The role of oxidative and nitrative stress and histone de-acetylation in rhinovirus induced acute exacerbations of Chronic Obstructive Pulmonary Disease

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ABSTRACT

Acute exacerbations are the major cause of morbidity and mortality in COPD and respiratory virus infection is believed to be a leading aetiology. However mechanisms by which viruses induce acute exacerbations are poorly understood.

This study reports the experimental inoculation with rhinovirus (RV) of COPD and non-obstructed smoking and non-smoking control subjects, of whom 9 COPD, 10 smoking and 11 non-smoking controls were later judged to have been successfully infected.

The hypothesis tested was that RV infection led to the induction of oxidative and nitrative stress which resulted in degradation of histone deacetylase (HDAC) enzymes in COPD subjects but not controls. Subsequent histone hyperacetylation would then result in prolonged inflammatory gene transcription and therefore generate clinical features of an acute exacerbation.

Following experimental RV inoculation the COPD subjects experienced excess lower respiratory tract symptoms and elevated virus load compared to the control groups. There was an associated acute inflammatory response and a greater burden of redox stress measured using the Griess and Potential of Antioxidant assays. HDAC2 activity was found to be reduced only in the COPD subjects following experimental RV infection.

These findings would suggest that RV induced oxidative and nitrative stress results in reduced HDAC2 activity in COPD subjects leading to increased inflammation and symptoms consistent with the clinical phenotype of a COPD exacerbation.

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Chapter 1: Background and statement of hypotheses

1.1 Introduction

Chronic obstructive pulmonary disease (COPD) is a disease with a global burden and the only common cause of death increasing in frequency, predicted to be the fourth leading cause of death worldwide by 2030. In 2005 3 million deaths (5% of total) worldwide were attributed to COPD. Cigarette smoke is a major aetiological factor; however in the developing world organic smoke and pollution contribute heavily to disease aetiology. The clinical phenotype is characterised by progressive airflow limitation with incomplete reversibility and pathological findings demonstrates airway inflammation predominantly with excess neutrophils, macrophages and CD8+ T cells.

Acute exacerbations of COPD (AECOPD) are the major unmet health need and are responsible for most morbidity, mortality and health care costs associated with COPD. Of the £1bn annual expenditure on COPD in the UK, 70% is consumed treating exacerbations. These are now the most common cause of UK hospital admissions, where overall 1 in 8 inpatient visits are attributed to COPD. There are great variations in rates across the UK, and these correlate with areas of most socioeconomic need. Respiratory infections are the leading cause of AECOPD; and viruses, particularly rhinoviruses, are detected in two thirds of these. An experimental model of rhinovirus 16 (RV16) induced COPD exacerbations has been established and is used in this study with the addition of a non-smoking control group in addition to the smoking controls.

The understanding of cellular mechanisms involved in AECOPD is poor, in part due to the difficulty in studying the condition. Here the experimental challenge model is used to study the role of oxidative and nitrative stress and the involvement of histone deacetylase (HDAC) enzymes in exacerbations. I have performed experimental RV16 infection by inoculating COPD subjects and control groups of both smokers and non-smokers without airflow obstruction. Samples were collected from the upper and lower respiratory tract using nasal lavage (NL), exhaled breath condensate (EBC), induced sputum (IS) and bronchoscopy.

Exacerbations of COPD due to virus infection last longer and are more severe that those without, they also lead to increased airway and systemic inflammation[1].

These differences cannot be attributed solely to the structural alterations in the lungs of patients with COPD because healthy smokers also experience exaggerated symptomatic responses after virus infection, with excess susceptibility and health care utilisation compared to non-smokers[2-4].

Chronic Obstructive Pulmonary Disease

1.2 Epidemiology

The World Health Organisation (WHO) estimates that globally there are 80 million sufferers of moderate to severe COPD. It is the only common cause of death increasing in frequency and predicted to be the fourth leading cause of death worldwide by 2030[5], with the number of deaths due to COPD increasing by 30% in the next 10 years. Despite a higher prevalence in men during the past five decades, COPD rates are now almost equal between genders - mainly due to increasing female smoking rates in high income countries and exposure to indoor smoke and air pollution in low income countries[6]. Disability from COPD is difficult to quantify and studies may be subject to bias but Murray and Lopez predict a global increase with COPD becoming the fifth leading cause of disability adjusted life years lost in 2020[7].

COPD is a disease of increasing age with relatively few cases diagnosed before the age of 40, however many cases in the population remain unidentified with sufferers not seeking medical attention despite persistent respiratory symptoms. The British Lung Foundation (BLF) report 'Invisible Lives'[8] estimates that there are 2.8 million sufferers in the UK who have yet to be diagnosed with COPD (described as the missing millions) and a study using survey data described COPD as 'predominantly undiagnosed' in adults in England[9]. Significant variations in prevalence exist both within and between countries which reflects a host of geographical and social factors and other complexities such as differences in smoking habits, medical diagnosis and resources between countries[10, 11]. Although an incurable disease treatment can slow progression and screening high risk populations is likely to be justified.

Although the Global initiative for Obstructive Lung Disease (GOLD) guidelines are widely accepted criteria for diagnosing COPD[12] these can lead to inaccuracies. The spirometric definition of obstruction is an $FEV_1/FVC < 0.7$ however use of this fixed ratio may lead to an over diagnosis of obstruction in elderly subjects[13] and an underestimate in younger subjects[14, 15]. These findings occur due to normal aging of the lung, predominantly the loss of elastic recoil resulting in a lower FEV_1. They

serve to emphasise the importance of the medical history and examination in making an accurate diagnosis of COPD.

1.3 Aetiology

Development of COPD

COPD typically develops insidiously over decades and results from a combination of genetic and environmental factors. The exposure of the lungs to noxious substances; chiefly smoke, dust and chemicals are the principle environmental cause. In high income countries tobacco smoke is the most frequently identified environmental risk factor, leading to 85-90% cases of COPD diagnosed. Many factors influence the effects of tobacco smoke including the age of smoking onset, the total exposure (pack year history) and the current smoking status. Second hand smoke is also a risk factor for disease development. In the UK a link to occupational and socioeconomic factors and the risk of disease development have been identified[16] but the mechanisms have not been fully elucidated. In developing countries indoor smoke exposure from cooking and heating with organic fuels; chiefly coal, straw, animal dung and crop residues are a major contributor to COPD[17, 18].

A recent review of COPD prevalence in non-smokers identified rates between 25-45%[19], which is considerably higher than previous studies. Risk factors and aetiological agents proposed include perinatal events and childhood illness, diet, fixed airway obstruction resulting from chronic asthma, human immunodeficiency virus (HIV) and recurrent pulmonary infections including tuberculosis (TB). Further research in this area is warranted as the presence of tobacco smoke exposure has become central to the diagnosis of COPD and a criteria for entry to most research studies; which may be leading to a selection bias against non-tobacco induced COPD.

1.4 Genetic influences

Alpha-1 antitrypsin (α1-AT) deficiency was first described in a group of patients with severe early onset emphysema in the 1960s[20] and it has now become the most widely recognised genetic abnormality leading to development of COPD. However the most common homozygous deficiency (PiZZ) for this anti-proteinase enzyme still only accounts for 1-2% of the total cases of COPD, emphasising the importance of environmental factors in disease aetiology. Given this fact it is perhaps surprising only a minority of smokers develop COPD. The fraction remains debated with original studies identifying 15%[21] but more recent longitudinal data suggests

between 20-50% of smokers will develop airway obstruction given sufficient follow up[22, 23].

Following the identification of α1-AT deficiency, twin studies demonstrated a familial link with the development of airway obstruction[24], however unfortunately subsequent larger genetic linkage studies have proved disappointing[25], failing to identity robust genetic risks for COPD. This is likely to reflect the multiple genetic influences responsible for the susceptibility to COPD – itself a complex and heterogeneous disease.

1.5 Diagnosis

The diagnosis of COPD depends on an appropriate medical history and examination followed by confirmation of an obstructed ratio $FEV_1/FVC < 0.7$ on spirometry, which is poorly reversible after bronchodilation. Dyspnoea, chronic cough and excessive sputum production with or without risk factors should prompt consideration of the diagnosis and severity of the disease is graded mild, moderate and severe according to the FEV_1[12]. Although spirometry forms the mainstay of diagnosis data from the USA suggests that this investigation is omitted in a large number of patients. However another study demonstrated that smoking rates act as a useful surrogate marker for spirometry in high risk populations and maybe used to in parts of the world where access to spirometry is limited[26-28].

1.6 Pathophysiology

COPD is an umbrella term for emphysema and chronic bronchitis, but the underlying disease mechanisms are diverse and complex leading to phenotypic heterogeneity. Pathologically loss of elastic recoil and lung tissue destruction (emphysema) combined with airway inflammation and mucus hypersecretion (chronic bronchitis) lead to ventilation perfusion (V/Q) mismatch and clinical symptoms.

1.7 Chronic bronchitis

Chronic bronchitis is defined clinically as the persistence of cough with sputum production for 3 months in at least two consecutive years; pathologically it consists of excessive mucus production and cellular inflammation of the bronchial walls. Both the larger (bronchial) and smaller (bronchiolar) airways can be affected to varying degrees in individuals, however an interesting autopsy study of young smokers who died outside hospital identified small airway disease before the development of clinically detectable lung disease[29]. An assessment of airways disease severity

using 3D computed tomography (CT) in COPD demonstrated that the FEV_1 correlated more strongly with reduced luminal area in the smaller airways than the larger airways; suggesting that distal disease contributes most to airway obstruction in COPD[30]. The chronic inflammation leads to a repair and remodelling process that thickens airway walls and leads to the irreversible bronchoconstriction which is pathognomonic of COPD[31].

1.8 Mucus hypersecretion

Under physiological conditions a thin layer of mucus lines the airways, acting as a primary defence to microbial infection, neutralising endogenous toxins (for example immune meditated cytokines) and facilitating removal of particulate matter through the mucociliary escalator. In disease excessive respiratory tract mucus production leads to airway obstruction, inflammation and increased susceptibility to infection.

In COPD both the quality and quantity of mucin production is altered; hypersecretion occurs through a combination of large airway mucous gland hyperplasia[32] and goblet cell metaplasia in the small airways[33]. Additionally levels of neutrophil elastase (NE) are elevated in chronic bronchitis and this proteolytic enzyme induces mucin production through direct inflammation and intracellular signalling via the protein kinase C (PKC) pathway[34]. Expression of the mucin genes MUC5AC and MUC5B are increased in COPD and the ratio between these is altered when compared to normal subjects with an excess of the negatively changed form of MUC5B[35] which may contribute to increased mucus viscosity.

1.9 Airway inflammation

Airway inflammation in chronic bronchitis is characterised by an increase in a wide range of cells types; including neutrophils, eosinophils and macrophages. The role of these cell types is discussed later. Cytotoxic T cells (CD8+) are found to be elevated in sputum[36], airway epithelium[33], submucosa[37] and the smooth muscle layer[38] of chronic bronchitis patients. Additionally studies of bronchial biopsies have identified an increased ratio of CD8+ to T helper cells (CD4+) cells in patients with chronic bronchitis[39].

Small airway remodelling is driven by a progressive increase in connective tissue and fibrous thickening of the airways[40] which has been shown to be proportional to the severity of airway obstruction. An inflammatory infiltrate formed from a broad range of cell types including neutrophils, macrophages and T and B lymphocytes are

thought to be responsible. A rodent model of airway inflammation utilising transgenic overexpression of the Th2 cytokine IL-10 demonstrated that several mechanisms were responsible[41] including enhanced fibrosis, mucus production and increased IL-13 release, but other studies have associated airways disease with IL-10 deficiency[42] care is therefore needed in interpreting this data. A bronchial biopsy study in small airway remodelling found over expression of vascular endothelial growth factor (VEGF), leading to enhanced vascularity and hypercellularity and providing evidence of another mechanism for disease development[43].

1.10 Emphysema

Emphysema is defined at an abnormal enlargement of airspaces distal to the terminal bronchiole accompanied by destruction of their walls with an absence of fibrosis. The exact mechanisms for this destruction remain unclear, it is known that emphysema occurs as a normal process of the aging lung[44], but the precise pathways by which environmental toxins induce lung destruction are unclear. Loss of physiological lung elastic recoil, small airway inflammation and destruction of lung vasculature are all considered important.

1.11 Proteolysis

The balance between proteases and antiproteases is essential for maintaining lung health and an excess of proteases was an early mechanism postulated for the development of emphysema. This hypothesis was supported by studies in hamsters where emphysematous changes were induced by neutrophil elastin and radiolabeled elastin was shown to be readily dissolved[45]. The premature development of emphysema in α1-AT deficiency also provides further evidence for the role of unchecked proteolysis in the development of emphysema where the increased cellularity with an excess of neutrophils and macrophages are associated with heightened neutrophil elastase and MMP release.

1.12 Loss of elastic recoil

As emphysema develops the elastin fibres in lung parenchyma are damaged, this results in a loss of the lungs physiological elastic recoil – a phenomenon of normal lung tissue to deflate after it has been inflated. Loss of elastic recoil occurs with ageing; in fact the process is predictable enough for age to be estimated from the pressure volume characteristics of a post-mortem lung[46]. This is a pertinent finding as one current hypothesis is to regard COPD as a disease of abnormal or premature lung ageing[47, 48], and an interesting study of facial skin wrinkles found this was a

risk factor for developing COPD and correlated with a reduced FEV_1[49]. The air liquid interface at the alveolar surface also has an influence on elastic recoil, where the normal tendency of alveolar sacs to collapse encourages lung deflation. Emphysema leads to a loss of alveolar surface area and in this way also reduces elastic recoil[50, 51]. So through these two separate mechanisms emphysematous processes reduce elastic recoil and contribute to airway obstruction.

1.13 Dynamic airway collapse

The peripheral bronchioles differ from larger airways, particularly as they lack internal rigidity and without cartilage in the walls rely on alveolar wall tethering to maintain patency. As alveolar units are destroyed by emphysematous processes, this support is lost and airways collapse more readily and at an earlier point in the cycle of expiration. This phenomenon of dynamic airway collapse is part of the process leading to fixed airways obstruction and air trapping characteristic of COPD.

1.14 COPD exacerbations

Exacerbations of COPD are responsible for the majority of morbidity and mortality in the disease and represent a major unmet health need. In the UK direct healthcare expenditure on COPD is £1bn but 70% of this is consumed by the management of exacerbations with unplanned acute care and hospital admission being the biggest draw on resources[52]. AECOPD are the most common cause of unplanned UK hospital admission, accounting for 1 in 8 episodes[53]. Exacerbations are associated with functional and physiological decline and are strong predictors of poor health status. They are feared by patients and carers and provide management challenges for healthcare providers. Additionally inequalities and variations exist in the provision of care for acutely ill COPD patients[54]; predicting prognosis is difficult and there is evidence of excess prognostic pessimism amongst doctors managing AECOPD[55]. There is evidence that exacerbations are associated with an accelerated loss of lung function, leading to a vicious cycle of increasing frequency of exacerbations and further pulmonary decline[56, 57]. In a longitudinal study assessing emphysema severity by CT, subjects who experienced exacerbations had increased emphysema progression compared to those who did not experience exacerbations[58]. Patients who experience frequent exacerbations have also been found to have reductions in health related quality of life scores when compared to in-frequent exacerbators, despite similar acute falls in lung function[59].

1.15 Diagnosis of exacerbations

Although usually readily diagnosed in clinical practice; exacerbations have been difficult to define in research practice and medical literature. Anthonisen *et al* established a definition of exacerbations in the mid 1980s based on the triad of increased dyspnoea, sputum volume and sputum purulence[60], this has been used extensively in research practice albeit with modifications. The GOLD and American Thoracic Society/European Respiratory Society (ATS/ERS) consensus statement is now widely accepted as the standard definition; where an exacerbation is *"an event in the natural course of the disease characterised by a change in the patient's baseline dyspnoea, cough and/or sputum that is beyond normal day-to-day variations, is acute on onset, and may warrant a change in regular medication in a patient with underlying COPD"[12, 61]*. The strength of this version is the emphasis that is placed upon a change in individual symptoms combined with the possible need for healthcare utilisation. The importance of a standardised definition is illustrated by a recent review article, where 51 studies of exacerbations were examined and 14 different definitions identified[62]. The effect of this redundancy between studies has been shown to change the relative risk for exacerbation rate and hazard ratio for time to first exacerbation from non-significant to significant and also has an impact on the effect size of interventions; stressing the urgent need for the use of a consistent definition in research studies[63].

1.16 Epidemiology of exacerbations

The frequency of exacerbations identified by studies range from 0.99 to 3.83 per person/year[64, 65], but the average frequency reported is 2 per person/year. This range reflects the heterogeneity of both the disease phenotype and study methodology. Studies in the East London Cohort have provided valuable insights into the epidemiology of exacerbations, and using diary cards have identified the highest exacerbation rates -2.92-3.36 per person/year. These have the advantage of capturing milder, self managed and unreported exacerbations[59, 66, 67]. These studies have also postulated that there is a sub-group of patients who suffer more frequent exacerbations; importantly this group suffered from worse respiratory symptoms, lower quality of life scores and more rapid decline in lung function[56] suggesting that identification of this group might allow targeting of healthcare resources and provide new insights into disease mechanisms.

Estimations of hospitalisation rates and mortality are complicated by many confounding factors; including disease severity, comorbidities and socioeconomic

conditions. But the literature suggests that Individuals can expect between 0.15 and 0.25 hospital admissions per year with an average length of stay 8.7 days[68]. Mortality rates between 2.5% and 24% have been reported in hospital inpatients[69, 70], but this increases to 43% in those requiring mechanical ventilation[71].

1.17 Aetiology of exacerbations

Respiratory tract infection by both bacteria and viruses are the leading cause of exacerbations, identified in up to 70%[72], however a wide range of non-infectious aetiologies have been identified including air pollution[73] by ozone and particulate matter[74], climatic factors including air temperature[66, 75] and cardiovascular events - particularly pulmonary embolism[76, 77]. In approximately 1/3rd of severe exacerbations a cause is not identified[78]. The role of infection in acute exacerbations is discussed in more detail later in this chapter.

Disease mechanisms in COPD exacerbations

1.18 Oxidative stress

Reactive oxygen species (ROS) are unstable pro-oxidant compounds with an unpaired electron, which have been implicated in a wide range of tissue injuries and inflammation[79]. Common examples include the oxygen metabolites superoxide (O_2^-), hydroxyl (OH^-) and peroxide ($O\text{-}O^{2-}$) where the unpaired valence shell electron can lead to extensive cellular damage. The process of oxidation involves a substance losing an electron, whereas compounds gaining an electron have undergone reduction. There is a significant burden of ROS in the lungs, the majority released from mitochondria as a by-product of normal metabolism, but also released by other cellular processes and immune responses such as phagocytosis. The measurement of oxidative stress in the lungs is problematic, mainly due to the instability and short life span of ROS and delays in collecting and processing respiratory samples. Strategies to measure oxidative stress have therefore included direct measurement of oxidative burden (e.g. NO or H_2O_2 in EBC), the physiological response to oxidative stress (e.g. antioxidants or enzymes in sputum/blood) and the effect of oxidative stress on proteins, lipids and DNA (e.g. lipid peroxidation products in EBC)[80-83].

1.19 Sources of oxidative stress

Cigarette smoking is a potent source of oxidants and measurements have found that a typical puff contains $4x10^{14}$ free radicals[84]. There is a complex mixture of chemical substances in cigarette smoke, and free radicals have been identified in

both the gas and tar phases. Within the gas phase highly reactive peroxyl and NO have been found at concentrations of 500-1000ppm[85]. Radicals in the tar component are more stable, but these subsequently form the epithelial lining fluid which produces ROS, particularly H_2O_2, for a longer period of time[86]. Endogenous production of ROS is increased in COPD. Inflammation leads to increased numbers and more active macrophages which produce an excess of O_2^- and H_2O_2[87]. In addition the airspaces of smokers contain higher levels of free iron which is able to generate OH through the Fenton and Haber-Weiss reaction[88]. Macrophages from COPD subjects have higher intracellular iron levels than smokers[89] and release more free iron *in vitro* than those from non-smokers[90]. ROS are also released from epithelial cells; type II alveolar cells have been shown to release similar amounts of O_2^- and H_2O_2 to macrophages[91] and *in vitro* were able to inactivate α1-AT[92]. Neutrophilia is frequently identified in COPD and these cells contribute significantly to oxidative stress, particularly during the respiratory burst when endogenously produced radicals are released in response to the presence of pathogens[93, 94].

1.20 Induction of oxidative stress

It has been proposed that COPD develops in a subset of smokers, in whom antioxidant mechanisms are overwhelmed and a high level of oxidative stress results[95]. There is considerable indirect evidence to suggest that oxidative stress is increased in the lungs in stable COPD and that during exacerbations this is increased still further, but the mechanisms behind this are poorly characterised. As described above, inflammatory cells appear to play an important part. With increasing cell number the oxidative burden worsens, mediated particularly through the respiratory (oxidative) burst which is produced by activated immune cells. Macrophages and neutrophils produce the highly active free radical O_2^- from oxygen under the influence of NADPH oxidase enzymes. In health this process benefits the immune system by killing pathogens. Indeed a deficiency of NADPH oxidase enzymes leads to chronic granulomatous disease and tissue destruction resulting from pathogen persistence. However in disease this illustrates a potential mechanism of increased ROS and particularly during an infective exacerbation of COPD when the immune system may be over stimulated. Neutrophilia is frequently found in COPD and in the face of infectious agents, neutrophils produce hypochlorous acid (HOCl) under the influence of myeloperoxidase enzymes, which also has potent oxidant effects. Once inflammation is established, oxidative stress itself can further propagate the inflammatory milieu by enhancing proinflammatory gene expression. This positive feedback occurs through the activation of oxidant

sensing transcription factors such as nuclear factor kappa light chain enhancer of activated B cells (NFκB) and activator protein-1 (AP-1)[96, 97].

Hydrogen peroxide (H_2O_2) is a derivative of free radicals and endogenously produced in neutrophil peroxisomes by dismutation (a reaction in which two distinct products are formed) of O_2^- and glycolate oxidase. Exogenous sources mainly include cigarette smoke and air pollution[98]. Endogenous H_2O_2 production is upregulated when neutrophils are exposed to cigarette smoke; studies in rabbit lung have demonstrated neutrophil activation and increased adhesion in the pulmonary circulation[99]. In EBC H_2O_2 is a direct measurement of oxidants and is increased in smokers[100] and COPD both when stable and during exacerbations[101].

Adenosine receptors actions are complex and can have both a pro- and anti-inflammatory effect; however in the lower airway in COPD they appear to have an association with airflow obstruction[102, 103]. On alveolar macrophages from COPD subjects adenosine receptors are down regulated and this has been shown to be mediated by oxidative stress, in a process ameliorated by the addition of the reducing agent N-acetyl cysteine (NAC)[104].

1.21 Nitrosative stress

Reactive nitrogen species (RNS) are also highly active in the lungs; derivatives of nitric oxide (NO) include nitrite (NO_2^-) and peroxynitrite ($ONOO^-$). NO is produced from arginine under the influence of nitric oxide synthase (NOS) which is present in both a constitutive and inducible form, the latter found in respiratory epithelium and macrophages in the lung. NO is also present in cigarette smoke at high concentrations (up to 100mg/L) where it acts as an oxidant[85].

Elevated levels of exhaled NO have been reported in stable COPD in some studies[105, 106], but others have not demonstrated either increased NO or iNOS[107]. The reasons for this contradiction are not clear, but may reflect the heterogeneity of inflammation in AECOPD or the combination of current and ex-smokers in the studied groups. Although Rutgers *et al* did not find increased NO concentration in the COPD subjects, the levels did correlate with sputum eosinophilia. Two studies of exhaled NO in exacerbations have found elevated levels at the time of hospital admission, which fell with recovery and correlated with improvements in FEV_1, demonstrating the potential role of oxidants in the aetiology of acute exacerbations[108, 109].

1.22 Tissue consequences of oxidative and nitrosative stress

Isoprostanes, which are prostaglandin like compounds, are produced by free radical catalyzed lipid peroxidation of essential fatty acids (chiefly arachidonic acid). They act indirectly as bronchoconstrictors and pro-inflammatory mediators and are produced independently of the cyclooxygenase enzyme. They can be used as markers of oxidative stress and to gauge the degree of damage to target proteins[110]. 8-isoprostane levels in EBC are increased in stable COPD[111] and still further at the time of exacerbation[112]. Plasma lipid peroxidation products are also increased[95], at least partly due to activation of NFκB. The levels of which are both elevated and associated with increased nuclear localisation in airway epithelial cells[113] of smokers and COPD subjects.

H_2O_2 causes direct protein damage and an important example is the loss of α1-AT anti elastase activity. This occurs through oxidation of methionine residues at position 351 and 358 which render the active site of the enzyme ineffective[114].

1.23 Oxidative stress in COPD

As discussed above oxidative stress markers are increased in COPD, in addition several studies have identified an association between airflow limitation and markers of oxidative stress linked to cellular inflammation in COPD[94, 115]. However there is a lack of longitudinal data with no studies focusing on this specifically and little direct evidence of causation of COPD by ROS. Several pathogenic mechanisms have been proposed however and these include worsening of the protease/antiprotease balance, epithelial cell damage, increased neutrophil sequestration mucus hypertrophy and increased apoptosis.

1.24 Antioxidant control

Counterbalancing the reactive oxygen and nitrogen species in the lung is the antioxidant system. This can be considered in terms of the enzymatic (super oxide dismutase [SOD], thiorodoxin and glutathione peroxide & S-transferase) or non-enzymatic (glutathione [GSH], ascorbate, urate, α-tocopherol and bilirubin) antioxidants. Oxidative stress occurs when the antioxidant system is unable to neutralise the burden of ROS/RNS.

An antioxidant is a substance able to delay the process of oxidation and several are released by the airway basal cells, suggesting they have an important role in the

neutralisation of ROS. These include secretion of SOD and expression of a transmembrane transporter responsible for the efflux of glutathione[116]. Damage to the respiratory epithelium not only induces oxidative stress, but also causes damage to the antioxidant defences.

Using antioxidant responses to assess oxidative load in COPD has been an effective strategy, particularly by measuring GSH which is an important antioxidant in the lungs. GSH levels are considerably higher in BAL from smokers and those with stable COPD when compared to non-smokers; reflecting the physiological response to increased oxidative stress. During acute exacerbations the levels of GSH were shown to fall in proportion to the severity of the exacerbation, presumably reflecting the increasing oxidative stress and subsequent overwhelming the anti oxidant system[96]. GSH in its reduced form is more prevalent in the lungs of chronic smokers[117] but ageing adversely affects the GSH response to cigarette smoke exposure[118], suggesting an age related decline in oxidant defences.

1.25 Epigenetics

Defined by Waddington in 1942, the term epigenetics combines the words epigenesis and genetics and encompasses the concept of heritable changes in gene expression that can occur without a change in DNA sequence. The field has become an area of intense study in recent years with recognition of its importance in both the normal and diseased state. Responses to environment, behaviour and pathology can be governed by epigenetic changes[119].

1.26 Histone activity

Histones are proteins which form the core of chromatin. They allow compaction of long lengths of DNA and play an important part in gene regulation. In 1964 the association between diffuse-extended DNA and active RNA was made, and conversely it was also shown that repressed DNA and condensed chromatin was not associated with mRNA activity[120]. Subsequently an association was made between an increase in histone acetylation and enhanced RNA polymerase II activity. Histones are divided into 6 major classes, and form an octamer around which the DNA is wound. It is now clear that histone modifications, particularly in the histone tail, can alter DNA exposure and hence gene transcription. Common examples of histone modifications are phosphorylation of serine residue 10 and acetylation of lysine residue 14 on histone H3 – this is regarded as a sign of active transcription. Studies in yeast have demonstrated that histones are acetylated in their resting state

and small changes in the number acetylated can result in biophysical changes, so only a minimal alteration in the number of lysines acetylated can rapidly change a gene from the active to inactive state and vice versa[121].

1.27 Histone acetylation

Acetylation of histone tails is catalysed by histone acetyltransferase (HAT) and conversely histone deacetylase (HDAC) deacetylates. Four classes of HDAC have been described; the majority of isoenzymes fall into class I and II with Silent Information Regulators (sirtuins) forming class III[122]. The discovery of HDAC 11 with unique characteristics necessitated creation of the fourth class[123]. HDACs are phosphoproteins and their control and activation is complex, but it appears that the activity is modified according to their phosphorylation status[124]. A study using lung tissue and alveolar macrophages identified an association between reduced HDAC activity and increasing clinical severity of COPD[125]. HDAC plays a pivotal role in repressing pro-inflammatory cytokines and reduced HDAC activity was associated with worse airway inflammation in severe COPD. mRNA expression of HDAC isoenzymes 2, 5 and 8 and HDAC2 protein levels showed graduated reductions with more severe COPD. Chromatin immuneprecipitation (ChIP) was used to measure histone 4 acetylation at the IL-8 promoter region where acetylation was associated with increased IL-8 mRNA and COPD severity. In GOLD stage IV COPD HDAC2 levels were reduced by 95% compared to normal controls. The mechanism of HDAC reduction is not known, but it is likely that oxidative and nitrative stresses are responsible. The increased oxidative stress in COPD and enhanced activity of reactive oxygen species and stress kinases result in HDAC degradation[126-128].

1.28 Modulation of HDAC activity

It is thought that the limited effectiveness of corticosteroids in COPD may be due to a deficiency of HDAC[129]. Steroids act, at least partly, by recruiting HDAC to repress inflammatory gene transcription. These compounds are therefore less effective in COPD when compared to disease states with normal or near normal HDAC levels; for example steroid responsive asthma.

It has been postulated that low dose theophylline may help to restore HDAC activity and act to potentiate the effect of steroids. In a study of macrophages from stable asthmatic subjects HDAC activity was increased following treatment with 4 weeks of theophylline[130], although the mechanisms behind this effect are not fully understood. Furthermore in a study of AECOPD using low dose theophylline it

appeared to enhance the anti-inflammatory effect of steroids and demonstrated improved HDAC2 activity in sputum samples[131]. The study was a single blind randomised design, lacked a placebo control and had relatively small subject numbers, however the findings appear to support the hypothesis that theophylline can restore HDAC2 activity *in vivo* and that HDAC2 deficiency is a mechanism for increased inflammation during exacerbations.

A recent study demonstrated that knockdown of a class III HDAC (sirtuin-SIRT1) using either oxidative stress, a sirtuin inhibitor or silencing RNA led to elevated matrix metalloproteinase-9 (MMP-9) levels. The finding that SIRT1 is a negative regulator of MMP-9 demonstrates a possible molecular mechanism for increased protease activity in COPD[132].

There has been interest in modulating HDAC activity with the use of specific inhibitors (HDACi), particularly using trichostatin A (TSA). The majority of clinical research has been in oncology as a potential chemotherapeutic agent, but some studies have investigated the role of HDACi in inflammation, and although reduced HDAC levels have usually been associated with worsening inflammation, the effects on HDAC inhibition in some studies have contradicted this[133]. This demonstrates that the links between HDAC activity and inflammation are not fully understood. Overall the effect of TSA at the protein level was to reduce IL-6 and TNF-α concentrations; the anti-inflammatory effect was found to be additive to that of dexamethasone[133].

An *ex vivo* study of cultured epithelial cells from COPD and control subjects with RV (rhinovirus 39) demonstrated an increase in inflammatory cytokines in COPD subjects compared to controls (IL-6 & IL-8). However there was no difference in HDAC2 or HDAC4 expression between the two groups, nonetheless there was a tendency to reduced expression of the transcription repressor SIRT1 in COPD subjects[134].

1.29 HDAC activity and immune function

Finally a potential link between HDAC activity and immune function has been postulated[135]. Should attenuated host defence responses be associated with reduced HDAC activity, this finding would have significant implications for AECOPD. In this study Rogers *et al* demonstrated that HDACi reduced expression of host defence genes including pattern recognition receptors, kinases and cytokines;

additionally an *in vivo* model of infection demonstrated that HDAC inhibition impaired macrophage function and natural host defence, resulting in increased susceptibility of mice to bacterial and fungal infections.

1.30 Inflammatory mediators

It is beyond the scope of this chapter to provide a full review for the many cytokines and chemokines which may be involved in the pathogenesis of COPD. However the following is a brief summary of the proteins that I have measured in the sputum and BAL supernatant in this study accompanied by a rationale for their study in the context of COPD and AECOPD. Several of the inflammatory mediators have been shown to be increased during AECOPD, but data has been obtained from a wide range of studies with different inclusion criteria and at different time points during an exacerbation.

1.31 Matrix metalloproteinase-9 (MMP-9)

Produced by macrophages, the matrix metalloproteinase (MMP) family of enzymes have a role in COPD pathogenesis by inducing emphysematous tissue damage. MMP-12 was the first of this family to be identified and found to be elastolytic when released from mouse macrophages activated by latex beads[136]. Subsequently MMP-12 knockout mice were shown to be resistant to emphysema after exposure to cigarette smoke[137] providing further evidence for their role in lung tissue destruction. MMP-9 is highly active in elastin degradation and both mRNA and protein levels were elevated in studies of BAL from COPD subjects compared to controls[138] and furthermore the biological activity of MMP-9 was increased in COPD subjects[139]. Counter balancing the effects of MMPs are tissue inhibitors of matrix metalloproteinases (TIMP) enzymes. These are also produced by macrophages, and the levels of these protective enzymes were found to be increased following *in vitro* stimulation of BAL macrophages from non smokers when compared to both healthy smokers and COPD patients[139]. MMP-9 levels are increased in AECOPD[140].

Mucus hypersecretion is pathognomonic of chronic bronchitis and in rodent models propenal – a simple aldehyde found in cigarette smoke - has been shown to induce epithelial damage and mucin production through activation of MUC5AC gene. Knockout mice experiments have indicated that this occurs through synthesis, secretion and activation of MMP-9[141], providing another mechanism for disease causation by this enzyme.

1.32 Granulocyte macrophage-colony stimulating factor (GM-CSF)

GM-CSF is a 127 amino acid cytokine with structural similarities to growth factors IL-2, IL-3 and IL-5. It acts by binding a heterodimeric receptor formed from an alpha and beta chain. GM-CSF receptor signalling is complex, reflecting the diverse actions of GM-CSF which include cell differentiation, activation and promotion of an inflammatory response. Transcription factors NFκB and PU.1 have both been shown to be activated by GM-CSF and the latter to induce terminal differentiation of alveolar macrophages[142]. GM-CSF has a positive feedback role in promoting inflammation in the lung, where it is produced by the majority of cell types present; it also acts as a chemoattractant for eosinophils.

Studies in COPD have demonstrated conflicting results on GM-CSF concentrations in sputum, but this maybe due to the rapid reduction in levels following internalisation of the GM-CSF-receptor complex[143]. More recent studies have identified elevated levels of GM-CSF in both BAL and sputum of stable COPD patients, which are significantly increased at exacerbation[144, 145]. As glucocorticosteroids mediate their response through HDAC, it has been postulated that the inability of these drugs to suppress GM-CSF in COPD may be due, at least in part, to HDAC2 deficiency.

Macrophages play a central role in COPD where their numbers are increased in both airways and parenchyma. By producing excess GM-CSF in response to cigarette smoke and virus or bacterial infection inflammatory cells are increased further and positive feedback is reinforced. This has led to interest in a therapeutic role for GM-CSF suppression. While untested in human subjects there are some data to support this rationale; particularly using drugs which are effective or show promise in COPD. Studies include an *in vitro* study of cilomilast, a novel phosphodiesterase 4 inhibitor (PDI), which reduced GM-CSF secretion in cells from induced sputum[146]. Other *in vitro* studies demonstrated reduced levels of inflammatory cytokines, including GM-CSF after exposure to formoterol and salmeterol in monocyte derived macrophages (MDM) [147] and the sirtuin activator resveratrol[148, 149] in smooth muscle cells and alveolar macrophages (AM) from COPD subjects. Resveratrol, isolated from red wine extract, has antioxidant and anti-inflammatory properties which may act through an alternative pathway to corticosteroids.

1.33 Interferon gamma (IFN-γ)

IFN-γ is a pivotal member of both the innate and adaptive immune systems. It is the only member of the type II interferon family and belongs to a group of proteins known as macrophage activating factors. It is a dimer formed from two interlocking 6 subunit alpha-helices. Produced by natural killer (NK) cells (innate) and CD4 and CD8 cells (adaptive), it is the archetypal cytokine of Th1 cells. IFN-γ has many biologically important actions, including the ability to inhibit virus replication, promote macrophage activity and activate inducible nitric oxide synthase (iNOS). There are no studies to date that have identified elevated levels of IFN-γ protein in COPD, but IFN-γ producing Th1 cells have been identified in increased numbers in COPD[150, 151].

1.34 Interleukin 10 (IL-10)

The anti-inflammatory cytokine IL-10 is produced predominately by monocytes and acts to down regulate Th1 cytokines, including IFN-γ, IL-2, TNF-α and GM-CSF. In response to virus infections it is released by cytotoxic T cells, suppressing NK cells and so damping down the innate immune response. In disease states associated with excess inflammation it has been hypothesised that IL-10 deficiency may play a role. Indeed, levels of IL-10 have been found to be reduced in sputum samples from COPD[42] patients compared to healthy non-smoking and smoking controls and genetic studies have identified IL-10 polymorphisms in α_1-antitrypsin deficient[152] and COPD populations[153]. Recombinant human IL-10 has been effective in controlling inflammatory bowel disease, supporting a possible role for use in COPD. However when IL-10 was over expressed in a rodent model airway inflammation was increased[41], suggesting that health may depend on optimum levels of IL-10.

1.35 Interleukin 12 (IL-12)

The biologically active heterodimer of IL-12 is IL-12p70, formed from p40 and p35 subunits. The expression of the p40 subunit is tightly controlled and released from activated macrophages and B cells. The cytokine is involved in regulation and differentiation of Th1 cells and therefore the maintenance of a Th1/Th2 balance. It can reduce the response to allergens and enhances production of IFN-γ and TNF-α. The role of IL-12 in COPD has not been well characterised but was found to be increased in EBC of COPD subjects[154]. However a genetic polymorphism study failed to identify differences in the rate of the IL-12 allele between control and COPD subjects[155]; furthermore in this study IL-27, which acts synergistically with IL-12

and is a closely related member of the IL-12 family appeared to have a protective role in COPD. A study of bacterial colonisation in COPD identified significantly higher IL-12 levels in subjects colonised with *H. influenza*[156] compared to those without, but not those with other bacterial species identified in the study, further illustrating the uncertainty of IL-12's role in COPD.

1.36 Interleukin 1 beta (IL-1β)

This proinflammatory cytokine is released from a variety of cells, particularly activated macrophages, as a pro-protein. It then undergoes caspase 1 dependent activation, having several biological effects similar to TNF-α. These include leukocytosis through neutrophil release and proliferation and activation of B cells, promotion of apoptosis and induction of cytokines including IL-8, IL-6, GM-CSF, TNF-α and IL-2. It also induces adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1). In COPD levels have been found to be elevated in both stable and exacerbated COPD in EBC[154] and IL-1β polymorphism studies have found a susceptibility association with COPD[157]. In cultured human bronchial epithelial cells enhanced IL-1β release and reduced glutathione (GSH) were found, demonstrating greater susceptibility to cigarette smoke and oxidative stress in COPD subjects[117].

An *in vitro* study of *C pneumoniae* infection of alveolar macrophages (AM) and peripheral blood mononuclear cells (PBMC) from COPD subjects demonstrated an imbalance of pro- and anti-inflammatory cytokines, with a six and fourfold respective increase in IL-1β secretion when compared to healthy controls[158]. This suggests a possible role for IL-1β in enhancing the inflammation seen during an infective exacerbation. Interest has focused on nuclear proteins which can act as carrier molecules and bind with high affinity to inflammatory cytokines, High-Mobility Group Box 1 (HMGB1) protein is an example recently studied in the context of COPD and IL-1β[159]. Here levels of this protein were found to be elevated in BAL from COPD subjects compared to healthy smokers and non-smokers, and IL-1β levels positively correlated in all smokers. Although in small numbers it was also shown that IL-1β-HMGB1 complexes were present in AM from COPD subjects. It was postulated that the presence of these complexes may be responsible for the prolongation of inflammation in COPD.

1.37 Interleukin 2 (IL-2)

IL-2 is a cytokine which promotes T cell activation and expansion of naive T cells when it is produced by activated T cells. It may also be able to activate AM and fibroblasts through receptors located on these cells; where it is able to enhance inflammation and possibly pulmonary fibrosis. IL-2 plays a central role in initiating the adaptive immune response and this is exploited as a target for therapeutic immunosuppression with drugs such as cyclosporin and tacrolimus.

There is evidence that T cell activity is abnormal in COPD and a study of peripheral blood T cell apoptosis found an increased rate in COPD subjects compared to controls and that differences in levels of IL-2 were not responsible for this[160]. Also a randomised double blind placebo controlled study of an immunomodulator drug, AM3, demonstrated restoration of IFN-γ levels and normalisation of PBMC proliferation in response to PHA which was not identified after addition of IL-2 to the culture[161]. Few studies have examined the role of IL-2 in COPD directly, however in a retrospective study of emphysema patients it was found that subjects with stable COPD had higher levels of IL-2 compared to a group with rapidly declining lung function[162].

1.38 Interleukin 6 (IL-6)

The pro-inflammatory cytokine IL-6 is produced by a wide range of cells both within and outside the immune system. It is active as an acute phase mediator and promotes a range of systemic responses to infection including inducing pyrexia. The production of IL-6 by AM following stimulation by PAMP illustrates the key role IL-6 plays in pulmonary host defence.

Interest in the role of IL-6 in COPD pathogenesis is considerable, it has been found to be elevated in serum and sputum of stable and exacerbated subjects and to be associated with reductions and a faster decline in lung function. Specifically following experimental infection with RV in asthmatic and COPD subjects IL-6 levels were found to be elevated in BAL and sputum respectively [163, 164]. In a cohort study of COPD exacerbations IL-6 levels in serum were elevated leading to an increase in plasma fibrinogen and suggesting a possible mechanism for an excess of thrombotic events seen following AECOPD[165]. There is evidence that IL-6 can up regulate C reactive Protein (CRP) at the transcriptional level and this is supported by a study designed to assess the reproducibility of IL-6 measurements in COPD patients over a year[166], where it was also shown that IL-6 and CRP levels correlated. As with

other cytokines; genetic polymorphism studies have identified links between populations of COPD subjects and specific inflammatory gene promoters; in a study by He[167] several IL-6 promoter polymorphisms were found to be associated with a greater decline in FEV_1 and increased susceptibility to COPD amongst smokers. However the precise role of IL-6 in COPD is not well understood, as illustrated by an *in vitro* study on the effect of commonly used drugs, which demonstrated that RV induced IL-6 levels were suppressed by pre-treatment with fluticasone but actually augmented by salmeterol exposure[168]. Given that β_2 agonists have many beneficial effects in both asthma and COPD more research is needed to clarify the role of IL-6 in this area.

1.39 Interleukin 8 (IL-8)

IL-8 was the first chemokine to be discovered and belongs to the CXC (α-chemokine) family. It is produced by a range of cell types, including macrophages, epithelial and endothelial cells and acts as a neutrophil and T cell chemoattractant through a G-protein coupled receptor. By activating neutrophils it facilitates release of stored enzymes and produces the respiratory burst through oxygen free radical and nitric oxide generation. By this mechanism it leads to host defence and tissue destruction which are the hallmarks of neutrophilic inflammation. IL-8 also promotes the formation of leukotriene B_4 (LTB_4) through activation of 5-lipoxygenase. LTB_4 is itself a powerful pro-inflammatory mediator, with chemoattractant properties. There is evidence of a positive feedback loop whereby elastase released from neutrophils under the influence of IL-8 stimulates epithelial cells to produce yet more IL-8 and LTB_4 further propagating the inflammatory response[169, 170].

In the airways IL-8 expression can be induced by a wide range of both immunological and non-immunological stimuli including cigarette smoke[171] and indeed higher levels of IL-8 are detected in BAL[172] and epithelial cells[173] from healthy smokers when compared to non-smokers. In COPD IL-8 is elevated still further when compared to controls and asthmatic subjects[174]. IL-8 levels have been found to correlate with the degree of airflow limitation; where levels in sputum show a negative correlation with the FEV_1/FVC [175] ratio and a positive correlation with the rate of decline in FEV_1[176]. The interaction of cigarette smoke extract (CSE) and RV infection has been studied *in vitro* using epithelial and BEAS2B cells and demonstrated that these two stimuli in combination augmented IL-8 induction by more than the additive effect of either alone and they promote IL-8 release through

different mechanisms[177]. Studies of IL-8 in EBC during exacerbations have produced conflicting results with IL-8 detectable in one but not in another despite similar methodology[178, 179]. So although it seems likely that IL-8 plays a central role in the development and proliferation of inflammation in COPD, a recent study to assess the potential role of sputum neutrophil count as a biomarker found only a weak correlation with serum IL-6 and IL-8[180] suggesting that there are many other factors which influence the inflammation in COPD. A blinded study of an IL-8 monoclonal antibody in COPD failed to demonstrate a benefit in lung function or exercise tolerance however[181], although there was a modest improvement in breathlessness.

1.40 Tumour necrosis factor alpha (TNF-α)

TNF-α is a pro-inflammatory cytokine and a member of the acute phase protein family which includes IL-1, IL-6, IL-8 and GM-CSF, many of which augment TNF-α release. It is produced predominantly by macrophages but also secreted from many other cell types in the respiratory tract including epithelial cells, T cells and mast cells. It plays a complex part in immune regulation with several functions identified; it is able to activate epithelial cells, macrophages and antigen-presenting cells (APC), induce apoptosis in areas of pulmonary inflammation, stimulate matrix metalloproteinases release from macrophages and induce adhesion molecules. TNF-α also exerts a pro-inflammatory role through IL-8 release, which is synergistically increased by the presence of CSE[182].

TNF-α has been shown to increase airway hyperresponsiveness and sputum neutrophilia in normal subjects[183] and is elevated in the sputum of smokers[184] and still further increased in COPD[174]. Additionally it is associated with systemic inflammation, muscle wasting and weight loss seen in COPD[73, 185]. There have been contradictory results from genetic studies, but a study in COPD identified those with a TNF-α polymorphism had a lower BMI and greater annual FEV_1 decline when compared to those without[186]. Despite hopes that removing excess TNF-α might provide a therapeutic role, anti TNF-α therapy has been disappointing in COPD and appears to be largely ineffective[187].

1.41 Eotaxin

Originally identified in BAL from allergic guinea pigs, Eotaxin is a member of the CC chemokine family and the most potent and selective eosinophil chemoattractant with

additional weak T cell chemotaxis properties. It is a ligand for several chemokine receptors, chiefly CC chemokine receptor 3 (CCR3), which is abundant on eosinophils. Interest has focused on asthma and allergic diseases but studies in COPD have demonstrated higher levels of eotaxin in BAL from stable COPD compared to controls[188] and those with rapid decline in lung function[162].

1.42 Eotaxin-3

Eotaxin-3 is closely related to Eotaxin and has similar properties, chiefly as an eosinophil chemoattractant. There is limited data on the role of Eotaxin-3 in both stable and exacerbations of COPD. In asthma increased concentrations have been found when compared to normal controls[189] and expression in bronchial biopsies is elevated following allergen challenge[190].

1.43 Interferon gamma-induced protein 10 (IP-10)

IP-10 is a CXC chemokine released from airway smooth muscle under the influence of IFN-γ and TNF-α, acting synergistically at transcription level[191]. It is a chemoattractant for immune cells, including macrophages, mast cells and T cells. Levels have been found to be elevated in COPD[192], steroid resistant asthma[193] and at the time of virus induced exacerbations of asthma[194]. A recent study in the East London COPD cohort identified serum IP-10 as a potential biomarker for RV induced exacerbations [195]. The study identified higher serum IP-10 levels in COPD subjects at baseline and demonstrated a correlation between sputum RV load and serum IP-10. This data suggests that IP-10 activity may be involved in immune mediated inflammation in COPD and could play a part in RV induced COPD exacerbations. A study of RV infected epithelial cells cultured *ex vivo* demonstrated increased IP-10 protein levels which were associated with higher vial titre and copy number[196]. Together results from these studies strengthen the evidence for IP-10 being an important mediator in RV induced COPD exacerbations.

1.44 Monocyte chemotactic protein 1 (MCP-1)

MCP-1 plays a role in the recruitment of monocytes, basophils and T-cells to sites of tissue injury and inflammation; it is secreted by both macrophages and monocytes. The MCP-1 receptor, CCR2, has been shown to mediate the activation of MAPK (Mitogen-activated protein kinase), a key kinase in the regulation of mucin in bronchial epithelium[197]. This mechanism may promote goblet hyperplasia and hence the pathogenesis of airway disease and interestingly there are elevated levels of MCP-1 in EBC, sputum, BAL and bronchial epithelium of smokers and patients

with COPD[198-200]. *In vitro* experiments have demonstrated an increase in MCP-1 release following RV infection of human monocytes and bronchial epithelial cells[201]. In a clinical study designed to identify biomarkers which correlate with Body mass index, Airflow obstruction, Dyspnoea, Exercise capacity scores (BODE)[202], MCP-1 was found to be elevated in COPD subjects compared to healthy smokers and correlated with FEV_1 and 6 minute walk test (6MWT)[203].

1.45 Monocyte chemotactic protein 4 (MCP-4)

MCP-4 is a member of the CC chemokine family and acts as a chemoattractant for monocytes, eosinophils, T-lymphocytes and basophils. It shares sequence identity and receptors with eotaxin[204] where its role in allergic disease has been confirmed by the demonstration of increased mRNA and protein levels in asthmatic BAL, sub-mucosa and epithelium when compared to airways of non-allergic control subjects[205, 206]. *In vitro* expression has been shown to be induced by IL-1β and TNF-α[205].

1.46 Macrophage derived chemokine (MDC)

MDC acts on the CCR4 receptor and is secreted by macrophages and dendritic cells. It has chemotactic activity for monocytes, natural killer and dendritic cells[207]. MDC expression is augmented by a wide range of mediators, specifically lipopolysaccharide, IL-1, TNF-α, IL-4 and IL-13; whereas IFNγ has inhibitory effects[208]. MDC has been found to be elevated in models of several lung diseases including pulmonary fibrosis, acute asthma and cigarette smoking. However in the smoking model, no increase in Th2 cells was identified, suggesting that MDC may act differently in inducing inflammation in the presence of cigarette smoke[209].

1.47 Macrophage inflammatory proteins alpha and beta (MIP-1α) and (MIP-1β)

Macrophage inflammatory proteins are chemokines released by macrophages; two major subtypes 'alpha' and 'beta' have been identified. They activate neutrophils and eosinophils and can stimulate the release of inflammatory cytokines including IL-1, IL-6 and TNF-α. MIP-1β is increased in chronic bronchitis patients [198] and levels have been shown to correlate with eosinophils in COPD patients sputum[210]. MIP-1α was increased in a mouse model of respiratory syncytial virus (RSV) infection and airway pollution with carbon black[211] and in a study of bronchial epithelium from subjects with mild COPD MIP-1α was also found to be increased[212].

1.48 Thymus and activation regulated chemokine (TARC)

Thymus and Activation Regulated Chemokine (TARC) belongs to the CC chemokine family, it is expressed primarily in the thymus but also in stimulated peripheral blood mononuclear cells. It is a key component in the recruitment of Th2 cells during the allergic response[213] and a specific ligand for CC chemokine receptor 4 (CCR4). In COPD, elevated mRNA expression of TARC has been found in the bronchial mucosa and bronchoalveolar lavage when compared to non-smoking controls[193]. Interestingly elevated TARC levels were also identified in non-obstructed smokers when compared to non-smoking controls, and this persisted after cessation of smoking[193]. In a murine study of RSV infection, TARC was found to be expressed and dependent on active virus replication[214]. TARC levels were also shown to be increased by Poly(IC) a synthetic analogue of viral dsRNA[215], despite this the role of TARC in human RV infection is poorly understood, particularly in COPD.

1.49 Cellular inflammation

Pathologically the hallmark of COPD exacerbations is an increase in airway inflammation above that found in stable disease. The mechanisms underlying this acute-on-chronic inflammation are likely to be central to disease understanding; however defining precisely the inflammatory changes occurring at exacerbation has proved difficult. There are conflicting studies in the literature, as discussed below, some identifying a predominance of neutrophils, some eosinophils and some macrophages. These inconsistencies highlight the difficulties of performing research studies during exacerbations of airway disease and are likely to reflect the range and complexities of study designs, for example differences in disease definition, severity and aetiology; subject smoking status and prescribed medications (particularly corticosteroids) and the time of sampling during the exacerbation. This is particularly important as the nature of inflammation is likely to change from onset of the exacerbation to recovery and with different aetiological agents.

1.50 Neutrophils

Through the release of elastase and the destruction of connective tissue, neutrophils have been regarded as a central player in COPD inflammation. Indeed both the number and function of neutrophils are altered in COPD; a study using radiolabeled fibronectin demonstrated enhanced chemotaxis and extracellular proteolysis by neutrophils from subjects with COPD when compared to smoking controls[216]. However there have been relatively few studies performed during exacerbations, at least partly due to the difficulty of obtaining samples or performing studies during

acute exacerbations. Where studies have been performed, the majority have demonstrated neutrophilia in biopsies, BAL[145, 217] and induced sputum[1] although some have not demonstrated any change in neutrophil number[218, 219]. During exacerbations the neutrophil chemoattractants ENA-78 and IL-8 have both shown to be increased[217, 220] and this is likely to be at least one mechanism of enhanced neutrophil number. The local tissue consequences of neutrophilic inflammation are increased mucus secretion through mucus gland hyperplasia and increased airway oedema and airway narrowing from vascular protein leakage. These changes have significant clinical sequelae as illustrated by the finding that increasing neutrophilic inflammation is associated with worsening airway obstruction[221].

1.51 Macrophages

The key role of macrophages in the pathogenesis of COPD has become evident in recent years. Numbers of macrophages are increased in active smokers and COPD patients[222] although absolute numbers appear unchanged during exacerbations; where neutrophils and eosinophils are known to increase[140]. Derived from blood, around 15% of circulating monocytes are destined to become either airway, interstitial or alveolar macrophages. Dendritic cells develop from the same pluripotent stem cell as macrophages and have features in common, but tend to possess enhanced antigen presenting cell activity and reduced phagocytic activity when compared to macrophages.

Macrophages have a broad range of functions, but central to this is their activity to kill bacteria and remove foreign and damaged tissue by phagocytosis. They also take part in the mucociliary transport system where they play a key part in pathogen clearance. The pathogenic effects of macrophages in COPD are likely to be mediated through both a loss of normal physiological function and an excessive release of toxic cytokines, resulting in tissue damage. An example of aberrant behaviour by alveolar macrophages in COPD is demonstrated by impaired phagocytic activity. *In vitro* studies of radio or fluorescently labelled bacteria have demonstrated reduced phagocytosis when compared to controls[223]. This finding may partly explain the increased incidence of bacterial infections and lower airway colonisation (particularly by *Haemophilus influenzae*) found in COPD. Of interest a further *in vitro* study demonstrated that treatment with the macrolide antibiotic azithromycin improved phagocytosis of apoptotic bronchial epithelial cells and neutrophils – suggesting a possible therapeutic role for this class of drug[224, 225].

Excessive elastase production in COPD was historically attributed to the neutrophil. But a shift of attention to the role of the macrophage has demonstrated excessive enzymatic release that can potentially lead to the development of emphysema[226]. The mediators which are implicated in this process are the family of MMP enzymes. First described by observing the degradation of elastin by mouse macrophages, MMPs are now recognised as central to the pathogenicity of COPD and observed to increase in exacerbations[136, 140].

As highly active immune cells, macrophages produce a wide range of inflammatory cytokines including IL-6, IL-8, TNF-α, IL-1β, MCP-1 and growth related oncogene alpha (GRO-α) all of which are increased in the sputum of COPD patients both in the stable and exacerbated state[174, 227]. These in turn enhance neutrophilic inflammation but emphasise the importance of macrophages in driving pathogenesis in COPD. Finally macrophages are also a major endogenous source of oxidants which are discussed later in detail but represent a mechanism of tissue damage in COPD.

1.52 Eosinophils

Although there is controversy surrounding the presence of eosinophilic inflammation in COPD, it has been detected during exacerbations[228] and in the stable state[229]. The importance of eosinophils in COPD is starting to be elucidated, with reductions of eosinophilic airway inflammation being associated with important clinical outcome measures including improvements in lung function, health status and exercise capacity[230-232]. In a study using targeted therapy to reduce eosinophil numbers, severe exacerbations were also reduced[233]. Interestingly, exacerbations where a virus was identified were associated with a sputum eosinophilia and independent of bacterial co-infection, this remained the best predictor of aetiology[1]. In a RV challenge study in healthy volunteers bronchial biopsies demonstrated tissue eosinophilia following infection[234], although these results were not statistically significant.

Driving eosinophil recruitment, cellular adhesiveness and survival are Th2 helper cell cytokines, particularly IL-5 and eotaxin[235]. Eosinophils have been shown to release a number of toxic granular products, specifically eosinophilic cationic protein (ECP), eosinophil peroxidase (EPO), eosinophil derived neurotoxin (EDN) and major basic protein (MBP). *In vitro* and *in vivo* experiments have demonstrated that the

activity of these degranulation products is related to tissue injury and although the majority of work has been performed in asthma it seems that overlapping pathology may be present in COPD[236]. Although a causal link between eosinophils and the pathogenesis of COPD has not been established, elevated ECP has been found in BAL[237], serum[238] and sputum[239, 240] of COPD subjects during both the stable and exacerbated state. Finally indirect evidence of tissue damage from eosinophilia in COPD was demonstrated in a study where ECP levels correlated with CT derived emphysema scores and an enhanced response to bronchodilation[241].

