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FACULTY OF MEDICINE

Virus-host interactions following experimental rhinovirus infection in airways disease

by

Dr Peter Adura

Thesis for the degree of Doctor of Medicine

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Abstract

Rhinoviruses (RVs), the commonest cause of the common cold, are a major cause of exacerbations of asthma, and smoking-induced chronic obstructive pulmonary disease (COPD). Asthmatics do not suffer more frequent RV infections, however they endure more severe and more prolonged lower respiratory tract symptoms during the common cold. In smokers, colds are both more prevalent, and induce more severe lower respiratory tract symptoms. Experimental RV infections have been used for a number of years as a model to explore various aspects of virus-host interactions in mild asthma that contribute to exacerbations of asthma. There is less experience with smokers (with or without COPD). The biology of severe asthma is different from that of mild asthma in several aspects. In this light it is inappropriate for our understanding of the mechanisms of virus-induced asthma exacerbations to be based on extrapolations and generalisations of findings from studies performed in mild asthma. Models of virus-induced exacerbations of asthma in moderate asthma, and ultimately in severe asthma, are needed. Similarly, models of virus-host interactions in smokers without COPD and ultimately with severe COPD are also needed. Biomarkers that predict clinical severity of virus-induced exacerbations are also required to identify asthmatics who are likely to suffer the most from virus-induced exacerbations of asthma, and therefore likely to benefit the most from novel and perhaps expensive therapies for virus-induced exacerbations of asthma. Given that virus-induced exacerbations of asthma and COPD lead to severe morbidity and healthcare consumption, it is important that experimental RV studies in these groups are carefully thought out to enable elucidation of mechanism of virus-host interactions without serious jeopardy to the health of the participants in such studies.

The overall aim of this thesis was to evaluate the safety and utility of the experimental RV infection model in moderate, corticosteroid-treated asthma, and to improve our understanding of the virus-host interactions during virus-induced asthma exacerbations; also, to expand the model's utility by applying it to smokers. The hypotheses were that it is feasible, and safe to experimentally induce a clinical RV illness in moderate asthma, and in smokers with or without COPD; that innate immunity can be induced and assessed in moderate asthma, and smokers following experimental RV infection; and that the *ex vivo* antiviral response to synthetic double-stranded RNA of peripheral blood of asthmatics is less than that of healthy controls, and is predictive of the severity of experimental rhinovirus-induced clinical illness.

A previously validated infection protocol with minor modifications used to inoculate the subjects. The modifications ensured that the inoculum was deposited exclusively to the nose. Symptoms of upper and lower respiratory tract infection were recorded as well as home, and departmental measurements lung function. The effects on airways inflammation were assessed in induced sputum, and by exhaled measurement of expired nitric oxide measurements. Infection was confirmed by quantitative polymerase chain reaction for RV16 in nasal lavage, and sputum, and by determining RV16 neutralising antibody titres in serum.. Innate immune responses were studied by quantifying the induction of virus-response genes interferon (IFN)β, IFNγ-inducible protein 10 (IP-10), myxoma resistance protein A (MxA), and 2'-5' oligoadenylate synthetase (OAS) in sputum, and peripheral blood, and by measuring IP-10 protein in nasal lavage and sputum. Finally, *ex vivo* induction of IFNβ by synthetic double-stranded RNA, polyinosinic:polycytidylic acid (poly (I:C)), was assessed before infection as a surrogate for virus-induced, TLR-3-mediated responses, and predictor of severity of clinical illness in asthmatics.

The inoculation induced $\geqslant$4-fold increase in serum RV16 neutralising antibody titres and/or RV16 detection in the airways. Infection caused measurable upper and lower respiratory tract symptoms. These symptoms were mild, well-tolerated and caused no unforeseen adverse effects. None of the participant required systemic steroids or hospitalization. Increased bronchodilator use was observed in the asthmatics . The infection caused a transient induction of the antiviral response genes mentioned above in the nose, lungs, and peripheral blood. *Ex vivo* antiviral gene expression in response to poly (I:C) negatively correlated with measures of clinical illness severity in asthma.

The studies indicated that experimental RV infection in these two groups is feasible, safe, and a valuable model for exploring mechanisms of virus-induced exacerbations of airways diseases in line with previous findings. Some of the findings were unexpected based on current understandings of virus-host interactions in these groups of subjects. Given the small number of subjects, and the lack of previous experimental RV infection in these subjects, some of the findings must be considered as preliminary and needing confirmation in future studies.

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Author declaration

I, ……Dr Peter Adura …..,

declare that the thesis entitled

……
Virus-host interactions following experimental rhinovirus infection in airways disease
……..

and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

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Experimental Rhinovirus Infection In Long-Term Smokers Am. J. Respir. Crit. Care Med. 185: A1445.

Experimental Rhinovirus-16 Challenge In Moderate Asthma Am. J. Respir. Crit. Care Med. 2010; 181: A5336.

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Abbreviations

AHR – airway hyperresponsiveness

BAL – bronchoalveolar lavage

BEC – bronchial epithelial cells

COPD – chronic obstructive pulmonary disease

DALY – disability-adjusted life year

ECP – eosinophil cationic protein

ELISA – enzyme-linked immuno-sorbent assay

eNO – exhaled nitric oxide

FEV_1 – Forced expiratory volume in 1 second

FVC – forced vital capacity

GAPDH – Glyceraldehyde-3-Phosphate Dehydrogenase

GINA – Global Initiative for Asthma

GM-CSF – granulocyte monocyte colony stimulating factor

GOLD – Global initiative for chronic Obstructive Lung Disease

ICAM-1 – intercellular adhesion molecule – 1

ICS – inhaled corticosteroids

IFN – interferon

IL – interleukin

IP-10 – interferon-gamma inducible protein-10

LABA – long-acting bronchodilator

LRT – lower respiratory tract

MHC – major histopathology complex

mRNA – messenger ribonucleic acid

MxA – Myxoma resistance protein A

NK – natural killer

OAS – 2'-5' oligoadenylate synthetases

PBMC – peripheral blood mononuclear cell

PC_{20} – provocation concentration of histamine of methacholine inducing a 20% decline in FEV_1

PCR – polymerase chain reaction

PEF – peak expiratory flow rate

REC – Research Ethics committee

RTI – respiratory tract infection

RV – rhinovirus

TNF – Tumour Necrosis Factor

tRNA – transport ribonucleic acid

UBC – ubiquitin

URT – upper respiratory tract

URTI – upper respiratory tract infection

Chapter 1 - Introduction

1.1 Burden of airways diseases of asthma and COPD

Asthma is a major global healthcare problem, with an estimated 300 million people of all ages affected. Estimates of its prevalence vary between 1% to 18% of the population in different countries (GINA, 2012). The social and economic burden of asthma is considerable. This is due to hospital admissions, cost of medications, time lost from work, negative impact on the quality of life of patients, and their families, and premature deaths (Hoskins, 2000). The Global Initiative for Asthma (GINA) currently recommends that asthma severity be classified based on the intensity of treatment required to achieve good asthma control (GINA, 2012; Taylor, 2008). This concept classifies asthma by severity as intermittent, mild, moderate or severe (Taylor, 2008). Intermittent asthma requires only inhaled bronchodilator treatment as needed. Mild asthma requires low-dose inhaled corticosteroid (ICS) or other low-intensity treatment. Moderate asthma requires low- to moderate-dose ICS with or without other extra treatment. Finally, severe asthma requires high-intensity treatment (Table 1.1). It is estimated that 70 – 77% of asthmatics would be classed as moderate to severe when their symptoms are carefully assessed (Fuhlbrigge, 2002).

Asthma severity	Lowest level of treatment required to achieve patient's best level of asthma control
Intermittent	Bronchodilator as needed
Mild	Low-dose ICS or other low-intensity treatment
Moderate	Low- to moderate-dose ICS and other extra treatment
Severe	High-intensity treatment (high-dose ICS and LABA ± oral corticosteroids and/or other extra treatment)

Table 1.1 Asthma Classification
Asthma classification by severity. Modified from Taylor *et al* (Taylor, 2008). Severity is based on the intensity of treatment required to control the patient's asthma once the diagnosis has been confirmed, co-morbidities treated, and inhaler technique and adherence to therapy have been optimized.

The reported prevalence of chronic obstructive pulmonary disease (COPD) varies between 4% - 10% in countries where it has been measured rigorously (Halbert, 2003). This high prevalence also has significant social and economic implications(1). The burden is due to healthcare resources devoted to diagnosis and medical management, the monetary consequences of disability, missed work, premature mortality, and caregiver or family costs resulting from the illness. It has been estimated that COPD was the 12th leading cause of years lost due to ill-health, disability or early death worldwide – measured in Disability-Adjusted Life Years (DALYs) in 1990, being responsible for 2.1% of DALYS at that time (Lopez, 2006). COPD is one of the most important causes of death in most countries. It has

been projected that by 2020, COPD could be the fifth leading cause of DALYs worldwide behind ischaemic heart disease, major depression, traffic accidents, and cerebrovascular disease, and the fourth leading cause of death worldwide by 2030 (GOLD, 2013). Its age-adjusted mortality is currently reported as between 4.4 to as high as 86.4 per 100,000 in Japan and Vietnam respectively (Mannino, 2007). The Global initiative for chronic Obstructive Lung Disease (GOLD) recommends that the severity of COPD should be classified according to the degree of airflow obstruction measured by spirometry after bronchodilator use(GOLD, 2013). The ratio of forced expiratory volume in one second (FEV_1) to forced vital capacity (FVC), FEV1/FVC, of less than 0.7 indicates the presence of airflow obstruction, while the severity of COPD is based on the degree of airflow obstruction as follows. In mild COPD, FEV_1 is 80% or more of predicted, in moderate COPD 50 – 79% of predicted, in severe COPD 30 – 49% of predicted and in very severe COPD 29% or less of predicted (Table 1.2).

COPD Severity	FEV_1/FVC ratio	Spirometry: FEV_1 as % predicted
Mild	< 0.7	$\geq$ 80
Moderate	< 0.7	50 – 79
Severe	< 0.7	30 – 49
Very severe	< 0.7	$\leq$ 29

Table 1. 2 COPD Classification
COPD classification. Based on spirometric classification of post-bronchodilator forced expiratory volume in one second (FEV_1). Modified from GOLD report 2013(GOLD, 2013).

1.2 Pathogenesis of asthma and COPD

Asthma is a chronic inflammatory condition of the airways, with reversible airflow obstruction, and airway hyperresponsiveness as its other hallmarks (Djukanovic, 2000). Its natural history involves recurrent exacerbations (also referred to as acute asthma or asthma attacks). Asthma exacerbations are episodes of progressive increases in one or more of the following of its symptoms: shortness of breath, cough, wheezing, or chest tightness which leads to increased use of quick-acting reliever medications. Symptoms can be mild, moderate or severe and depending on the severity, can necessitate hospitalisation. These exacerbations are often precipitated by viral infections of the upper airways such as the common cold (Johnston, 1995; Wark, 2001). Like asthma, COPD is a chronic inflammatory disease of the airways(Quint, 2007) whose natural history includes exacerbations. Exacerbations of COPD are episodes of progressive increases in one or more of the

symptoms of dyspnoea, cough, and sputum production. These too are often precipitated by viral upper respiratory tract infections (URTI) (Seemungal, 2000).

Airway inflammation is a central pathological process in these two highly prevalent diseases and there has been many investigations attempting to fully characterise the inflammatory processes (Pizzichini, 2002; Wenzel, 2006; Green, 2002; Thomson, 2004 2240 ;Taylor, 2006). Induced sputum has been established as a useful, safe, and non-invasive tool for sampling of the lower airways (Pizzichini, 2002) because it contains the inflammatory cells and cytokines involved in the pathophysiology of asthma and COPD. Sputum induction involves the inhalation of nebulised hypertonic saline and collection of the sputum expectorated during the procedure. Lower airway inflammation is assessed using induced sputum. Inflammatory phenotypes are identified which form the basis of approaches to both research and clinical management of asthma. Based on the types of cells found in the induced sputum by immunohistological staining and cell counts, the inflammatory phenotype of asthma can be classified into eosinophilic, neutrophilic, and pauicigranulocytic (Wenzel, 2006). Each phenotype has a different response to treatment. Eosinophilic asthma, in which eosinophils form >3% of the inflammatory cell population in the induced sputum, is most amenable to treatment with ICS. Neutrophilic asthma, in which neutrophil infiltrates predominate, is seen most commonly in patients with severe disease. This phenotype is less responsive to corticosteroid therapy than eosinophilic asthma(Green, 2002), and the presence of an underlying neutrophilic pathology could also explain the poor response to ICS seen in some patients with asthma who smoke(Thomson, 2004). Paucigranulocytic asthma appears to exist in the absence of an identifiable influx of inflammatory cells such as eosinophils, neutrophils, or lymphocytes. It is not clear whether the absence of infiltrating cells is factual or whether the samples missed the presence or location of inflammation. Some of these patients remain symptomatic on high doses of ICS. Exhaled nitric oxide levels have been shown to be high in subjects with eosinophilic asthma and to decrease with appropriate treatment. Episodes of clinical exacerbations are heralded by increased levels of exhaled nitric oxide in such patients (Taylor, 2006).

Respiratory abnormalities in COPD comprise of airway inflammation, largely irreversible airflow obstruction, destruction of lung parenchyma, small airway fibrosis, and periods of exacerbations (GOLD, 2013). It is now accepted that individual susceptibility coupled with exposure to inhaled noxious substances leads to the development of COPD (Mannino, 2007). Globally, cigarette smoke is the commonest noxious agent. In high-income countries, 73% of COPD mortality is related to smoking, with 40% related to smoking in nations of low, and middle income. Exposure to biomass smoke, occupational exposure to

dust and fumes, history of pulmonary tuberculosis, history of chronic asthma, outdoor air pollution, and poor socioeconomic status are the most important risk factors in non-smokers (Salvi, 2009). In the smoking-induced airway inflammation of COPD, a marked increase in number of neutrophils and macrophages in induced sputum, bronchoalveolar lavage and bronchial biopsies is evident (Keatings, 1996; Pesci, 1998;). Neutrophil and macrophage numbers are correlated with COPD disease severity (Keatings, 1996). Neutrophil numbers also correlate with the rate of decline in lung function (Stanescu, 1996). Corticosteroids are ineffective in suppressing airway inflammation in patients with COPD (Keatings, 1997; Culpitt, 1999)

Overlaps and similarities in the clinical presentation as well as underlying inflammatory phenotypes of asthma and COPD are found for the following reasons. Individuals with asthma may also be exposed to air-borne noxious agents leading to COPD-type airway inflammation (Thomson, 2004). Bacterial and viral infections lead to exacerbations (Seemungal, 2000; Johnston, 1995; Johnston, 1996). In some patients, asthma and COPD may co-exist, while in others, chronic asthma is a risk factor for the development of COPD phenotype (Silva, 2004). A sputum eosinophil count above 3% can be seen asthma (Wenzel, 2006) and COPD (Pizzichini, 1998; Brightling, 2000; Chanez, 1997). Evidence that the presence of sputum eosinophilia is predictive of an objective response to corticosteroid treatment in COPD is emerging, suggesting that eosinophilic airway inflammation is functionally important in some subjects with COPD (Brightling, 2005) and asthma (Green, 2002).

1.3 Importance of virus-induced exacerbations in asthma and COPD

Asthma exacerbations are often precipitated by viral infections of the upper airways such as the common cold (Johnston, 1995; Wark, 2001). Asthmatics suffer longer-lasting and more severe lower respiratory tract (LRT) symptoms from common colds (Corne, 2002), causing predictable common cold-induced peaks in hospital admissions every year(Johnston, 1996). Corne *et al* (Corne, 2002) prospectively studied 76 cohabiting couples, one of which had atopic asthma. They observed that people with atopic asthma are not more likely to acquire a natural rhinovirus infection given the same exposure than healthy individuals, but suffer from more frequent LRT involvement, leading to more severe and longer-lasting LRT symptoms following rhinovirus infections.

The picture is remarkably similar in COPD. The common cold is also the cause of much morbidity in COPD, with rhinoviruses (RV) as the most common cause of virus-induced exacerbations (Seemungal, 2001; El-Sahly, 2000; Rohde, 2003; Hutchinson, 2007), 50% of colds leading to an exacerbation in all COPD subjects (Hurst, 2005). Seemungal *et al* (Seemungal, 2001) studied the effects of respiratory viral infection on the time course of COPD exacerbations. The study monitored changes in systemic inflammatory markers in stable COPD and at exacerbation in 83 patients with COPD who were asked to record daily peak expiratory flow rate and any increases in respiratory symptoms. Nasal samples and blood were taken for respiratory virus detection by culture, polymerase chain reaction and serology. Plasma fibrinogen and serum interleukin-6 (IL-6) were determined at stable baseline and exacerbation. Colds were found to occur up to 18 days before 64% of exacerbations. Viruses were detected in 39.2% of COPD exacerbations. The majority of these were rhinoviruses. Viral exacerbations were associated with increased dyspnoea, a higher total symptom score at presentation, and a longer median symptom recovery period of 13 days (compared to 6 days in non-viral exacerbations).

Every year, there are predictable peaks in asthma and COPD exacerbations when there are more viral infections prevalent in the community (Johnston, 1996; Moineddin, 2008). Johnston *et al* (Johnston, 1996) conducted a time-trend analysis, comparing the seasonal patterns of respiratory infections and hospital admissions for asthma in adults and children. During a one year study, 108 school-age children monitored upper and lower respiratory symptoms and took peak expiratory flow rate (PEF) recordings. From children reporting a symptomatic episode or a decrease in PEF, samples were taken for detection of viruses. A total of 232 respiratory viruses were detected. The rates of upper respiratory infection (URI) were compared with the rates of asthma hospital admissions for the same time period for the hospitals serving the areas from which the cohort of school children was drawn. Strong correlations were found between the seasonal patterns of URI and hospital admissions for asthma for paediatric and adult admissions. URI and admissions for asthma were more frequent during periods of school attendance than during school holidays. This study thus demonstrated that upper respiratory viral infections are strongly associated in time with hospital admissions for asthma in both children and adults. Rhinoviruses were the major pathogen implicated, and the majority of viral infections and asthma admissions occurred during school attendance. Moineddin *et al* (Moineddin, 2008) on the other hand, investigated seasonal and temporal patterns in primary care visits for respiratory diseases over a 10-year period. All age groups were included in the retrospective cross-sectional study. Clear and statistically significant seasonal patterns for the overall population were

observed for asthma and COPD. Primary care physician visits peaked in the spring and fall every year corresponding to respiratory syncytial virus and influenza circulations.

1.4 Central role of interferons in antiviral defence

An actively multiplying virus uses the host cell's very own synthesis machinery for its own multiplication, while the cell simultaneously sets in motion innate defence mechanisms for inhibiting viral replication (Pestka, 1987) (Figure 1.1). Virus replication could be didactically summarised as involving transcription of the virus genetic information into mRNA, translation of the mRNA into peptides and proteins, processing of the synthesised proteins, and assembly and release of virus particles. Successful antiviral defence involves disrupting these processes without compromising cell survival. At the cellular level, the most efficient defence leads to elimination of the virus from the cell and cell survival. Less successful defence leads to apoptotic cell death or worse still, cell lysis. Interferons (IFNs) are central in this innate defence. Viral infection leads to the induction, production, and release of IFNs from the affected cell. The released interferon induces further IFN production, induces the expression of IFN-stimulated genes, and induces the production of antiviral products (Pestka, 1987).

Type I IFNs (IFNα, IFNβ) and type III intereferons, (IFNλ) are produced by all nucleated mammalian cells while type II interferon (IFNγ) is produced by immune cells (namely by the T cells) in response to viral infection and other stimuli. The IFNs so released act in an autocrine and paracrine fashion as an anti-viral warning system. Paracrine action warns neighbouring cells that there is in "an enemy in their mist". These pre-warned cells go into a state of preparedness by the induction of inactive enzymes such as myxoma resistance protein A (MxA), protein kinase (PKR) and 2' – 5'oligoadenylate synthetases (OAS) amongst others (Pestka, 1987; Stark, 1998). These inactive enzymes become activated in the presence of double-stranded RNA. The activation of PKR (by activated OAS) triggers the activation of a pathway that ultimately leads to the inhibition of the translation of mRNA (Pestka, 1987). The OAS use adenosine triphosphate (ATP) in 2'-specific nucleotidyl transfer reactions to synthesize 2', 5'-oligoadenylates. The latter activate latent RNase L, which results in mRNA and tRNA degradation and the inhibition of viral replication (Justesen, 2000). MxA is an IFN-induced 76 kDa protein which harbours GTPase activity (Horisberger, 1992), accumulates in the cytoplasm of IFN-primed cells and shows antiviral activity against members of several virus families (Mundt, 2007) as a transcription inhibitor by a mechanism that is not well known. This multi-pronged attack on the processes required for virus-

replication is akin to stepping through a booby-trapped door for the virus. This antiviral state also stops some of the essential cell processes. When this antiviral defence succeeds, the infecting virus is destroyed and the cell survives. When this shutdown is prolonged, the cell undergoes apoptotic cell death, contributing to eradication of the virus infection without inducing an inflammatory process. Inefficient innate defence leads to lytic cell death with the release of a multitude of virus copies and induction of inflammation (Wark, 2005; Uller, 2010). Interferon-gamma-induced protein 10 (IP-10) (also known as chemokine ligand 10, CXCL10) is secreted by bronchial epithelial cells, monocytes, lymphocytes, and neutrophils in response to IFNγ and Tumour Necrosis Factor (TNF)-α(1). This chemokine is a ligand for the CXCR3 receptor and serves as a selective chemo-attractant for both activated T lymphocytes and natural killer (NK) cells. Levels of IP-10 in serum have been shown to be a reliable marker of rhinovirus-induced exacerbation of COPD (Alexopoulou, 2001) and asthma (Wark, 2007).

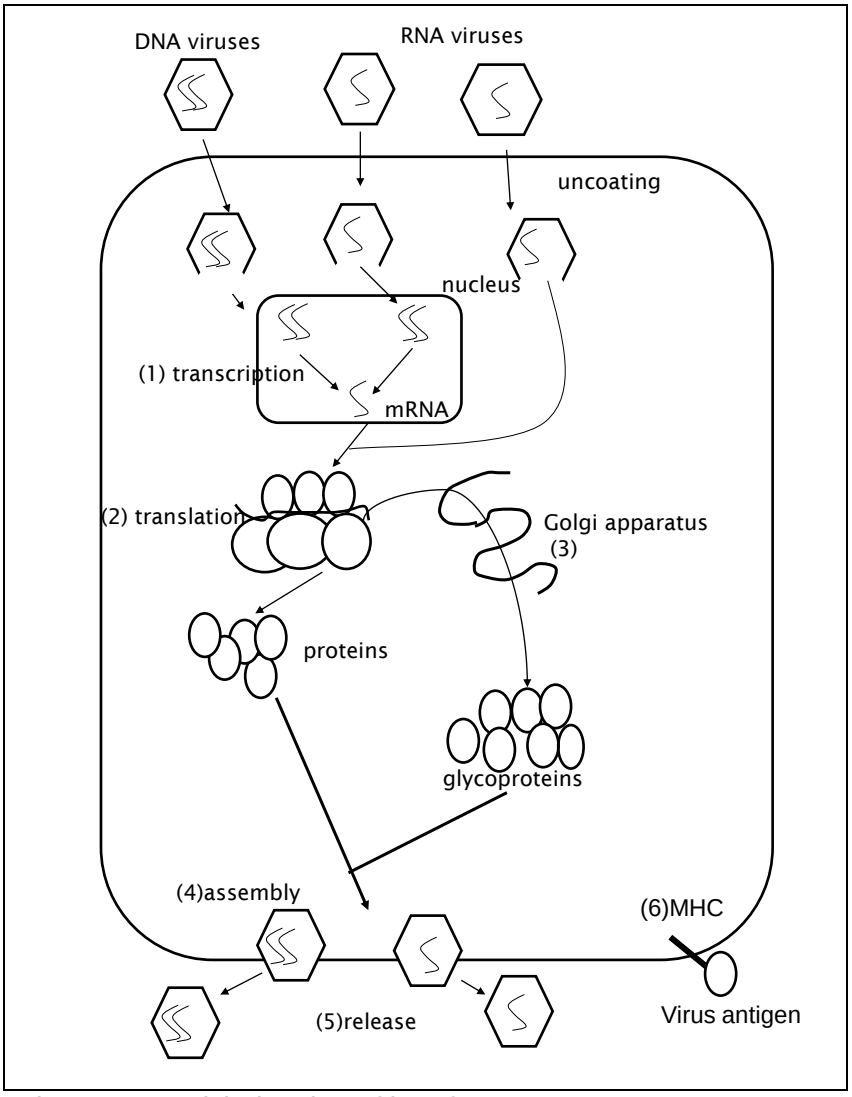


Figure 1.1 Antiviral action of interferons

Interferons induce genes with the following antiviral actions in the target cells. (1) Transcription inhibitors (e.g. Myxoma resistance protein A). (2) Translation inhibitors (e.g. 2' – 5' Oligoadenylate synthetases degradation of mRNA and tRNA; protein kinase inhibition of mRNA translation) (3) protein processing inhibitors (e.g. glycosyltransferase which blocks glycosylation). (4, 5) Virus particle assembly and release inhibitors (e.g. glycosyltransferase cause membrane changes that block virus budding). (6) MHC: major histopathology complex induction – which participates in cell surface antigen presentation leading to cytotoxic T cell lysis of virus infected cells. Adapted from Medical Microbiology, 4th edition, Edited by S Baron. Published by The University of Texas Medical Branch at Galveston, 1996.

1.5 Interferon-deficiency in asthma and COPD

Evidence from *in vitro* studies has suggested that asthmatics have a deficient interferon response to viruses. This has been demonstrated using epithelial cells obtained by bronchoscopic brushings (Wark, 2005), bronchoalveolar lavage (BAL) cells (Contoli, 2006) and peripheral blood mononuclear cells (PBMC) (Papadopoulos, 2002). Wark *et al* (Wark, 2005) employed fibreoptic bronchoscopy to collect BEC from steroid-naive and steroid-treated asthmatics, which were then exposed to RV16 in culture, along with BEC from healthy subjects as control. Exposure to RV16 led to infection of the BEC cultures and induction of antiviral responses. Assessment of IFNβ gene expression and IFNβ protein production revealed low levels at baseline by healthy and asthmatic BEC. After 8 h of RV16 infection BEC from healthy subjects had a 3.6 times higher induction of INFβ mRNA compared to 0.3 times in uninfected BEC. This level of IFNβ mRNA induction was 10-fold higher than that in asthmatic BEC. This was coupled with higher levels of virus multiplication and inflammatory cytokine release from the asthmatic BEC compared to BEC from healthy controls. Exposure of the asthmatic BEC to exogenous IFNβ protein increased their antiviral response to RV16, limiting virus replication to levels observed in the BEC from healthy subjects. This implies that the virus-induced production of IFNβ is deficient in asthmatic BEC while downstream signalling is preserved. Contoli *et al*'s work (Contoli, 2006) with IFNλ showed similar results. Virus infection-induced IFNλ gene expression *in vitro* by BEC of atopic (steroid-naive and steroid-treated) asthmatics was 10-fold lower than that by BEC of healthy subjects, with a corresponding lower IFNλ protein release. Lower interferon induction and production correlated with higher levels of virus replication in those cells. There was also a negative correlation between *in vitro* IFN-λ induction and severity of clinical illness when the same subjects were later experimentally challenged *in vivo* with RV16. RV16 is a laboratory strain of rhinovirus that has been used successfully in several *in vivo* experimental infection studies (Contoli, 2006; Message, 2008; Gern, 2007; Mallia, 2011). Bronchoalveolar lavage cells from the same subjects showed a similar deficient IFNλ production in response to RV16. These studies raised the prospect that exogenous interferon treatment may have therapeutic utility in preventing or treating virus-induced exacerbations of asthma (Wark, 2005). In their work, Papadopoulos *et al*(Papadopoulos, 2002) exposed PBMC to RV *in vitro* and measured IFN-gamma expression in the supernatant at several time points. Expression of IFN-gamma by PBMC from atopic asthmatic subjects was significantly lower than from healthy subjects.

BEC and BAL cells from COPD are more susceptible to rhinovirus infection and exogenous IFN has been shown to protect BEC from COPD from viral infection (Schneider, 2010; Haywood, 2010; Mallia, 2011). Schneider *et al* (Schneider, 2010) cultured BEC from

COPD and healthy controls *ex vivo* and assessed their response to RV39. BEC from COPD subjects were more susceptible to viral infection with significantly higher viral load than the normal cells. Mallia *et al* (Mallia, 2011)performed an experimental RV challenge in COPD subjects during which the subjects also underwent bronchoscopy and BAL cells were collected. The viral load in the BAL was assessed and found to be significantly higher than that from healthy controls. It was also found that lower levels of IFN were produced by BAL cells from COPD subjects relative to those from healthy controls. Accumulated evidence from both *in vivo* and *in vitro* studies suggests that smoking impacts on innate immunity. Bronchial epithelial cells (BEC) represent the primary site of rhinovirus (RV) infection in the lower airways(Papadopoulos, 2000 58) and *in vitro* studies have shown that BEC from smokers without COPD are more susceptible to RV16 relative to cells from non-smoking controls(2). *Ex vivo* exposure of BECs to cigarette smoke extract has been found to blunt the response of airway epithelial cells to RV infection, leading to increased viral replication by interfering with the IFN pathway and consequent activation of antiviral genes (Eddleston, 2011). Ongoing smoking is associated with an increased risk of lower airway involvement with sputum production, wheezing, and pain on respiration (Nicholson, 1996) following RV infection. Haywood *et al*(Haywood, 2010) demonstrated that exogenous IFN is protective to BEC from smokers with or without COPD against RV16 infection. Deficient innate antiviral defence which can be overcome with exogenous IFNβ is therefore evident in smoking-induced COPD.

1.6 The unmet need of virus-induced exacerbations of asthma and COPD

Although the natural history of virus-induced exacerbations of asthma and COPD have the potential to be altered by several therapeutic strategies, specific clinically proven therapeutic options are limited (Varkey, 2008; Jackson, 2010). The backbone of clinical guidelines for the management of asthma and COPD exacerbations is glucocorticosteroids (GINA, 2012; GOLD, 2013). However, several authors have demonstrated that glucocorticosteroids lack efficacy against virus-induced exacerbations of these two conditions (Harrison, 2004; Grunberg, 2001; Reddel, 1999; Cameron, 2006). Harrison *et al* (Harrison, 2004) studied 390 individuals with asthma who were at risk of an exacerbation by monitoring their morning peak flow and asthma symptoms for up to 12 months. When peak flow or symptoms started to deteriorate, participants added a corticosteroid or placebo inhaler to their usual corticosteroid for 14 days to produce a doubling or no change in dose. During 12 months, 11% of 192 and 12% of 198 in the active and placebo groups, respectively, started oral

prednisolone. The study therefore demonstrated that doubling the dose of inhaled corticosteroid when asthma control starts to deteriorate (which, as already stated, is most commonly due to RV infections) is not protective. Grunberg *et al* (Grunberg, 2001) investigated whether treatment with inhaled corticosteroids prevents rhinovirus-induced worsening of lower airway inflammation. They selected 25 atopic patients with mild asthma who underwent experimental RV16 infection, while receiving double-blind, placebo-controlled treatment with inhaled budesonide 800 mcg twice a day, starting two weeks before infection. RV16 infection induced worsening of airway inflammation in asthma, which was not affected by inhaled corticosteroids in keeping with the limited protection of inhaled corticosteroids against acute asthma exacerbations. Reddel *et al* (Reddel, 1999) demonstrated that good asthma control on ICS does not protect asthmatics against sudden loss of lung function during a virus-induced exacerbation. Similarly, Cameron *et al* (Cameron, 2006) investigated the incidence of common infectious agents in acute exacerbations of COPD requiring ventilation, with a focus on respiratory viruses. The study prospectively recorded 107 episodes of intensive care unit admissions for invasive or non-invasive ventilation in 105 patients in a study conducted over 3 years. Upper and lower respiratory tract samples were assessed for pathogens by polymerase chain reaction (PCR). A virus was identified in 43% of all admissions, being the sole organism in 33% of cases and part of a mixed infection in 10%. A probable bacterial aetiology was found in only 23%. There was no statistically significant difference in clinical characteristics or outcomes between the subjects with or without virus infections, suggesting systemic glucocorticosteroids which are the mainstay of medical therapy for exacerbations of COPD are not effective against virus-induced exacerbations of COPD.

1.7 Antiviral therapies and airways disease

The pathobiology of virus infections would suggest that it is theoretically possible to interfere with every step of the infectious cycle of respiratory tract viruses from virus attachment, virus entry and uncoating, gene translation, replication, and onward to virus release. However, with the exception of the neuraminidase inhibitors for influenza infection, few approaches have met with success thus far (Whitton, 2005; Jackson, 2010) (Table 1.3).

Table 1.3

Drug	Proven benefit	Asthma exacerbations	COPD exacerbations
Inhaled zanamivir (neuraminidase inhibitor) (Murphy, 2000)	Shortened duration of influenza symptoms. Reduced severity of influenza illness. Improved night sleep disturbance. Reduced the incidence of complications requiring antibiotics and a change in respiratory medication.	Not addressed – despite testing in asthmatics	Not addressed – despite testing in COPD
Nasal ruprintrivir (a protease inhibitor) (Hayden, 2003	Reduction of common cold symptoms in healthy adults	Not tested	Not tested
Oral pleconaril (capsid-binding antiviral) (Hayden, 2003)	Reduction of common cold symptoms in healthy adults	Not tested	Not tested
Tremacamra (soluble ICAM-1 receptor) (Turner, 1999)	Marginal benefit in cold symptoms, viral replication, and development of clinical colds	Not tested	Not tested
Intranasally administered IFNα2 in healthy volunteers(Scott, 1982; Hayden, 1988)	Dose-dependent improvement in symptom scores and reduced viral shedding	Not tested	Not tested

Table 1. 3 Antiviral therapy in respiratory tract viruses.

Most of these therapies have not addressed the specific question of virus-induces exacerbations of asthma and/or COPD. It was reported that Zanamivir does not adversely affect pulmonary function in patient with COPD or asthma(Murphy, 2000).

There are several difficulties inherent in attempts to investigate naturally occurring infections of which virus-induced exacerbations of airways diseases are no exceptions. These include heterogeneous aetiologies of the infections, variation in time from onset to investigation, effects of treatment, and difficulties in the optimal timing for both baseline and acute sampling. Therein lays the utility of experimental rhinovirus infection studies which are not subject to such difficulties.

1.8 The need to extend the experimental rhinovirus infection model

1.8.1 Asthma

Abundant evidence exists to support the consensus that asthma is a heterogeneous condition with many clinical phenotypes (Anderson, 2008; Wenzel, 2006) classifiable by severity as mild, moderate and severe as discussed in section 1.1. Most asthmatics are found to have moderate or severe persistent disease when their symptoms are carefully assessed (Fuhlbrigge, 2002) and receive the full impact of virus-induced asthma exacerbations (Johnston, 1996). However, the model has mainly been used to elucidate many aspects of virus-induced exacerbations in subjects with mild disease as already mentioned (Bardin, 1994;de, 2003;Mosser, 2005;Message, 2008;DeMore, 2009). A body of evidence exists, that the biology of severe asthma is different from that of mild asthma in several aspects(Chanez, 2007): small airway involvement, association with increased risk of hospitalisation and death, lower prevalence of atopy, higher prevalence of NSAID-induced asthma, a link with lower biosynthetic capacity for lipoxins, poor response to corticosteroids, increased smooth muscle in the airways, large and small airway thickening, and poor control of clinical illness in many patients with severe asthma. In this light it is inappropriate for our understanding of the mechanisms of virus-induced asthma exacerbations to be based on extrapolations and generalisations of findings from studies performed in mild asthma. Models of virus-induced exacerbations of asthma in moderate asthma, and ultimately in severe asthma, are needed.

