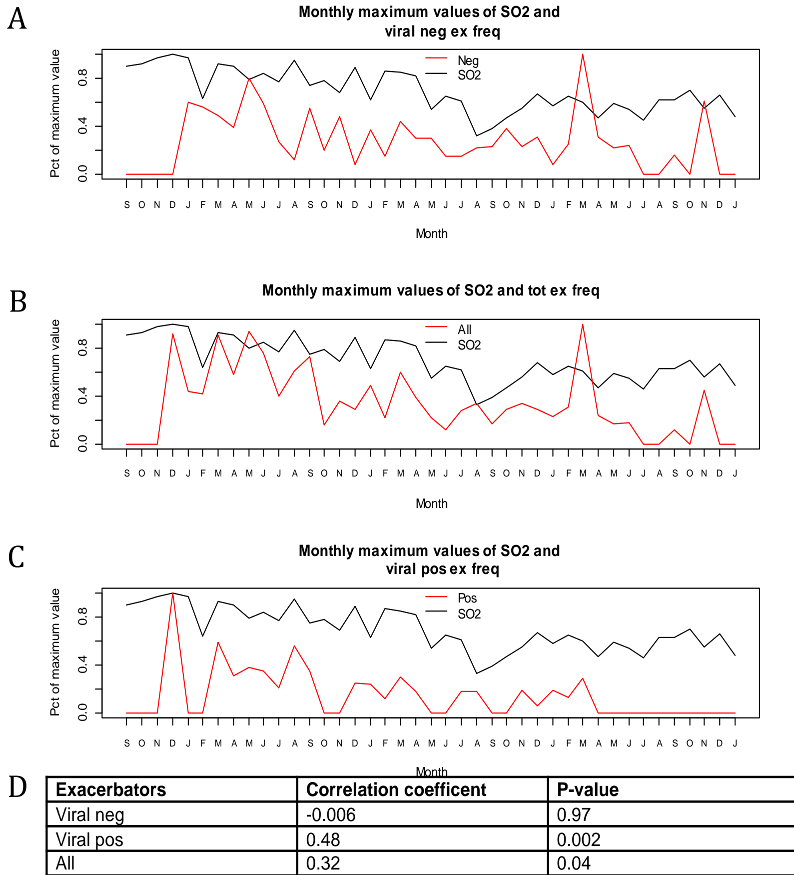


Figure 16. Factors which discriminate exacerbation months from exacerbation-free months in the Guangzhou COPD cohort.
The following steps were repeated 1000 times for all available clinical and biomarker variables, each with the 161 exacerbation months (months in which a given patient had an exacerbation) and a new random set of 161 exacerbation-free months (months in which for a given patient, an exacerbation did not occur): 1. The most significant variables for discriminating between exacerbation months and exacerbation-free months were selected; 2. The selected variables from step 1 were assessed for their ability to correctly classify an exacerbation or an exacerbation-free month. The overall error rate for the classifications (using cross validation) was 29%. Shown in the graph are the 6 variables that correctly classified exacerbations vs. stable disease >50% of the time. (CXCL11 = CXC motif chemokine ligand 11; IL_1b = interleukin-1β; EXACT max cough, EXACT total = symptom scores derived from EXACT-PRO)



Exacerbators	Correlation coefficent	P-value
Viral neg	-0.006	0.97
Viral pos	0.48	0.002
All	0.32	0.04

Figure 17. The relationship between air pollution (SO_2) and exacerbation frequency in the Guangzhou COPD cohort.
Monthly maximum SO_2 values (black lines) and exacerbation frequencies (red lines) expressed as proportion of the maximum observed value. A: Virus negative exacerbations only; B: All exacerbations; C: Virus positive exacerbations only; D: Correlation coefficient and p-value for the time profile of SO_2 levels versus exacerbation frequency.

5. Conclusions

The main conclusions from this thesis are:

Paper I

Phenotypic functional genomics screening using RNAi can be used to successfully identify targets and pathways which can be modulated to affect RV replication in NHBE cells.