1.53 T lymphocytes

The role of T cells in exacerbations of COPD is not well understood. In the stable state there are increased numbers of cytotoxic CD8+ cells and a recent study in exacerbations found a fall in the CD4+/CD8+ cell ratio compared to the stable state[242], suggesting that CD8+ cells increase in number during an exacerbation. However another study failed to show an increase in CD4+ or CD8+ cells in bronchial biopsies from COPD subjects compared to smokers (although this may reflect the low number of subjects in the control group)[243]. It has also been proposed that CD8+ T cells and the chemokine 'regulated upon activation, normal T cell expressed and secreted' (RANTES) may interact to promote apoptosis of virus infected cells[39]; this acts a possible explanation for the severity of virus induced exacerbations and the more rapid decline in lung function in those COPD patients who suffer frequent exacerbations[56], as increased apoptosis could lead to more tissue destruction and worsening emphysema.

1.54 Infection and AECOPD

Infection is the most common cause of COPD exacerbations; implicated as the aetiological agent in over 80% of acute exacerbations[244]. Bacteria have traditionally been regarded as the leading cause, but with new molecular techniques enabling improved detection of respiratory viruses, interest in their role has flourished. In a study of hospitalised patients with an acute exacerbation, an infective agent identified in 78% of cases; 30% bacterial, 23% virus and 25% co-infection with both virus and bacteria[1].

1.55 Viruses

It has long been known that infection with a respiratory virus worsens symptoms in patients with airway disease; a year-long observational study demonstrated that respiratory virus infection resulted in an excess of lower respiratory tract (LRT)

symptoms in subjects with chronic bronchitis compared to healthy controls[245]. The mechanisms by which virus infection induces an exacerbation remain unclear, however in exacerbations associated with the presence of common cold symptoms the severity and recovery time of exacerbations was increased[246] and had elevated inflammatory markers[218] such as IL-6. Exacerbations in which viruses are detected are more common in the winter months[247] when levels of respiratory viruses are greater in the community and temperatures lower[248] and these are more likely to result in hospitalisation. Respiratory viruses which are frequently associated with exacerbations include RV, influenza, coronaviruses and RSV.

Investigations into the potential role of respiratory viruses in acute exacerbations were originally performed using serology and culture; in these studies virus detection rates ranged from 26-39%[249, 250]. However when more sensitive molecular techniques have been employed detection rates have increased, particularly for RVs which are relatively resistant to culture methods[251]. The first study to employ the polymerase chain reaction (PCR) in COPD detected respiratory viruses in approximately 40% of exacerbations[252], the majority of which were RVs, the finding was repeated when again RVs were associated with the majority of acute exacerbations requiring hospital admission[253], where overall in 58% of exacerbations a RV was detected.

1.56 Bacteria

Bacterial infection is frequently implicated in the cause of AECOPD where the most commonly detected species are *Haemophilus influenzae*, *Moraxella catarrhalis*, *Streptococcus pneumoniae* and *Staphylococcus aureus*. However controversy surrounding the aetiological role of bacterial infection in acute exacerbations has existed for several decades[254, 255]. Although bacteria are detected in sputum from over 50% of exacerbations[256] there is a higher than expected rate in the stable state, 25-30% in several studies[1, 257]. Treating exacerbations with antibiotics is beneficial when compared to placebo, but the effect is less than might be expected if acute bacterial infection was causing the majority of exacerbations. The balance between host defence and pathogen virulence is central to the process. It has been postulated that either an increase in bacteria load or the acquisition of a new bacterial strain leads to an exacerbation, and this later hypothesis is supported by work from Sethi where acquisition of a new strain of *H. influenzae* was associated with worsening of symptoms[258]. Historical studies using only simple bacterial culture would not have detected this change in strain. Finally patient factors

influence species and detection rates, for example disease severity is associated with increased bacterial infection rates at exacerbation, particularly when the FEV_1 falls below 50% predicted. In these patients the bacterial species identified are also different with higher levels of *H. influenzae* and *Pseudomonas aeruginosa* infection[259] particularly in critically ill patients*[260]*.

1.57 Co-infection

The concept that bacterial infection can occur secondary to an antecedent virus infection has been present for many years[261], however no studies have demonstrated a causal relationship. The lag period between virus and subsequent bacterial infection may result in difficulty in virus detection and few studies employ longitudinal methodology. There is evidence of reduced bacterial phagocytosis following RV infection[262] and this would provide one mechanism for secondary bacterial infection.

Whether bacterial infection occurs as a consequence or coincidence a recent study demonstrated that co-infection was a serious event. In this study of hospitalised patients with an exacerbation, 25% were found to be co-infected with both virus and bacteria. This group had more marked lung function impairment and longer hospitalisation when compared to those with no infection[1]. This suggests that where virus and bacteria are present simultaneously they act synergistically to worsen airway inflammation and increase exacerbation severity, this field merits urgent further research in view if these interesting findings and the high number of patients affected.

1.58 Rhinoviruses

Rhinovirus is a member of the Picornovirus family and is a positive strand RNA virus of which over 100 serotypes exist. It is the most frequent cause of the common cold, responsible for up to 80% in the autumn[263] and despite the seasonal peak from September to April it is detected throughout the year[264]. Transmitted through aerosols, fomites and hand to hand contact, they are unable to survive in low pH environments and have an optimal growth temperature of 33^oC.

Historically detection of RV has been problematic due to the high number of serotypes and difficulty in performing culture, but PCR technology has allowed more rapid and sensitive detection to be possible. It was thought that replication could only take place in the upper airway (rhino: nose, virus: poison) although now replication in

the lower airway has been shown to occur[265]. Divided into major and minor groups, RV16 is a member of the former whose cellular receptor is ICAM-1. Infection induces cellular apoptosis but they cause less direct cell death and inflammation than many other respiratory viruses such as influenza. To date no vaccine has been developed due to the large number of serotypes, but this therapeutic strategy remains of research interest. In addition a group of RV has recently been identified based on sequence analysis data[266]. The RV-C group has been identified in up to 50% of RV infections in young children and maybe associated with a more severe clinical illness.

1.59 Common cold

In normal healthy subjects a common cold induces mild upper respiratory tract symptoms and an increase in airway responsiveness[267]. However in the elderly and young children infection can induce more severe lower respiratory tract symptoms[268, 269], although this is rare. The major harm from RV infection occurs in the setting of airways disease and was originally demonstrated in asthma, both in adults[270] and children[271]. The evidence for COPD has been discussed elsewhere. In smoking subjects there is evidence of increased severity and frequency of respiratory infection in smokers(Kark and Lebiush 1981; Cohen, Tyrrell et al. 1993; Arcavi and Benowitz 2004). The mechanisms for this are not clear, but a study in mice identified impairments in innate immunity following cigarette smoke exposure. At least partly explained by suppression of apoptosis following exposure to virus pathogen associated molecular patterns (PAMP)[272].

1.60 Mechanism of RV induced inflammation

The precise mechanisms of RV induced inflammation are not known. Replication of RV in epithelial cells has been identified in both *in vitro* and *in vivo* studies[273, 274] and then subsequently in macrophages[275]. Many mediators have been shown to be increased in RV infection, including IL-1β, IL-6, IL-8, IL-10, IL-11, TNF-α, RANTES, MPO and ECP[276-278]. RV infection has less cytopathic effects than many other viruses and so it seems likely that much of the inflammatory response occurs as a result of the innate inflammatory response, mediated by increases in the mediators described above. It is possible that in the face of increased inflammation present in COPD this leads to an additional influx of cells and excess symptoms.

1.61 RV induced oxidative stress

In vitro experiments have demonstrated induction of intracellular oxidant production within 20 minutes of RV infection; this rapid production of O_2^- release mirrors the time course of NFκB activation[279]. It was also shown that reduced glutathione inhibits RV induction of ICAM-1 and TNF-α in a dose dependent manor. Glutathione was only found to exert this effect in its reduced state but not in its oxidised state, suggesting that redox mechanisms are central to the effect.

The severity of RV infection and levels of IL-8 in nasal lavage have been found to correlate. In an attempt to investigate the mechanisms for this, the BEAS2B respiratory epithelial cell line was infected with RV[280]. The study demonstrated elevated levels of H_2O_2 suggesting a role of oxidative stress in the pathogenesis of RV infection. Addition of the anti-oxidant NAC inhibited RV induced NFκB activation and IL-8 release, demonstrating that reduction of oxidative mediators may reduce the immune mediated effects of RV infection[280].

A study of primary epithelial cells from COPD patients cultured *ex vivo* showed an increase in oxidative stress genes following RV infection[134]. In addition mRNA for DUOX 1 & 2, which are H_2O_2 generating enzymes, was found to be increased. This suggests a possible mechanism for increased oxidative stress in COPD. However in another study, levels of DUOX 1 & 2 from bronchial brushings were reduced in COPD patients[281]. The reason for this contradiction is not clear but may relate to differences in severity of disease or smoking status between the study subjects.

Although oxidative stress may occur as the result of RV infection and be responsible for an excess inflammatory response, there is also evidence that nitrosative stress plays a part in the normal host defence response. NO has antiviral properties and is induced *in vitro* and *in vivo* by RV infection[282], suggesting a pivotal role in health as part of the normal innate immune response. Supporting this an experimental RV infection of healthy volunteers the expression of iNOS was increased and this correlated with exhaled NO, virus clearance and reduced symptom scores[283]. The mechanism of this effect is not fully known, but NO is known to inhibit RV induced production of IP-10. This action has been found to be through blocking virus activation of NFκB and interferon regulatory factors (IRF). It has been suggested that NO donors may provide a therapeutic approach to virus induced exacerbations in COPD[284].

1.62 Experimental RV infection

There is a long history of human experimental rhinovirus infection. It has been performed in healthy volunteers to study transmission, infectivity, serological responses and immune mechanisms[285, 286]. Additionally human challenge has been used to evaluate potential antiviral therapies[287, 288]. Numerous studies have also been performed in asthmatic patients to gain insights into mechanisms of exacerbation. These have demonstrated correlation between airway inflammation and virus load, NO induction and Th2 cytokine excess[289, 290]. However there has only been one published study of experimental RV infection in COPD[164], and the insights gained from this study and the absence of adverse events suggest it seems likely that this methodology will prove useful in the future.

The introduction to this thesis has illustrated the significant magnitude of disease burden from COPD across the globe, with particular emphasis on the central role of acute exacerbations in the morbidity and mortality associated with the disease. An enormous amount of human suffering occurs as a result, especially from infection with RVs, which have been shown to be a major aetiological factor in exacerbations of airways disease. In this study the unique experimental RV induced COPD exacerbation model will be used to increase knowledge and understanding of the mechanisms involved in virus induced exacerbations of COPD. By focusing on the relationship between oxidative and nitrative stress and epigenetic factors this body of work investigates an area which has not been studied previously. These studies and the use of this model in the future may provide inroads towards developing treatments that could improve outcomes in this burdensome disease.

1.63 Hypotheses

The central hypotheses to be tested are that RV infection leads to (i) increases in markers of oxidative and nitrative stress in induced sputum supernatant, (ii) reduced activity of HDAC2 isoenzymes in macrophages isolated from sputum and BAL and (iii) increases in pro inflammatory cytokines in sputum and BAL supernatant and that these effects are exaggerated in COPD subjects when compared to both non-smoking and smoking non-obstructed controls. In addition it is hypothesised that non-smoking controls experience reduced lower respiratory tract symptoms and lower total virus load compared to COPD subjects and smokers when experimentally infected with RV. The proposed mechanism is that RV induced oxidative stress leads to ubiquitination and subsequent proteasomal degradation of HDAC2.

Therefore to investigate this hypothesis, groups of COPD and smoking and non-smoking control subjects were recruited and experimentally infected with RV16. Samples were collected to enable confirmation of virus infection and spirometry and symptom scores were recorded to enable identification of features representative of an exacerbation in COPD subjects.

Subsequently pro-inflammatory cytokines and chemokines were measured to both confirm and explore the degree of excess inflammation in COPD subjects compared to controls. Finally the HDAC2 activity was measured in sputum and BAL macrophages and oxidative and nitrative stress measured in samples collected from study subjects.

Chapter 2: Materials and methods

2.1 Outline of clinical experimental design and procedures

Experimental challenge of subjects with RV16 was performed in a similar manner to the pilot and validation study of COPD exacerbations performed at Imperial College, London[164, 291]. Briefly baseline samples of exhaled breath condensate (EBC), nasal lavage (NL), induced sputum (IS), blood and fibre optic bronchoscopy (FOB) were obtained prior to infection and repeated on subsequent visits. Subjects attended for 8 visits 3, 5, 7, 9, 12, 15, 21 and 42 days post infection. Sampling and the study timetable are illustrated in Figure 2.1. Lung function and symptom data were also recorded throughout the study. In addition to COPD subjects, two non-obstructed control groups of smoking and non-smoking subjects were recruited. The aim was to recruit 12 subjects per group (36 in total).

2.2 Inclusion and exclusion criteria for study subjects

During the study the safety and well being of all volunteers was the main priority, the criteria for participating have been maintained from an initial pilot study performed at St Mary's hospital[291]. In this study it was first shown that experimental RV infection of COPD subjects was safe and induced features of an acute exacerbation. The study presented here was the first to include a non-smoking control group, and the inclusion criteria for participating was identical to smoking controls except for the smoking pack year history which was changed to 0.

Inclusion criteria

2.3 COPD

Subjects in the COPD group were recruited according to the following criteria:

- Age 40-75 years
- Medical history or clinical diagnosis of COPD (GOLD stage II)
- No history of asthma or allergic rhinitis
- No atopy on skin testing
- Cumulative smoking history of over 20 pack years
- Post-bronchodilator FEV_1 $\leq$80% and $\geq$50% predicted for age and height
- Post-bronchodilator FEV_1/FVC ratio less than 70%
- β-agonist reversibility of less than 12% predicted FEV_1 and 200ml
- Absence of current or previous history of significant respiratory disease (except COPD)

- Absence of significant systemic disease

2.4 Non-smoking controls

- Age 40-75 years
- No history or clinical diagnosis of COPD
- No history of asthma or allergic rhinitis
- Not atopy on skin testing
- No smoking history
- Post-bronchodilator FEV_1 >80% predicted for age and height
- Post-bronchodilator FEV_1/FVC ratio >70%
- Absence of current or previous history of significant respiratory disease
- Absence of significant systemic disease

2.5 Smoking controls

- As non-smoking controls, with the exception of smoking history:
    - Cumulative smoking history of over 20 pack years

Exclusion criteria

2.6 COPD

- Any clinically relevant or significant systemic disease
- Pregnant or nursing women
- COPD exacerbation within the previous 8 weeks
- Treatment with oral or inhaled steroids now or in the previous 3 months, current use of long-acting β-agonists, nasal spray, anti-histamine, leukotrienes or tiotropium
- Shortness of breath score at screening over 1 or total lower respiratory tract score over 7
- Antibodies to rhinovirus 16 in a titre >1:2

Although not specifically included as inclusion or exclusion criteria, practical considerations were considered prior to subject's enrolment in the study; for example distance of travel availability between work hours. Additionally administration of the seasonal influenza vaccine in the previous year was recorded for all subjects.

The inclusion and exclusion criteria for study subjects are included below and summarised in table 2.1.

Inclusion (✓) & exclusion (X) criteria	COPD	Controls	
		Smoking	Non-Smoking
Age 40-75 years	✓	✓	✓
Clinical diagnosis of COPD, GOLD stage II	✓	X	X
History of asthma or allergic rhinitis	X	X	X
Atopic on skin testing	X	X	X
Cumulative smoking history (pack years)	>20	>20	0
Post-bronchodilator FEV_1 (% predicted)	≤80 & ≥50	>80	>80
Post-bronchodilator FEV_1/FVC ratio (%)	<70	>70	>70
β-agonist reversibility (% predicted FEV_1 & ml)	<12% & 200		
Absence of history of significant respiratory disease	(excluding COPD)	(including COPD)	(including COPD)
Significant systemic disease	X	X	X
Any clinically relevant abnormality on screening medical	X	X	X
Pregnant or nursing women	X	X	X
AECOPD or common cold in last 8 weeks	X	X	X
Treatment with oral/inhaled steroids in last 3month, current use of LABA, nasal spray, anti-histamine/-leukotrienes, tiotropium	X	X	X
SOB score at screening >1 or total LRT score >7	X	X	X
Serum antibodies to rhinovirus 16 in a titre >1:2	X	X	X

Table 2.1 Inclusion and exclusion criteria for subjects recruited into the study, (Long acting beta agonist (LABA), Shortness of breath (SOB) and Lower respiratory tract (LRT)

Individual subjects were required to attend for 12 visits over the duration of the study. Bronchoscopy could only be performed on Monday, Wednesday or Friday in conjunction with established NHS sessions at St Mary's Hospital. Figure 2.1 outlines the overall study protocol, with individual study visits shown in boxes across the time line of the project. Ethical approval for the study was obtained from St Mary's Local Research Ethics Committee; Patient information sheet and REC letter in appendix, (study number 07/H0712/138).

Summary of study visits and procedures performed

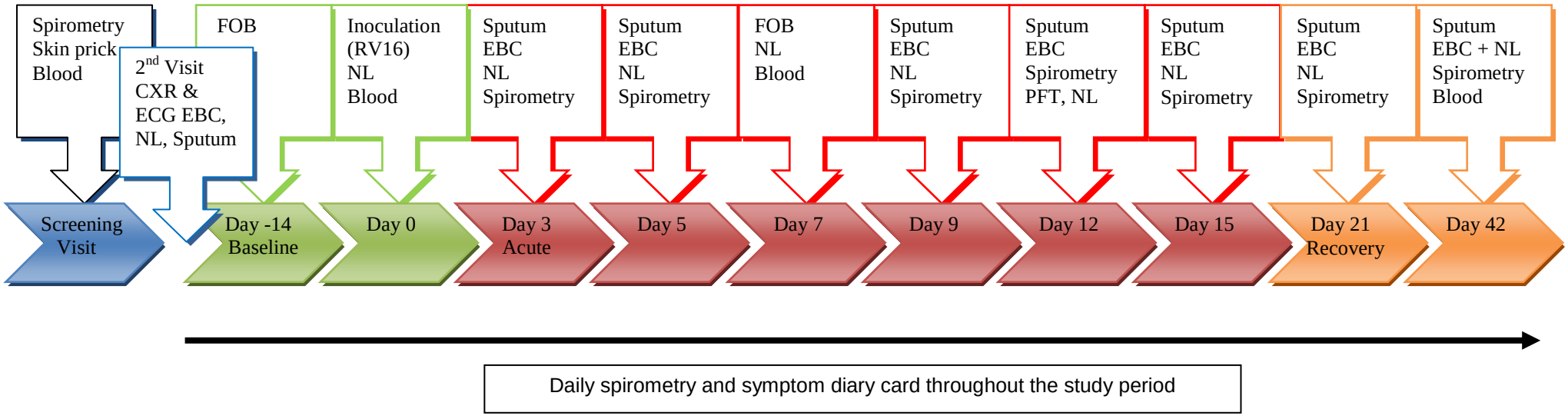


Figure 2.1 Outline of study visits and timetable. Screening (blue), baseline (green), acute (red) and recovery (orange) phases shown

FOB: Fibre Optic Bronchoscopy
EBC: Exhaled Breath Condensate
NL: Nasal Lavage
PFT: Full Pulmonary Function Tests
CXR: Chest X-ray
ECG: Electrocardiogram

2.7 Subject screening

Subjects were recruited through advertisements in local newspapers and on the internet. Attempts to recruit through hospital outpatient clinics, general practitioners surgeries or support groups were largely unsuccessful, mainly due to the disease severity of COPD patients in these settings. Initial contact with volunteers was made by telephone and a *pro forma* screening questionnaire (see example in appendix) completed, this enabled exclusion of unsuitable subjects and the formation a database for volunteers interested in future research projects. In total approximately 1550 subjects were screened by telephone. Of those 176 attended hospital clinic in person for full screening. Approximately 45% of subjects were subsequently excluded after being found to be RV16 seropositive.

2.8 Clinic screening visit

Individuals attended St Mary's Hospital, London for a standardised clinic screening visit. A Patient Information Sheet was provided and informed consent obtained from all subjects prior to enrolment in the study. A full medical history and examination were performed followed sequentially by clinic spirometry, skin prick tests and venepuncture. Results obtained that were not compatible with the inclusion/exclusion criteria ended the consultation.

2.9 Pulmonary function testing

Spirometry was performed on each clinic visit and during screening in accordance with the BTS/ARTP guidelines[292]. Measured using a Micro Medical MicroLab spirometer (Micro Medical, Kent, UK), subjects inhaled maximally then performed a forced exhalation to forced residual capacity (FRC), the best of 3 manoeuvres was recorded. In addition full pulmonary function testing in St Mary's Hospital lung function laboratory was performed at baseline and on day 12 or 15 post-inoculation by Association for Respiratory Technology and Physiology (ARTP) accredited NHS staff according to established protocols. At the baseline test FEV_1 reversibility was assessed by administration of 200μg of salbutamol using a metered dose inhaler (MDI) and large volume spacer with spirometry repeated after 15 minutes. Subjects also recorded spirometry values daily at home using a portable Micro DL spirometer (Micro Medical, Kent, UK).

2.10 Skin prick testing

Skin prick testing was used to define the atopic status of volunteers. Those with one or more positive reactions were excluded from the study. A drop of aeroallergen

(specifically grass pollen, house dust mites, cat hair, dog hair, Aspergillus fumigatus, Alternaria alternate, Cladosporium herbarum, mugwort, silver birch and 3 trees) was placed on the labelled inner forearm and lancets used to make a small scratch. Histamine and 0.9% saline were included as positive and negative controls respectively. Positive reactions were defined as a weal 3mm greater than the negative control. No subjects were taking antihistamine medications or had used topical medication on the arm.

2.11 Venepuncture

Screening and baseline blood samples were collected and then on days 7 and 42 post infection. On day 7 full blood count (FBC) and C-reactive protein (CRP) analysis was performed in accredited NHS laboratories. On day 42 serum was obtained to assess status of RV16 serology. The protocol was subsequently amended in the final stages so that blood was collected additionally on days 9, 12, 15 to enable investigation of serum markers following RV infection. Peripheral blood mononuclear cells (PBMC) were collected and processed as described below on days 0, 7 and 42. Serum separation was performed by centrifuging a 10ml plain Vacutainer® tube of blood at 3500g (4500rpm) for 10 minutes and aliquoted in 6 x 200µL aliquots for storage at –80^{o}C.

2.12 RV16 serology testing

RV16 serology testing was performed on heat inactivated (56^{o}C in water bath for 30minutes) serum from the screening and day 42 visit. RV16 neutralising antibody levels were assessed using a HeLa cell monolayer in a 96 well plate. 50µL of serum was serially diluted from 1:2 to 1:64 followed by the addition of 50µL of RV16 stock virus containing 100 tissue infective dose 50% ($TCID_{50}$) to each well. The plate was shaken for 1 hour at room temperature. Finally 100µL of freshly stripped HeLa cells at a concentration of 2 x $10^{5}ml^{-1}$ were added, before incubating the plate at 37^{o}C for three days. Virus positive (RV16 and cells, no serum) and negative (media and cells, no serum) controls were included in each plate and neat serum (no RV16) was also included. A typical plate layout and picture of completed assay is shown in figure 2.2. Antibody titre was defined by the greatest serum dilution where cytopathic effect (CPE) was identified. Seroconversion occurred when antibodies were present with at least a 4-fold increase in titre from baseline. During screening, subjects were excluded from the study if an antibody titre ≤1:2 was found.

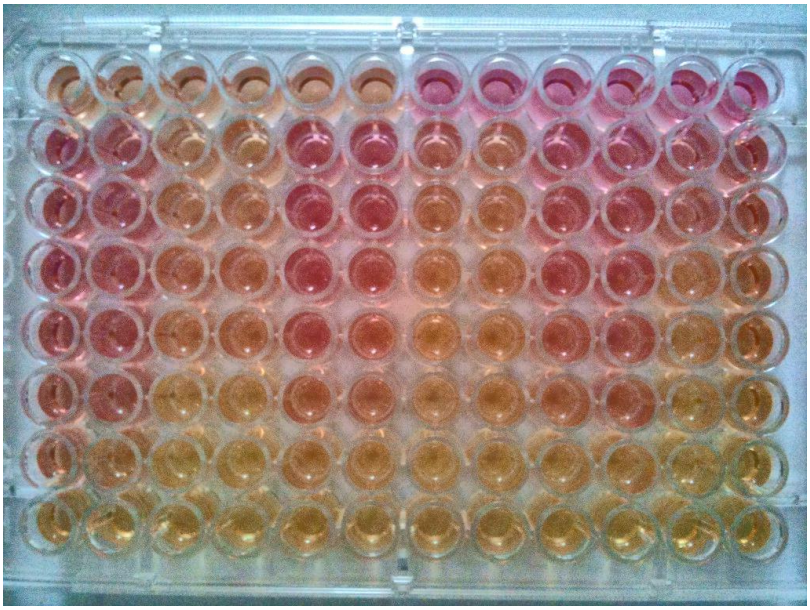


Figure 2.2a Illustration of typical serology plate after completed assay, showing red wells from cytopathic effect (CPE) of virus and yellow wells from cellular monolayer

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	M	M	M	M	M	M	RV	RV	RV	RV	RV	RV
B	1:64		1:64		1:64		1:64		1:64		1:64	
C	1:32		1:32		1:32		1:32		1:32		1:32	
D	1:16		1:16		1:16		1:16		1:16		1:16	
E	1:8		1:8		1:8		1:8		1:8		1:8	
F	1:4		1:4		1:4		1:4		1:4		1:4	
G	1:2		1:2		1:2		1:2		1:2		1:2	
H	S	S	S	S	S	S	S	S	S	S	S	S

Figure 2.2b Illustration of typical serology plate layout as shown in figure 2.2a, Columns 1-2, 3-4, 5-6, 7-8, 9-10, 11-12 represent individual subjects (S - neat serum, M - media alone, RV - virus alone, 1:2 to 1: 64 serial serum dilution)

2.13 Experimental infection with RV16

The RV16 inoculum has been used previously and details of safety testing and isolation from volunteers are published[267]. It is considered a representative strain of major group rhinoviruses, responsible for inducing common colds in the community. A 2μL volume of virus inoculum, approximately equivalent to 10 $TCID_{50}$, was thawed and diluted with 1.98ml of normal (0.9%) saline. Inoculation was performed in a specified clinical room by nasal inhalation using a DeVillbiss 286 atomizer. Subjects were administered the dose using two puffs per nostril. As an amendment to the protocol, in the last 16 subjects 250μL of 0.9% saline was used to irrigate the atomizer after RV administration in attempt to increase successful infection rates and wash residual RV from the atomizer.

2.14 Symptom scores

Subjects were asked to complete an upper and lower respiratory tract symptom score chart every day. These were commenced approximately 2 weeks prior to the baseline bronchoscopy through until study completion on day 42.

2.15 Upper respiratory tract symptoms

The scoring system for upper respiratory tract (URT) symptoms was based on the Jackson criteria, which has been validated in an experimental infection study and used in many subsequent experimental RV infection studies[293-295]. An example of the diary card used is shown in figure 2.3. Symptoms (sneezing, headache, malaise, chills, nasal discharge, nasal obstruction, sore throat, cough and fever) were graded daily on a scale of 0 to 3, (where the criteria ranged from: 0 = no symptoms to 3= severe symptoms). A clinical cold was considered to be present if 2 of the following 3 criteria were present:

- A cumulative symptom score of at least 14 over a 6-day period
- The subjective impression of a cold
- Rhinorrhoea present on at least 3 days

Please score symptoms in the boxes as shown.

0 = no symptoms **1** = mild **2** = moderate **3** = severe

Date						
Day number						
Sneezing						
Runny nose						
Blocked or stuffy nose						
Sore throat or hoarse voice						
Headache or face pain						
Generally unwell						
Chill, fever or shivery						
Cough						
Total score						
Medications (name and dose)						
1.						
2.						
3.						
4.						
Spirometry						
Peak flow (PEFR)						
FEV_1						
FVC						

Figure 2.3 Example of URT diary card used for daily symptom recording

2.16 Lower respiratory tract symptom scores

Most definitions of an AECOPD used in the literature[59, 296, 297], including the GOLD guidelines[12], focus on a change in patients baseline symptoms beyond the normal day-to-day variation to make a diagnosis of an exacerbation. Study subjects were asked to complete a daily LRT symptom score card which was based on 5 typical symptoms of an AECOPD (breathlessness, wheeze, cough, sputum production and sputum quality). Each variable was evaluated on a rating-scale with higher scores indicating more severe symptoms, breathlessness and wheeze scored 0 to 4 and the other parameters scored 0 to 3. The possible individual total daily score ranged from 0 to 17. An example of the LRT card and scoring scheme is shown in figure 2.4. An exacerbation was defined by an increase in total LRT score

over baseline level of at least 2 points on 2 consecutive days. At the time the original RV16 COPD exacerbation model was established there was no validated measures of day-to-day variation in symptoms in the literature, but these have now been published[298, 299] and are broadly similar to the one used in this study.

	0	1	2	3	4
BREATHLESSNESS	Not breathless at rest or on exertion	Breathless on moderate exertion (eg Walking quickly)	Breathless on mild exertion (eg Walking on level ground)	Breathless on minimal exertion (eg Getting washed)	Breathless at rest
WHEEZE	None	Present on moderate exertion	Present on mild exertion	Present on minimal exertion	Present at rest
COUGH	None	Mild (eg morning only)	Moderate	Severe	
SPUTUM PRODUCTION	None	Small amount of sputum (<30ml)	Moderate amount of sputum (30 – 100ml)	Large amount of sputum (>100ml)	
SPUTUM QUALITY	None	Mucoid (clear)	Mucopurulent (yellow)	Purulent (green)	

Date						
Day number						
BREATHLESSNESS						
WHEEZE						
COUGH						
SPUTUM PRODUCTION						
SPUTUM QUALITY						
Total score						

Figure 2.4 Example of LRT diary card used for daily symptom recording

2.17 Correcting for baseline symptom scores

There are variations in the way that individual subjects score and perceive their symptoms despite the use of a standard rank scoring system for symptoms. In addition some subjects, particularly those with COPD, experienced increased symptoms prior to infection when compared to the control group subjects. Finally we predicted that the baseline or acute bronchoscopy may induce additional symptoms in subjects. Therefore baseline symptoms were corrected for in the following ways:

- The total daily score for both upper and lower respiratory tract symptoms from day 1 to 42 were corrected for individual baseline symptoms, achieved by subtracting the mean score of day -3 to 0 from post-infection results.

- The total URT score between days 7 to 12 was corrected for the effects of the acute bronchoscopy (on day 7) by subtracting the mean score from days -14 to -9.

- In addition the daily symptom scores were calculated in two week blocks as shown in figure 2.5. The pre-baseline and baseline phase were defined as the points 14 days prior, and then post, baseline bronchoscopy (on day -14). The two week phase from day 0 to day 14 was termed the acute phase, with the subsequent 14 day period the recovery phase. The final two week period of the study was defined the convalescent phase.

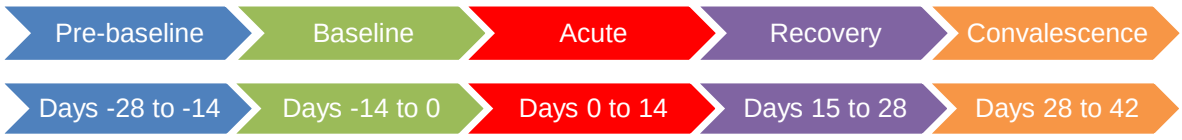


Figure 2.5 Division of study time course into two week blocks to examine upper and lower respiratory tract scores

2.18 Safety criteria

In order to identify any subjects in the study who were becoming excessively unwell the following safety criteria were established for defining a severe exacerbation[164]:

- An increase in shortness of breath score by 2 points or more on 2 consecutive days.
- An increase in wheeze score by 3 points or more on 2 consecutive days.

- A fall in FEV_1 or PEF of 40% or more from baseline.
- The subject developed a subjective feeling of severe exacerbation and wished to have treatment.
- The study doctor decided that the subject had a severe exacerbation and required treatment.

If a subject fulfilled any 2 of the first 3 criteria or either of the last 2 they would be defined as having a severe exacerbation, withdrawn from the study and medical treatment initiated on the clinical judgement of the investigators.

2.19 Nasal lavage

A nasal lavage specimen was collected on all study visits, primarily for virus detection. With the subjects head extended and soft palette closed, up to 10ml of sterile normal saline was instilled into each nostril using a Pasteur pipette. Subjects then gently blew their nose into a sterile universal container. Lavage fluid was stored on ice and then vortexed and aliquoted into 500μL volumes and stored at –80^{o}C.

2.20 Induced sputum

Induced sputum was collected by inhalation of hypertonic saline using an ultrasonic nebuliser (DeVilbiss U3000 Ultraneb) in line with European Respiratory Society (ERS) guidelines[300]. The majority of subjects tolerated 5% saline, but occasionally it was necessary to use 3% or 4% saline in intolerant subjects. The FEV_1 was recorded and subjects rinsed their mouth prior to commencing induction. After 10 minutes subjects ceased inhalation and were asked to report any adverse symptoms (for example chest tightness or breathlessness) and if either these or a fall in FEV_1 of >20% was recorded then the procedure was stopped. Where no adverse events occurred the procedure was continued until an adequate sputum sample was collected or the subject felt unable to continue.

Sputum samples were placed on ice and processed in the laboratory directly after collection, broadly using established protocols. Briefly the total sample was poured onto a Petri dish and weighed, sputum plugs were selected from saliva by macroscopic inspection using a Pasteur pipette and forceps. Two plugs were removed and stored unprocessed at –80^{o}C for subsequent virus and bacterial studies. A further aliquot was selected for microbiological analysis, weighted and diluted 1:1 by weight with 0.1% dithiothreitol (DTT, Sigma, UK) (1% stock in distilled water diluted to 0.1% in phosphate buffered saline [PBS]) to facilitate

homogenisation. This sample was mixed well and transferred to St Mary's Hospital Microbiology Laboratory for semi-quantitative bacteriology assessment.

The remaining sample was diluted with a volume of 4 times 0.1% DTT by weight, before mixing thoroughly and filtering via a 70µm cell strainer to remove mucus and debris. The same volume of PBS was added before centrifuging at 453g (1500rpm) for 10 minutes at 4^{o}C. The sputum supernatant was then aliquoted and stored at –80^{o}C. The cell pellet was washed and resuspended in PBS before centrifuging again (conditions as before). Finally the cells were resuspended in 1ml of simple macrophage media (Gibco, Invitrogen) and counted by haemocytometer with trypan blue staining to obtain total cell count. Sufficient cells were removed to make up to 6 cytospin slides, at a concentration of 20 000 cells per slide, using the Shandon cytospin at 400rpm for 5 minutes. Slides were air dried and 2 stained using the Shandon Diffquick kit. 4 slides were stored unstained at –20^{o}C. The remaining sputum cells were diluted with 3ml of macrophage media, distributed across wells of a modified polystyrene flat bottom adhesive culture plate (Falcon, Becton Dickinson, USA) and incubated for 2 hours at 37^{o}C and 5% CO_2 to facilitate macrophage separation by adhesion to the well base. After 2 hours adherent cells were washed from the well base using a cell scraper and centrifuged (18.8g for 7 minutes) to obtain a cell pellet which was stored immediately at –80^{o}C.

2.21 Bacteriology

Bacteriological analysis was performed according to the following protocol by NHS staff in an accredited microbiological laboratory. 10µl of the homogenate was inoculated onto a plate of chocolate agar, blood agar + optochin disc, CLED agar and Sabouraud agar and spread with a loop for discrete colony detection. 100µl of the homogenate was added to 5ml sterile saline and mixed for a 1:100 dilution. 50µl of the 1:100 dilution was added to 5ml sterile saline, the final dilution being 10^{-4}. 20µl of the 10^{-4} dilution was inoculated onto a blood and chocolate agar and spread over the entire surface with a loop. The blood and chocolate agars were incubated at 37^{o}C in CO_2, the CLED and Sabouraud agars at 37^{o}C without CO_2. Interpretation of the 10^{-4} dilution cultures was as follows (cfu – colony forming units):

0-2 colonies	10^6 cfu/ml
2-20 colonies	10^7 cfu/ml
20-200 colonies	10^8 cfu/ml
>200 colonies	10^9 cfu/ml

Only potentially pathogenic bacteria were counted and identification with subsequent sensitivity testing performed. Normal flora was reported as such.

2.22 Bronchoscopy

Bronchoscopy was performed in the endoscopy unit at St Mary's Hospital according to BTS guidelines[301]. Informed consent was obtained from fasted subjects who all received 2.5mg of nebulised salbutamol and 0.5mg of ipratropium bromide prior to commencing the procedure. Subjects were offered sedation with intravenous midazolam, titrated to a maximum dose of 10mg. Topical lignocaine was applied to the nasopharynx, vocal cords (2ml of 4%) and large airways (12ml of 2%). Blood pressure, heart rate and oxygen saturations were monitored throughout the procedure by a dedicated independent observer. Oxygen was administered via nasal cannula according to pulse oximeter readings. Samples were collected in the following order: (i) bronchial brushings, (ii) bronchoalveolar lavage (BAL) & (iii) bronchial biopsies. After the procedure subjects remained nil by mouth and were observed for two hours, then provided with transportation home and contact telephone number with 24 hour availability.

Bronchoscopy sample collection

2.23 Bronchial brushings

Six 10mm (BC-202D-5010 Olympus, Japan) disposable cytology brushes were used to collect epithelial cells from the left lower lobe sub-segmental bronchi. Five brushes were immediately washed and transferred in warmed media for further processing in the laboratory, the sixth brush was snap frozen whole unprocessed on dry ice before transferring to liquid nitrogen storage.

2.24 Bronchoalveolar lavage

BAL was performed by irrigation of the left upper lobe bronchus using up to 240ml of sterile 0.9% saline. Aspirated BAL fluid was collected into a plastic trap before transferring to polypropylene tubes for transport to the laboratory on ice.

2.25 Bronchial biopsies

Six bronchial biopsies were taken from the segmental and sub-segmental bronchi of the right middle and lower lobe using 1.8mm Radial Jaw (1523, Boston Scientific, USA) serrated needle free biopsy forceps. Four biopsies were placed immediately in 4% paraformaldehyde and two were snap frozen on dry ice.

2.26 Cell sample processing

BAL and PBMC cells were processed and subsequently stimulated in the laboratory immediately after collection.

2.27 BAL

The sample was vortexed briefly and the total volume collected recorded. 6ml was aliquoted (unprocessed & unfiltered) and frozen at –80^{o}C for subsequent virus studies. The remaining sample was filtered through a 100μm cell strainer and then centrifuged at 453g (1500rpm) for 10 minutes at 4^{o}C. The supernatant was stored in 1ml aliquots at –80^{o}C and after washing; the cells resuspended in 1ml of simple macrophage media (Invitrogen, UK), prior to counting total cell number using trypan blue and a haemocytometer. Cells for cytospin slides and macrophage separation (minimum 4 x 10^6 cells) were removed using the same method for sputum described above. Any remaining BAL cells were used for *ex vivo* stimulation. These were diluted to a concentration of 2 x 10^6 per ml and 1ml added to a 50ml Falcon tube for stimulation in the following conditions in this priority order (LPS: lipopolysaccharide, PHA: phytohaemagglutinin):

- Media
- RV16
- Filtered RV16
- LPS 10μg/ml
- PHA 10μg/ml
- LPS 0.1μg/ml
- PHA 1μg/ml

1ml of RV, filtered RV (FRV) or media was added to cells and incubated for 1 hour (virus infection time) followed by centrifuging and resuspending the cells in their final conditions; media, LPS or PHA (RPMI media with 10% FCS, 3% HEPES and 1% Penicillin/Streptomycin). After incubating for 48 hours supernatant was stored at –80^{o}C and cells frozen in 350μL RLT lysis buffer; where sufficient cells were obtained, an additional time point at 24 hours was added.

2.28 Peripheral blood mononuclear cells (PBMC)

Freshly collected blood in lithium heparin Vacutainer® tubes was diluted 1:1 with sterile PBS. This mixture was added carefully drop wise to 3.5ml of histopaque in 15ml Falcon tubes which were centrifuged at 1320g (2500rpm) for 30 minutes at room temperature with accelerator and brake switched off. The PBMC layer was

removed with a Pasteur pipette before washing cells in basic media. Cells were then counted, stimulated and harvested in the same was as BAL cells described above.

2.29 Cryopreservation of cells

Excess PBMC were stored in fetal calf serum (FCS) and 20% dimethyl sulfoxide (DMSO) added drop wise at a concentration of 5 x 10^6 per ml before freezing at –80^{o}C for 24hours before transfer to liquid nitrogen.

2.30 PCR

A freshly frozen (-80 ^{o}C) unprocessed sputum plug was diluted prior to PCR analysis. The plug was thawed and sample volume made up to 1ml using nuclease free water followed by vigorous pipetting and vortexing to break up sample. This was then made up to 5ml of nuclease free water and re-frozen at (-80 ^{o}C).

2.31 PCR for other respiratory viruses

To exclude incidental co-infection with respiratory pathogens other than RV, the presence of adenoviruses, coronaviruses, human metapneumovirus, influenza, parainfluenza, RSV, *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* was excluded using PCR with the random hexamer methods as previously published[252, 302].

2.32 Quantitative PCR for picornavirus

Quantitative PCR (qPCR) was performed to detect the amount of picornavirus in NL, sputum and unfiltered BAL stored as described at -80^{o}C. RNA was extracted from samples using the QIAamp virus RNA mini kit (Qiagen) with reverse transcription performed using the omniscript RT kit (Qiagen) and addition of random hexamer primers as per manufacturer's instructions. Biosystems ABI Prism 7700 sequence detection system was used with AmplitaqGold DNA polymerase, a picornavirus specific primer pair (forward oligo 5'- GTG AAG AGC CSC RTG TGC T-3', reverse oligo 5'-GCT SCA GGG TTA AGG TTA GCC-3') and a FAM/TAMRA labelled picornavirus probe (FAM-TGA GTC CTC CGG CCC CTG AAT G-TAMRA). A master mix was made up consisting of Qiagen quantitect probe mix, forward primer (50nM), reverse primer (300nM), probe (100nM) and RNase inhibitor. 23µL of PCR mastermix was added to 2µL cDNA in each well of a 96 well Taqman plate.

The thermal cycle conditions used were: 50^{o}C 2 minutes, 95^{o}C 10 minutes then 45 cycles x 95^{o}C 15s / 55^{o}C 20s / 72^{o}C 40s. Fluorescence data was collected for each

cycle and the cycle number (Ct) at which fluorescence rose above threshold was determined (threshold used for RV in data analysis was 0.09). Negative extraction (water), negative PCR (template only) and positive extraction (RV16 stock) was included. A standard curve was produced on each qPCR plate processed by including 2µL of RV plasmid serially diluted 10 fold from 10^7 to 10^0 copies. Results were expressed for each sample in terms of copies/ml for nasal lavage and BAL and copies/ml for sputum by reference to the standard curve and taking into account both dilution factors inherent in processing to RNA.

2.33 Sputum and BAL cell counts

Cytospin slides from induced sputum and BAL were prepared and stained as described above. These were blinded and the first hundred cells per microscope field view counted.

2.34 Meso scale discovery platform

The Meso Scale Discovery (MSD) platform (Maryland, USA) was used to measure chemokines and cytokines in sputum and BAL supernatant; performed following manufactures guidelines. The technique enables quantitative detection of between 1 and 9 mediators per well in a 96 well plate format using a Multi-spot® technique. This technology utilises a capture antibody attached to each spot within the well which enables measurement of multiple mediators simultaneously. Electrochemiluminescence of detection antibody is then recorded with an internal high sensitivity camera (Sector imager).

Briefly, the protocol requires addition of 25µL of blocking solution to each plate well before incubation (RT at ~600rpm) for 30 minutes. Following plate washing either 25µL of unknown sample or standard was added to the plate, which was then incubation for period of 2 hours. Repeat washing is followed by addition of 25µL of detection antibody and incubation for 1 hour. Finally 150µL of read buffer is added and the plate passed through the Sector imager for reading. The MSD assay has been used in sputum supernatant from COPD subjects and a range of mediators shown to be detectable[303].

2.35 ELISA

ELISA was performed in *ex vivo* BAL samples for interferon alpha and beta according to manufactures guidelines (Invitrogen, UK).

2.36 Potential of antioxidant assay (PAO)

The Potential of Antioxidant (PAO) assay (Japan Institute for the Control of Aging, Japan) was used to assess the total antioxidant capacity in EBC, sputum and BAL supernatant. In this process the cupric ion (Cu^{++}) is reduced by any types of antioxidant present in the substrate to the copper (1) ion (Cu^{+}). This in turn reacts with bathocuprine to induce a chromatic change, detectable on a plate reader at a wavelength of 480nm. The assay is used as an indirect measure of oxidative stress; as oxidative stress increases the detectable antioxidant is reduced.

2.37 Griess assay

The Griess assay was performed to measure nitrative stress in sputum, BAL supernatant and EBC. The assay is a modification of that first described by Peter Griess in 1879 and relies on a diazotization reaction. Griess reagents were made in-house by adding 10g of sulphanilamide (S9251, Sigma, UK) to 950ml distilled water with 50ml phosphoric acid (H_3PO_4) (Griess 1) and 5g of napthylethyl-endiamide dihydrochloride (N9125, Sigma, UK) added to 1L distilled water (Griess 2). All analysis was performed with the same batch of reagents stored at 4^{o}C in light protective containers. A nitrate standard curve was prepared using sodium nitrite (S2252, Sigma, UK) at concentrations from 1mM, 500µM, 250µM, 125µM, 62.5µM, 31.25µM, 15.63µM 7.82µM 3.91µM 1.96µM, 1µM to 0µM (PBS alone).

Briefly an equal volume of Griess reagents 1 & 2 were mixed and allowed to equilibrate, 100µL of standard or sample was added to duplicate wells of a 96 well plate before addition of 100µL complete (combined) Griess reagent. Top standard changes to a dark purple instantly, then shake plate for 5 minutes before recording colourimetric values at 550nm on a standard plate reader. Data was analysed by linear regression to the standard curve.

2.38 HDAC2 immunoprecipitation and activity

The HDAC2 isoenzyme was isolated from macrophage pellets obtained from sputum and BAL samples. Immunoprecipitation (IP) using the HDAC2 antibody (Insight Biotect, UK) and PureProteome® Protein A magnetic beads (Millipore, USA) was performed. HDAC activity was measured using an HDAC activity assay (Cayman Chemicals, USA). Briefly the protocol involved addition of radioimmunoprecipitation assay (RIPA) buffer to the macrophage pellet obtained from cellular adhesion described above. This was incubated for 30 minutes before combination with the HDAC2 antibody and overnight mixing on a sample roller. Magnetic beads were then

added for 2 hours of incubation prior to bead removal. Finally analysis of HDAC2 activity was performed in the protein sample and the supernatant used to calculate total protein concentration using the bicinchoninic acid assay (BCA). The BCA was performed according to published methods[304]. Results of HDAC2 activity were corrected for sample protein levels.

2.39 My role in the project

I undertook all of the clinical work involved in the study, including obtaining ethical approval, recruiting and screening subjects and collecting the samples. Later in the study I had the assistance of a research nurse which enabled more rapid recruitment. I performed the laboratory analysis for RV16 serology, to improve sensitivity all serology assays were performed in duplicate with a research technician, Dr A Telcian. I inoculated all the subjects and attended all the study visits. I performed all bronchoscopies myself with the assistance of nursing and medical staff. The formal lung function testing was carried out by respiratory technicians in the pulmonary function laboratory at St Mary's Hospital. I performed all the cell culture work including growing the virus used for *in vitro* studies; specifically this involved *ex vivo* stimulation and storage of BAL and PBMC samples. I performed all the processing of the BAL, PBMCs and sputum samples, the latter with some assistance of a research technician. The microbiological analysis of sputum for bacteria was carried out in the accredited microbiology laboratory at St Mary's Hospital. The qPCR and PCR for other respiratory viruses was performed by technicians in our laboratory. I performed all the MSD, PAO and Griess analysis in the study. HDAC2 IP and activity assays were performed by researchers collaborating on the project at the Royal Brompton Campus. I performed statistical analysis with assistance of Dr M Bafadhel, PhD student

2.40 Statistical analysis and software used

Statistical analysis was performed using SPSS version 18 (Chicago, IL) and PRISM version 5 (San Diego, CA). All parametric and non-parametric data is presented as mean (standard error of the mean) and median (interquartile range), log transformed data is presented as the geometric mean (95% confidence interval). One way analysis of variance (one way ANOVA) was used to compare parametric means across groups. Chi squared analysis was used to compare proportions. For comparison of clinical and mediator changes between baseline and study visits the paired T-test was used and the Mann Whitney U test for non-parametric comparisons. Correlations of clinical characteristics, mediator analysis and

experimental assays were assessed by Pearson's correlation (r) coefficient for parametric data and Spearman's coefficient (rs) for non-parametric data. A p value of < 0.05 was taken as the threshold of statistical significance.

Study visits were assumed to be independent of each other. This enabled analysis of clinical characteristics, inflammatory mediators and experimental assay results using linear regression. Validation of study visit independence was performed by demonstrating that: (i) virus load in the sputum and nasal lavage was significantly different on study visits for all subjects and for subject groups; (ii) the intra-class coefficient between visits was not significant indicative of no repeatability of virus load on study visits; and (iii) multiple regression analysis modelling that demonstrates that study visit was not a significantly independent predictor of virus load measured in the sputum or lavage of subjects.

Microsoft Office Excel spreadsheet and Access database were used to record all study results and to investigate related parameters. EndNote X2 (Thomson Reuters) was used for bibliographic database management.

Chapter 3: Clinical characteristics of study population

3.1 Introduction

In this chapter the demographic data and clinical characteristics of subjects recruited into the study are presented. The upper and lower respiratory tract symptom scores and lung function results are shown at baseline and following RV16 infection; with relationships between them described. The hypothesis studied in this chapter is that (i) Upper respiratory tract (URT) and lower respiratory tract (LRT) symptom scores and airway obstruction are increased in COPD subjects compared to controls following RV16 infection, (ii) Smoking subjects experience an excess of URT & LRT symptom scores and falls in lung function compared to non-smoking subjects following RV16 infection and (iii) Non-infected subjects do not experience changes in URT & LRT symptom scores or lung function following unsuccessful RV16 inoculation.

Results

3.2 Recruitment of subjects

Subjects were recruited through respiratory clinics and advertising in local newspapers between January 2008 and December 2010. Subject recruitment is illustrated in the consort diagram, figure 3.1.

3.3 Withdrawal of study subjects

The majority of subjects that were not eligible to participate in the study were screened out by telephone interview. The most common reason for this was disease severity in the COPD group, particularly as many subjects were using medications listed in the exclusion criteria (frequently inhaled corticosteroids). Amongst control subjects the majority withdrew from the study because of the duration of the project and the time commitment required or because they did not wish to have a flexible bronchoscopy. A substantial proportion of subjects (42%) who were otherwise suitable were excluded as they had detectable RV16 antibodies. There were 50 subjects who were serology negative and suitable for the study who withdrew. The main reason that subjects withdrew is that they were unable to commit the time required to participate or they rescinded consent after screening.

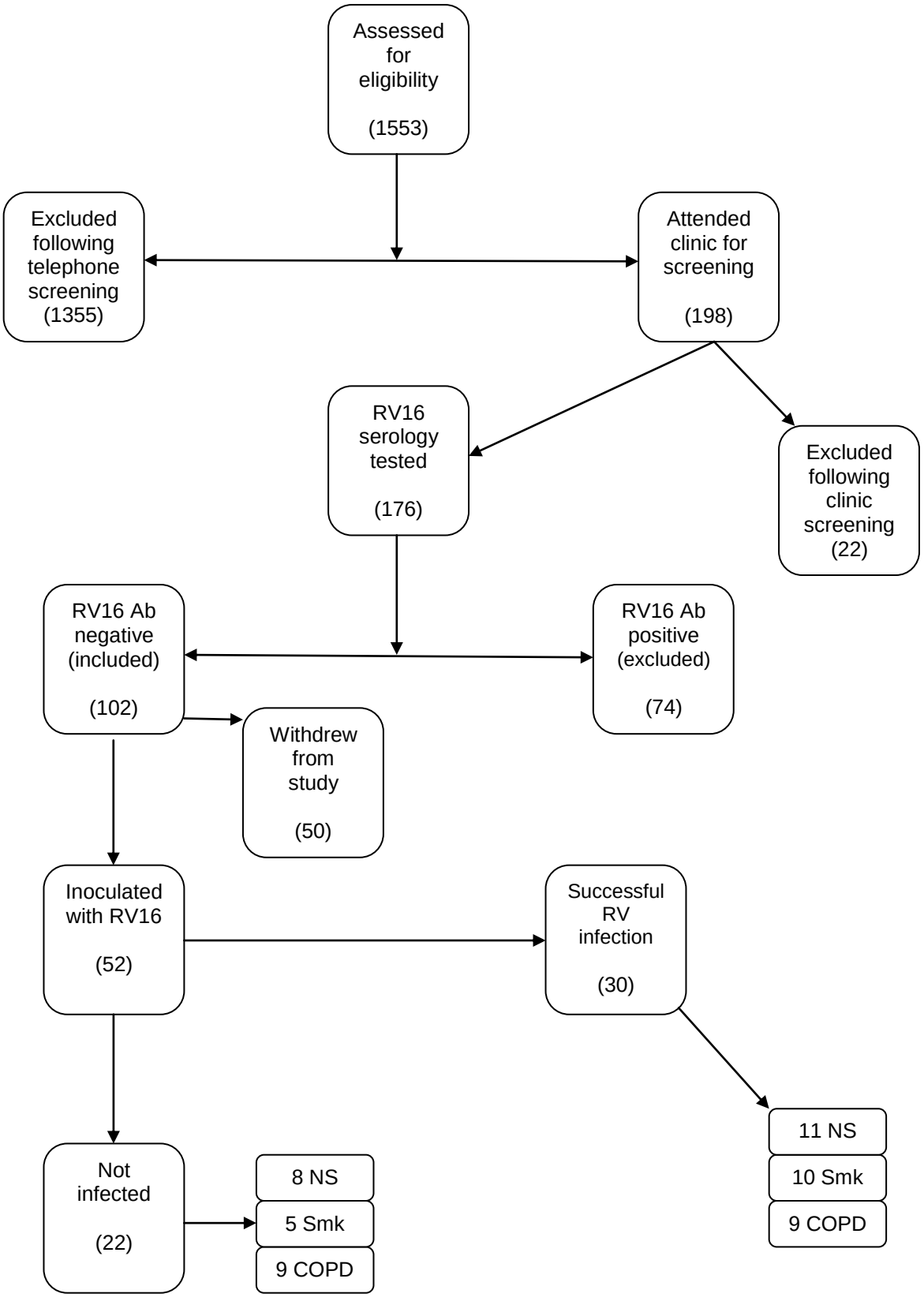


Figure 3.1 Consort diagram of experimental RV infection in COPD study, number shown = total subjects included in each group (NS non-smoking control, Smk smoking control)

3.4 Demographics of all study subjects

There were 52 subjects (19 non-smoking controls, 15 smoking controls and 18 COPD subjects) recruited for experimental RV16 inoculation. The clinical characteristics of this study group are shown in table 3.1.

	Non smokers N=19	Smokers N=15	COPD N=18	p value
Male, n (%)	9 (47)	8 (53)	13 (72)	0.286
Age*	59 (46 to 71)	54 (41 to 66)	59 (44 to 72)	0.076
Current smokers, n (%)	0 (0)	13 (87)	14 (78)	<0.001
Ex-smokers, n (%)	0 (0)	2 (13)	4 (22)	<0.001
Pack year history*	0 (0 to 0)	32 (21 to 51)	39 (25 to 57)	<0.001
Body mass index, kg/m^2	25.1 (1.0)	26.9 (1.0)	25.8 (1.1)	0.475
FEV_1 (L)	3.06 (0.22)	3.14 (0.17)	2.01 (0.10)	<0.001
FEV_1 % predicted	106 (4)	99 (3)	65 (2)	<0.001
FVC (L)	3.86 (0.26)	4.08 (0.23)	3.55 (0.21)	0.331
FVC % predicted	110 (4)	105 (4)	91 (2)	<0.001
FEV_1/FVC, %	79 (1)	77 (2)	58 (2)	<0.001
Peak expiratory flow (L/min)	464 (25)	455 (24)	372 (21)	0.048
SpO_2 at rest, %†	97 (3)	98 (2)	96 (3)	0.280

Table 3.1 Clinical baseline characteristics for all subjects entering the study. Data presented as mean (standard error of the mean) unless stated. * mean (range), † median (interquartile range), (p: one way ANOVA or Chi squared test)

3.5 Defining successful RV infection

Successful experimental infection of subjects was deemed to have occurred if any one of the following criteria was met:

- Detection of ≥ 150 RV RNA copies by real time PCR (RT-PCR) in either: (i) nasal lavage, (ii) sputum or (iii) BAL samples.
- An RV16 neutralising antibody titre of at least 1:4 from serum collected 6 weeks after inoculation.

The level of 150 copies was established based on the sensitivity and lower limit of detection in the qPCR assay. This was similar to the lower limit used in previous RV challenge studies in our department.

3.6 Successfully RV infected subjects

Of the 52 subjects challenged with RV16, 30 (11 non-smoking controls, 10 smoking controls and 9 COPD subjects) were judged to have been successfully infected. The clinical characteristics of these subjects are shown in table 3.2. There were no differences in the proportions of subjects in each study group who were challenged with RV16 and then found to have been successfully infected; 11 of 19 (58%) non-smokers, 10 of 15 (66%) smokers and 9 of 18 (50%) of COPD subjects, (chi square p=0.356). On the criteria of frequency of infection no study group demonstrated an increased susceptibility to experimental RV infection.

3.7 Demographics of successfully RV infected subjects

Successfully RV infected subjects were age and sex matched between the COPD and both control groups but post hoc analysis showed that non-smokers were older than non-smoking controls. Cigarette smoke exposure between non-obstructed smoking controls and COPD subjects was also matched for pack year history (mean pack year difference 7, p=0.155). In accordance with the screening criteria the FEV_1 % predicted and FEV_1/FVC ratio were significantly different between the groups (p<0.001). Non-smoking and smoking control subjects were matched for lung function, with no statistical difference in FEV_1 or % predicted FEV_1 (FEV_1 mean difference -0.212, 95% CI -0.800 to 0.376, p=0.648; % predicted FEV_1 mean difference 5.9, 95% CI -4.7 to 16.5, p=0.364).