1.8.2 Smoking-induced COPD

There is less experience with the model in COPD. Mallia *et al* successfully infected COPD subjects in a first study of its kind (Mallia, 2011). The experimental infection needs to be replicated, and other aspects of the virus-induced exacerbations of airway symptoms elucidated.

1.9 What were the potential risks? Was it appropriate to pay participants?

The experimental RV infection model has helped elucidate many aspects of virus-induced exacerbations of asthma. The experience in smoking-induced COPD is more limited. Ways need to be found of further extending the usefulness of the model. However, there are potential risks to be taken into consideration while conducting such studies.

The most important risk is the induction of a severe virus-induced illness. There is also the risk of exposing contacts of the experimentally infected individuals to the virus, especially those with a weakened immune system. Finally, there was the potential for the virus to be spread to other users of the research facility in which the study was conducted. A carefully thought-out protocol would address these potential risks and allow such a study to be safely performed.

Given the potential risks involved, careful consideration was given to payments the volunteers were to be paid and how this was likely to be perceived by the REC and volunteers. The question of payments to research participants is not new – whether it is ethical to pay, and if it is what level of payment is acceptable. The pros and cons of payments to research participants are as follows (Grady, 2005).

1.9.1 Pros of payments to research participants

- Payment facilitates the timely recruitment of an adequate number and type of subject.

- Payment may be important to research to the extent that it encourages participation.

- The offer of money as reimbursement for time or expenses incurred could help to make research participation a revenue-neutral activity for participants. In this regard, money may enable individuals to take part who otherwise could not afford to participate or who are not willing to make a financial sacrifice in order to do so.

- Payment could help recruit individuals who believe they should be fairly compensated for their time and effort.

- Money can also serve as a recruitment incentive, especially if the amount offered is high enough to attract subjects to research and overcome inertia, lack of interest, as well as financial and other barriers.

- Money may also be helpful in achieving the goals of racial, ethnic, gender, and social diversity of subjects participating in biomedical research.

- Money may as an incentive may help to not make biomedical research the reserve for sensation seeking, or desire for attention provided to participants.

- Huge sums of money may be perceived as coercive, or serve as undue inducement to participate in a given study.

- Some participants take part in research then donate the money they are paid for their time an inconvenience to charity.

1.9.2 Cons of payments to research participants

- When subjects understand the likelihood of direct medical benefit to be remote or non-existent, the offer of money might be the only reason they volunteer to participate.

- Distrust in experimental medicine could be exacerbated by an offer of money.

- Subjects attracted by money may be less interested in evaluating or understanding study details, reading the consent form, or attempting to understand the goals, purposes, and risks associated with a study. This attitude might hinder the efforts to produce high quality data from research.

- Money can impair judgment or compromise voluntary decision making.

- Money may influence subjects to lie or conceal information that if known would disqualify them from enrolling or continuing as participants in a research project.

- Some worry that payment might be more attractive to individuals with low socioeconomic status, and thus the payment of subjects could result in a disproportionate research burden on this population.

- The potential risks were identified and steps taken to minimize these, and the REC review included the risks involved and the remuneration. Continuing to question the motives of the subjects who volunteered to participate in these studies may therefore seem like unwarranted paternalism.

1.10 Biomarkers of virus-induced exacerbation of asthma

1.10.1 Properties of a biomarker

A biomarker is defined by the Biomarkers Definition Working Group as 'a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention'(Frank, 2003) . Finding an acceptable biomarker is, however, an ideal that is often quite difficult to attain. Biomarkers are classified into three types – type 0, type I and type II(Naylor, 2003). Type 0 biomarkers measure the natural history of a disease. Type I biomarkers indicate the effects of an intervention, while type II are surrogate markers.

In this era of personalised evidence-based medical care, biomarkers are essential to healthcare providers to identify patients early, increase treatment efficacy, reduce adverse events and improve clinical development (Amado, 2008). This is becoming increasingly feasible because of advances in areas such as genomics and imaging, molecular medicine and improved trial design.

There is also a demand for good biomarkers from many of the stakeholders in healthcare, all from their respective perspectives. Patients are exercising their right to be informed and asking for evidence of efficacy of the therapeutic options available to them. Clinicians want to be certain that the right treatment is given to the right patient. Healthcare funders want evidence for cost-effectiveness and lastly, regulators are also now requiring biomarker data to support approval applications.

Certain conditions need to be met by a good biomarker. The biomarker needs to reflect the likely site of action of the drug or disease. The biomarker has to be detectable in adequate quantity and the quality of sample for the intended assay has to be high. The biomarker has to be obtained using a procedure that requires minimal intervention, and must generate statistically robust data consistent with the assay. If a peripheral blood biomarker can be shown to exhibit all these qualities, it would be an ideal biomarker.

Several difficulties may arise in the search for a good biomarker. Clinically relevant human tissue samples are not routinely collected as part of the diagnosis for most diseases (for example, in neurodegenerative conditions). Identifying disease tissue can be problematic (for example, psychiatric illness). When tissue is available, sample size may be very limited (for example, a needle biopsy). Finally, limited availability of clinical cohorts makes validation difficult, and clinical data can be limited and difficult to collect (for example, in post-mortem diagnoses).

Naylor proposed a Decision Wheel of Biomarker Discovery(Naylor, 2005). This is presented in figure 3.1 with minor modifications, and consists of the following:

1. The scientific question to be answered –for example, whether it is a hypothesis- or discovery-driven endeavour.

2. The purpose of the biomarker– within the context of the scientific question under consideration, what is the required output from the biomarker dataset? For example, are the biomarkers being used in a simple 'go' or 'no go' decision making process, or are they being used to understand a mechanism of biological action?

3. Experimental design – predicated on 1 and 2, issues such as what is the statistically significant number of samples needed and what are the appropriate controls (both positive and negative)?

4. Organism, tissue, cell or body fluid selection – the scientific question and the information needed from the biomarker output will determine the selection of the biological system to be studied. For example, a biomarker of response to treatment of an oncological illness must relate to the pathobiology of the disease. An example of this is serum levels of M protein in multiple myeloma which correlate with disease activity (NICE, 2007; Richardson, 2003).

5. –Omic, panomic, imaging, clinical chemistry, physiology or systems biomarker selection are all factors that must also be taken into consideration in selecting a biomarker. In relation to these, sample size, cost and time factors must also be considered before arriving at a decision on a molecular class of markers or determining if imaging will provide more pertinent information about the process under investigation. An example of this is in multiple myeloma, where radiological hotspots and/or serum M protein level is used to assess disease progression and/or response to therapy (NICE, 2007; Richardson, 2003).

6. Single biomarker versus a panel of biomarkers. As an example, several mutations are known to impact on the response of breast cancer to treatment (Gianni, 2005). Oncotype DX® (developed by Genomic Health, Inc, Redwood City, USA) is an example of a panel of prognostic biomarkers. It incorporates the PCR results of the expression of a 21-gene panel in breast cancer tissues. A computer analyses the expression of the panel of 21 genes to determine a Recurrence Score. The Recurrence Score is a number between 0 and 100 that corresponds to a specific likelihood of breast cancer recurrence within 10 years of the initial diagnosis. With this information, it is possible for doctors and patients to make more informed decisions about breast cancer treatment options.

7. If a panel of biomarkers is the obvious choice, the next task is to decide on the optimum number. Less than 10 may be cheaper and more appealing to healthcare funders. On the other hand, more than 10 may be information-rich, robust, but very expensive. As an example, Oncotype DX® (Genomic Health, Inc, Redwood City, USA) costs approximately US$4,000 per test. This is considered to be cost-effective in the USA, Canada and Japan by providing additional information that helps doctors tailor treatment to the individual patient (Hornberger, 2005; Tsoi, 2010; Kondo, 2008; Lyman, 2007) but may not be so in other countries.

8. Identity versus molecular signature. The expectation of a biomarker ranges from a molecular signature of structurally unidentified markers to a panel of identified biomarkers specific for the disease process being evaluated(Naylor, 2003). In other words, the choice may be between a simple molecular signature (without any of the biomarkers being identified) or a well characterised and identified panel.

9. Validation question: commercial or internal – the rigour of validation is determined by whether it is an internal process (for example, toxicity of a drug in animals), or the biomarkers are used as part of a clinical toolkit (for example, disease diagnosis). For example, a set of specific but unidentified biomarkers may signify a hepatotoxic response in rats to a new compound being evaluated, and armed with such limited information, the decision can be made to halt development(Naylor, 2003).

10. Validation and utilisation – biomarkers are subject to validation based on the answer to 9. Type 0 biomarkers can be validated longitudinally, in a well-defined patient population against a gold standard. Type I should be validated in parallel with the drug candidate, while type II must be shown to be relevant to both the mechanism of action of the drug and to the pathobiology of the disease.

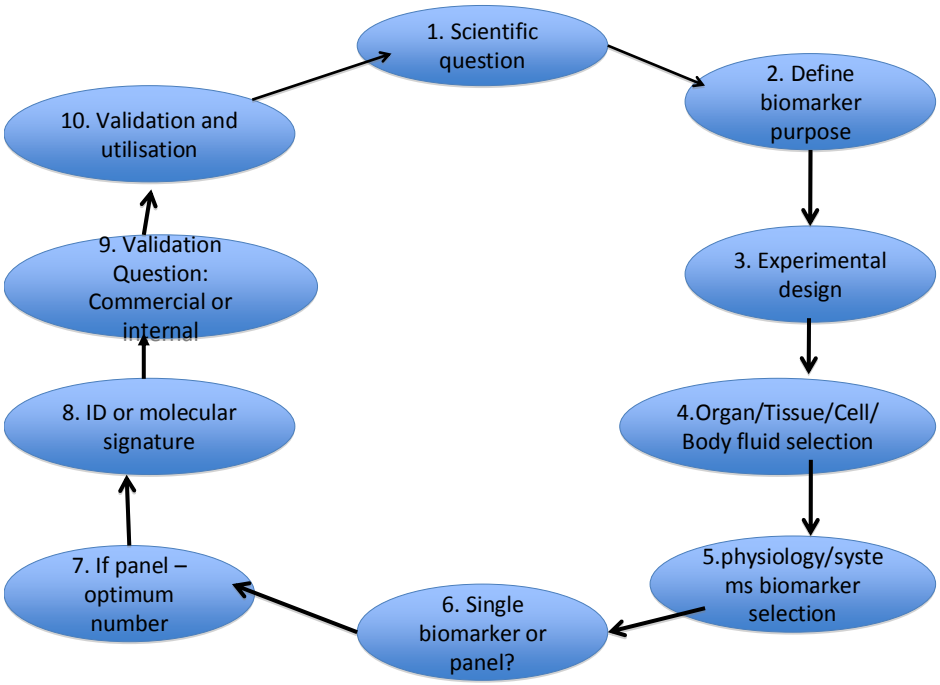


Figure 1.2 Decision Wheel of Biomarker Discovery
A relatively simple, compartmentalised series of steps that make up the 'Decision Wheel of Biomarker Discovery'. A careful and thoughtful decision has to be made to address the key issues raised within each step. The 'Decision Wheel' is designed to systematize the process of determining the optimal biomarker(s) for the scientific question under scrutiny

In this era of personalised medical care, biomarkers are essential to healthcare providers to identify patients early, increase treatment efficacy, reduce adverse events and improve clinical development (Amado, 2008). All of this is now possible because of advances in areas such as genomics and imaging, molecular medicine and improved trial design. Some biomarkers have been identified that predictive of outcomes in previous experimental rhinovirus infections. .

1.10.2 Predictors of clinical outcomes in previous experimental rhinovirus infection studies

Asthmatics suffer more severe and more prolonged lower respiratory tract symptoms compared with non-asthmatics(Corne, 2002), and experimental data exists about the deficiencies of the asthmatic lung's response to virus(Wark, 2005;Contoli, 2006;Message, 2008). Efforts have been made to elucidate the factors that may serve as biomarkers of asthmatics' response to rhinoviruses and potentially other respiratory viruses.

In a study by Grünberg *et al* (Grunberg, 1997), the effect of experimental RV16 infection on airway inflammation was investigated by analysing induced sputum. Twenty-

seven non-smoking atopic, mildly asthmatic subjects were enrolled in a placebo-controlled parallel study. RV16 (n = 19) or it's diluent (n = 8) was nasally administered. Sputum inductions were performed on all subjects at entry into the study, and on days 2 and 9 after inoculation. Airway responsiveness to histamine (expressed as PC_{20} which is the concentration of histamine causing a 20% decline in FEV_1) was measured on days 4 and 11, as well as cell counts expressed as a percentage of non-squamous cells, and eosinophil cationic protein (ECP) levels in sputum. A significant decrease in PC_{20} at day 4 ($p = 0.02$), which was no longer significant at ay 11 ($p = 0.19$) was found. The decrease in PC_{20} correlated significantly with the increase in ECP in the first week ($r = 0.60$) and with the decrease in the percentage of eosinophils in the second week after inoculation ($r = 0.58$). These results indicate that levels of ECP and eosinophil numbers in sputum could be indirect indicators i.e. biomarkers of airway hyperresponsiveness in rhinovirus infections in atopic asthmatic subjects.

Message *et al* (Message, 2008)hypothesized that in asthmatic subjects RV infection would induce clinical, physiologic, and pathologic lower airway responses typical of an asthma exacerbation and that these changes would be related to virus replication and immune responses. To test this hypothesis, the production of IFN-γ, IL-4, IL-5, and IL-13 by CD4+ cells in peripheral blood and BAL from healthy and asthmatic volunteers was assessed at baseline by intracellular cytokine staining. They also assessed the response of BAL cells from the same volunteers to RV16, LPS and PHA *ex vivo*. The study has been summarised in section 1.7.5. The observed strong relationships between induction of Th1 cytokines and clinical outcomes of experimental RV infection indicate that these cytokines measured in BAL and peripheral blood have potential utility as biomarkers of clinical outcomes of rhinovirus infection in asthmatics.

Similarly, Contoli *et al* (Contoli, 2006) assessed expression of mRNA encoding IFNλ, and production of IFNλ protein by BAL cells from healthy individuals and asthmatics in response to RV and LPS *in vitro*. The same volunteers were then experimentally inoculated with RV16 *in vivo*. Both expression of mRNA encoding IFNλ, and production of IFNλ protein were found to be significantly lower in asthmatics. IFN-λ protein production in BAL cells infected *in vitro* with RV16 was significantly inversely correlated with severity of maximal percentage reduction of FEV_1 from baseline recorded over the 2-week period post infection when subjects were subsequently experimentally infected with RV16 *in vivo* ($r = 0.65$, $p < 0.03$). IFNλ protein production in BAL cells stimulated *in vitro* with LPS was significantly inversely correlated with severity of maximal percentage reduction of FEV1 from baseline recorded over the 2-week period post infection ($r = 0.64$, $p < 0.03$), again indicating that *ex*

vivo production of the cytokine IFNλ could be a reliable biomarker of *in vivo* response to a rhinovirus infection in asthmatics.

Parry *et al* (Parry, 2000) conducted a study to determine the relationship of RV-specific responses in PBMCs to the outcome of experimental infection with RV16. Twenty-two subjects with either allergic rhinitis or asthma were inoculated with RV16. Virus-induced proliferation and cytokine production were determined on PBMCs obtained at baseline and then again 7 and 28 days after inoculation. Several subjects had proliferative responses to RV16 at baseline, and RV-specific proliferative responses were inversely correlated with RV shedding after inoculation. In addition, there was a negative correlation between baseline RV-induced IFNγ secretion *ex vivo* and peak RV shedding during the cold. RV-specific lymphocyte responses such as vigorous proliferation or IFNγ secretion were associated with reduced viral shedding after inoculation, suggesting that *ex vivo* mononuclear cell responses to RV could predict the individual viral shedding during experimentally induced, and perhaps naturally acquired, RV infections in subjects with respiratory allergy or asthma.

Not all studies that set out to investigate plausible biomarkers of clinical outcomes of RV infection in asthmatics were able to report significant correlations with increased asthma morbidity. One such study is that by Gern *et al* (Gern, 2000). To test the hypothesis that rhinovirus-induced immune responses influence the outcome of RV infections, 22 subjects with allergic rhinitis (n = 7) or asthma (n = 15) were inoculated with RV16 in this study. Induced sputum was repeatedly sampled over the next 14 days. Respiratory symptoms, sputum neutrophil counts, G-CSF levels, and mRNA encoding IFNγ and IL-10 were assessed. None of the study subjects reported wheezing or increased use of short-acting bronchodilators associated with the induced colds, and there were no cold-related changes in mean FEV_1 for the group as a whole. Changes in sputum neutrophils and G-CSF with RV16 infection were relatively modest. No relationship was found between changes in sputum neutrophils, G-CSF, or IL-8 and alterations in lung function. The lack of correlation could have been due to the relatively small changes in lung function, sputum cellularity, and cytokines.

In summary, the above studies indicate that certain mRNA/gene expression profiles have potential utility as biomarkers of asthmatics' response to rhinoviruses. A test on peripheral blood whose development is hypothesis-driven, from a parallel design study, that produces an Identified, specific mRNA profile, would therefore be a good biomarker of experimental, and ultimately naturally acquired, rhinovirus-induced exacerbations of asthma. This would be a type II biomarker (i.e. based on pathobiology of the disease process) that ultimately forms part of the clinical tool kit in the management of the unmet need of virus-

induced exacerbations of asthma. It would be able to predict which asthmatics who are likely to suffer the most from virus-induced exacerbations of asthma, and therefore likely to benefit the most from novel and perhaps expensive therapies for virus-induced exacerbations of asthma.

1.11 Aims of DM Thesis

The overall aim of this thesis was to evaluate the safety and usefulness of the experimental rhinovirus infection model in moderate, corticosteroid-treated asthma, and to improve our understanding of the mechanisms of virus-induced asthma exacerbations secondary to steroid-independent inflammation; also, to expand the model's usefulness by applying it to smokers. The specific aims were:

I. to explore the feasibility and safety of an experimental rhinovirus infection in moderate, inhaled glucocorticosteroid-treated asthma;
II. to replicate the findings by Mallia *et al*(Mallia, 2011) regarding the feasibility and safety of an experimental rhinovirus infection in smokers with or without COPD;
III. to assess the mechanisms involved in responses to an experimental RV infection in the 2 groups above; and
IV. to assess the *ex vivo* response of peripheral blood cells to dsRNA in moderate asthma and healthy controls, and to assess the utility of these antiviral responses as predictors of the severity of clinical illness during virus-induced asthma exacerbation.

The hypotheses were:

I. it is feasible and safe to experimentally induce a clinical RV illness in moderate asthma and smokers with or without COPD;

II. innate immunity can be induced and assessed in moderate asthma and smokers with or without COPD following experimental RV infection;

III. the *ex vivo* antiviral response to synthetic dsRNA, of peripheral blood of asthmatics is lower than that of healthy controls and is predictive of the severity of virus-induced clinical illness.

In summary, rhinoviruses are a major cause of exacerbations of asthma and COPD. Experimental RV infection studies have been used as a model to investigate mechanisms of virus-induced exacerbations in asthma and COPD. To increase the model's usefulness,

ways need to be found of employing it in moderate asthma controlled with ICS and in smokers, which is the aim of the work described in the following chapters.

Chapter 2 - Materials and Methods

2.1 Protocol development and ethical considerations

The protocols were developed in accordance with the International Conference on Harmonisation Guideline for Good Clinical Practice (ICH GCP), the standards set out by the Research Governance Framework, and the Ethical principles that have their origin in the Declaration of Helsinki. The study protocols for experimental RV infection were developed in collaboration with Synairgen research ltd.

The asthma study was approved by the Southampton and South West Hampshire Research Ethics Committee B (REC Number: 08/H0504/52). The REC meeting at which this first protocol was considered, was attended by me (as the Chief Investigator, who had written the first draft of the protocol), and the CEO of Synairgen. Their ethical and safety concerns were addressed at the meeting so the REC gave a favourable opinion following which the Research and Development (R&D) department of the Southampton University Hospitals NHS Trust, who co-sponsored the study with the University of Southampton, also gave their approval (RHM MED 0811). The R&D departments of Imperial College London NHS trust also gave their approval for the study to be conducted at the St Mary's Hospital, London (Reference 08/BA/003).

The protocol for experimental RV infection in moderate asthmatics was then adapted by Synairgen for the experimental RV infection in smokers. This protocol was reviewed by me prior to submission to the REC for approval. The smokers' study was approved by the Isle of Wight Portsmouth & South East Hampshire Research Ethics Committee B (REC Number: 09/H0501/56). Following REC approval, the Research and Development (R&D) department of the Southampton University Hospitals NHS Trust also gave their approval (RHM MED 0885). The study was sponsored by Synairgen Research Limited.

2.1.1 Relevant ethical principles taken into consideration based on the Declaration of Helsinki:

The scientific questions to be answered by these studies could not be answered by other means that would not involve human subjects.

A regards the safety of research subjects, the subjects' symptoms scores were received twice daily, and the secure webpages that displayed this information were checked on a daily basis. Also, the program sent out alert e-mails to designated e-mail addresses if there was no response to the SMS request for symptom scores. Every time a subject failed to respond to the SMS request for symptom scores, he or she was called for their safety by a nurse.

With regards to appropriate compensation and treatment for subjects who are harmed as a result of participating in research, all the NHS Trusts, the University of Southampton, and the medical staff involved in the research all had the necessary insurance policies in place.

With regards to the importance of the objective versus the risks and burdens to the research subjects, all subjects recruited into the study had been on the treatment they were on at the time for at least 8 weeks, and had not attended emergency department, had asthma-related hospital admission, or systemic steroids for the treatment of an asthma exacerbation in the preceding 12 months. This minimized the risk of an asthma exacerbation during the study.

With regards to assessment of predictable risks and burdens to the individuals and groups involved in the research in comparison with foreseeable benefits to them and to other individuals or groups affected by the condition under investigation, there was no direct benefit to the subjects for taking part in these studies. This was made clear to all subjects when they were consented. However, the research was expected to add to the general understanding of the effects of rhinovirus infections in people with airways disease.

With regards to measures to minimise the risks, the greatest risk foreseeable was a severe exacerbation of asthma. Twice-daily monitoring of the symptoms was in place which would pick up any severe increase in symptoms between the 5 visits to the research unit following inoculation. Also, procedures were put in place to minimise the RV16 infection spreading to other subjects using the research facility at which the clinical work was carried out. The infected subjects used a separate rear entrance, they did not mix with other volunteers in common areas, and the surfaces in the procedure room were cleaned as soon as their visits were completed.

With regards to when the risks are found to outweigh the potential benefits or when there is conclusive proof of definitive outcomes, physicians must assess whether to continue, modify or immediately stop the study, one subject was infected at a time, so that if the

infection proved to be more severe than anticipated, the study could be stopped, thereby exposing a minimum number of subjects to the higher than anticipated risk.

Vulnerable groups and Individuals were not considered for inclusion into these studies.

The information leaflets approved by the REC contained information regarding funding, sponsors, institutional affiliations, potential conflicts of interest, payments to subjects, provisions for treating and/or compensating subjects who may be harmed as a consequence of their participation in these studies. It was made clear that they were academic project, but that they were contributing to the drug-development activities of a commercial sponsor namely, Synairgen. It was explained to the subjects that the payment was not for any potential risk involved, but for the time they were committing to the studies, and for the inconveniences that the trial procedures and visits might cause them.

With regards to the requirement to seek REC approval for substantial changes to the protocol, one substantial amendment was made to the protocol for experimental infection in asthmatics to facilitate recruitment. The level of RV16 neutralizing antibody titre allowable into the study was changed from no-one with any level of RV16 neutralizing antibody being recruited, to recruiting subjects with RV16 neutralizing antibody titre of ≤1:2. The amendment was approved by REC before it was implemented. After the end of the study a final report was submitted to the REC.

Every precaution was taken to protect the privacy of research subjects and the confidentiality of their personal information.

Each potential subject was adequately informed of the aims, methods, potential risks of the study and the discomfort it may entail, post-study provisions and any other relevant aspects of the study. The potential subject were informed of the right to refuse to participate in the study or to withdraw consent to participate at any time without reprisal, and with no obligation to give their reasons for withdrawal. This information was included in the information leaflets sent out to the volunteers and also reiterated in face-to-face discussions with the researchers during the process of obtaining informed consent.

After ensuring that the potential subject has understood the information, a physician sought the subject's freely-given informed consent in writing. A copy of the consent form was given to the subjects, and another copy filed in their medical notes.

No plans were made to store the samples collected from these studies in a biobank. However, subjects were made aware of, and consented for a situation whereby their samples may be used in research that is not a directly related to the main questions been addressed in these studies.

2.2 Design of the experimental rhinovirus infection studies

Experimental rhinovirus 16 (RV16) infections in asthma have been well defined. The experience in smoking-induced COPD is more limited. They are an established method of studying the pathological processes involved in exacerbations of lower airways diseases such as asthma and COPD precipitated by viral upper airway infections. Modifications to the usual methods employed to induce these experimental infections in previous studies were made for the following reasons. The first modification was to use a lower dose of virus than has been used in previous studies because this approach would more closely replicate the conditions in naturally acquired infections whereby a small dose of virus inoculated to the eyes or nose of susceptible subjects by way of aerosols or hands that have come in contact with virus-containing secretions, multiplies and then spreads.

The second modification made was to use a method of inoculation that delivered the inoculum to the nasal cavity only. Again, this approach more closely replicated the conditions in naturally acquired infections as opposed to previous experimental RV infection studies in which the method of inoculation generated small-sized aerosols with the ability to reach the lower airways.

Naturally acquired common colds are known to have a low infectivity rate. The low rate may be explained by studies which have revealed that there is a directly proportional relationship between the transmission of a rhinovirus (RV) and the number of hours a susceptible individual spends with an infected host(Jennings, 1987). RV transmission to 50% of susceptible individuals is achieved only after 200 hours (about eight days) of exposure(Jennings, 1967), most RV types may spread to a large percentage of a small, concentrated population (e.g. within a family, amongst pupils from a classroom) (Beem, 1969; Monto, 1968), but the great majority of outbreaks spread poorly or not at all within the larger, less concentrated population (e.g. neighbouring families, the school at large) (Gwaltney, 1968). Similarly, in previous experimental RV infections, clinical infections were not achieved in all susceptible subjects. An inoculum that has been used successfully in previous studies in which clinical infections have developed consistently in about 80% of

susceptible subjects when inoculated with 1000TCID$_{50}$ of RV16(Gern, 2007) used . It was therefore not guaranteed that the inoculation with a lower dose would lead to a similar rate of clinical infection among susceptible subjects. It was therefore important to monitor the rate at which subjects developed clinical colds in these studies with these modifications. A dose-escalation was therefore incorporated into the study design whereby five susceptible subjects were inoculated with a 100TCID$_{50}$ of RV16 and observed for signs of a clinical upper respiratory tract infection (URTI) with the anticipation that if the inoculation was successful, at least 4 would develop an URTI. If this was not the case, then the dose was to be increased to 1000TCID50 and a further 5 subjects inoculated and observed. A final dose of 10,000TCID50 was to be tried in 5 final subjects if necessary. Clinical URTI were observed in all 5 of the first subjects inoculated with 100TCID$_{50}$, so that dose was used to perform experimental *in vivo* RV16 in asthmatic subjects with moderate asthma. This was followed by the use of the same dose to perform another experimental RV infection in smokers COPD. Both studies were single group, unblinded studies.

All subjects attended the research unit on nine occasions (Figure 2.1). Subjects were first screened for suitability. If they were suitable and clinically stable they were inoculated with RV16 virus via the nasal route and followed over a period of 14 days. Subjects recorded daily upper and lower respiratory tract symptoms and spirometry (forced expiratory volume in one second (FEV_1) and peak expiratory flow rate (PEF)) measured with a portable spirometer. Sputum induction was performed on the day of inoculation, and further attempts were made at all subsequent visits to the research unit. Measurements of lung function were performed at screening, on the day of infection and all subsequent visits to the research unit. Blood sampling was performed during screening and on days 0, 3, 5, 7, 10 and 13 or 14. The final visit was on or after day 35 for a blood sample to measure convalescent antibody titres.

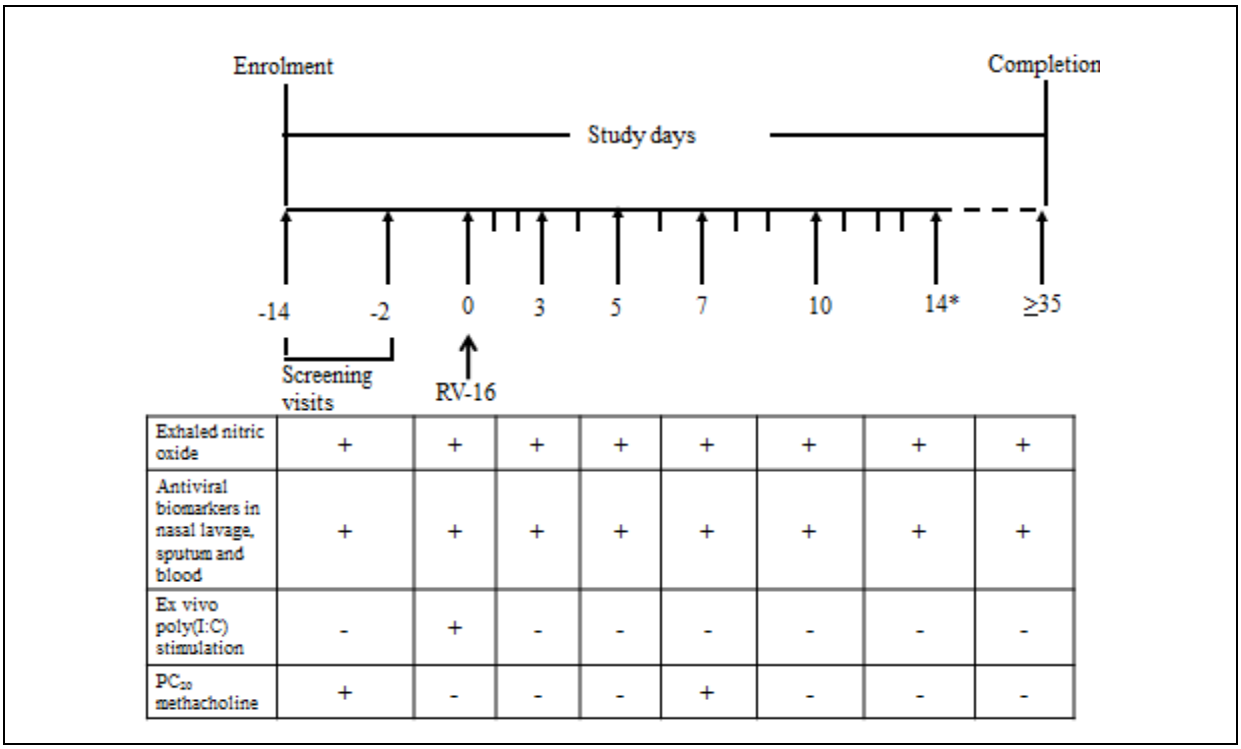
2.3 Inclusion criteria for the virus challenge studies

2.3.1 Asthma study

Male and female subjects aged 18 to 60 years were screened for participation in the asthma study. They all had a physician diagnosis of asthma, were on regular inhaled corticosteroids (ICS), had normal lung function with $FEV_1 \geq 80\%$ of predicted and transfer factor for carbon monoxide (TLCO) >80% of predicted. They were also required to have a history of virus-induced exacerbation of asthma on the treatment they were on at the time of screening, and

a positive Skin Prick Test to common aeroallergens. An upper limit of beclometasone dipropionate ≤1000 mcg daily, or equivalent was set for the dose of ICS.

Figure 2.1



Exhaled nitric oxide	+	+	+	+	+	+	+	+
Antiviral biomarkers in nasal lavage, sputum and blood	+	+	+	+	+	+	+	+
Ex vivo poly(I:C) stimulation	-	+	-	-	-	-	-	-
PC_{20} methacholine	+	-	-	-	+	-	-	-

Figure 2.1 Study schedule for the experimental rhinovirus infection studies. Short vertical arrows identify the study days while long vertical arrows identify the visits to the research unit. Inoculations were performed on day 0. *Day 13 for the smokers' study and day14 for the asthma study. Blood for e*x vivo* stimulation with poly(I:C) was collected on study day0. PC_{20} methacholine challenge was not performed in the smokers.

2.3.2 Study in smokers

Male and female subjects aged 45 to 70 years were screened for participation in the smokers' study. They did not have a previous diagnosis of COPD or any other respiratory illness and were not on regular ICS or other inhaled therapy. The lower limit of lung function was a post-bronchodilator FEV_1 $\geq$50% of predicted.

2.4 Exclusion criteria for the virus challenge studies

Subjects were excluded if there was any medical condition, including findings in the medical history or in the pre-study assessments, that constituted a risk or a contraindication for the participation of the subject in the study or that could have interfered with the study objectives, conduct or evaluation. A high titre of anti-RV16 antibodies in the serum (cut-off >1:2), acute infection or disease within 6 weeks prior to screening were all reasons for exclusion. Pregnancy - either current or planned - over the duration of the study was also reason for exclusion. Subjects who were taking part in clinical trials or had received an investigational medicinal product within 12 weeks for small molecules or within 6 months for biological therapeutics were also excluded. Subjects who were living with people with a weakened immune system including but not limited to young children, the elderly, and those with severe ischaemic heart disease were also excluded.

2.4.1 Asthma

In the asthma study, in addition to the above, lung disease other than asthma was also reason for exclusion, as well as an emergency department visit due to acute asthma in the preceding 12 months except if their treatment had been subsequently stepped up. This was to limit the likelihood and a moderate or severe asthma attack following experimental rhinovirus infection.

2.4.2 Smokers

A history of lung disease other than COPD GOLD stage 1 or 2 was reason for exclusion from the study. Subjects with a history of significant cardiovascular diseases including but not limited to ischaemic heart disease, heart failure and leaking or replacement valves were also excluded.

2.4.3 *Ex vivo* stimulation with poly(I:C)

Subjects with a history of respiratory tract infection within 6 weeks, chronic lung or immunological conditions were excluded.

2.5 Skin prick testing

This was performed using sterile microlancets to introduce common aeroallergens into the skin. The allergens tested for were D Pterronyssinus, D Farinae, house dust mite, mixed grass pollen, cat dander and dog fur (ALK Abello, Hørsholm, Denmark). Histamine (ALK Abello, Hørsholm, Denmark) was used as the positive control and normal saline as the negative control. The diameter of the weal was measured twice at right angles 15 minutes after application. A positive response was considered a weal 3 mm or more in diameter, or at least as large as the positive control.

2.6 Assessments of lung function

Two to seven days before inoculation, on the day of inoculation and over the following two-week period, subjects used a handheld spirometer (PIKO-1, Ferraris Respiratory, Herts, UK) twice daily. The asthmatics were asked to perform the spirometry before taking their regular twice-daily inhaled medication whenever possible. Three forced respiratory manoeuvres were always performed and the device kept a record of the best FEV_1, forced vital capacity (FVC), and peak expiratory flow rate (PEF) obtained. In addition**,** these three parameters were measured on a spirometer in the pulmonary function laboratory at enrolment and during the visits to the research unit on days 0, 3, 5, 7, and, 10. Final lung function tests were performed on day 13 for the smokers' study and day14 for the asthma study.