Inhibition of LSS inhibits RV replication in primary NHBE cells via a mechanism which involves the induction of 24(S),25 EC but not LXR agonism. The anti-viral effect was observed against serotypes of both the major (RV16) and minor (RV2) groups, indicating broad spectrum anti-RV activity. In addition, we demonstrated that RV-induced IFNβ production is enhanced by LSS inhibition, suggesting that this mechanism has a dual role, inhibiting viral infection both directly and indirectly through promoting anti-viral host defense. Inhibition of LSS is thus a promising target for the prevention of RV-triggered exacerbations of asthma and COPD.

Paper II

When given following symptomatic colds or flu, inhaled IFNβ-1a was not able to prevent the occurrence of severe asthma exacerbations but did significantly improve lung function. Reasons for the lack of effect on exacerbations may be in part due to the start of treatment being too late. Exploratory subgroup analysis suggested that patients with high blood eosinophils or low IL-18 relative change at treatment baseline, have the greatest improvement in lung function by IFNβ-1a. Overall, the results suggest that inhaled IFNβ-1a could be a valuable therapy for the prevention of asthma worsening following viral infections, but that further research into treatment regimen and responder populations is required to maximise the potential for treatment benefit.

Papers III-IV

COPD patients in China were successfully followed up for 2 years, with capturing of exacerbations during that period. The results of the study support a relationship of exacerbations with viral and bacterial infections. Furthermore,

increased levels of the air pollutants, PM_{10} and SO_2, are also associated with exacerbations, with a potential interaction between SO_2 and viral infections at exacerbation.

Low levels of sputum hBD-2 in stable disease are associated with the future occurrence of exacerbations. At exacerbation, the presence of a respiratory virus is related to low levels of hBD-2. Finally, viral exacerbations are associated with increased levels of CXCL10, CXCL11 and IFNγ in serum and/or sputum, supporting the results of previous studies. Overall, these findings suggest that COPD patients with impaired innate anti-microbial defense through low hBD-2 levels are at increased risk of exacerbation, especially in the context of an acute respiratory viral infection. Therapeutic approaches which boost hBD-2 levels in the airway may prove to be useful for the prevention of COPD exacerbations.

6. Future Perspectives

Taken together, this thesis has identified factors which play an important role in the complex relationship between respiratory viruses and their human hosts in the development of exacerbations of asthma and COPD. A wide-ranging approach has been utilized in the research performed, from the mechanistic *in vitro* studies described in **Paper I**, through a randomized, placebo-controlled clinical trial in **Paper II,** to a longitudinal observational study of **Papers III-IV**.

Over the last 5-10 years, significant developments have been made in understanding host-Picornavirus interactions, with the discovery of how Picornaviridae such as RV manipulate intracellular lipid structures such as the Golgi apparatus to facilitate viral replication. As discussed in Sections 1.5.2.3 and 4.1, several proteins involved in lipid metabolism have been described to be hijacked by Picornaviridae. However, our finding in **Paper I** that LSS could be one such component is novel.

In investigating the mechanism by which LSS inhibition exerts an anti-viral effect, we discovered that 24(S),25 EC is both induced by partial LSS inhibition and is anti-viral in its own right. One of the known effects of 24(S),25 EC is to stimulate LXR, which in turn drives cholesterol efflux through transporters such as ABCA1. Using either an LXR agonist or antagonist, we could exclude LXR agonism as the mechanism through which LSS inhibitors exert an anti-viral effect. The other known effects of 24(S),25 EC are to inactivate SREBP by sequestering it in the ER membrane, a transcription factor which drives the expression of multiple key components of cholesterol biosynthesis, and to directly inhibit HMG-CoA reductase (234). In **Paper I**, the role of those mechanisms has not been explored.