	Non Smokers N=11	Smokers N=10	COPD N=9	p value
Male, n (%)	4 (37)	4 (40)	6 (67)	0.351
Age*	62 (53 to 71)	53 (41 to 66)	60 (44 to 72)	0.014°
Current smokers, n (%)	0 (0)	10 (100)	8 (89)	<0.001
Ex-smokers, n (%)	0 (0)	0 (0)	1 (11)	<0.001
Pack year history*	0 (0 to 0)	32 (21 to 51)	39 (25 to 50)	<0.001•
Body mass index, kg/m^2	24.2 (1.2)	26.0 (1.3)	26.6 (1.8)	0.449
FEV_1 (L)	2.70 (0.17)	3.01 (0.20)	2.08 (0.15)	0.005°
FEV_1 % predicted	103 (4)	98 (4)	69 (2)	<0.001°
FVC (L)	3.48 (0.20)	3.88 (0.30)	3.41 (0.27)	0.405
FVC % predicted	109 (4)	103 (5)	91 (3)	0.031°
FEV_1/FVC, %	78 (1)	78 (2)	61 (2)	<0.001°
Peak expiratory flow (L/min)	410 (27)	435 (38)	368 (25)	0.295
SpO_2 at rest, %†	97 (4)	98 (4)	96 (3)	0.579

Table 3.2 Clinical baseline characteristics for all subjects successfully infected with RV16. Data presented as mean (+/- SEM) unless stated, *mean (range), †median (interquartile range) (p shown in table - one way ANOVA) (°p<0.05 controls vs COPD, •p=0.16 Smk vs COPD, Tukey analysis)

3.8 Symptom scores uncorrected for baseline and effects of bronchoscopy

As described in chapter 2, Material and Methods, subjects recorded daily upper and lower respiratory tract symptom scores from two weeks prior to the baseline bronchoscopy until study completion on day 42. The raw uncorrected total symptom scores in two week blocks are displayed in the following section. The data has been presented in two week blocks to allow investigation of the effect on symptoms scores of study procedures and to minimise the effect of day to day variations. Each of the two week phases contain separate interventions as described in the Materials and Methods chapter. Uncorrected scores are presented here to enable direct comparison of each phase of the study, which has been divided into pre-baseline, baseline, acute, recovery and convalescence phases.

3.9 Upper respiratory tract symptom scores in infected and non-infected subjects

To assess the effect of successful RV16 infection on symptom scores, the URT score in two week blocks for all subjects who were judged to either have been successfully infected or not infected are shown in figure 3.2. In a two way ANOVA comparison when the two groups were compared, there was a significant increase in the scores between infected subjects versus non-infected only during the acute fortnight (mean difference -26, 95% CI -48 to -4, $p<0.05$). There was no difference between the groups when any other two week time block was compared ($p>0.05$).

In infected subjects only, the mean score during the acute phase was significantly higher than in any other two week interval, ($p<0.0001$, one way ANOVA, with Tukey's multiple comparison analysis). In non-infected subjects Tukey's analysis determined that the pre-baseline and acute phase score were significantly different using a one way ANOVA analysis, $p<0.05$.

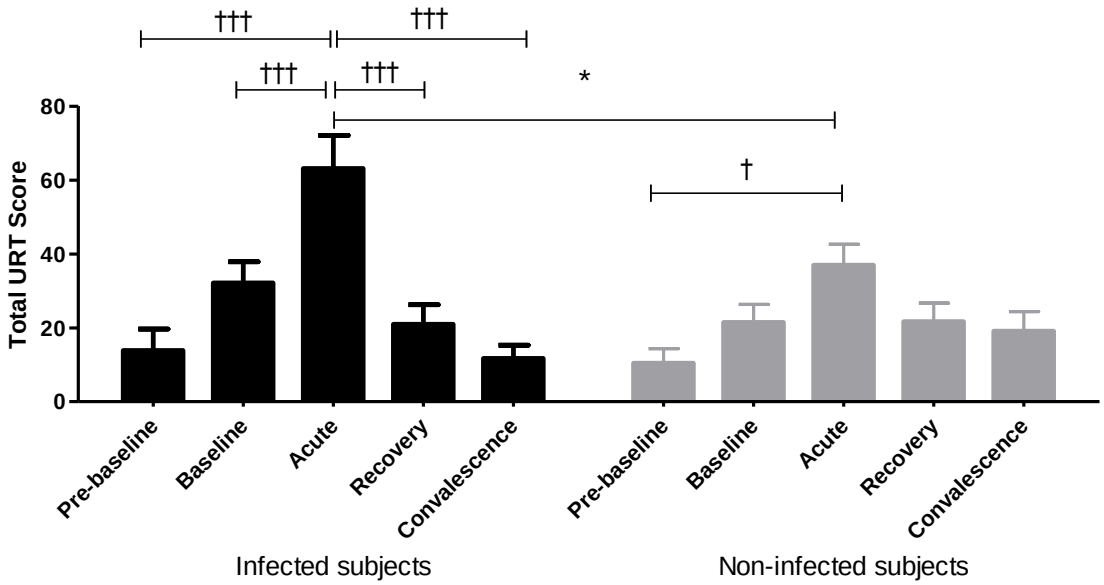


Figure 3.2 Total mean two week phase URT score in all infected and non-infected subjects. Higher scores were recorded in infected subjects compared to non-infected only during the acute phase. In infected subjects acute phase scores were higher than any other phase assessed. Data presented mean +/- SEM, (*p<0.05, two way ANOVA, †††p<0.0001, †p<0.05 one way ANOVA)

3.10 Upper respiratory tract symptom phase scores in infected and non-infected study groups

To investigate whether there are differences between individual study groups in URT symptom severity either at baseline, or at any of the four other two week phases of the study, the results of URT scores in two week blocks have been presented for non-smokers, smokers and COPD subjects and comparison between groups at each phase performed by one way ANOVA. In the non-infected subjects there was no significant difference between any of the study groups at any time point, figure 3.3.

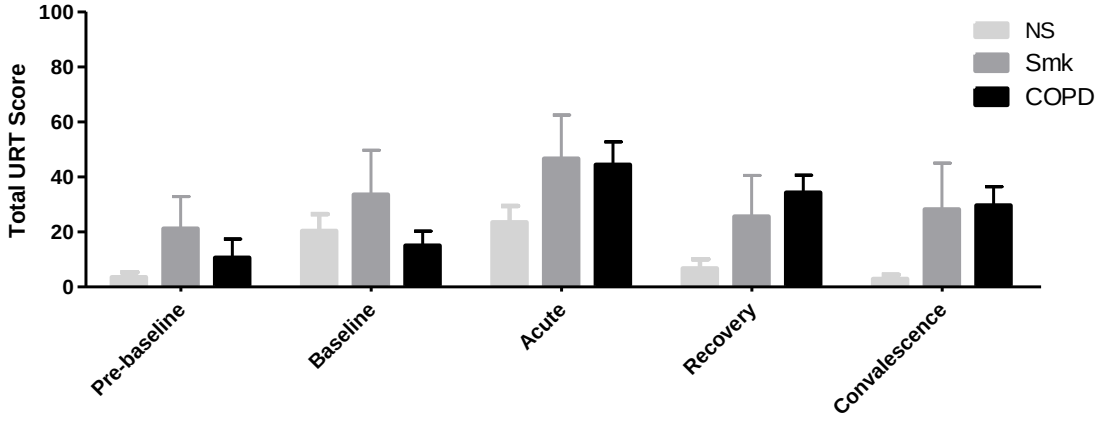


Figure 3.3 Total mean two week phase URT score in non-infected subjects. No significant difference between groups at any time period. Data presented mean +/- SEM, (one way ANOVA, p>0.05)

When the URT score from infected subjects was examined by individual study group, there was no difference between the scores at any time period, figure 3.4 (one way ANOVA, p>0.05).

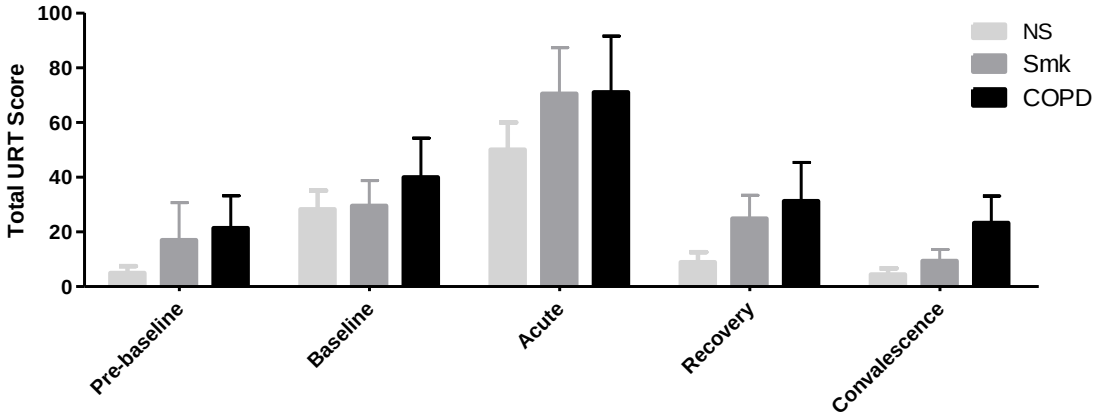


Figure 3.4 Total mean two week URT phase score in infected subjects. No significant difference between groups at any time period. Data presented mean +/- SEM, (one way ANOVA, p>0.05)

3.11 Lower respiratory tract symptom phase scores in infected and non-infected subjects

The two week block LRT scores for all infected and non-infected subjects are shown in figure 3.5. This demonstrated that only during the acute phase was there a difference between infected and non-infected subjects. There was a higher mean acute phase score in infected subjects compared to non-infected subjects, (unpaired t test, total score 44 vs 38 $p<0.001$).

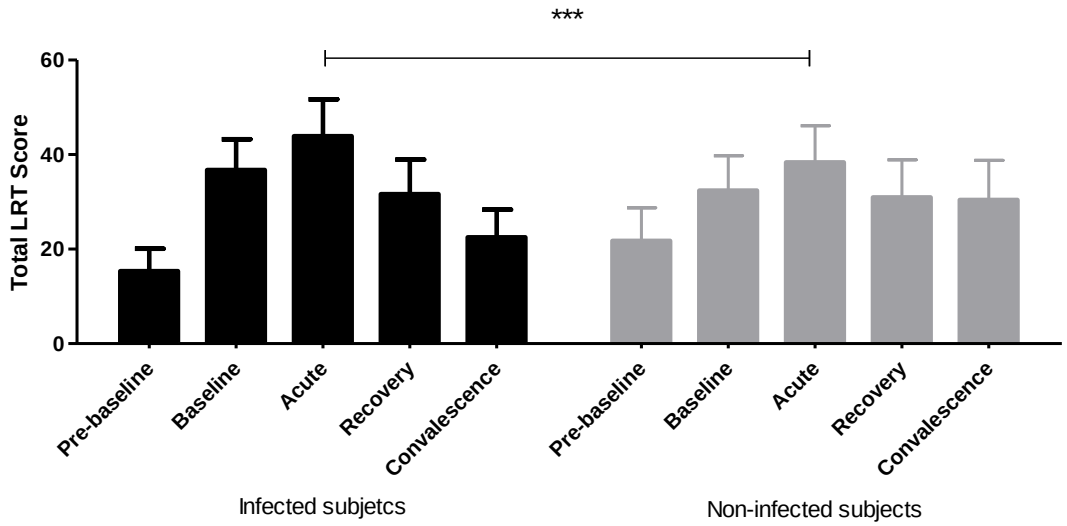


Figure 3.5 Total mean two week LRT phase score in all combined infected and non-infected subjects. Higher score recorded in infected subjects during the acute phase only. Data presented mean +/- SEM, (unpaired t test, ***$p<0.001$)

3.12 Lower respiratory tract symptom phase scores in infected and non-infected study groups

When the two week phase LRT scores in non-infected subjects was considered, no difference between groups during the pre-baseline and baseline time periods was recorded. However during the acute, recovery and convalescent period the COPD subjects had higher scores than non-smoking controls in a two way ANOVA analysis and Bonferroni's post test, figure 3.6.

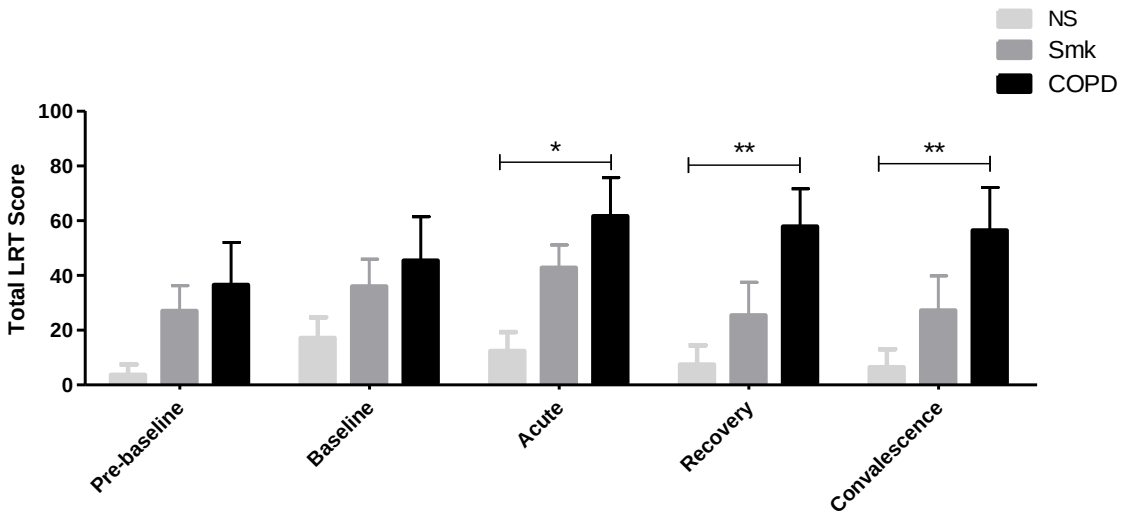


Figure 3.6 Total mean two week phase LRT score in non-infected subjects. Significant difference between non-smoking and COPD subjects at acute, recovery and convalescence phase, no significant difference between smoking and COPD subjects at any time point, (two way ANOVA, *p<0.05, **p<0.01)

In infected subjects there was no difference in LRT phase scores between the three study groups during the pre-baseline and baseline period. During the acute phase COPD subjects had significantly greater scores than both control groups; with no difference between smoking and non-smoking controls during this time period. In the recovery and convalescence period LRT scores in COPD subjects remained higher than in non-smoking controls, figure 3.7.

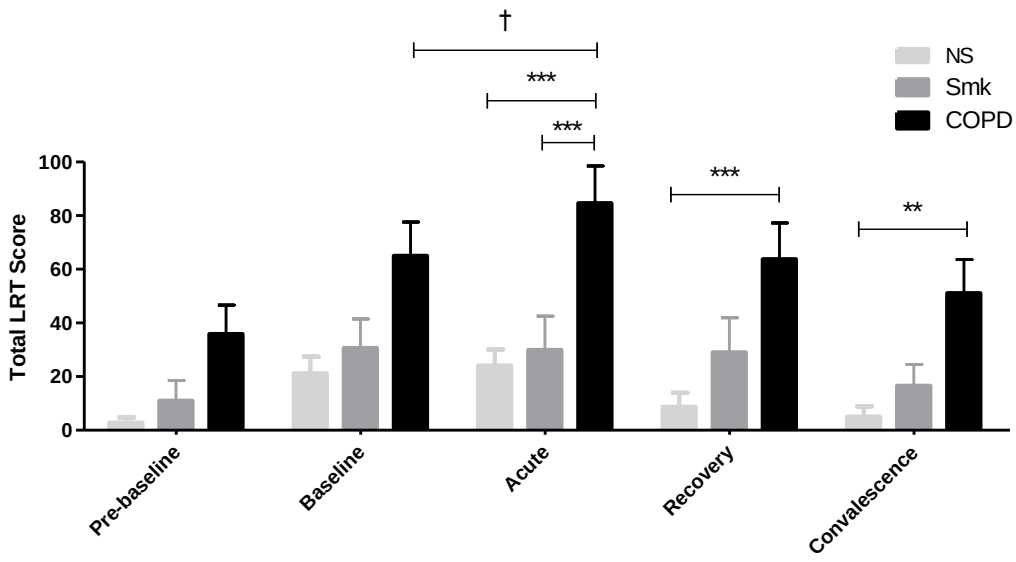


Figure 3.7 Total mean two week phase LRT score in infected subjects. No difference between control groups at any time point, greater scores in COPD subjects as shown. (**p<0.01, ***p<0.001, two way ANOVA) Increase in COPD subjects score between baseline and acute periods, (paired t test, †p<0.05)

A significant increase in LRT scores from baseline following RV infection only occurred in the acute phase in COPD subjects, when baseline was compared to the acute period the mean scores increased from 65 to 85, p=0.015, (paired t test). In control subjects there was no difference in mean score between baseline and acute time periods, figure 3.7.

3.13 Daily symptom scores corrected for baseline and effects of bronchoscopy

Daily upper and lower respiratory tract total symptom scores were corrected for pre-infection levels, by subtracting the mean of symptom scores from days -3 to 0. This was performed to eliminate baseline symptoms and enable between group analyses. Additionally URT scores were corrected for the effects of the post infection bronchoscopy between days 7 and 12 by subtracting mean day -14 to -9 score. Only URT symptoms were corrected for the effects of bronchoscopy, as there was no evidence of an effect on LRT scores and correction resulted in a dip below preceding day's values. A similar pattern was identified in previous RV exacerbation studies in COPD. Henceforth only corrected total URT and LRT scores are discussed. The results of these are summarised below.

3.14 Daily corrected upper respiratory tract symptom scores in infected and non-infected subjects

URT scores in both RV infected and non-infected subjects are illustrated in figure 3.8 and demonstrate that successfully infected subjects had significantly greater URT scores than those who were not infected. This was evident on days 2 to 7 using an unpaired t test between infected and non-infected subjects. Despite no evidence of RV infection there was a significant rise in symptom scores following experimental inoculation in non-infected subjects, the paired t test analysis confirmed this on all visits after inoculation until day 11. The pattern of this increase was similar in both infected and non-infected subjects. In those subjects with confirmed infection there was significantly increased URT symptoms scores from inoculation until day 20, analysed using a paired t test from baseline. The peak increase in URT symptom scores occurred on day 5 (mean difference (95%CI) URT score on day 5 from baseline 4.52 (2.31 to 6.72), $p<0.001$).

3.15 Daily corrected upper respiratory tract symptom scores in infected subjects between groups

To assess the effect of RV infection on URT symptom scores in individual groups, figure 3.9 illustrates the time course in non-smokers, smokers and COPD subjects. The peak URT score (mean difference URT score, 95%CI) occurred on day 4 in non-smoking controls 4.70 (1.44 to 7.97), p=0.01; on day 5 in smoking controls 6.61 (0.29 to 12.92), p=0.04 and on day 3 in COPD subjects 3.94 (-0.21 to 8.08), p=0.060.

There was a statistically significant difference in mean URT scores between the three study groups on day 15 (p=0.030, one way ANOVA); determined with post hoc Tukey's analysis to be between non-smokers and smokers (mean difference -2.6, 95% CI -5.1 to -0.1, p=0.035).

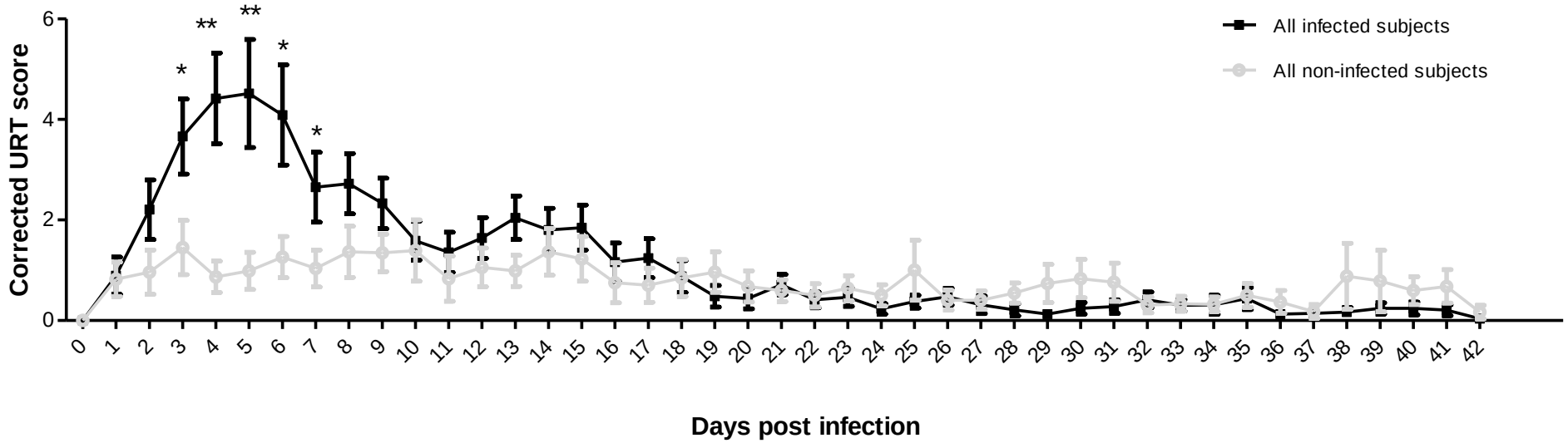


Figure 3.8 Corrected total daily URT symptom scores for all infected and non-infected subjects following RV challenge. Data presented as mean +/- SEM, (unpaired t test between infected and non-infected subjects, *p<0.05, **p<0.01)

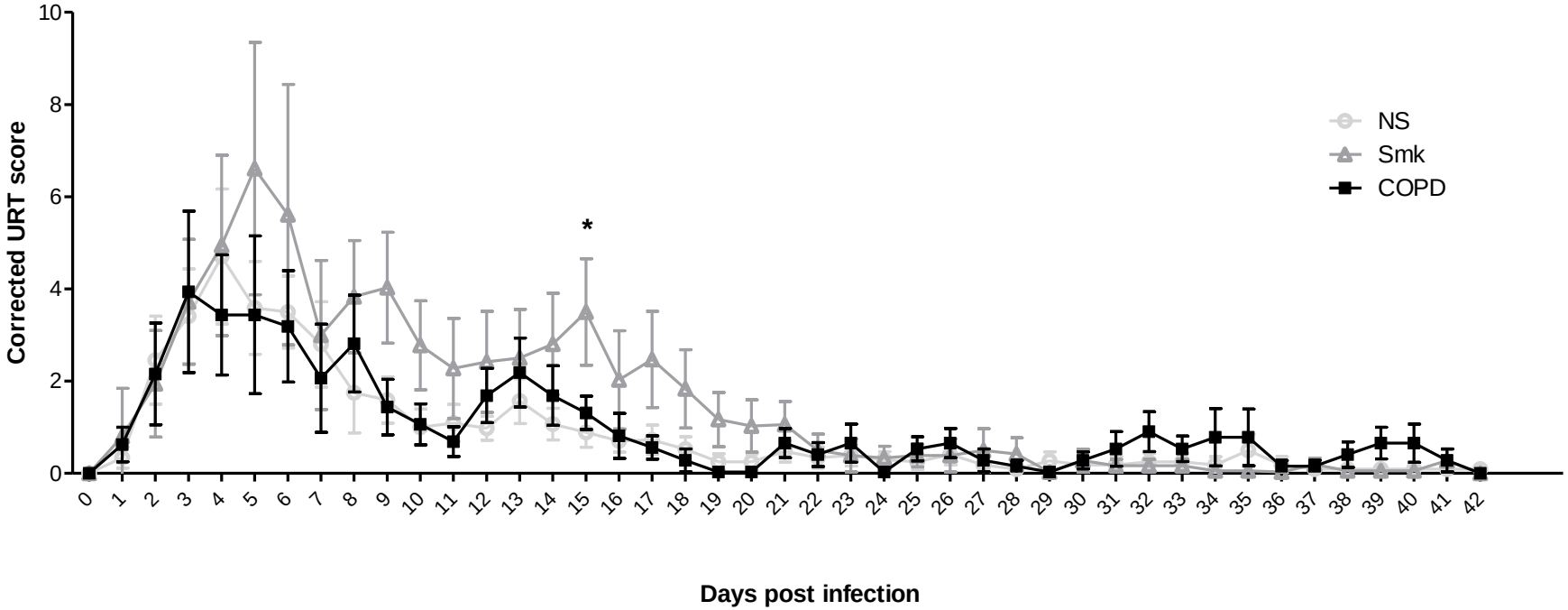


Figure 3.9 Corrected total daily URT symptom scores for infected subjects by group following RV challenge, significant difference between groups on day 15 in smoking subjects. Data presented as mean +/- SEM, (one way ANOVA, *p<0.05)

To investigate the duration of increased URT symptoms following RV infection the time taken for scores to return to baseline has been calculated. This was performed using Kaplan-Meier analysis for the time in days of URT symptom scores to return to baseline. This was not different between the three study groups, p=0.64, log rank test, figure 3.10. It took until day 17, 18 and 21 for half of the subjects in the non-smoking, COPD and smoking groups respectively to return to baseline scores.

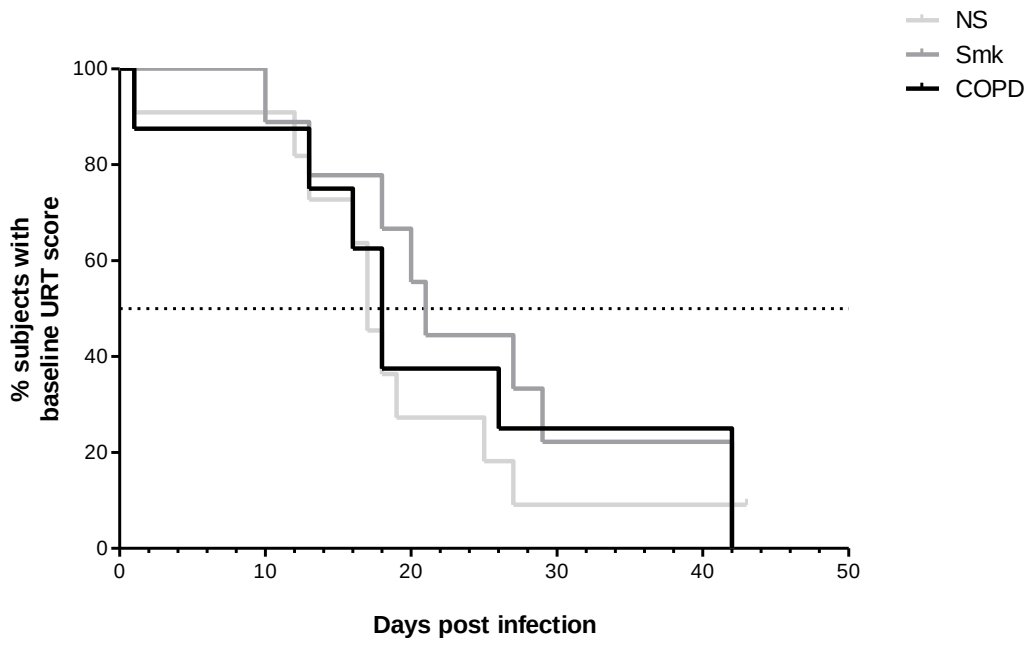


Figure 3.10 Time to taken to return to baseline for URT symptom scores in all infected subjects classified according to study group. Dashed line indicates time at which 50% of the subjects returned to baseline symptom scores, (log rank test, p=0.64)

3.16 Daily corrected lower respiratory tract symptom scores in infected and non-infected subjects

The time course of total LRT scores in both infected and non-infected subjects is shown in figure 3.11. There was a trend to a higher mean LRT score in subjects who were successfully infected compared to non-infected subjects, but this difference was not significant at any time point when analysed with an unpaired t test.

3.17 Daily corrected lower respiratory tract symptom scores in infected subjects by group

In infected subjects a one way ANOVA analysis showed a significant difference between the mean scores of the three groups on days 2, 3, 14, 15, 18, 19, 34, 35 and 36, figure 3.12. Only on day 3 was this associated with a significantly higher LRT scores between COPD subjects and non-smoking (mean difference 1.99, 95% CI 0.58 to 3.40; p=0.004) and smoking controls (mean difference 1.80, 95% CI 0.22 to 3.38; p=0.022), when analysed using Tukey's test.

The area under the curve (AUC) for the cumulative LRT scores between days 0 and 7 was significantly higher in the COPD group compared to non-smoking and smoking controls, AUC mean (SEM) 15.3 (3.4), 4.8 (1.5), and 6.6 (6.6) respectively, p=0.017, assessed using one way ANOVA. This data demonstrates that acute RV infection induces symptoms of an exacerbation in COPD subjects during the first week of infection. Additionally this rise is independent of the possible effects of bronchoscopy and pre-morbid symptoms as the scores have been corrected for baseline values.

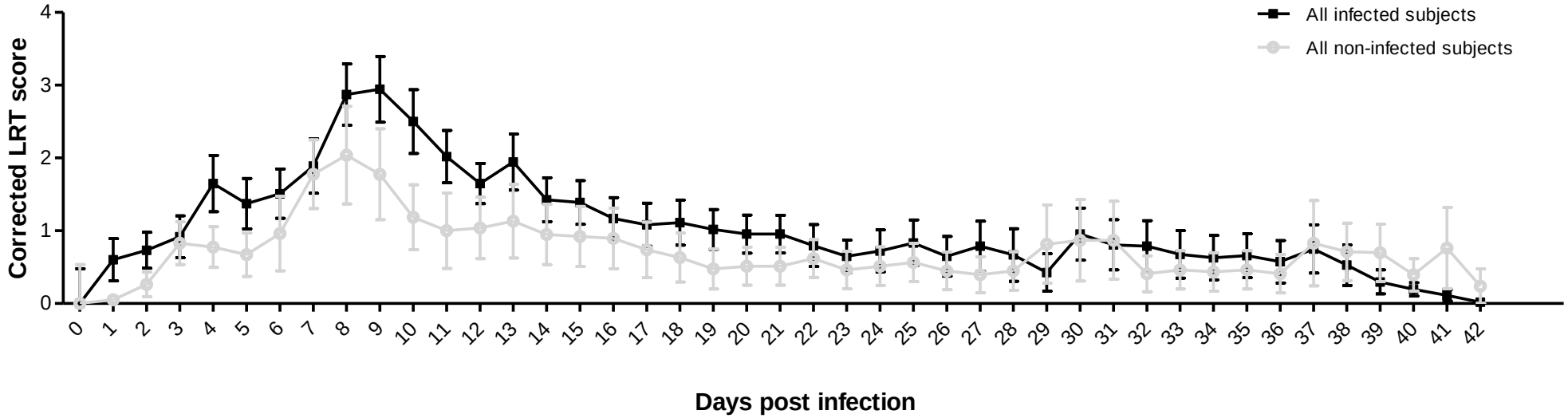


Figure 3.11 Corrected total daily LRT symptom scores for all infected and non-infected subjects following RV challenge, no significant difference between infected and non-infected subjects at any time point. Data presented as mean +/- SEM, (unpaired t test, $p>0.05$)

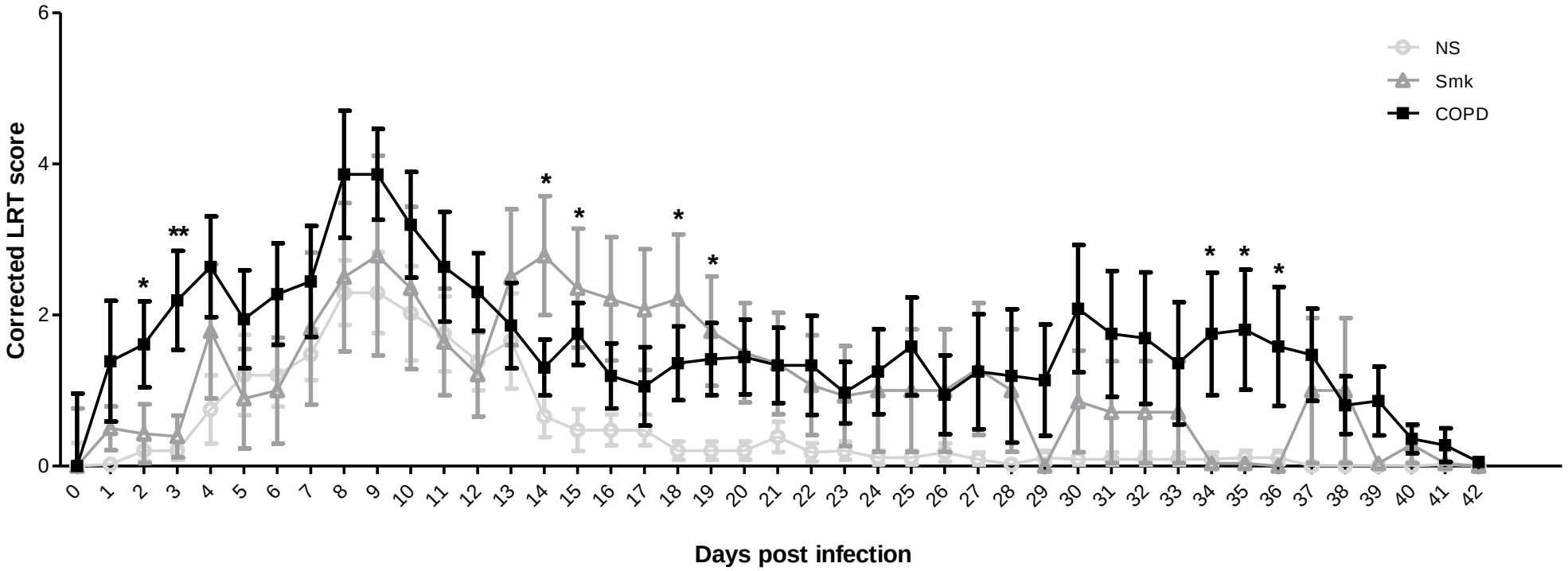


Figure 3.12 Corrected total daily LRT symptom scores for infected subjects by group following RV challenge, significant between group differences indicated on days 2, 3, 14, 15, 18, 19, 34, 35 & 36. Data presented as mean +/- SEM, (one way ANOVA, *p<0.05, ** P<0.01)

In order to evaluate any between group differences in the time taken for symptoms to return to baseline levels, a Kaplan Meier plot is shown in figure 3.13. This illustrates the prolongation of LRT symptoms in COPD subjects compared to both smoking and non-smoking controls, p=0.05 log rank test. The time taken for 50% of subjects to return to their baseline LRT score was 15, 22 and 35 days in non-smoking controls, smoking controls and COPD subjects respectively.

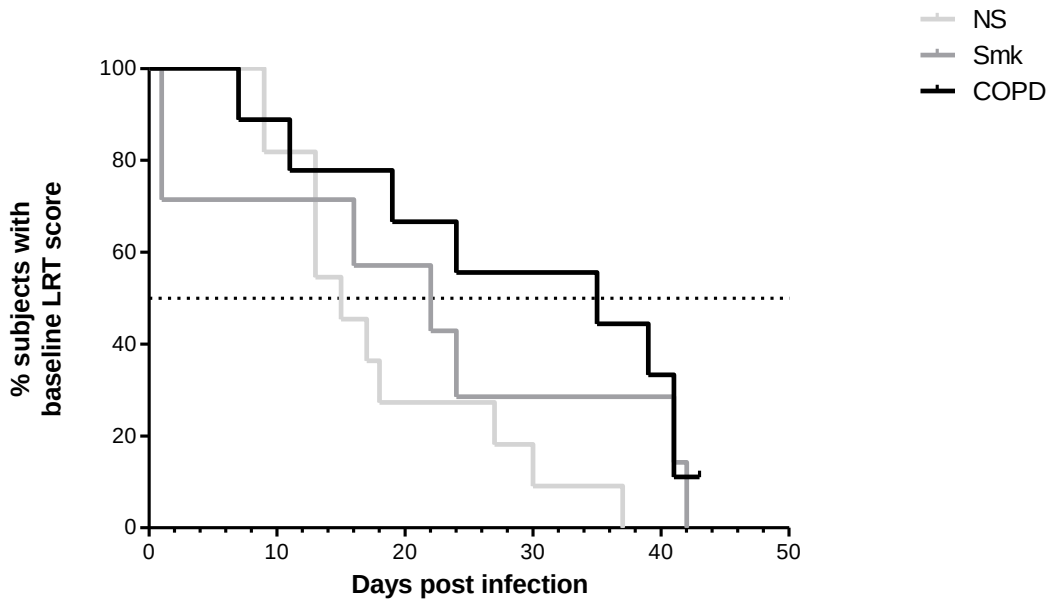


Figure 3.13 Time taken for return to baseline LRT symptom scores for infected subjects in individual study groups. Dashed line indicates time at which 50% of the subjects returned to baseline symptom score, (Log rank test, p=0.05 COPD vs controls)

3.18 Daily lower respiratory tract score domains in all infected subjects

When the individual domains of the LRT score were analysed the mean scores in individual domains were small and correcting these for baseline levels resulted in fractional numbers. The scoring system is formed from integers and so the reliability and clinical utility of this data was uncertain. Therefore individual domains of the LRT score have been presented uncorrected for baseline values.

The composite LRT score comprises scores from five individual domains, namely breathlessness, wheeze, cough, sputum production and sputum quality as described in Material and Methods, chapter 2. The score of all individual domains in successfully infected subjects over the time course of the study is shown, figure 3.14. This illustrates a trend to greater burden of symptoms arising from cough in the first two weeks following RV infection and from the sputum quality domain after day 24. Further conclusions cannot be drawn about individual domains from this data as there is considerable overlap in scores; however it is possible to observe that the rise in total LRT score following RV infection is driven by increases in all five domains.

3.19 Daily lower respiratory tract score domains in study groups

The scores for the three study groups in the breathlessness, wheeze, cough, sputum production and sputum quality domains are shown in figures 3.15 to 3.19. The AUC for scores between day 0 and 7 was calculated for each study group in the five LRT domains. These were significantly higher in the COPD group compared to the control subjects for 4 of the 5 domains. Specifically AUC for breathlessness (7.1, 0.2 and 2.8; p=0.003), cough (9.4, 3.3 and 5.2; p=0.031), wheeze (7.1, 0.05 and 1.4; p=0.0003) and sputum production (8.2, 1.9 and 4.2; p=0.006) was higher in the COPD group that the non-smoking and smoking controls respectively. Whilst only in sputum quality scores was there no significant difference between the AUC of the study groups.

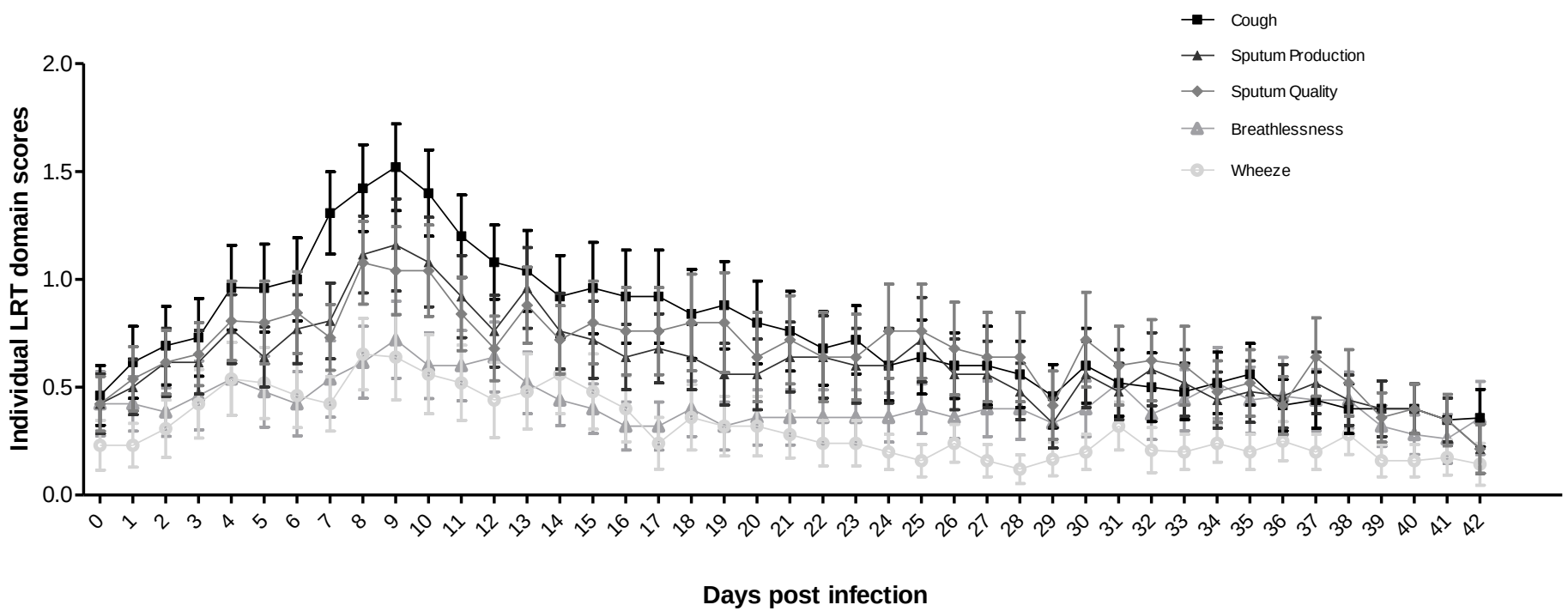


Figure 3.14 Individual domains of LRT score in all infected subjects. Data presented as mean +/- SEM, (one way ANOVA, p>0.05)

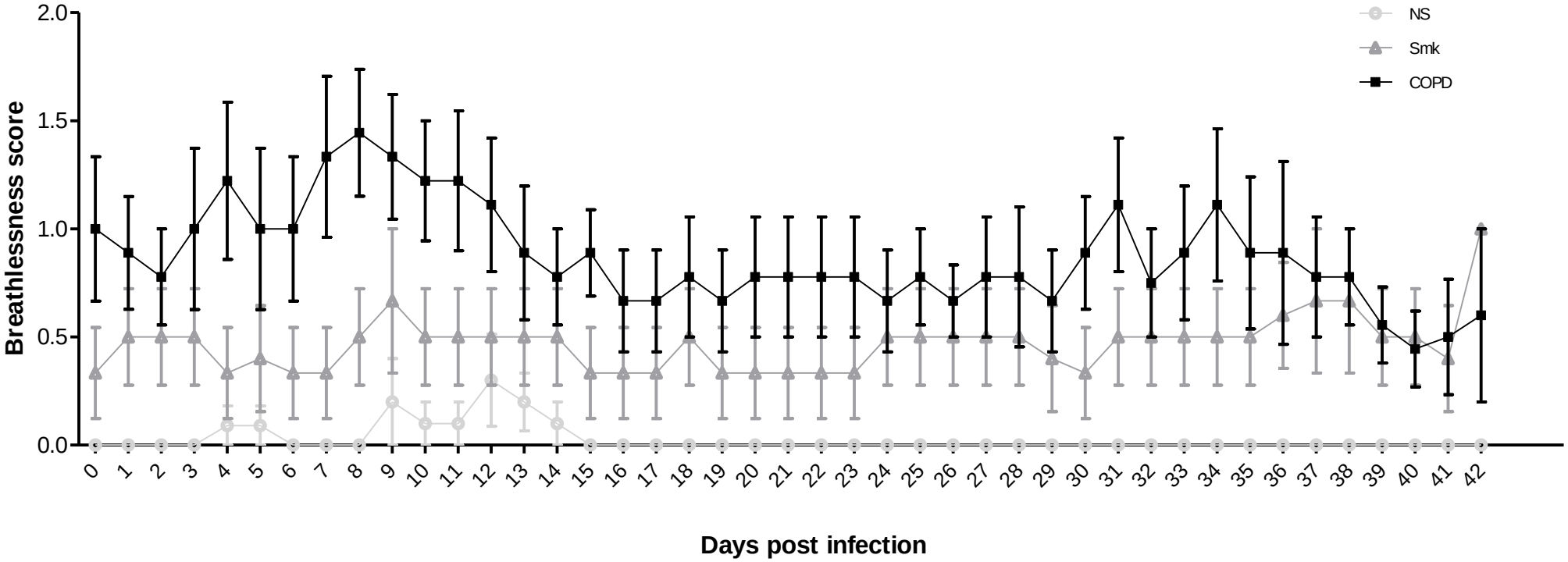


Figure 3.15 Daily breathlessness scores in infected study groups. Data presented as mean +/- SEM, (one way ANOVA, p>0.05)

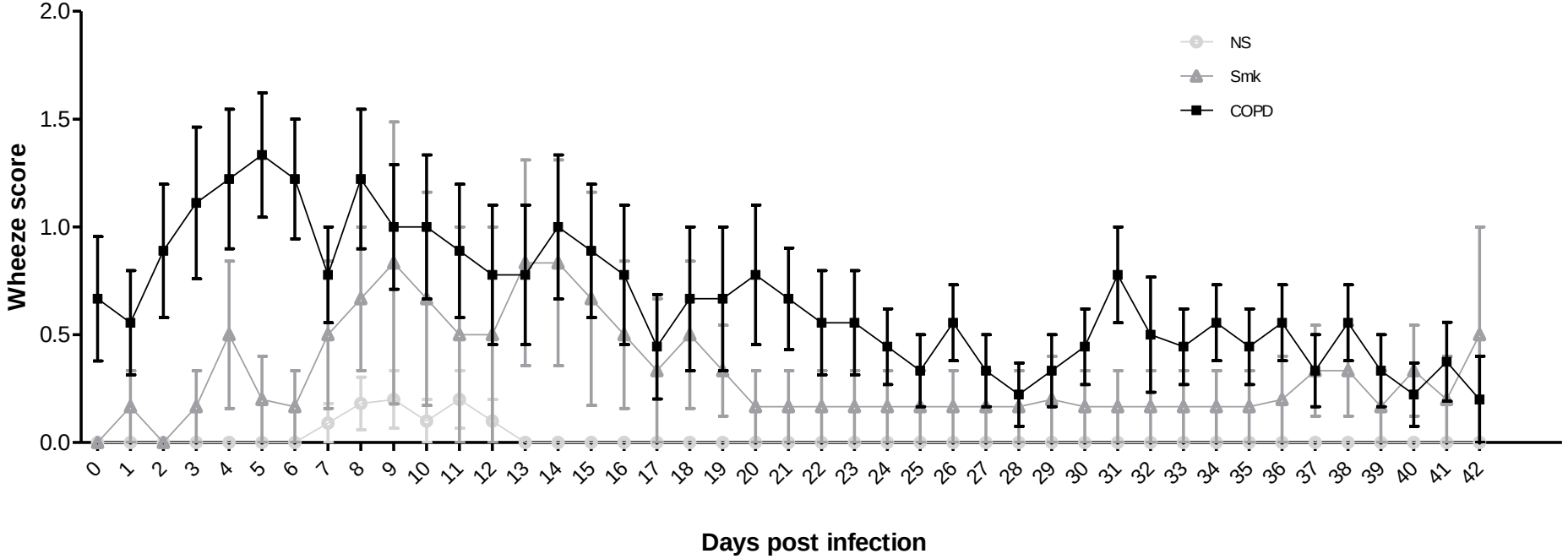


Figure 3.16 Daily wheeze scores in infected study groups. Data presented as mean +/- SEM, (one way ANOVA, p>0.05)

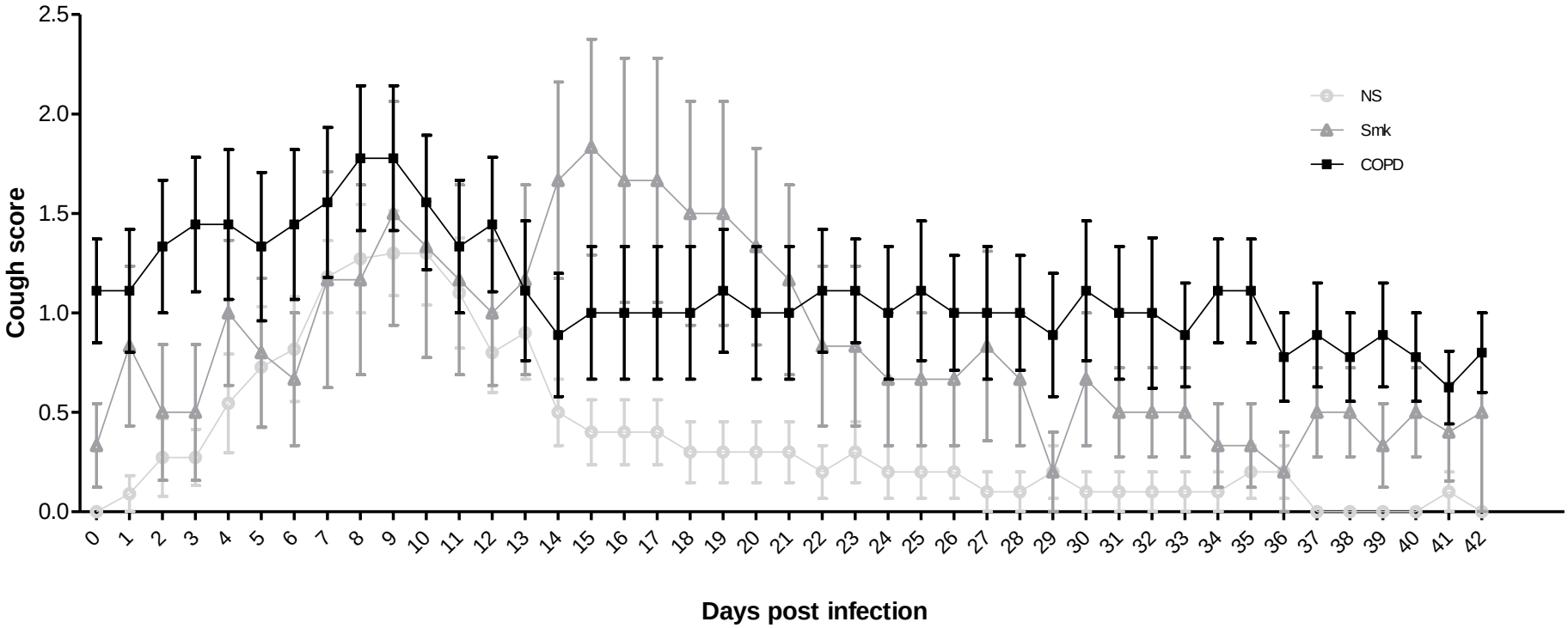


Figure 3.17 Daily cough scores in infected study groups. Data presented as mean +/- SEM, (one way ANOVA, p>0.05)

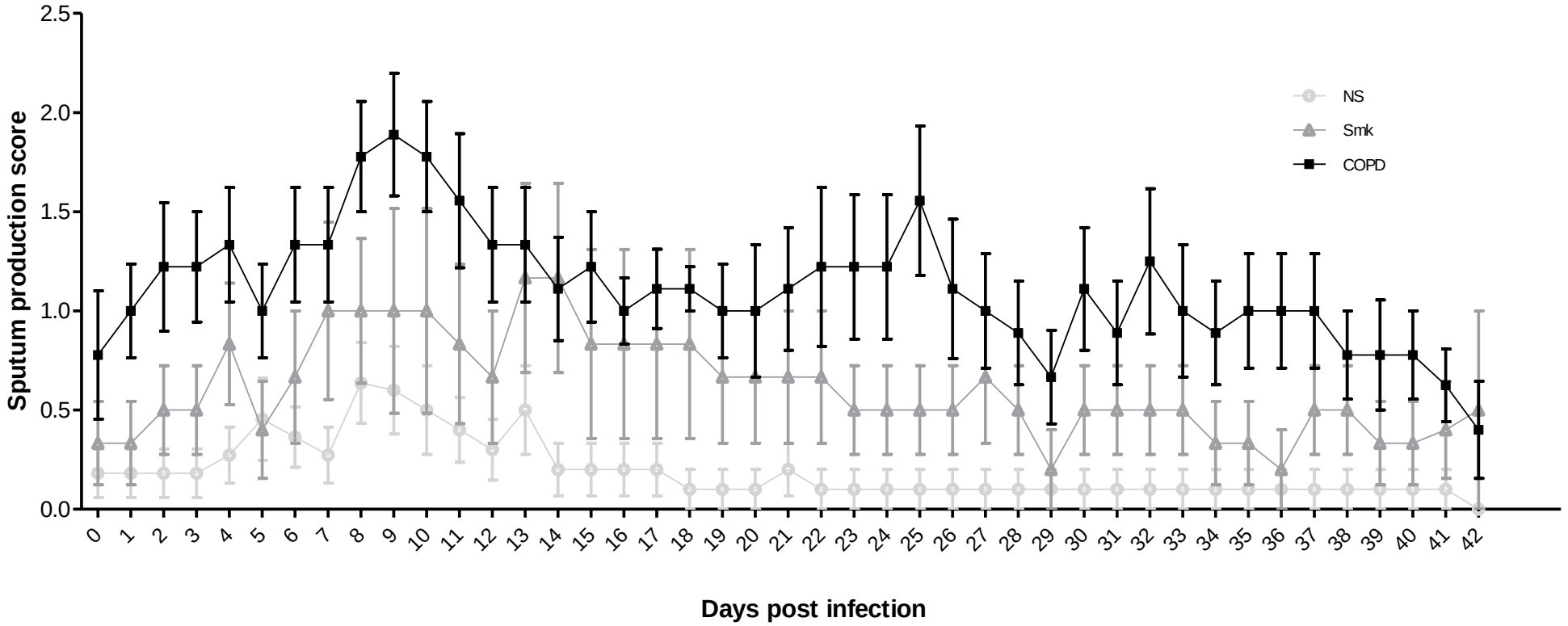


Figure 3.18 Daily sputum production scores in infected study groups. Data presented as mean +/- SEM, (one way ANOVA, p>0.05)

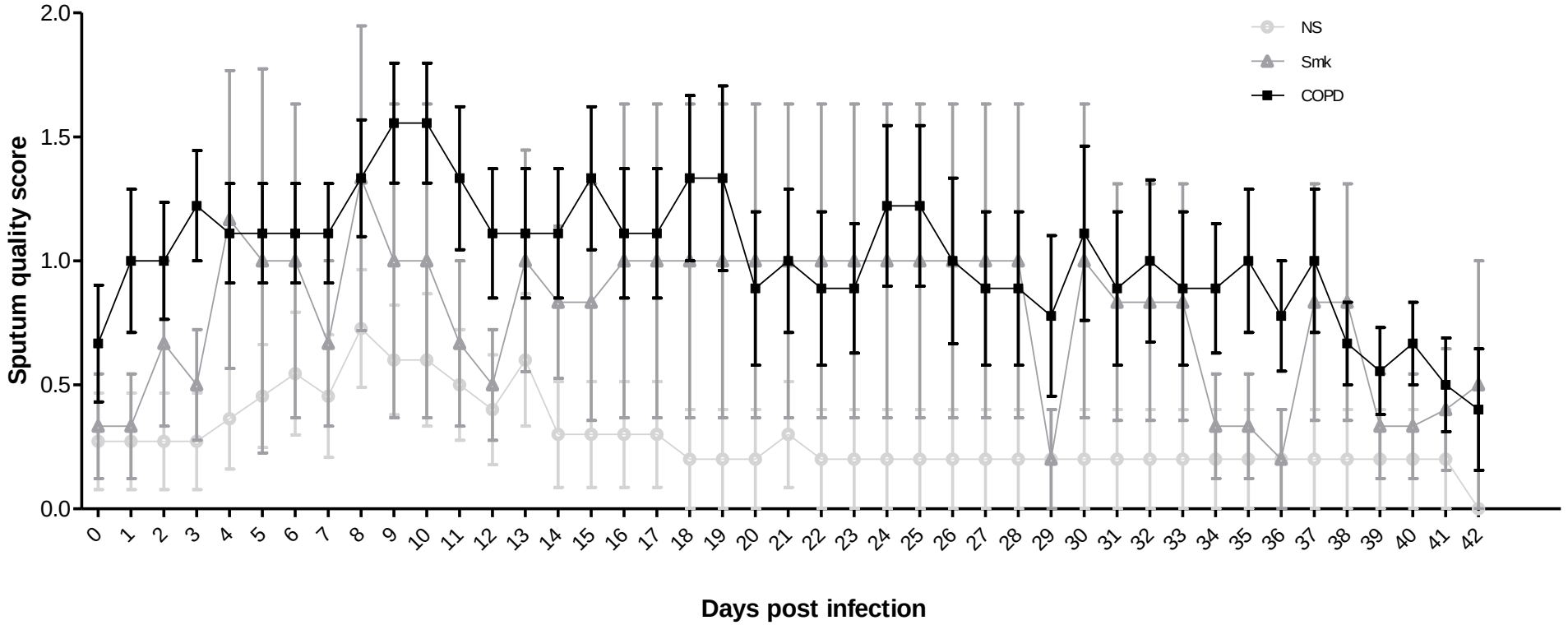


Figure 3.19 Daily sputum quality scores in infected study groups. Data presented as mean +/- SEM, (one way ANOVA, p>0.05)

3.20 Lung function results in infected and non-infected subjects

Full lung function testing using the helium dilution technique was performed on all subjects at baseline and day 12 (+/- 3 days) following RV16 infection at St Mary's Hospital pulmonary function laboratory. Testing was performed according to BTS/ERS criteria by ARTP accredited technicians. Subjects also performed spirometry on all scheduled clinic visits prior to sputum induction and completed daily peak flow and spirometry measurements at home.

The results of formal lung function testing from all subjects enrolled in the study are shown in table 3.3. Data obtained on day 12 has been presented in both infected and non-infected subjects, when these results were compared only the TLC was found to differ between the groups; being lower in infected compared to non-infected subjects (mean difference TLC 0.99L, 95% CI 0.17 to 1.82; p=0.039). There was no significant difference in any of the other parameters (p>0.05).