Bronchial hyperresponsiveness was tested using a standard methacholine (Metapharm Inc, Coral Springs, USA) provocation test as reported previously (Djukanovic,

2004 297). Results were expressed as the cumulative dose of methacholine required to cause a 20% drop in FEV_1 (PC_{20}).

Transfer factor of the Lung for Carbon Monoxide (TLCO) was measured at screening, on the day of inoculation and on day 7 post inoculation. The single breath-hold He dilution method was used according to ATS/ERS guidelines. Two values within 10% of each other from an acceptable technique were documented per visit. The results were expressed as a percentage of predicted, not corrected for haemoglobin (Hb) because Hb is not expected to change within a 2-week period.

2.7 Measurement of exhaled nitric oxide

Exhaled nitric oxide (eNO) was measured using a table-top portable device (NIOX MINO^{TM}, Solna, Sweden) according to ATS/ERS guidelines, i.e. the machine was setup to measure eNO in the last 3 seconds of a 10s period during which the subject exhaled at a rate of 50mls per second. One measurement of acceptable quality was recorded per visit.

2.8 Assessment of the potency of RV16

Regular assessment of viral potency was performed in HeLa cells (Imperial College, London, UK) based on the ability of a serially diluted virus suspension to induce cytopathic cell death in a $TCID_{50}$ assay. These potency tests were purely for testing that biological activity remained consistent throughout. The dose to be used for inoculation was calculated based on the results from the originating laboratory.

2.9 Administration of rhinovirus

The inoculum dose was 1 µl of stock virus diluted in 250 µl of normal saline delivered using the Mucosal Atomizer Device (MAD^{TM}, Wolfe Tory Inc, Salt Lake City, USA). The MAD is designed to deliver a precise volume to the nasal mucosa in a controlled manner. It generates particles with 30 – 100 µm size. Subjects were instructed not to sneeze or blow their nose for at least 30 minutes after inoculation. As mentioned in section 2.3, this method of administration is a modification of previously established methods(2). The 30 – 100 µm sized particles were expected to deposit exclusively in the upper airways(Newman, 1985 2786).

2.10 Assessment of upper and lower respiratory tract symptoms

All symptom scores were collected by a short messaging service (SMS) designed specifically for these studies by (Clinical SMS Ltd, Bournemouth, UK). Two Requests for symptom scores were sent out twice daily by central computer over the commercial mobile telecommunications network to the subject's cell phone. Subjects who did not have a mobile phone were provided with one. Two requests in the morning between 07:00 and 10:00, one for URT, and the other for LRT symptom scores. Two further requests were sent out in the evening between the hours of 17:00 and 21:00. The convenient hour in the morning and in the evening was agreed with the subject beforehand. They had been instructed that the symptom scores related to the preceding 12-hour period. They had also had written instructions to refer to if there was a need. The request for scores included a unique digital identifier that had to be included in the reply to enable it to be recognised and logged accurately - for the right subject and in the right order. Two reminders an hour apart were sent out if a recognisable SMS had not been received by then. The system was programmed to reject any SMS sent outside this time window in order to keep the responses as real-time as possible, and to log a missing value. The responses were monitored on a daily basis. In addition the programme sent out e-mails to designated e-mail addresses if a sudden increase in symptoms was reported or replies were not received within the allowed time window so that subjects were contacted, their well-being assessed, and any technical glitches addressed.

Cold symptom scores (headache, malaise, chills/feverishness, runny nose, blocked nose, sneezing, sore throat and cough) as defined by the Jackson cold score(Jackson, 1958 237) that is widely used in experimental virus infection studies(Fleming, 1999 985) were collected twice-daily. These symptoms were scored as on a 4-point scale as none (0), mild (1), moderate (2), or severe (3). A note was also made of the day when they felt they had developed a cold, regardless of the sum of symptoms scores recorded.

At the same time, subjects scored twice-daily their lower respiratory tract symptoms. Asthmatic subjects were asked about breathlessness, chest tightness, wheeze, and cough. These symptoms were scored as on a 4-point scale as none (0), mild (1), moderate (2), or severe (3). The smokers were asked about the symptoms of breathlessness, cough and sputum production. These symptoms were scored as on a 4-point scale as none (0), mild (1), moderate (2), marked (3), or severe (4). This is a slight modification of the Breathlessness, Cough, and Sputum Scale (BCSS) validated by Leidy *et al*(Leidy, 2003 2788). In addition,

asthmatic subjects recorded the use of their reliever medication and any episodes of nocturnal awakening.

2.11 Nasal lavage collection and processing

Nasal lavage is an established method used to collect upper respiratory tract samples following experimental RV infections for the purposes of virology and assessment of virus-induced immune and response. Nasal lavage was performed in these studies for the same reason on days 0, 3, 5, 7, 10, and 13 (14 for the asthmatics) post-inoculation using a modification of a previously described method(Greiff, 1990 955). An olive-shaped hollow stainless steel device was introduced to the nostril providing a seal. To this was attached to a 5 ml syringe containing the lavage fluid (normal saline). The nasal lavage was performed by pushing and pulling on the plunger several times. The subjects blew any fluid left in the nostril into a funnel placed in a sterile universal container. The nasal lavage was filtered through a 100 µm cell strainer and then centrifuged (400 g for 10 minutes at 4 °C). The cell-free supernatant was divided into aliquots and stored at -80°C, for subsequent determination of RV16 viral RNA, interferon-gamma inducible protein-10 (IP-10).

2.12 Sputum induction and processing

Induced sputum is a safe method for sampling the lower which was therefore used in these studies to assess lower airway viral load and immune responses following experimental rhinovirus infection. Sputum induction was carried out using the guidelines of the European Respiratory Society (ERS) Task Force(Pizzichini, 2002 953), applying the modified method suitable where necessary. Sputum was collected, sputum plugs selected, and then diluted in DTE(Efthimiadis, 2002 2790) (5 mM final concentration) with protease inhibitors. The diluted sputum was filtered through a 100 µm cell strainer and centrifuged (450 g for 10 minutes at 4 °C). Cell-free supernatants were stored at -80°C for subsequent analysis in light protected tubes. Cytospins were made from the cell pellet and any remaining cells (if sufficient in quantity) were lysed in RLT buffer (QIAGEN, Crawley, UK) for subsequent mRNA analysis.

2.13 Sampling and processing of peripheral blood

Peripheral blood was collected using standard phlebotomy techniques. Samples for serological tests were allowed to clot at room temperature for 30-45 min. Samples were then spun and the serum collected, divided into aliquots and stored at -80^{0}C for antiviral antibody titres at a later date. Blood samples dedicated for gene expression analysis were collected directly into PAXgene tubes (BD Biosciences, Oxford, UK) (Perera, 2007) and stored at -80^{o}C for mRNA extraction at a later date.

2.14 Viral load estimation in nasal lavage and sputum

Qualitative-PCR (qPCR) was used to assess viral load in nasal lavage and sputum. Viral RNA extraction and purification was performed using QIAamp viral RNA columns buffer (QIAGEN Crawley, UK) (Myatt, 2004), using specific primers and probes specifically designed to detect RV16 (Primer Design, Southampton, UK). The virus copy numbers were expressed as copies per unit volume of supernatant. Virus copy numbers were estimated by relating the PCR signal to a standard curve.

2.15 Measurements of cytokines

IP-10 (CXCL10) protein concentrations were measured in in sputum and nasal lavage samples using a commercial ELISA kit (R&D, Abingdon, UK) after the samples were serially diluted. Serial dilutions accompanied by spiking experiments were used to determine the optimum dilution required to produce reliable results in duplicates. IP-10 levels were then determined by interpolation from a standard curve.

2.16 Assessment of gene expression in sputum and blood cells

Antiviral responses were assessed by measuring the induction of the expression of mRNA encoding for antiviral response genes by qPCR. Gene expression was normalised to reference genes Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) and ubiquitin (UBC). Samples were run in duplicate for the PCR. The arithmetic mean of all 4 cycles of quantification (Cq) values for the reference genes was used to normalise the target gene data. The ΔCq was calculated by subtracting the arithmetic mean of the reference genes Cq from the target gene Cq. The $\Delta\Delta$Cq was calculated by subtracting the ΔCq at baseline (V3)

from the samples ΔCq. This was converted to a fold induction by $2^{\Delta\Delta Cq}$. IFNβ was at low abundance at V3 (day 0), therefore the Cq value for baseline samples was very high/not detectable and the values were not reliable. This led to difficulties in analysing the data as a fold induction compared to baseline. To overcome this issue, Cq values below the limit of quantification (LOQ) of 35 have been assigned an arbitrary value of half LOQ, i.e. a Cq value of 36 for IFNβ gene analysis. Gene expression for antiviral biomarkers interferon-beta (IFNβ), IP-10, Myxoma resistance protein A (MxA) and 2'-5' Oligoadenylate synthetase (OAS) were assessed, and their expression was compared to that of GAPDH and UBC as reference genes.

After mRNA extraction, the concentration of mRNA in each sample was determined using a spectrophotometer (NanoDrop 2000c, Thermoscientific, Wilmington, USA). This measurement made it possible to determine the volume of the sample required to provide 1 µg of mRNA for reliable PCR.

2.17 *Ex vivo* stimulation of whole blood with polyinosine-polycytidylic acid

Peripheral blood was collected into 4 EDTA tubes (3 ml/tube) per subject. Poly(I:C) 100 µg/ml (Invivogen, San Diego, USA) was added to 2 of the samples, and diluent (sterile 0.9%Nacl, as control) was added to the other 2. Samples were then incubated at 37°C and 5% CO_2. One poly(I:C)-treated sample, and one control samples were removed from the incubator at 2h, and the other pair of samples were removed a 6h. 2.5 ml from each sample were added to PAXgene tubes (The PAXgene tubes were then inverted 10 times then frozen at -80^{o}C until required for mRNA extraction for gene expression analysis. When the samples were removed from the freezer, they were left to stand at room temperature for at least 4 h during which time they were inverted several times intermittently before mRNA was extracted with RNeasy spin columns (QIAGEN, Crawley, UK) by following the manufacturer's instructions.

2.18 Statistical Analysis

Data distributions were assessed for normality. The mean value and 95% confidence interval were used to summarise the data when the assumption of normality was deemed valid. Where appropriate the data was log-transformed to achieve normality.

Symptom scores were collected twice daily and the average symptom score for the day was calculated. If only one score was available, this was used. In order to assess the maximum deterioration in symptoms experienced by each participant during the 14 days after RV16 inoculation, the change from baseline to peak symptom value was calculated. This change, along with the day of occurrence of peak symptom value, was summarised using the mean, 95% confidence interval and corresponding P value. Comparisons between baseline and peak measurements were performed using paired t-tests. Correlation between variables was assessed using Pearson's correlation coefficient.

Mean values over time were assessed using repeated measures analysis of variance (ANOVA). If significant differences in mean values were found, trend analysis was performed by fitting polynomial contrasts in order to determine whether the changes over time detected with ANOVA were linear or quadratic (i.e. an increase followed by a decrease). Where appropriate, then mean (CI) baseline and peak values were calculated and the mean (CI) difference between these two values assessed using a 2-tailed t-test. Where differences between the median and the mean were large indicating skewed distribution, the data was log-transformed to enable parametric tests to be used.

Missing values were inputted by the following rules to enable statistical analysis where required. For subjects who had data missing for day 14, the value from the previous day was used; for other study days, the missing values were replaced with an average of the value before and after the missing value. Missing values for gene expression at other occasions were assigned the group mean. A value of 0.5 the lower limit of detection was assigned samples in which viral RNA levels were undetectable to enable log transformation and analysis. For fraction expired nitric oxide, 2 subjects did not have a measurement at baseline. The first value obtained following infection was also taken as baseline.

Statistical analysis was performed using SPSS (release 19.0.0) and Graphpad Prism (version 6.0).

2.19 Discussion

The inclusion and exclusion criteria were intended to select a group of subjects who were likely to development a clinical infection that would be measureable, but not so severe as to be detrimental to their health. Endpoints were chosen to be relevant to a clinical presentation of an exacerbation of symptoms of lower airway illness, and mechanisms of innate

responses to rhinovirus infections. Finally, the laboratory tests, data collection tools and methods of data analysis that were relevant to the endpoints were selected.

A full discussion of these methods is given in Chapter 6 – General discussion.

Chapter 3 - *Ex vivo* antiviral response of whole blood to polyinosine-polycytidylic acid

3.1 Introduction

IP-10 (CXCL10) is a biomarker of virus-induced exacerbations of airways disease such as asthma and COPD. In the clinical setting, most exacerbations of asthma and COPD are caused by rhinovirus (Seemungal, 2000; Johnston, 1996). Levels of IP-10 protein in serum have been shown to be a reliable marker of rhinovirus-induced exacerbation of COPD (Quint, 2010) and asthma (Wark, 2007).

IP-10 is part of the innate response to viruses. It is produced by is secreted by bronchial epithelial cells, monocytes, lymphocytes, and neutrophils in response to IFNγ, Tumour Necrosis Factor (TNF)-α (Quint, 2010), and dsRNA both by IFN-dependent and independent pathways during virus infections (Spurrell, 2005). It has chemotactic and effector activity for T lymphocytes, NK cells, monocytes, and eosinophils (Spurrell, 2005).

Defective innate antiviral response has been demonstrated in asthmatics and smoking-induced COPD. Evidence from *in vitro* studies has suggested that asthmatics have a deficient interferon response to viruses. This has been demonstrated using bronchial epithelial cells (BEC) obtained by bronchoscopic brushing (Wark, 2005), bronchoalveolar lavage cells (BALC) (Contoli, 2006) and peripheral blood mononuclear cells (PBMC) (Papadopoulos, 2002). Similarly, BEC and BALC from smoking-induced COPD are more susceptible to rhinovirus infection (Schneider, 2010). Clinically, asthmatics suffer from more severe and more prolonged lower airway symptoms following viral airways infections(Corne, 2002 50), and cigarette smoking is similarly associated with a significant increase in the risk of lower airway complications from respiratory viral infections (Nicholson, 1996).

Ex vivo stimulation of TLR-3 with polyinosine-polycytidylic acid (poly(I:C)) is a surrogate for virus-induced immune responses. It has been used as such to elicit a cytokine response in BEC from subjects with asthma and healthy volunteers. Deficient IFNβ mRNA expression from the asthmatic BEC was observed (Uller, 2010), in line with the finding of a deficient IFNβ gene expression in response to RV infection (Wark, 2005).

The aims were to

1. compare the *ex vivo* antiviral gene expression of PBMC from healthy subjects in response to poly(I:C) to that of moderate asthmatics before experimental RV infection

2. to look for correlations between the *ex vivo* antiviral gene expression of PBMC from moderate asthmatics and their subsequent response to an experimental RV infection

3.1.1 Hypothesis

The hypothesis were that

1. *ex vivo* innate response of peripheral blood to dsRNA is deficient in moderate asthma, and

2. *ex vivo* innate response to prior to RV infection dsRNA correlates with clinical response to experimental infection in moderate asthma

3.2 Methods

A method of reliably inducing an innate antiviral response by TLR-3 stimulation in whole blood *ex vivo* with poly(I:C) was established. Briefly, blood was collected into EDTA tubes and treated with 100 mcg/ml of poly(I:C) (Invivogen, San Diego, USA) or diluent (sterile 0.9% NaCl) added as control. Samples were then incubated at 37°C and 5% CO2 for up to 6h. Treated and control samples were removed from the incubator at 2h and 6h time points and added to PAXgene tubes (QIAGEN, Crawley, UK). PAXgene tubes were then frozen at -80^{o}C until required for mRNA extraction for gene expression analysis by qPCR. Blood was first drawn from moderate asthmatics with a previous history of virus-induced exacerbations before they underwent experimental RV infection. The induction of IP-10 mRNA expression in the blood cells of the moderate asthmatics was used to calculate the sample size of healthy control subjects whose peripheral blood underwent similar *ex vivo* treatment for comparison. The healthy control subjects were age-matched with the moderate asthmatics.

See chapter 2 for a detailed description of methods.

3.3 Results

Pilot data (data not shown) indicated 2h time point for assessment of early mRNA expression, and 6h time point for later responses. This was confirmed by the responses seen in these 2 groups.

3.3.1 Induction of antiviral response genes at 2h and 6h time points

A significant up-regulation of mRNA was observed in the control samples for some of the genes at 6h compared to 2h. In healthy controls (HC), this was significant in IP-10 ($P < 0.01$) (Figure 3.1A) and IFNβ ($P = 0.01$) (Figure 3.2A). Similar observations were made in the moderate asthmatics (MA) for IP-10 ($P < 0.01$) (Figure 3.1B) and IFNβ (Figure 3.2B) only. Further analyses were limited to the poly(I:C)-induced mRNA expression above and beyond that induced by the incubation process.

Similar patterns of induction of mRNA expression were observed between HC and MA. Exposure to poly(I:C) led to up-regulation of mRNA for all 4 genes assessed. After 2h of exposure, IP-10 (Figure 3.1A, 3.1B) and IFNβ (Figure 3.2A, 3.2B) mRNA were significantly up-regulated in both HC and MA ($P < 0.01$), mean (CI) 17 (6.4, 44) and 4.7 (2, 11) times respectively. MxA mRNA was significantly down-regulated mean (CI) 0.77 (0.63, 0.95) times in the MA ($P = 0.02$) (Figure 3.3B) but not in the HC ($P = 0.61$) (Figure 3.3A), while OAS mRNA was not significantly up-regulated in neither HC ($P = 0.51$) nor MA ($P = 0.85$). After 6h of exposure further up-regulation of IP-10 was observed in both HC (Figure 3.1A) and MA (Figure 3.1B) mean (CI) 14 (3.8, 49), and 99 (32, 4100) times respectively ($P < 0.01$). MxA was upregulated 3.5 (1.3, 9.5) times in HC ($P = 0.02$) (Figure 3.3 A) and 9.1 (3.7, 22) times in MA (Figure 3.3B). OAS was up-regulated mean (CI) 3.1 (1.2, 7.7) times in HC 5.8 (3.2, 11) times in MA ($P < 0.01$) Figure 3.4). IFNβ mRNA expression (Figure 3.2) had returned to mean (CI) 1.8 (0.97, 3.3) ($P = 0.06$) above control samples in HC. However, it was still significantly up-regulated in the MA - mean (CI) 3.3 (2.2, 5.0) times ($P < 0.01$).

Figure 3.1

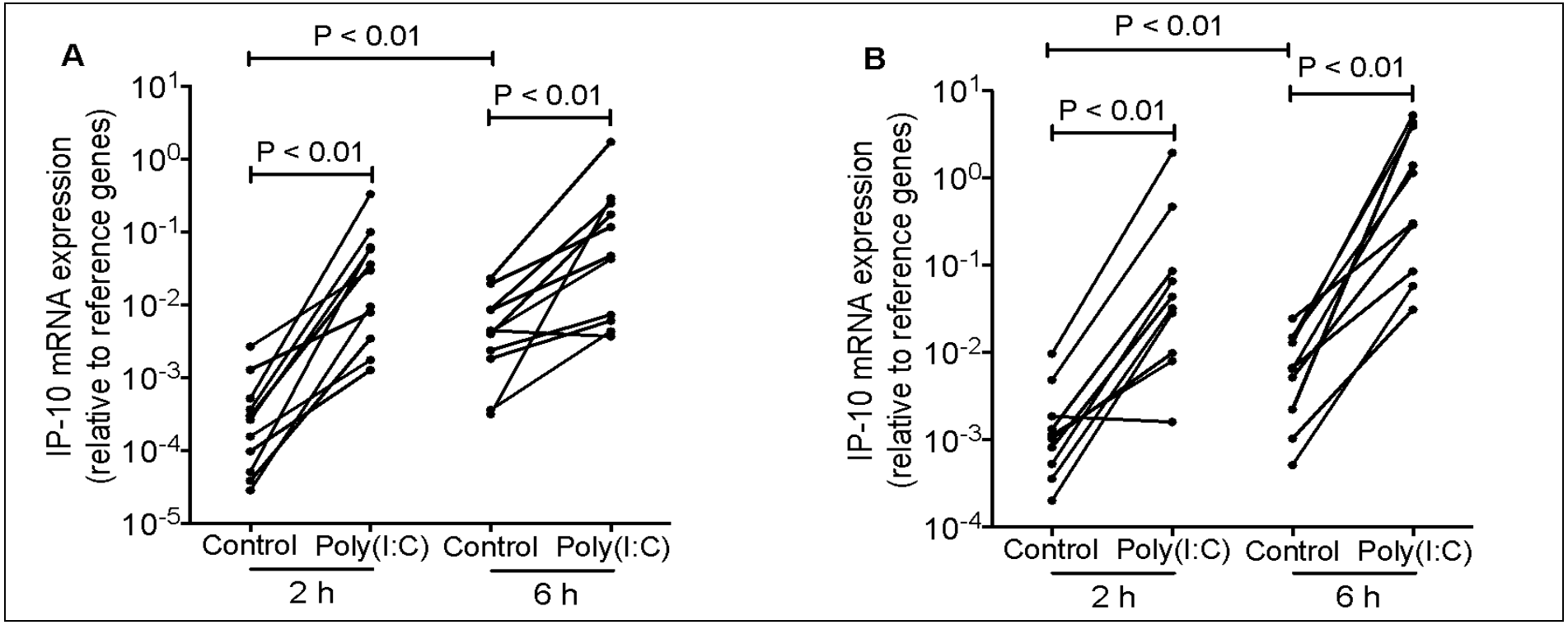


Figure 3.1 *Ex vivo* induction of IP-10 gene expression in whole blood in response to TLR-3 stimulation with poly(I:C) relative to reference genes GAPDH and UBC. mRNA levels between poly(I:C)-treated and control samples were compared after 2 h and 6 h of incubation. **A**: In healthy controls. IP-10 gene expression increased mean (CI) 17 (6.4, 44) times in control samples at 6 h relative to the 2 h control samples ($P < 0.01$). Poly(I:C) led to a mean (CI) increase of 85 (25, 290) times at 2 h ($P < 0.01$), and mean (CI) 14 (3.8, 49) times at 6 h ($P < 0.01$). **B**: In moderate asthmatics. IP-10 gene expression increased mean (CI) 4.7 (2, 11) times in control samples at 6 h relative to the 2 h control samples ($P < 0.01$). Poly(I:C) led to a mean (CI) increase of 38 (11, 130) times at 2 h ($P < 0.01$), and mean (CI) 99 (32, 310) times at 6 h ($P < 0.01$).

Figure 3.2

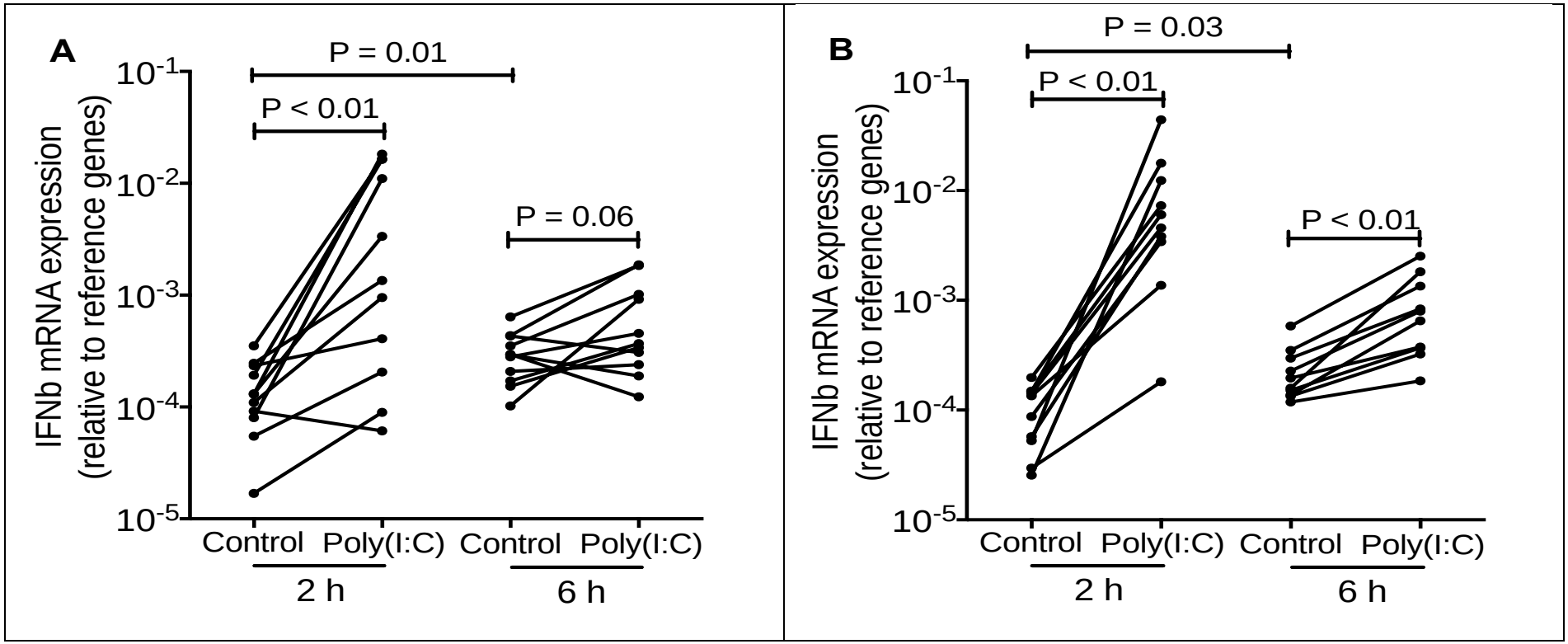


Figure 3.2 *Ex vivo* induction of IFNβ gene expression in whole blood in response to TLR-3 stimulation with poly(I:C) relative to reference genes GAPDH and UBC. mRNA levels between poly(I:C)-treated and control samples were compared after 2 h and 6 h of incubation. **A**: In healthy controls. IFNβ gene expression increased mean (CI) 2.3 (1.3, 4.1) times in control samples at 6 h relative to the 2 h control samples (P = 0.01). Poly(I:C) led to a mean (CI) increase of 13 (3.9, 45) times at 2 h (P < 0.01), and mean (CI) 1.8 (0.97, 3.3) times at 6 h (P = 0.06). **B**: In moderate asthmatics. IFNβ gene expression increased mean (CI) 2.5 (1.1, 5.4) times in control samples at 6 h relative to the 2 h control samples (P = 0.03). Poly(I:C) led to a mean (CI) increase of 57 (19, 170) times at 2 h (P < 0.01), and mean (CI) 3.3 (2.2, 5.0) times at 6 h (P < 0.01). .

Figure 3.3

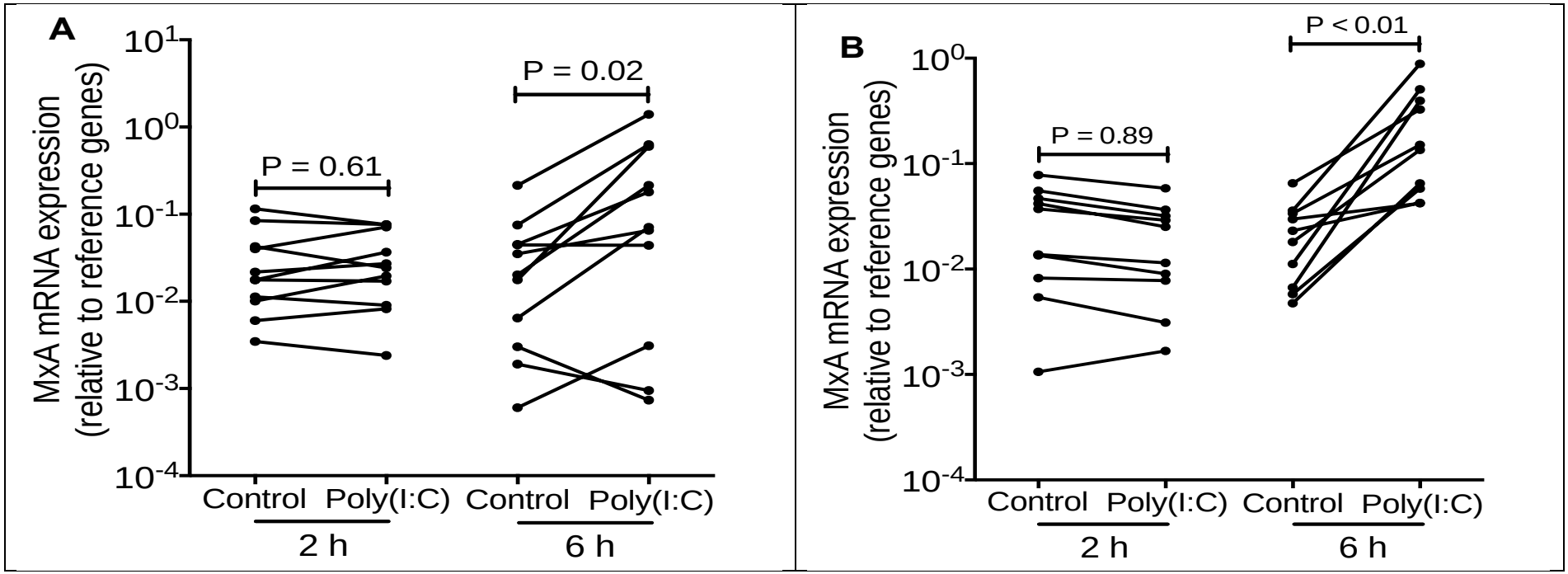


Figure 3.3 Ex vivo induction of MxA gene expression in whole blood in response to TLR-3 stimulation with poly(I:C) relative to reference genes GAPDH and UBC. mRNA levels between poly(I:C)-treated and control samples were compared after 2 h and 6 h of incubation. **A**: In healthy controls. Poly(I:C) did not lead to a significant increase in MxA gene expression at 2h - mean (CI) increase of 1.1 (0.79, 1.5) (P 0.61). However, the mean (CI) increase in MxA gene expression at 6 h was significant 3.5 (1.3 9.5) (P = 0.02). **B**: In moderate asthmatics. Similarly, there was no significant Poly(I:C)-induction of MxA gene expression at 2 h - mean (CI) 0.77 (0.63, 0.95) times, (P = 0.89). However, there was a mean (CI) increase in MxA gene expression at 6 h of 9.1 (3.7, 22) times (P < 0.01).

Figure 3.4

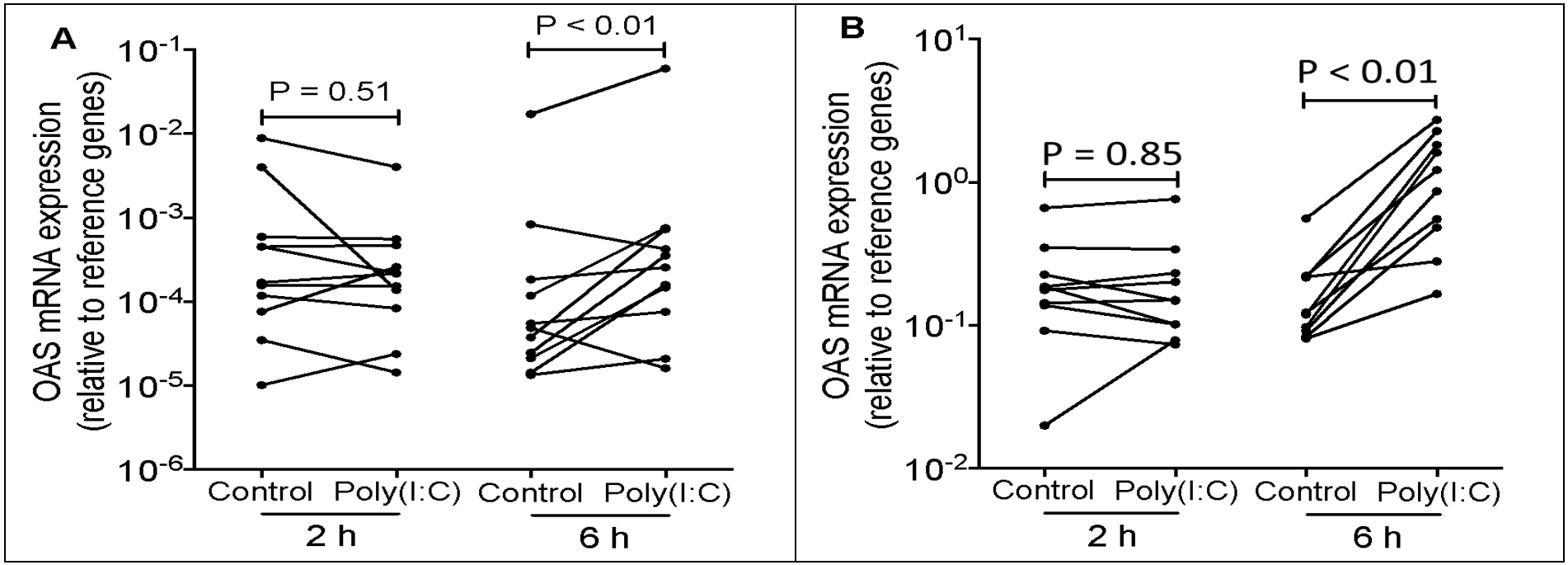


Figure 3.4 *Ex vivo* induction of OAS gene expression in whole blood in response to TLR-3 stimulation with poly(I:C) relative to reference genes GAPDH and UBC. mRNA levels between poly(I:C)-treated and control samples were compared after 2 h and 6 h of incubation. **A**: In healthy controls. Poly(I:C) did not lead to a significant increase in OAS gene expression at 2h - mean (CI) 0.71 (0.32, 1.6) (P = 0.51). However, the mean (CI) increase in OAS gene expression at 6 h was significant 3.1 (1.2 7.7) (P < 0.01). **B**: In moderate asthmatics. Similarly, there was no significant Poly(I:C)-induced MxA gene expression at 2 h - mean (CI) difference of 1.0 (0.70, 1.5), (P = 0.85). However, the mean (CI) increase in MxA gene expression at 6 h was significant 5.8 (3.2, 11) (P < 0.01).

Next, comparisons were made between the expression of these innate response genes in HC and MA at 2 h and 6 h. OAS mRNA expression was not significantly induced at 2 h in HC (P = 0.51) and in MA (P = 0.85) so no comparison was made. At 6 h the induction was not significantly different between HC and MA - the mean (CI) difference was 1.9 (0.66, 5.4) times (P = 0.21) (Figure 3.5). After 2 h of incubation, both HC and MA showed a significant induction of IP-10. However, there was no significant difference between induction of IP-10 in HC and MA. The mean (CI) difference was 0.44 (0.086, 2.3) times (P = 0.31) (Figure 3.6A). After 6 h of incubation, both HC and MA showed a significant induction of IP-10. The mean (CI) induction of IP-10 was higher in MA by 7.3 (1.5, 3.7) times (P = 0.02) (Figure 3.6A). After 2 h of incubation, both HC and MA showed a significant induction of IFNβ. However, there was no significant difference between induction of IFNβ in HC and MA. The mean (CI) difference was 4.3 (0.9, 20) times (P = 0.06) (Figure 3.7 A). After 6 h of incubation, a significant induction of IFNβ was only observed in MA. However, this was not significantly different from the induction in HC – mean (CI) 1.9 (0.93, 3.7) times (P = 0.08) (Figure 3.7B). After 2 h of incubation, neither HC nor MA showed a significant induction of MxA. After 6 h of incubation a significant induction of IP-10 was observed in both HC and MA. Induction in MA was higher, but was not significantly different from the induction in HC – mean (CI) 2.6 (0.73, 9.1) times (P = 0.13).

MA subjects then underwent experimental RV infection. Comparisons were then made between *ex vivo* innate antiviral responses and clinical responses to experimental RV infection. Correlation was observed between *ex vivo* induction of IFNβ mRNA expression and severity of cold symptoms, but not asthma symptoms. This correlation and its significance are discussed in chapter 4 .