The finding in **Paper I** that LSS inhibition leads to an enhancement of RV-induced IFNβ production is consistent with another report by York et al suggesting an inverse relationship between cholesterol biosynthesis and type I IFN responses (238). However, we have not investigated the mechanisms driving this IFNβ enhancement and whether the mechanism is similar to that described in the previous report by York et al. Our results do, however, suggest that induction of 24(S),25 EC may be involved in the IFNβ enhancement and, given that York et al demonstrated the importance of ER components in their findings, it is intriguing to speculate that the actions of 24(S),25 EC at the ER membrane might lead to the same or similar mechanisms.

As suggested above, further study on the role of LSS in RV replication should focus on the mechanism(s) by which 24(S),25 EC drives both the direct anti-viral effect and the IFNβ-enhancing effect, e.g. by investigating the effect of knocking down or knocking out SREBP or STING. Furthermore, the spectrum of RV species affected by this mechanism needs to be explored further, with particular attention paid to RV-C. Finally, the role of LSS and other recently-described mechanisms of viral hijack, such as the PI4KIIIβ-induced re-organisation of the Golgi apparatus to form viral replication organelles, would be worthy of exploration.

In **Paper II**, we evaluated the efficacy of inhaled IFNβ, perhaps one of the best characterized components of anti-viral innate immunity, for the prevention of severe asthma exacerbations following symptomatic colds or flu. The lack of efficacy on severe exacerbations in the INEXAS trial does not in any way suggest that IFNβ is not an important anti-viral defense protein, nor does it suggest that viral infections are not important triggers of exacerbations. On the contrary, it was clear that asthma worsening occurred following cold or flu symptoms, but the worsening may have peaked too soon for this therapeutic dosing regimen to successfully prevent it. The effect of IFNβ-1a on lung function was a promising finding, which suggests that with optimisation of treatment regimen, efficacy could be improved. Future study of inhaled IFNβ-1a in asthma should evaluate different treatment regimens, e.g. prophylaxis (year-round, seasonal or post-exposure) vs therapeutic, for example utilizing the well-established experimental viral challenge models that are available.

The INEXAS trial was focused on asthma patients and it is possible that this mechanism may be efficacious for the prevention of COPD exacerbations. A trial in COPD similar in design to the INEXAS trial is currently in progress, and it will be intriguing to understand whether the same timing issues may apply in COPD patients, or if the longer duration and secondary bacterial infections which occur provide a greater window of opportunity for a efficacy (179). As discussed in Section 1.6.2, the relationship between type I IFN and risk of bacterial infections is somewhat unclear, especially in humans, and this trial may provide the opportunity to gain some understanding of how well the reported mouse *in vivo* studies translate to humans.

In an exploratory analysis, we found that patients with either high blood eosinophils or low IL-18 relative change from screening, tended to exhibit the best lung function effect of inhaled IFNβ-1a. As discussed in **Paper II,** a biological rationale can be made for these subgroups, however further confirmation in larger, better-powered studies would be required before these subgroups can be regarded with any confidence as potential responder populations.

Whether or not the INEXAS trial cohort is a truly virus-susceptible population has been discussed in **Paper II**. There appeared to be no relationship between the magnitude of the endogenous IFN response (as measured by CXCL10 levels) and efficacy, and it is not known whether the patients studied were in any way "IFN deficient". Patients were, however, required to have had a history of exacerbations related to upper respiratory tract infections. As mentioned in Section 3.3.1, our recent epidemiological work in the West Sweden Asthma Study cohort has identified a potential "viral exacerbator phenotype". Further profiling of patients exhibiting this phenotype is under way and might lead to improved methods for the identification of the patients most susceptible to viral exacerbations (and, in turn, perhaps more likely to respond to inhaled IFNβ-1a or other similar anti-viral therapies).