	Day 0	Day 12	
	Study entry	Non-infected subjects	Infected subjects
FEV_1 (L)	2.69 (0.13)	2.82 (0.26)	2.55 (0.13)
FEV_1 % predicted	93 (4)	92 (7)	92 (4)
FEV_1/FVC ratio, %	67 (2)	65 (3)	67 (2)
MEF_{50} % predicted	65 (6)	68 (11)	66 (6)
TLC, L	6.3 (0.2)	6.9 (0.4)	5.9 (0.2)*
TLC, % predicted	105 (5)	107 (5)	103 (2)
ResV, L	2.3 (0.1)	2.3 (0.2)	2.1 (0.1)
ResV, % predicted	105 (5)	106 (11)	101 (6)
FRC, L	3.5 (0.2)	3.7 (0.3)	3.3 (0.2)
FRC, % predicted	110 (6)	114 (10)	108 (5)
D_LCO, ml CO/min/mmHg	6.7 (0.3)	7.0 (0.6)	6.4 (0.3)
D_LCO, % predicted	78 (3)	77 (6)	75 (3)
KCO, ml CO/min/mmHg/L	1.18 (0.04)	1.13 (0.10)	1.14 (0.04)
KCO, % predicted	81 (3)	80 (8)	76 (3)

Table 3.3 Full lung function parameters in all subjects at baseline and those successfully and not successfully infected with RV at day 12. Data presented as mean (SEM). MEF_{50} Maximal expiratory flow at 50%; TLC Total lung capacity; ResV Residual volume; FRC Functional residual capacity; D_LCO Carbon monoxide diffusion capacity; KCO Carbon monoxide diffusion capacity corrected for alveolar volume (*$p<0.05$)

3.21 Formal lung function in infected subjects

The parameters recorded during formal lung function testing pre- and post infection are shown in the three study groups in table 3.4. In the non-smoking control group there was no significant change in any measurement between baseline and the day 12 visit. Following RV16 infection airway obstruction increased in smokers; with a significant fall in FEV_1/FVC ratio (mean difference change from baseline 3.4%, CI 95% 0.3 to 6.4, p=0.034). In COPD subjects there was a significant decrease in the absolute and percent predicted FRC (0.3%, 95% CI 0.6 to 3.3, p=0.031) and (10%, 95%CI 1 to 20, p=0.042) on day 12 compared to baseline. Neither of these groups had changes in other lung function measurements.

	Non smokers		Smokers		COPD	
	Day 0	Day 12	Day 0	Day 12	Day 0	Day 12
FEV_1 (L)	2.80 (0.20)	2.83 (0.20)	2.90 (0.19)	2.79 (0.22)	1.97 (0.18)	1.97 (0.15)
FEV_1 % predicted	111 (4)	112 (4)	100 (3)	97 (4)	65 (5)	66 (3)
FEV_1/FVC ratio, %	72.6 (1.6)	73.5 (2.0)	72.7 (1.0)	69.3 (1.6) *****	58.5 (2.6)	57.8 (2.7)
TLC, L	5.7 (0.3)	5.7 (0.3)	5.8 (0.4)	5.9 (0.5)	6.2 (0.4)	6.1 (0.3)
TLC, % predicted	103.7 (5.0)	105.4 (4.7)	102.9 (3.8)	104.7 (3.2)	97.3 (5.7)	98.4 (4.1)
RV, L	1.76 (0.14)	1.85 (0.10)	1.90 (0.21)	2.06 (0.29)	2.72 (0.24)	2.59 (0.25)
RV, % predicted	84.0 (6.2)	89.6 (5.6)	97.7 (9.1)	105.3 (12.5)	119.6 (11.0)	114.7 (9.8)
FRC, L	3.4 (0.4)	3.3 (0.3)	2.6 (0.2)	2.8 (0.3)	4.0 (0.4)	3.7 (0.4) *****
FRC, % predicted	119 (15)	116 (7)	90 (7)	98 (9)	122 (11)	112 (11) *****
D_LCO, ml CO/min/mmHg	7.0 (0.5)	7.2 (0.5)	5.8 (0.5)	5.7 (0.4)	6.2 (0.4)	6.0 (0.4)
D_LCO, % predicted	87.8 (3.9)	90.8 (3.5)	65.8 (4.6)	65.6 (3.2)	69.1 (3.7)	67.1 (3.6)
KCO, ml CO/min/mmHg/L	1.31 (0.07)	1.26 (0.06)	1.02 (0.06)	1.04 (0.05)	1.12 (0.06)	1.09 (0.05)
KCO, % predicted	88.1 (4.4)	85.1 (5.2)	65.7 (3.2)	66.6 (2.4)	79.8 (4.8)	77.6 (3.9)

Table 3.4 Full lung function data in successfully RV infected subjects by study group. Data presented as mean (SEM), (paired t test analysis; *p<0.05)

3.22 Clinic spirometry: FEV_1

Spirometry was performed by trained staff according to BTS criteria on each scheduled clinic visit. Absolute and percent predicted values were analysed for all subjects who were judged to have been successfully infected with RV. Changes in spirometry readings between baseline and subsequent clinic visits have also been calculated.

Figures 3.20 and 3.21 demonstrate the absolute and % predicted FEV_1 for each study group over the study time course. This illustrates clearly the between group difference at baseline and reflects graphically the screening criteria.

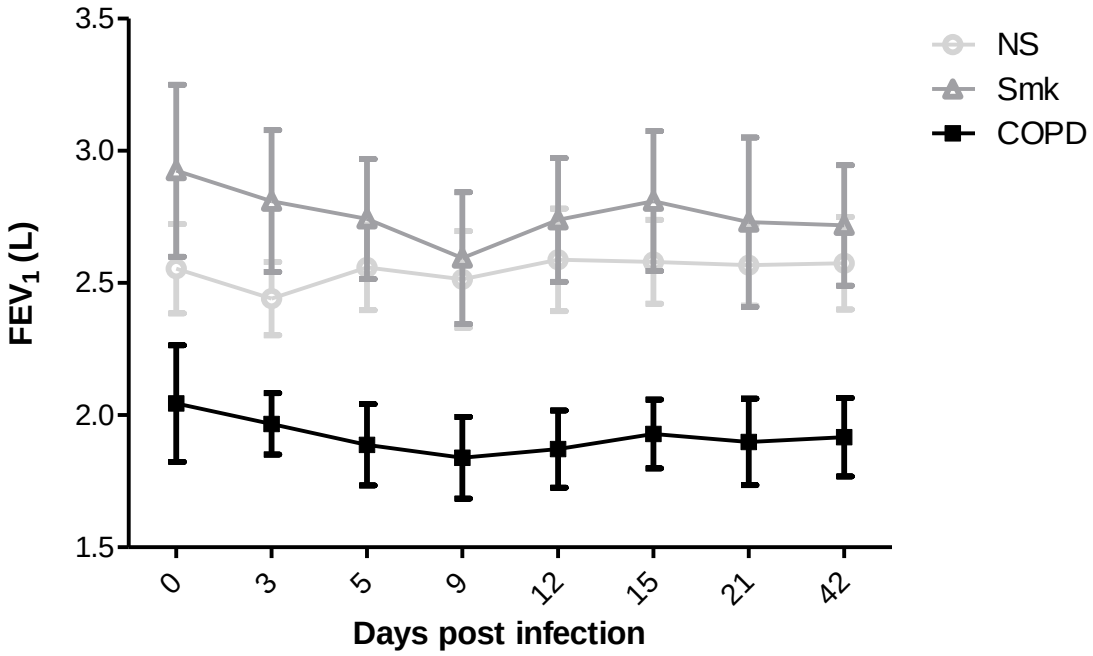


Figure 3.20 Time course of FEV_1 values recorded during clinic spirometry in study groups. Data presented as mean +/- SEM, (one way ANOVA, $p<0.001$ between controls and COPD, $p>0.05$ NS and Smk)

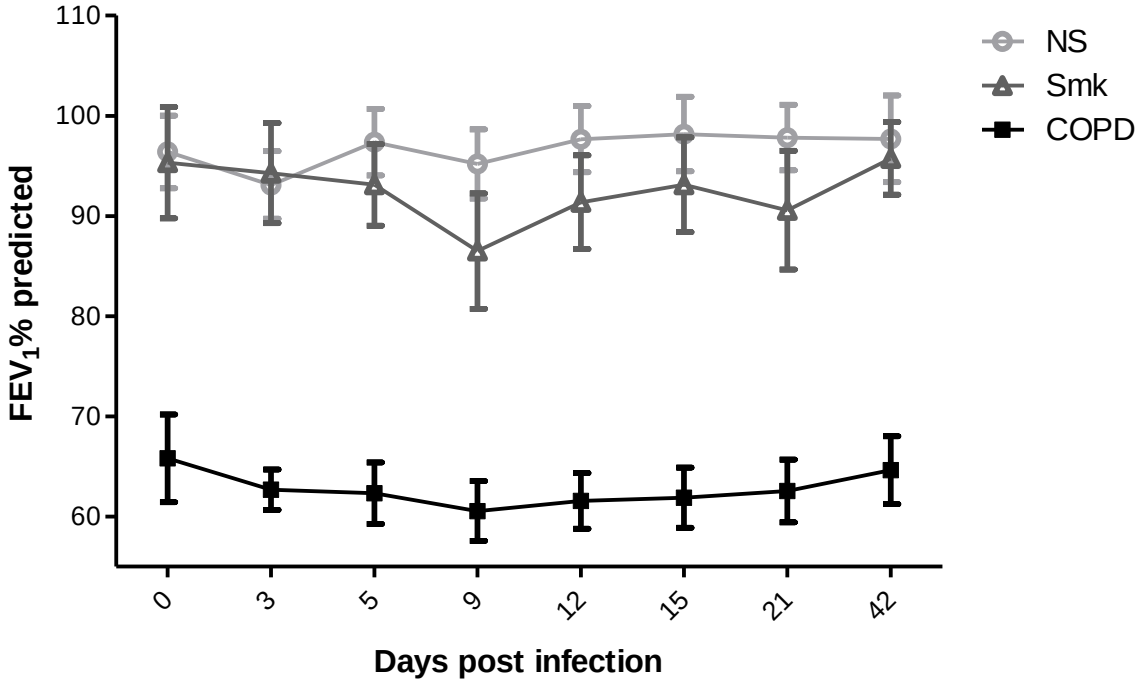
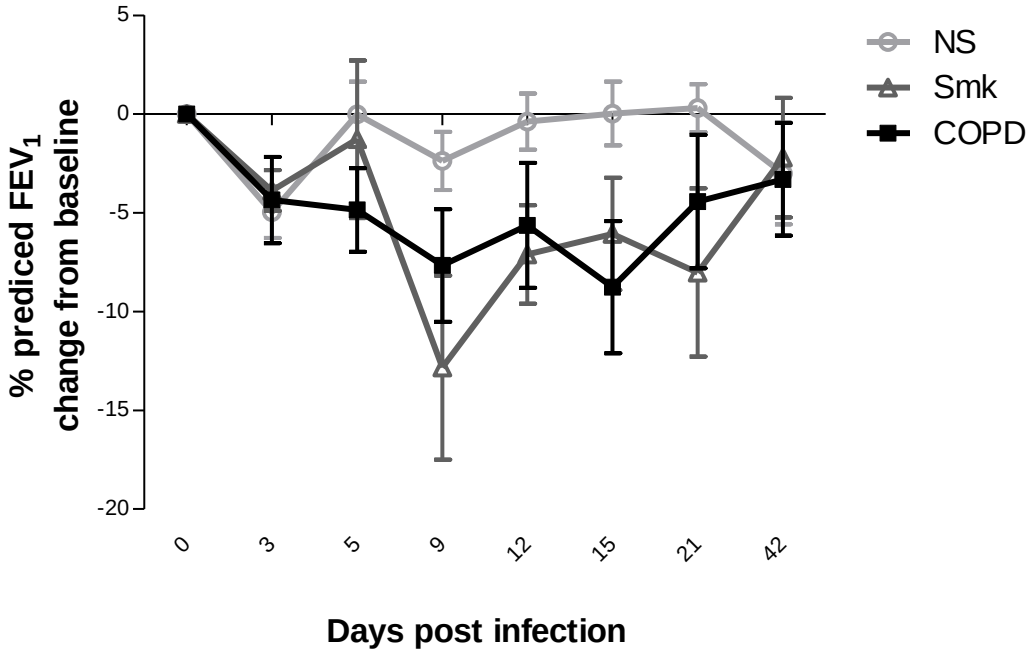


Figure 3.21 Time course of % predicted FEV_1 recorded during clinic spirometry in study groups. Data presented as mean +/- SEM, (one way ANOVA, p<0.001 between controls and COPD, p>0.05 NS and Smk)

3.23 FEV_1 change from baseline

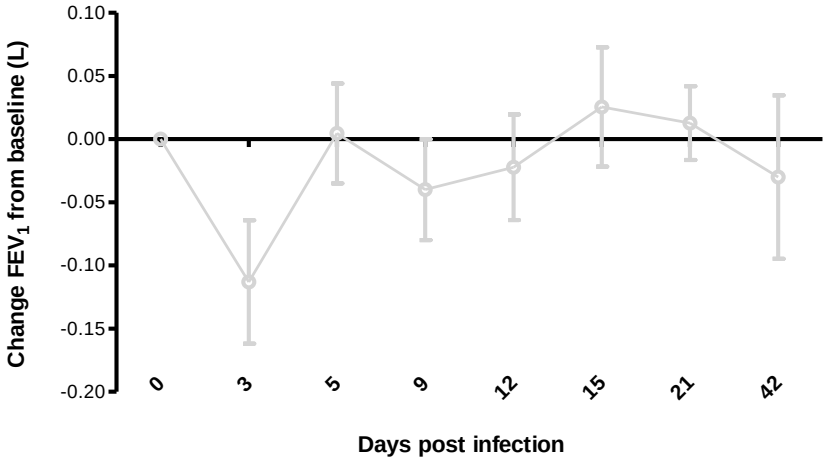
The change in percent predicted FEV_1 from baseline in each study group is shown in figure 3.22. There was an equal fall of almost 5% in all study groups on day 3. This was a significant drop in non-smoking controls (mean % FEV_1 change from baseline -3.9%, 95% CI -7.4 to -0.3, p=0.035, paired t test). In COPD subjects the peak significant fall occurred on day 15 (mean % FEV_1 change from baseline -8.8%, 95% CI -16.7 to -0.9, p=0.034, paired t test) and although not statistically significant occurred on day 9 in smoking controls (mean % FEV_1 change from baseline -10.9%, 95% CI -22.1 to 0.2, p=0.054, paired t test).

The % FEV_1 change from baseline was analysed between groups using one way ANOVA. This did not demonstrate any significant differences between the % predicated FEV_1 change in study groups at any time point.

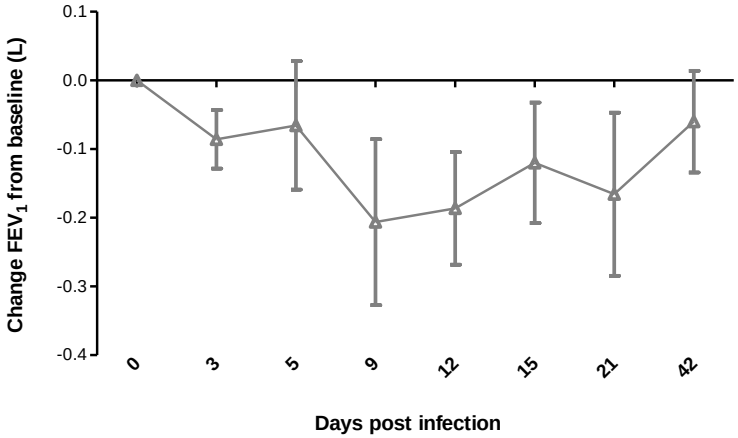


Figures 3.22 Percent predicted FEV_1 change from baseline in study groups over time course of study. Data presented as mean +/- SEM, (one way ANOVA, p>0.05)

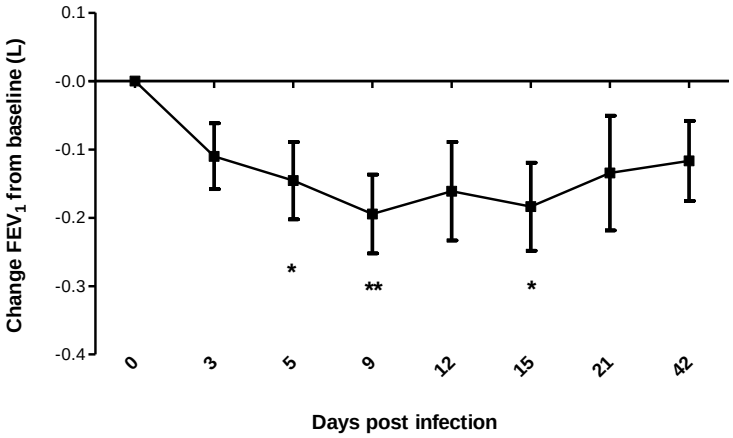
To evaluate the effect of RV infection on lung function in the three study groups the change in absolute FEV_1 from baseline has been plotted in figure 3.23. In control groups (smokers and non-smokers) there was no significant change at any time point. However in COPD subjects there was a significant fall on day 5 (mean difference -146mL, 95% CI -276 to -15, p=0.033), day 9 (mean difference -194mL, 95% CI -327 to -61, p=0.010) and day 15 (mean difference -184mL, 95% CI -337 to -31, p=0.025), (paired t test).



Figures 3.23a Non-smoking controls



Figures 3.23b Smoking controls



Figures 3.23c COPD subjects

Figures 3.23 Change from baseline in FEV_1 values over time course of study in (a) non-smoking controls (b) smoking controls and (c) COPD subjects. Data presented as mean +/- SEM, (paired t test change from baseline, *p<0.05, **p<0.01)

3.24 Clinic spirometry – FVC

The absolute and % predicted FVC over the time course of the study are shown in figures 3.24 and 3.25 respectively. There was no significant difference in absolute FVC between study groups across the time points. The % predicted FVC was lower in COPD subjects reflecting the study entry criteria. These illustrate the fall in FVC in cigarette smoke exposed subjects compared to constant levels in non-smokers following RV infection.

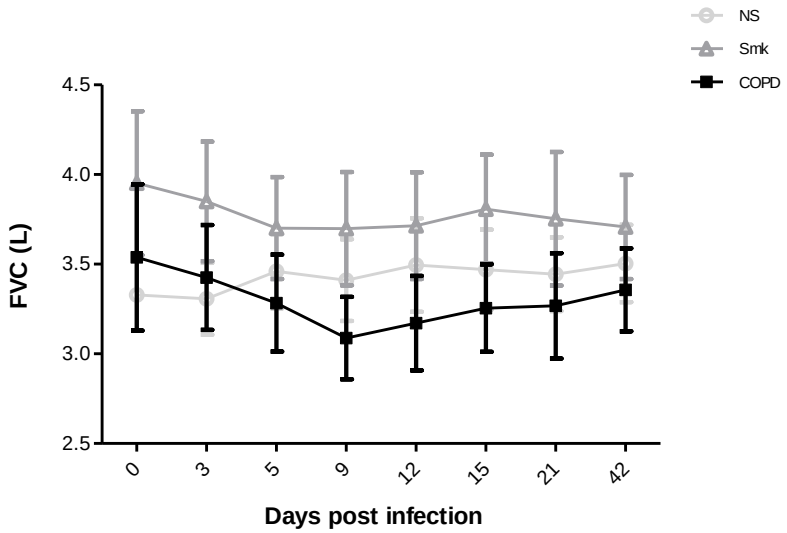


Figure 3.24 Change in absolute FVC values over time course of study in study groups. Data presented as mean +/- SEM, (one way ANOVA, p>0.05)

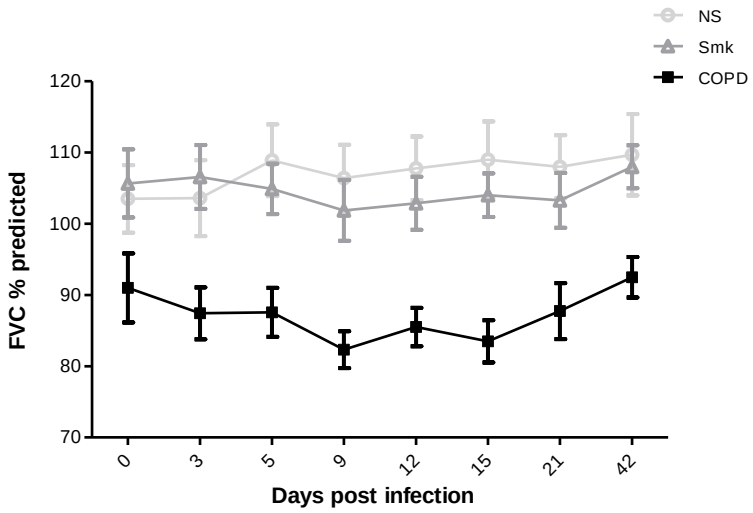
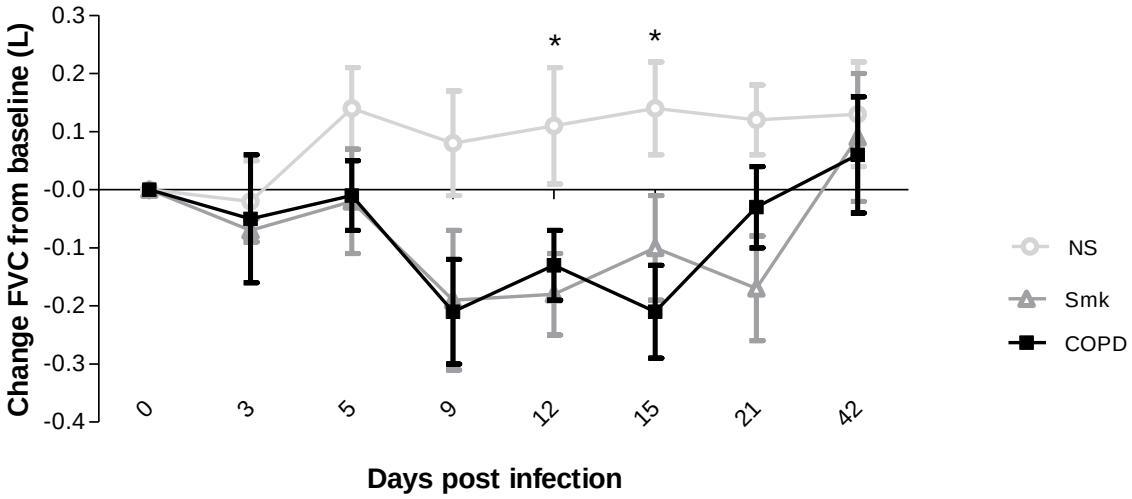


Figure 3.25 Change in percent predicted FVC over time course of study in study groups. Data presented as mean +/- SEM, (one way ANOVA, p<0.001 between controls and COPD, p>0.05 NS and Smk)

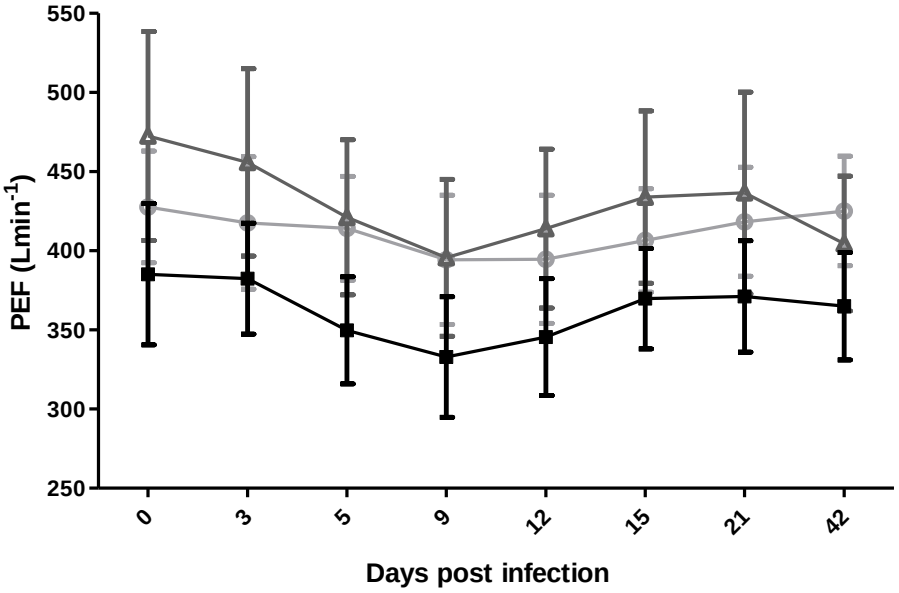
The change from baseline in FVC is shown in figure 3.26. The overall trend following RV infection in non-smokers showed no change in FVC. In smoking and COPD subjects FVC fell from day 5 to day 21 with a similar magnitude in each group. There was a statistical difference between the mean change from baseline in FVC in the study groups on day 12 (p=0.034) and day 15 (p=0.025) using one way ANOVA. This did not reach statistical significance on day 9 (p=0.06).



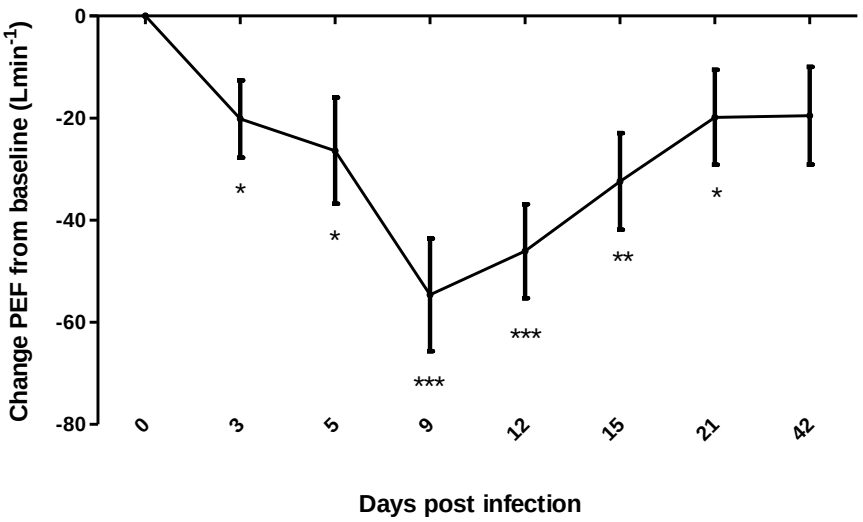
Figures 3.26 Change from baseline in absolute FVC over time course of study in study groups. Data presented as mean +/- SEM, (one way ANOVA, *p<0.05)

3.25 Clinic spirometry – PEF

Peak expiratory flow (PEF) was performed in addition to spirometry on clinic visits. The results of absolute values in each group over the study time course is shown in figure 3.27. This demonstrates a considerable overlap in results between all three groups, but a trend to lower mean levels in the COPD subjects and highest levels in smoking controls. When all study subjects were considered there was found to be a significant fall from baseline at all time points except day 42. This illustrates a significant increase in airway resistance for 21 days following RV infection in this study population, figure 3.28.

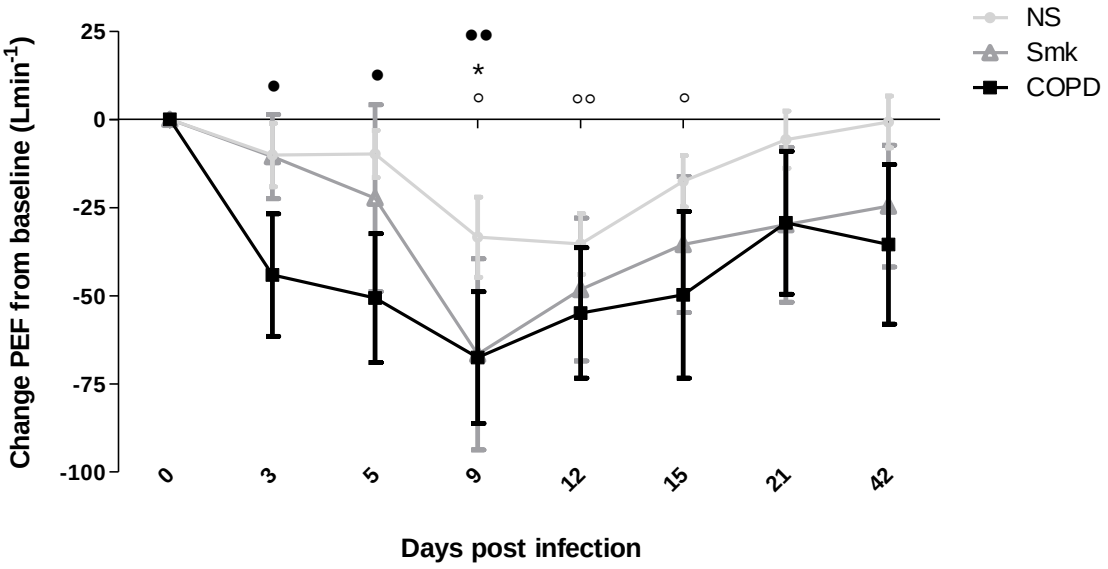


Figures 3.27 Time course of PEF in study groups. Data presented as mean +/- SEM, (one way ANOVA, p>0.05)



Figures 3.28 Change from baseline in PEF in all RV infected study subjects. Data presented as mean +/- SEM, (paired t test change from baseline, *p<0.05, **p<0.01, ***p<0.001)

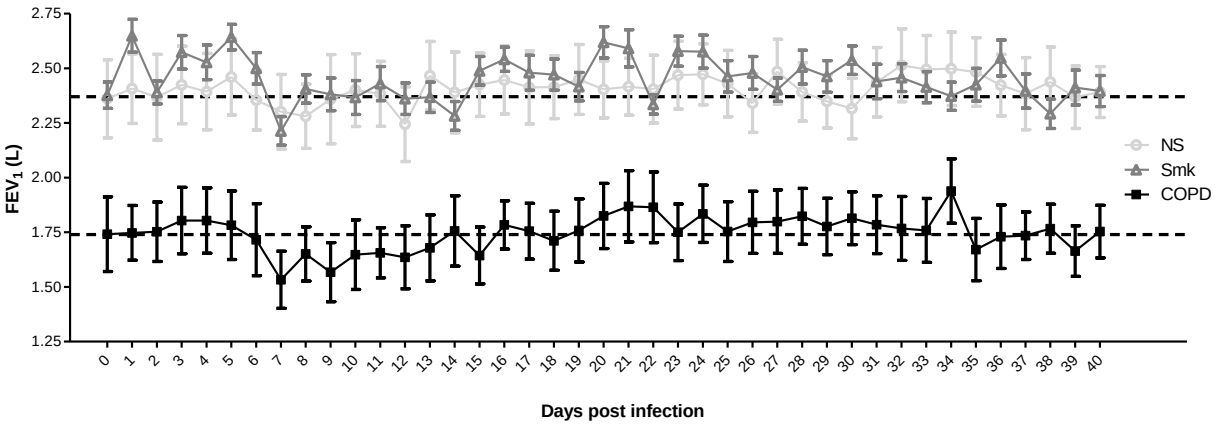
When the change from baseline was analysed in individual study groups the fall was significant in non-smokers on day 9 (p=0.017), 12 (p=0.003) and 15 (p=0.037); in smoking controls on day 9 (p=0.04) and in COPD subjects on days 3 (p=0.043), 5 (p=0.024) and 9 (p=0.007) (paired t test). The overall trend demonstrates greatest fall from baseline in COPD subjects and least in non-smoking controls, figure 3.29.



Figures 3.29 Change from baseline in absolute PEF over time course of study in study groups. Data presented as mean +/- SEM, (paired t test change from baseline, NS °p<0.05, °°p<0.01, Smk *p<0.05, COPD •p<0.05, ••p<0.01)

3.26 Home spirometry

Subjects were asked to perform spirometry daily from recruitment to day 42 of the study using a hand held spirometer. The FEV_1 values recorded from this data are displayed in figure 3.30. The lower FEV_1 in COPD subjects is evident with a decline from mean baseline levels occurring at day 6. It took until approximately day 19 for the mean FEV_1 to return to baseline values in COPD subjects compared to day 8 in control subjects. The baseline levels have been indicated on the figure for control and COPD subjects with a dotted line. The values in control subjects appear to rise in the first week after RV infection. From approximately day 20 they are higher than baseline levels in all subject groups. This data represents interesting trends but was subject to considerable intra subject variation.



Figures 3.30 Time course of FEV_1 values in study subjects recorded from home spirometry. Dashed line represents mean FEV_1 at baseline. Data presented as mean +/- SEM, (one way ANOVA, $p<0.001$ between controls and COPD, $p>0.05$ NS and Smk)

3.27 Sputum total cell counts

Results of all non-epithelial cells counted in sputum samples are displayed in this section. These were counted blind to the infection status of the subjects. The total cell count in all infected subjects combined was significantly higher when analysed using an unpaired t test on days 9 and 12 than in non-infected subjects. There was no difference between the two groups at other time points, figure 3.31.

When individual subject groups were analysed using a one way ANOVA it was found that the mean total sputum cell count was significantly different on days 9, 12 and 15. It was greatest in COPD subjects on days 12 and 15, but there was a trend to higher levels in COPD subjects throughout the study, and lowest in non-smokers. In smoking controls the count was greater than non-smokers only on day 9, figure 3.32.

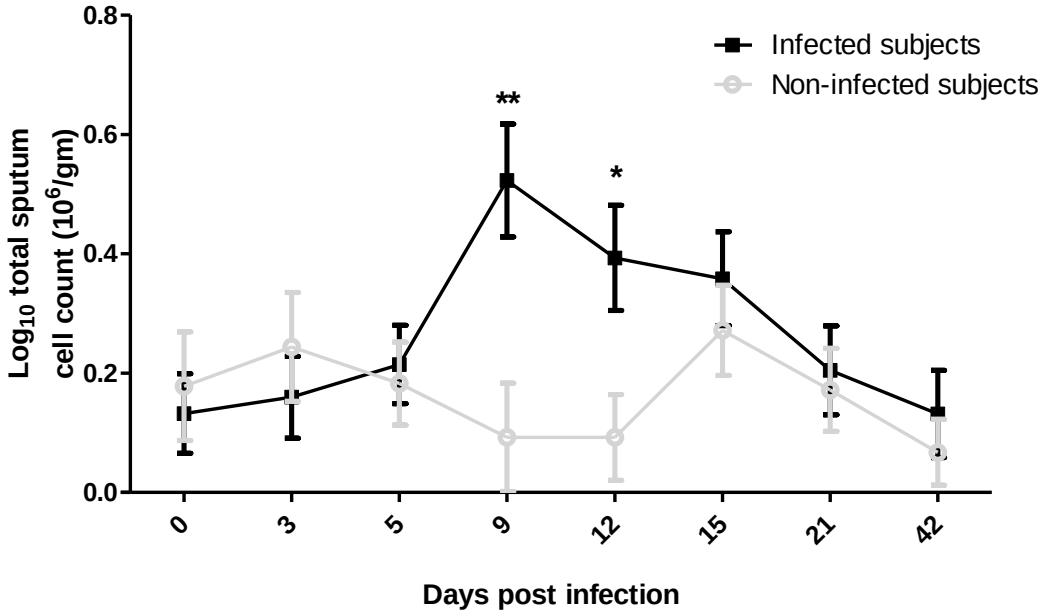


Figure 3.31 Total non-epithelial sputum cell count in RV infected and non-infected subjects. Higher cell counts recorded on days 9 and 12 in infected subjects. Data presented as mean +/- SEM, (unpaired t test, *p<0.05, **p<0.01)

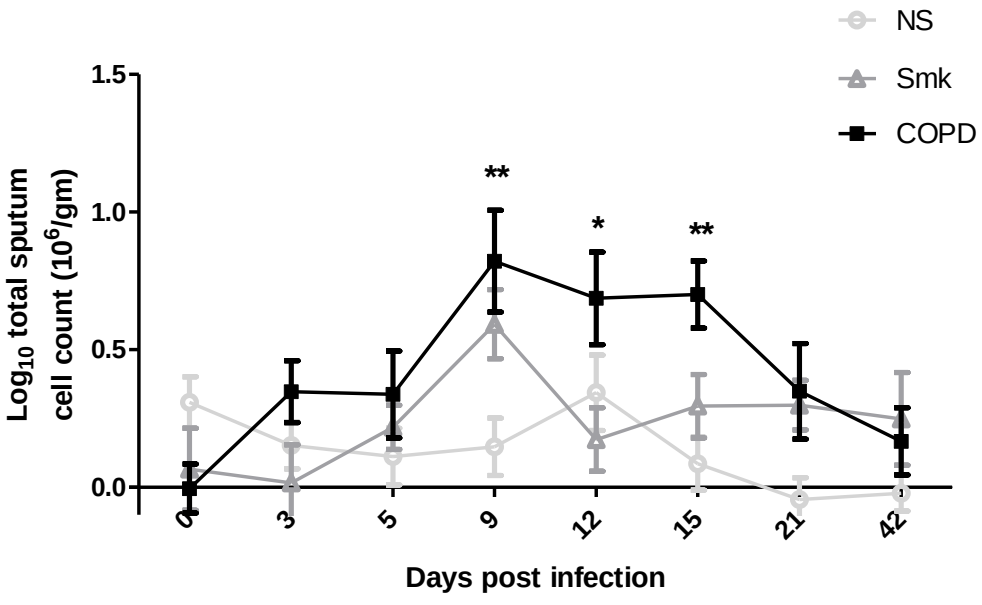


Figure 3.32 Total non-epithelial sputum cell counts in RV infected subjects by study group. Significant mean group difference in counts on days 9, 12 and 15. Data presented as mean +/- SEM, (one way ANOVA, *p<0.05, **p<0.01)

3.28 BAL volume and total cell counts

The volume of BAL fluid recovered during bronchoscopy was analysed to evaluate between or within group differences. Excessive variations in recovery of BAL could lead to confounders in recovery of cells or mediators measured in these samples. The same volume of saline was instilled during each bronchoscopy performed, 240ml. Despite a small trend to lower BAL recovery in the COPD group, there was no significant difference either between groups or within groups at different time points, figure 3.33. Analysis of mediators measured in BAL will be presented later in this work, chapter 5. Methods of correcting for dilution or variation in cell count vary and are not standardised; here they have not been corrected for either the cell count or the BAL volume.

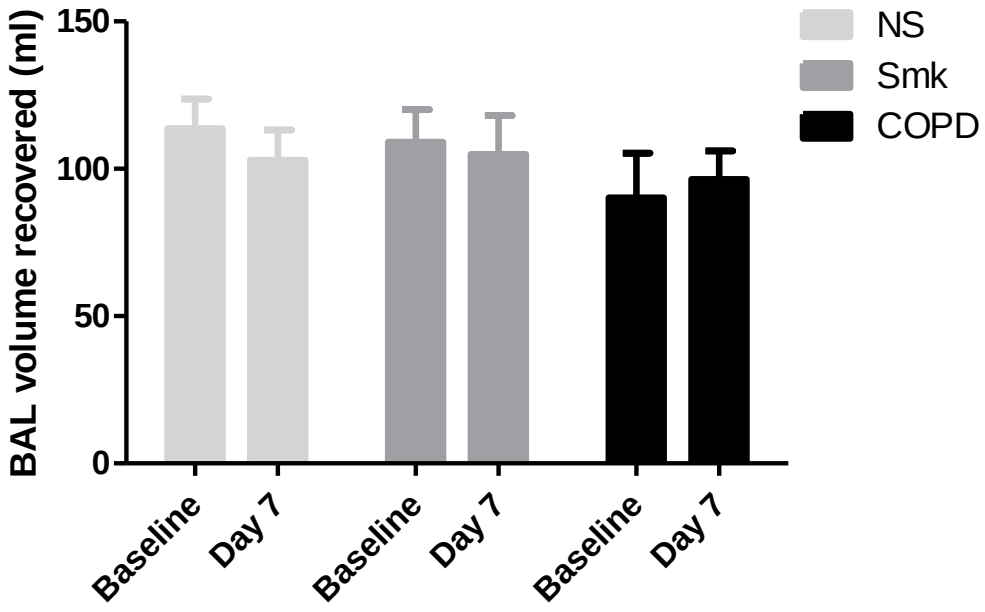


Figure 3.33 Volume of BAL recovered during baseline or acute bronchoscopy in infected subject groups. Data presented as mean +/- SEM. No difference between groups at either time point (one way ANOVA) and no difference at time points in individual groups (paired t test)

BAL cell counts from infected and non-infected subjects were then examined, figure 3.34. No significant difference was found between the infected and non-infected groups at baseline. However on day 7 only the cell count in infected subjects had increased significantly from baseline, ($p=0.012$, paired t test).

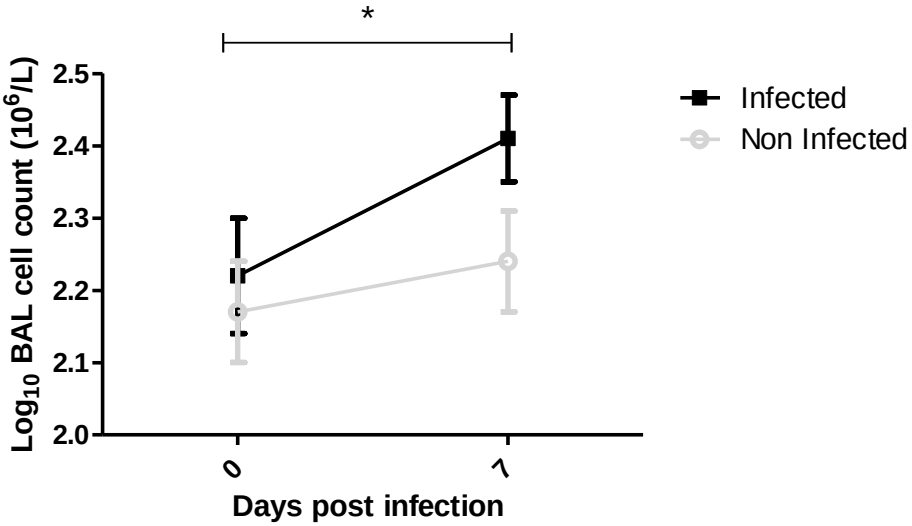


Figure 3.34 Total BAL cell count at baseline and day 7 in all infected and non-infected subjects combined. Cell count increased significantly only in infected subjects. Data presented as mean +/- SEM, (paired t test, *p<0.05)

3.29 BAL cell count by infected study group

BAL cell counts from infected subjects in the three study groups were then analysed to identify between group differences pre- and post infection. There was a difference between the mean cell counts of all three groups at baseline, p=0.034, (one way ANOVA) but not at day 7. Tukey analysis identified that smoking controls had a significantly higher average cell count than non-smoking controls, p=0.019 at baseline, figure 3.35. On day 7 there was a trend to lower counts in the non-smoking controls with similar mean cell count in COPD and smoking subjects.

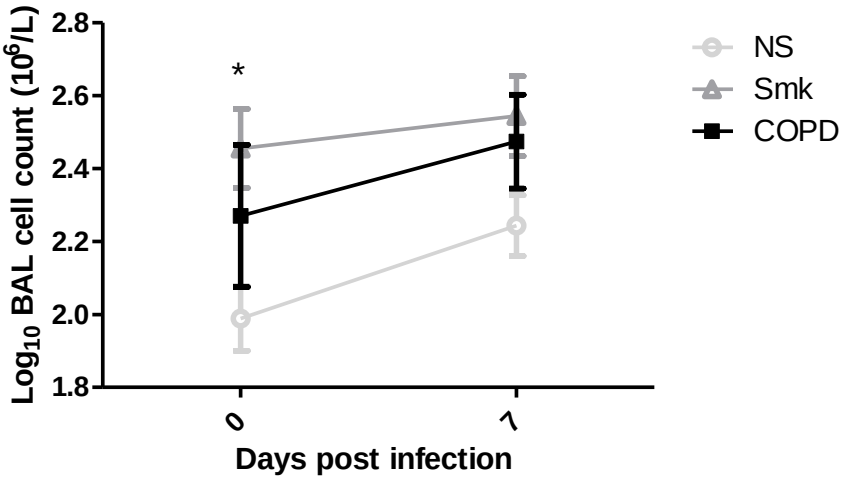
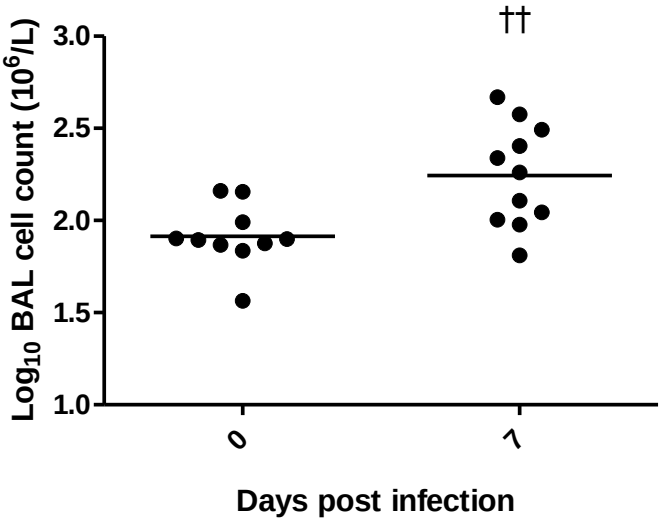
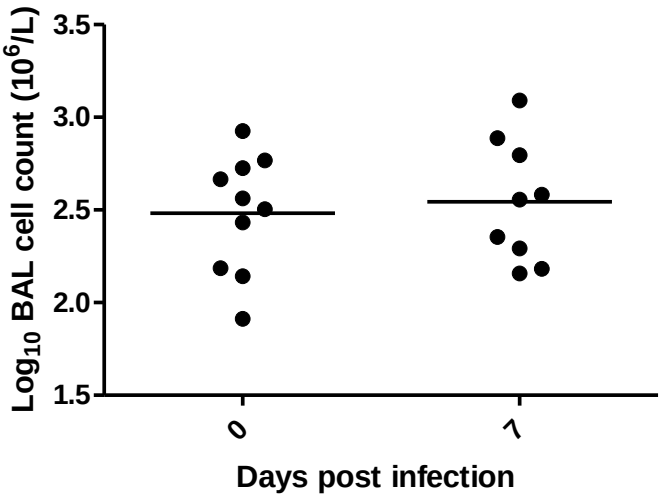


Figure 3.35 Total BAL cell count at baseline and day 7 in infected groups, difference between groups at baseline only. Data presented as mean +/- SEM, (one way ANOVA, *p<0.05)

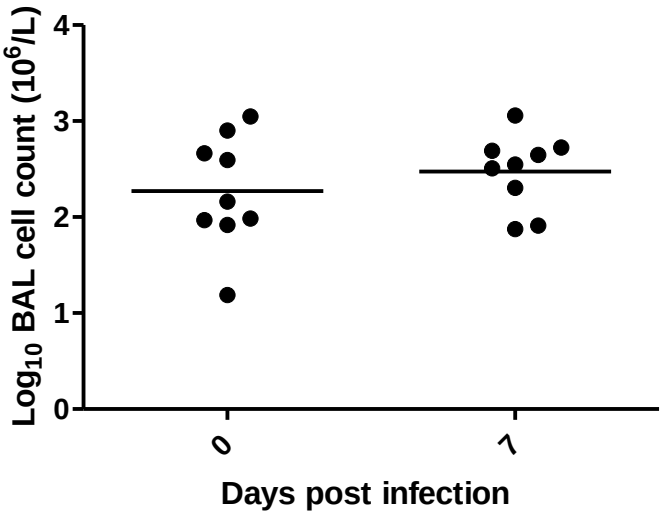
The change in BAL total cell count between baseline and day 7 in individual study groups was then analysed, figure 3.36. There was a significant increase in the mean cell count in non-smoking controls between baseline and day 7, p=0.006. In COPD and smoking subjects there was a small increase in the total cell count at day 7 compared to baseline but this was not significant, (paired t test).



Figures 3.36a Non-smoking controls



Figures 3.36b Smoking controls



Figures 3.36c COPD subjects

Figure 3.36 Total BAL cell count at baseline and day 7 in infected (a) non-smoking subjects, (b) smoking subjects and (c) COPD subjects, significant increase from baseline only in non-smokers. Data presented as mean +/- SEM, (††p<0.01, paired t test)

3.30 Effect of season on infection rates

To investigate if the time of year that subjects were experimentally inoculated with RV16 had an influence on the rates of successful infection, the total number of infected and non-infected subjects were calculated per month, figure 3.37. This data represents the culmination of the period between July 2008 and August 2010. The highest rate of successful infection was achieved during January (3/3 subjects) followed by August (8/11 subjects).

However because there was an uneven distribution of inoculation episodes over the year and to related infection rates to respiratory virus seasons the infection rates were calculated by quarter, figure 3.38. There was no significant difference between quarter 4 & 1 (October to March) and quarter 2 & 3 (April to September) in a chi squared analysis, p=0.29. There was a trend to differences between the groups, with the number of successful infections being greater during the spring and summer, and a small excess of unsuccessful infections occurring during autumn and winter.

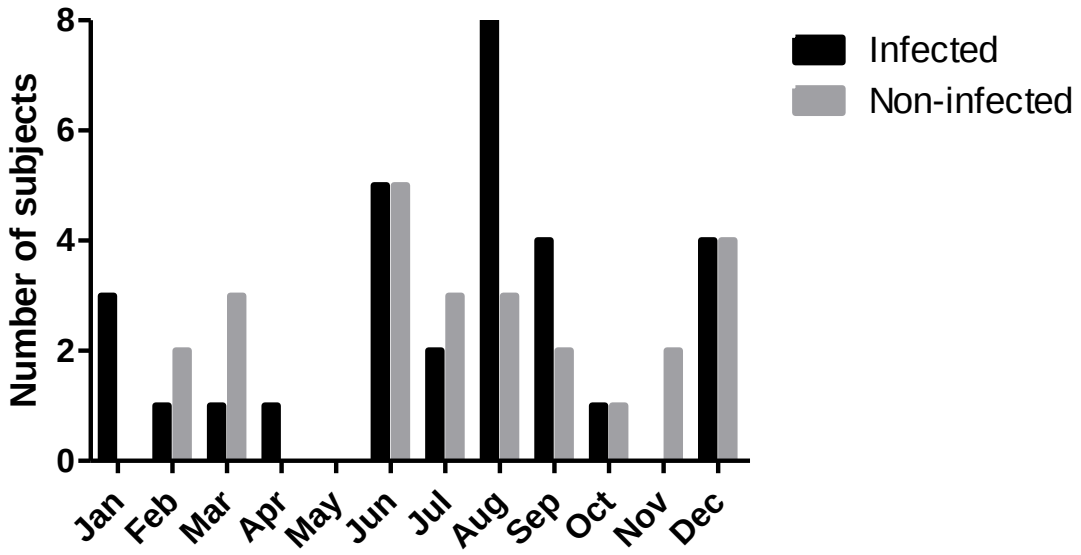


Figure 3.37 Number of successfully infected and non-infected subjects by month of experimental RV16 inoculation

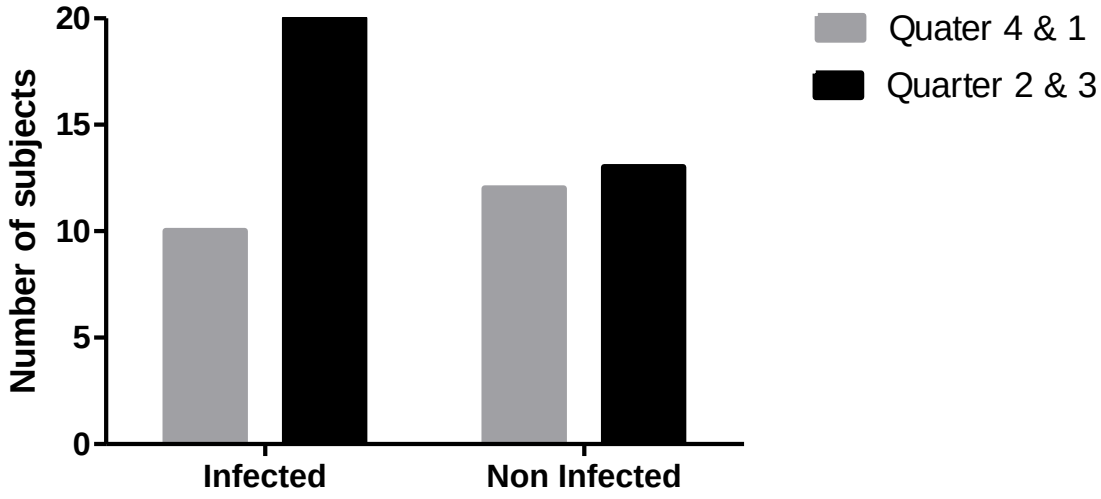


Figure 3.38 Total number of infected and non-infected subjects inoculated with RV16 by season, October to March (quarter 4 & 1) and April to September (quarter 2 & 3), (p=0.29, Chi square)

3.31 Chapter discussion

In this chapter results for a considerable amount of demographic, symptomatic and lung function data has been presented. The overall screening and recruitment statistics for the study are also shown. This illustrates the large number of subjects who were screened to take part in the study but were subsequently not eligible. Although there was a large dropout rate between screening and inoculation, the study population met the inclusion criteria and are representative of the study groups defined. The demographic data presented for successfully infected subjects shows that the COPD and control groups were age and sex matched although between control groups the non-smoking subjects were older than smoking controls. There was no difference in baseline spirometry between the two control groups. The mean FEV_1 at baseline in the COPD group was 2.08L (69% predicted), demonstrating the relatively mild disease severity of this group. The criteria for defining successful RV infection has been maintained from previous studies[164, 291], although due to variations in qPCR technique the lower limit of RV detection was greater in this study. It is not possible to define infection in an experimental study with complete certainty, but there are several reasons to be confident that the definition used in this study was accurate. Those subjects judged to have been infected all met more than one of the parameter for defining infection and the majority met them all. When independent makers such as total sputum cell count were analysed, they were found to be significantly greater in the infected subjects compared to non-infected subjects. This provided further evidence of the accurate definition of RV infection in the study.

Although not intended to form a sham infection group, the non-infected subjects can provide evidence of potentially confounding factors involved in the study; such as the placebo effect of experimental infection, nasal inoculation and the sample collection procedures involved in the project. The factors which may have predisposed subjects not to become infected with RV have not been specifically examined in this study; however there are no demographic, symptomatic or spirometric differences between these groups to suggest a mechanism for resistance to infection.

3.32 RV infection leads to increased upper and lower respiratory tract symptoms

Initially symptom scores were analysed in five two week blocks over the entire time course of the study. This was done to minimise ‘noise’ due to day to day variation in scores and to enable comparison of the effect of key study events between time

periods. Specifically the pre-baseline scores represent a period of stable symptoms with no study interventions. The baseline period included the first bronchoscopy and the acute period incorporated RV challenge, bronchoscopy and sampling in clinic. Recovery and convalescent periods included only occasional clinic sampling. As both the successfully and unsuccessfully infected subjects underwent the same procedures the effect of RV infection on upper and lower respiratory tract symptoms can be compared. Additionally comparison between time periods within groups makes assessment of the potential impact of a study procedure possible. Finally this provides justification for only correcting URT scores for the impact of bronchoscopy on day 7, because when non-infected subjects were considered there is an increase between pre-baseline and baseline URT scores not seen in LRT scores.

Only during the acute period was a difference seen in symptom scores between infected and non-infected subjects, with statistically greater upper and lower respiratory tract scores in infected subjects compared to non-infected. This provides additional evidence that the infected and non-infected groups have been correctly defined, as LRT scores did not form part of the criteria for defining infection and no subject was defined as being successfully infected or not only on the basis of URT scores alone. The validity of the scoring systems used is also supported by this finding as subjects in which RV was not detected had significantly lower scores than in those who it was.

3.33 Upper and lower respiratory tract symptoms in non-smoking controls

This is the first RV challenge study of COPD subjects to include a non-smoking control group. Analysis of the differences in symptom scores between these two groups has not been possible previously. When the two week block data for URT scores was analysed there was no statistical difference between these two groups at any time point. However there was a trend to higher scores in COPD subjects in every two week block. URT symptoms have been reported in COPD although are not a well recognised feature of the disease. A study in Sweden randomly screened approximately 8500 subjects in the population with a postal questionnaire and reported nasal symptoms in 33% of those who replied, amongst self reported asthmatics and COPD patients this rose to 45% and 40% respectively[305]. Two later studies in the East London COPD cohort reported nasal symptoms of 75% and 88% in COPD subjects, although these studies lacked a control group, the authors were able to conclude that nasal symptoms are common in COPD patients and have an adverse impact on quality of life[306, 307]. Interestingly it has been reported that

the incidence of nasal symptoms increase with age[308], and these symptoms typically occur as a result of degenerative changes, comorbidities or medication side effects. The background rate of URT symptoms reported by non-smokers in the study presented here may reflect the increased age in this group and the findings by Montnemery *et al* of nasal symptoms in up to a third of the population. When non-infected non-smoking controls are reviewed it is again evident that bronchoscopy has a higher than expected impact on URT symptoms, indicated by the increase from pre-baseline to baseline scores. The effect of alternate day nasal lavage and induced sputum is noted by comparing baseline to acute time periods, and this had little impact on total scores. If this is extrapolated to the infected subjects it offers support to the hypothesis that the increase between baseline and acute periods is due to RV infection to the greatest extent and not the sampling procedures per se. The main limitation of these conclusions is that the potential placebo effect on subjects of RV infection cannot be measured, and may have contributed to an excess of symptom reporting. Post RV infection scores over the full time course of the study show no statistical difference in URT symptoms between non-smoking controls and COPD subjects. Between days 4 to 7 the trend is for mean score in non-smokers to be greater than COPD subjects but at all other time points in the study this pattern is reversed.

LRT scores in non-smokers were less in all two week blocks compared to both other groups, and statistically less that COPD subjects during the acute, recovery and convalescent time periods. When the daily LRT scores were studied there was a lower mean score in non-smoking controls throughout the full time course of the study and this was significantly reduced on days 2, 3, 15, 18 and 19 compared to smoking controls and COPD subjects. The data presented in this chapter therefore confirms previous findings in RV challenge studies in COPD of exaggerated symptoms consistent with an exacerbation compared to smoking controls[164]. However the inclusion of a non-smoking control group provides additional information, including evidence that LRT symptoms are exaggerated and prolonged in non-obstructed cigarette exposed subjects compared to non-smokers.