Figure 3.5

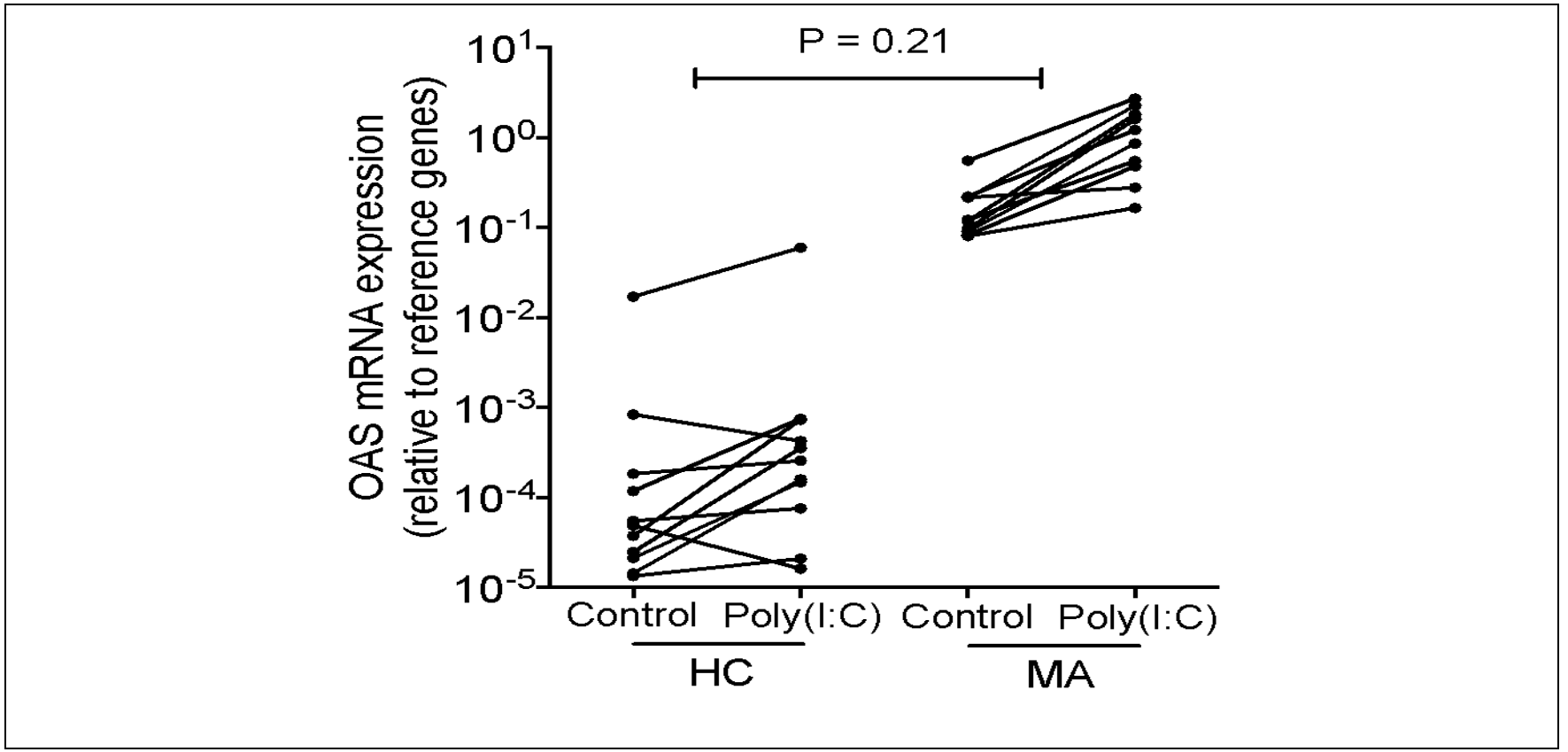


Figure 3.5 *Ex vivo* induction of OAS gene expression in whole blood in response to TLR-3 stimulation with poly(I:C) relative to reference genes GAPDH and UBC. mRNA levels between poly(I:C)-treated and control samples were compared after 6 h of incubation. Both groups showed a significant induction of OAS. However, there was no significant difference between induction of OAS in healthy controls (HC) and moderate asthmatics (MA). The mean (CI) difference was 1.9 (0.66, 5.4) times (P = 0.21).

Figure 3.6

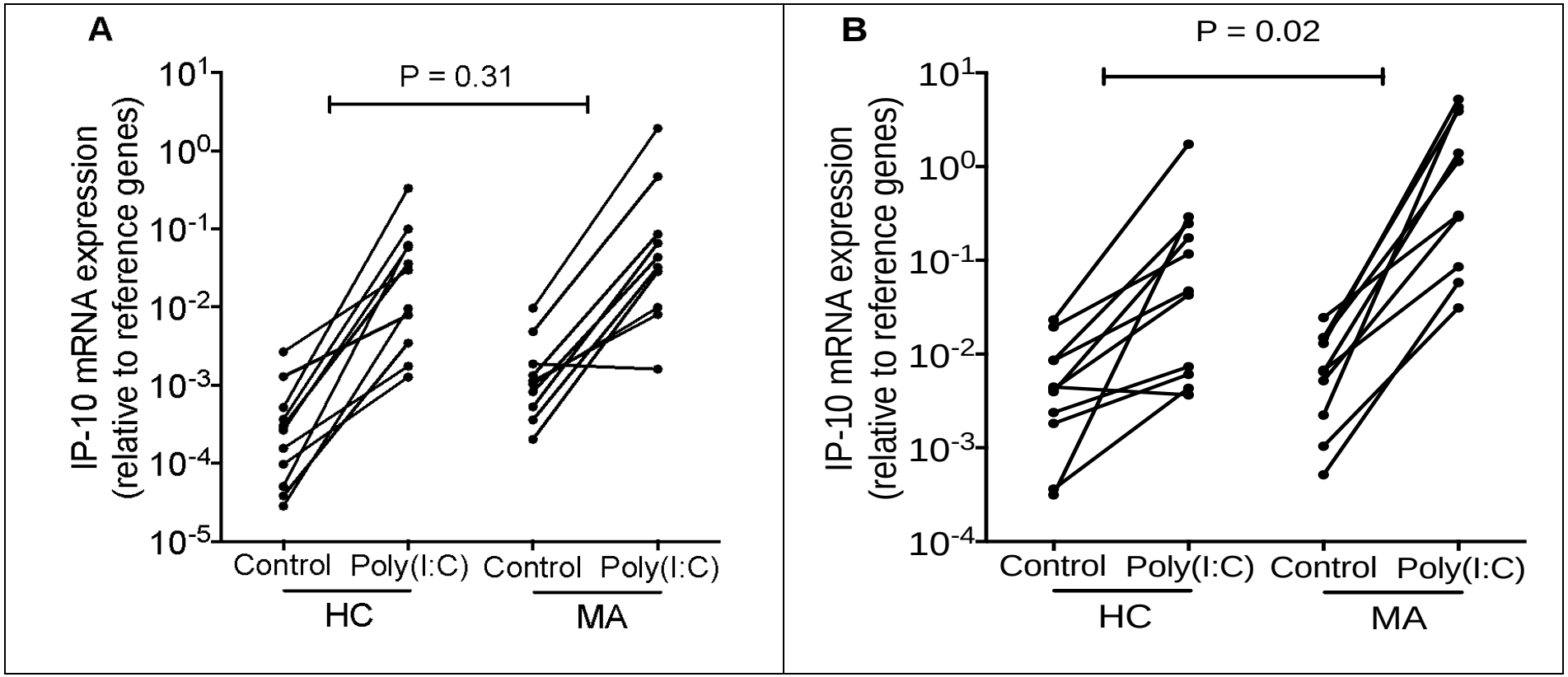


Figure 3.6 *Ex vivo* induction of IP-10 gene expression in whole blood in response to TLR-3 stimulation with poly(I:C) relative to reference genes GAPDH and UBC. mRNA levels between poly(I:C)-treated and control samples were compared. A: After 2 h of incubation. Both healthy controls (HC) and moderate asthmatics (MA) showed a significant induction of IP-10. However, there was no significant difference between induction of IP-10 in HC and MA. The mean (CI) difference was 0.44 (0.086, 2.3) times (P = 0.31). B: After 6 h of incubation. Both HC and MA showed a significant induction of IP-10. The mean (CI) induction of IP-10 was higher in MA by 7.3 (1.5, 3.7) times (P = 0.02).

Figure 3.7

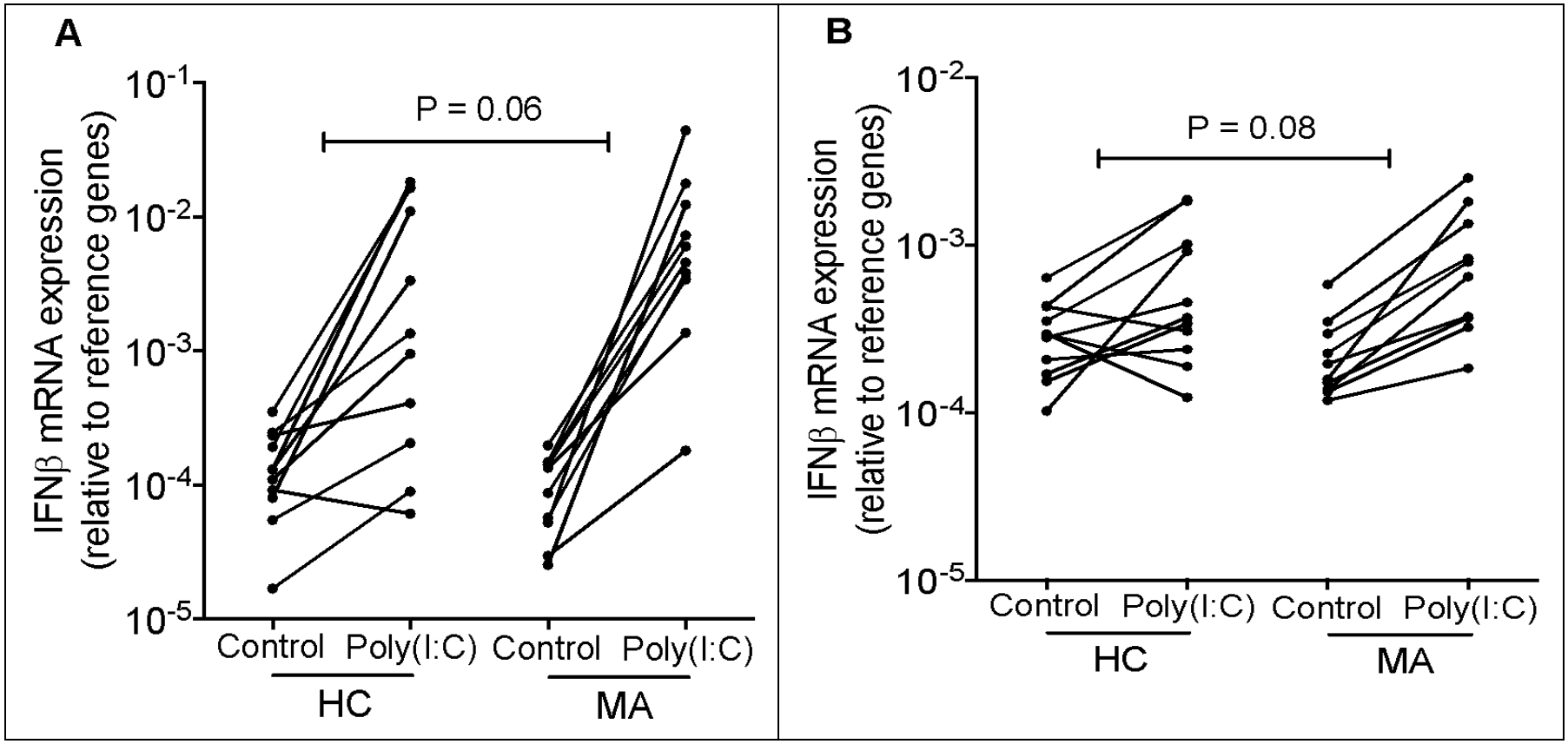


Figure 3.7 *Ex vivo* induction of IFNβ gene expression in whole blood in response to TLR-3 stimulation with poly(I:C) relative to reference genes GAPDH and UBC. mRNA levels between poly(I:C)-treated and control samples were compared. A: After 2 h of incubation. Both healthy controls (HC) and moderate asthmatics (MA) showed a significant induction of IFNβ. However, there was no significant difference between induction of IFNβ in HC and MA. The mean (CI) difference was 4.3 (0.9, 20) times (P = 0.06). B: After 6 h of incubation. A significant induction of IP-10 was only observed in MA. However, this was not significantly different from the induction in HC – mean (CI) 1.9 (0.93, 3.7) times (P = 0.08).

Figure 3.8

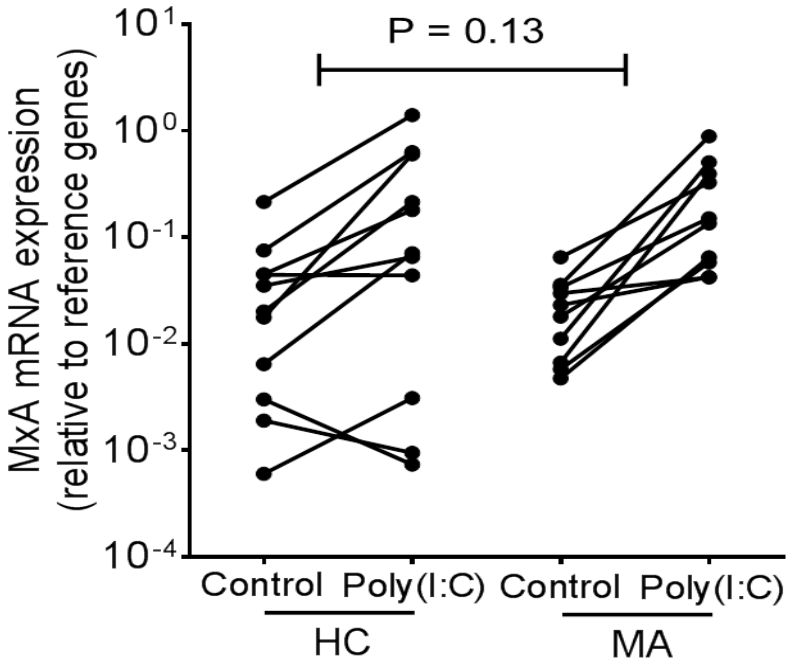


Figure 3.8 *Ex vivo* induction of MxA gene expression in whole blood in response to TLR-3 stimulation with poly(I:C) relative to reference genes GAPDH and UBC. mRNA levels between poly(I:C)-treated and control samples were compared. After 2 h of incubation, neither healthy controls (HC) nor moderate asthmatics (MA) show a significant induction of MxA (graph not shown). After 6 h of incubation a significant induction of IP-10 was observed in both HC and MA. Induction in MA was higher, but was not significantly different from the induction in HC – mean (CI) 2.6 (0.73, 9.1) times (P = 0.13).

3.4 Discussion

Double-stranded RNA (dsRNA) is produced by most viruses, including rhinoviruses as an intermediary during their replication (Guidotti, 2001). It is recognised as a 'danger signal' by TLR-3 (Guidotti, 2001). This recognition triggers innate immune antiviral responses such as the secretion of type I interferons (IFNα and IFNβ) within a few hours (Wark, 2005; Gern, 2003). Poly(I:C) is a synthetic dsRNA that acts as a surrogate of viral infection. This property of poly(I:C) has been utilised to study the biological effects of virus infections such as IFNβ production from BEC (Uller, 2010), RANTES and IL-8 from BEC (Gern, 2003; Konno, 2002). The concentrations of poly(I:C) used in these experiments ranged from as low as 1 ng/ml (Konno, 2002) to as high as 100 mcg/ml (Alexopoulou, 2001). This property of poly(I:C) was employed, and the expression of IFNß gene in whole blood in response to 100 µg/ml of poly(I:C) was assessed. Similar induction was seen on peripheral blood for IFNβ, OAS, and MxA. It is only the induction of IP-10 at 6 h that was observed to be significantly higher in MA compared with HC.

Deficient innate response to RV in asthma has been demonstrated in BEC (Wark, 2005), BALC(Contoli, 2006), and PBMC(Papadopoulos, 2002). Papadopoulos *et al*(Papadopoulos, 2002) investigated the secretion patterns of cytokines from PBMC from normal and atopic asthmatic subjects on exposure to RV at 24h and 48h time points. They found production of IFN-γ and IL-12 from PBMC of normal subjects after exposure to RV16 was significantly higher than in asthmatic subjects at both time points. The reverse was found with the levels of IL-10. These findings demonstrated the defective innate antiviral response in asthmatic subjects is not restricted to the lung in asthma. IP-10 mRNA expression in asthmatics was observed to be higher than that in healthy controls in response to poly(I:C), while there was no significant difference in the expression of IFNβ. However, due to the set-up of this study, it was not possible to assess protein synthesis. These findings however, need to be taken into consideration in future research.

It is believed that the increased susceptibility of the asthmatic lung to infection contributes to the association between respiratory virus infections and exacerbations (Contoli, 2006; Message, 2008; Wark, 2005). It was therefore investigated whether *ex vivo* whole blood antiviral responses to poly (I: C) are related to clinical and biological outcomes following experimental infection. Antiviral gene expression was found to correlate significantly with measures of severity of the cold but not to predict the severity of asthma symptoms, possibly because the deficient responses of the lung epithelium to virus (Wark, 2005) play a more important role in defining how asthmatics respond to infection; or possibly

because responses of whole blood may not be the same as the responses of airway mononuclear cells.

Chapter 4 - Experimental rhinovirus 16 infection in moderately severe asthmatics on inhaled corticosteroids

4.1 Introduction

The majority of asthma exacerbations are associated with respiratory virus infections, most frequently rhinoviruses (RV), the main cause of the common cold (Nicholson, 1996; Wark, 2002; Johnston, 1996). They are believed to result from worsening airway inflammation driven by the spread of virus infection from the upper to the lower airways (Harrison, 2004; Fleming, 1999). Virus-induced exacerbations occur despite good symptom control with inhaled corticosteroids (the mainstay of asthma treatment), and doubling the dose of inhaled corticosteroids does not prevent exacerbations (Harrison, 2004).

Experimental rhinovirus (RV) infection is a valuable tool for studying virus-induced exacerbations where RV is applied to the nose from where it spreads to the lower respiratory tract (LRT) and the effects on symptoms, lung function and inflammation are assessed (Fleming, 1999; Grunberg, 1999; Grunberg, 1997; Grunberg, 1997; Halperin, 1985; de Gouw, 1998; de Kluijver, 2003; Grunberg, 2001; Gern, 2000; Parry, 2000; Gern, 1997; Contoli, 2006; Message, 2008; Jarjour, 2000; Lemanske, 1989; DeMore, 2009). Studies to date have demonstrated variable, usually mild, increases in asthma symptoms (Fleming, 1999; Grunberg, 1999), AHR, airflow obstruction and bronchodilator use (Grunberg, 1999; Halperin, 1985; de Kluijver, 2003; Grunberg, 2001). Biological effects have been observed in bronchial biopsies (presence of virus (Gern, 1997; Papadopoulos, 2000; Mosser, 2005) reduced eosinophil numbers in the epithelium (Grunberg, 2001), increased mucosal lymphocytes (Fraenkel, 1995), BAL (induction of antiviral/interferon inducible genes or their products (Contoli, 2006; Message, 2008; Jarjour, 2000)), induced sputum (no significant changes in sputum eosinophil counts (Message, 2008; Grunberg, 1997), increased neutrophils (Message, 2008; Grunberg, 1997)) and exhaled breath (increased exhaled nitric oxide (eNO)) (de Gouw, 1998).

All studies to date have involved corticosteroid-naïve asthmatics. In one study (Grunberg, 2001) pre-treatment with inhaled corticosteroids failed to prevent a T-cell response to virus infection, pointing to corticosteroid-insensitive mechanisms of virus-induced inflammation. In this study the aims were

- to assess the feasibility and safety of experimental RV16 infection in moderate asthmatics on regular treatment with inhaled corticosteroids as a model,

- to evaluate antiviral responses mechanisms, and

- to explore *ex vivo* responses before experimental infection to a viral mimetic as biomarkers of clinical response during an experimental RV infection.

The hypotheses were that

- it is feasible and safe to induce an experimental RV16 infection in moderate asthmatics on regular treatment with inhaled corticosteroids

- the experimental infection would lead to a mild evaluable exacerbation of asthma

- *ex vivo* response of peripheral blood to ploy(I:C) before infection would predict clinical response to an experimental RV infection.

A previously validated infection protocol (Lemanske, 1989) was applied with minor modifications. Symptoms of upper respiratory tract (URT) infection and asthma and home and departmental measurements of forced expiratory volume in one second (FEV_1) were recorded. AHR was measured and the effects on airways inflammation assessed in induced sputum and by eNO measurement. Infection was confirmed by quantitative polymerase chain reaction (qPCR) for RV16 in nasal lavage and sputum and by determining serum anti-RV16 titres. Innate immune responses were studied by quantifying the induction of virus-response genes interferon (IFN)ß, IFNγ inducible protein 10 (IP-10), myxoma resistance protein A (MxA), and 2'-5' oligoadenylate synthetase (OAS) in sputum and peripheral blood and by measuring IP-10 protein in nasal lavage and sputum. Finally, *ex vivo* induction of IFNß by synthetic double stranded RNA, poly(I:C) was assessed before infection as a surrogate for virus-induced, TLR-3-mediated responses (Uller, 2010; Gern, 2003) and predictor of severity of infection.

4.2 METHODS

4.2.1 Study Design and subjects

Eleven non-smoking moderate asthmatics(Taylor, 2008) without RV16 immunity were selected from 44 screened subjects (Table 4.1) and infected with RV16 (Figure 2.1) from a stock used previously(Gern, 2000;Parry, 2000;DeMore, 2009;Mosser, 2005) applied at 10-fold lower dose ($100TCID_{50}$) as a safety precaution. Subjects recorded daily cold symptoms (headache, malaise, chills/feverishness, runny nose, blocked nose, sneezing, sore throat and cough, defined by the Jackson cold score)(Jackson, 1958), asthma symptoms (breathlessness, chest tightness, wheeze and cough), reliever bronchodilator use and nocturnal awakening and also the day they felt they developed a cold. All scores were collected by a short messaging service.

4.2.2 Lung Function and eNO

Daily FEV_1 and peak expiratory flow rate (PEF) were measured by a handheld spirometer (PIKO-1) with additional departmental measurements. Bronchial hyperresponsiveness was tested before and 7 days after inoculation. eNO was measured by NIOX MINO^{TM}.

4.2.3 Sample Collection and Processing

Nasal lavage was performed as reported (Greiff, 1990) and supernatants assessed for RV16 RNA and IP-10. Sputum was induced (Pizzichini, 2002), prior to infection with further attempts at subsequent visits. Cytospins were prepared (Efthimiadis, 2002) and remaining cells lysed in lysis buffer for gene expression analysis, while supernatants were assayed for RV16 viral RNA and IP-10. Subjects gave blood 6 times: samples for gene expression analysis were collected into PAXgene tubes (Perera, 2007) separate from samples for serum and for poly(I:C) stimulation.

Table 4.1

Subjects	Age (yr)	Gender (M/F)	Smoking history (pack years)	Baseline FEV_1[#] (% predicted)	BDP-equivalent daily dose of ICS (µg)
1	35	M	0	3.26 (86)	400
2	29	F	0	2.04 (81)	400
3	45	F	0.75	2.73 (105)	400
4	38	F	0	2.75 (85)	1000
5	29	F	0	2.55 (83)	200
6	36	M	0	5.65 (129)	400
7	45	F	0	3.45 (104)	400
8	41	M	0	5.21 (118)	800
9	39	F	0	3.26 (106)	1000
10	53	F	1	2.58 (98)	2000*
11	20	M	0	4.09 (87)	100
Median (Mean)	38 (37.3)	4M/7F		3.26 (98) (3.42 (98))	400 (645)

Table 4.1 Subject demographics. BDP: beclomethasone dipropionate. ICS: inhaled corticosteroids. [#]litres. An error in conversion to BDP at screening led to the inclusion of this subject in the study.

4.2.4 Virus Detection in Nasal Lavage and Sputum

RNA extraction was performed using QIAamp viral RNA columns (Myatt, 2004). Virus shedding in nasal lavage and sputum was quantified by qPCR using RV16 specific reagents. Analysis of sputum samples was performed in both cellular and fluid phases, while analysis of nasal lavage was only performed in the fluid phase.

4.2.5 Assessment of Innate Immune Responses

IP-10 was measured in nasal lavage and sputum supernatants by ELISA. Expression of antiviral genes for IFNß, IP-10, MxA and OAS in sputum cells was evaluated by qPCR. For systemic responses, mRNA from whole blood was assessed for IFNß, IP-10, MxA and OAS expression.

4.2.6 *Ex vivo* stimulation of whole blood as a predictor of responses to infection

Before infection, blood was collected in EDTA, treated with 100µg/ml poly(I:C) or 0.9% NaCl for 2 h and stored in PAXgene tubes at -80^{0}C until analysis of IFNß expression by qPCR.

4.2.7 Statistical Analysis

Distributions were assessed for normality; where appropriate the data was log-transformed. Suitable measures were used to summarise central tendencies. Maximum deterioration in symptoms during the first 14 days, change from baseline to 'worst' symptom value was calculated. To understand the variation in lung function measurements and the magnitude of the extreme observations, changes from baseline to 'best' and 'worst' values were calculated. The absolute differences (in change from baseline) between the extreme values were tested by paired t-test. Mean values for the study population following inoculation were assessed using

repeated measures analysis of variance (ANOVA) and if significant differences were found, trend analysis was performed by fitting polynomial contrasts to determine linear or quadratic trends in mean score over time. Statistical analysis was performed using SPSS and Graphpad Prism.

The study received ethical approval from Southampton and South West Hampshire Research Ethics Committee B.

See chapter 2 for detailed description of methods.

4.3 Results

4.3.1 Safety of experimental infection in moderate asthma

After infection, all the subjects had increased URT and asthma symptoms which were accompanied by lung function changes and increased bronchodilator use, but none of the subjects had a serious adverse event, withdrew because of safety concerns, or required hospitalization/emergency department visit or treatment beyond extra use of bronchodilators.

4.3.2 Evidence of infection

Subjects were considered successfully infected if they fulfilled at least 1 of the following criteria (Table 4.2): a) sero-conversion (>4-fold increase in anti-RV16 neutralising antibodies in serum during convalescence), b) shedding of virus detected by qPCR of nasal lavage and c) virus detection by qPCR in sputum. Thus, all subjects were found to be successfully infected, with 8 of 11 subjects fulfilling at least 2 criteria for infection and 6 having evidence of spread to LRT. The virus load seemed to decline faster in nasal lavage than in sputum (Figure 4.1).

4.3.3 Cold and asthma symptoms

The mean (CI) pre-inoculation cold symptom score was 0.9 (0.0, 1.7). The mean (CI) peak post-inoculation cold score was 9.5 (7.9, 11.1). The cold symptoms peaked mean (CI) 3.7 (2.9, 4.6) days post-inoculation (Figure 4.2A). ANOVA showed significant differences over time ($P < 0.01$) and trend analysis showed a significant quadratic trend ($P < 0.01$). Most subjects (6 of 11) additionally reported the development of a cold on day 2, two subjects reported colds on day 3, the remaining two subjects on days 4 and 6, whilst one subject recorded increased URT symptoms but did not specifically report a cold. The mean (CI) of differences between baseline and peak URT symptom scores (Figure 4.3A) was 8.6 (7.3, 9.9), ($P < 0.01$).

Table 4.2

Subject	a. Anti-R V16 antibody		b. Virus copy numbers in nasal lavage (day post inoculation)	c. Virus copy numbers in sputum/µl (day post-inoculation)	Fulfilled criteria for infection
	Baseline	Convalescent			
1	1.7	6.7	85 (10)	24,765(3)	a,b,c
2	1	4.8	742 (3)	101,516(5)	a,b,c
3	1	11.3	1,073 (3)	n.d.	a,b,
4	1	16	n.d.	n.d.	a
5	0	6.7	144 (3)	33,838(10)	a,b,c
6	0	4	373 (3)	n.d.	a,b
7	0	32	12 (5)	n.d.	a,b
8	0	4	10,239 (5)	3,612 (5)	a,b,c
9	0	1.2	6,201 (3)	546 (14)	b,c
10	0	8	1,324 (5)	84 (5)	a,b,c
11	0	1.4	14 (5)	6 (3)	b,c

Table 4. 2 Evidence of RV16 infection. The criteria for infection were: a) sero-conversion (≥4-fold increase in RV16 neutralising antibody titre in serum during convalescence), b) virus detected by qPCR in nasal lavage samples (shown as virus copy number per µl of supernatant, and c) virus detected by qPCR in sputum supernatant (shown as virus copy numbers per µl of supernatant). The highest virus copy number and the day on which this was detected are shown for illustrative purposes. n.d.: virus not detected.

Figure 4.1

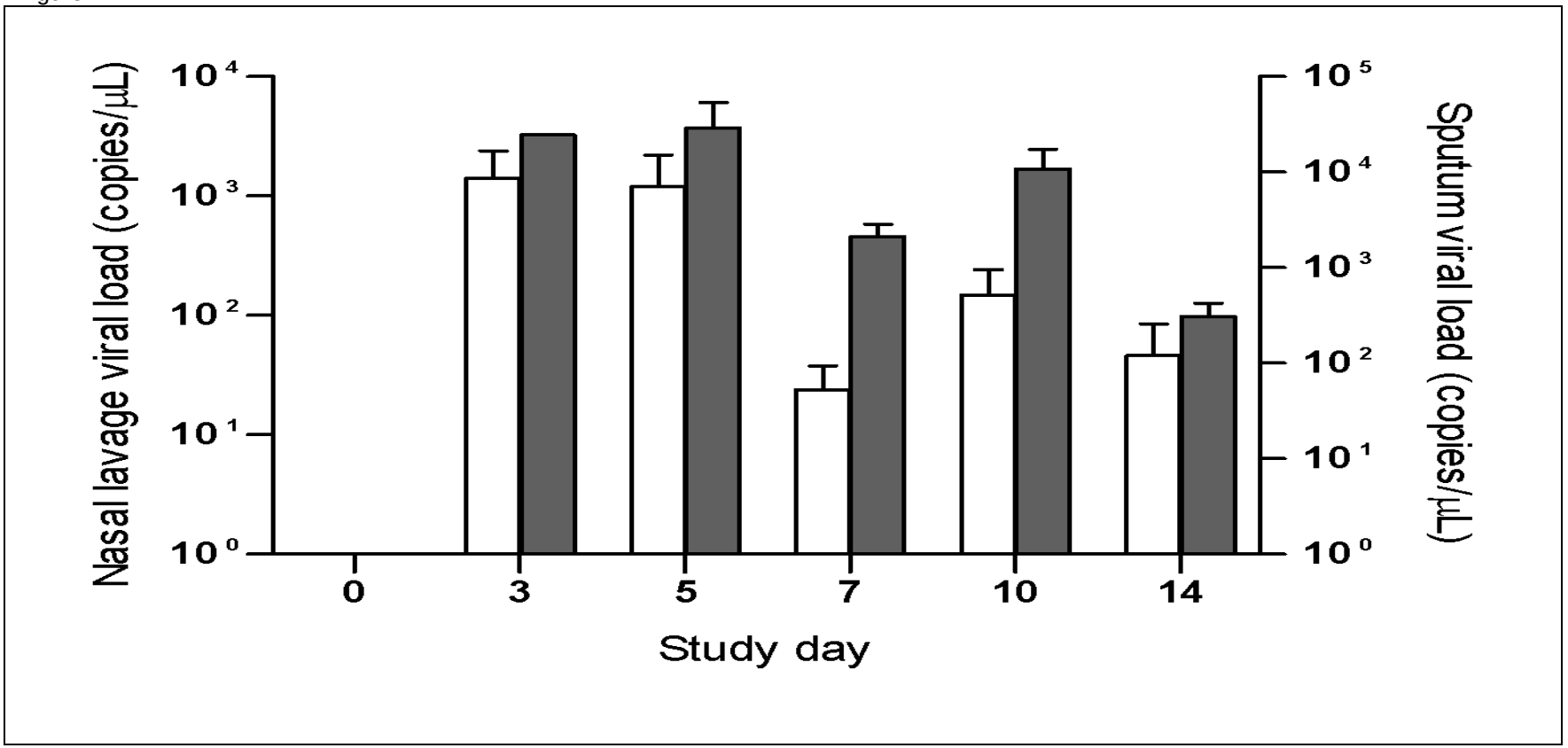


Figure 4.1 Viral load in the airways. Viral load in the upper airway, i.e. nasal lavage (clear bars, left y-axis), and lower respiratory tract, i.e. induced sputum (full bars, right y-axis) shown as mean (SEM) virus copy numbers per µl of sample supernatant.

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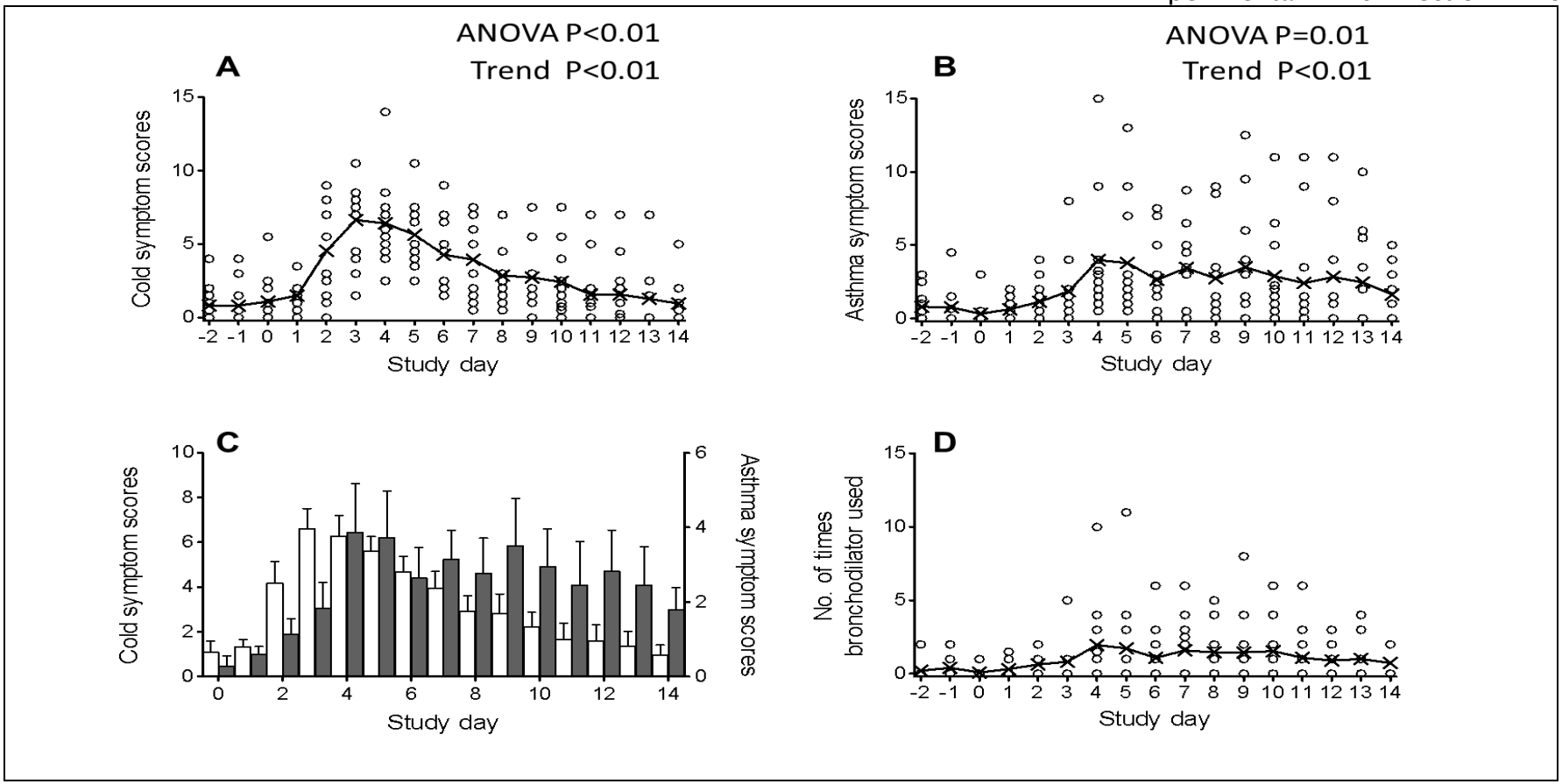

Figure 4.2 Cold and asthma symptom scores and bronchodilator use. A: Individual daily cold symptom scores (open circles) and means (x) are shown. There was a significant change in cold symptom scores over time following inoculation (ANOVA, P < 0.01) which followed a quadratic trend (P < 0.01). B: Individual daily asthma symptom scores (open circles) and means (x) are shown. There was a significant change in asthma symptom scores over time following inoculation (ANOVA, P = 0.01), which followed a quadratic trend (P < 0.01). C: Cold symptoms (left Y-axis, open bars) peaked mean (CI) 3.7 (2.9, 4.6) days post-inoculation. Asthma symptom scores (right Y-axis, full bars) peaked mean (CI) 5.8 (4.0, 7.6) days post-inoculation, mean (CI) 2.1 (0.3, 3.9) days after peak cold symptoms). Mean (SEM) are shown. **D**. Number of times short-acting bronchodilator was used per day does not show a significant change over time.