On a technical note, a challenge in understanding the pharmacokinetic/pharmacodynamic relationship in trials of IFNβ-1a is that the sensitivity of the currently-available assays is insufficient to permit reliable quantification of IFNβ. This means that a surrogate biomarker such as CXCL10 has to be used to assess drug response. Future development of inhaled IFNβ-1a would benefit from recent advancements in immunoassay technologies which provide sensitivity down to fg/ml concentrations. Increased sensitivity to detect IFNβ may also provide a method to identify "IFN-deficient" patients via blood sampling, something which is also currently not possible with the sensitivity of the currently-available assays. At the time of writing, no IFNβ assay has been commercialized using the more sensitive assay technologies, although efforts should be made to do this.

We have shown in our Chinese COPD cohort that low sputum hBD-2 levels are associated with risk of exacerbation. It could be that these findings are by chance, or alternatively are cohort- or region- specific. The results therefore need to be replicated in additional cohorts of frequent and infrequent exacerbators, to ensure that the finding is applicable across multiple cohorts and parts of the world. As discussed in Sections 1.6.3 and 4.3, hBD-2 is just one of many AMPs and the role of others should be explored further using samples from the Guangzhou and other similar studies and thus building on, for example, the findings of Mallia et al.

Although we have identified a relationship between hBD-2 levels in sputum and a clinical outcome (i.e. exacerbation risk), the mechanism responsible for these low levels of hBD-2 have not been explored. For example, it is not known whether it is the induction of hBD-2 mRNA expression that is defective or alternatively some post-translational effect such as increased proteolytic cleavage. The latter seems likely since COPD lungs have been shown to have higher levels of proteases such as cathepsins and neutrophil elastase. Such proteases could be measured in the sputum to determine whether their levels correlate with hBD-2

measured in the same samples. A direct effect of NE and other proteases on cleaving hBD-2 could be investigated *in vitro,* e.g. using western blotting and bacterial infection assays to confirm effect on hBD-2 activity.

It would be intriguing to investigate whether there is any relationship between the IFN response and hBD-2 levels. In **Paper IV**, we show that virus positive exacerbations are characterized both by low hBD-2 and high CXCL10, CXCL11 and IFNγ, but we have not shown whether hBD-2 levels and IFN response biomarkers are mechanistically associated with one another. This could also be investigated in *in vitro* models of viral infection, or indeed viral/bacterial co-infection, using either submerged AEC or ALI cultures. Using such models, the relative roles of hBD-2 and IFN can be investigated by either measuring the endogenous response to viral/bacterial infections or by determining the effect of pre-treatment with exogenous hBD-2 on viral/bacterial host interactions.

Paper III illustrates the breadth of data available from the Guangzhou COPD study, which permits extensive multivariate analysis of the different kinds of data obtained. Using such multivariate models in an unbiased way could allow the identification of the most important determinants of exacerbation risk. Preliminary, as-yet unpublished multivariate analyses have shown that viral infections and PM_{10} are among the most important determinants of exacerbation risk. Of the soluble biomarkers measured in sputum and serum, sputum hBD-2 stands out as one of the strongest and interestingly does not correlate with any of the pro-inflammatory biomarkers measured (not shown). It will be extremely valuable to complete these multivariate analyses and to confirm the preliminary findings.

When analyzing air pollutants (PM_{10} and SO_2) over time, we see a relationship with exacerbation frequency. More sophisticated modelling of the pollution data is planned in order to strengthen these findings. The relationship between SO_2 levels and the frequency of virus-positive exacerbations is intriguing. In addition to confirming this finding with more sophisticated modelling, *in vitro* viral/bacterial infection models like the ones described above could be utilized to determine the effect of SO_2 on virus/bacteria-host interactions.

A common theme emerges through the somewhat diverse studies presented in this thesis: the IFN response is central, not only to the intricate intracellular virus-induced events that occur involving lipid shuttling and metabolism, but also to the downstream consequences of infection at the tissue and organ level, likely playing a key role in both anti-bacterial and anti-viral host defense. Despite the extraordinary complexity of the interaction between the virus and its host, this thesis demonstrates that key drivers of this interplay can be identified, manipulated and, hopefully, developed into future medicines for the prevention of asthma and COPD exacerbations.

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Appendix