3.34 RV infection increased lower respiratory tract symptoms only in COPD subjects

When symptom scores in two week blocks was analysed, the total LRT scores had a trend to be greater in the COPD subjects at all time points in infected and non-infected subjects. The scores were significantly higher in COPD subjects compared

to non-smoking controls in the acute, recovery and convalescent periods for both infected and non-infected subjects. The difference between these two groups in non-infected subjects would not be predicted, but may be explained by several factors. There is a modest increase in LRT scores in the COPD group between baseline and the acute period which is likely to reflect the influence of frequent sampling and the scores then remain unchanged until the end of the study. However in non-smoking controls the trend is for scores to fall from baseline to convalescence, reflecting minimal effect of frequent lower airway sampling in non-smokers compared to controls. In addition subject numbers are even smaller in the non-infected group, making interpretation of the findings less reliable.

In those subjects who were infected successfully, only during the acute period was the LRT scores significantly higher in the COPD subjects compared to both control groups. Additionally only COPD subjects had a significant increase in symptoms between the baseline and acute time periods. In combination this data supports the hypothesis that RV infection leads to increased LRT symptom scores in COPD subjects and not controls, and that during a two week period after infection features of an exacerbation are present. These two findings were not present in the non-infected subjects.

There are potential limitations to self reported symptom score data. Subjects may not report symptoms consistently over the duration of the study. Initial enthusiasm or close attention to symptoms in the early part of the study may lead to over reporting, but at later time points fatigue or familiarity with symptoms may lead to under reporting. Individual subjects perception of symptoms is likely to vary considerably and despite attempts to standardise the scoring system with written definitions there was evidence from individuals diary cards of wide variations in symptom scoring. The total number of successfully infected subjects was lower than anticipated and the small numbers are likely to have an impact on the reliability and reproducibility of symptom scores, also reducing the opportunity to identify statistically significance differences. Given these limitations it is possible to conclude that LRT symptom scores increase only between the baseline and acute period in COPD subjects and that this provides symptomatic evidence of an exacerbation similar in nature to those reported in naturally occurring exacerbations.

In addition data presented in chapter 4, Virology and Bacteriology, demonstrated that both upper and lower respiratory tract symptom scores correlate strongly with virus

load in nasal lavage and sputum respectively. Although discussed in detail later, this adds considerable biological credibility to the symptom scores described in this chapter.

3.35 RV infection resulted in impaired spirometry

Formal lung function testing was performed on one occasion after RV infection and did not demonstrate differences compared to baseline in COPD subjects. The absolute falls in lung function tended to be greater in smoking subjects; both in airway obstruction (FEV_1/FVC ratio) and FEV_1. However when clinic spirometry was considered, only the COPD group demonstrated a significant fall in FEV_1 compared to baseline levels, with absolute falls of 146ml, 194ml and 183ml on days 5, 9 and 15 respectively. This was identified on days 5, 9 and 15 and demonstrates the benefit of repeated longitudinal measurements in this study. Interestingly the FEV_1 in COPD subjects fell significantly from baseline prior to the bronchoscopy on day 7, offering supporting evidence that any subsequent changes were not the result of sample collection procedures – specifically repeated nasal lavage, induced sputum and a bronchoscopy.

Peak flow fell significantly compared to baseline in all RV infected groups on day 9. In COPD subjects the PEF was reduced at earlier time points (days 3 & 5) by 44 and 50 $Lmin^{-1}$ respectively and the maximum fall compared to baseline occurred on day 9, with a mean decline of 67$Lmin^{-1}$. Only non-smokers had falls in PEF at later time points. The relationship between changes in PEF and FEV_1 in the COPD subjects raises further questions. It is possible that earlier falls in PEF may represent changes in larger airways followed by small airway abnormalities and reduced FEV_1 later in the time course. There was a trend to falls in FVC from baseline in smoking and COPD subjects and an increase in non-smokers. The peak fall in COPD was 210ml on days 9 and 15 and 190ml in smoking subjects on day 9.

Data collected from home spirometry contained considerable daily intra subject variation, and therefore its validity could be questioned. However it is seen that the trend is for greater and more prolonged fall in FEV_1 only in COPD subjects in data collected this way. In control subjects the values either remained similar or increased compared to baseline. It is not clear why spirometry results recorded in a domiciliary and clinic setting should vary so much, other than due to the supervision and encouragement of clinical staff, but this has limited further analysis of this data.

Overall the spirometric data from this study supports the concept that experimental RV infection results in a fall in peak flow in all subjects and markers of obstruction in smoke exposed subjects. Additionally as only the COPD group had a significant fall in FEV_1 from baseline levels these subjects have spirometric features consistent with an exacerbation after RV infection.

3.36 Total sputum and BAL cell counts - Confirmation of successful infection

Comparing the total non-epithelial sputum cell counts in infected and non-infected subjects demonstrated significantly higher counts on days 9 and 12 in infected subjects. In BAL there was a significant increase in cell count between baseline and day 7 samples only in RV infected subjects. Total cell counts in these samples did not form part of the criteria for defining successful RV infection and therefore the finding of increased cell counts only in infected subjects adds independent support to the definition used for successful RV infection. This conclusion is based on the assumption that higher cell counts would be found in RV infected subjects reflecting increased inflammation in these subjects compared to the non-infected.

3.37 Sham RV infection

Although not initially intended to form a control group – the non-infected subjects can be considered as a sham infection group as they underwent identical sampling and study procedures to those later found to have been infected. Identification of successful infection was performed after subjects had completed the study and so subjects and investigators were effectively blinded to infection status during study execution. A weakness of previous virus challenge studies has been the lack of a sham infection control group; studies have typically disregard non-infected subjects from analysis of the primary outcome[164], although others have included a saline control arm[309]. However the increase of approximately 1 point in URT and LRT symptom scores between days 0 and 7 in non-infected subjects demonstrates the considerable perception of symptoms by study participants. Enrolled subjects were highly motivated and their awareness of symptoms is likely to have been heightened during this period, which may have contributed to over reporting or perception of symptoms.

The study protocol did not include a sham infection group from the outset and non-infected subjects were not randomised to receive active RV16 inoculum or not. Important limitations should be considered before regarding these subjects as a reliable control group. There may be unmeasured environmental or genetic factors

which have induced resistance to infection, and therefore made comparison of biological markers with infected subjects unreliable. Previous studies have reported differences in susceptibility to respiratory virus infection measured by many factors including psychological stress[309], sleep efficiency[310] and alcohol consumption[3], illustrating the range of potential confounding factors.

Despite the potential limitations discussed of using non-infected subjects as a control group future work to investigate potential mechanisms of resistance to RV infection might be justified. The inclusion of a double blinded sham control group in the protocol of future studies would enable comparisons with successfully infected subjects. This would be particularly useful to investigate biological responses to RV infection while eliminating potential confounding factors from sample collection, perception of illness and bias from study investigators, but necessitates the use of considerable extra time, finances and personnel.

3.38 Sputum total cell counts

When the sputum cell count between groups was examined in the infected subjects it was found that the COPD group had a trend to higher levels throughout the entire time course. There was a significant difference between means of the three groups on days 9, 12 and 15; these were significantly greater in COPD subjects on days 12 and 15. This reflects a greater burden of lower airway inflammation in these subjects, and offers further evidence of features of an exacerbation in COPD subjects following RV infection. Only in non-smoking subjects did the BAL cell count increase significantly between baseline and post infection sampling. This group had the lowest counts at baseline, and the increase is likely to reflect the normal immune response to infection.

Comparison with published data

3.39 Lower RV infection rate than anticipated

A greater than expected number of subjects who entered the study were not subsequently defined as being successfully infected with RV16. In the previous RV challenge study of COPD subjects the successful infection rate was 88%[164], compared to a rate of 58% in this study. The reason for this difference is not known, and may be multifactorial. These could include differences in inoculum titre or effectiveness of delivery, or may reflect variations in host immunity in a separate group of subjects.

The method of experimental inoculation was the same in both studies; an identical atomizer (DeVillbiss 286, UK) was used and the technique of experimental infection was the same, although performed by different individuals. The successful infection of subjects was evenly distributed throughout the whole recruitment period, suggesting that there was not a delay in learning or becoming proficient in the technique of inoculation. The RV16 inoculum used in both studies was prepared from the same stock. However this has undergone a series of dilutional steps in order to define the lowest infective dose[291]. These aliquots were stored in normal saline at –80^{o}C for a period of years and although original RV16 stock had been stored for decades at –80^{o}C this had been in a glycerol based vehicle. The differences in storage methods may have led to degradation of the inoculum over time, thus contributing to reduced infection rates in this study.

The definition of successful infection and the qPCR technique was maintained between both studies; however there are other possibilities for the discrepancy between infection rates. Although RV16 serology was tested in all subjects prior to inoculation, a different batch of *in vitro* RV16 was used to test serology between studies and this may have resulted in incomparable titres being recorded. Additionally a degree of immune cross reactivity may exist between RV serotypes, it is therefore possible that seasonal RV immunity derived from naturally occurring colds conferred increased immunity on the subjects recruited in the more recent study. Finally it is not known if the season in which experimental RV inoculation is performed has an impact on the rates of successful infection. Data presented in this chapter did not identify a statistically significant difference between infection rates and the seasons, but there was a trend to higher infection rates during spring and summer suggesting that successful infection maybe more difficult during the time of year when community respiratory virus infections are more common. This indicates that an effect similar to herd immunity may be contributing during periods of high respiratory tract infections in the community.

3.40 Inclusion of non-smoking control subjects

This is the first RV challenge study performed in COPD subjects which has included a non-smoking non-obstructed control group. Healthy non-smoking subjects have participated in RV challenge studies of asthma[290, 293] and to investigate the mechanisms[3, 309] and treatments[311, 312] of the common cold. However few studies have enrolled subjects over the age of 50 years or included a population of the age range recruited to this study (40 to 75 years). The mean age of non-smoking

controls was 62 years and therefore older than those in most previous RV challenge studies. It is known from community based studies of respiratory virus infections that the elderly experience an increased burden of symptoms and mortality compared to younger subjects[313, 314]. The data in this chapter reporting increased LRT symptom scores and falls in spirometry following RV infection is supported by published studies. However this data is the first to enable an accurate description of the time taken for symptoms to return to baseline levels and a comparison with otherwise healthy smoking subjects.

The inclusion of a non-smoking control group in this study adds power and enables the investigation of the confounding effects of cigarette smoke exposure. It is recognised that individuals who smoke experience increased symptoms following respiratory virus infection compared to non-smokers[2-4]. Comparisons between the non-smoking and smoking control group in an otherwise well matched population enables evaluation of the effect of cigarette exposure follow RV infection. Surprisingly LRT symptoms reached a similar peak in the early time points following inoculation, but tended to return to baseline more quickly in non-smoking controls.

Additionally comparison between the non-smoking control group and COPD subjects enables investigation of the effect of RV infection in older subjects with no respiratory illness and those with a serious respiratory disease, effectively allowing direct comparison between the symptoms of a common cold and an exacerbation. Because smokers experience an excess burden of symptoms, non-smoking subjects are arguably a more appropriate control group for comparison with COPD subjects, particularly those who are ex-smokers. In this study 8 of 9 COPD subjects and all smoking controls were active smokers. Due to the time taken to recruit subjects, no attempt was made to manipulate this fraction. However if greater time and financial resources were available, the inclusion of matched ex-smoking COPD and non-obstructed ex-smokers with active smoking COPD and non-obstructed subjects would be preferable. Comparison between the groups would enable analysis of the effects of smoking and remove a potential confounding factor from the present study protocol.

3.41 Lower respiratory tract symptom scores

There is only one published study with similar longitudinal symptom data to that presented in this chapter[164]. LRT scores were significantly greater in COPD subjects than smoking controls in both studies following RV infection. However the

work presented is chapter has the additional benefit of an age matched non-smoking control group. Comparison with this group has identified an increased LRT symptom burden in non-obstructed smokers, demonstrating the adverse effect of cigarette smoking on health status following RV infection. Particularly as in this study non-smoking controls were older than smoking controls. Observational studies have identified similar findings[2, 4], as has a respiratory virus challenge study[3].

It is of note that LRT symptoms were reported in control subjects following RV infection, albeit at a lower rate than in COPD subjects. Age is likely to contribute to this finding; community studies of RV infection in subjects over 40 years have reported LRT symptoms at a rate of approximately 65% compared to 34% in subjects under 40[264, 314]. In the data presented in this chapter the COPD and individual control groups were age matched, but in the study by Mallia *et al* COPD were older than smoking controls. This may represent another reason for more modest between group differences identified in the present study compared to Mallia[164].

The similarity between published data and that described here provides evidence that this group of experimentally infected COPD subjects experienced an exacerbation. The LRT scores in these subjects increased following RV infection beyond normal day to day variation, which forms an integral part of commonly accepted exacerbation definitions[12]. Specifically most published COPD exacerbation studied use symptomatic definitions modified from Anthonisen criteria[296], namely an increase in two major criteria (dyspnoea, sputum purulence or sputum volume) or one major and one minor criteria (nasal discharge, wheeze, sore throat, cough or fever) for at least two consecutive days[56, 66, 246, 252, 315, 316] and in patients admitted to hospital[317-319]. The subjects in this group experienced significant increases in breathlessness, cough, wheeze and sputum production following RV infection and it can therefore be concluded that they experienced LRT symptoms similar in effect to those occurring in naturally occurring exacerbations.

3.42 Spirometry

There are few published studies which present prospective spirometry data from an exacerbation and many use a time point following the exacerbation as a surrogate for baseline. Additionally variations in spirometric methodology exist, with both domiciliary and clinic data or pre- and post bronchodilator results published. These factors make accurate comparison with the results presented in this chapter difficult.

The range of fall in FEV_1 in published studies is from approximately 25 to 275ml depending on the severity of the exacerbation[246, 318, 320-322].

Interestingly two studies of hospitalised patients identified similar changes to those presented here, with an FEV_1 fall of 170ml and peak drop on day 14[320, 322]. So it can be concluded that the changes in FEV_1 identified in this study are broadly similar to those found in published studies.

In this experimental RV challenge study PEF fell by a maximum of 67$Lmin^{-1}$ on day 9 in the COPD group, this is larger than in most published studies, where PEF falls of up to 9-36$Lmin^{-1}$ during exacerbations have been reported[218, 246, 323]. In the only previous RV challenge study in COPD the PEF fell by 121$Lmin^{-1}$ on day 9 in COPD subjects[164]. However the baseline peak flow was greater in this study population. The effect of bronchodilators and the time point of sampling during the course of the exacerbation again makes peak flow data difficult to compare; but the falls in this study population are greater or similar to those in the published literature.

FVC fell by a similar amount in smoking controls and COPD subjects in this study; this is similar to changes reported in a RV challenge study with identical methodology. Mean falls reported in other studies are around 350ml to 530ml[322, 323], but these probably reflect more severe exacerbations than those induced in this study.

Overall the changes described in spirometry in this study are similar to those in published studies; this supports the conclusion that the COPD subjects in this study experienced an exacerbation following RV challenge.

3.43 Sputum and BAL counts

Sampling of the lower airway during naturally occurring COPD exacerbations is problematic, particularly because mild episodes can be difficult to identify in the community and those with more severe disease are often too unwell to undergo sputum induction and particularly bronchoscopy for research purposes alone. Where sputum sampling has been performed in the research setting, results are contradictory. In some studies no increase in cell numbers from baseline to exacerbation have been identified[218, 219] while in others an increase at the time of exacerbation has been found[140, 324].

No consensus on the nature of cellular inflammation in exacerbations has emerged, although the majority have reported neutrophilia[1, 217, 220], others have identified eosinophilia[1, 228]. This is perhaps surprising given the burden of disease from COPD and the number of studies in the literature. The finding reflects the intrinsic heterogeneity of COPD and the broad spectrum of current COPD definitions. Two other factors which are likely to have a particularly potent effect on the nature of cellular inflammation identified during an exacerbation are the broad ranges of aetiologies and exposure to treatments, particularly inhaled steroid therapy, both pre- and peri- exacerbation. However the finding of significant increases in sputum cell count in this work reflects increased inflammation in the lower airway and is broadly supported by findings of increased inflammation in the airway reported in the literature. In BAL this study identified an increase in cell numbers in all infected subjects after RV exposure, but only in non-smoking controls was there a significant increase with a trend to increasing numbers in smoking and COPD groups. Published studies have reported an increase in BAL cell numbers during exacerbation compared to stable state[96, 145]. The lack of statistically significant increase in BAL total cell count from baseline to exacerbation in this study is likely to reflect the small sample size, and the trend to increased numbers is supported by those findings in published literature.

3.44 Conclusion

In this chapter it has been shown that a group of study subjects were successfully infected with RV after experimental challenge. Within this group 9 COPD subjects developed symptomatic, spirometric and inflammatory changes consistent with those of a naturally occurring exacerbation.

The work supports previous studies that have identified RV as a potential aetiological factor in AECOPD. There was an increase in severity and duration of LRT scores in COPD subjects compared to controls, with greater falls from baseline in spirometry in these subjects. The magnitude of these was similar to that in published studies. The results presented here also demonstrate increased lower airway inflammation in COPD subjects compared to controls.

When compared to published data, the features of an exacerbation presented in the chapter are broadly similar and support the conclusion that data obtained from subjects in this model can be considered comparable to that which would be found in naturally occurring RV induced exacerbations.

Chapter 4: Virology and bacteriology

4.1 Introduction

In this chapter I present results of RV detection by quantitative PCR (qPCR) in NL, sputum and BAL and detection of bacteria in sputum samples. The association between these will be examined and correlations between RV load and symptoms, lung function and sputum cell count illustrated. The hypothesis examined is that RV load is greater in COPD subjects compared to controls and that this leads to an increased incidence of secondary bacterial infection with exaggerated respiratory symptoms and inflammation in COPD subjects.

Results

4.2 Virus load in all infected subjects

When all successfully infected subjects were examined; the virus load was found to peak on day 3 in nasal lavage and fall at each time point until returning to baseline levels by day 21. In sputum, the peak virus load occurred on day 9, with levels also returning to baseline by day 21. Figure 4.1 illustrates the relationship between sputum, nasal lavage and BAL virus load, with peak levels of 7.40, 5.88 and 3.80 log_{10} copies/ml respectively. There was a delay of 6 days between sampling time points for peak levels in the upper and lower airway. This may reflect the time taken for RV to infect the lower airway after nasal replication. The magnitude of virus load overall was greatest in sputum. The lower limit of virus detection (log_{10} copies/mL) was 4.7 in sputum, 2.9 in nasal lavage and 1.1 in BAL; this reflects the dilution of samples during processing and analysis.

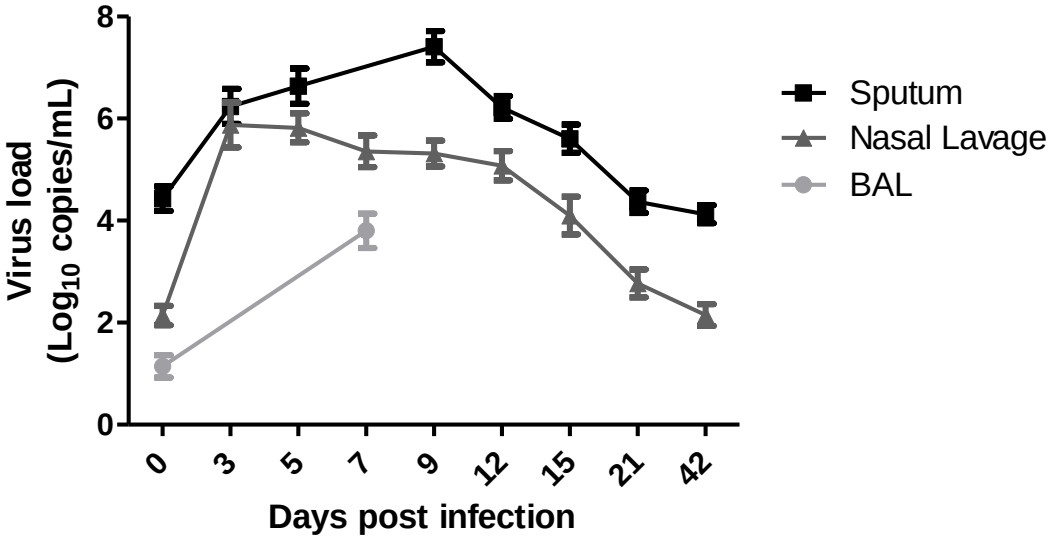


Figure 4.1 Time course of virus load in sputum, nasal lavage and BAL of all successfully infected subjects. Data presented as mean +/- SEM

Figure 4.2 and 4.3 show nasal lavage and sputum virus load over the time course of the study in all infected subjects. They demonstrate a significant increase from baseline levels on every time point sampled until day 15. By days 21 and 42 the virus load is unchanged from baseline levels in both nasal lavage and sputum.

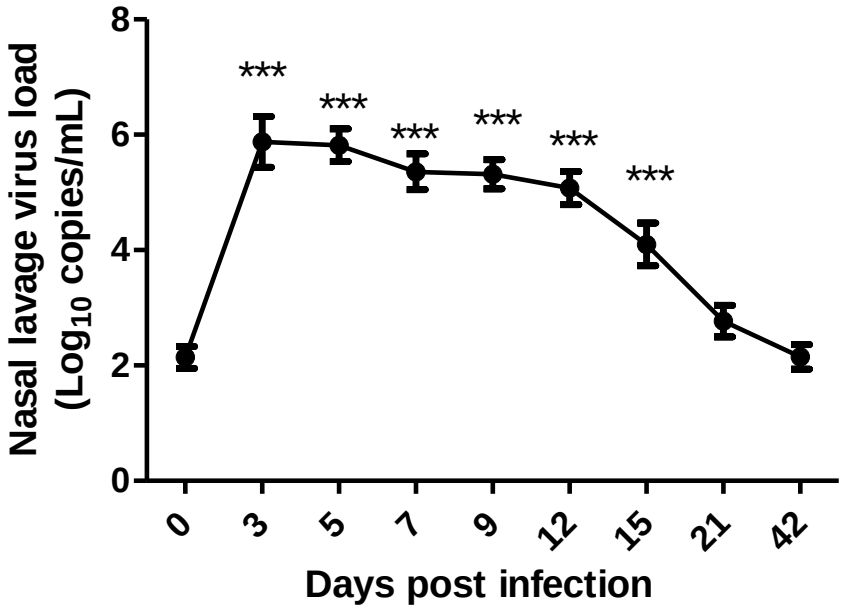


Figure 4.2 Time course of virus load in nasal lavage of all successfully infected subjects. Significant increase when compared to baseline at time points shown. Data presented as mean +/- SEM, (paired t test, ***p<0.001)

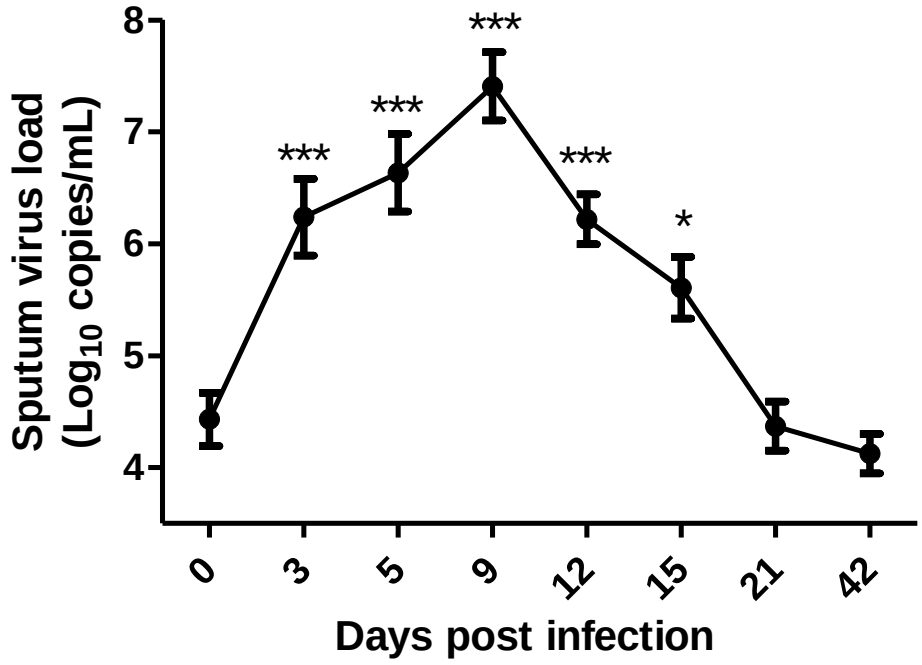


Figure 4.3 Time course of virus load in sputum of all successfully infected subjects. Significant increase compared to baseline at time points shown. Data presented as mean +/- SEM, (paired t test, *p<0.05, ***p<0.001)

Two time points are available for bronchoscopy shown in figure 4.4. The virus load was found to increase significantly following RV infection, from mean (SEM) of 1.16 (0.23) to 3.89 (0.34) Log_{10} copies/mL, (p<0.001).

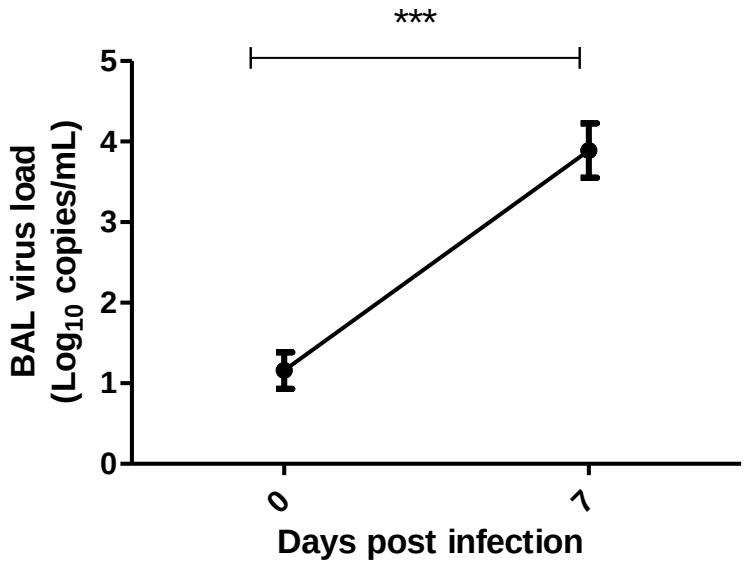


Figure 4.4 Pre and post inoculation virus load in BAL of all successfully infected subjects, significant increase compared to baseline on day 7. Data presented as mean +/- SEM, (paired t test, ***p<0.001)

4.3 Nasal lavage virus load in study groups

When the individual study groups were examined the pattern of virus load in nasal lavage was found to be similar between COPD subjects and controls, figure 4.5, and showed no statistical difference between groups.

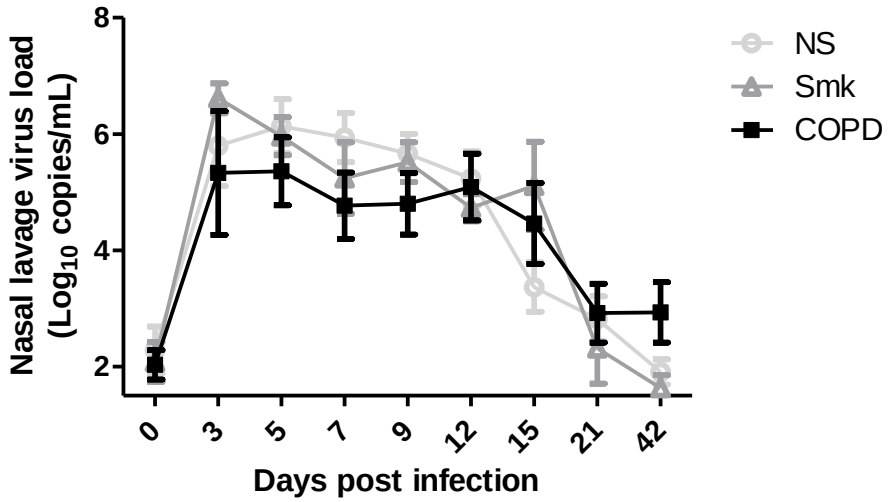


Figure 4.5 Time course of virus load in nasal lavage of all successfully infected subjects by study group. Data presented as mean +/- SEM, (one way ANOVA, p>0.05)

Figure 4.6 presents the change from baseline in nasal lavage for virus load in the individual study groups. There was a significant increase in nasal lavage virus load in the non-smokers from days 3 to 12 inclusive, with peak virus load detected on day 5. In both smoking controls and COPD subjects the virus load was increased from days 3 to 15 inclusive; representing a delay of a three day interval for virus clearance compared to non-smoking controls. The peak level occurred on day 3 in the smoking group and day 5 in COPD subjects.

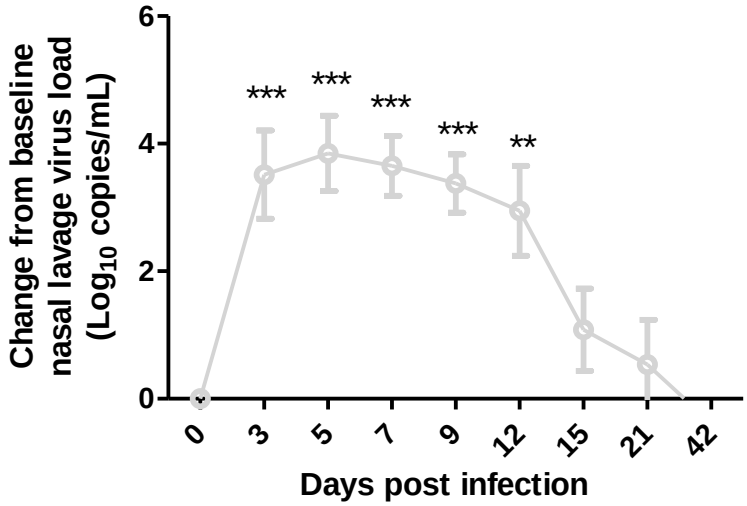


Figure 4.6a Non-smoking controls

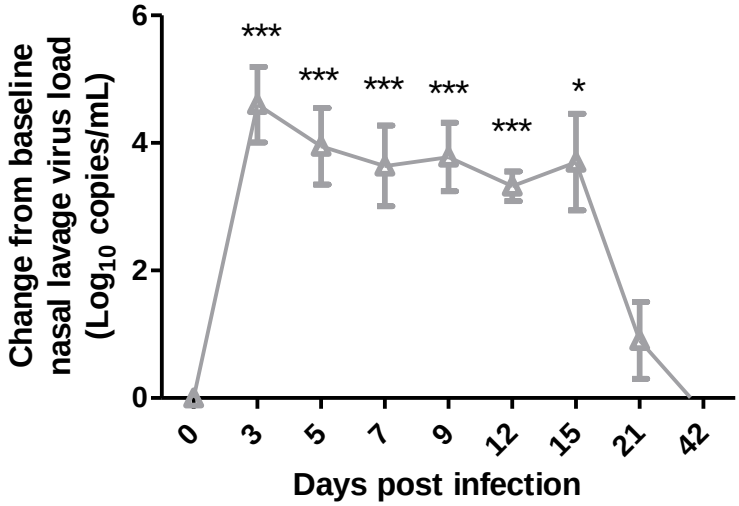


Figure 4.6b Smoking controls

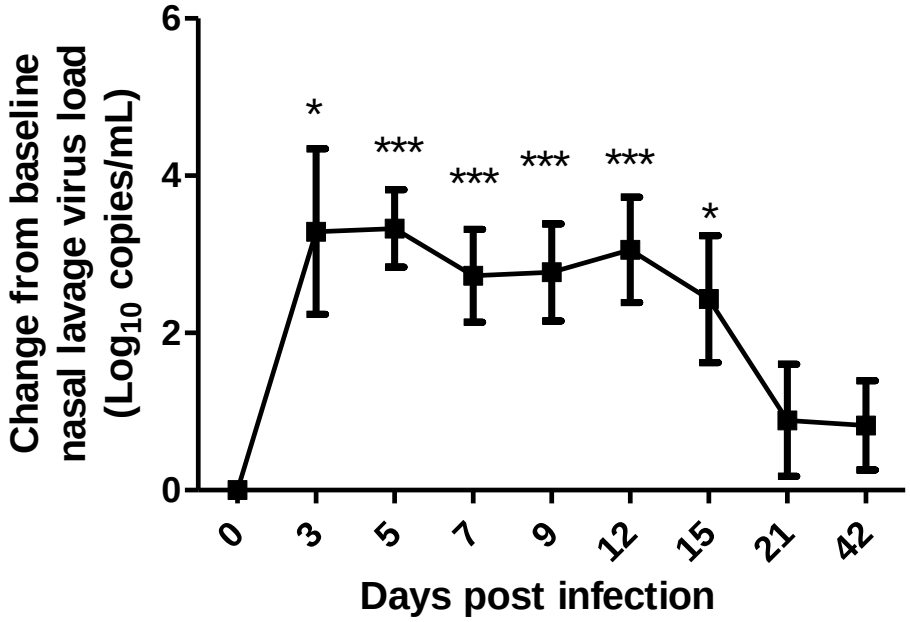


Figure 4.6c COPD subjects

Figure 4.6 Time course of change from baseline in virus load from nasal lavage in (a) non-smoking controls, (b) smoking controls and (c) COPD subjects. Data presented as mean +/- SEM, (paired t test analysis from baseline to study time points, *p<0.05, **p<0.01, ***p<0.001)

4.4 Sputum virus load in study groups

The virus load in sputum from individual groups is shown in figure 4.7. This illustrates an increase after infection with a peak on day 9. The magnitude was greater in the COPD group throughout the study with a trend to higher levels at most time points, and significantly higher levels on day 12 compared to both control groups (one way ANOVA, p=0.034). On day 15 the sputum virus load was greater in COPD subjects compared to non-smokers (Post hoc Tukey's analysis, p=0.046).

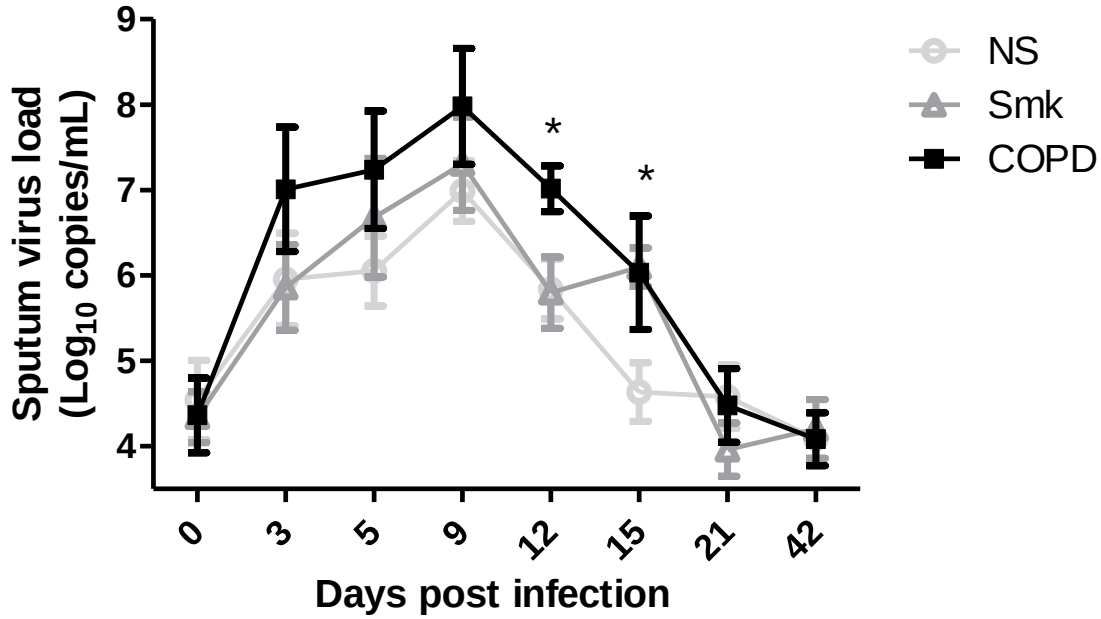


Figure 4.7 Time course of virus load in sputum of all successfully infected subjects by study group. Data presented as mean +/- SEM, (one way ANOVA between groups at study time points, *p<0.05, *Day 12 COPD vs NS and COPD vs Smk, *Day 15 COPD vs NS)

The change from baseline in sputum virus load is displayed in figure 4.8. This was increased significantly in non-smokers from days 5 to 9. In the smoking control and COPD group the levels were significantly higher from days 3 to 12 inclusive.

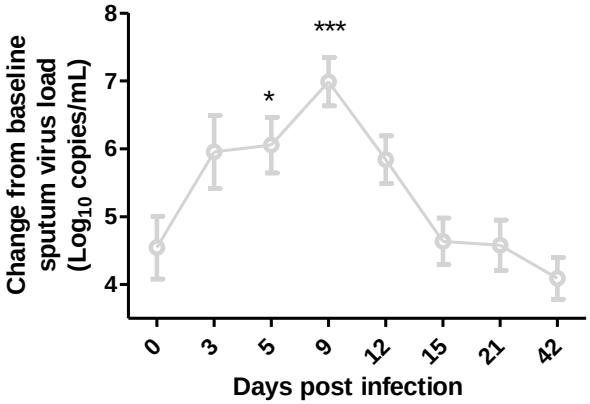


Figure 4.8a Non-smoking controls

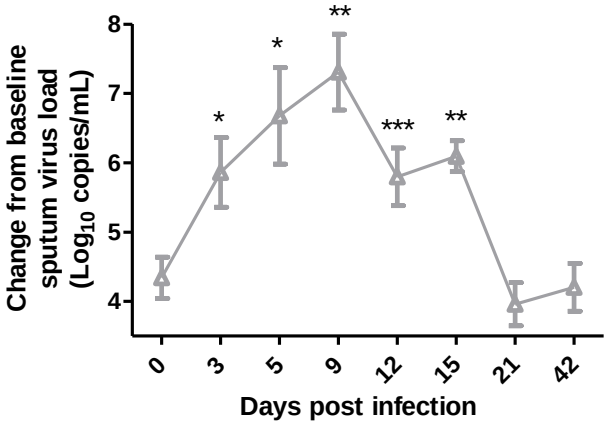


Figure 4.8b Smoking controls

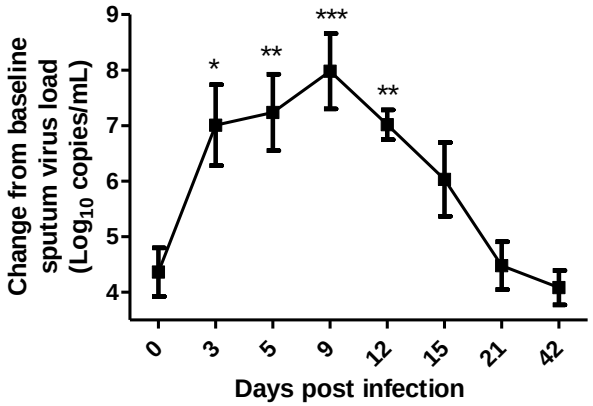


Figure 4.8c COPD subjects

Figure 4.8 Time course of change from baseline in virus load from sputum samples in (a) non-smoking controls, (b) smoking controls and (c) COPD subjects. Data presented as mean +/- SEM. (paired t test, *p<0.05, **p<0.01, ***p<0.001)

There was no difference in the virus load in BAL between the three study groups on either day 0 or 7 when analysed using a one way ANOVA, figure 4.9. There was a significant increase for all study groups from baseline to day 7 using a paired t test analysis, the fold increase was 3.4 in non-smoking controls (p=0.001), 2.5 in smoking controls (p<0.001) and 2.3 in COPD subjects (p=0.020).

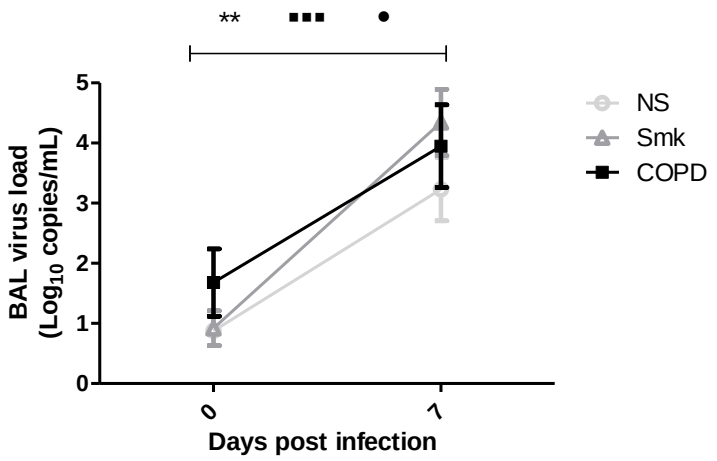


Figure 4.9 Pre and post-inoculation virus load in BAL from successfully infected subjects by study group. Data presented as mean +/- SEM, (paired t test increase from baseline, **p<0.01 non-smoking controls, ■■■p<0.001 smoking controls and •p<0.05 COPD subjects)

4.5 Sputum bacterial detection in all study subjects

Semi-quantitative detection of bacteria in sputum was performed as described in the Material and Methods chapter. No bacteria were detected in baseline sputum samples for any successfully RV infected subjects. Following inoculation 11 of the 30 RV infected subjects had bacteria detected in any sputum sample over the time course of the study. Of the three study groups there were 6 COPD, 2 smoking controls and 3 non-smoking controls with a bacterial isolate, of these, pathogenic bacteria were detected in 100%, 0% and 33% of study groups respectively, (as described in table 4.1).

To investigate the association between RV and bacterial detection, the time course of virus and bacterial loads in all subjects have been plotted in figure 4.10. This demonstrates a peak in virus load at day 9 followed by a peak in bacterial CFU load at day 15. Although the absolute numbers are low, the pattern illustrated is of increasing bacterial load in parallel to falling virus load.

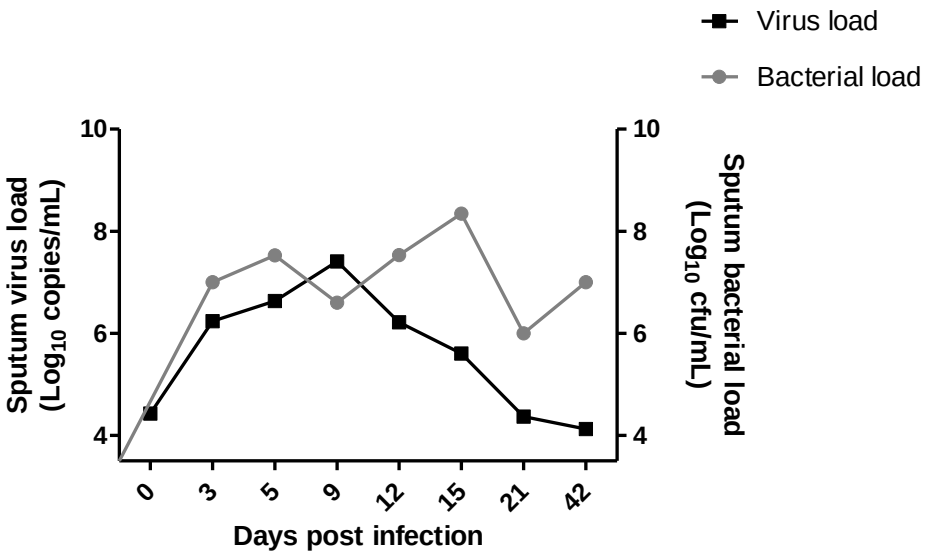


Figure 4.10 Time course of virus load and bacterial load in sputum of all study subjects

4.6 Relationship between virus load and clinical parameters of disease severity

As demonstrated in figure 4.10 the peak virus load occurred on day 9. How this relates to bacterial culture in subject groups was investigated by analysing the bacterial status (both pathogenic and non-pathogenic) of all sputum samples from the study groups from day 9 onwards. Any subject that had bacteria detected on or after day 9 was defined as "bacteria positive" and those with none detected were defined "bacteria negative". There were significantly more bacteria positive subjects in the COPD group compared to the controls, (chi square p=0.04). Figure 4.11 illustrates this data.

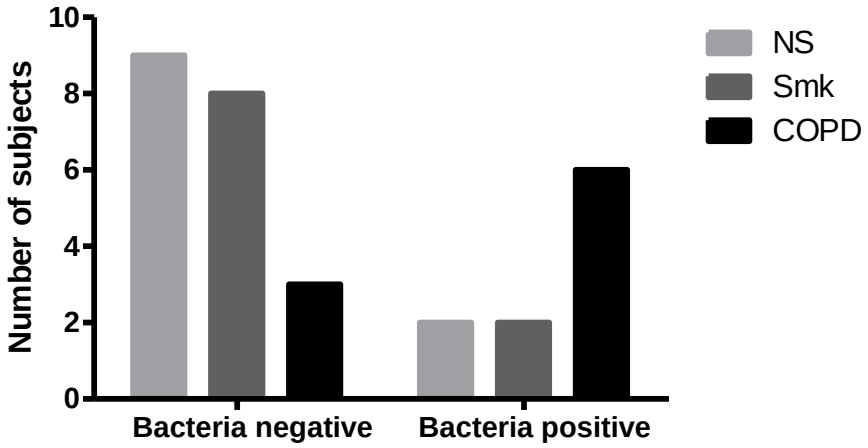


Figure 4.11 Number of subjects in each study group with bacteria negative or bacteria positive sputum samples at any study time point after day 9 inclusive, (chi square, p=0.04)

4.7 Detection of pathogenic bacteria in study groups

Bacteria positive samples were then classified as either “pathogenic” or “non-pathogenic” according to species frequently associated with respiratory illness and shown in table 4.1. This shows the number of sputum samples in which any bacteria were detected in each of the three study groups. COPD subjects had significantly more pathogenic bacteria in sputum samples when compared to controls, p=0.001 using chi squared analysis, table 4.1.

	Non smokers N=6	Smokers N=3	COPD N=8
Pathogenic bacteria (Haemophilus influenza, Staphylococcus aureus, Streptococcus pneumoniae)	1	0	8
Non-pathogenic bacteria (Coliform, Coagulase negative Staphylococcus, Enterococcus, Beta Haemolytic Streptococcus)	5	3	0

Table 4.1 Definition of “pathogenic” or “non-pathogenic” bacteria and number of positive sputum samples by study group (chi square analysis, p=0.001)

4.8 Virus and bacterial load in COPD subjects

The virus and bacterial load in COPD subjects over the time course of the study has been shown to examine the temporal relationship that exists in figure 4.12. The peak virus and bacterial load occurred on day 9 and day 15 respectively. There was a temporal delay between the two measurements, with increasing bacterial counts occurring at a time of falling virus load. There was no correlation between sputum virus and bacteria load in all subjects (r=0.16, p=0.551) or in COPD subjects (r=-0.08, p=0.855) in corresponding sputum samples collected at the same time.

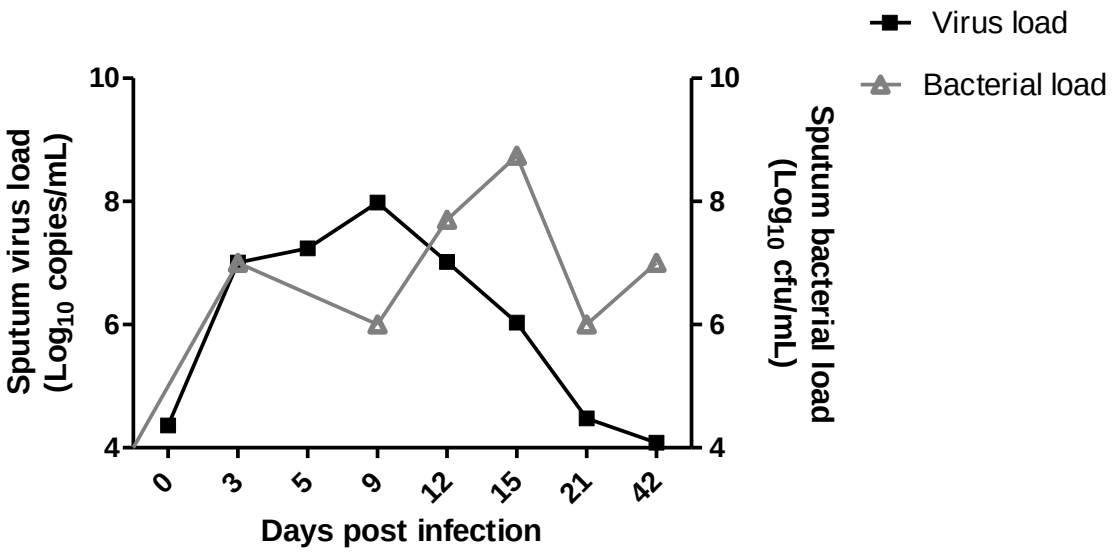


Figure 4.12 Time course of virus load and bacterial load in sputum of COPD subjects only

4.9 Correlation of nasal lavage virus load and URT symptom scores in all subjects

To explore the relationship between RV infection and reported subjective symptoms, total URT symptom score was correlated with virus load in nasal lavage at each time point that the two parameters were recorded simultaneously. In all infected subjects, URT and nasal lavage virus load had a highly significant positive correlation, ($r=0.42$, $p<0.001$), figure 4.13. This data suggests that the severity of cold symptoms is related to the burden of virus load and supports the validity of symptom scores displayed in chapter 3.

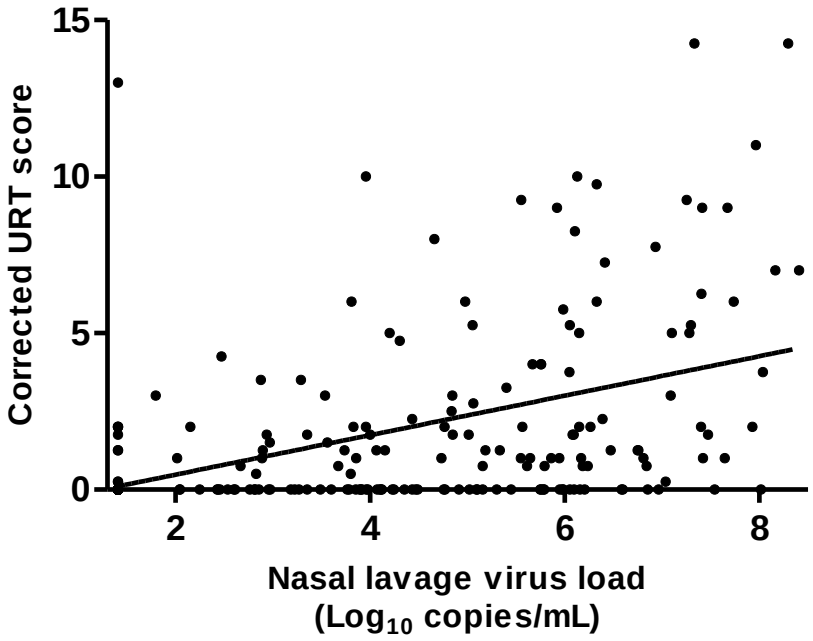


Figure 4.13 Correlation of nasal lavage virus load and corrected URT scores in all infected subjects combined, (r=0.42, p<0.001)

4.10 Correlation of nasal lavage virus load and URT symptom scores in study groups

When individual study groups were examined it was found that the correlation between these parameters was maintained in all groups, figure 4.14. It was highly significant (p<0.001) in non-smoking and COPD subjects, with the strongest correlation identified in COPD subjects (r=0.52) and weakest in smoking subjects (r=0.31).

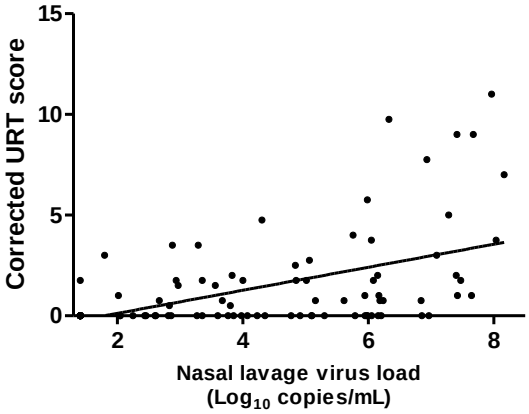


Figure 4.14a Non-smoking subjects, (r=0.50, p<0.001)

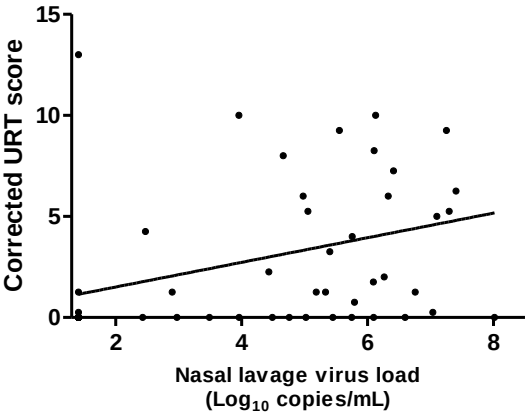


Figure 4.14b Smoking subjects, (r=0.31, p=0.022)

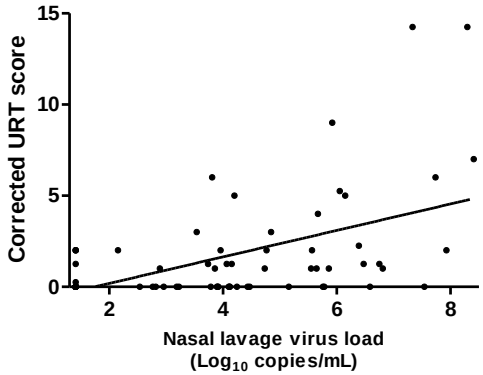


Figure 4.14c COPD subjects, (r=0.52, p<0.001)

Figure 4.14 Correlation of nasal lavage virus load and corrected URT scores in (a) non-smoking, (b) smoking and (c) COPD subjects

4.11 Correlation of sputum virus load and LRT symptom scores

Next we analysed the relationship between virus load in sputum and LRT symptom scores. There was a significant correlation between total LRT symptom score and virus load in sputum, ($r=0.50$, $p<0.001$), at each time point that the two parameters were recorded simultaneously. This was similar to the pattern observed in the upper airway, figure 4.15.

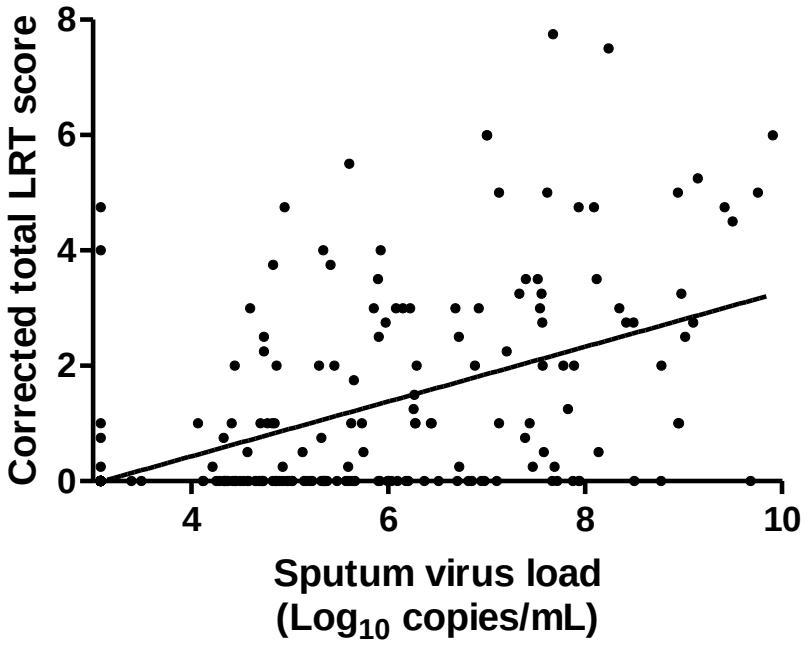


Figure 4.15 Correlation of sputum virus load and corrected LRT scores in all subjects ($r=0.50$, $p<0.001$)

Individual subject groups were analysed for the relationship between LRT scores and sputum virus load. There remained a positive correlation between the two parameters in each of the groups. This was weakest in the smoking control group ($r=0.35$) and strongest and most significant in COPD subjects ($r=0.63$, $p<0.001$). This matches the findings identified in URT scores; and demonstrates that only the COPD group had a strong positive correlation between virus load and symptom scores in both the upper and lower airway.

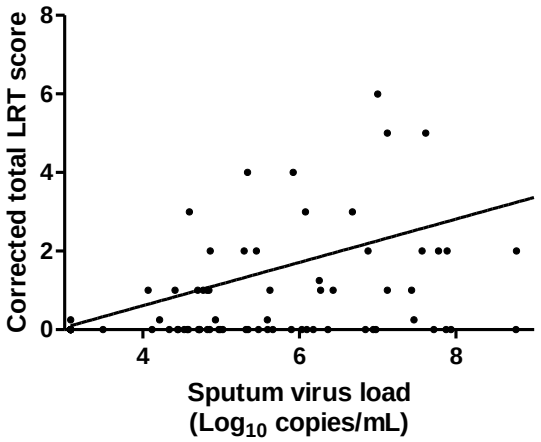


Figure 4.16a Non-smoking controls (r=0.38, p=0.001)

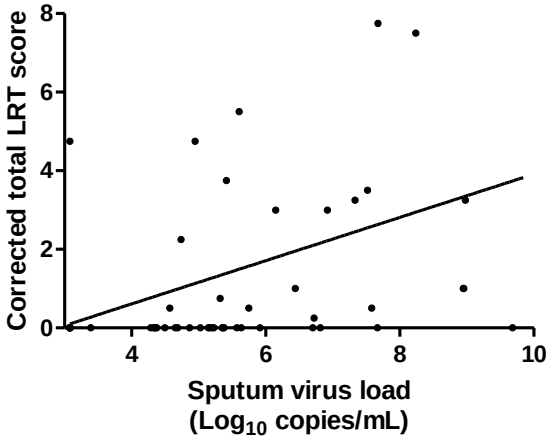


Figure 4.16b Smoking controls (r=0.35, p=0.012)

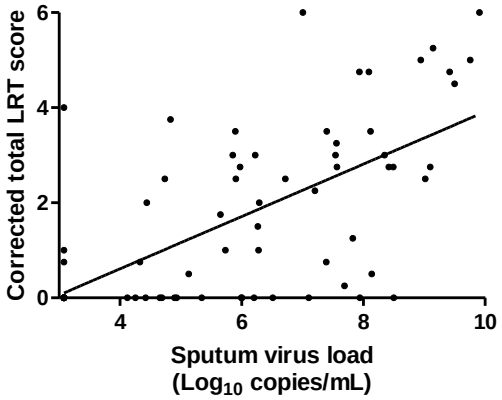


Figure 4.16c COPD subjects (r=0.63, p<0.001)

Figure 4.16 Correlation of sputum virus load and corrected LRT scores in (a) non-smoking subjects, (b) smoking subjects and (c) COPD subjects

4.12 Correlation between virus load and pro-inflammatory cytokines

The full results and discussion of mediators measured using the MSD assay in BAL and sputum are presented in the following chapter; however the results of correlations between inflammatory cytokines and chemokines with virus load in sputum have been included here. This allows investigation of the association between RV infection and airway inflammation. When the virus load and cytokine concentrations in sputum samples were correlated a consistent pattern was identified, with positive correlations between virus load and increased cytokine levels. For all pro-inflammatory cytokines this was strongest in COPD subjects compared to controls, where the relationship was frequently not significant.