Figure 4.3

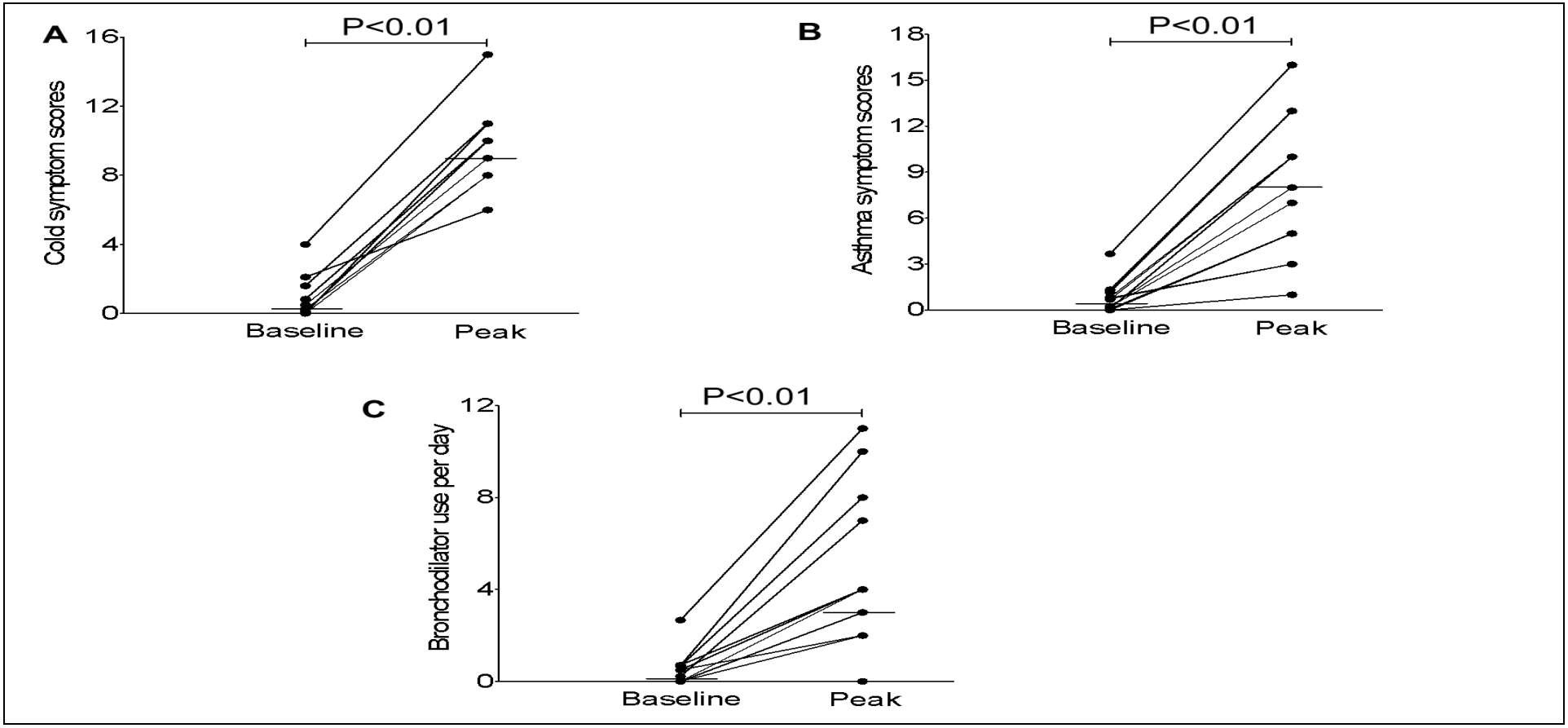

Figure 4.3 Change in cold symptom scores. Baseline cold symptom score was mean (CI) 0.9 (0.0, 1.7). The mean (CI) peak cold score was 9.5 (7.9, 11.1). The mean (CI) difference was 8.6 (7.3, 9.9), ($P < 0.01$). **B**: Change in asthma symptom scores. Baseline asthma symptom score was mean (CI) 0.8 (0.1, 1.5). The mean (CI) peak asthma score was 7.8 (4.5, 11.1). The mean (CI) difference was 7.0 (4.2, 9.8) ($P < 0.01$). **C**: Change in bronchodilator use per day. Baseline bronchodilator use was mean (CI) 0.2 (-0.1, 0.5), and the mean (CI) peak use was 3.6 (1.4, 5.9). The mean (CI) difference was 3.2 (1.4, 4.9) times per day ($P < 0.01$).

The mean (CI) pre-inoculation asthma symptom score was 0.8 (0.1, 1.5). The mean (CI) peak post-inoculation asthma score was 7.8 (4.5, 11.1). Asthma symptoms peaked mean (CI) 5.8 (4.0, 7.6) days post-inoculation (Figure 4.2B) and ANOVA showed significant differences over time (P = 0.01). Trend analysis showed a significant quadratic trend (P < 0.01). The mean (CI) of differences between baseline and peak asthma scores (Figure 4.3B) was 7.0 (4.2, 9.8) (P < 0.01). ANOVA of bronchodilator use following infection (Figure 4.2D) showed no significant differences over time (P = 0.25). However, the mean (CI) maximum increase in bronchodilator use from baseline during the study period (Figure 4.3C) was 3.2 (1.4, 4.9) median 3 P < 0.01. The mean (CI) period between peak cold and peak asthma symptoms (Figure 4.2C) was 2.1 (0.3, 3.9) days. After peaking, the asthma symptoms declined less rapidly than the cold symptoms.

4.3.4 Innate immune responses

4.3.4.1 **Nasal lavage**

Analysis of IP-10 protein was restricted to 7 of 11 samples because the lavage method in the other four subjects who were recruited at the Imperial College, London was sufficiently different to raise concern about effects on mediator measurements. ANOVA showed significant differences over time (P < 0.01) with a significant quadratic trend (P < 0.01) (Figure 4.4A). Baseline IP-10 concentration was mean (CI) 31 (-4.1, 66). The mean (CI) peak IP-10 concentration was 280 (200, 360). The mean (CI) difference was 250 (160, 340) (P < 0.01) (Figure 4.4B). There was a significant change in concentration of IP in sputum over time following inoculation (ANOVA, P < 0.01) which followed a quadratic trend (P < 0.01) (Figure 4.4C) Baseline IP-10 concentration was mean (CI) 190 (91, 380). The mean (CI) peak IP-10 concentration was 2100 (540, 8500). The mean (CI) difference was 12 (3.4, 39) (P < 0.01). (Figure 4.4B).

4.3.4.2 Sputum

ANOVA on concentrations of IP-10 in sputum showed they changed significantly over time (P < 0.01) and followed a significant quadratic trend (P < 0.01) (Figure 4.4C). Baseline IP-10

concentration was mean (CI) 190 (91, 380). The mean (CI) peak IP-10 concentration was 2100 (540, 8500). The mean (CI) difference was 12 (3.4, 39) ($P < 0.01$) (Figure 4.4D).

Only 4 of the 11 subjects produced sputum of sufficient quality for RNA analysis of antiviral response genes IFN β , MxA, IP-10 and OAS (Figure 4.5). Given the small sample size, the fact that there were missing data at several time-points and that IFN β gene expression was detected at baseline in only one subject, these data were not subjected to statistical analysis. Nevertheless, the overall pattern observed was suggestive of an increase in gene expression during the infection and a decline during recovery.

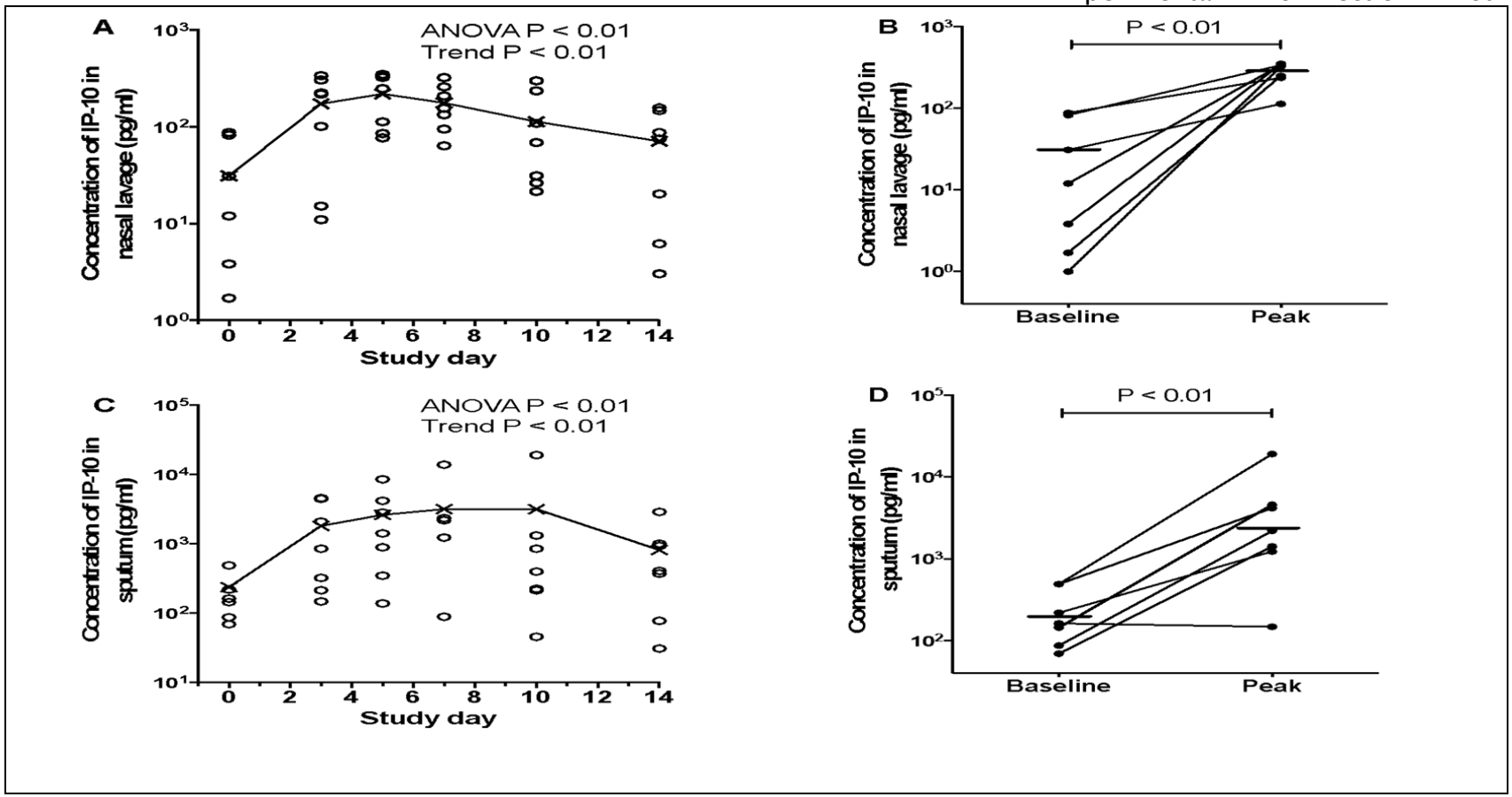

Figure 4.4. Changes in IP-10 in response to infection. A: Individual values for concentration of IP-10 in nasal lavage (open circles) and means (x) are shown. There was a significant change in concentration of IP-10 in nasal lavage over time following inoculation (ANOVA, P < 0.01) which followed a quadratic trend (P < 0.01). B: Change in IP-10 concentration in nasal lavage. Baseline IP-10 concentration was mean (CI) 31 (-4.1, 66). The mean (CI) peak IP-10 concentration was 280 (200, 360). The mean (CI) difference was 250 (160, 340) (P < 0.01). C: Individual values for concentration of IP-10 in sputum (open circles) and means (x) are shown. There was a significant change in concentration of IP in sputum over time following inoculation (ANOVA, P < 0.01) which followed a quadratic trend (P < 0.01). D: Change in IP-10 concentration in sputum. Baseline IP-10 concentration was mean (CI) 190 (91, 380). The mean (CI) peak IP-10 concentration was 2100 (540, 8500). The mean (CI) difference was 12 (3.4, 39) (P < 0.01).

Figure 4.5

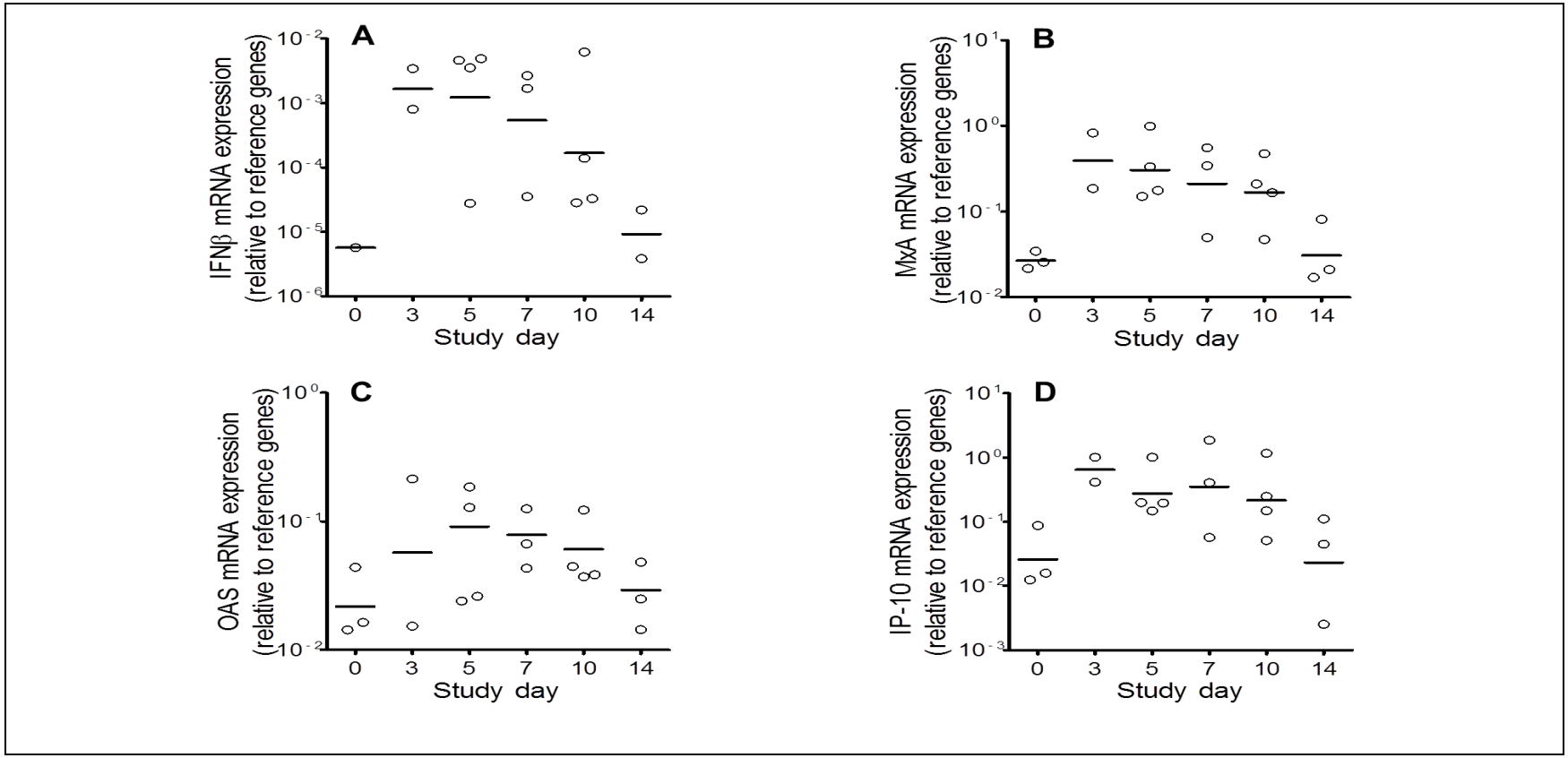

Figure 4.5 Expression of IFNB (panel A), MxA (panel B), OAS (panel C), and IP-10 (panel D) in sputum cells. Individual values (open circles) and geometric means (horizontal lines) are shown.

4.3.4.3 Blood

ANOVA analysis of antiviral gene expression in peripheral blood cells showed induction of genes for MxA (Figure 4.6A), OAS (Figure 4.6B), IP-10 (Figure 4.6C), and IFNβ (Figure 4.6D) which were significantly different over time ($P < 0.01$, $P < 0.01$, $P = 0.01$, and $P = 0.04$, respectively). All four genes followed a significant quadratic trend ($P = 0.01$, $P < 0.01$, $P = 0.04$, and $P = 0.01$, respectively).

4.3.5 Gene expression in response to *ex vivo* stimulation with poly(I:C)

IFNβ gene expression in response to poly(I:C) was assessed in 10 of the 11 subjects, one subject being unable to donate blood, and this showed average 75-fold up-regulated IFNβ gene expression (Figure 4.7A). There was a significant inverse correlation (Pearson's correlation coefficient, $r = -0.84$, $P < 0.01$) between the *ex vivo* induction of IFNβ and symptoms of cold (Figure 4.7B) but no other measure of clinical or biological response.

4.3.6 Lung function

The increase in asthma symptoms was not associated with significant increasees in airflow obstruction measured at home, or in the laboratory, expressed as FEV_1 or PEFR (Figure 4.8). ANOVA showed no statistically significant differences over time of the mean FEV_1 measured in the department ($P = 0.09$) (Figure 4.8A) and the home morning FEV_1 ($P = 0.61$) (Figure 4.8B). ANOVA also showed no statistically significant differences over time of the mean PEF measured in the department ($P = 0.21$) (Figure 4.8C) and the morning PEF recorded at home ($P = 0.67$) (Figure 4.8D).

4.3.7 Bronchial reactivity

One subject did not undergo the second methacholine challenge for her own safety because her FEV_1 on that day was 62.7% of predicted. The geometric mean (range) cumulative PC_{20} values at baseline (1.7 (0.2-113.6 mg/ml) and day 7 (3.5 (0.25 – 66.6) mg/ml)

were not significantly different (P = 0.18) (Figure 4.9) in the 9 subjects who underwent the challenge.

4.3.8 Exhaled NO

ANOVA of measurements of eNO showed that these did not vary significantly over time (P = 0.58) (Figure 4.10).

Figure 4.6

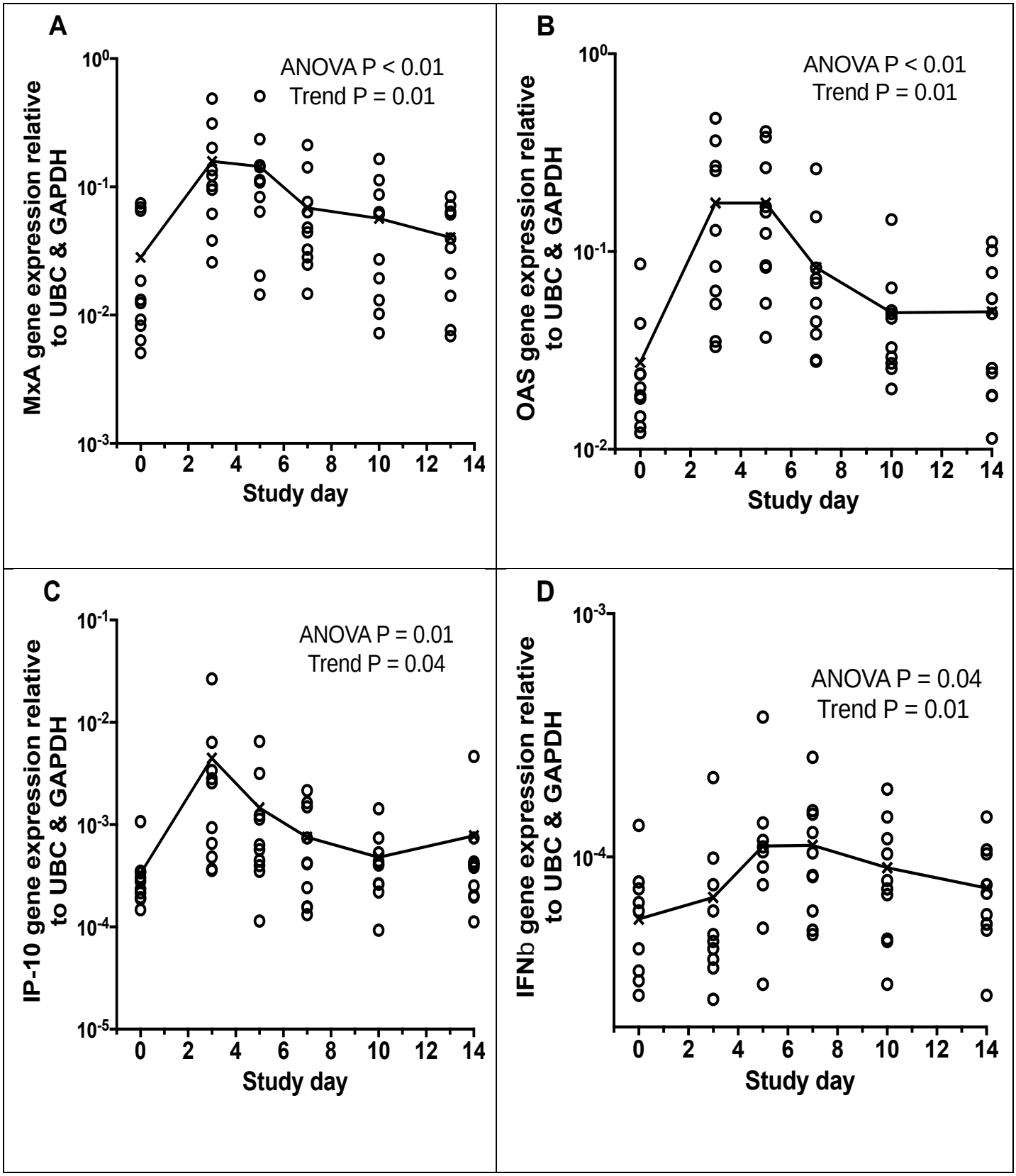

Figure 4.6 Induction of innate response genes in peripheral blood in response to infection. Individual values for mRNA (open circles) and means (x) relative to reference genes GAPDH and UBC are shown. There was a significant change in MxA (panel A), OAS (panel B), IP-10 (panel C), and IFNβ (panel D) mRNA over time following inoculation (ANOVA, P < 0.01, P < 0.01, P = 0.01, and P = 0.01 respectively) which followed a quadratic trend (P = 0.01, P = 0.01, P = 0.04, and P = 0.01 respectively

Figure 4.7

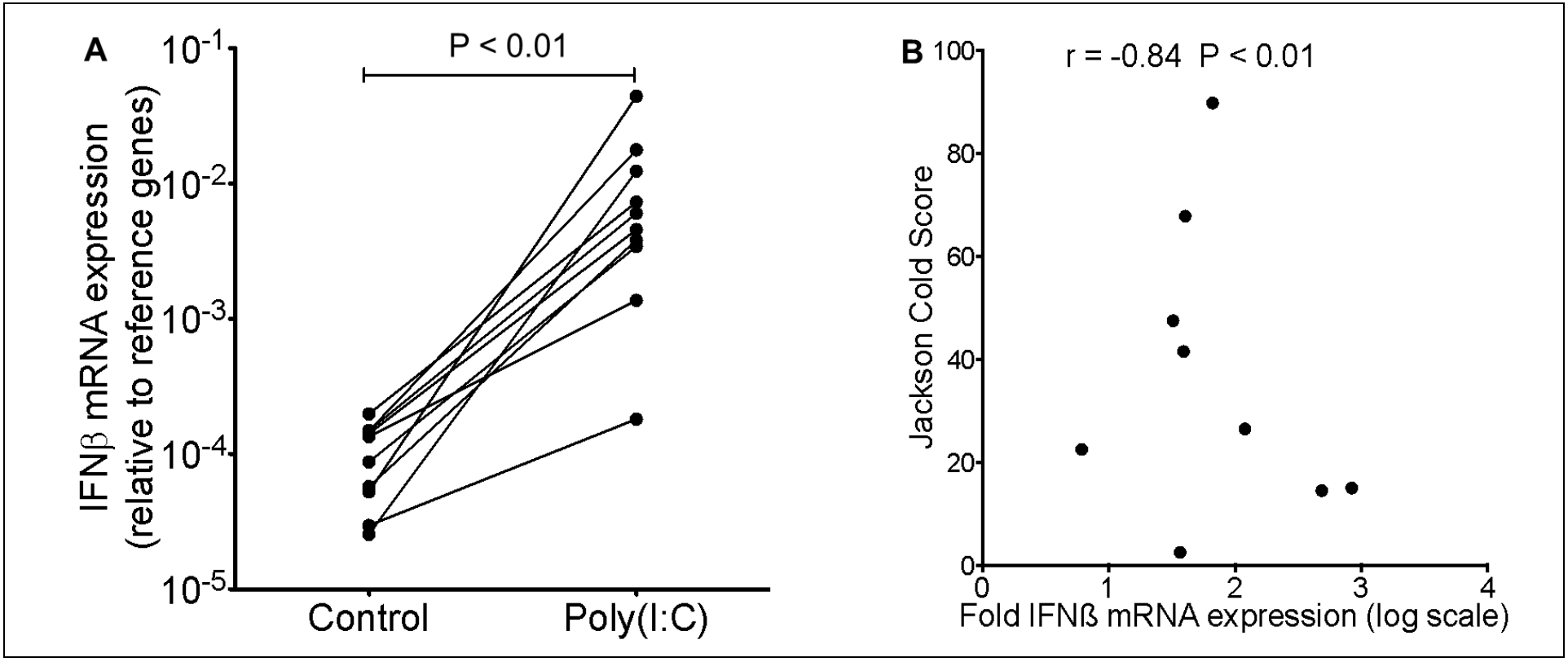

Figure 4.7 *Ex vivo* up-regulation of IFNβ gene expression in whole blood after stimulation with the TLR-3 stimulating viral mimetic poly(I:C) correlated with cold symptom scores. **A**: Signifcant induction of IFNβ mRNA was in the moderate asthmatics' peripheral blood before inoculation (P < 0.01). **B**: A negative correlation between the extent of IFNβ mRNA expression and cold symptom scores following inoculation was observed (Pearson's coefficient r = -0.84, P < 0.01).

Figure 4.8

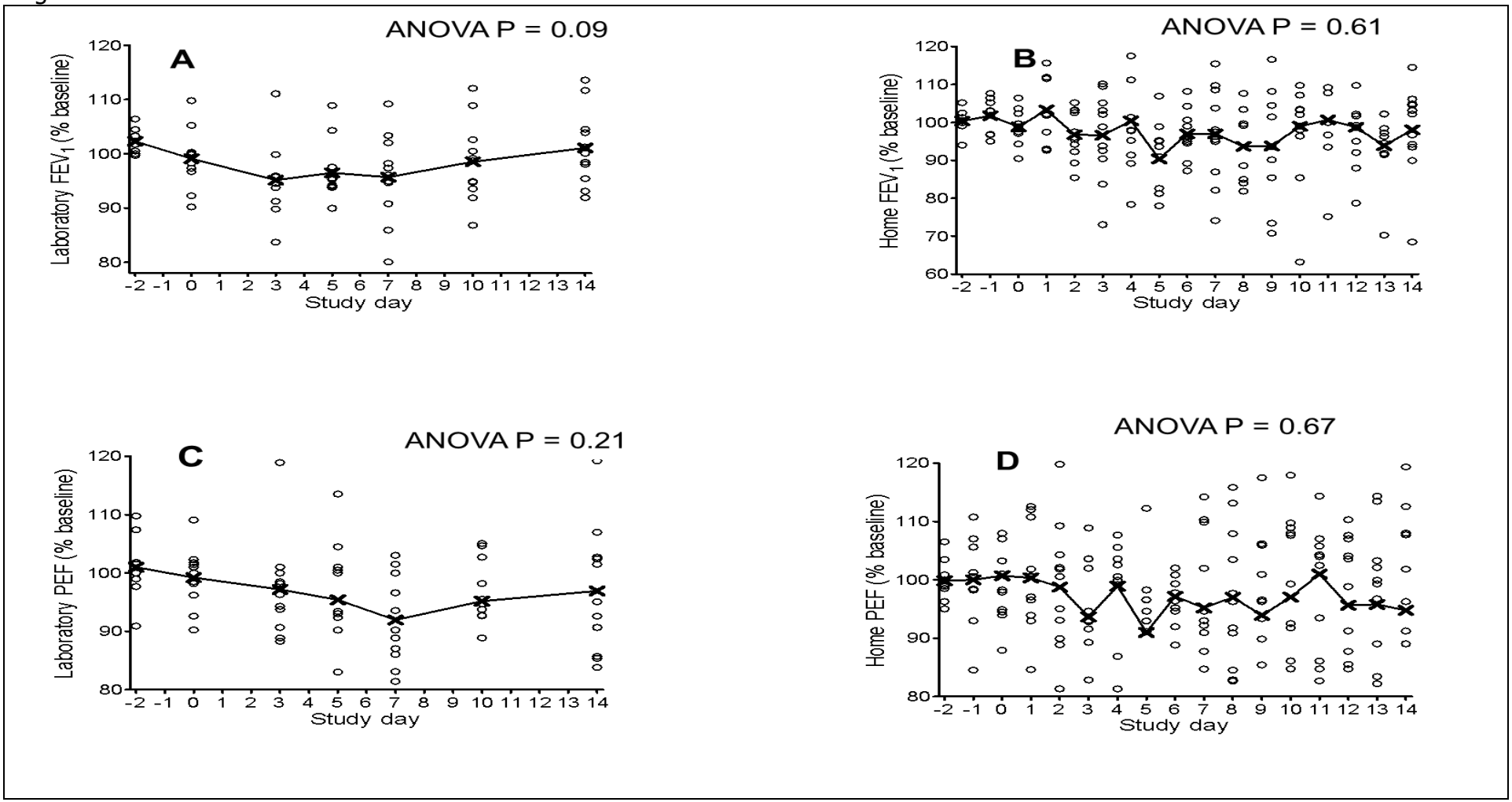

Figure 4.8 Assessment of airflow obstruction following infection. The group mean changes in FEV_1 measured in the department laboratory (panel A) and at home in the morning with a handheld device (panel B) were not significantly different following infection (P = 0.09, and P = 0.61 respectively). Similarly, group mean changes in PEF measured in the department laboratory (panel C) and at home in the morning with a handheld device (panel D) were not significantly different following infection (P = 0.21, and P = 0.67 respectively).

Figure 4.9

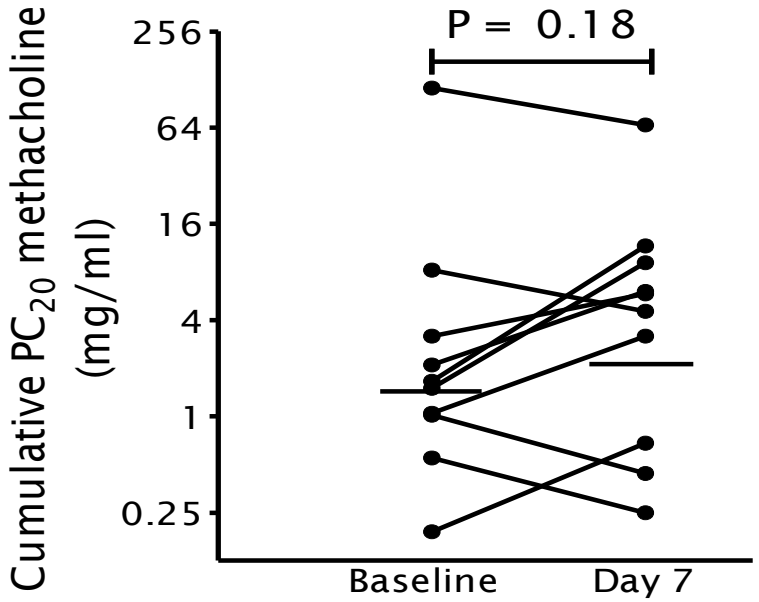

Figure 4.9 Bronchial reactivity expressed as the cumulative dose of methacholine causing a 20% decline in FEV_1 (PC_{20}) measured at baseline and 7 days after RV16 inoculation. Horizontal bars represent geometric means. Mean baseline (CI) PC_{20} was 1.7 (0.52, 5.6) mg/ml, mean (CI) PC_{20} at day 7 was 3.5 (1.1, 12) mg/ml. The mean difference between baseline and day 7 PC_{20} was 0.6 (0.28, 1.3) (P = 0.18) mg/ml.

Figure 4.10

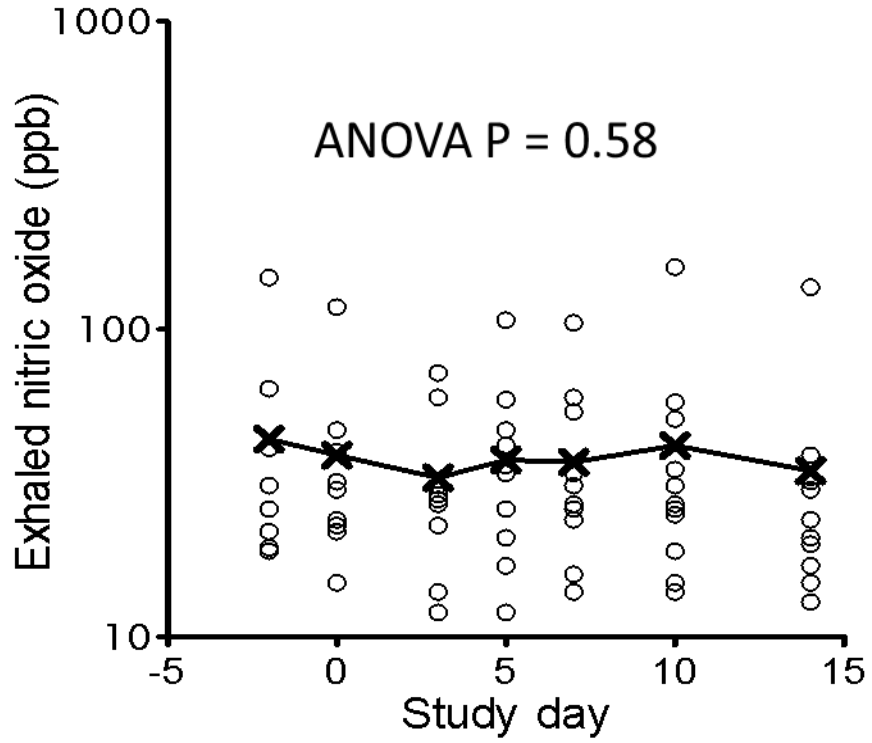

Figure 4.10 Exhaled nitric oxide following infection. Individual values (open circles) and means (x) are shown. There was a significant change in exhaled nitric oxide over time (ANOVA, P = 0.58).

4.4 Discussion

This is the first study to demonstrate the safety of experimental RV infection in asthmatics requiring inhaled corticosteroids. The increased asthma symptoms and observed innate immune, anti-viral responses in nasal lavage, sputum and blood suggest that studies in this population have the potential to further our understanding of virus-induced exacerbations. The viral load and antiviral responses seen in sputum suggested spread of infection from the URT to the LRT. The viral load was temporally related to cold and increased asthma symptoms that were associated with increased use of reliever medication which probably accounts for the lack of sustained falls in FEV_1 and PEF. Finally, the degree of up-regulation of antiviral gene expression in blood by the viral mimetic poly(I:C) prior to experimental infection correlated with measures of severity of cold but not asthma symptoms.

Recognising that this was the first experimental infection in patients at risk of severe exacerbations, a cautious study design was implemented. All subjects had to be well controlled prior to inoculation and they were followed up very closely during the study, assisted by the messaging service (SMS) developed specifically for the purpose of this study, with a central computer sending out requests and collecting subjects' replies twice daily. We used RV16, a strain used safely in previous studies and shown *in vitro* to replicate to a similar extent but to induce relatively lower levels of inflammatory cytokine release and epithelial cell death (Wark, 2009) than other strains. Whilst we applied the same stock of virus used in previous studies in asthma(Gern, 2000;Parry, 2000;DeMore, 2009;Mosser, 2005), for reasons of safety we chose an inoculation dose 10 times lower than in previous studies, allowing in the design (see chapter 2)) for dose escalation if this was ineffective. However, this was not necessary since all subjects developed cold symptoms at this dose. As a final precaution, we used a delivery device (see chapter 2) which generates 30 - 100 μm particles. This restricted delivery to the nose given that aerosols $\geqslant$16 μm result in 100% deposition in the nasal passages(Newman, 1985), in contrast to the DeVilbiss atomizer used in most previous studies(Lemanske, 1989) that generates small enough aerosol (as low as 5 μm) immediately to reach the LRT. Thereby, the infection protocol mimicked more closely natural infection and limited direct lung exposure during challenge.

The magnitude of symptoms and physiological and biological responses in previous studies involving corticosteroid-naïve, mild asthmatics (de Kluijver, 2003; Contoli, 2006; Message, 2008; DeMore, 2009; Mosser, 2005; Bardin, 2000) have varied significantly, likely reflecting differences in patient susceptibility. In our study, similar variability was observed. Asthma symptoms increased transiently, but no subject withdrew because of safety or

adverse events. To be of value, experimental infection should lead to significant changes in asthma-relevant endpoints. In the current study the observed changes were comparable to those previously reported (Fleming, 1999; Grunberg, 1999) Asthma peak symptoms followed the peak cold symptoms by mean 2.1 days. Correspondingly, URT and LRT symptoms were associated with nasal and sputum viral loads peaking at days 3 and 5, respectively. Antiviral biomarkers in nasal lavage (IP-10) and sputum (MxA, OAS, IP-10) followed a similar pattern, presumably in response to infection. Interestingly, blood responses (MxA, OAS, IP-10, and IFNß) followed the pattern of nasal lavage and not sputum responses, although it should be noted that the three compartments were not sampled daily. Thus, a window of therapeutic opportunity appears to exist between the onset of URT and LRT symptoms, allowing this model to be used to test therapies for virus-induced asthma exacerbations.

The increase in asthma symptoms was not accompanied by reduced lung function observed previously(Grunberg, 1999;Halperin, 1985;de, 2003;Grunberg, 2001) and, unlike the study by Grunberg *et al*(Grunberg, 1999) there was no significant change in bronchial hyperresponsiveness, possibly due to long-term inhaled corticosteroids (Kerstjens, 1994). Morning FEV_1 fell by mean 15.6%, although the days when the peak falls were recorded varied considerably between patients, suggesting varying dynamics of the inflammatory response. This degree of airflow obstruction has been reported before (Grunberg, 1999; Message, 2008). The lack of significant fall in FEV_1 is likely due to a clinically relevant increased use of bronchodilators from mean 0.4 to a peak of 3.2 times per day. Consequently, statistical analysis of lung function data remains challenging as its true relationship with viral infection within patients over time is difficult to demonstrate. In this study we chose also to analyse maximum decreases in lung function as this was deemed to be a clinically meaningful. In order to put some perspective on the observation of the 'extreme' value, we also analysed the maximum increase in lung function. This revealed that, whilst not statistically significant, on average maximum decrease was greater than maximum increase. Greater variation in home measurements (compared to laboratory) resulted in more extreme values being observed and a relatively smaller difference between the maximum increase and decrease. Detection of statistically significant differences was difficult due the small sample size and hence it is appropriate to place onus instead on the summary estimate and 95% confidence intervals.

It is believed that the increased susceptibility of the asthmatic lung to infection contributes to the association between virus infections and exacerbations (Contoli, 2006; Message, 2008; Wark, 2005). We investigated, therefore, whether *ex vivo* whole blood antiviral responses to the viral mimetic poly(I:C) are related to clinical and biological

outcomes. We found that antiviral gene expression correlated significantly with measures of severity of the cold but did not predict the severity of the asthma symptoms or the anti-viral responses in the airways, possibly because the deficient responses of the lung epithelium to virus (Wark, 2005) play a more important role in defining how asthmatics respond to infection.

This study has limitations. First, there was no placebo or healthy control group, so the pre-inoculation readouts have served as the basis for comparisons. Also, it has not been possible to assess cellular and mediator response in the lower airways prior to the detection of virus in sputum. Furthermore, we detected viral RNA by PCR without distinguishing between live and inactivated virus; however, detection of viral RNA and infectious virus has correlated closely in previous studies (DeMore, 2009). The study design did not make it possible to look for viraemia, given the strong induction of antiviral response genes in the systemic circulation and the demonstration of viraemia in some children during RC-associated exacerbations. For some read-outs (e.g. innate immunity in sputum) measurement was possible in a small number of subjects; these observations should, therefore, be seen as preliminary. Finally, although all study participants were stable before infection, one cannot completely exclude the possibility that some of the observed changes could be due to variability that characterises asthma.

In summary, this study has important implications for further research, first and foremost showing that it is feasible to apply a small dose of virus exclusively into the nose and, thereby, safely induce a mild exacerbation in susceptible subjects with moderately severe asthma in whom exacerbations are more clinically significant than in those previously studied. The changes in lung function and induction of innate immunity observed in the study suggest that this is a valuable model for studying exacerbation mechanisms and for testing novel therapies.

Appendix

The results of this chapter have been accepted for publication in the European Respiratory Journal, as the following Letter to the Editor

Dear Editor:

Experimental rhinovirus 16 infection in moderate asthmatics on inhaled corticosteroids

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The majority of asthma exacerbations are associated with respiratory virus infections, mostly rhinoviruses (RVs)(1), due to enhanced inflammation in the airways(2), and these occur despite symptom control with inhaled corticosteroids (ICS)(3). Experimental RV infection is a valuable tool for studying virus-induced exacerbations(2,4), but has to date involved corticosteroid-naïve asthmatics only. We have, therefore, modified a validated infection protocol(4) to inoculate 11 subjects whose asthma was well-controlled with ICS. As this was the first experimental infection in patients at risk of severe exacerbations, a cautious study design was implemented. All subjects were followed-up twice daily by SMS during the study. We used RV16, a strain used safely in previous studies, which replicates *in vitro* to a similar extent but induces less inflammation and cell death(5) than other strains. We also chose a 10-fold lower inoculation dose of the same stock used in previous studies(6). The design allowed for dose escalation if necessary (this proved to be unnecessary as all subjects developed cold symptoms at this dose). As a final precaution, the delivery device generated 30-100 μm particles, restricting delivery to the nose - aerosols ≥16 μm are deposited in the upper respiratory tract (URT)(7) - thus mimicking closely natural infection, i.e. limiting direct lung exposure during inoculation.

Symptoms of URT infection, asthma, and measurements of lower respiratory tract (LRT) function were recorded post-inoculation. Infection was confirmed by quantitative polymerase chain reaction (qPCR) for RV16 in nasal lavage and sputum and by determining serum anti-RV16 titres: a ≥4-fold increase in titres in convalescent serum or shedding of RV16 in the airways was evidence of successful infection. We also studied innate immune responses by extracting mRNA from sputum cell pellets and whole blood cells and quantifying the induction of virus-response genes interferon (IFN)β, C-X-C motif chemokine 10 (CXCL10), myxoma resistance protein A (MxA), and 2'-5' oligoadenylate synthetase (OAS). Furthermore, CXCL10 protein was measured by ELISA in nasal lavage and sputum supernatants. Finally, blood was collected before RV16 infection, treated with poly(I:C) at a dose of 100 μg/ml (Invivogen, San Diego, USA) or 0.9% NaCl as control for 2 hr and stored in PAXgene tubes (QIAGEN, Crawley, UK) for IFNβ gene expression analysis by qPCR as a

surrogate for virus-induced, TLR-3-mediated responses(8) and to assess the value of this assay as a predictor of severity of infection.

All 11 subjects were successfully infected without any serious adverse effects, withdrawal because of safety concerns, or requirement for treatment beyond extra bronchodilator use. All developed increased cold symptoms (Figure 1A), peaking mean (CI) 3.7 (2.9, 4.6) days post-inoculation. ANOVA showed significant symptom differences over time ($P < 0.01$) with a significant quadratic trend ($P < 0.01$) and the mean (CI) of differences between baseline and peak URT symptom scores was 8.6 (7.3, 9.9), ($P < 0.01$). Asthma symptoms (Figure 1A) peaked mean (CI) 5.8 (4.0, 7.6) days post-inoculation. ANOVA showed significant differences over time ($P = 0.01$), with a significant quadratic trend ($P < 0.01$). The mean (CI) of differences between baseline and observed peak values were 7.0 (4.2, 9.8), ($P < 0.01$) for asthma scores and 3.2 (1.4, 4.9), ($P < 0.01$) for bronchodilator use. The mean (CI) period between peak cold and peak asthma symptoms (Figure 1A) was 2.1 (0.3, 3.9) days. In contrast, there was no significant reduction in lung function post-inoculation.

ANOVA of CXCL10 concentrations in nasal lavage in 7 of 11 subjects showed significant differences over time ($P < 0.01$), with a significant quadratic trend ($P < 0.01$) (Figure 1B). Comparison of baseline and peak CXCL10 concentrations showed a significant mean (CI) increase of 247 pg/ml (155, 339), ($P < 0.01$). ANOVA of CXCL10 concentrations in sputum showed significant differences over time ($P < 0.01$), with a significant quadratic trend ($P < 0.01$) (Figure 1C). Comparison of log-transformed peak against baseline concentrations showed a mean (CI) increase of 1.1 (0.5, 1.6) log, ($P < 0.01$) i.e., mean (CI) increase of 12-fold (3, 40). ANOVA of MxA, OAS, CXCL10, and IFNβ gene expression showed significant difference over time ($P < 0.01$, $P < 0.01$, $P = 0.01$, and $P = 0.04$, respectively), with a significant quadratic trend for all assessments ($P = 0.01$, $P < 0.01$, $P = 0.04$, and $P = 0.01$, respectively). CXCL10 gene expression is shown in Figure 1D as an example.

Ex vivo IFNβ gene expression in blood cells in response to poly(I:C) stimulation, assessed in 10 of the 11 subjects, was up-regulated mean 75-fold, with a significant inverse correlation (Spearman's correlation coefficient, r_s = -0.72, P = 0.04) between the *ex vivo* induction of IFNβ and cold symptoms, but not with asthma symptoms or the biological response.

This is the first study to demonstrate the safety and feasibility of experimental RV infection in asthmatics requiring ICS, which paves the way for larger studies in more severe asthmatics. The increased asthma symptoms and antiviral responses in nasal lavage, sputum and blood suggest that such studies can be used to understand the disease mechanisms in this population for whom exacerbations present a greater risk than the type of asthmatic studied to date. Such studies also have the potential utility for testing new antiviral or anti-inflammatory drugs. However, we recognise that the mild nature of exacerbation induced is, to an extent, a limitation; thus, FEV_1 did not drop significantly, possibly because of increased use of bronchodilators by an average of 3 puffs per day. Thus this model cannot be used as a surrogate for more severe exacerbations and these will have to be studies in a natural setting on community-acquired exacerbations. Any experimental challenge in patients with asthma needs to demonstrate safety and the current study shows that this can be achieved whilst producing disease-relevant readouts.

To be valuable, experimental infection should induce significant changes in asthma-relevant endpoints. The virus shedding and antiviral responses seen in sputum suggested spread of infection from the URT to the LRT. Virus shedding was temporally related to cold and increased asthma symptoms that were associated with increased bronchodilator use (from mean 0.4 to a peak of 3.2 times per day), which probably accounts for the lack of sustained fall in lung function. Unlike in steroid-naïve asthmatics(2), there was no significant change in bronchial hyperreactivity, possibly due to long-term ICS(9). Peak asthma symptoms followed peak cold symptoms by mean 2.1 days. Correspondingly, URT and LRT symptoms were associated with nasal and sputum viral loads peaking at post-inoculation days 3 and 5, respectively. Antiviral biomarkers in nasal lavage (CXCL10) and sputum (MxA,

OAS, CXCL10) followed a similar pattern, presumably in response to infection. Interestingly, blood responses (MxA, OAS, CXCL10, IFNβ genes) followed the pattern of nasal lavage and not sputum responses. Thus, a window of therapeutic opportunity is apparent between URT and LRT symptoms; accordingly, this model has utility for testing of therapies for virus-induced asthma exacerbations.

The increased susceptibility of the asthmatic lung to infection contributes to the association between virus infections and exacerbations(2,10). We investigated, therefore, whether *ex vivo* poly(I:C)-induced antiviral responses by whole blood cells relate to clinical and biological outcomes. We found that antiviral gene expression correlated significantly with cold severity without predicting asthma symptom severity or the antiviral responses in the airways, possibly because the deficient lung epithelial responses to virus(10) play a more important role in defining how asthmatics respond to infection.

This study has limitations. First, there was no control group. Furthermore, we detected viral RNA by PCR without distinguishing between infectious and inactivated virus; however, detection of viral RNA and infectious virus has correlated closely in previous studies(2,6). Assessments of sputum, and nasal lavage were possible in a small number of subjects; these observations should, therefore, be seen as preliminary. Finally, although all study participants were stable before infection, some of the observed changes could possibly be due to the variability that characterises asthma. Nevertheless, we believe that this study provides proof of concept for the use of experimental virus infection model in the study of chronic asthmatics whose disease requires the use of ICS.

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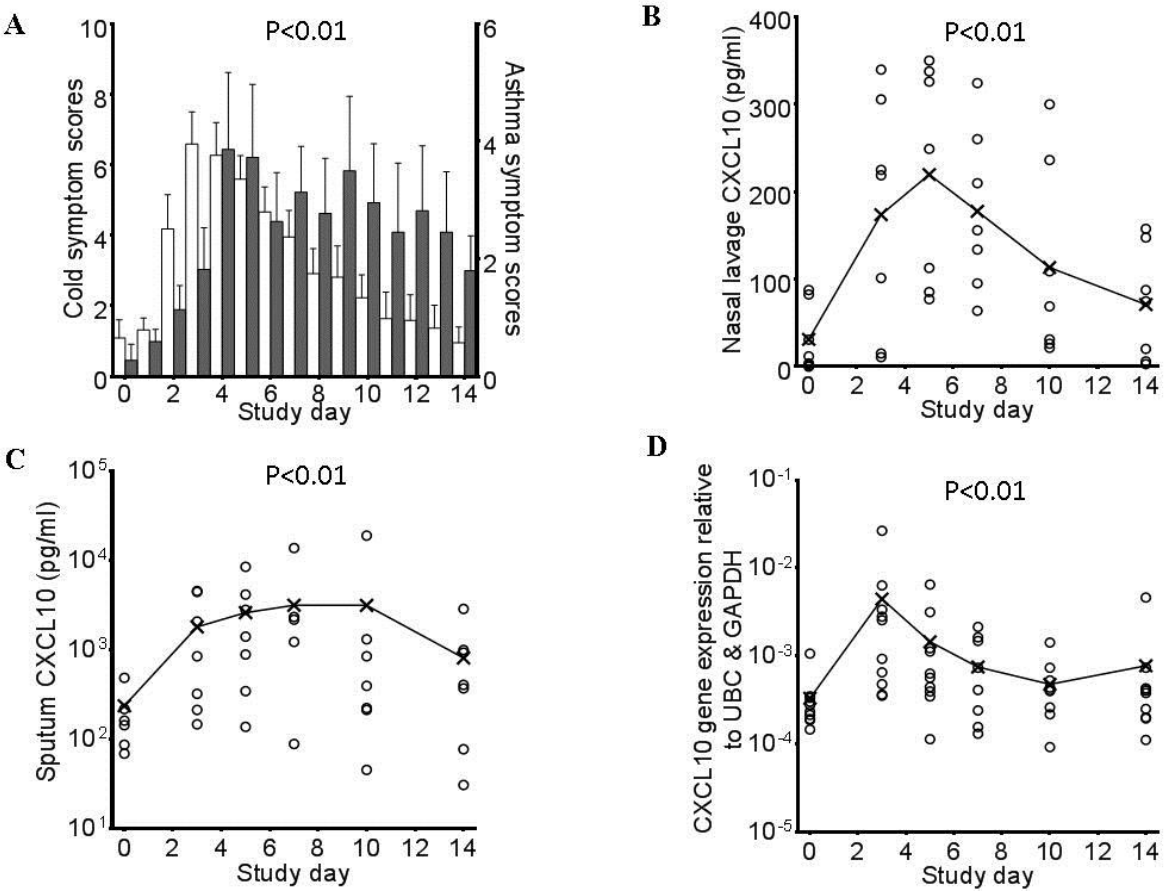

Figure 1. Key clinical and biomarker results of the study. A: Both cold (left y-axis, clear bars) and asthma (right y-axis, shaded bars) symptom scores increased significantly ($p < 0.01$ by ANOVA and quadratic trend). B: C-X-C motif chemokine (CXCL10) concentrations in nasal lavage. C: CXCL10 concentrations in sputum. D: Induction of CXCL10 gene in peripheral blood cells. All the changes were significant ($p < 0.01$), as shown by ANOVA and quadratic trend analysis. The data in panel A are shown as means and standard errors of the mean, in panels B, C and D they are shown as individual values (open circles), with means shown as x.

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Chapter 5 - Experimental rhinovirus 16 infection in smokers

5.1 Introduction

It is now accepted that individual susceptibility coupled with exposure to inhaled noxious substances leads to the development of chronic obstructive pulmonary disease (COPD). Globally, exposure to cigarette smoke is the commonest noxious agent that leads to COPD. Respiratory abnormalities of smoking-induced COPD could be summarised as comprising of airway inflammation, largely irreversible airflow obstruction, destruction of lung parenchyma, and periods of exacerbations (GOLD, 2013; Mannino, 2007). Asymptomatic smoking-induced airways pathology may remain undiagnosed in many subjects in earlier stages. Prevalence surveys carried out in a number of countries using standardised methods including spirometry estimate that up to 25% of adults aged 40 years and over may have airflow obstruction classifiable as COPD (Menezes, 2005; Chapman, 2006; Lopez, 2006; Buist, 2005).

Bronchial epithelial cells (BEC) represent the primary site of rhinovirus (RV) infection in the lower airways (Papadopoulos, 2000). *In vitro* experiments have shown that BEC from long-term smokers without COPD are more susceptible to RV16 relative to cells from non-smoking controls (Haywood, 2010). Exposure to cigarette smoke has been found to blunt the response of airway epithelial cells *in vitro* to RV infection, leading to increased viral replication by interfering with the IFN pathways and consequent activation of antiviral genes (Eddleston, 2011). The central role of interferons in antiviral defence has already been alluded to (see Chapter 1 Introduction). Ongoing smoking is associated with a significant increase in the risk for respiratory viral infections (Eddleston, 2011), and lower airway complications of RV infection (Nicholson, 1996). About half of common cold infections lead to exacerbation of COPD (Seemungal, 2000). The vast majority of such colds are caused by rhinovirus infections.

RV infections are therefore important events in long-term smokers with or without COPD, but experimental models to study the mechanisms involved in the pathobiology of such infections are lacking. Mallia *et al* (Mallia, 2011) have successfully carried out an experimental rhinovirus challenge study in smokers with normal lung function and with GOLD Stage 2 COPD (FEV_1 between 50% and 80% of predicted). They showed that infecting long-term smokers (with and without COPD) was safe. The RV infection led to both upper and lower airway symptoms and also caused on average a 9% decrease in FEV_1 from

baseline, thus representing a mild exacerbation (Mallia, 2011). These findings have not been replicated. It is important that these findings are replicated, and other aspects of virus-host interactions evaluated to increase our understanding of the mechanisms involved in virus-induced exacerbation of airway inflammatory diseases caused by long-term exposure to cigarette smoke such as COPD.

This study sought to replicate the findings of Mallia *et al* by carrying out an experimental RV infection in long-term smokers, to further explore virus-host interactions following experimental RV16 infection in smokers, and thus explore the potential of experimental RV16 infection in smokers as a model for studying mechanisms of virus-induced exacerbations of COPD. In this study mainly individuals with normal lung function were selected, with a minority having mild COPD. The hypotheses were

- it is feasible, and safe to carry out an experimental rhinovirus infection in long-term smokers;
- such an infection would lead to measurable upper and lower respiratory tract symptoms; and
- the infection would induce an evaluable innate antiviral response.

5.2 Methods and materials.

5.2.1 Study Design and subjects

Twelve subjects with >10 packet-year smoking history without RV16 immunity were selected from 55 screened subjects (Table 5.1) and infected with RV16 (Figure 2.1) from a stock used previously (Gern, 2000; Parry, 2000; DeMore, 2009; Mosser, 2005) but applied at 10-fold lower dose ($100TCID_{50}$). Subjects recorded daily cold symptoms (headache, malaise, chills/feverishness, runny nose, blocked nose, sneezing, sore throat and cough, defined by the Jackson cold score) (Jackson, 1958), lower airway symptoms (breathlessness, cough, and sputum scores (BCSS)) (Leidy, 2003), and they also recorded the day they felt they had developed a cold. All scores were collected by a short messaging service.

5.2.2 Lung Function and eNO

Daily FEV_1 and peak expiratory flow rate (PEF) were measured by a handheld spirometer (PIKO-1) with additional departmental measurements. eNO was measured by NIOX MINO^{TM}, and gas transfer by a Collins CPL machine (Ferraris Respiratory, Hertford, UK).

5.2.3 Sample Collection and Processing

Nasal lavage was performed as reported (Greiff, 1990) and supernatants were assessed for RV16 RNA. Sputum was induced (Pizzichini, 2002) prior to infection with further attempts at subsequent visits. Cytospins were prepared (Efthimiadis, 2002) and remaining cells lysed in lysis buffer for gene expression analysis, while supernatants were assayed for RV16 viral RNA and IP-10. Subjects gave blood 6 times and samples for gene expression analysis were collected into PAXgene tubes (Perera, 2007) separate from samples for serum.

5.2.4 Virus Detection in Nasal Lavage and Sputum

RNA extraction was performed using QIAamp viral RNA columns (Myatt, 2004). Virus shedding in nasal lavage and sputum was quantified by qPCR using RV16

Table 5.1

Subject	Age (yr)	Gender (M/F)	Smoking history (pack years)	FEV_1/FVC ratio	Baseline FEV_1 (% predicted)	TLCO (% predicted)
1	51	M	13	75	4.09 (106)	92
2	50	M	46	74	2.73 (115)	80
3	56	F	27	87	4.13 (110)	77
4	47	M	53	85	4.54 (110)	97
5	45	M	15	74	3.17 (95)	80
6	60	M	44	66*	3.29 (92)	56
7	68	M	66	68*	2.14 (80)	75
8	47	M	32	84	3.68 (93)	70
9	50	F	52	73	2.53 (91)	62
10	58	M	30	77	3.57 (105)	86
11	60	M	48	62*	2.57 (74)	96
12	52	M	37	66*	2.88 (78)	62
Median (Mean)	51.5 (53.7)	2F/10M	40.5 (38.6)	74 (74.3)	94 (95.8)	78.5 (77.8)

Table 5. 1 Subject demographics

specific reagents. Analysis of sputum samples was performed in both the cell and fluid phases, while analysis of nasal lavage was performed in the fluid phase alone.

5.2.5 Assessment of Innate Immune Responses

IP-10 was measured in sputum supernatants and serum by ELISA. Expression of antiviral genes for IP-10, MxA and OAS in sputum cells was evaluated by qPCR. For systemic responses, mRNA from whole blood was assessed for IP-10, MxA and OAS expression.

5.2.6 Statistical Analysis

Distributions were assessed for normality; where appropriate the data was log-transformed. Suitable measures were used to summarise central tendencies. Maximum deterioration in symptoms during the following 13 days, and change from baseline to 'worst' symptom value was calculated. To understand the variation in lung function measurements and the magnitude of the extreme observations, changes from baseline to 'best' and 'worst' values were calculated. The absolute differences (in change from baseline) between the extreme values were tested by paired t-test. Mean values for the study population following inoculation were assessed using repeated measures analysis of variance (ANOVA) and if significant differences were found, trend analysis was performed by fitting polynomial contrasts to determine linear or quadratic trends in mean values over time. Statistical analysis was performed using SPSS and Graphpad Prism.

The study was approved by a National Research Ethics Committee (REC No 09/H0501/56).

Detailed methods and materials are described in Chapter 2.

5.3 Results

5.3.1 Evidence of infection

Subjects were considered successfully infected if they fulfilled at least 1 of the following criteria (Table 5.2): a) sero-conversion (≥4-fold increase in RV16 neutralising antibodies in serum during convalescence), b) shedding of virus detected by qPCR of nasal lavage and c) virus detection by qPCR in sputum. Thus, 10 of the 12 subjects were found to be successfully infected, with 9 of 10 subjects fulfilling at least 2 criteria for infection, and 5 having evidence of spread to LRT. Virus shedding in the sputum was apparent by day 3 following inoculation (Figure 5.1). Further analysis was restricted to the 10 subjects who showed evidence of infection.

5.3.2 Safety of experimental infection

After infection, all the subjects had increased upper and lower airway symptoms, but none of the subjects had a serious adverse event and none withdrew because of safety concerns or required hospitalization/emergency department visit or treatment.

5.3.3 Cold and lower respiratory tract symptoms

The 10 subjects who showed evidence for infection developed cold symptoms (Figure 5.2A), which peaked mean (CI) 4.5 (2.9, 6.1) days post-inoculation. ANOVA showed significant differences over time ($P < 0.01$) and trend analysis showed a significant quadratic trend ($P < 0.01$). Most subjects (4 of 10) additionally reported the development of a cold on day 2, two subjects reported colds on day 4, one subject on days 5, whilst three subjects recorded increased URT symptoms but did not specifically report a cold. Mean (CI) baseline cold symptom score was 0.6 (0.1, 1.1). Mean (CI) peak cold score was 7.5 (5.5, 9.6). The mean (CI) difference between baseline and peak cold scores was 7.9 (5.9, 9.9) (2-tailed t-test, $P < 0.01$). (Figure 5.2B).

Table 5.2

Subject	a. Anti-RV16 antibody		b. Virus copy numbers in nasal lavage/µl (day post-inoculation)	c. Virus copy numbers in sputum/µl (day post-inoculation)	Criteria for infection fulfilled
	Baseline	Convalescent			
1	0	26.9	176(7)	43(5)	a,b,c
2	0	>32	n.d.	4,006(7)	a,c
3	0	>32	5(10)	n.d.	a,b
4	1.7	1.7	n.d.	n.d.	-
5	0	4	4(13)	n.d.	a,b
6	1	>32	n.d.	n.d.	a
7	1	1.2	n.d.	n.d.	-
8	0	13.5	2(5)	n.d.	a,b
9	0	3.4	2250(3)	n.d.	a,b
10	1.2	>32	14829(3)	56,1139(3)	a,b,c
11	0	0	76(3)	14,970(3)	b,c
12	1.4	>32	1956(3)	9,253(3)	a,b,c

Table 5. 2 Evidence of RV16 infection The criteria for infection were: a) sero-conversion (≥4-fold increase in RV16 neutralising antibodies in serum during convalescence), b) virus copy numbers in the nose as detected by qPCR of nasal lavage samples (number per µl of supernatant, c) virus copy numbers in sputum supernatant (number per µl of supernatant). n.d.: virus not detected.

Figure 5.1

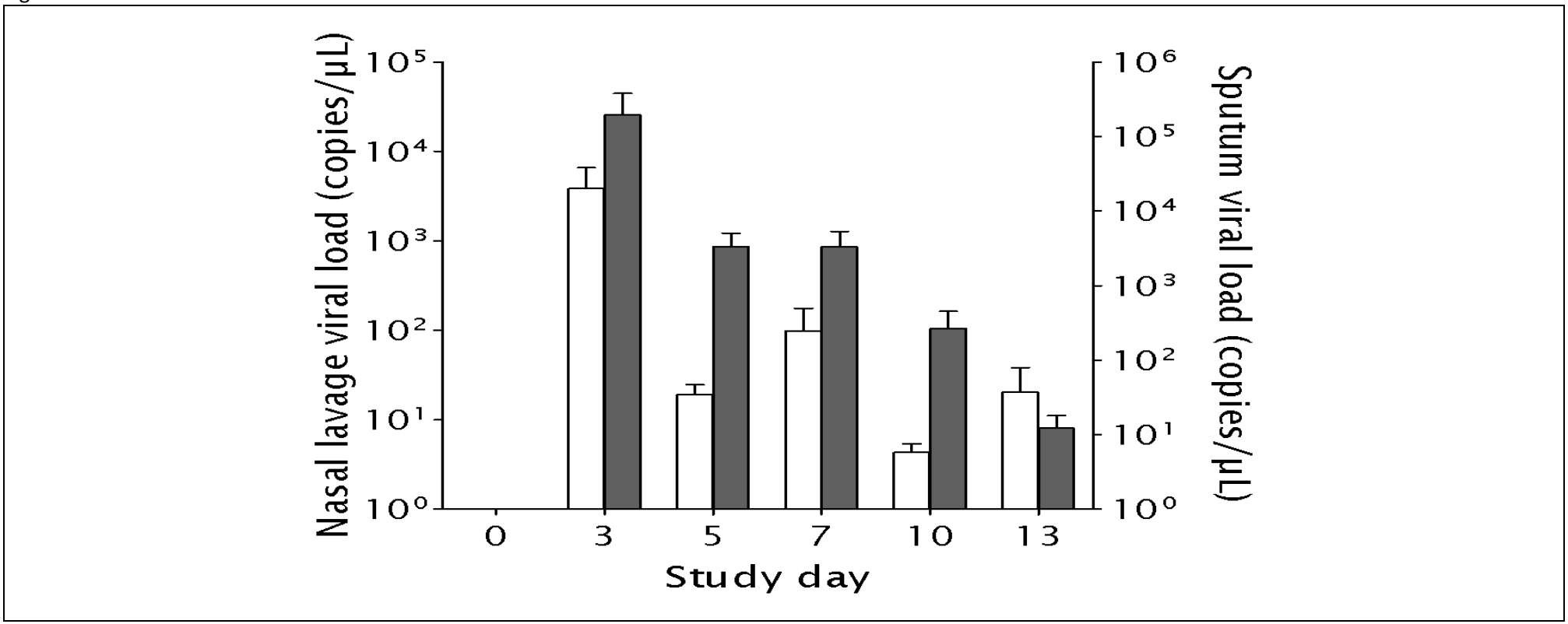

Figure 5.1 Viral load in the airways. Viral load in the upper airway, i.e. nasal lavage (clear bars, left y-axis), and lower respiratory tract, i.e. induced sputum (full bars, right y-axis) shown as mean (SEM) virus copy numbers per µl of sample supernatant. Study days are shown on the x-axis. Nasal inoculation with rhinovirus was performed on day 0. Nasal lavage and sputum induction were performed before inoculation on day 0, and on days 3, 5, 7, 10 and 13.

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Figure 5.2

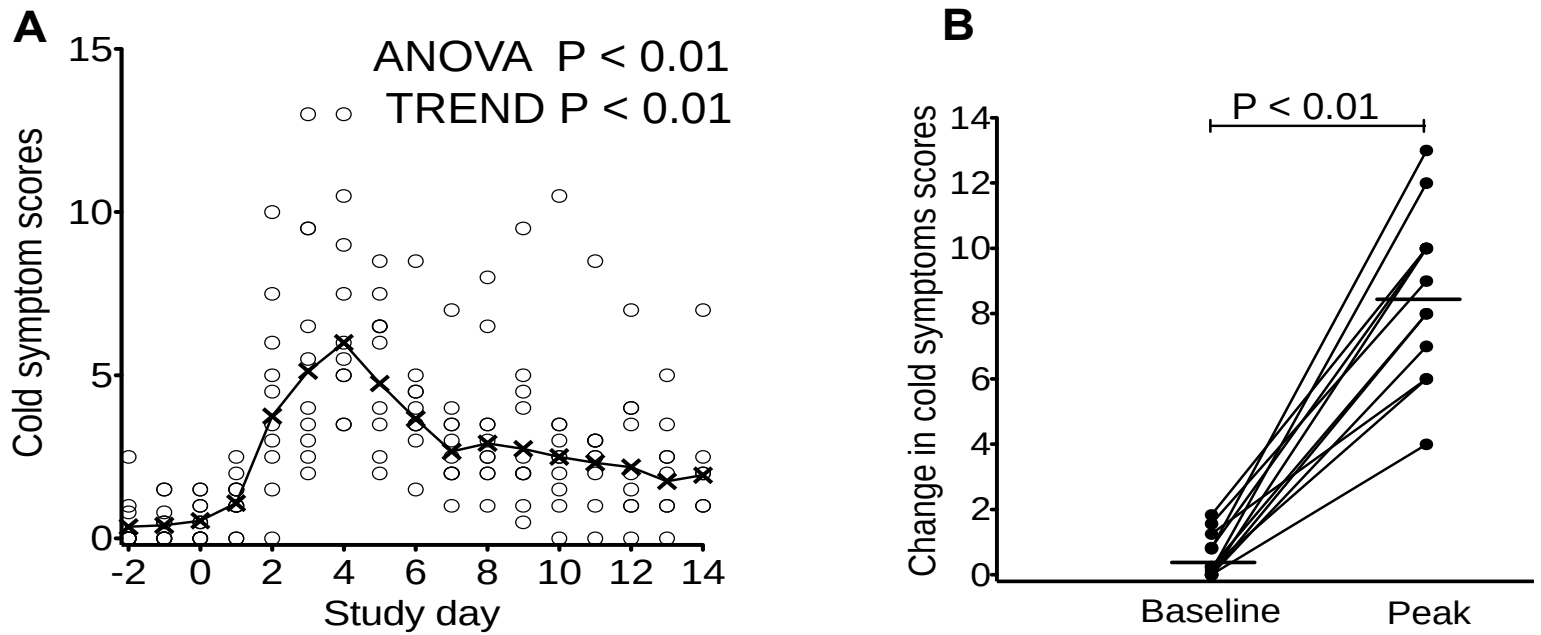

Figure 5.2 Cold symptom scores. A: Individual values (open circles) and means (x) are shown of daily cold symptom scores. ANOVA showed significant change in cold symptom scores over time (P < 0.01), with a significant quadratic trend (P < 0.01). Cold symptoms peaked mean (CI) 4.5 (2.9, 6.1) days post-inoculation. B. Change in cold symptom scores relative to baseline. Mean (CI) baseline cold symptom score was 0.6 (0.1, 1.1). Mean (CI) peak cold score was 7.5 (5.5, 9.6). The mean (CI) difference between baseline and peak cold scores was 7.9 (5.9, 9.9) (2-tailed t-test, P < 0.01).