4.13 Sputum virus load and TNF-α

The correlation between TNF-α and virus load is shown in figure 4.17 for all subjects combined and in figure 4.18 for individual groups; the relationship was highly significant with a Pearson coefficient of 0.52 in COPD subjects. However in non-smoking and smoking controls the association was not statistically significant, r=-0.01, p=0.90 and r =0.05, p=0.68 respectively.

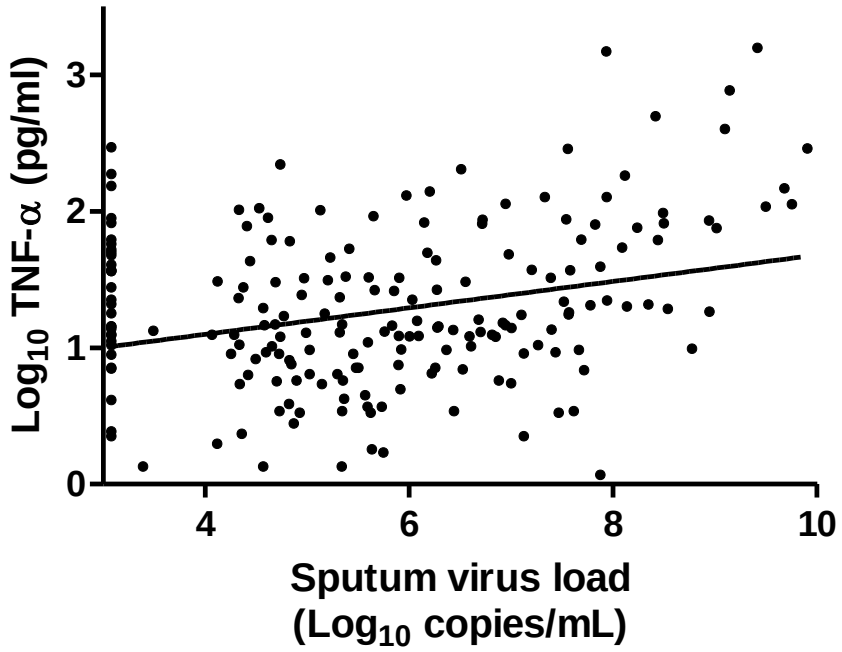


Figure 4.17 Correlation of sputum virus load and sputum TNF-α concentration in all study subjects combined (r=0.27, p<0.001)

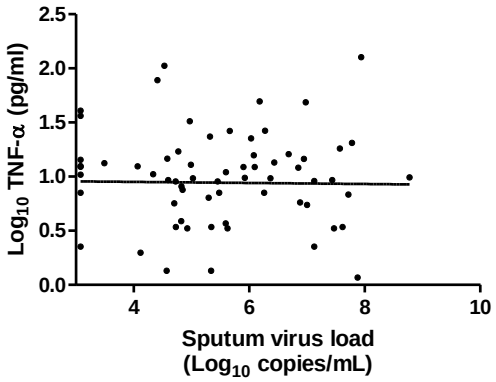


Figure 4.18a Non-smoking controls (r=-0.01, p=0.904)

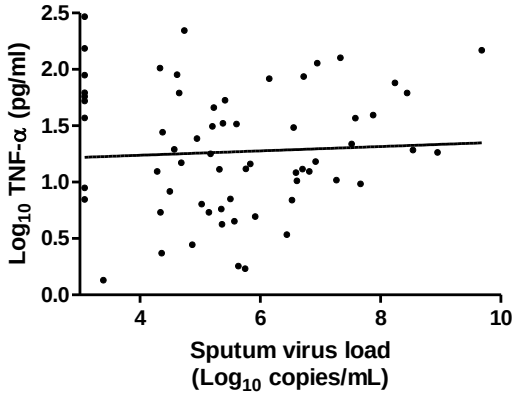


Figure 4.18b Smoking controls (r=0.05, p=0.681)

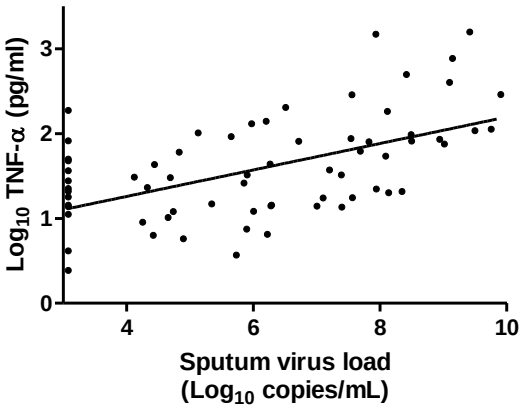


Figure 4.18c COPD subjects (r=0.52, p<0.001)

Figure 4.18 Correlation of sputum virus load and sputum TNF-α concentration in (a) non-smoking controls, (b) smoking controls and (c) COPD subjects

4.14 Sputum virus load and IL1-β

When the relationship between virus load and IL1-β was examined a similar pattern was found, with a strong association only in COPD subjects and not in controls. The correlation coefficients for COPD subjects and non-smoking and smoking controls was (r=0.47, p<0.001), (r=0.23, p=0.054) and (r=0.19, p=0.126) respectively. Figure 4.19 shows this for all subjects combined and COPD subjects alone.

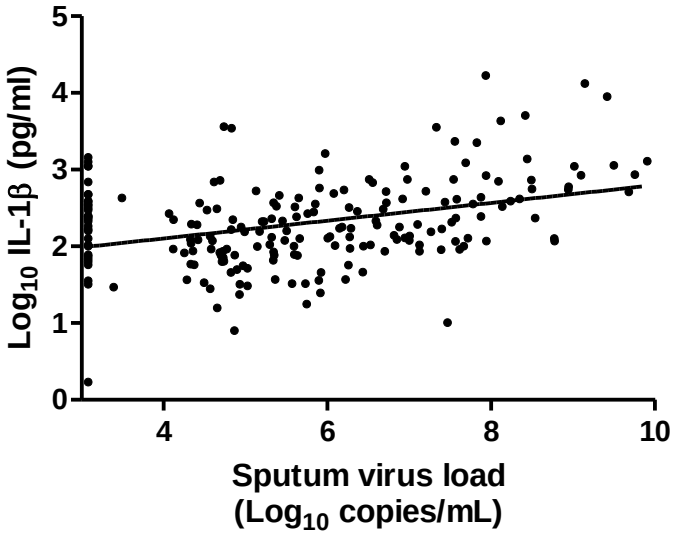


Figure 4.19a All subjects combined (r=0.36, p<0.001)

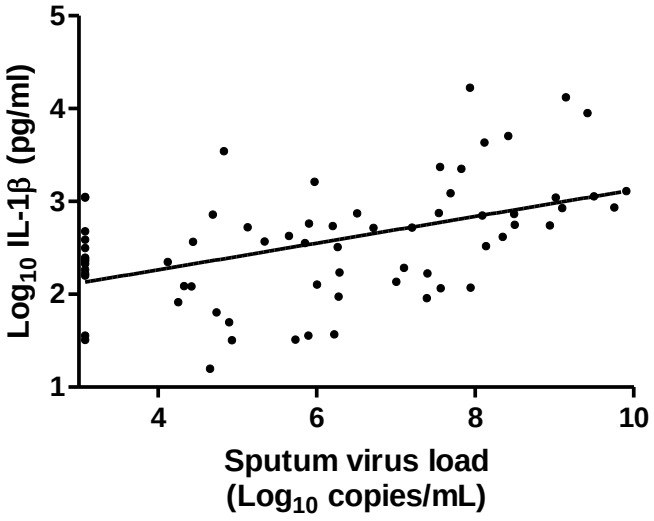


Figure 4.19b COPD subjects (r=0.47, p<0.001)

Figure 4.19 Correlation of sputum virus load and sputum IL-1β concentration in (a) all subjects combined and (b) COPD subjects

The results of all correlations between pro-inflammatory cytokines measured using the MSD assay and sputum virus load are shown in table 4.2. These have been ranked by Pearson coefficient in COPD subjects, and illustrates that this group demonstrates the strongest association between virus load and inflammatory mediators.

	Non-smoking controls		Smoking controls		COPD	
	Coefficient (r)	p-value	Coefficient (r)	p-value	Coefficient (r)	p-value
TNF-α	-0.02	0.904	0.05	0.681	0.52	<0.001
IL-1β	0.23	0.051	0.19	0.126	0.47	<0.001
IL-10	0.04	0.722	-0.04	0.751	0.38	0.001
IL-8	0.20	0.099	0.07	0.558	0.38	0.002
IL-6	0.23	0.060	0.05	0.695	0.36	0.002
IL-2	0.17	0.158	0.04	0.776	0.35	0.003
IL-12	0.02	0.873	-0.04	0.749	0.25	0.039
IFN-γ	-0.15	0.226	-0.02	0.891	0.23	0.056
GM-CSF	0.04	0.727	-0.08	0.526	0.21	0.080

Table 4.2 Pearson's correlation coefficient for sputum virus load and inflammatory cytokines in the three study groups

4.15 Correlation between virus load and chemokines

Virus load in sputum was correlated with chemokine mediators measured using the MSD assay. A strong positive correlation was found for IP-10 in all subjects, however for all mediators there was consistently a stronger correlation in the COPD group compared to controls, table 4.3. Sputum virus load and IP-10 is shown in figure 4.20 for all subjects combined and COPD subjects alone, $r=0.49$, $p<0.001$ and $r=0.54$, $p<0.001$ respectively. In the individual control groups the results were $r=0.48$, $p<0.001$ and $r=0.44$, $p<0.001$ for non-smoking and smoking controls.

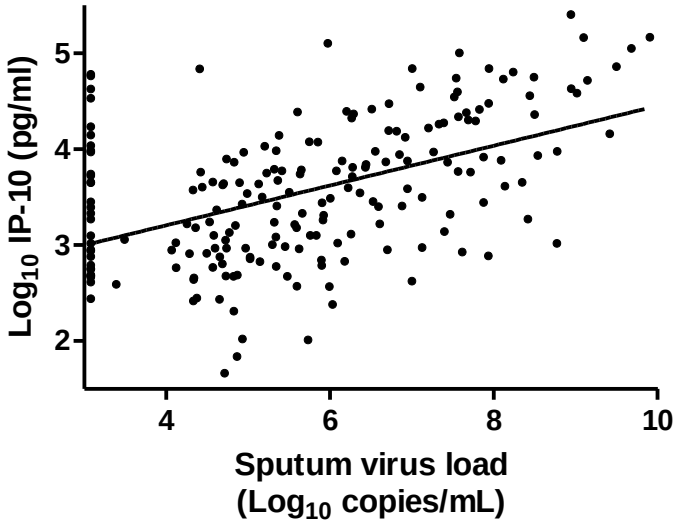


Figure 4.20a All subjects combined (r=0.49, p<0.001)

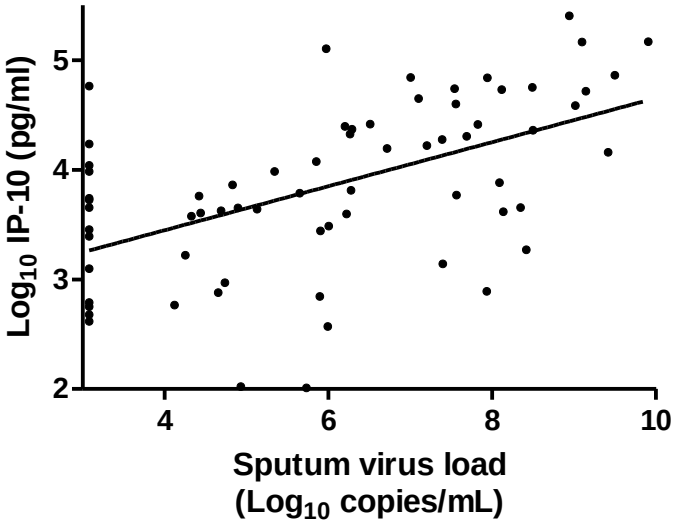


Figure 4.20b COPD subjects (r=0.54, p<0.001)

Figure 4.20 Correlation of sputum virus load and sputum IP-10 concentration in (a) all subjects combined and (b) COPD subjects

A significant positive correlation between MCP-1 and virus load was found in COPD subjects, this was stronger than in control subjects, figure 4.21.

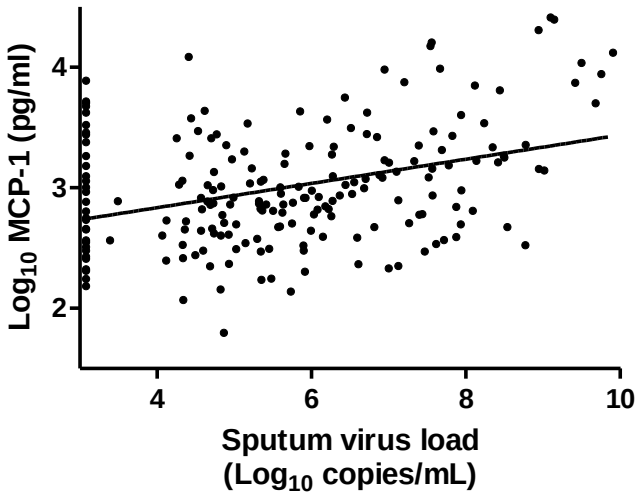


Figure 4.21a All subjects combined (r=0.36, p<0.001)

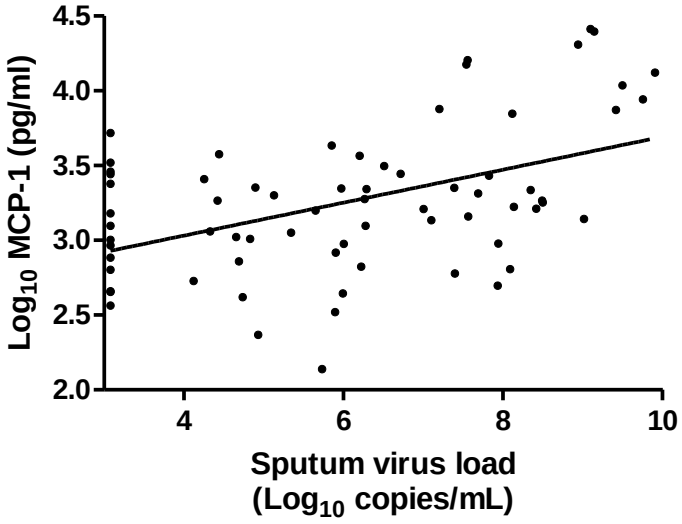


Figure 4.21b COPD subjects (r=0.47, p<0.001)

Figure 4.21 Correlation of sputum virus load and sputum MCP-1 concentration in (a) all subjects combined and (b) COPD subjects

The correlations between chemokines measured using the MSD assay and virus load are shown in the table. COPD subjects have a stronger correlation than control subjects for all parameters measured, the coefficient being greater than 0.3 for IP-10, MCP-1, Eotaxin and MIP-1β and less than 0.3 for MDC, MCP-4, TARC and Eotaxin-3.

	Non-smoking controls		Smoking controls		COPD	
	Coefficient (r)	p-value	Coefficient (r)	p-value	Coefficient (r)	p-value
IP-10	0.48	<0.001	0.44	<0.001	0.54	<0.001
MCP-1	0.27	0.027	0.20	0.118	0.47	<0.001
Eotaxin	0.20	0.103	0.32	0.011	0.39	<0.001
MIP-1β	0.21	0.081	0.19	0.130	0.33	0.005
MDC	0.25	0.039	0.23	0.074	0.28	0.020
MCP-4	0.09	0.470	0.08	0.542	0.28	0.021
TARC	0.24	0.053	0.20	0.120	0.28	0.022
Eotaxin-3	-0.00	0.987	-0.01	0.956	0.12	0.312

Table 4.3 Pearson's correlation coefficient for sputum virus load and chemokines in the three study groups

Chapter discussion

4.16 Virus load in nasal lavage and sputum

Following experimental inoculation the virus load detected in nasal lavage rose in all groups, with values significantly greater than baseline from days 3 to 15. The peak virus load occurred on day 3. There was no significant difference in nasal lavage virus load between the study groups at any time point. In sputum the virus load was significantly higher than baseline levels in all three subject groups on days 5 and 9. In smoking controls and COPD subjects the levels were also significantly increased on days 3 and 12 compared to baseline. There was a trend to higher virus load in sputum in COPD subjects throughout the study, with a significantly greater mean virus load on day 12 compared to both control groups. There was a delay of 6 days between peak virus load in nasal lavage and sputum, occurring on days 3 and 9

respectively. There are several reasons why this may have occurred. Virus was introduced via the nose on day 0; with the peak virus load detected in nasal lavage on the first subsequent occasion this was performed. The falling virus load identified after this time point is likely to represent immune clearance and virus shedding from the nose. The highest rate of virus replication may occur in the period immediately after inoculation, but this depends on the dose of inoculum delivered. However as sampling was not performed on days 1 and 2 it is not possible to comment exactly on the time period of this. In sputum there is a gradual increase in virus load. The delay between the peak virus loads is likely to occur because of the time taken for virus to be transferred from the upper to lower airway. Given the natural site of RV infection is the upper airway; an alternative hypothesis is that the host immunological response to RV infection is heightened in the upper airway compared to the lower, resulting in more rapid and effective clearance of virus from nasal lavage than sputum. Finally, despite attempts to minimise upper airway contamination by asking subjects to rinse their mouth with water following sputum induction prior to sputum induction and carefully selecting sputum plugs there remains likely to be virus from the upper airway detected in sputum samples and may reflect the relatively rapid rise in sputum virus load during the early time course of the study.

When the three study groups were analysed individually, COPD subjects had a trend to higher mean virus load in sputum throughout the study, which was significantly greater on days 12 and 15. Additionally COPD and smoking controls had significantly higher sputum virus load compared to baseline on more time points than non-smoking controls. The reasons for this are not known, but likely to reflect either impaired virus clearance or enhanced virus replication in these subjects. This could occur as the result of deficient immunological responses in subjects exposed to cigarette smoke leading to prolongation and excessive virus detection, the greater levels in COPD subjects suggest that this mechanism is exaggerated in these subjects compared to smokers. Cigarette exposure induces airway inflammation and it is possible this interferes with mechanism of innate immunity. In a study by Mallia *et al* deficient interferon beta levels in COPD subjects compared to smoking controls were identified, representing a possible mechanism for increased virus load in COPD subjects. However no similar data exists comparing smoking and non-smoking subjects[164].

4.17 Bacterial infection

Increased bacterial detection in sputum samples collected at time points after peak virus load, suggests a possible causative effect of RV on secondary bacterial infection. The peak bacterial load detected with semi-quantitative analysis was found on day 15, a time point 6 days later than the peak virus load in sputum and 12 days after the peak in nasal lavage. The rates of sputum samples with bacteria detected are higher than expected, because at baseline none of the subjects had bacteria identified sputum samples. By defining subjects as bacteria positive or negative after the peak of RV infection, an investigation of the effect of RV infection on bacterial rates was possible. It was found that significantly more sputum samples from COPD subjects had bacteria detected than samples from controls. In addition, the species of bacteria identified in the COPD subjects were exclusively those recognised as respiratory pathogens. In subjects from the control groups where bacteria were identified these were most likely to be commensal bacteria or those with uncertain respiratory pathogenicity. None of the subjects required treatment after detection of bacteria in their sputum samples. This suggests that although bacteria were detected, the impact on health status in the COPD subjects was unclear.

The role of bacterial infection in exacerbation of COPD is also not fully understood. Traditionally thought to be a leading cause of exacerbations, bacteria have been detected in the stable state of up to 30**%** of COPD subjects[325] with higher rates in studies of increased disease severity[1]. The high rates of bacterial detection in sputum of COPD subjects in this experimental RV infection study illustrates that they may indeed play a part in exacerbations. However the detection of bacteria at time points after the peak of upper airway symptoms may have detracted focus from the investigation of the role of viruses in the pathogenesis of AECOPD. This could explain why virus infection has been under reported as an aetiological agent in exacerbations and why secondary bacterial infection has not been studied in COPD. Indeed detected bacteria may represent innocent bystanders in the course of the exacerbation.

There was no correlation between virus and bacterial load when this was performed. However this is not unexpected as the time course between the two parameters is not concordant and at times of high virus load there was minimum bacteria detected. Further investigation of the association between virus and bacteria is again limited by the number of subjects with bacteria detected, but in future studies, with greater

numbers identification of an association between peak virus load and the likelihood of developing bacteria positive sputum samples could be performed.

4.18 Correlation between virus load and symptom scores

Strong correlations between both upper and lower respiratory tract symptoms and corresponding virus loads were demonstrated in this chapter. These were found to be most significant and strongest in COPD subjects, with Pearson's correlation coefficients of r=0.52 and r=0.63 for URT and LRT symptom scores respectively. These findings are both biologically plausible and add weight to the hypothesis that RV infection has a causative role in driving the symptoms of exacerbation. The symptom score results have been corrected for baseline levels and therefore accurately reflect the disease burden during the period of RV infection. A potential confounding factor in the strong correlation only in the COPD might have been a higher total score as a consequence of background symptoms. However this has been eliminated by baseline correction of scores prior to data analysis.

The positive correlation identified between virus load in nasal lavage and URT symptom scores in all infected subjects provides useful evidence to also support the validity of the questionnaire used in the study, furthermore this supports the hypothesis that greater URT symptoms are experienced at times of greater virus load.

4.19 Correlation between virus load and cytokines and chemokines

The measurement of inflammatory cytokines and chemokines was performed in sputum by the MSD platform as described in the Material and Methods chapter. The results of these assays and the concentrations of the mediators over the time course of the study are presented and discussed in detail in chapter 5. However the correlation between the concentration of these mediators in sputum and virus load have been included in this section. These results are included here to illustrate the possible consequences of changes in virus load on the mediators studied and to enable further discussion of the role of virus infection in study subjects, particularly those with COPD in whom higher sputum virus loads and stronger correlations between virus load and pro-inflammatory cytokines were seen.

The results are consistent across several mediators and show a stronger positive correlation in COPD subjects compared to a weaker or absent correlation in controls. These results are intriguing; they suggest that RV infection is driving inflammation in

the airways of COPD subjects and that this relationship is not present in controls. The data supports the hypothesis that increased inflammation is induced in the airways of COPD subjects compared to controls by RV infection and that this leads to the excess of symptoms observed in this group. This suggests direct induction of host defence mechanisms in COPD subjects in excess of a normal response to RV infection, whilst airway inflammation in healthy controls may not necessarily be driven directly by virus load.

Although the strong correlations between pro-inflammatory cytokines and virus load in COPD subjects do not prove causality it does offer significant support to the concept that exacerbations result from RV infection. The control subjects did not demonstrate significant correlations for any of the inflammatory mediators frequently associated with exacerbations. Concentrations of the inflammatory cytokines IL-1β, TNF-α, IL-6 and IL-8 in sputum were found to peak on day 9 (data presented in following MSD results chapter) which coincides with the peak of virus load in sputum presented in this chapter and this is borne out by the significant linear correlations identified. Additionally this powerful temporal relationship further suggests that virus infection may be responsible for increased levels of IL-1β, TNF-α, IL-6 and IL-8. The mechanism of action leading to a significant correlation between pro-inflammatory cytokines and virus load exclusively in the sputum of COPD subjects has been investigated in this chapter. The data in this chapter has also shown increased virus loads in the sputum of COPD subjects, having demonstrated increased LRT symptoms consistent with an exacerbation in the previous chapter.

The three cytokines most strongly correlated with virus load in COPD subjects were TNF-α, IL-1β and IL-8. These are recognised mediators of inflammation and elevated levels have been measured during exacerbations of COPD. However the anti-inflammatory cytokine IL-10 was also found to correlate with virus load. The association with virus load and IL-10 identified in COPD subjects suggests that in response to an increase in airway inflammation an appropriate IL-10 driven anti-inflammatory response can be produced.

In all subjects a positive correlation between IP-10 and virus load existed and this was the only mediator examined in which healthy controls have a relationship nearly as strong as COPD subjects. IP-10 has been shown to be a strong marker for virus associated exacerbations in asthma and COPD[194, 195, 326]. These findings

support the concept that IP-10 has a normal role in host immunity to RV infection. For the mediators MDC, MCP-4, TARC and Eotaxin-3, there was a significant but weak correlation with virus load in COPD subjects, suggesting that they do not play a central role in virus induced inflammation during exacerbations.

4.20 Comparison with published data

An experimental RV challenge of COPD subjects has been performed previously[164]. However this is the first study to include a non-obstructed non-smoking control group. This enables the analysis of the confounding effect of cigarette smoke following RV infection. Healthy smoking subjects are known to have an increased symptom burden following respiratory virus infection[2-4] and this has been borne out by the symptom data shown in this study. A possible mechanism is illustrated in this chapter; high virus loads were found in smoking subjects compared to non-smoking controls. In contrast to the previous study[164], virus load in nasal lavage from COPD subjects did not demonstrate significantly higher values compared to controls. However, unlike the previously published work, sampling was not performed on day 6 which was the only day on which a significant difference was found. In contrast to other RV challenge studies, the data presented in this chapter is the first to demonstrate significantly greater virus load in the sputum of COPD subjects compared to non-smoking controls.

The relationship between upper and lower airway virus load has been poorly reported in the literature, due to difficulties in obtaining samples over a prolonged time period, and delays between upper airway infection and development of LRT symptoms. In a study of naturally occurring exacerbations there was higher viral recovery from sputum than nasal lavage samples[218, 253]. This is supported by the data shown in this chapter with increasing sputum virus load at a time of greater LRT symptoms. However in previously published work by Rohde *et al*, it is likely that the peak virus load in nasal samples was missed in their study of hospitalised patients, whereas the frequent sampling of the upper airway in this experimental challenge model demonstrates an earlier peak in nasal virus load. The duration of RV detection in my study is comparable with a longitudinal study in children, where virus was detected up to 21 days after infection[327]. Finally in a study of asthmatics designed to examine the relationship between virus load in the upper airway and lower airway symptoms, no association was identified[328]. This suggests that exaggerated LRT symptoms in COPD subjects can still occur despite no differences between virus load in the upper airway between COPD subjects and controls.

The role of RV infection in exacerbations of COPD has become increasingly evident in recent years. The availability of PCR has resulted in respiratory viruses being identified in approximately 40% of exacerbations and around 2/3rd are due to RVs[1, 252]. Detection or viruses at the time of exacerbations has provided evidence that these have an aetiological role in exacerbations. The establishment of an experimental RV induced exacerbation model in COPD, however also provides causative evidence for this hypothesis. The finding in this study that COPD subjects challenged with RV develop features of an exacerbation further supports this hypothesis. In addition data presented in this chapter is the first to demonstrate an association between several inflammatory mediators and virus load. This strong correlation only in COPD subjects will add further evidence to the published literature that RV infection results in exacerbation.

4.21 Relationship between RV and bacterial infection

Many studies have identified respiratory infections as the most common cause of exacerbations in COPD[1, 329], however recently the differential roles of bacteria and viruses in the aetiology of exacerbations has been debated. The traditional belief that bacterial infection led to exacerbations has been questioned after the discovery of high levels of bacteria in stable COPD[325]. Additionally new molecular techniques have allowed detection of viruses during exacerbations of airways disease and in COPD RV are the virus type most commonly identified[315]. Interestingly there are no *in vivo* reports in the literature of bacterial infection occurring after RV infection, despite reports of RV infection increasing susceptibility to bacterial infection *in vitro*[262, 330, 331].

Studies of naturally occurring exacerbations have identified the presence of both RVs and bacteria[1, 332], however these studies only sampled at a single time point and therefore the temporal relationship between the agents cannot be investigated. Although when the identification of co-infection with bacteria and viruses occurred this was associated with an increased severity of exacerbations[1]. The temporal relationship between peak virus and bacterial load on days 9 and 15 respectively in my study supports the concept that RV infection results in secondary bacterial infection in COPD subjects at a higher rate than in controls subjects.

An important question the data in this chapter poses is whether bacteria are present at baseline and not detected by microbiological culture, or whether they are acquired during the time course of the study. The former would suggest that bacteria are in

stable state in the respiratory tract of COPD subjects and RV infection disrupts the host defence balance leading to bacterial overgrowth. While the second concept suggests that RV infection creates an environment in which acquisition and growth of pathogenic bacteria is favoured. There is published evidence that bacteria present in the airways are not detected by standard microbiological culture techniques[333]. Thus it is likely that pathogenic bacteria were present in the airways of COPD subjects in this study and subsequent RV infection led to alteration in the host immune response leading to further growth of bacteria to detectable levels.

Work by Sethi *et al* report that the acquisition of a new bacterial strain led to an exacerbation in a longitudinal study of COPD patients[258]. Higher than expected levels of bacterial detection in the stable state can be explained by the data presented in this study, as exacerbations were characterised by a change in strain type between the stable and exacerbated state. This however does not provide an explanation for the relationship between RV infection and bacterial detection, but does indicate that a stable balance may exist between host and bacteria which is disrupted by RV infection, leading to subsequent bacterial overgrowth.

In my study there was a second peak in symptoms in COPD subject which although small may be attributed to secondary bacterial infection. A prospective study of AECOPD identified RV in 20% of exacerbations and bacteria in 70%; those with co-infection had higher bacterial loads and increased symptoms than those with bacteria alone. Serum IL-6 was also greater in this group[334]. Finally another study identified COPD subjects who were colonised with bacteria and found that nasal IL-8 and bacteria load were increased[335], in addition upper and lower airway IL-8 levels were positively correlated.

4.22 Prophylactic antibiotic therapy in COPD

There has been interest in using long term prophylactic antibiotics in stable COPD to reduce the incidence of exacerbations. However early studies did not identify a benefit and this led to a Cochrane review[336] which concluded there was a small benefit but insufficient evidence to support their routine use in clinical practice. A well conducted randomised study using erythromycin did find a reduction in exacerbations compared to the placebo group[337], but was underpowered. The benefits of erythromycin in this study appeared to be through an anti-microbial effect rather than the class related anti-inflammatory effect. As individuals were likely to have experienced an average of 2-3 common colds per year, the benefit of prophylactic

antibiotics may be through reducing the incidence of secondary bacterial infections, however to date no studies have investigated this mechanism. Finally a recent randomised trial of azithromycin as prophylaxis for AECOPD over a one year period also identified a benefit over placebo with reduction in exacerbation frequency in the treatment group. However there are several uncertainties about the study. The incidence of AECOPD was lower than expected in the placebo group and there was an increase in side effects and minimal improvement in respiratory quality of life questionnaires in the treatment group. Additionally there was a high dropout rate from both arms of the study and worse than expected compliance, again leading to doubts over the reliability and power of the study[338].

This data suggest that macrolide antibiotics may have a beneficial role in prophylaxis against exacerbations. Whether the effects or macrolide therapy are anti-microbial or anti-inflammatory remain poorly understood whilst the role of viruses in these exacerbations have not been studied.

4.23 Bacterial species detected in COPD group

When the species of bacteria detected following the peak virus load was analysed there were significantly more pathogenic bacteria identified in the COPD group compared to controls. This difference between the two groups raises interesting questions. In the data presented in this chapter the three species identified in COPD subjects were *H. influenza, S. aureus* and *S. pneumoniae* and in published studies similar species have been identified. In a study investigating the association between sputum purulence and bacterial infection during an AECOPD, species were found in the following frequencies: *H. Influenza, M. catarrhalis* and *S. pneumoniae*, additionally this was applicable across a range of COPD disease severities[325]. Studies which have associated AECOPD with the acquisition of a new bacterial strain have isolated either *H. influenza M. catarrhalis* and *S. pneumoniae*[258]. In other studies *S. aureus* has been reported as a cause of AECOPD, however less frequently than those described above[339, 340].

The identification of *H. influenza* and *S. pneumoniae* in this RV challenge study is therefore supported by evidence from published studies, however no *M. catarrhalis* was found in sputum samples following RV infection. There are several possible explanations for this. It is possible that RV infection does not predispose to infection with *M. catarrhalis*, particularly if *M. catarrhalis* associated exacerbations were due to acquisition of the bacteria rather than reactivation of a colonised agent. An

alternative possibility is that the milder disease severity of the subjects in this study reduced their risk of *M. catarrhalis* infection, however data from Stockley *et al* would appear not to support this argument[325]. However the modest number of subjects with bacteria detected in my study is the most likely explanation.

Although mechanisms of bacterial infection have not been investigated specifically in this study, it would be an important area of future work. Given the modest differences in clinical, spirometric and inflammatory markers between COPD subjects and controls at baseline, the striking differences in bacterial species identified between the two groups appears to be of utmost importance in the pathogenesis of RV induced exacerbations.

4.24 Relationship between virus load and symptom scores

Studies that have specifically investigated the association between RV and LRT symptom scores have typically been in immunocompromised subjects[341, 342]. In these studies RVs have been detected during the stable state, and levels above 10^5 virus copies/ml in nasal lavage were associated with symptoms of illness. During AECOPD virus detection has been associated with fever[253] but studies of the correlation between increased virus load and symptom scores are lacking.

The significant correlation between virus load and symptom scores presented in this chapter was not identified in previous RV challenge studies from COPD subjects[164, 290]. This may reflect differences in the method of data analysis used. In the study presented here all time points for either nasal lavage or sputum collection were correlated with upper or lower respiratory tract symptoms for the corresponding day. In the published study only the peak virus load was used or peak in LRT symptom scores, this results in significantly fewer correlating points and importantly does not take into account periods during the study when lower virus load is associated with a reduced symptom burden. This illustrates the difficulty of comparing published studies with each other; in this case even though identical study protocols were used different methods of data analysis resulted in variations in results.

4.25 Relationship between virus load and cytokines

No studies with a direct relationship between sputum cytokines and virus load in COPD subjects have been published. A study by Aaron *et al* identified increases in TNF-α, IL-8 and MPO during AECOPD compared to stable state; but concluded that there were no differences in inflammatory mediators between exacerbations where

an infectious agent was or was not identified[343]. A cautious interpretation of these findings is required as virus was only detected in 3 subjects and so the reliability of this statement is not clear.

An *in vitro* study of RV infection in cultured tracheobronchial tissue from COPD and control subjects demonstrated higher virus titre and copy numbers in COPD tissue, which was associated with increases in IL-6 and IL-8 concentrations[134]. Additionally Schneider *et al* reported increased interferon beta (IFN-β) mRNA production in *ex vivo* COPD tissue compared to control samples but was not able to detect IFN-β protein in media following RV infection[134]. Interestingly other studies have suggested IFN-β deficiency as a possible mechanism in RV induced COPD exacerbations[164].

Although this data does not allow direct comparison with the positive correlation between sputum virus load and inflammatory cytokines reported in this chapter it offers *in vitro* evidence supporting this *in vivo* data. The unique nature of an experimental challenge study allows collection of samples over the full time course of a virus infection and data presented in this chapter illustrates the power of relating this to changes in immunological and inflammatory markers.

4.26 Conclusion

The results of virus and bacteria studies from nasal lavage and sputum in the study group have been presented in this chapter. The virus load was found to be similar in upper airway samples between the three study groups, but COPD subjects had an elevated and prolonged virus burden in the lower respiratory tract compared to controls. This is likely to explain the excess of lower respiratory symptoms in this group.

A greater number of sputum samples grew bacteria in the COPD group during their exacerbation compared to control subjects. The second striking factor was that all bacteria identified in the COPD group were recognised respiratory pathogens whereas the opposite was true in controls. Although this data has not identified a causal link or investigated potential mechanisms between RV and bacterial infection, the results have provided strong supporting evidence for this hypothesis. However this is an important field in which future work should focus.

Chapter 5: Measurement of immune mediators in sputum and BAL

5.1 Introduction

The Meso Scale Discovery platform was used to measure a range of cytokines and chemokines as described in chapter 2, Materials and Methods. The results of selected proinflammatory cytokines and mediators of macrophage and virus related inflammation have been included in this chapter. Only data from sputum and BAL supernatant of successfully RV infected study subjects are presented in this chapter. Differences between groups have been analysed using one-way ANOVA and changes from baseline with the paired t test.

Results

5.2 Sputum supernatant mediators

All MSD assays in sputum supernatant were performed in duplicate wells and the mean concentration calculated, data displayed is corrected for the 9 fold dilution of the sputum sample during laboratory processing.

5.3 Pro-inflammatory cytokines in sputum supernatant

The proinflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α have been included in this section, with mediator concentrations in each study group over the time course of the study shown and changes from baseline in the three study groups.

5.4 IL-1β in sputum supernatant

The time course of IL-1β in individual study groups is shown in figure 5.1. There were no differences between groups in sputum IL-1β levels at baseline prior to infection. There was a significant difference between the mean concentration of IL-1β between groups on day 9 (one way ANOVA, p=0.002: post hoc Tukey's analysis difference between COPD and non-smoking controls, p=0.002) and 15 (one way ANOVA, p=0.008; post hoc Tukey's analysis difference between COPD and non-smoking controls, p=0.006).

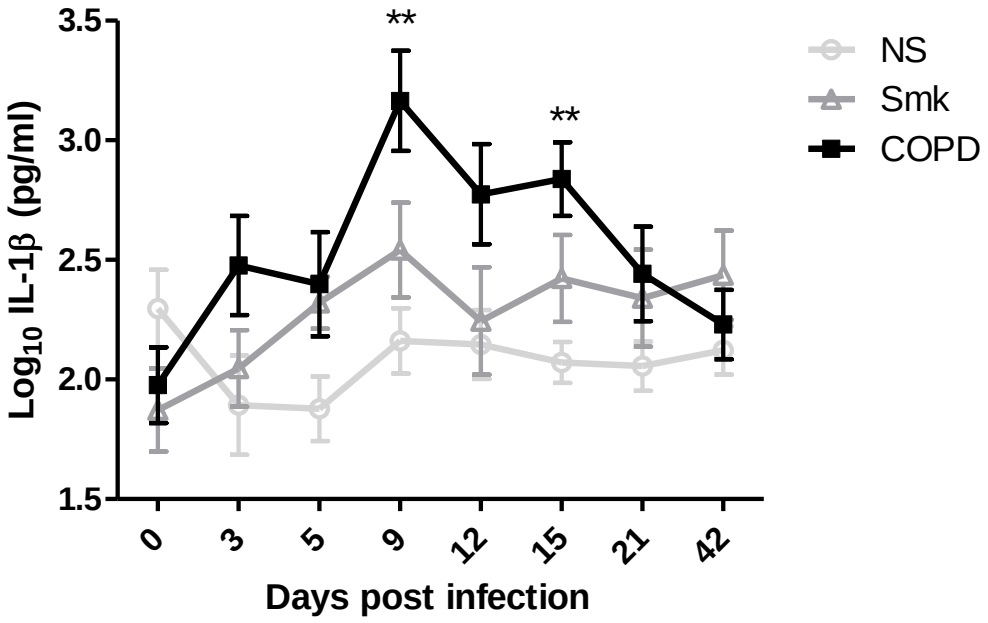


Figure 5.1 Time course of IL-1β concentration in sputum supernatant of individual study groups. Data presented mean +/- SEM, one-way ANOVA analysis between the three study groups, (**p<0.01, **Day 9 COPD vs NS, **Day 15 COPD vs NS)

Changes from baseline in individual study groups are shown in figure 5.2, with significant changes for non-smoking controls on day 5 and 21; smoking controls on days 5, 9 and 15 and 42; and COPD on days 9 to 42 inclusive.

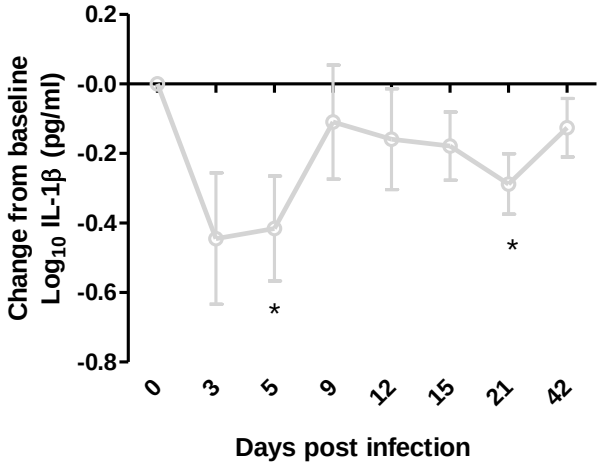


Figure 5.2a Non-smoking controls

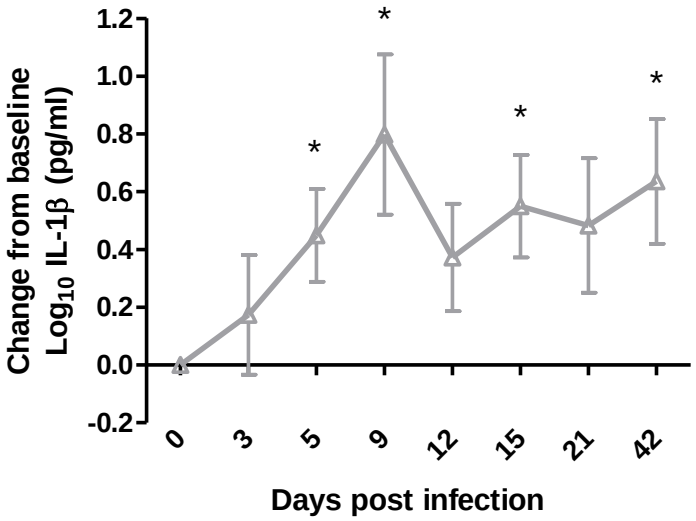


Figure 5.2b Smoking controls

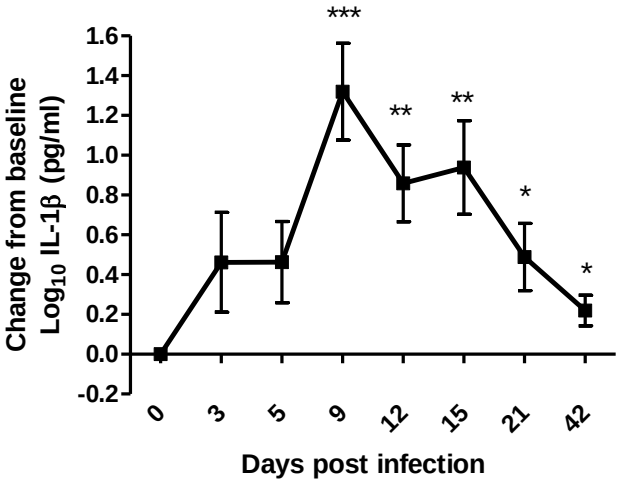


Figure 5.2c COPD subjects

Figure 5.2 IL-1β changes from baseline for (a) non-smoking controls, (b) smoking controls and (c) COPD subjects. Data presented as mean +/- SEM, using paired t test analysis for comparison between baseline and study visit (*p<0.05, **p<0.01, ***p<0.001)

5.5 IL-6 in sputum supernatant

The time course of IL-6 in individual study groups is shown in figure 5.3. There was no difference in sputum IL-6 levels between groups at baseline (p=0.748). There were significant differences in the mean concentrations of IL-6 between the three study groups on all study visits after infection. This demonstrated significant differences between non-smoking controls and smoking controls in addition to differences between non-smoking controls and COPD subjects.

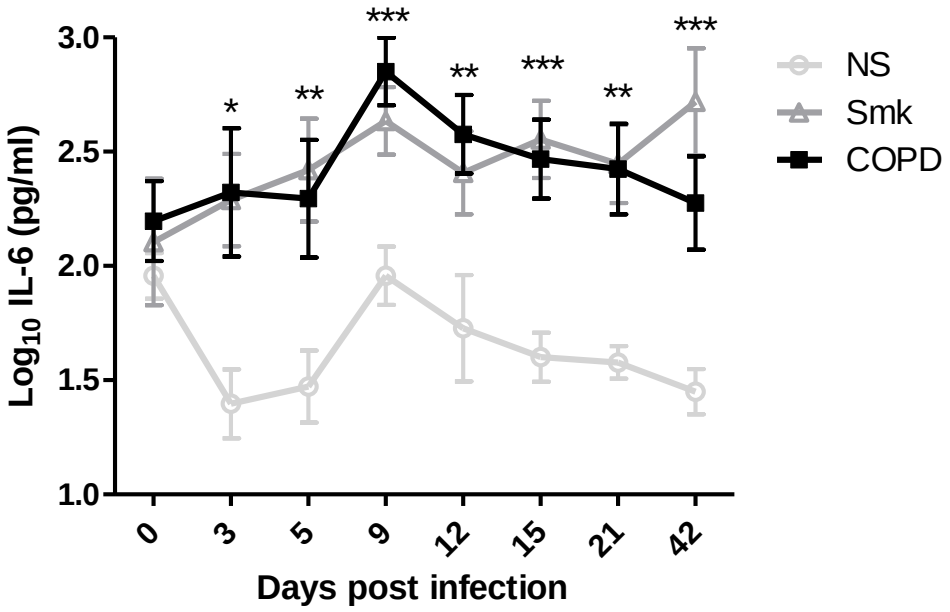


Figure 5.3 Time course of IL-6 concentration in sputum supernatant of individual study groups. Data presented mean +/- SEM, one-way ANOVA analysis between the three study groups (*p<0.05, **p<0.01, ***p<0.001)

Changes from baseline in individual study groups are shown in figure 5.4. There were significant falls from baseline on days 3 (paired t test, p=0.024), 15 (p=0.044) and 42 (p=0.012) in non-smoking controls. In COPD subjects there was a significant increase from baseline on day 9 alone (paired t test, p=0.010). In smoking controls there were no significant increases from baseline at any time point.

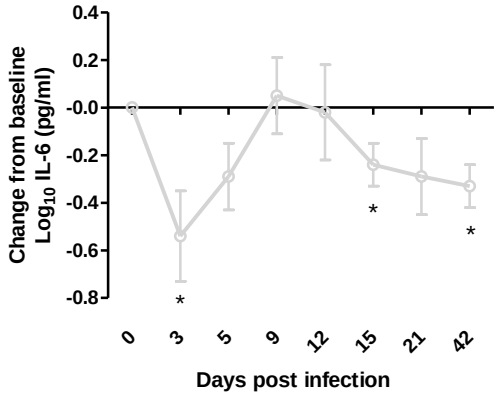


Figure 5.4a Non-smoking controls

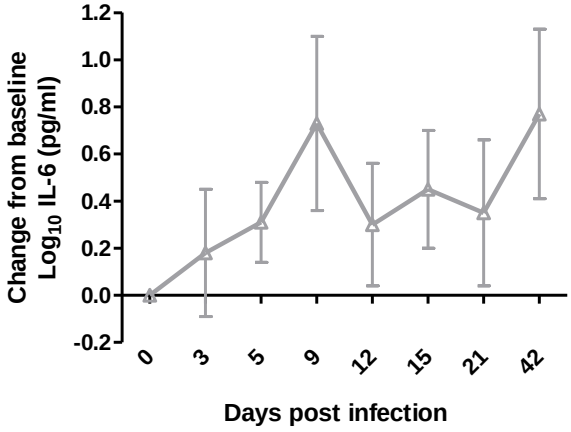


Figure 5.4b Smoking controls

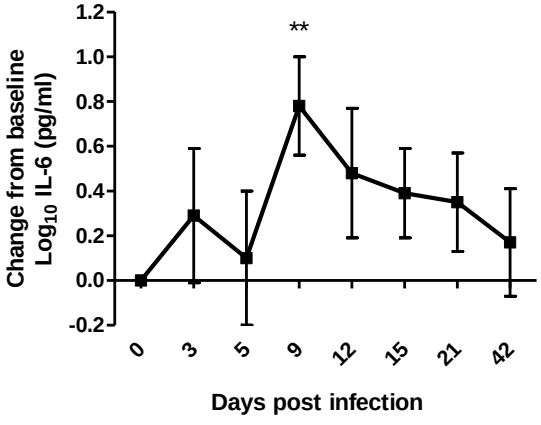


Figure 5.4c COPD subjects

Figure 5.4 IL-6 changes from baseline for (a) non-smoking controls, (b) smoking controls and (c) COPD subjects. Data presented as mean +/- SEM, using paired t test analysis for comparison between baseline and study visit (*p<0.05, **p<0.01)

5.6 IL-8 in sputum supernatant

The time course of IL-8 in individual study groups is shown in figure 5.5. There was no difference in sputum IL-8 levels between groups at baseline (p=0.623). There were significant differences in the mean concentrations of IL-8 between the three study groups on days 9 to 42 inclusive. Tukey's analysis showed that on days 9 and 12 this difference was between non-smoking controls and COPD subjects (p=0.001 and p=0.017 respectively) and on days 15, 21 and 42 the difference was between non-smoking controls and smoking controls (p=0.048, p=0.041 and p=0.001 respectively) in addition to non-smoking controls and COPD subjects (p<0.001, p=0.022 and p=0.010 respectively).

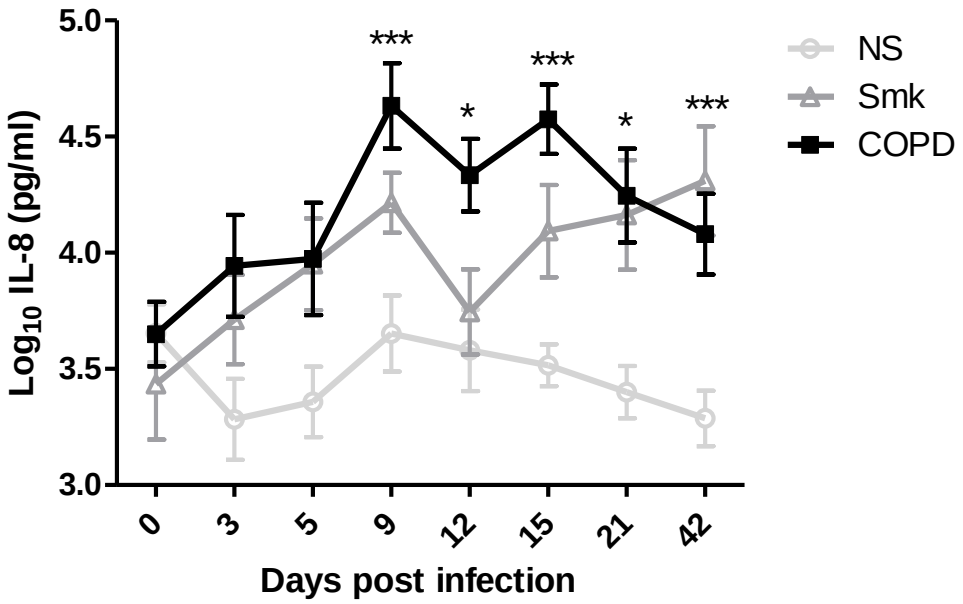


Figure 5.5 Time course of IL-8 concentration in sputum supernatant of individual study groups. Data presented mean +/- SEM, one-way ANOVA analysis between the three study groups (*p<0.05, ***p<0.001)

Changes from baseline in individual study groups are shown in figure 5.6. In smoking controls there were significant increases from baseline on days 5, 9, 15 and 42 (paired t test, p=0.011, p=0.038, p=0.011 and p=0.012 respectively). In COPD subjects there was a highly significant increases from baseline in sputum IL-8 levels from days 9 to 42 inclusive (paired t test, p<0.01). In non-smoking controls there were no significant increases from baseline at any time point.

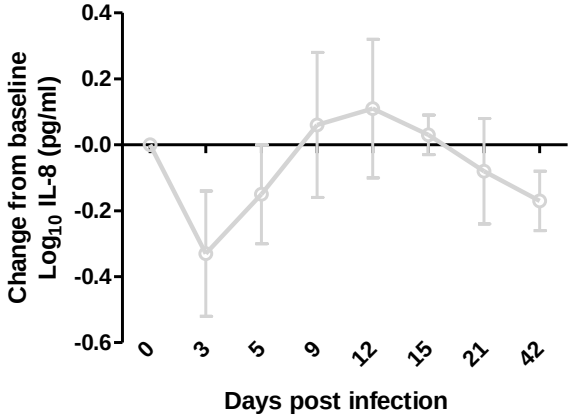


Figure 5.6a Non-smoking controls

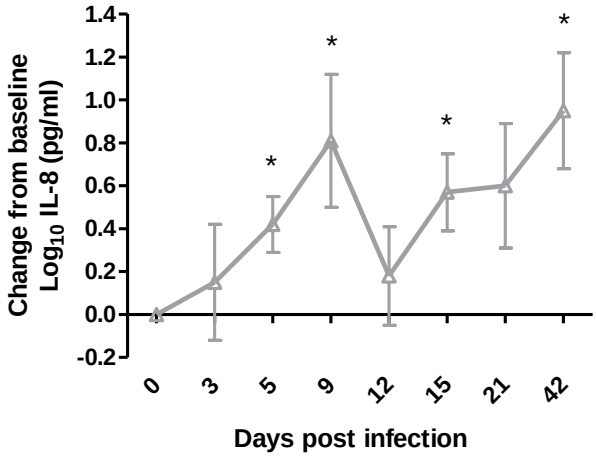


Figure 5.6b Smoking controls

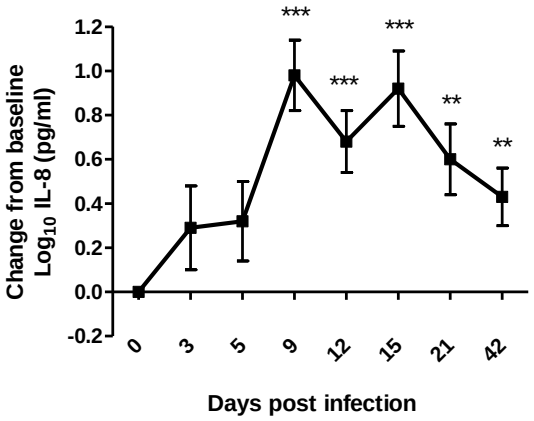


Figure 5.6c COPD subjects

Figure 5.6 IL-8 changes from baseline for (a) non-smoking controls, (b) smoking controls and (c) COPD subjects. Data presented as mean +/- SEM, using paired t test analysis for comparison between baseline and study visit (*p<0.05, **p<0.01, ***p<0.001)

5.7 TNF-α in sputum supernatant

The time course of TNF-α in individual study groups is shown in figure 5.7. There was no difference in sputum TNF-α levels between groups at baseline (p=0.252). There were significant differences between the means of the three study groups on days 3 to 15 when analysed using a one way ANOVA. Tukey's analysis demonstrated that at each of these time points this difference was between non-smoking controls and COPD subjects and on day 9 there was also a difference between smoking controls and COPD subjects (p=0.037).

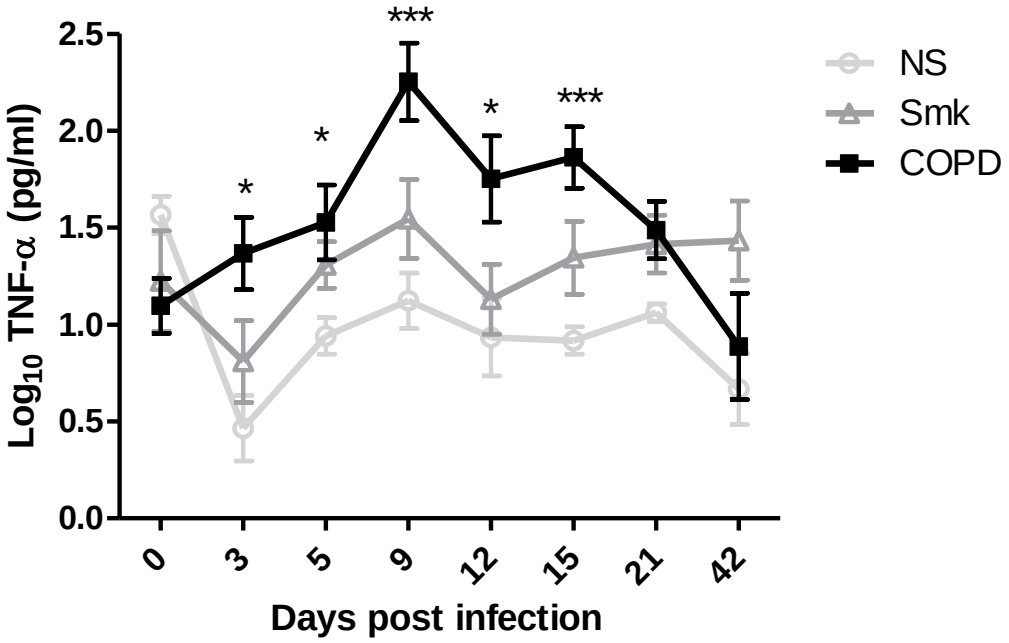


Figure 5.7 Time course of TNF-α concentration in sputum supernatant of individual study groups. Data presented mean +/- SEM, one-way ANOVA analysis between the three study groups (*p<0.05, ***p<0.001)

Changes from baseline analysis using the paired t test for non-smoking controls showed significant reduction on days 3, 5, 15, 21 and 42. No significant changes from baseline in smoking controls at any time points and significant increases in sputum TNF-α from baseline at days 5 to 15 inclusive in COPD subjects, figure 5.8.