The lower respiratory tract symptoms (BCSS) peaked mean (CI) 5.8 (4.0, 7.6) days post-inoculation and ANOVA showed significant differences over time (P = 0.01). Trend analysis showed a significant quadratic trend (P < 0.01) (Figure 5.3A). . Mean (CI) baseline score was 0.5 (0.2, 1.0). Mean (CI) peak score was 4.2 (3.0, 5.3). Mean (CI) difference between baseline and peak score was 3.5 (2.4, 4.7) (2-tail t-test, P < 0.01). (Figure 5.3B) When assessed separately, breathlessness, cough, and sputum scores all showed significant differences over time, with a significant quadratic trend (Figures 5.3C and 5.4A and C). The mean (CI) baseline breathlessness score was 0.0 (0.2, 0.1). The mean (CI) peak score was 0.9 (0.3, 1.5) (2-tailed t-test, P < 0.01) (Figure 5.3D). Mean (CI) baseline cough score was 0.4 (0.0, 0.7). Mean (CI) peak score was 1.9 (1.3, 2.5). The mean (CI) difference between baseline and peak was 1.5 (1.0, 2.1) (2-tailed t-test, P < 0.01) (Figure 5.4B). Mean (CI) baseline sputum score was 0.2 (0, 0.4). Mean (CI) peak score was 1.5 (1.1, 1.8). The mean (CI) difference between baseline and peak was 1.3 (0.8, 1.7) (2-tailed t-test, P < 0.01) (Figure 5.4D). In most subjects the cold symptoms peaked before LRT symptoms. The mean (CI) period between peak cold and peak LRT symptoms was 0.7 (-0.3, 1.7) days.

5.3.4 Exhaled nitric oxide

Exhaled nitric oxide (eNO) levels increased following infection. ANOVA showed significant differences over time (P = 0.01). (Figure 5.5). The increase from baseline in eNO following infection was mean (CI) 15.3 (7.5, 23.0) ppb (P < 0.01).

5.3.5 Spirometry

ANOVA showed no statistically significant differences over time of the mean FEV_1 measured in the department (P = 0.94) (data not shown) and the morning FEV_1 monitored at home (P = 0.90) (data not shown). ANOVA also showed no statistically significant differences over time of the mean PEF measured in the department (P = 0.20) and the morning PEF monitored at home (P = 0.77) (data not shown)

5.3.6 Gas Exchange

Transfer factor of the Lung for Carbon Monoxide (TLCO) was measured at baseline and at every visit during the study and expressed as a percentage of the baseline. ANOVA showed no statistically significant differences over time (P = 0.29)

5.3.7 Innate immune responses

5.3.7.1 Sputum

Only 4 of the 10 subjects were able to consistently produce sufficient sputum of adequate quality (< 20% squamous cells) for RNA extraction from sputum cells. In these samples induction of IP-10 (CXCL10) mRNA expression was observed (Figure 5.6A). These were no subjected to ANOVA given the small number of samples and missing values. Measurement of IP-10 (CXCL10) protein was possible in 5 of the 10 subjects (Figure 5.6B). These data were not subjected to ANOVA given the small number of samples coupled with missing values. However, comparison of baseline against peak CXCL10 protein concentrations showed a significant mean (CI) increase of 2.0 (0.1, 3.9) log (P = 0.04) (Figure 5.6C), i.e., mean (CI) increase of 100 (1.3, 790) times in response to infection.

Adequate sputum samples were obtained from 5 of the 10 subjects for differential cell counts. The increase in LRT symptoms was associated with an increase in the percentage of neutrophil counts in sputum (Figure 5.9). These data were not subjected to ANOVA given the small number of samples.

Figure 5.3

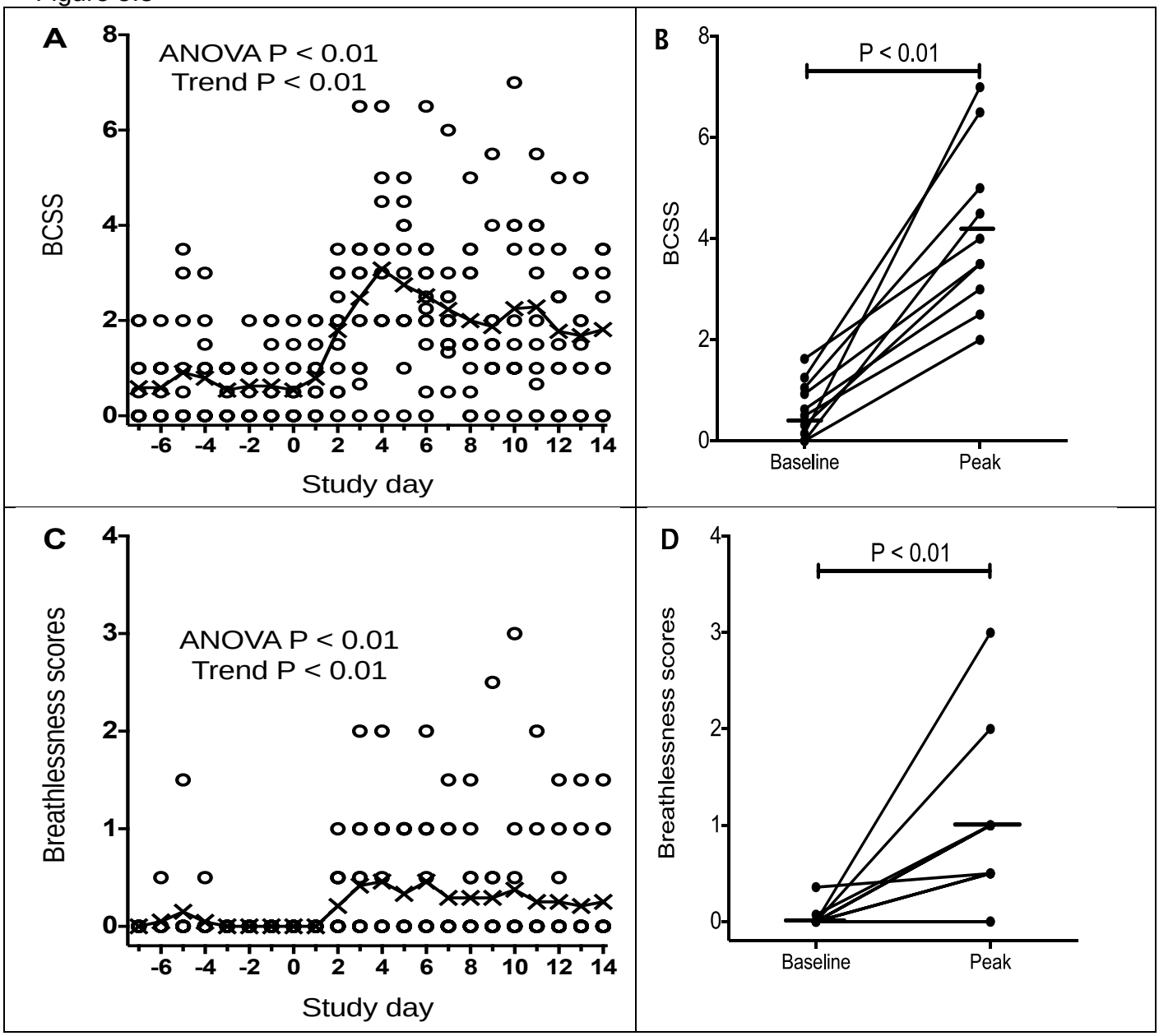

Figure 5.3 Breathlessness, cough and sputum scores. **A**: Daily breathlessness, cough and sputum scores (BCSS). Individual values are shown as open circles, means are shown as x. These changed significantly over time (ANOVA P < 0.01). Trend analysis showed quadratic trend (P < 0.01). **B**: Change in BCSS. Individual values are shown as full circles, means are shown as horizontal bars. Mean (CI) baseline score was 0.5 (0.2, 1.0). Mean (CI) peak score was 4.2 (3.0, 5.3). Mean (CI) difference between baseline and peak score was 3.5 (2.4, 4.7) (2-tail t-test, P < 0.01). **C**: Breathlessness scores. Individual values are shown as open circles, means are shown as x. These changed significantly over time (ANOVA P < 0.01). Trend analysis showed quadratic trend (P < 0.01). **D**: Change in breathlessness score. Individual values (full circles) and means (horizontal bars) are shown. The mean (CI) baseline score was 0.0 (0.2, 0.1). The mean (CI) peak score was 0.9 (0.3, 1.5) (2-tailed t-test, P < 0.01).

Figure 5.4

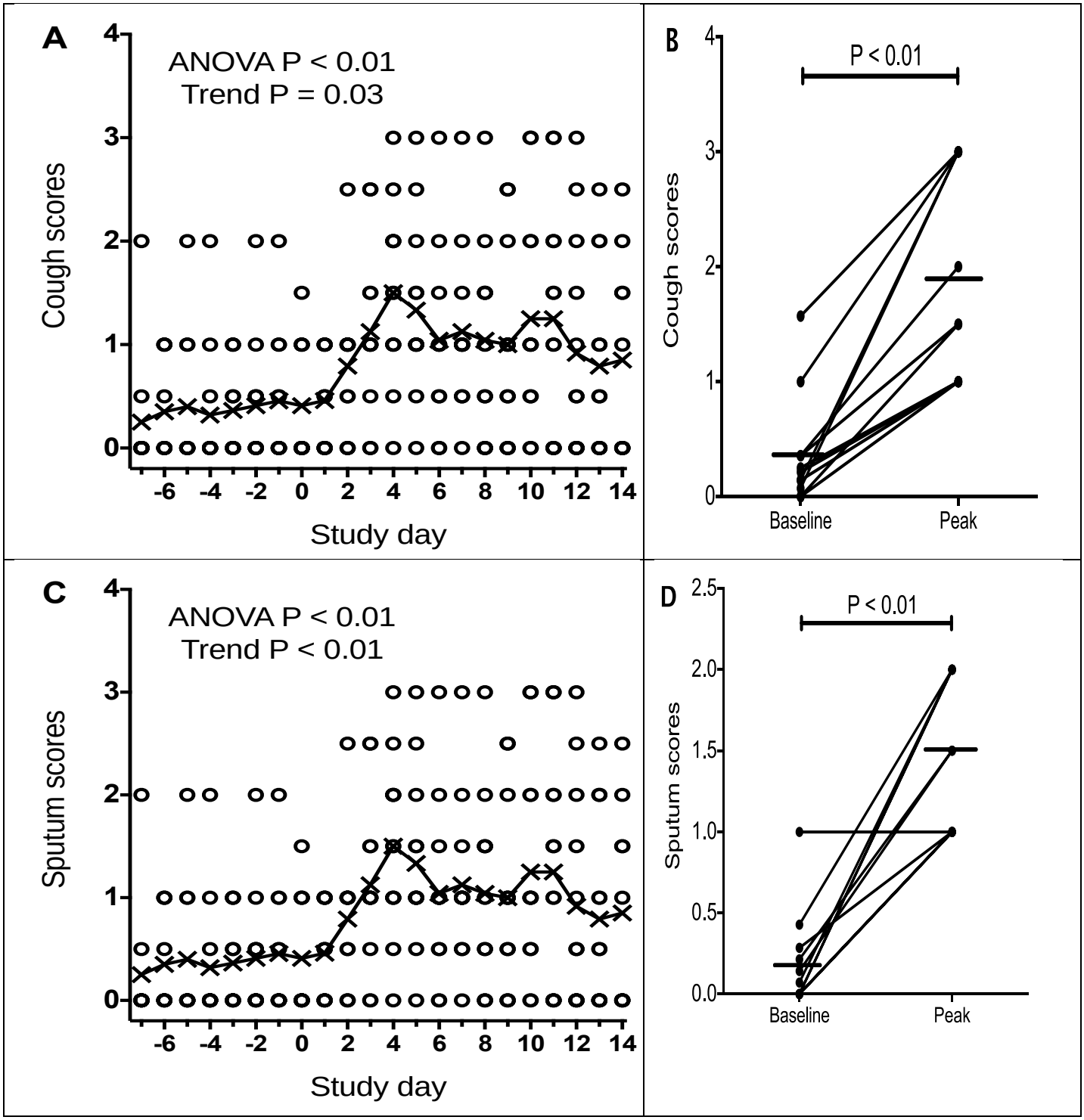

Figure 5.4 Changes in cough and sputum scores. **A**: Daily cough scores. Individual values are shown as open circles, means are shown as x. These changed significantly over time (ANOVA P < 0.01). Trend analysis showed quadratic trend (P = 0.03). **B**: Change in cough scores. Individual values are shown as full circles, means are shown as horizontal bars. Mean (CI) baseline score was 0.4 (0.0, 0.7). Mean (CI) peak score was 1.9 (1.3, 2.5). The mean (CI) difference between baseline and peak was 1.5 (1.0, 2.1) (2-tailed t-test, P < 0.01). **C**: Sputum scores. Individual values are shown as open circles, means are shown as x. These changed significantly over time (ANOVA P < 0.01). Trend analysis showed quadratic trend (P < 0.01). **D**: Change in sputum score. Individual values are shown as full circles, means are shown as horizontal bars. Mean (CI) baseline score was 0.2 (0, 0.4). Mean (CI) peak score was 1.5 (1.1, 1.8). The mean (CI) difference between baseline and peak was 1.3 (0.8, 1.7) (2-tailed t-test, P < 0.01).

Figure 5.5

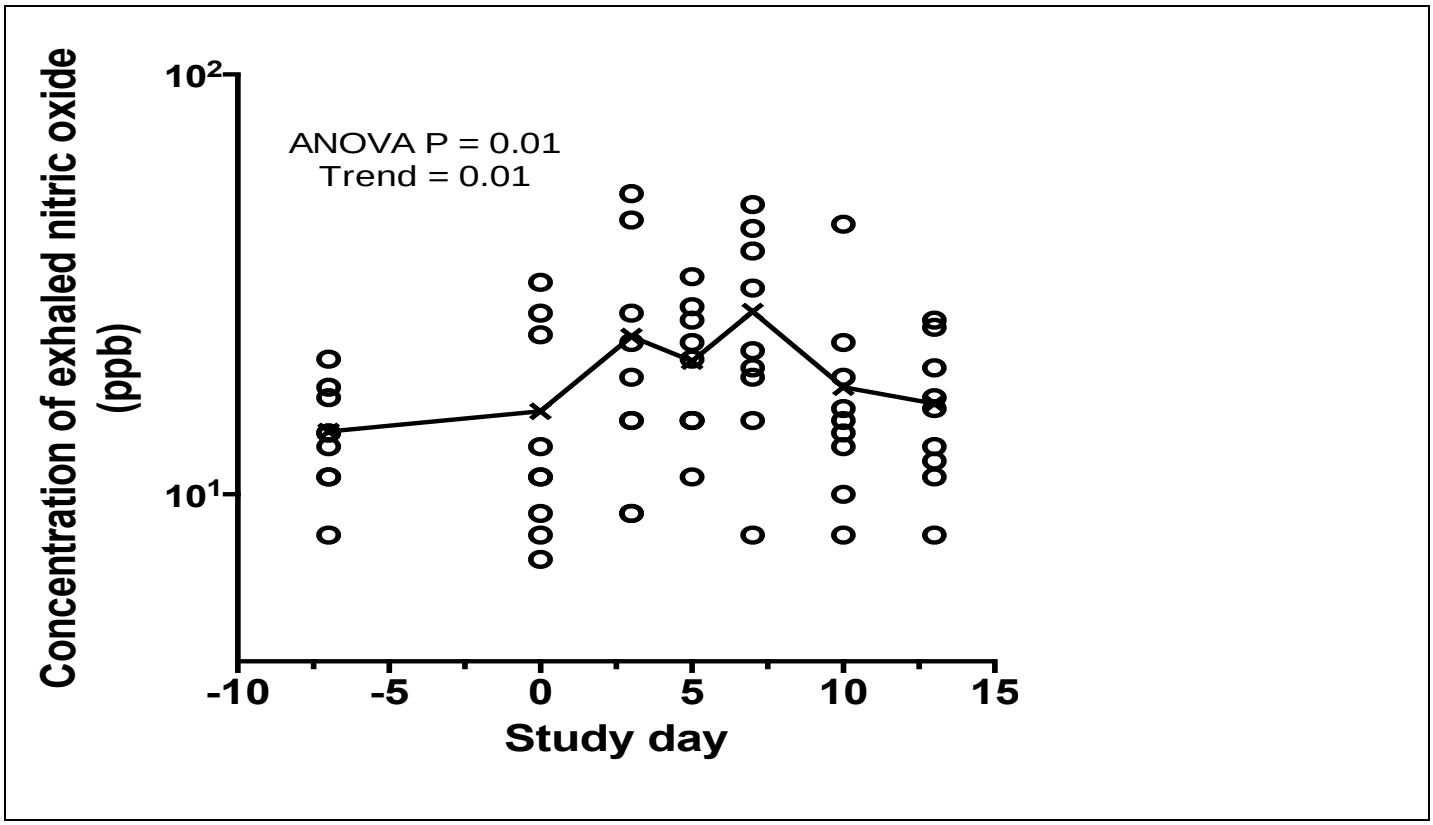

Figure 5.5 Concentration of nitric oxide in exhaled breath changed significantly over time following infection (ANOVA, P = 0.01) with a significant quadratic trend (P = 0.01). Individual values (open circles) and means (x) are shown...

Figure 5.6

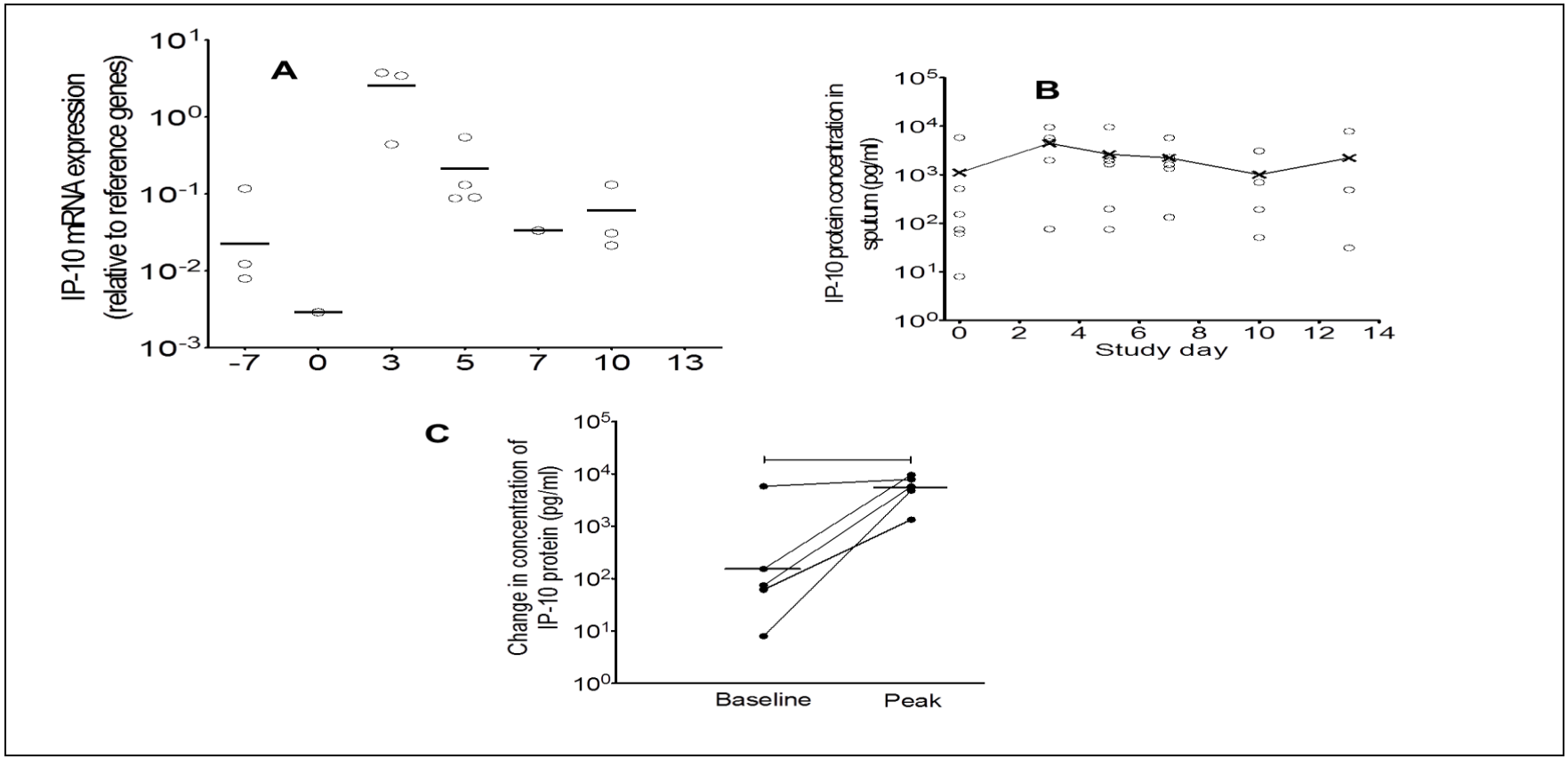

Figure 5.6. Induction of innate response in sputum. **A**: IP-10 mRNA relative to reference genes GAPDH and UBC. Individual values (open circles) and geometric means (horizontal bars) are shown. **B**: IP-10 protein levels in sputum. Individual values (open circles) and means (x) are shown. mRNA and protein levels were not subjected to ANOVA given the small numbers and missing values. **C**: Change in IP-10 protein concentration in sputum. Individual values (full circles) and geometric means (horizontal lines) are shown. Mean (CI) baseline concentration was 170 (13, 2200) pg/ml. Mean (CI) peak concentration was 4900 (1900, 13000) pg/ml. The mean (CI) difference between baseline and peak concentration was 29 (2.9, 300) pg/ml (2-tailed t-test, P = 0.01).

Figure 5.7

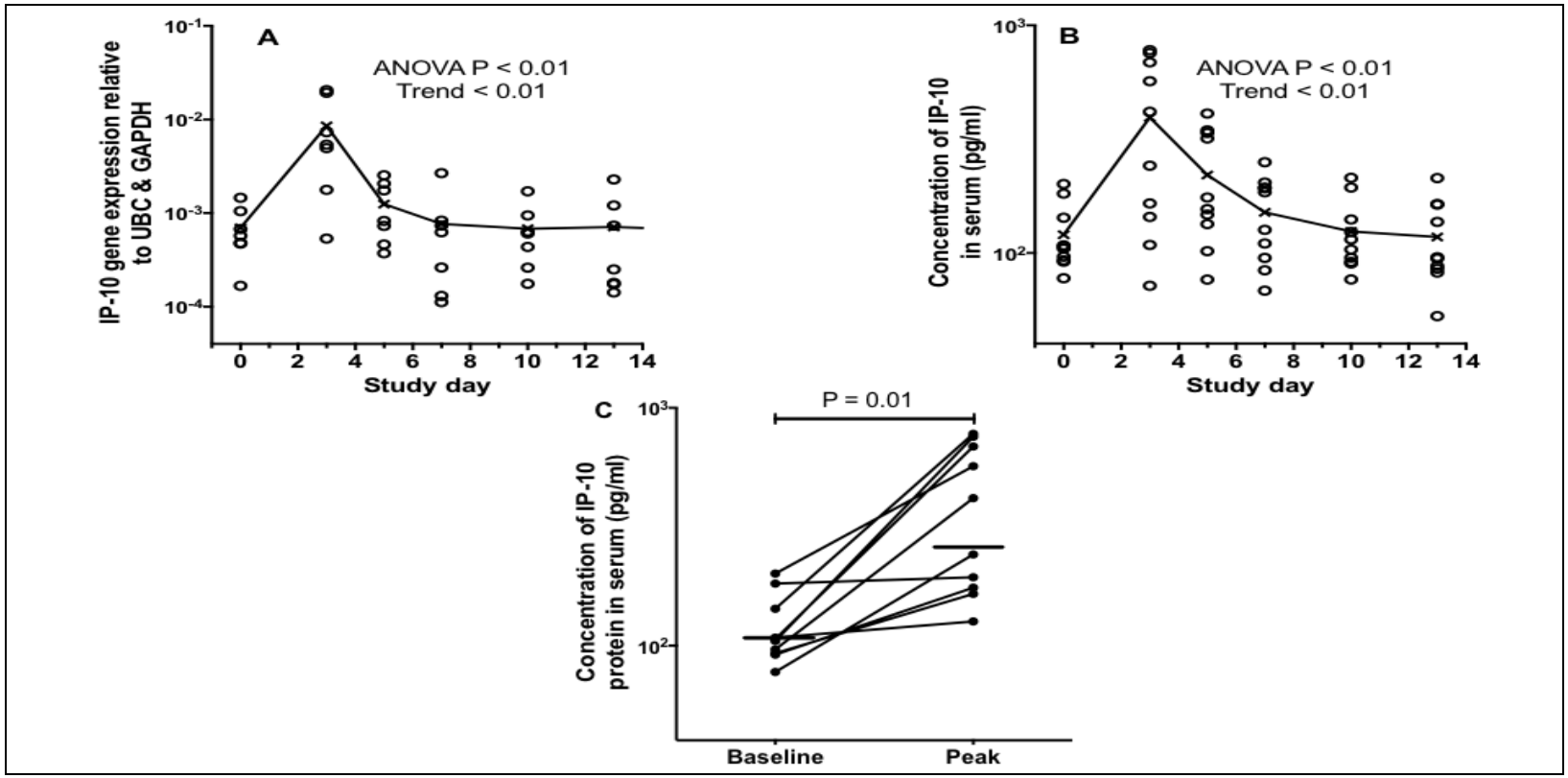

Figure 5.7. Systemic expression of IP-10 in response to RV16 infection. Individual values (open circles) and means (x) are shown in A and B. Both IP-10 mRNA (panel A) and serum IP-10 protein concentration (panel B) showed significant change over time following infection (ANOVA, P < 0.01) with a quadratic trend (P < 0.01). C: Mean (CI) baseline IP-10 protein concentration was 110 (92, 140) pg/ml, mean (CI) peak – 330 (200, 550) pg/ml. Mean (CI) difference between baseline and peak IP-10 protein concentration was 150 (53, 430) pg/ml (2-tailed t-test, P = 0.01). Individual values are shown as full circles, means as horizontal lines.

Figure 5.8

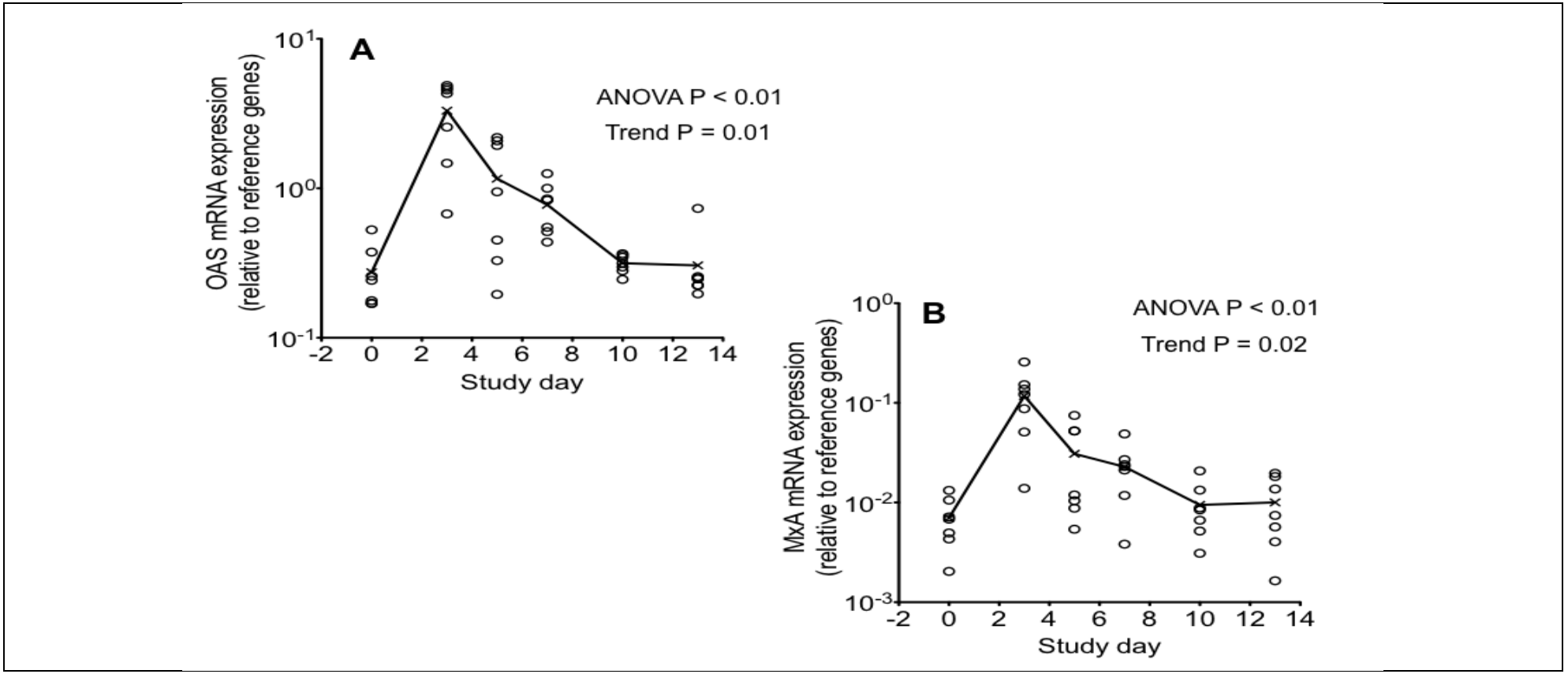

Figure 5.8 Systemic antiviral gene expression in response to RV16 infection. Systemic expression of OAS (A) and MxA (B) in response to RV16 infection. Individual values (open circles) and means (x) are shown as open circles. ANOVA indicated significant change in mRNA for both following infection (P < 0.01) with a significant quadratic trend (P = 0.01, and P = 0.02 respectively).

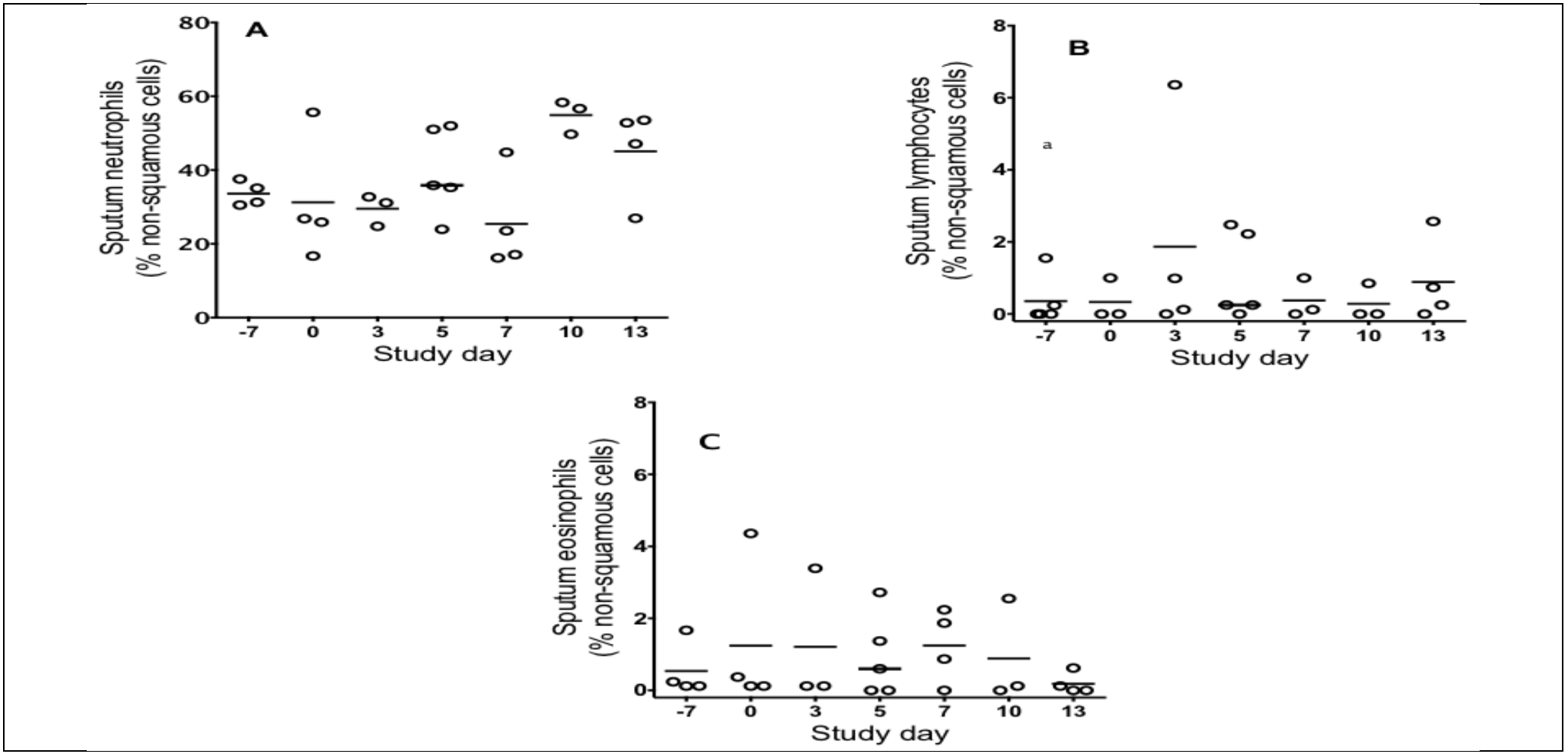

Figure 5.9 Sputum cell counts as a percentage of non-squamous cells neutrophils (A), lymphocytes (B), and eosinophils (C). Individual values (open circles) and means (horizontal lines) are shown. No statistical analysis has been made on these given the small numbers and missing values.

5.3.7.2 Blood

ANOVA of antiviral gene expression in peripheral blood cells showed significant induction of genes for OAS (Figure 5.8A), MxA (Figure 5.8B) and IP-10 (Figure 5.7A), over time ($P < 0.01$ for all three). Induction of MxA and OAS genes followed a significant quadratic trend ($P = 0.01$ and $P = 0.02$ respectively, $P < 0.01$ for IP-10). Both IP-10 mRNA and serum IP-10 protein concentration (Figure 5.7B) showed significant change over time following infection (ANOVA, $P < 0.01$) with a quadratic trend ($P < 0.01$). Mean (CI) baseline IP-10 protein concentration was 110 (92, 140) pg/ml, mean (CI) peak – 330 (200, 550) pg/ml. Mean (CI) difference between baseline and peak IP-10 protein concentration was 150 (53, 430) pg/ml (2-tailed t-test, $P = 0.01$) (Figure 5.7C).

5.4 Discussion

The current study has expanded on the study by Mallia *et al* (Mallia, 2011) and further explored the potential of experimental RV16 infection in smokers as a model to study exacerbation mechanisms. There are therefore important similarities and differences with the study by Mallia *et al.* The similarities include the clinical observations, the small dose of virus used in the inoculation, and the assessment of airway inflammation. This study has expanded on the study by Mallia *et al* by assessing the innate immune responses in the airways and the systemic circulation, and by the infection procedure that introduced the virus only into the nasal cavity.

There are a number of similarities between this study, and the work of Mallia *et al* (Mallia, 2011) which was the first study of this kind in this population. Firstly, both studies used RV16 as the challenge virus based on its safety profile as stated in the asthma study (chapter 4). Secondly, this study enrolled mainly smokers with normal lung function, with a minority having mild COPD. Mallia *et al* targeted subjects with smoking-induced COPD as the main population, and used smokers with normal lung function as the control group. Thirdly, in both studies clinical infection was induced with a lower dose of RV16 virus than used in previous studies although the doses of virus used in the two studies cannot be directly compared due to the inherent variability of the $TCID_{50}$ as a unit of virus dose (see chapter two for more details). Fourthly, induction of upper and lower airway symptoms was observed without a detrimental loss of lung function or other serious adverse event in both

studies. In the study by Mallia *et al,* a small transient decline in FEV_1 (mean 9%) was observed in the COPD subjects, but not in the smokers with normal lung function. Lastly, Mallia *et al* observed a significant increase in the sputum neutrophils over baseline on Days 5, 9, 12, and 15 in the subjects with COPD, whereas there was no significant increase in sputum neutrophils in the control subjects. In this study however, there were too few sputum samples coupled with missing values to allow for statistical analysis of cell counts (Figure E 5-5). Taken together, these observations indicate the safety and potential of experimental RV16 infection in smokers as a model to induce lower airway symptoms and study mechanisms involved.

The observations by Mallia *et al* have been expanded on in several ways. While the inoculation method employed in the study by Mallia *et al* using the DeVilbiss atomizer would have generated 5µm particles capable of reaching the lower airways, the device used in this study (the same device as in the experimental RV infection in asthma, Chapter 4) generates particle of a size expected to be deposited only in the upper airways. In this way, the virus was allowed to multiply in the upper airways and then spread to the lower airways akin to a naturally acquired cold. Significant increases in the levels of exhaled nitric oxide (eNO) following infection were observed in keeping with the findings by de Gouw *et al* in asthma (de Gouw, 1998). This is likely secondary to the recruitment of eosinophils and other immune cells in response to the LRT infection. This is in contrast to Mallia *et al* who did observe significant changes in inflammatory cytokines such as IL-6, IL-8, and TNF-α in neither induced sputum nor BAL fluid of COPD and control subjects. The significant increases in the levels of eNO are in contrast with the observations in the experimental RV infection in asthma. High levels of eNO are seen in eosinophilic airway inflammation(Taylor, 2006), and the fact that the asthmatics in the study had ICS-controlled disease may explain why they did not have significantly raised levels of eNO – they may not have been able to mount an eosinophilic response even though experimental viral infections have been observed to increase eosinophil counts in the epithelium of subjects with mild allergic asthma(Pizzichini, 1998). Also, whereas the immune response of the subjects to experimental RV infection in the airways and in the systemic circulation were assessed in this study, Mallia *et al* focused on the inflammatory response. An increase in the expression of mRNA for the IP-10 gene and increased concentrations of IP-10 protein levels (Figure 6) in sputum were observed. Increased induction mRNA expression for the MxA, and OAS genes was also noted (data not shown). However, formal statistical tests for significance could not be carried out because of small sample size and missing values. A significant induction of expression of mRNA for the IP-10 gene was observed in the systemic circulation, accompanied by a significant increase in IP-10 protein levels. Smoking has been shown to

blunt the immunological response to RV infections (Eddleston, 2011), which explains the clinical observations of increased lower airway symptoms in smokers following RV infections (Nicholson, 1996). These observations indicate that experimental RV infection is a useful tool to further investigate the mechanisms involved.

In the experimental RV16 infection in asthma (described in chapter 4), all the 11 subjects who underwent inoculation met the criteria for the development of an RV16 infection. In this study, only 10 of the 12 who underwent inoculation met the same criteria. However, smoking is associated with increased prevalence and severity of the common cold than in non-smokers (Arcavi, 2004; Cohen, 1993; Blake, 1988; Kark, 1981) whereas asthmatics are not more susceptible than non-asthmatics but suffer more severe and more prolonged symptoms (Corne, 2002). This observation is in a small number of subjects, so must be considered as preliminary and requiring further study.

This study has limitations similar to those of the experimental RV infection in asthma. First, the study lacks a placebo or healthy control group, so the pre-inoculation readouts have served as the basis for comparisons. Virus detection in the airways was by PCR, which detects virus RNA without distinguishing between live and inactivated virus. For some read-outs measurement was possible in only a small number of subjects (e.g. assessment of innate responses in sputum); the observations made should, therefore, be seen as preliminary and. Finally, although all participants in this study were clinically stable before infection, one cannot completely exclude the possibility that at least some of the observed changes, could be due to variability between measurements.

In summary, the findings in this study have helped to create a basis for further research into virus-host interactions in smoking-induced airways diseases such as COPD. First and foremost, they have shown that it is safe and feasible to apply a small dose of virus exclusively into the nose and, thereby, safely induce mild LRT symptoms in susceptible subjects. The induction of innate immune responses observed in the study suggest that this is a valuable model for studying exacerbation mechanisms and for testing of novel therapies of virus-induced exacerbations of lower airway symptoms. More studies are required in this area to further elucidate these mechanisms.

Chapter 6 - General discussion

6.1 Introduction

Experimental rhinovirus (RV) infections have been used for a number of years as a model to explore various aspects of virus-host interactions in mild asthma that contribute to exacerbations of asthma and lately, exacerbations of COPD. The experimental RV infections described in this thesis sought to contribute to the understanding of the mechanisms involved. To this end, the model was applied in moderate asthmatics on long-term treatment with inhaled corticosteroids (ICS) for the first time. A body of evidence exists, that the biology of severe asthma is different from that of mild asthma in several aspects(Chanez, 2007): small airway involvement, association with increased risk of hospitalisation and death, lower prevalence of atopy, higher prevalence of NSAID-induced asthma, a link with lower biosynthetic capacity for lipoxins, poor response to corticosteroids, increased smooth muscle in the airways, large and small airway thickening and poor control of clinical illness in many patients with severe asthma. In this light it is inappropriate for our understanding of the mechanisms of virus-induced asthma exacerbations to be based on extrapolations and generalisations of findings from studies performed in mild asthma. Models of virus-induced exacerbations of asthma in moderate asthma, and ultimately in severe asthma, are needed. The model was also applied to smokers with or without COPD who were not receiving treatment for any airways disease. Accumulated evidence from both *in vivo* and *in vitro* studies suggests that smoking impacts on innate immunity. Bronchial epithelial cells (BEC) represent the primary site of RV infection in the lower airways(Papadopoulos, 2000 58) and *in vitro* studies have shown that BEC from smokers without COPD are more susceptible to RV16 relative to cells from non-smoking controls(1). *Ex vivo* exposure of BECs to cigarette smoke has been found to blunt the response of airway epithelial cells to RV infection, leading to increased viral replication by interfering with the IFN pathway and consequent activation of antiviral genes (Eddleston, 2011). Ongoing smoking is associated with lower airways manifestations such as sputum production, wheeze, and pain on respiration following RV infection(Nicholson, 1996). RV infections are therefore important events in smokers with or without COPD, but experimental models to study the mechanisms involved in the pathobiology of such infections are lacking. These studies aimed to extend the use of the experimental RV infection model, first by applying it to moderate asthmatics requiring regular treatment with ICS to control symptoms and maintain lung function, and to expand its utility in smokers who have been investigated with the model in one single study to date.

As mentioned in chapters 3 and 4, the studies indicated that experimental RV infection in the two groups is feasible, safe, and a valuable model for exploring mechanisms of virus-induced exacerbations of airways disease in line with previous findings. In carrying out these studies, several novel modifications were made to the methods used in previous studies. Furthermore, some of the findings were unexpected based on current understandings of virus-host interactions in these groups of subjects. Some of the findings must therefore be considered as preliminary and needing confirmation in future studies by virtue of the fact that the subject population has not been investigated in this manner before, the small number of participants, and the lack of a control group.

6.2 Methods

6.2.1 General inclusion/exclusion criteria

An RV16 neutralising antibody titre of 1:2 was selected because it was reckoned that such a low titre was likely to be non-specific cross-reacting antibodies, which would not confer protective immunity. Subjects with a recent acute infection required a washout of 6 weeks or more before they were screened for participation to ensure that the symptoms, lung function and immune status had returned to baseline at enrolment.

Rhinovirus infections are not known to cause embryo-foetal toxicity, however pregnancy is an altered immune state. Female subjects were therefore enrolled into the study provided they were neither pregnant nor planned to become pregnant over the duration of the study. A pregnancy test was performed before enrolment of female subjects, and at the end of the study.

Subjects to whom an investigational drug had been administered required a 12-week washout prior to entry into the study if the drug was a small molecules, and 6-month washout if the drug was a biological therapy. This was to avoid any confounding effect the investigational drug might have on the subject's response to a rhinovirus infection, or on the endpoints.

Volunteers who were living with people with a weak immune system such as young children, the elderly, and those heart, liver, or kidney failure, or on immunosuppressive therapy were excluded in order to avoid the risk of the latter acquiring the RV16 infection.

6.2.1.1 Asthma

The age restriction of 18 - 60 for the asthmatic subjects was to avoid age-related differences such as diminished immune responses in older subjects. Regular treatment with ICS indicated that the subjects had chronic persistent asthma which, coupled with a preserved lung function ($FEV_1 \geq 80\%$ of predicted and transfer factor for carbon monoxide TLCO >80% of predicted) indicated that their asthma was moderate in severity. The dose of ICS of BDP $\leqslant$ 1000 mcg was chosen because a higher dose would indicate that a subject's disease was difficult to control. The preserved lung function also meant that the subjects were more likely to cope with a potential loss of lung function induced by the experimental RV infection. The subjects were also required to have demonstrable reversible airflow obstruction or a positive methacholine challenge test. A history of increased asthma symptoms during a common cold while on the treatment they were on at the time of screening, that had not been severe enough to warrant emergency department treatment, hospital admission or systemic steroids in the 12 months prior to screening. This set of criteria were intended to increase the likelihood of the subjects developing increased lower airway symptoms during the experimental RV infection, which would however not be so severe as to warrant additional therapy. Subjects who has suffered an exacerbation of their asthma in the preceding 12 months which had resulted in a visit to the Emergency Department, were excluded because the risk of another severe exacerbation triggered by the experimental RV infection.

A positive Skin Prick Test to common aeroallergens was required to ensure that the subjects all had atopic asthma, and so were homogenous in that respect, but also because atopy also increases susceptibility to rhinovirus infections(Bardin, 1994). Similarly, Subjects with a history of any lung disease other than asthma were excluded to maintain homogeneity of the group.

In summary, the inclusion and exclusion criteria were intended to facilitate selection of a group of subjects who were likely to development a clinical infection that would be measureable, but not so severe as to be detrimental to their health.

6.2.1.2 Smokers

Male and female subjects aged 45 to 70 years, with a $\geqslant$10 pack-year smoking history were screened for participation in the smokers' study. They did not have a previous diagnosis of COPD or any other respiratory illness. This was intended to select for subjects who had smoked long enough to have developed significant smoking-related inflammatory changes in the airways, which were not severe enough to have led to a diagnosis of overt COPD. It was

anticipated that some of them would have spirometry classifiable as COPD. An FEV_1 of $\geqslant$ 50% predicted was required so that the subjects were more likely to cope with a potential loss of lung function induced by the experimental RV infection. Because smoking is a risk factor for cardiovascular disease, a directed history was taken at during screening, and subjects with symptoms suggestive of cardiovascular illness were excluded.

6.2.1.3 *Ex vivo* stimulation with poly(I:C)

The healthy control subjects were aged-matched to the asthmatics so that age would not be a confounding factor for between-group differences.

6.2.2 Sample size considerations

The number of subjects included in the experimental RV infection studies were not based on power calculations. Sample size calculations require that the size of the anticipated change be known with its variance. LRT symptoms, or changes in lung function following experimental RV infection were the 2 potential endpoints to be used for sample size calculations. However, previous studies used an inoculation technique that introduced the virus to the lower airway unlike the MAD device employed in these studies. Therefore it was not certain that LRT symptoms or lung function changes were going to be observed. However, it was anticipated that 10 – 15 subjects would be enough to assess trends if there were any to be seen.

The number of healthy controls enrolled for *ex vivo* stimulation of whole blood with poly(I:C) was based on sample size calculation that took into account the induction of IP-10 mRNA expression in response to poly(I:C) stimulation of whole blood from moderate asthmatics *ex vivo*. The sample size was calculated to be enable the detection of IP-10 mRNA expression that differed by 50% from that observed in moderate asthmatics, with a similar variance, 0.05 significance, and a power of 80%.

6.2.3 Polymerase chain reaction (PCR)

PCR's major advantage in estimating virus load is that it is sensitive enough to detect small amounts of genetic material that it would not have been possible to detect by other means. The advent of PCR made it possible for virus to be identified in samples collected from subjects with respiratory symptoms which was not possible previous methods. Such studies laid the groundwork for the recognition of the importance of central role played by viruses in

exacerbations of asthma and COPD. However, the detection of viruses by PCR has one major limitation – it is not possible to say whether or not the detected virus material is from viable virus particles or not. It has therefore not been possible to tell for how long a period the subjects continued to shed virus which was not inactivated by the host immune response.

The synthesis of mRNA in response to either RV16 or dsRNA is the first step in the innate immune response. The changes in this response in the presence of the immune challenge gives an insight into the responsiveness of the innate immunity. However, ultimately, a gene's function is carried out by the protein synthesized following translation of mRNA into a protein. In the intricate biological processes involved in gene expression and function, there are several steps between the synthesis of mRNA and it's translation into a protein that is ready to do perform its biological function. It is not therefore possible to tell for certain the ultimate efficiency and duration of the innate immune response. However previous studies have shown good correlations between mRNA induction and protein synthesis as well as it's correlations with efficiency of antiviral response (Wark, 2005).

The amount of mRNA extracted from each sample varies. Some may contain significantly less mRNA than others. There are two main reasons for this - inadequate sample collection, and inefficient mRNA extraction. Only 2.5 ml of blood are required per PAXgene tube. The tubes are prefilled with a reagents in a clear, colourless fluid that changes to the colour of blood even if the volume of blood added is less than the recommended 2.5 ml. There is no marker on the side of the tube to indicate the level to which a 2.5 ml volume of blood added would raise the contents. This, coupled with any problems a particular sample may have experienced during storage and extraction results in a significant variability in the concentration of mRNA in each sample at the end of the extraction procedure. The concentration of mRNA was therefore estimated with a spectrophotometer after extraction. With this information, a volume of sample required to provide 1 μg of mRNA was added to each well at PCR.

6.2.4 Assessments of lung function

The home-based assessment of FEV_1 and PEF is very useful because it enables continuous assessment of airflow obstruction. This would highlight significant loss of lung function early so that safety measure could be implemented if necessary, and would also give a an indication of changes in lung function relative to other markers such as symptoms and virus shedding. However, it also means that the forced spirometry manoeuvres required are performed at home without oversight from the research staff, and as such may not be consistently of acceptable quality. This was reflected in the observation that there was

greater between-test variability for each individual in the home-based spirometry compared to the laboratory measurements. Each value recorded by the device had a date and time stamp. However, there was no way of telling whether anyone else apart from the volunteer had used the handheld device.

6.2.5 Virus inoculation dose and procedure

The device used in these studies to inoculate the subjects was a commercially available atomizer, designed to generate a spray with aerosol particle size 30 – 100 µm. It is therefore a certainty that the inoculation procedure did not introduce virus particles to the lower airways, as aerosol particle size of ≥16 µm have a 100% deposition in the nasal passages(Newman, 1985). Inoculation procedures employed in previous studies (Lemanske, 1989; Fleming, 1999; Grunberg, 1999; Grunberg, 1997; Grunberg, 1999; Halperin, 1985; de Gouw, 1998;de Kluijver, 2003; Grunberg, 2001; Gern, 2000; Parry, 2000; Gern, 1997; Contoli, 2006; Message, 2008; Jarjour, 2000; Lemanske, 1989; DeMore, 2009; Mallia, 2011) used the DeVilbiss atomiser which is able to generate aerosol particles as small as 5 µm in size which are able to reach the lower airways. This was a deliberate effort to ensure that the virus reached the lower airway where it would multiply and lead to symptoms. The detection of the virus in sputum implies that the virus multiplied in the nose and subsequently spread to the lungs. This is akin to naturally acquired infections whereby RV are picked up in hand-to-hand and fomite-to-hand contact and introduced to the eyes or nose(Gwaltney, 1982). This variation to the experimental rhinovirus infection model is important because it provides researches with the opportunity to test an intervention that is initiated following exposure to the virus, but before the virus takes hold in the lower airways, in subjects with asthma or COPD.

Another novel outcome from these studies was successful induction of an experimental infection with an inoculation dose that was 10 times lower than the dose of the same stock and strain of RV used to inoculate subjects successfully in previous studies. Virus potency was checked on a regular basis throughout the study and was found to be maintained. These potency tests were purely for testing that biological activity remained consistent throughout. The dose to be used for inoculation was calculated based on the results from the originating laboratory because $TCID_{50}$ results are not comparable between laboratories. There are several reasons for this including the cell line used for the $TCID_{50}$ assay, the cell line's susceptibility to the virus being tested, the cell line's senescence/passage, cell culture techniques employed, and inter-operator variability. This

may explain the wide range of $TCID_{50}$ doses used in previous experimental RV infection studies ranging from 10 to 240,000 $TCID_{50}$(Mallia, 2011;Fleming, 1999;Grunberg, 1999;Grunberg, 1997;de Gouw, 1998;Grunberg, 2001;Parry, 2000;Mosser, 2005;de, 2003). The laboratory of origin at the University of Wisconsin School of Medicine, Madison, USA had assessed the stock to contain 10^5 $TCID_{50}$ per ml, and used 10 µl (= 1000 $TCID_{50}$) per inoculation successfully in their studies(Gern, 2007;DeMore, 2009;Mosser, 2005;Gern, 2000;Parry, 2000). 1 µl (= 100 $TCID_{50}$) of that stock per inoculation was used in these studies. The design of the asthma study allowed for an escalation of the dose of virus 100 $TCID_{50}$ had not proved to be effective in inducing an infection. However, it turned out that all asthmatic subjects developed cold symptoms at this dose, so there was no need to escalate the dose. The same dose was then used to infect the smokers too. In this way, these two studies have demonstrated that it is possible to reliably, and safely induce an experimental rhinovirus infection in asthmatics and smokers with or without COPD with 10 times lower dose than in previous studies, and that lower airway symptoms would be induced even when the inoculation procedure delivers the inoculum exclusively to the nasal cavity.

6.3 Important findings

6.3.1 Experimental Rhinovirus Infection in moderate asthmatics

This was the first study to demonstrate that experimental RV infection is safe in asthmatics whose asthma is persistent, and severe enough to require regular ICS to control the background airway inflammation in order to achieve clinical stability (i.e., to maintain symptom control and normal lung function). This was therefore a population at greater risk of clinically significant exacerbations caused by respiratory virus infections. However, the RV16 strain used in the challenge has been shown to induce relatively lower levels of inflammatory cytokine release and cell death during infection of respiratory cells *in vitro* (Wark, 2009) compared to other strains. In the study by Wark *et al*(Wark, 2009), RV16 induced a lower level of apoptosis as well as IL-6 and IFN-γ release than clinical strains, but similar virus copy numbers were released into the supernatant, indicating that RV16 may replicate quite well in a permissive host without inducing as much a pathological response as clinical strains of rhinovirus. Therefore, none of the subjects suffered a serious adverse event, nor did any require a step up in treatment beyond the increased use of bronchodilators for respiratory symptom relief.

In both inoculation studies, one subject was inoculated at a time and followed up in contrast to other experimental virus studies such as respiratory syncytial virus (DeVincenzo, 2010) where subjects are inoculated as a cohort and quarantined in co-habitation during the acute infection. The co-habitation probably ensures that those subjects who did not catch the infection from the experimental inoculation would become infected with infectious material emanating from the other participants. This arrangement whilst ensuring maximum clinical infection, might obscure the true rate of successful experimental infection at a given dose of virus. Also, incubation periods and other time-relevant measures for the group as a whole may not be accurate.

The use of a short-messaging service over the commercial telephone network ensured that the symptom scores were collected in real-time thus circumventing the well documented recall bias and unreliability associated with paper-based data collection(Verschelden, 1996). The use of commercial mobile telephone networks to collect clinical study data, and also to send prompts avoided potentially missing data, greatly improved compliance and reliability while having a minimal impact on the daily routines of the subjects.

Detection of virus in the lower airways at day 3: Virus was detected in the lower airway on day 3 post-inoculation in both experimental RV infection studies. Sputum sampling was not feasible on a daily basis. This was the first day on which sputum sampling was undertaken post-infection. It is therefore not possible to be certain the earliest point at which virus appeared in the lower airway and to relate this to lower airway symptoms.

A tandem rise in upper and lower respiratory tract symptoms as observed. Moreover, in the smokers, upper and lower airway symptoms peaked at the same time. Similar observations were made during the experimental RV infection in COPD and smoking controls carried out by Mallia *et al* (Mallia, 2011). This coupled with the presence of the challenge virus in the lower airways indicate that there is no window between upper and lower airway symptoms. This has implications for therapies aimed at treating exacerbations of conditions such as asthma and COPD. For such treatments to be efficient, they would have to be given at the at the very earliest upper airway symptoms of respiratory virus infection. This is in line with the concept of the global airway, which is supported by observations that treatment of upper airway inflammation indirectly improves asthma symptoms and decreases bronchial hyperresponsiveness (Watson, 1993).

6.3.2 Experimental Rhinovirus Infection in smokers

Subjects with a significant smoking history who did not have a diagnosis of lung disease were targeted for recruitment into this study with the knowledge that asymptomatic smoking-induced airways pathology may remain undiagnosed in many subjects in earlier stages. Prevalence surveys carried out in a number of countries using standardised methods including spirometry estimate that up to 25% of adults aged 40 years and over may have airflow obstruction classifiable as COPD (Lopez, 2006). In keeping with this observation, that 4 out the 11 subjects who were inoculated had spirometry classifiable as COPD. Again, this is observation is in a small number of participants so cannot lead to firm conclusions.

A significant increase in exhaled nitric oxide (eNO) levels following experimental RV infection was observed in the smokers but not in the asthmatics. High levels of eNO are seen in eosinophilic airway inflammation(Taylor, 2006), and the fact that the asthmatics in the study had ICS-controlled disease may explain why they did not have significantly raised levels of eNO. They may not have been able to mount an eosinophilic response, given that experimental viral infections have been observed to increase eosinophil counts in the epithelium of subjects with mild asthma (Pizzichini, 1998). Few sputum samples coupled with missing values meant that correlations between sputum eosinophil counts and concentrations of eNO could not be sought. However, these are important observations and must be taken into consideration in future research.

6.3.3 *Ex vivo* stimulation of whole blood

The ability to use whole blood for *ex vivo* immune responses has a couple of advantages. It eliminated the need to separate the immune cells from whole blood before they can be subjected to the appropriate experimental conditions. This is important because as it was seen from the control samples, mRNA may be induced *ex vivo* without any specific treatment which could happen during the processing of blood to separate its components prior to TLR-3 stimulation. Also, stimulation of whole blood would elicit a response from the blood cells while the blood is in its constitutive state. We believe that such response would be more reflective of the *in vivo* response.

Interferons have a central role in antiviral defence of all mammalian cells. One testament to this fact is the observation that although it cannot replicate well in extraneural tissues *in vivo*, poliovirus is able to replicate in monolayer cells of primary kidney cell culture because of the loss of rapid IFN inducibility during the cultivation process (Yoshikawa, 2006).

BEC(Wark, 2005), BAL cells(Contoli, 2006), and PMBCs(Papadopoulos, 2002) of asthmatics have been observed to be deficient in IFN production *in vitro*, which has been accepted as an explanation for the clinically observed increased susceptibility of the asthmatic lung to RV infections(Corne, 2002). Significant *ex vivo* induction of IFNβ mRNA expression in response to poly(I:C) from the asthmatics relative to healthy subjects was observed (Figure 3.2), however the degree of induction at 2h and 6h time points was not significantly different between healthy controls and asthmatics. It was not possible to assess IFNβ protein synthesis in this study. It is feasible that there are differences in protein synthesis i.e. the differences in peripheral blood may arise post-transcription which need to be explored in future studies.

The *ex vivo* stimulation of whole blood with poly(I:C) produced an induction of IFNβ mRNA expression that showed an inverse relationship with cold symptom scores, but not asthma symptoms. The innate response as assessed by mRNA expression of antiviral response genes followed the pattern of innate response assessed in the nasal lavage in both *in vivo* experimental RV infections. Taken together, these observations indicate that systemic immune response to common colds is similar to the nasal response, but separate from the local immune response in the lungs, despite the fact that the symptoms in the upper and lower airways go hand-in-hand.

6.4 Comparisons between asthma and COPD

Asthma and COPD have several overlaps and similarities between them. Individuals with asthma may also be exposed to air-borne noxious agents leading to COPD-type airway inflammation(Thomson, 2004). Airflow obstruction is a characteristic of both diseases. In some patients, asthma and COPD may co-exist, while in others, chronic asthma is a risk factor for the development of COPD phenotype(Silva, 2004). A sputum eosinophil count above 3% can be seen in asthma(Wenzel, 2006) and COPD(Pizzichini, 1998;Brightling, 2000;Chanez, 1997). Evidence that the presence of sputum eosinophilia is predictive of an objective response to corticosteroid treatment in COPD is emerging, suggesting that eosinophilic airway inflammation is functionally important in some subjects with COPD (Brightling, 2005) and asthma (Green, 2002). Bacterial and viral infections lead to exacerbations of both COPD (Seemungal, 2000) and asthma (Johnston, 1995; Johnston, 1996). The susceptibility to virus-induced exacerbations is a huge unmet need for both conditions.

There are important differences too between asthma and COPD. Airflow obstruction in COPD is not largely reversible neither spontaneously nor with the administration of $beta_2$-

receptor agonists. This is a key distinguishing factor in the clinical characteristics of the 2 conditions. Another important difference stems from the risk factors. Asthmatics have an inherent genetic predisposition to allergic reaction to substances in the environment, which manifests with clinical symptoms very early in life. COPD on the other hand, is a manifestation of chronic exposure to noxious airborne substances to which the subject is susceptible. As such, COPD manifests clinically after the age of 40. A non-smoker who is under 40 years old who presents with recurrent viral bronchitis during the winter is likely to end up with a diagnosis of asthma, while a similar presentation in a smoker who is over 40 years old is likely due to COPD. The pathology of COPD involves the activation of lung proteases which lead to the loss of lung parenchyma. This loss of surface area for gas transfer manifests as low TLCO in formal tests. Asthmatic lung on the other hand do not under a loss of lung parenchyma, and contrary to COPD, asthmatics tend to have a higher than predicted TLCO for reasons that are not well-understood (Knudson, 1990).

In both studies there was no significant loss of lung function observed from the close monitoring at home on handheld devices and measurement on a departmental desktop spirometer. However both sets of subjects reported significant increases in lower airway symptoms that showed a quadratic trend over the two weeks following infection. This indicates that validated, patient-reported outcomes may be the best type of tools to employ in assessments of subjects with airways disease during virus-induced exacerbations. This is to be welcomed, and not seen as a drawback because it is in keeping with every day clinical experience whereby patients' symptoms are the drivers of healthcare usage for the unmet need of virus-induced exacerbations of asthma and COPD.

Therefore, the approach to a challenge study that adds to the current knowledge of virus-induced clinical exacerbations of COPD would necessarily be different. The study described here was aimed at smokers with and without COPD – having established that smoking is the single most important noxious agent to which exposure leads to COPD in the developed world(GOLD, 2013), and that smoking induces a deficient antiviral defence state in the lungs(see section 1.5). These subjects would by definition not have clinically significant smoking induced pathology, so are not expected to be on any COPD-directed treatment. Recruiting them would be targeted via a significant smoking history. The other factors mentioned above would all have to be taken into consideration, namely the use of a laboratory strain of RV (such as RV16) that have been shown to induce relatively lower levels of inflammatory cytokine release and cell death during infection of respiratory cells *in vitro*; the use of a low dose virus; and inoculum delivered exclusively to the upper airways.

A successful clinical model of RV-induced exacerbation in persistent, glucocorticosteroid-treated asthma and smokers would be important for two primary reasons. It will be a platform for further studies of virus-induced, glucocorticosteroid-resistant inflammation. It will also serve as a platform to test much needed new therapies for the un-met need of virus-induced exacerbations of asthma and smoking-induced COPD.

Recruitment in these studies was aimed at subjects who were likely to show pathophysiological changes that would reflect mechanisms of virus-induced exacerbations. On the other hand, it was important for them to be controlled asthmatics, or not to have severe COPD to minimise to risk of severe morbidity following experimental rhinovirus infection.

$TCID_{50}$ results are not comparable between laboratories. There are several reasons for this including the cell line used for the $TCID_{50}$ assay, its susceptibility to the virus being tested, its senescence/passage, cell culture techniques employed and inter-operator variability. This may explain the wide range of $TCID_{50}$ doses used in previous RV challenge studies ranging from 10 to 240,000 $TCID_{50}$(Mallia, 2011;Fleming, 1999;Grunberg, 1999;Grunberg, 1997;de Gouw, 1998;Grunberg, 2001;Parry, 2000;Mosser, 2005;de, 2003). The laboratory of origin at the University of Wisconsin School of Medicine, Madison, USA had assessed the stock to contain 10^5 $TCID_{50}$ per ml, and used 10 µl per inoculation successfully in their studies (Gern, 2007;DeMore, 2009;Mosser, 2005;Gern, 2000;Parry, 2000). 1 µl of that stock per inoculation was used in these studies.

The responses were monitored on a daily basis. In addition the programme sent out e-mails to designated e-mail addresses if a sudden increase in symptoms was reported or replies were not received within the allowed window so that subjects were contacted as a safety measure.

6.5 Limitations of current research

The greatest limitation in these RV challenge studies was the absence of a healthy control or sham inoculation group. In order to circumvent this, pre-infection observations were used for comparison as well as peak and trough values of observations made during the studies wherever this analysis was deemed appropriate. Therefore, some of the findings in these studies have to be regarded as preliminary, to be confirmed in future research involving control groups.

It could not be determined with certainty when the lower airways became infected because sputum samples were not taken in a daily basis. To overcome this while avoiding daily sputum inductions, a future study design might involve sputum inductions on varying study days for the participants in such a way that taken together, an attempt at sputum induction for the study group as a whole would have been made during each of the first three days.

In the *ex vivo* whole blood challenge, only gene transcription i.e. expression of mRNA for the genes of interest was assessed. It was not possible to assess the quality or quantity of the corresponding protein from the translation of the mRNA into protein which is the ultimate gene product. It is feasible therefore that epigenetic phenomena may further differentiate the asthmatics' from the healthy subjects' innate responses to poly(I:C), the surrogate viral infection.

Analysis of sputum samples was limited by the small number of subjects who could consistently produce sputum. In future studies, sputum induction may be included into the screening procedures if sputum analysis is a key readout. Only subjects who can produce sputum would then be enrolled. Due to small numbers and missing values sputum cell counts and mRNA expression in sputum cells could not be assessed reliably so that observations in those samples have to be regarded as preliminary, to be confirmed in future research. Sputum scores rose significantly in the smokers post-infection. It is therefore possible that a future study which involves home collection of spontaneous sputum samples might improve sample size.

6.6 Potential future directions

Up to 40% of western populations are atopic. Allergic asthmatics represent a subpopulation of atopics. This high prevalence of atopy would indicate that this trait may have conveyed a survival advantage to those who carried it, allowing it to persist and to propagate in the population. Given the allergy to cat hair that is frequently encountered, it may be that those atopic individuals could sense lairs/haunts of feline predators in hunter-gatherer communities over several generations. This would have been a warning that would have made a difference between life and death (thus providing a survival advantage and preserving atopy in the population) as the atopic individual would be warned to either change course to avoid an unwanted encounter, or to be more alert in anticipation. At present, however, atopy clearly has a downside – atopics have an exaggerated response to respiratory tract

infections compared to non-atopics (Bardin, 1994). Smoking also conveys a susceptibility to common colds. Increased healthcare consumption by atopic asthmatics and smoking-induced COPD patients spikes predictably at time every year at times when there is increased circulation of upper respiratory tract infections in the community such as following school holidays (Johnston, 1996) and during the winter months(Moineddin, 2008). There are no effective vaccines, against the common cold viruses (commonest amongst which are rhinoviruses) to prevent these infections so ways need to be found to elucidate the mechanisms involved in virus-induced exacerbations, and hopefully ways to soften the impact of respiratory viruses on respiratory diseases. Experimental RV infections will continue to play a role in the decades to come.

Only subjects with a previous history of virus-induced exacerbations of asthma were recruited into the asthma study, however it was required that their asthma symptoms be controlled with ICS for safety reasons. They experienced a mild exacerbation of asthma – increase in asthma symptoms without significant loss of lung function. However, it is in severe asthma that models of virus-induced exacerbations of asthma are ultimately needed i.e. in those who receive the full impact of virus-induced asthma exacerbations (Johnston, 1996). Future studies should aim to test the experimental RV infection model perhaps in asthmatics with less well-preserved lung function say 80 – 60% of predicted, who have a history of virus-induced exacerbations, but do not have a history of detrimental loss of lung function during acute exacerbations requiring emergency room treatment, hospital admission, or systemic corticosteroids in the preceding 12 months while on the current therapy. Atopic subjects without asthma could be used as controls in such a study.

The observations in the long-term smokers are in line with the findings by Mallia *et al* (Mallia, 2011). It is therefore appropriate that a future study should take the modifications to the inoculation procedure and apply them to a group of subjects with GOLD stages 1 and 2 COPD, while using smokers with normal spirometry as controls.

6.7 Summary

In summary, the findings in these studies have important implications for further research into virus-host interactions in airways diseases. First and foremost, these observations have demonstrated that it is safe and feasible to apply a small dose of virus exclusively into the nose and, thereby, safely induce a mild exacerbation of asthma in susceptible subjects with moderately severe asthma in which exacerbations are more clinically significant than in

patient groups previously studied. Secondly, they have demonstrated that lower airway symptoms can be reliably induced in long-term smokers in the same way. The airway symptoms coupled with the induction of local and system innate immune responses observed in these studies suggest that this is a valuable model for studying mechanisms, and testing of novel therapies of virus-induced exacerbations or airways diseases.

Chapter 7 - List of References

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