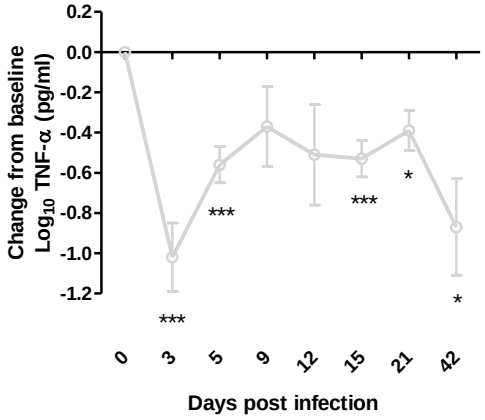


Figure 5.8a Non-smoking controls

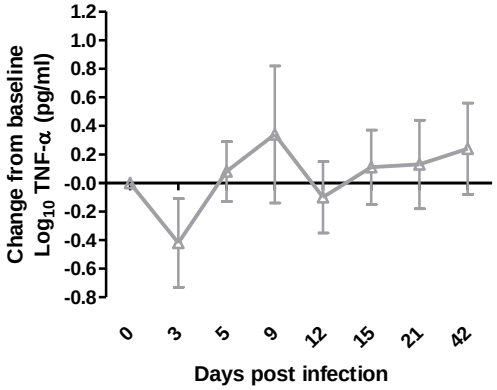


Figure 5.8b Smoking controls

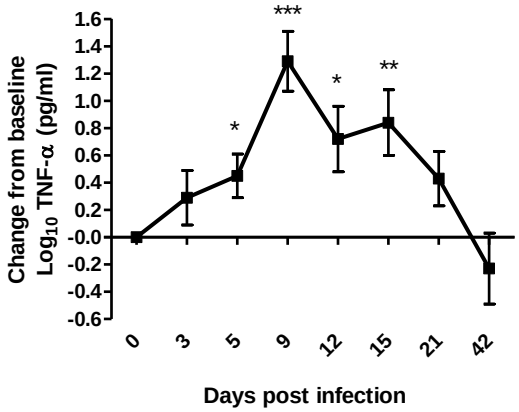


Figure 5.8c COPD subjects

Figure 5.8 TNF-α changes from baseline for (a) non-smoking controls, (b) smoking controls and (c) COPD subjects. Data presented as mean +/- SEM, using paired t test analysis for comparison between baseline and study visit (*p<0.05, **p<0.01, ***p<0.001)

Overall the pattern observed post RV infection was similar for IL-1β, IL-6, IL-8 and TNF-α in each study group over the time course of the study. There were significant differences between the mean cytokine concentrations of all three study groups on days 9 and 15, with highest levels measured in COPD subjects. Only the COPD group had a significant increase from baseline in all four pro-inflammatory cytokines on day 9.

5.8 Pro-inflammatory chemokines in sputum supernatant

The pro-inflammatory chemokines and mediators related to macrophage activity MIP-1β, GM-CSF and MCP-1 have been included in this section.

5.9 MIP-1β in sputum supernatant

The time course of study groups is shown in figure 5.9. There was no difference in sputum MIP-1β levels between groups at baseline (p=0.238). Only on day 9 was there a significant difference between the means of the three study groups when analysed using a one way ANOVA, p=0.007. Tukey's analysis demonstrated differences between non-smoking controls and COPD subjects (p=0.014) and smoking controls and COPD subjects, (p=0.017).

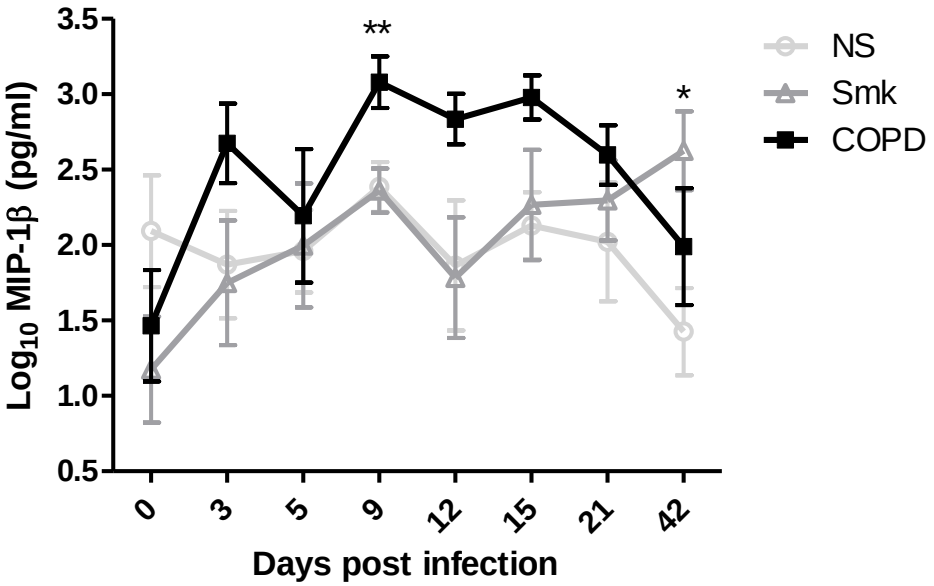


Figure 5.9 Time course of MIP-1β concentration in sputum supernatant of individual study groups. Data presented mean +/- SEM, one-way ANOVA analysis between the three study groups (**p<0.01)

Changes from baseline analysis using the paired t test for non-smoking controls showed no significant change at any time points. In smoking controls MIP-1β increased significantly from baseline on days 9, 15, 21 and 42 and in COPD on days 3 and 9 to 21 inclusive, figure 5.10.

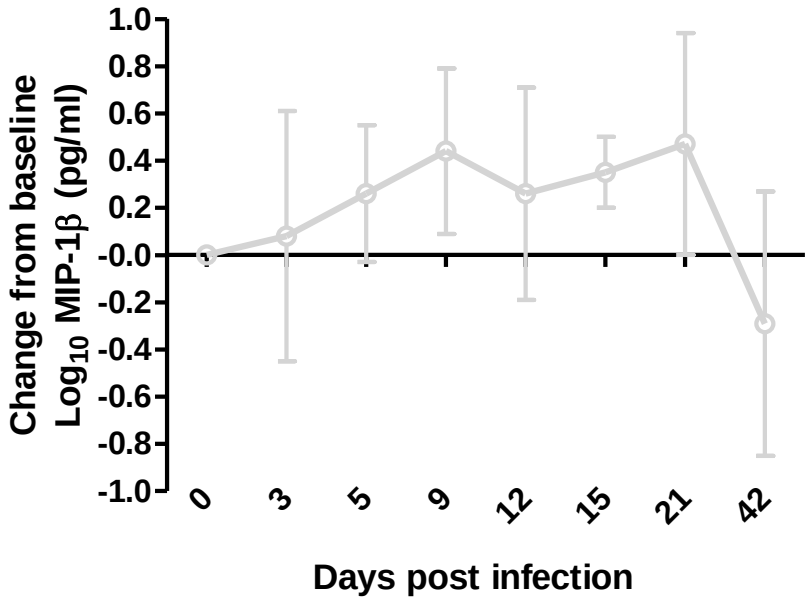


Figure 5.10a Non-smoking controls

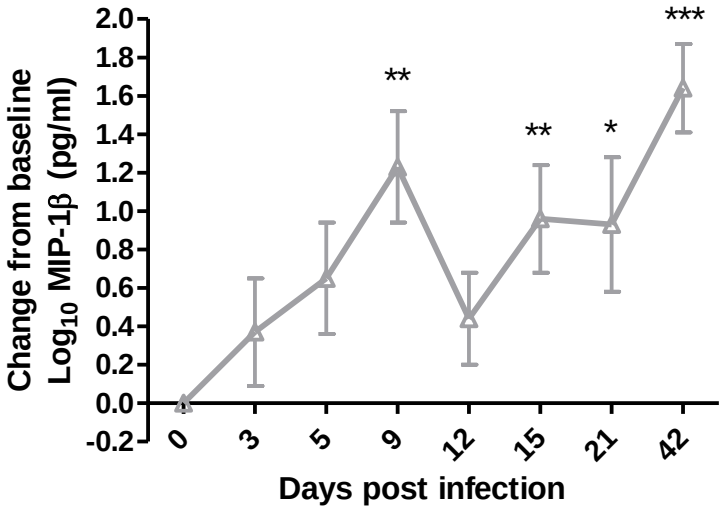


Figure 5.10b Smoking controls

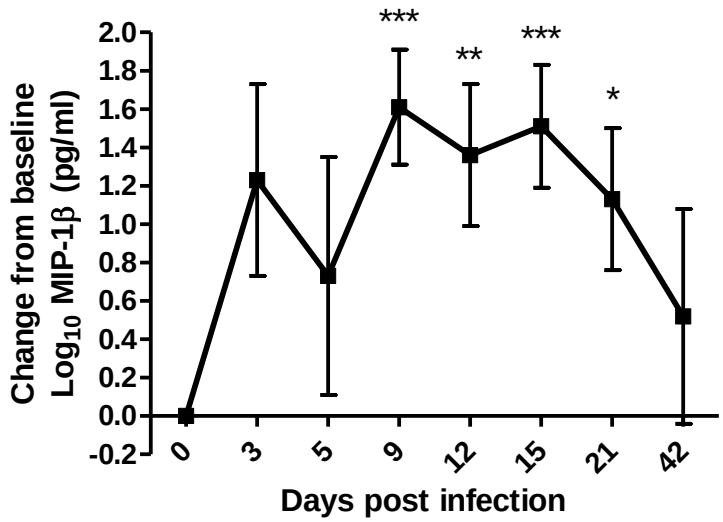


Figure 5.10c COPD subjects

Figure 5.10 MIP-1β changes from baseline for (a) non-smoking controls, (b) smoking controls and (c) COPD subjects. Data presented as mean +/- SEM, using paired t test analysis for comparison between baseline and study visit (*p<0.05, **p<0.01, ***p<0.001)

5.10 GM-CSF in sputum supernatant

GM-CSF levels were not significantly different at baseline between the three groups (p=0.871). The time course of GM-CSF in individual study groups is shown in figure 5.11. On days 5 to 42 inclusive there were significant differences between the means of the three study groups. Tukey's post hoc test showed differences between non-smoking controls and COPD subjects on all of these time points and between smoking controls and COPD on day 15 only (p=0.023). On days 21 and 42 there were significant differences between the two control groups.

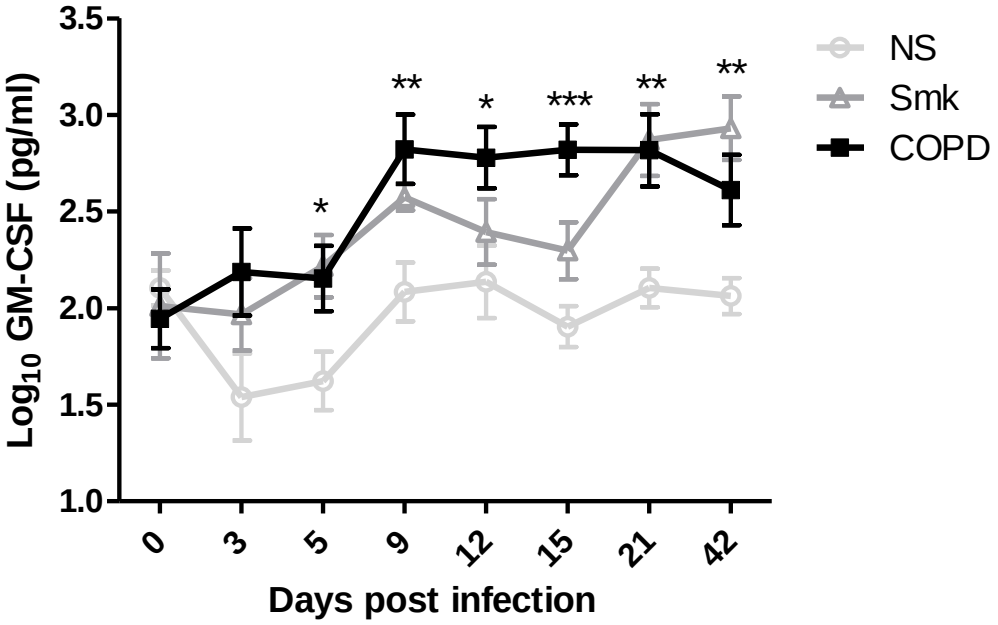


Figure 5.11 Time course of GM-CSF concentration in sputum supernatant of individual study groups. Data presented mean +/- SEM, one-way ANOVA analysis between the three study groups (*p<0.05, **p<0.01, ***p<0.001)

Changes from baseline using the paired t test showed significant increases in the COPD subjects on days 9 to 42 inclusive and in smoking controls on days 21 and 42, figure 5.12.

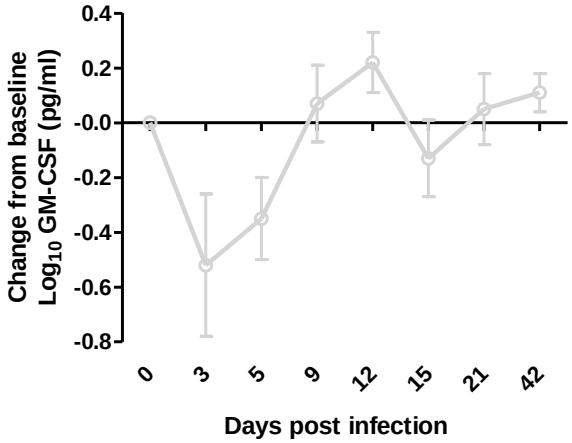


Figure 5.12a Non-smoking controls

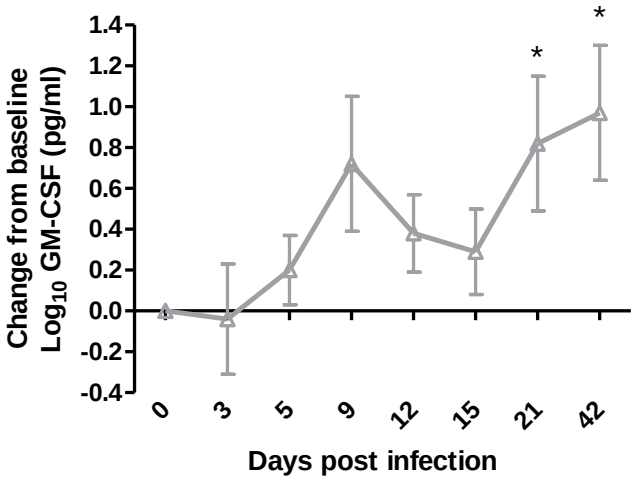


Figure 5.12b Smoking controls

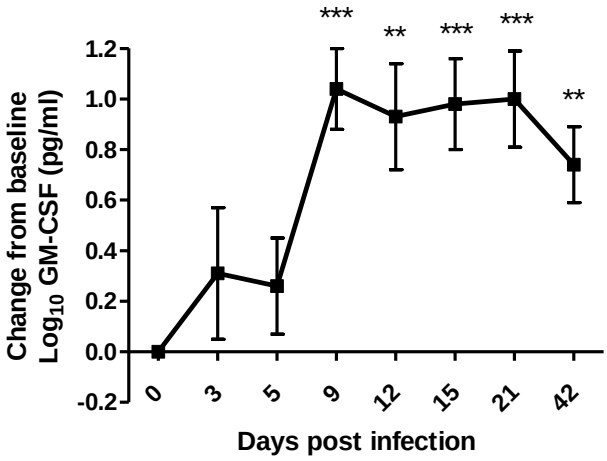


Figure 5.12c COPD subjects

Figure 5.12 GM-CSF changes from baseline for (a) non-smoking controls, (b) smoking controls and (c) COPD subjects. Data presented as mean +/- SEM, using paired t test analysis for comparison between baseline and study visit (*p<0.05, **p<0.01, ***p<0.001)

5.11 MCP-1 in sputum supernatant

The time course of GM-CSF in individual study groups is shown in figure 5.13. MCP-1 levels were not significantly different at baseline between the three groups (p=0.108). On days 9, 12 and 42 there were significant differences between the means of the three study groups. Tukey's post hoc test showed day 9 differences between non-smoking controls and COPD subjects (p<0.001) and smoking controls and COPD subjects (p=0.014). On day 12 the differences were between smoking controls and COPD subjects (p=0.028) and on day 42 the difference was between the two control groups (p=0.007).

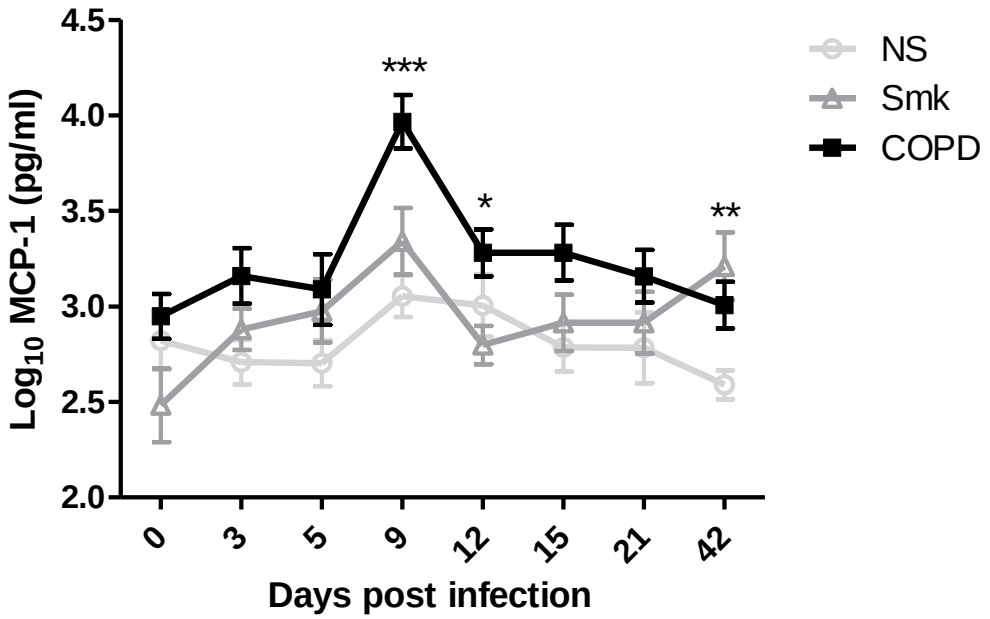


Figure 5.13 Time course of MCP-1 concentration in sputum supernatant of individual study groups. Data presented mean +/- SEM, one-way ANOVA analysis between the three study groups (*p<0.05, **p<0.01, ***p<0.001)

Changes from baseline using the paired t test showed significant increases in the COPD subjects on days 9 to 15 inclusive and in smoking controls on days 5, 9, 15 and 42, figure 5.14.

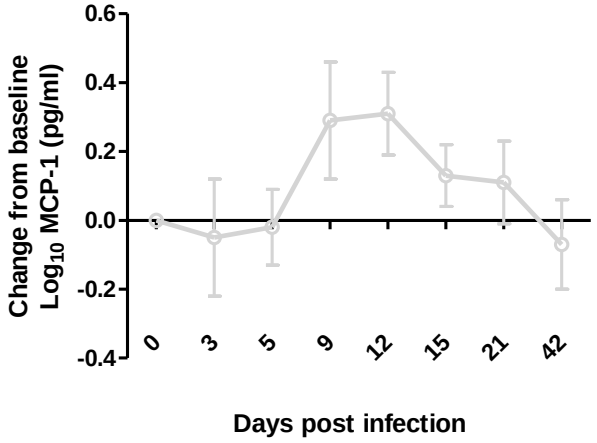


Figure 5.14a Non-smoking controls

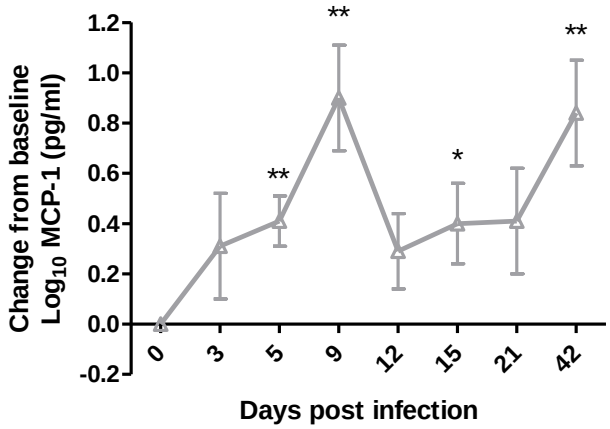


Figure 5.14b Smoking controls

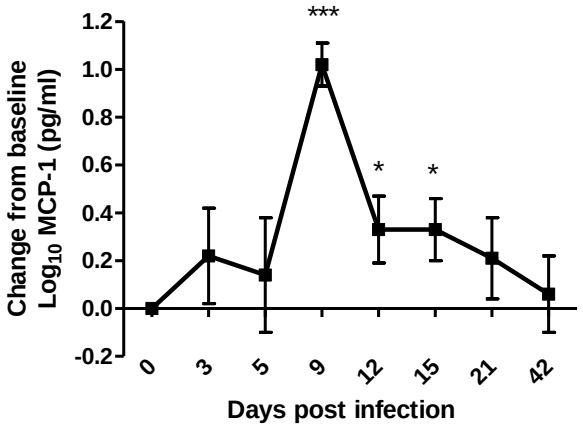


Figure 5.14c COPD subjects

Figure 5.14 MCP-1 changes from baseline for (a) non-smoking controls, (b) smoking controls and (c) COPD subjects. Data presented as mean +/- SEM, using paired t test analysis for comparison between baseline and study visit (*p<0.05, **p<0.01, ***p<0.001)

5.12 MMP-9 in sputum supernatant

The time course of the collagenase MMP-9 in individual study groups is shown in figure 5.15. MMP-9 levels were not significantly different at baseline between the three groups (p=0.108). On day 9 there were significant differences between non-smoking controls and COPD subjects (Tukey's post hoc, p=0.013). On day 15 there was a difference between non-smoking controls and COPD subjects in addition to smoking controls and COPD subjects (p=0.013 and 0.031 respectively).

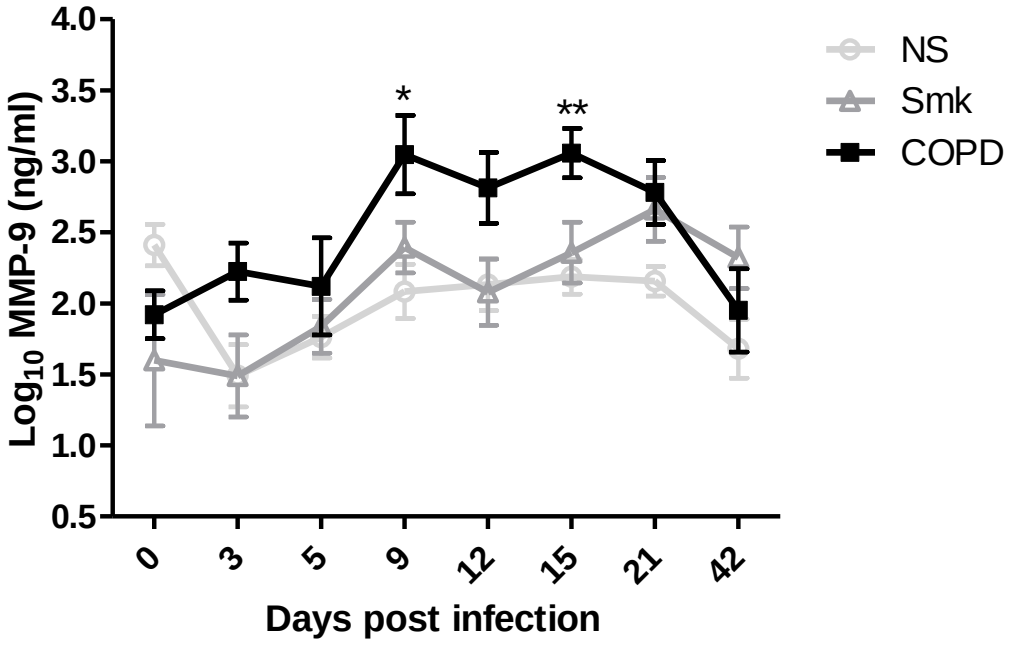


Figure 5.15 Time course of MMP-9 concentration in sputum supernatant of individual study groups. Data presented mean +/- SEM, one-way ANOVA analysis between the three study groups (*p<0.05, **p<0.01)

A significant change from baseline using the paired t test was found in non-smoking controls on days 3, 5 and 42; in smoking controls on day 15 and in COPD subjects on days 9 to 21 inclusive, figure 5.16.

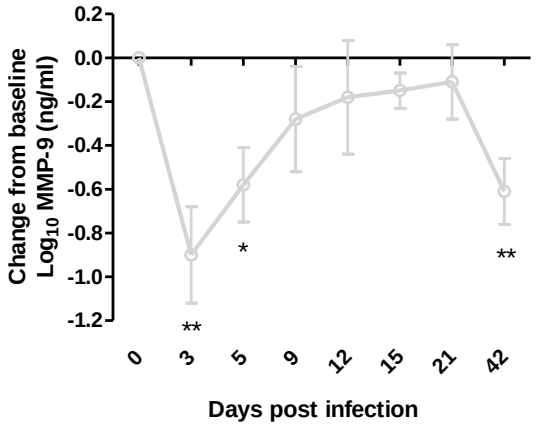


Figure 5.16a Non-smoking controls

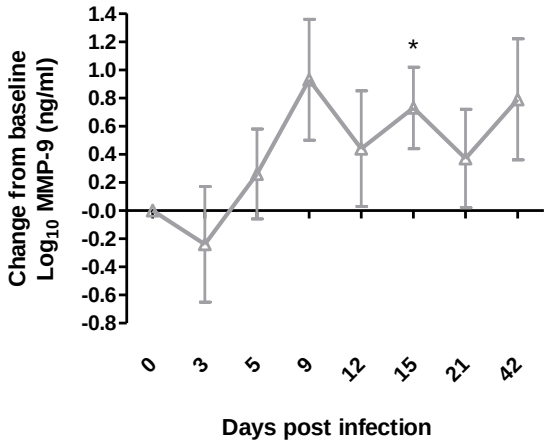


Figure 5.16b Smoking controls

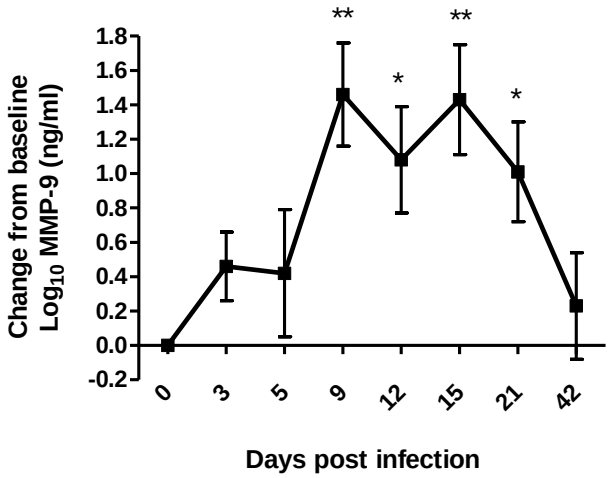


Figure 5.16c COPD subjects

Figure 5.16 MMP-9 changes from baseline for (a) non-smoking controls, (b) smoking controls and (c) COPD subjects. Data presented as mean +/- SEM, using paired t test analysis for comparison between baseline and study visit (*p<0.05, **p<0.01)

In summary MMP-9, MIP-1β, GM-CSF and MCP-1 were all found to change over the time course of the study in a similar pattern within each of the three study groups. There were trends to higher levels in COPD subjects at most time points throughout the study, with significantly higher levels on day 9 for all four mediators listed above. Only COPD subjects demonstrated a significant increase from baseline levels on days 9, 12 and 15.

5.13 Virus related cytokines in sputum supernatant

The results of IP-10 and IFN-γ concentrations in sputum supernatant have been selected to illustrate the differences between groups of virus related mediators.

5.14 IP-10 in sputum supernatant

Levels of IP-10 were not significantly different between the three groups at baseline (p=0.059). The time course of IP-10 in individual study groups is shown in figure 5.17. On days 9, 15 and 42 there was a significant difference between the means of the three study groups when analysed with one way ANOVA (p<0.001, p=0.003 and p=0.013 respectively). On days 9 and 15 there was a difference between the non-smoking controls and COPD subjects and on days 15 and 42 there was a difference between the two control groups.

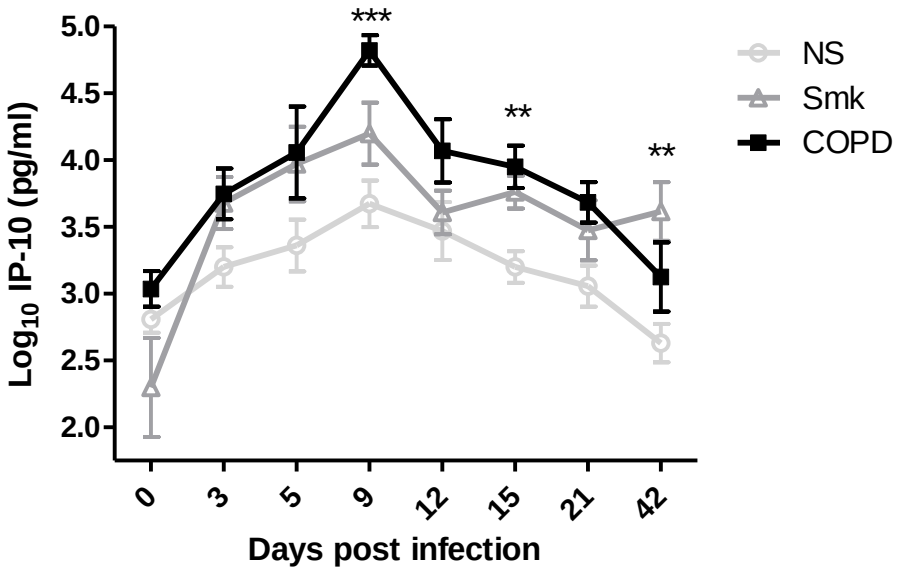


Figure 5.17 Time course of IP-10 concentration in sputum supernatant of individual study groups. Data presented mean +/- SEM, one-way ANOVA analysis between the three study groups (**p<0.01, ***p<0.001)

IP-10 concentrations were significantly increased compared to baseline in all three study groups on days 5 to 15 inclusive. Additionally in smoking controls, increases from baseline were identified on days 3, 21 and 42 and on day 21 in COPD subjects, figure 5.18.

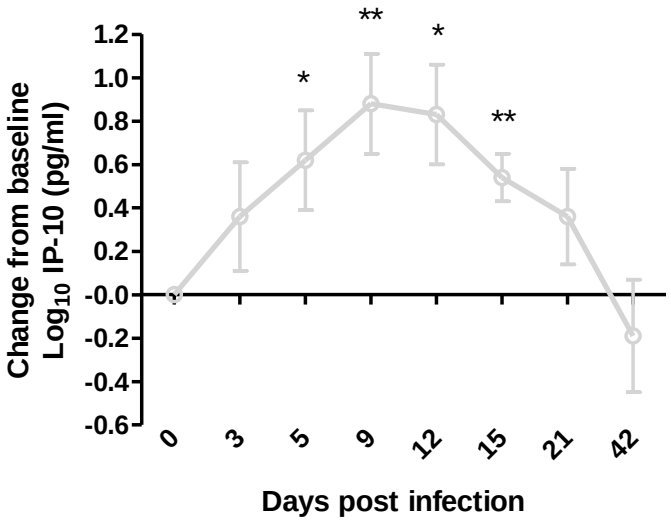


Figure 5.18a Non-smoking controls

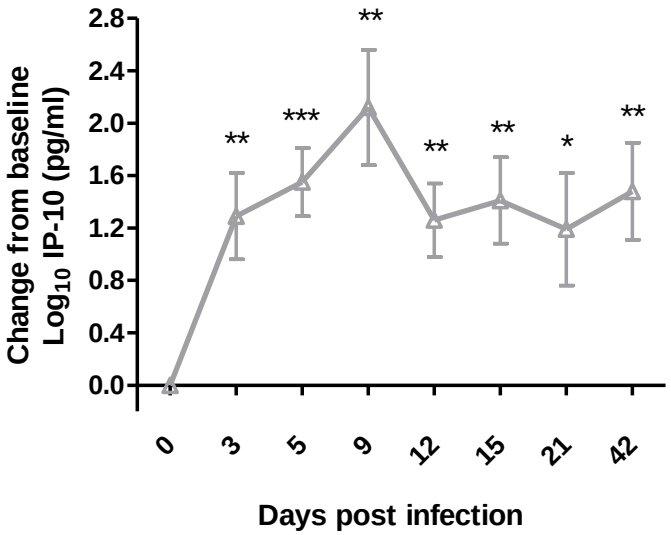


Figure 5.18b Smoking controls

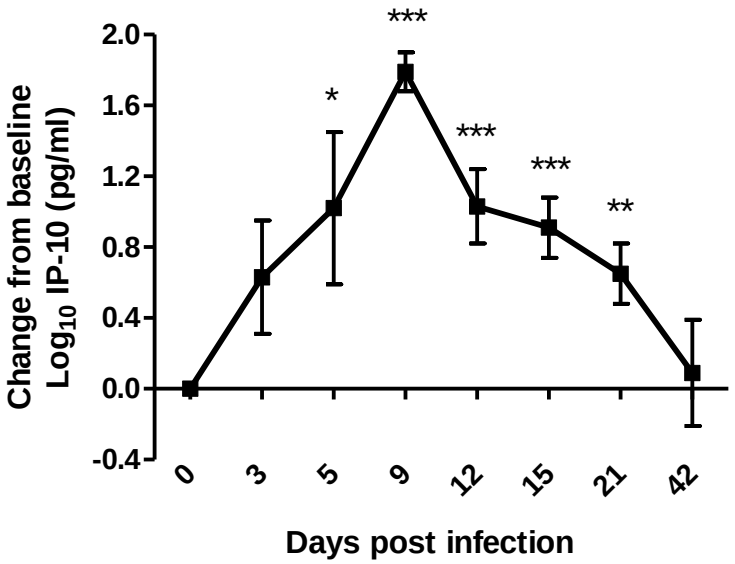


Figure 5.18c COPD subjects

Figure 5.18 IP-10 changes from baseline for (a) non-smoking controls, (b) smoking controls and (c) COPD subjects. Data presented as mean +/- SEM, using paired t test analysis for comparison between baseline and study visit (*p<0.05, **p<0.01, ***p<0.001)

5.15 IFN-γ in sputum supernatant

The time course of IFN-γ in individual study groups is shown in figure 5.19. Levels of IFN-γ were not significantly different between the three groups at baseline (p=0.203). There was a significant difference between the means of the three study groups on days 9, 12, 15 and 21 when analysed using one way ANOVA (p=0.002, p=0.012, p=0.012 and p=0.021 respectively). Post hoc Tukey's analysis confirmed that on days 9, 12 and 15 the differences were between the two control groups and the non-smoking and COPD groups.

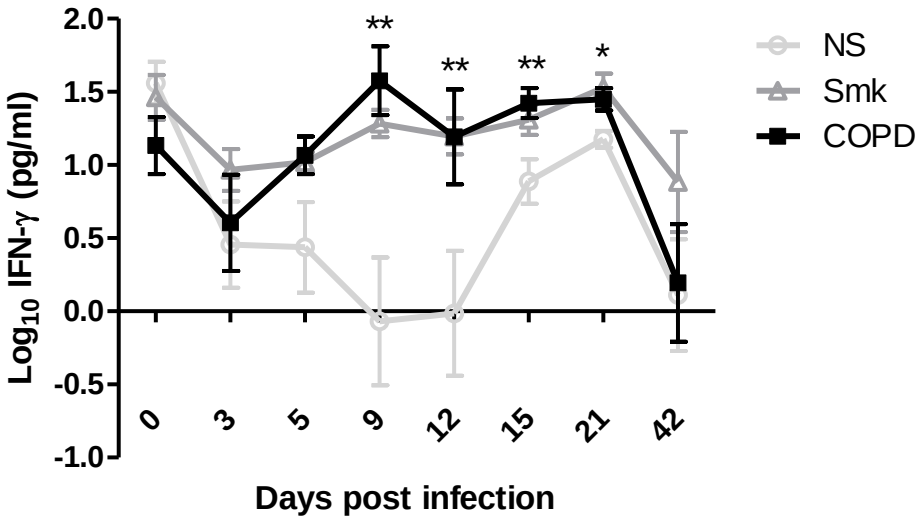


Figure 5.19 Time course of IFN-γ concentration in sputum supernatant of individual study groups. Data presented mean +/- SEM, one-way ANOVA analysis between the three study groups (*p<0.05, **p<0.01)

There was no difference in IFN-γ concentrations between the smoking controls and COPD subjects on these time points. Changes from baseline are shown in figure 5.20. This demonstrated reduction in IFN-γ concentrations in the control groups and increased levels compared to baseline in the COPD subjects.

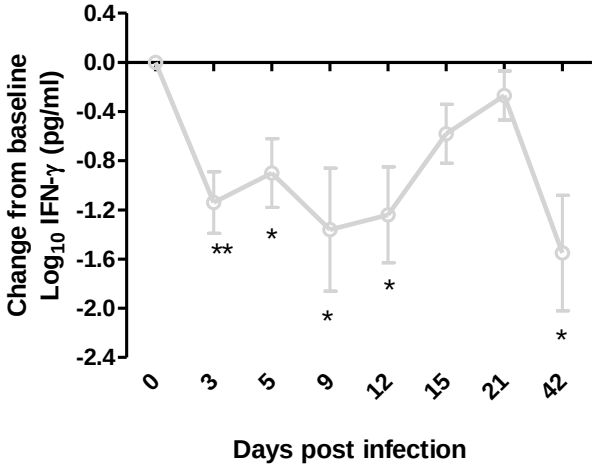


Figure 5.20a Non-smoking controls

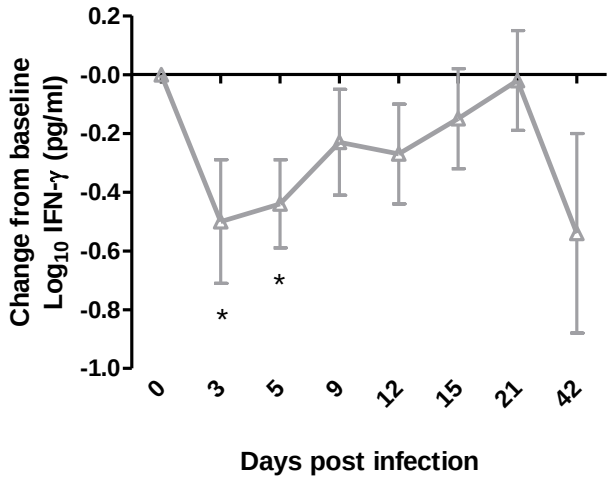


Figure 5.20b Smoking controls

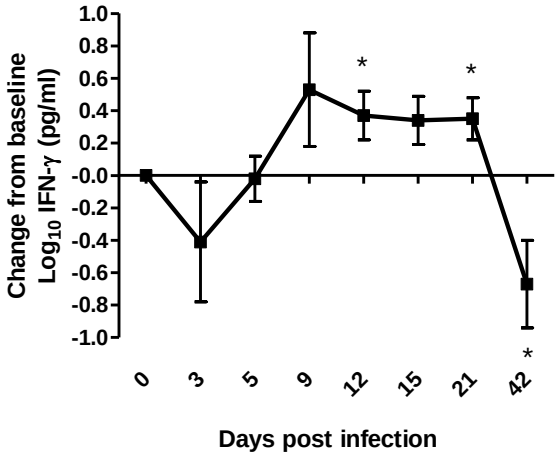


Figure 5.20 (a) non-smoking controls, (b) smoking controls and (c) COPD subjects

Figure 5.20 IFN-γ changes from baseline for (a) non-smoking controls, (b) smoking controls and (c) COPD subjects. Data presented as mean +/- SEM, using paired t test analysis for comparison between baseline and study visit (*p<0.05, **p<0.01, ***p<0.001)

5.16 Eosinophil related mediator in sputum supernatant

Eotaxin-3 in sputum supernatant

Baseline levels of Eotaxin-3 were not significantly different between the three groups (p=0.914). The time course of Eotaxin-3 in individual study groups is shown in figure 5.21. On days 5, 9, 15, 21 and 42 there was a significant difference between the means of the three study groups when analysed with one way ANOVA. Differences

were detected between the two control groups on days 5, 15, 21 and 42, and between non-smoking controls and COPD subjects on all these time points. Compared to baseline, Eotaxin-3 concentrations were significantly increased on day 15 in non-smoking controls, days 5, 15, 21 and 42 in smoking controls and on all post infection time points in COPD subjects, figure 5.22

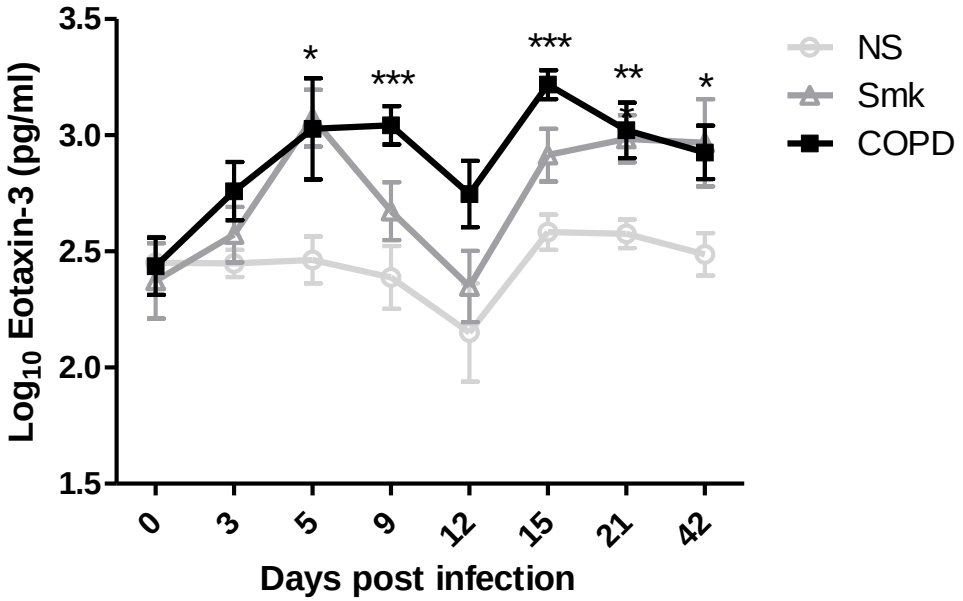


Figure 5.21 Time course of Eotaxin-3 concentration in sputum supernatant of individual study groups. Data presented mean +/- SEM, one-way ANOVA analysis between the three study groups (*p<0.05, **p<0.01, ***p<0.001)

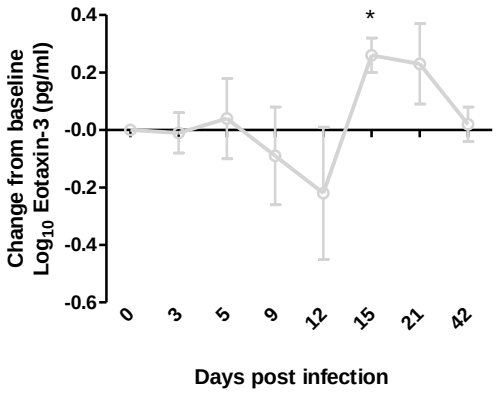


Figure 5.22a Non-smoking controls

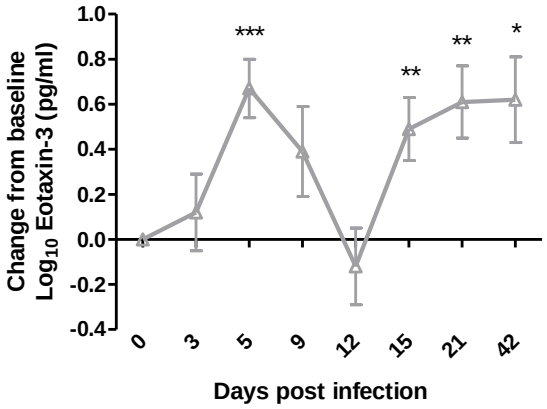


Figure 5.22b Smoking controls

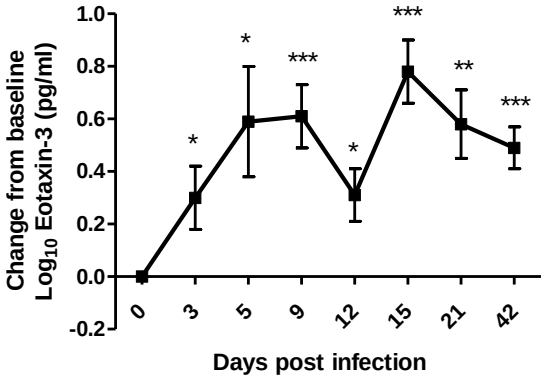


Figure 5.22c COPD subjects

Figure 5.22 Eotaxin-3 changes from baseline for (a) non-smoking controls, (b) smoking controls and (c) COPD subjects. Data presented as mean +/- SEM, using paired t test analysis for comparison between baseline and study visit (*p<0.05, **p<0.01, ***p<0.001)

5.17 BAL Supernatant Mediators

All MSD assays in BAL supernatant were performed in duplicate wells and the mean concentration calculated. Correcting for both dilution and variations in the volume of BAL samples recovered is controversial, with several methods recognised in the literature. Here to maintain consistency throughout the study the same volume of saline was instilled during each bronchoscopy. There was no significant difference in the total volume of BAL recovered either between or within groups, data presented in chapter 3. No correction has been made for the BAL cell count. Analysis of differences between groups at baseline and day 7 were performed using one way ANOVA. Changes from baseline to day 7 in each of the three study groups was performed with a paired t test for each mediator presented.

5.18 Pro-inflammatory cytokines in BAL

Results of the proinflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α in BAL supernatant have been included in this section. The pattern between groups and across the time course of the study was similar for each of those examined, however only IL-1β had significant differences between the three study groups.

5.19 IL-1β in BAL

BAL IL-1β levels were significantly different between groups at baseline (p=0.001) and day 7 (p=0.010) these differences were between the two control groups, figure 5.23. There was no significant increase from baseline to day 7 in any of the study groups.

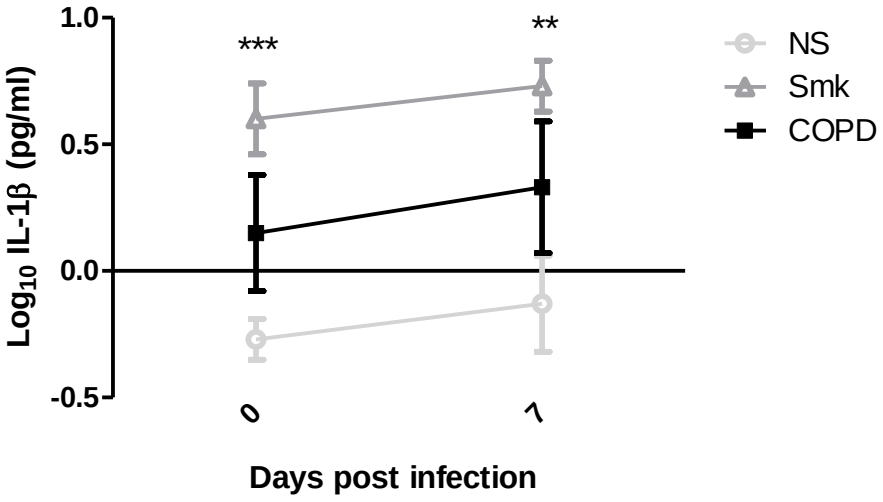


Figure 5.23 Pre and post-infection IL-1β concentration in BAL supernatant of individual study groups. Data presented mean +/- SEM, one-way ANOVA analysis between the three study groups (**p<0.01, ***p<0.001)

5.20 IL-6 in BAL

The concentrations of BAL IL-6 were not significantly different between the three study groups at baseline or day 7 and no changes from baseline were detected in individual study groups, figure 5.24.

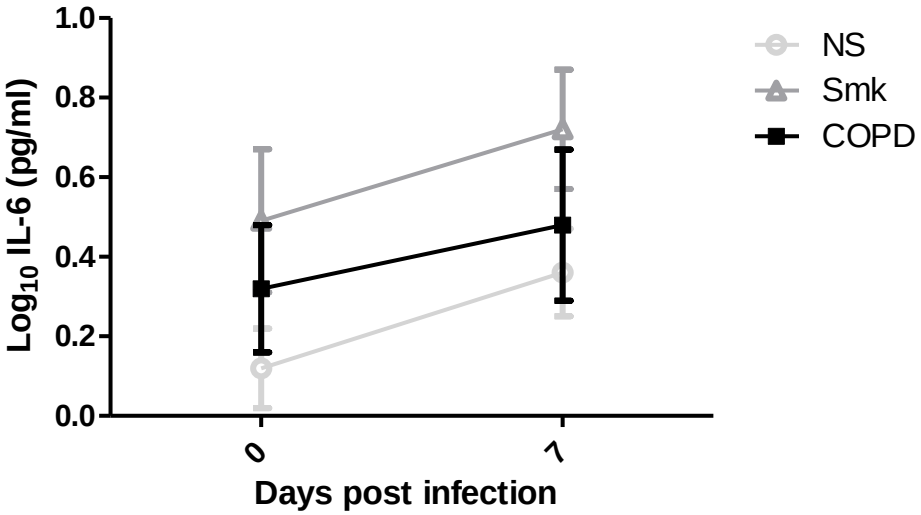


Figure 5.24 Pre and post-infection IL-6 concentration in BAL supernatant of individual study groups. Data presented mean +/- SEM, one-way ANOVA analysis between the three study groups

5.21 IL-8 in BAL

The concentrations of BAL IL-8 were not significantly different between the three study groups at baseline or day 7 and no changes from baseline were detected in individual study groups, figure 5.25.

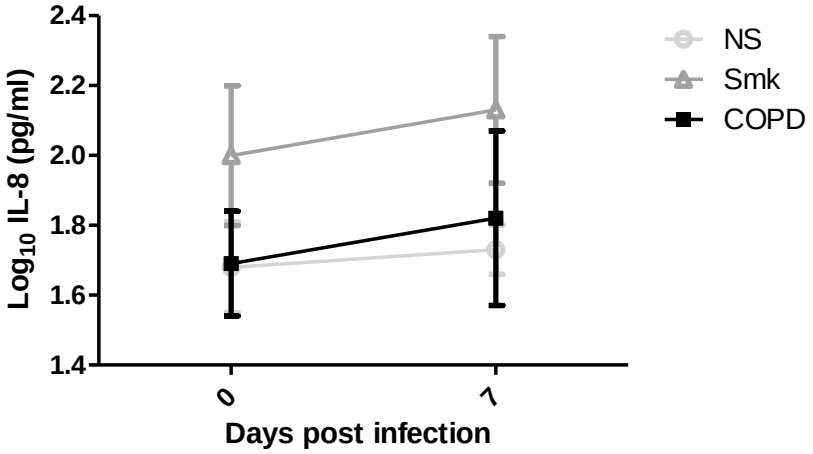


Figure 5.25 Pre and post-infection IL-8 concentration in BAL supernatant of individual study groups. Data presented mean +/- SEM, one-way ANOVA analysis between the three study groups

5.22 TNF-α in BAL

The BAL concentration of TNF-α was not different between the three study groups at baseline or day 7. There was a significant increase in TNF-α concentrations from baseline in non-smoking controls (p=0.006) and COPD subjects (p=0.024), figure 5.26



Figure 5.26 Pre and post-infection TNF-α concentration in BAL supernatant of individual study groups. Data presented mean +/- SEM, one-way ANOVA analysis between the three study groups. Paired t test for change from baseline: **p<0.01 non-smoking controls and •p<0.05 COPD subjects

5.23 Pro-inflammatory chemokines in BAL

The macrophage related mediators MIP-1β, GM-CSF and MCP-1 have been shown below.

5.24 MIP-1β in BAL

The concentrations of BAL MIP-1β were not significantly different between the three study groups at baseline or day 7 and no changes from baseline were detected in individual study groups, figure 5.27.

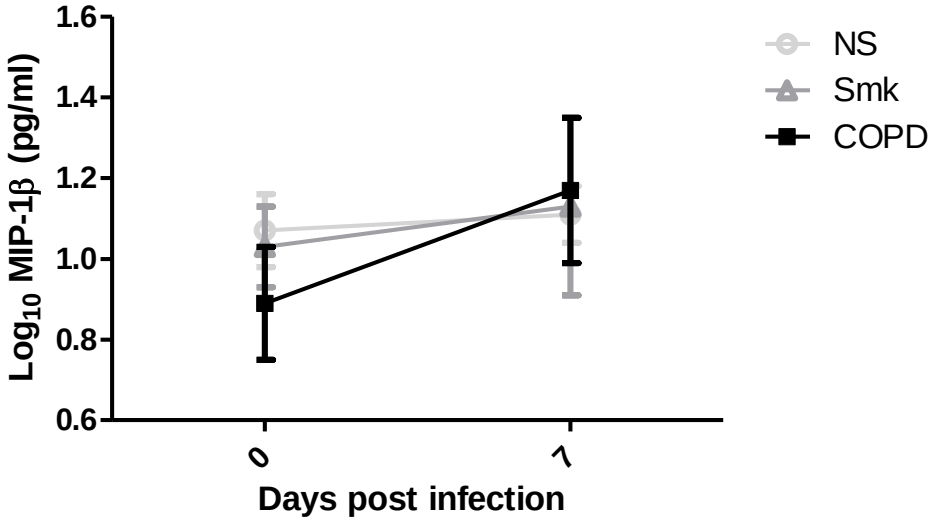


Figure 5.27 Pre and post-infection MIP-1β concentration in BAL supernatant of individual study groups. Data presented mean +/- SEM, one-way ANOVA analysis between the three study groups

5.25 GM-CSF in BAL

The BAL concentration of GM-CSF was not different between the three study groups at baseline or day 7. There was a significant increase in GM-CSF concentrations from baseline in COPD subjects (p=0.044), figure 5.28.

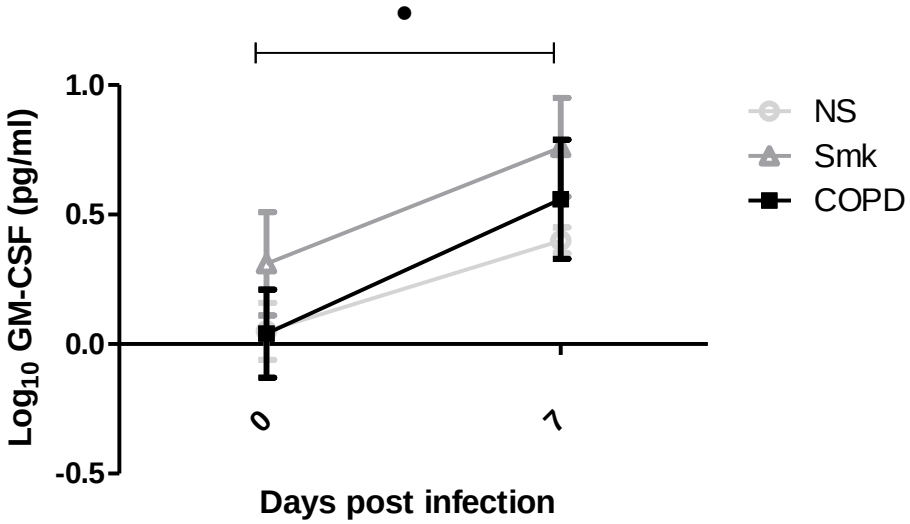


Figure 5.28 Pre and post-infection TNF-α concentration in BAL supernatant of individual study groups. Data presented mean +/- SEM, one-way ANOVA analysis between the three study groups. Paired t test for change from baseline: •p<0.05 COPD subjects

5.26 MCP-1 in BAL

The concentrations of BAL MCP-1 were not significantly different between the three study groups at baseline or day 7 and no changes from baseline were detected in individual study groups, figure 5.29.

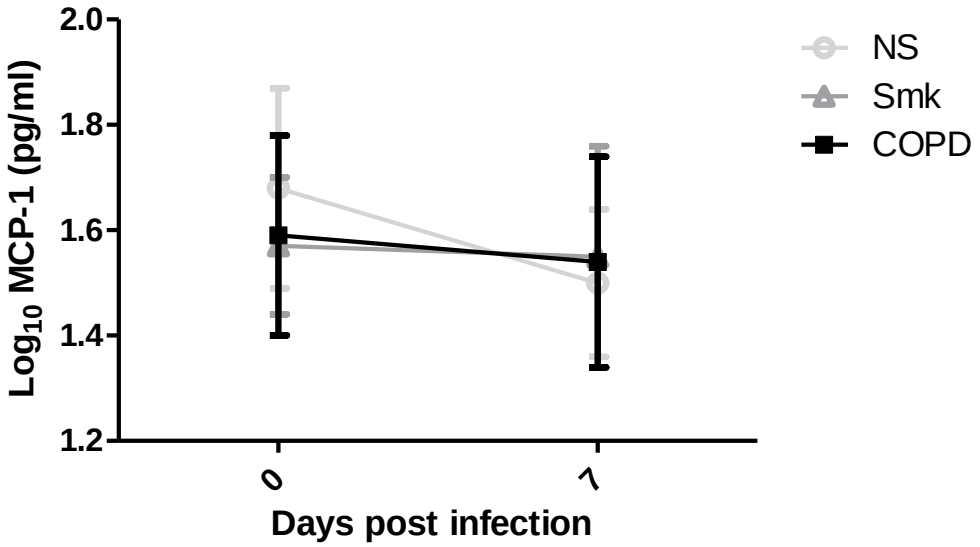


Figure 5.29 Pre and post-infection MCP-1 concentration in BAL supernatant of individual study groups. Data presented mean +/- SEM, one-way ANOVA analysis between the three study groups

5.27 MMP-9 in BAL

There was no difference in MMP-9 levels between the study groups at either baseline or day 7. From baseline MMP-9 levels increased significantly in all three study groups (paired t test, non-smoking controls p=0.008, smoking controls p=0.032 and COPD subjects p=0.005), figure 5.30.

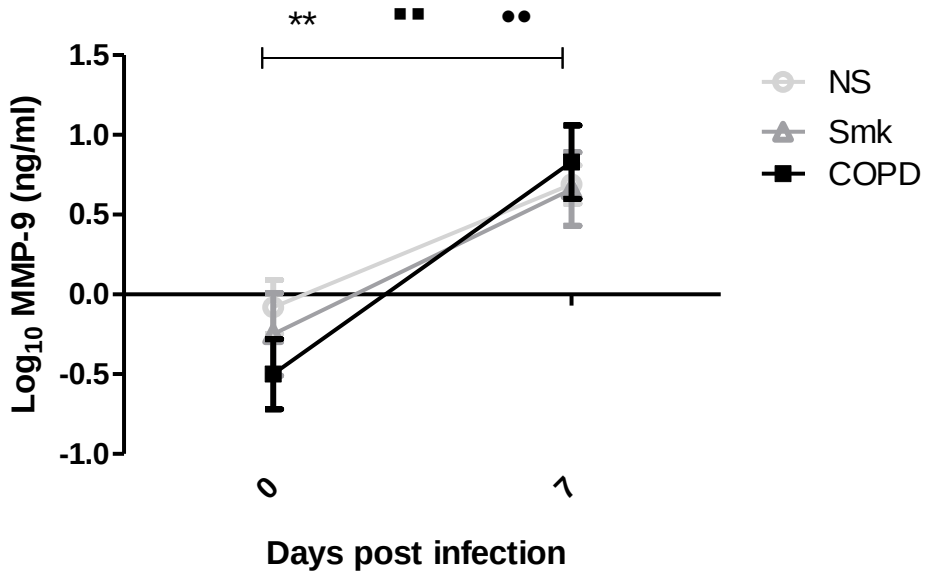


Figure 5.30 Pre and post-infection MMP-9 concentration in BAL supernatant of individual study groups. Data presented mean +/- SEM, one-way ANOVA analysis between the three study groups. Paired t test for change from baseline: **p<0.01 non-smoking controls, ▪▪p<0.01 smoking controls and ¨p<0.01 COPD subjects

5.28 Virus related cytokines in BAL

The results of IP-10 and IFN-γ concentrations in BAL supernatant are shown.

5.29 IP-10 in BAL

The concentrations of BAL IP-10 were not significantly different between the three study groups at baseline or day 7 and no changes from baseline were detected in individual study groups, figure 5.31.

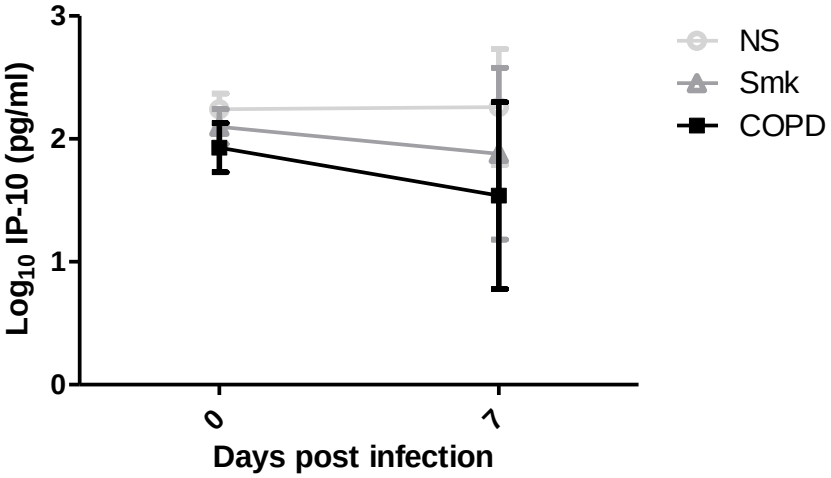


Figure 5.31 Pre and post-infection IP-10 concentration in BAL supernatant of individual study groups. Data presented mean +/- SEM, one-way ANOVA analysis between the three study groups

5.30 IFN-γ in BAL

IFN-γ concentrations were not significantly different between the three study groups at baseline or day 7. Only COPD subjects had a significant increase from baseline, p=0.038, figure 5.31.

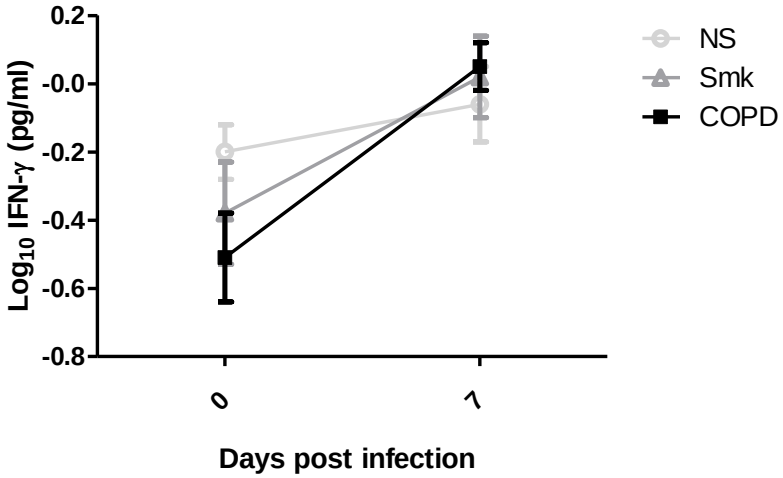


Figure 5.32 Pre and post-infection IFN-γ concentration in BAL supernatant of individual study groups. Data presented mean +/- SEM, one-way ANOVA analysis between the three study groups. Paired t test for change from baseline: *p<0.05 COPD subjects

5.31 Eosinophil related mediators in BAL

The results of Eotaxin and Eotaxin-3 concentrations in BAL supernatant are shown.

5.32 Eotaxin in BAL

BAL Eotaxin levels were significantly different between groups at baseline (p=0.023) but not at day 7. The difference at baseline were between non-smoking controls and COPD subjects (p=0.043) and smoking controls and COPD subjects (p=0.033). There was no significant increase from baseline to day 7 in any of the study groups, figure 5.33.

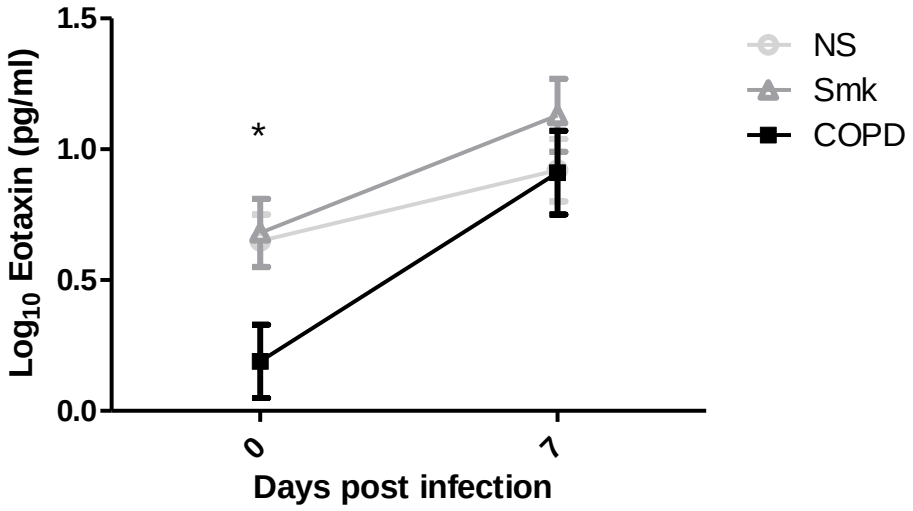


Figure 5.33 Pre and post-infection Eotaxin concentration in BAL supernatant of individual study groups. Data presented mean +/- SEM, one-way ANOVA analysis between the three study groups.

5.33 Eotaxin-3 in BAL

The concentrations of BAL Eotaxin-3 were not significantly different between the three study groups at baseline or day 7 and no changes from baseline were detected in individual study groups, figure 5.34.

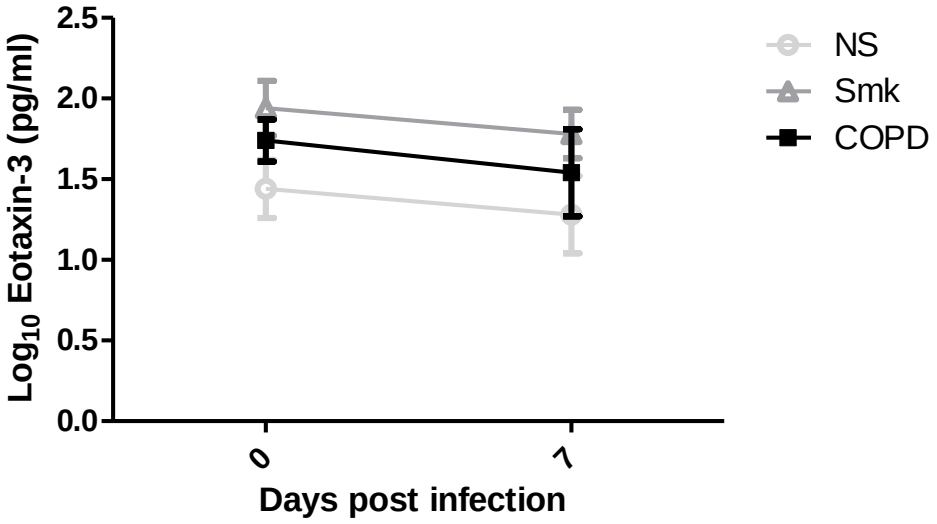


Figure 5.34 Pre and post-infection Eotaxin-3 concentration in BAL supernatant of individual study groups. Data presented mean +/- SEM, one-way ANOVA analysis between the three study groups

5.34 Correlation of pro-inflammatory cytokines and LRT scores

In chapter 4 it was shown that the virus load in nasal lavage and sputum correlated strongly with URT and LRT symptom scores in COPD subjects, ($r=0.52$, $p<0.001$) and ($r=0.63$, $p<0.001$) respectively. To evaluate the association between inflammatory markers in sputum and their possible effect on clinical symptoms, the inflammatory cytokines and LRT scores has been correlated in this section.

When all subjects were considered in combination IL-1β and the total LRT score were found to have a positive correlation ($r=0.38$, $p<0.001$), figure 5.35. In individual groups the relationship was strongest in COPD subjects ($r=0.44$, $p<0.001$), followed by smoking controls ($r=0.37$, $p=0.006$); however the relationship was not maintained in non-smoking controls ($r=0.03$, $p=0.788$), figure 5.36. This is likely to reflect minimal changes in LRT scores in non-smoking controls during the time course of the study.

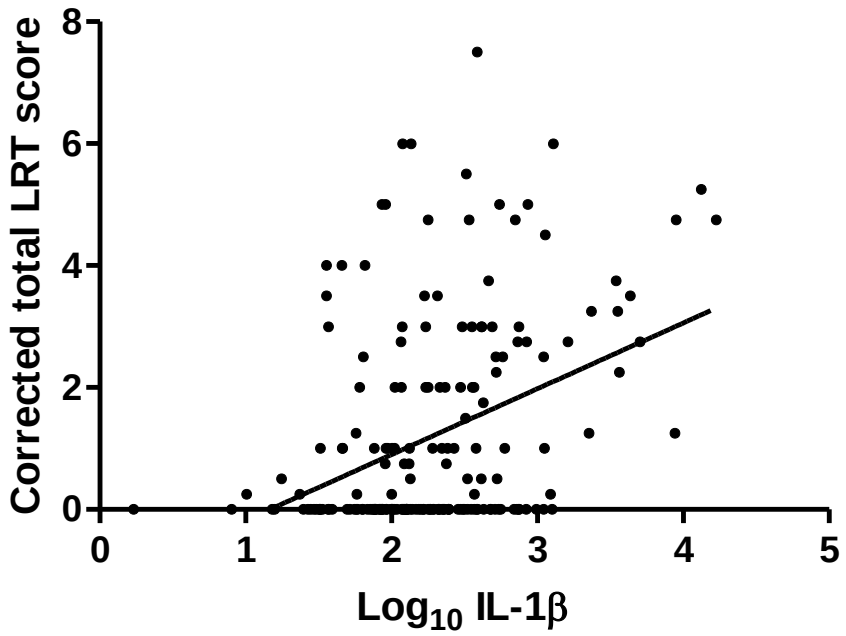


Figure 5.35 Correlation of sputum IL-1β concentration and corrected LRT symptom scores in all subjects combined (r=0.38, p<0.001)

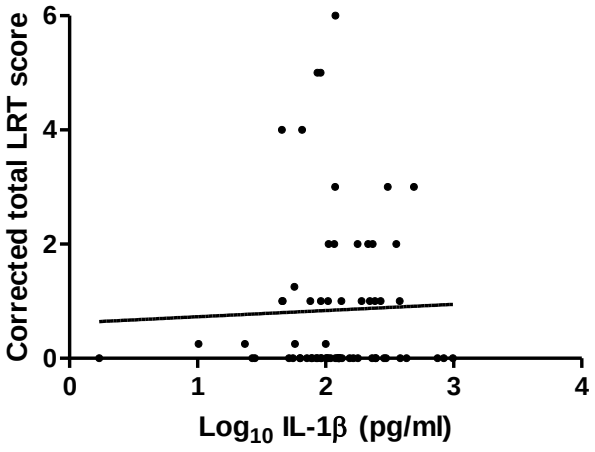


Figure 5.36a Non-smoking controls (r=0.03, p=0.79)

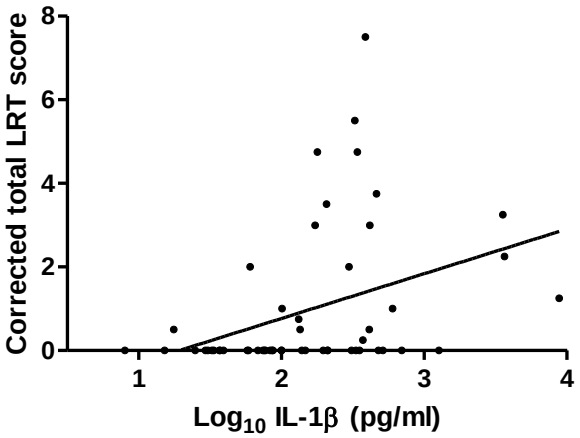


Figure 5.36b Smoking controls (r=0.37, p=-0.006)

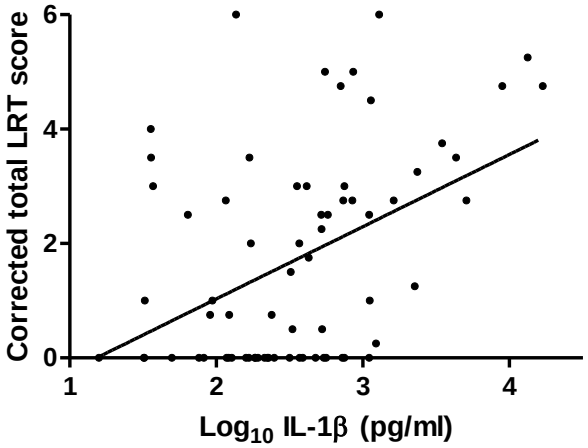


Figure 5.36c COPD subjects (r=0.44, p<0.001)

Figure 5.36 Correlation of sputum IL-1β concentration and corrected LRT symptom scores in (a) non-smoking controls, (b) smoking controls and (c) COPD subjects

A similar pattern was identified when the correlation between TNF-α and LRT scores was examined. In all subjects combined the relationship was shown to correlate positively (r=0.34, p<0.001), figure 5.37a but this is again driven mainly by COPD subjects (r=0.44, p<0.001), figure 5.37b. Only in non-smoking controls was there no correlation between the two parameters (r=-0.08, p=0.525).

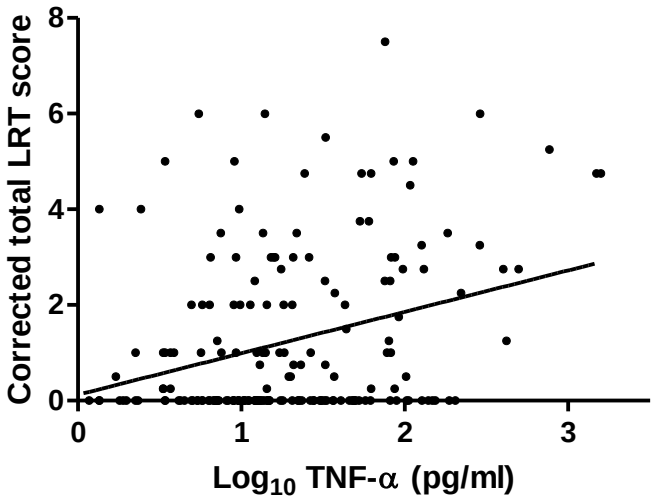


Figure 5.37a All subjects combined (r=0.34, p<0.001)

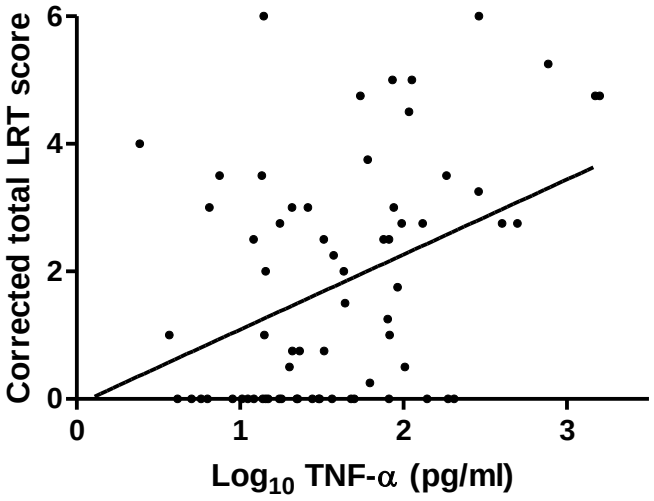


Figure 5.37b COPD subjects (r=0.44, p<0.001)

Figure 5.37 Correlation of sputum TNF-α concentration and corrected LRT symptom scores in (a) all subjects combined and (b) COPD subjects

The mediators that were found to have the strongest correlation with LRT scores in COPD subjects were TNF-α and IL-1β. These two markers were also found to correlate most strongly when the relationship between LRT scores and sputum viral load was examined. Further evidence that increasing concentrations of inflammatory cytokines drives an increase in LRT symptoms is provided in table 5.1. Here the correlation coefficient is found to be greater for all mediators measured in COPD subjects compared to the study population.

	Non-smoking controls		Smoking controls		COPD	
	Coefficient (r)	p-value	Coefficient (r)	p-value	Coefficient (r)	p-value
TNF-α	-0.08	0.525	0.33	0.017	0.44	0.001
IL-1β	0.03	0.788	0.37	0.006	0.44	0.001
IL-10	-0.02	0.854	0.20	0.155	0.40	0.001
IFN-γ	-0.24	0.046	0.20	0.147	0.36	0.002
IL-8	-0.10	0.410	0.38	0.005	0.35	0.003
IL-2	-0.03	0.820	0.36	0.009	0.33	0.005
IL-6	-0.05	0.671	0.23	0.096	0.29	0.016
IL-12	-0.14	0.238	0.30	0.029	0.26	0.027
GM-CSF	-0.12	0.343	0.28	0.045	0.24	0.049

Table 5.1 Pearson's correlation coefficient for LRT symptom scores and inflammatory cytokines in the three study groups

5.35 Correlation of chemokines and LRT scores

Calculation of the association between total LRT scores and sputum chemokines was performed to investigate the strength of the relationship; data for all chemokines measured in the MSD assay are presented in this section. MCP-1 and IP-10 were found to have the greatest correlation coefficient with symptoms score.

The correlation between MCP-1 and total LRT score is shown in for all subjects ($r=0.32$, $p<0.001$), figure 5.38a and for the COPD group ($r=0.39$, $p<0.001$), figure 5.38b.

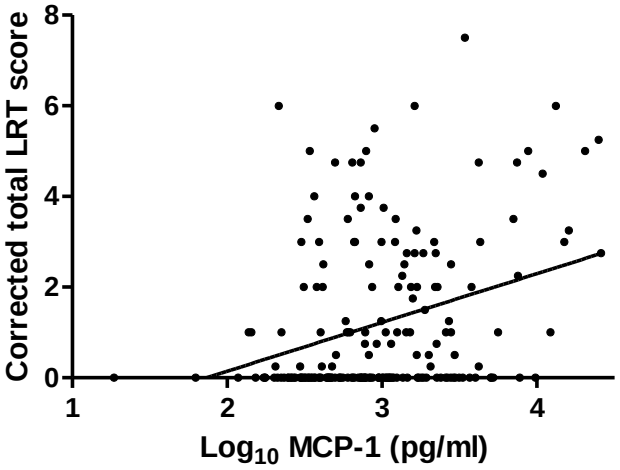


Figure 5.38a All subjects combined (r=0.32, p<0.001)

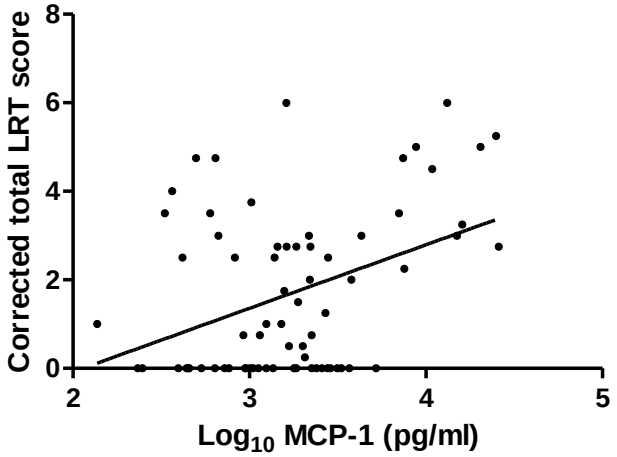


Figure 5.38b COPD subjects (r=0.39, p<0.001)

Figure 5.38 Correlation of sputum MCP-1 concentration and corrected LRT symptom scores in (a) all subjects combined and (b) COPD subjects

When the correlation between IP-10 and LRT score was investigated the results were highly significant for all subjects combined, figure 5.39a. The COPD group had the strongest association (r=0.38, p=0.001), figure 5.39b compared to non-smoking (r=0.16, p=0.186) and smoking (r=0.40, p=0.004) controls. The data for all subjects combined and the COPD group are shown, figure 5.39.

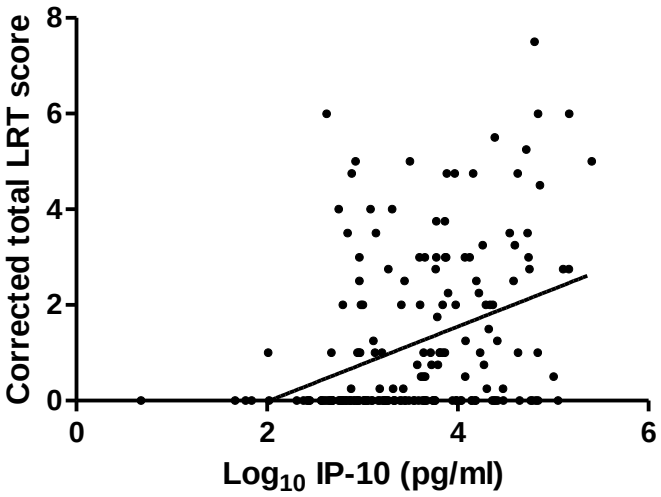


Figure 5.39a All subjects combined (r=0.37, p<0.001)

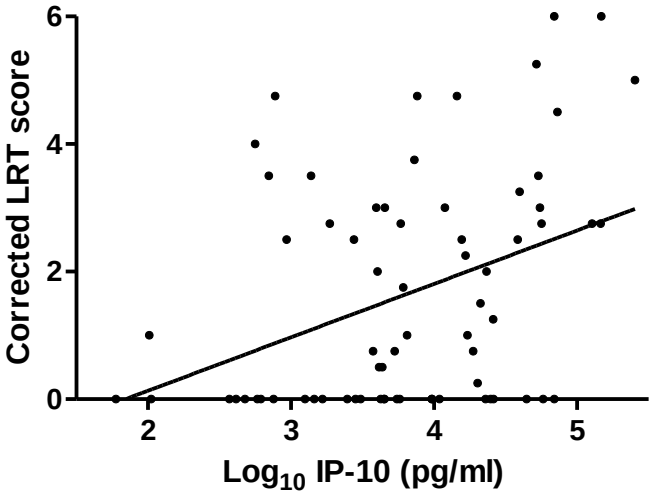


Figure 5.39b COPD subjects (r=0.38, p=0.001)

Figure 5.39 Correlation of sputum IP-10 concentration and corrected LRT symptom scores in (a) all subjects combined and (b) COPD subjects

In addition to LRT symptom scores concentrations of sputum MCP-1 and IP-10 were found to correlate most strongly with sputum virus load, chapter 4. The Pearson's coefficient for correlations between all measured chemokines and LRT symptom scores is shown in table 5.2. The COPD group demonstrated the strongest association for all parameters measured when compared to controls.

	Non-smoking controls		Smoking controls		COPD	
	Coefficient (r)	p-value	Coefficient (r)	p-value	Coefficient (r)	p-value
MCP-1	0.00	0.968	0.27	0.053	0.39	0.001
IP-10	0.16	0.186	0.40	0.004	0.38	0.001
Eotaxin	0.14	0.255	0.35	0.012	0.37	0.002
MCP-4	-0.09	0.478	0.32	0.019	0.30	0.010
MDC	0.16	0.182	0.30	0.032	0.29	0.014
MIP-1β	-0.12	0.314	0.35	0.011	0.27	0.023
Eotaxin-3	-0.20	0.099	0.33	0.016	0.23	0.056
TARC	0.09	0.466	0.23	0.104	0.16	0.196

Table 5.2 Pearson's correlation coefficient for LRT symptom scores and chemokines in the three study groups

5.36 Anti-inflammatory cytokine (IL-10) results

IL-10 is regarded as an anti-inflammatory cytokine, sputum and BAL IL-10 results have been included in this final section of the chapter. The results of mean sputum IL-10 concentration from individual study groups is shown in figure 5.40. There were significant differences between the group means on days 5, 9, 12, 15, 21 and 42. At baseline there was a trend to lower mean concentrations in the COPD group; following infection the levels increased in this group until a peak on day 9. In control subjects the levels fall immediately following infection. In non-smokers they remain significantly lower on days 3, 5 and 15 following infection. However in smoking controls they increased again to a peak similar to COPD subjects on day 21.

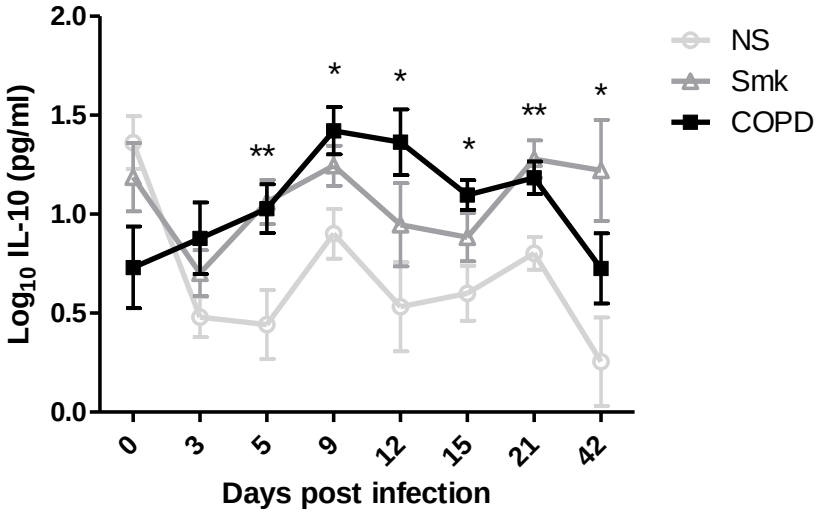


Figure 5.40 Time course of IL-10 concentration in sputum supernatant of individual study groups. Data presented mean +/- SEM, one-way ANOVA analysis between the three study groups (*p<0.05, **p<0.01)

5.37 IL-10 in BAL

BAL IL-10 levels were significantly different between groups at baseline (p=0.034) but not at day 7. The difference at baseline were between smoking controls and COPD subjects (p=0.027). There was a significant increase from baseline to day 7 in COPD subjects (p=0.024), figure 5.41.

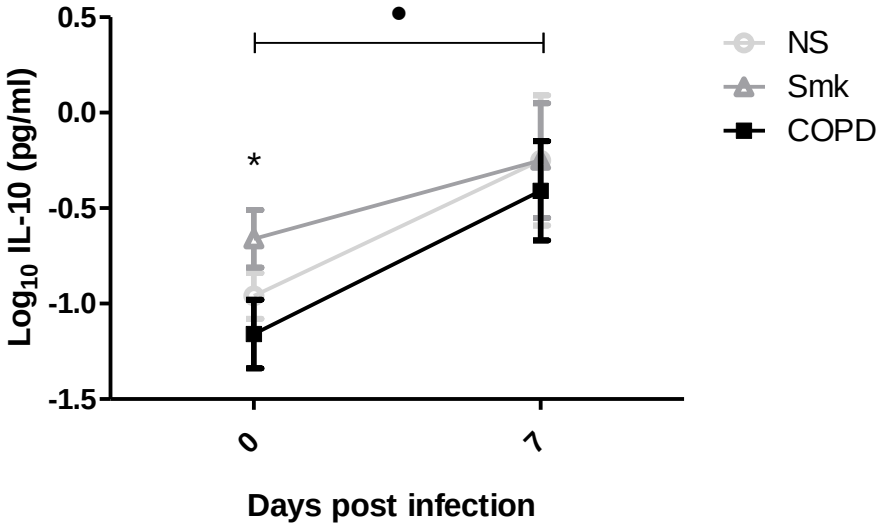


Figure 5.41 Pre and post-infection IL-10 concentration in BAL supernatant of individual study groups. Data presented mean +/- SEM, one-way ANOVA analysis between the three study groups (*p<0.05). Paired t test for change from baseline: •p<0.05 COPD subjects

5.38 Chapter discussion

These results demonstrate that the MSD assay can be used to measure cytokines and chemokines in sputum and BAL supernatant from *in vivo* samples following RV challenge. They also illustrate that a wide range of mediators can be measured with a minimal (25μL) sample volume. This utilised a new technique in our laboratory and to our knowledge is the first to measure samples from an RV challenge study. There is however published data using the MSD technique in sputum supernatant from COPD subjects[303].

The major strengths of this data come from its longitudinal nature, with repeated measures in sputum samples from the same subjects over time. The quality of the data appeared technically accurate with small between well variations, biologically plausible findings and consistent patterns identified in different mediators.

Exaggerated cytokine and chemokine response in COPD subjects

5.39 Sputum pro-inflammatory cytokines

In sputum, the pro-inflammatory cytokines IL-1β, IL-8 and TNF-α were elevated in COPD subjects compared to controls on at least two study time points (days 9 and 15). In smoking controls, the concentration of IL-1β, IL-6 and IL-8 increased in post-infection samples compared to baseline, however the absolute levels were lower than the COPD group. In non-smokers the levels of inflammatory mediators remained static or even fell after RV exposure. These findings are consistent with published studies and provide evidence of increased inflammation in COPD during an RV induced exacerbation. This is the first data comparing inflammatory mediators during virus infection in COPD with smoking and non-smoking controls and also demonstrates that inflammatory responses to virus infection are enhanced in COPD.

Factors controlling *in vitro* regulation of inflammatory cytokines may be responsible for the elevated levels identified in COPD subjects in this study, and the central hypothesis of this work is that reduced HDAC2 activity is a key factor responsible for this.

5.40 Sputum pro-inflammatory chemokines

The patterns of change in concentration seen following RV infection are similar in MMP-9, MIP-1β, GM-CSF and MCP-1 for all groups over the time course of the study. There was a trend to higher concentrations in the COPD subjects compared

to the controls throughout, and these were significantly higher on at least day 9 or 15. These findings also mirror those described in pro-inflammatory cytokines. On days 9, 12, and 15 only the COPD had a significant increase in post infection levels compared to baseline. The significantly greater levels identified in COPD subjects suggests that these may be important mediators in RV induced exacerbations. This data suggests that these mediators are involved in the pathogenesis of an exacerbation, as they reflect the clinical and inflammatory pattern with such a strong temporal correlation and the levels are greatest in the COPD subjects.

5.41 Virus and eosinophil related mediators

Eotaxin-3 and IP-10 were found to increase following RV infection, again these demonstrated a similar behaviour in COPD subjects, with significantly higher levels on days 9 (IP-10) and days 9, 12 and 15 (Eotaxin-3). The results for IP-10 are consistent with those expected, as IP-10 has been found to increase in naturally occurring COPD exacerbations, and has been postulated as a possible biomarker specifically for RV induced exacerbations[195]. However there have been few studies that have examined the changes in Eotaxin and Eotaxin-3 during exacerbations of COPD, particularly as interest has focused on its role in asthma and allergic diseases. However despite the selection of non-atopic subjects for inclusion into this study Eotaxin-3 levels rise in all groups after RV infection, and to a greater extent in COPD. There is inconsistency in the nature of inflammation identified in COPD, but some studies have identified eosinophilia in 30% of exacerbations[228]. This may therefore offer an explanation for the elevated levels of Eotaxin-3 found in COPD subjects.

5.42 BAL samples

Somewhat unexpectedly the pattern in BAL was different to that found in sputum and described above. Although COPD subjects had the highest concentration of mediators in sputum; in BAL the concentration in smoking controls was greater than in COPD for all mediators described. Only COPD subjects had an increase from baseline levels following RV16 infection in IL-10 and GM-CSF. TNF-α increased in both COPD and non-smoking controls whilst MMP-9 increased from baseline to day 7 in all three study groups.

Several possible mechanisms for differences between sputum and BAL exist. Samples reflect constituents of different airway compartments with induced sputum predominantly from large airways of the bronchial tree and BAL from alveolar and

small airway compartments. Alveolar macrophages in COPD subjects may function less efficiently and therefore release lower levels of mediators whereas the cells upregulated as part of the inflammatory process (particularly neutrophils) produce excess mediators in the airways of COPD subjects. Tissue destruction due to emphysema may result in lower levels of inflammatory cytokines found in BAL samples from COPD subjects, whereas smoking controls with no emphysematous change have increased alveolar inflammation.

RV replication has been shown to occur in the lower respiratory tract, but low virus loads were found in BAL compared to sputum, suggesting that the larger airways are the main site of RV infection and hence chemo and cytokine production. Although there is only one post infection time point for BAL sputum samples taken both before and after this demonstrate elevated mediators suggesting that it is not related to the sampling time point of the acute (day 7) BAL. The data does not suggest that the procedure of bronchoscopy itself is leading to increased inflammation in sputum of COPD subjects as at time points prior to bronchoscopy (especially day 5) the mediators show a trend to higher levels than in smokers.

5.43 Inflammatory mediators correlate with symptom scores

To investigate which cytokines contribute to clinical illness, I examined the relationships between sputum mediators and LRT symptom scores. It was found that IL-1β and TNF-α had a strong positive correlation with symptoms only in the COPD subjects. This suggests that these pro-inflammatory mediators have a role in driving RV infection and lower airway symptoms during an exacerbation. The findings are surprisingly strong for many other mediators measured (IL-10, IFN-γ, IL-8 and IL-2) suggesting that they too either have a role in inducing or in responding to inflammation during an AECOPD.

5.44 Anti-inflammatory cytokine (IL-10) increases in exacerbation

The pattern of IL-10 following infection is different between the study groups. In COPD subjects the concentration increases from a low baseline but in controls appears to fall. This may be a response to exaggerated inflammation in the COPD subjects that is not seen in the other groups.

5.45 Limitations

Spiking studies using known concentrations of mediators was not performed in sputum samples collected as part of this study. However published data has shown that sputum samples processed with DTT using a similar method to the samples collected in this study is valid for most mediators[303]. The sensitivity of the MSD assay was reduced for Eotaxin, Eotaxin-3 and IFN-γ detection when DDT was present, however the data contains repeated measures with samples processed in the same way, so information over the time course of the study remains useful. The final concentration of DTT in sputum samples used in this study were checked with MSD technical staff prior to performing the assay and their opinion was that these were too low to interfere with assay sensitivity.

Sputum samples analysed in this study have produced rewarding data on time courses of change following RV infection, often with greatest between group differences on day 9. However in general data in BAL samples did not demonstrate between group differences and this may reflect the sampling process. Many mediators were close to the lower limit of detection in BAL and therefore its usefulness in the MSD assay may be questioned.

5.46 Comparison with published studies

The results described in this chapter cannot be compared directly with published literature as there are no studies which report a similar range of mediators over the time course of an exacerbation. However many studies have reported on inflammatory cytokines in the sputum and BAL of stable COPD and at a single time point during an exacerbation.

5.47 Pro-inflammatory cytokines

Methods of obtaining samples from stable COPD subjects to compare with exacerbation state have varied between studies; some obtaining a pre-exacerbation and some a post-exacerbation sample. Samples collected post-exacerbation risk bias if subjects have not returned to baseline levels or insufficient time has lapsed following the exacerbation or treatment has been administered. Those obtaining samples pre-exacerbation require recruitment of a large defined population, who subsequently experience an exacerbation. This can lead to over emphasis or investigation of a subject cohort, leading to bias from an unrepresentative population.

Studies employing both methods have found mediators that are increased in the stable state of COPD have all been shown to increase in sputum and BAL during an exacerbation particularly TNF-α, IL-8, IL-6 and GM-CSF[145, 218, 343-345]. In the results presented in this chapter all of these mediators increased above baseline but only in the COPD subjects. Few studies have investigated the time taken for inflammatory markers to return to baseline levels, but one study identified increased systemic inflammation and elevated symptoms scores 35 days after an exacerbation in 23% of their study cohort[346].

In a study designed to investigate the relationship between the upper and lower airway a study by Hurst *et al* identified a correlation between upper and lower airway concentrations of IL-8[335]. In the data reported in this chapter cytokines from nasal lavage have not been measured, but these may reveal interesting results if assayed in the future. It is recognised that COPD patients have an excess of nasal symptoms and increased concentrations of inflammatory cytokines may be associated with this. The demonstration of a correlation between upper and lower inflammatory markers is of interest because sampling of the upper airway is likely to prove more practical in clinical practice. However the study by Hurst et al did not demonstrate a correlation between upper and lower airway IL-6 levels, so further studies are required in this area to understand the reasons for this.

A further study linking breathlessness and plasma cytokines showed that IL-6 and IL-8 were increased during an AECOPD compared to samples collected during the recovery period[318]. This study failed to identify changes in TNF-α concentrations between exacerbation and recovery sampling. However comparisons with data presented in this chapter is limited because differences are likely to exist between plasma and sputum samples.

5.48 Macrophage mediators

It has been postulated that elevated concentrations of MMP-9 are a mechanism of damage in COPD. An *in vitro* study using alveolar macrophages from COPD subjects and smoking and non-smoking controls supported this hypothesis[139]. The data from this RV challenge study demonstrating greater levels in COPD subjects appears to support this; however no published studies have yet identified increased sputum MMP-9 levels during an exacerbation *in vivo*. A study using sputum samples collected during stable COPD did not identify differences in MMP-9 or IL-1β

concentrations between COPD and control subjects, whereas IL-8 levels were elevated[347]. Again comparisons with published studies are limited by the lack of longitudinal studies during COPD exacerbations. However the increase in MMP-9 presented in this chapter suggests that these mediators are involved in the pathogenesis or are the sequelae of exacerbations.

Induced sputum samples from COPD patients have been found to have significant increases in MPO and IL-8, and non-significant increases in GM-CSF and ECP during severe exacerbations[144]. This study by Tsoumakidou *et al* lacked a control group and the subject numbers were relatively small which may indicate why increases in GM-CSF were not significant. Overall however these severe exacerbations appear to support the findings in the experimental exacerbation data presented here.

5.49 Virus mediators

In an *in vitro* and human challenge study of healthy volunteers investigating IP-10 production by epithelial cells following RV infection, it was concluded that IP-10 was released in a RV dose dependent fashion[348]. The finding that IP-10 increased in the airway of healthy volunteers following RV infection led to the postulation that this may be an important mediator in RV induced exacerbations of airway disease. The data presented in this chapter is the first to do so in COPD subjects and supports the findings by Spurrell *et al*[348].

5.50 Inflammatory mediators correlate with symptom scores

No published studies have directly correlated sputum cytokines with respiratory tract symptoms. Inherent in the definition of an exacerbation has been an increase in symptoms in those studies where inflammatory cytokines have been measured. Obtaining a direct correlation between specific markers and symptoms may lead to identification of those cytokines with greater importance in the pathogenesis of AECOPD or even identification of different phenotypes of exacerbation. Many studies have attempted to identify a biomarker to confirm an exacerbation[349], define the aetiology[195] or grade severity[350] but these have not used sputum cytokines.

5.51 Conclusion

The data presented in this chapter is novel and interesting. No reports in the literature have included the number of inflammatory mediators measured over the time course of an exacerbation as documented here. The picture of sputum inflammatory markers increasing above baseline levels in sputum, and in excess of control groups, is seen predominantly in the COPD group during the study time course. This tended to peak between days 9 and 15 and correlated with the peak in symptoms, particularly in the COPD group. The consistent finding of stronger correlations between cytokines and symptoms in the COPD group compared to the controls suggests that these mediators are driving features of exacerbations in COPD subjects.

Chapter 6: The role of histone deacetylase enzyme activity in rhinovirus induced COPD exacerbations

6.1 Introduction

Investigations of the inflammatory mechanisms responsible for COPD have led to interest in enzymes controlling gene expression, particularly the gene repressive effect of the histone deacetylase (HDAC) group. These can act to obscure or expose transcriptionally active sites on DNA by controlling histone binding. Their anti-inflammatory effect is thought to be mediated through reducing DNA transcription and hence inflammatory gene expression. It has been found that the activity HDAC enzymes is reduced in subjects with stable COPD and that this correlates with the severity of airflow obstruction[125]. This finding represents a potential mechanism for steroid resistance found in COPD[129]. Although little clinical data exists, particularly during exacerbations, a randomised study using treatment with theophylline, an HDAC activator, identified reduced levels of TNF-α and IL-8 in the sputum of the treatment group 3 months after an exacerbation[131]. There were no clinical outcomes measured.

In this chapter longitudinal data of HDAC activity in both sputum and BAL over the time course of the study is presented. The hypothesis to be tested is that HDAC activity is (i) reduced in both sputum and BAL macrophages from COPD subjects and that (ii) following rhinovirus infection this falls further when compared to non-obstructed control subjects.

Results

HDAC2 isoenzyme activity was measured as described in the Materials and Methods chapter and all data shown is corrected for the volume of protein obtained in each sample. This was measured using the Bradford protein assay. In addition only data from successfully RV infected subjects is presented. All data presented has been log transformed prior to analysis with parametric tests.

Sputum macrophage HDAC2 activity assays results

6.2 Sputum HDAC2 activity at baseline in study groups

To investigate differences between the study groups prior to RV infection the activity of HDAC2 at baseline in the three study groups are shown, figure 6.1. This does not demonstrate a significant difference between the groups (one way ANOVA, p=0.095). The activity in the COPD group has a trend to the highest activity of the three groups. There was no difference in HDAC2 activity between the two control groups at baseline.

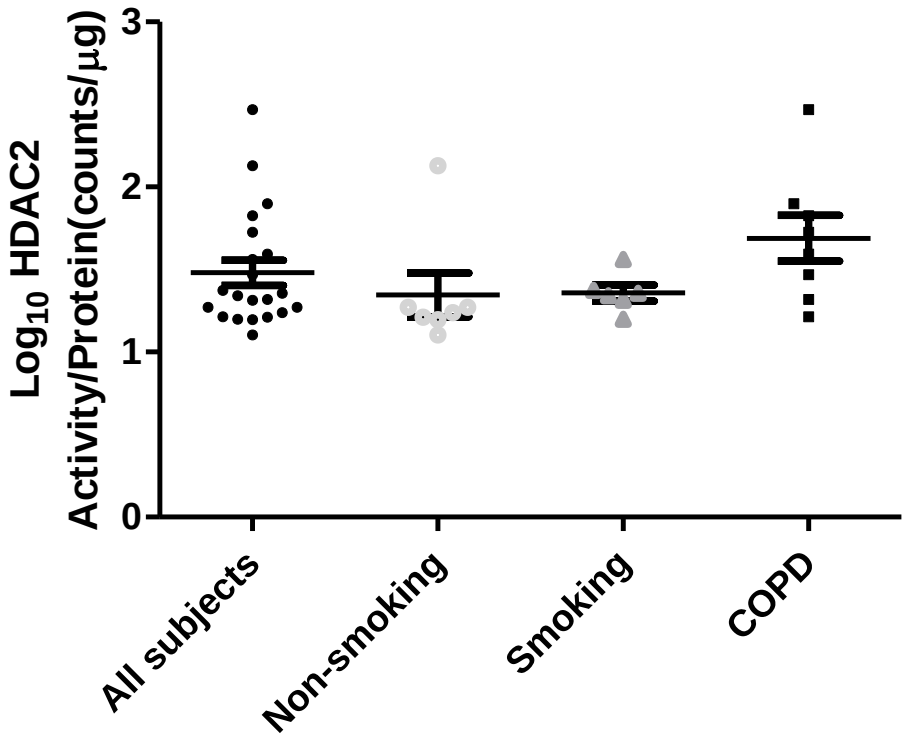


Figure 6.1 HDAC2 activity in sputum macrophages at baseline in individual subject groups. Data presented mean +/- SEM, (one way ANOVA, p=0.095)

6.3 Time course in sputum HDAC2 activity in infected subjects

To evaluate the effect of RV infection in the whole study population the level of HDAC2 activity in sputum supernatant on scheduled study visits is plotted in figure 6.2. When compared to baseline there is a significant and maximal fall in HDAC2 activity on day 9 following RV infection, (paired t test, p=0.007). There is a trend to increasing levels of HDAC2 activity from day 9 to day 15 when there is a return to baseline levels.

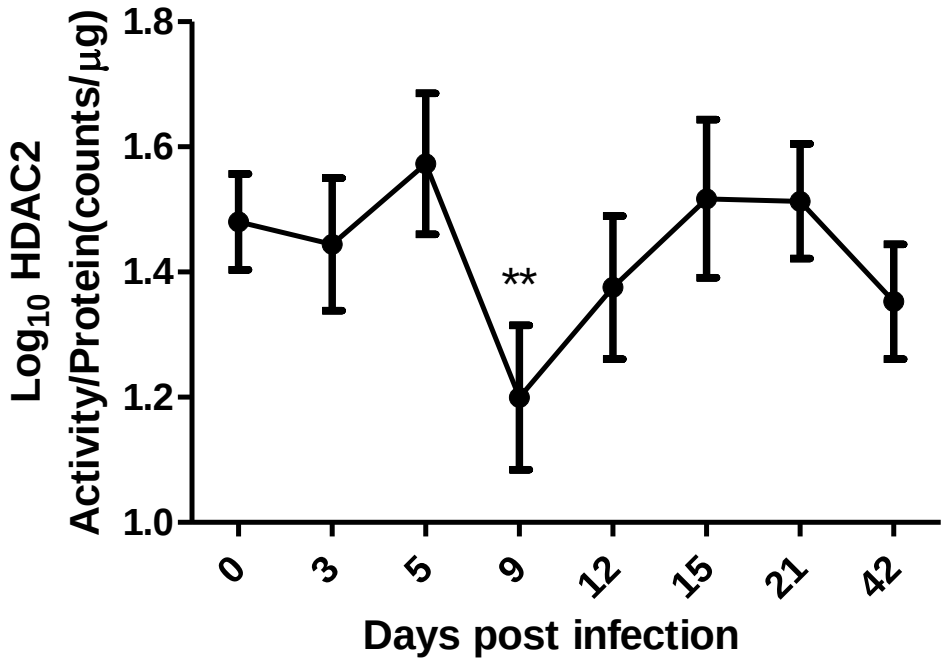


Figure 6.2 Time course of sputum macrophage HDAC2 activity in all RV infected subjects. Data presented mean +/- SEM, (paired t test, **p<0.01)

6.4 Time course in sputum HDAC2 activity in study groups

Sputum HDAC2 activity across the time course of the three study groups is shown in figure 6.3. There was no statistical difference between the groups at any of the time points when analysed with one way ANOVA. However there was a trend to lower absolute levels in the COPD group compared to controls following RV infection. In control groups there was similar activity between the two groups, but a trend to reduced activity in smoking controls compared to non-smoking subjects following RV infection.

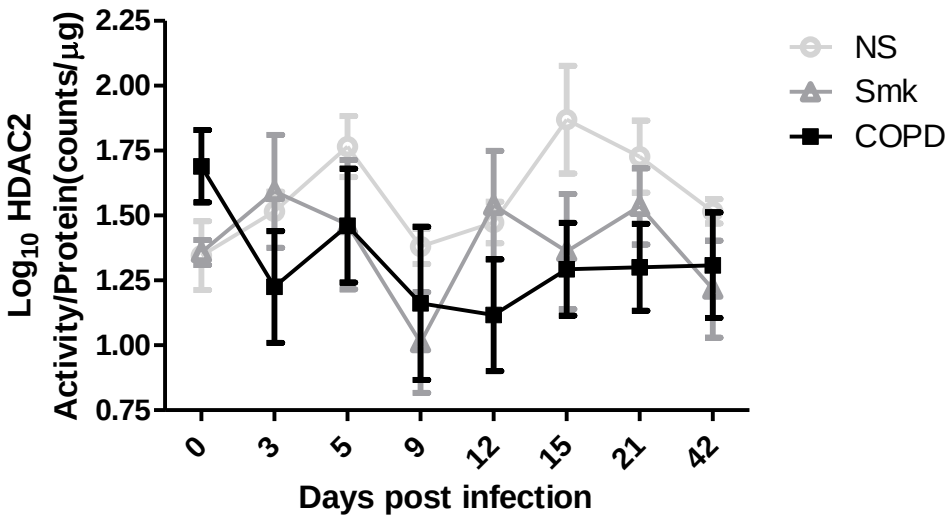


Figure 6.3 Time course of sputum macrophage HDAC2 activity in individual groups. Data presented mean +/-SEM with no difference between groups demonstrated, (one way ANOVA)

In order to further investigate the overall levels of HDAC2 activity in each group, the area under the curve (AUC) for each individual in the study over the whole time course was calculated and presented as mean (SEM) AUC for study groups in figure 6.4. This does not demonstrate a difference between the three study groups when analysed with one way ANOVA; but does confirm the trend to higher levels of HDAC2 activity in non-smoking subjects and lower levels in COPD subjects.

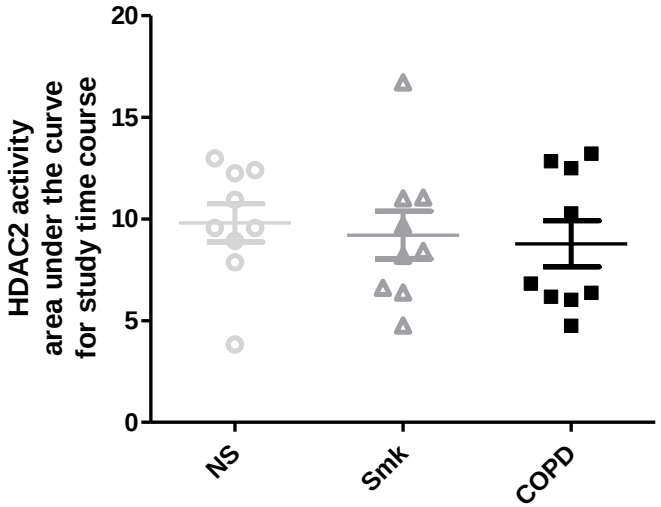


Figure 6.4 Plot of area under the curve for sputum macrophage HDAC2 activity over total study time course in individual groups. Data presented mean +/- SEM. No difference between groups demonstrated, (one way ANOVA, p=0.798)

6.5 Change from baseline in sputum HDAC2 activity

An important measurement of the impact RV infection had on HDAC2 activity is derived by calculating the change from baseline on subsequent study visits. The results of baseline HDAC2 activity was therefore subtracted from measurements obtained on each study day. Results of the change from baseline in HDAC2 activity from the three study groups is presented for the study time course, figure 6.5.

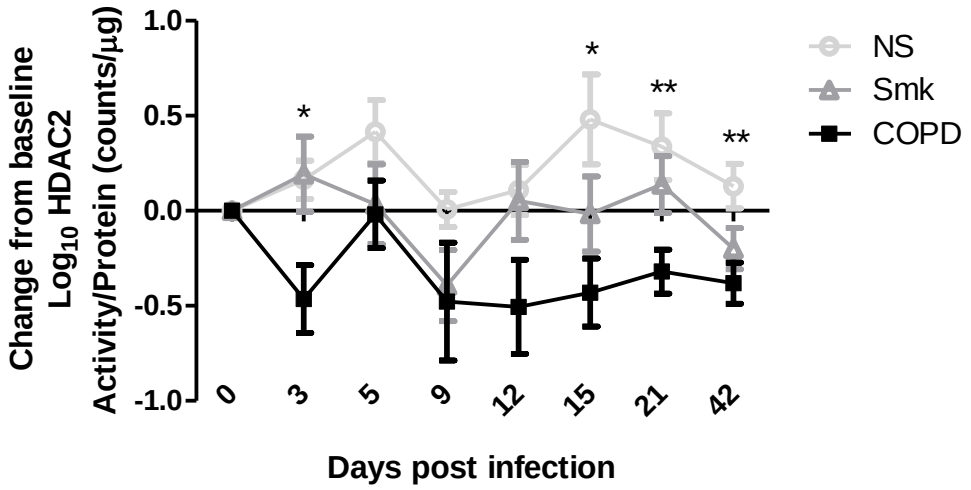


Figure 6.5 Time course of change of sputum macrophage HDAC2 activity from baseline in study groups. Data presented mean +/-SEM, (one way ANOVA, *p<0.05, **p<0.01)

The change from baseline was found to differ in the individual groups, with increased activity in non-smoking controls, no difference in smoking controls and a loss of activity in COPD subjects, from day 3 onwards. There was a significant difference between the group means on days 3, 15, 21 and 42. On day 3 this is driven by a fall in activity in the COPD group compared to controls and at the later time points by a fall in activity in the COPD group and an increase in the non-smoking controls.

6.6 Change from baseline in individual study groups

The changes in HDAC2 activity from baseline in individual groups have been plotted, figure 6.6. It was found that in non-smoking controls (figure 6.6a) there was an increase in HDAC2 activity at early time points following infection; with significantly increased levels on day 5 ($p<0.05$). Activity returns to baseline levels at day 9 and then there is a non-significant trend to increased levels with a second peak at day 15.

In smoking controls (figure 6.6b) there was no significant change from baseline at any time point following infection; there is a trend to lower HDAC2 activity on day 9. In COPD subjects (figure 6.6c), there is a significant fall in HDAC2 activity at all time points following infection, except on day 5. The greatest fall from baseline occurs on day 12. Activity increases after this time point but has still not returned to baseline levels by day 42. This represents a complete reversal of the pattern seen in non-smoking subjects and is an exaggeration of that seen in smoking controls.

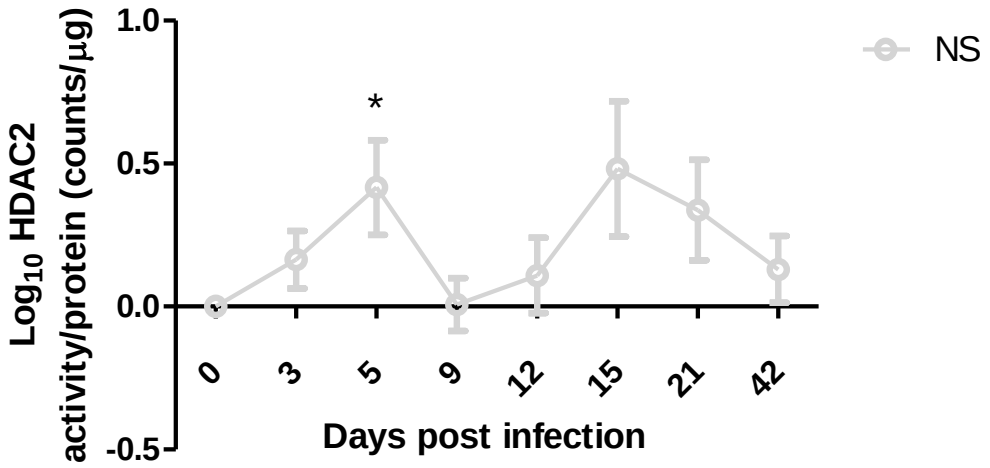


Figure 6.6a Non-smoking controls

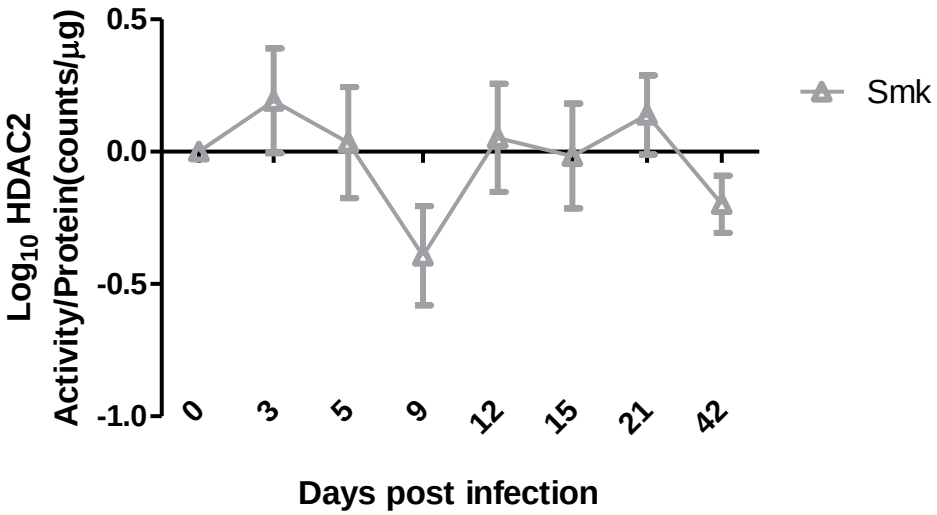


Figure 6.6b Smoking controls

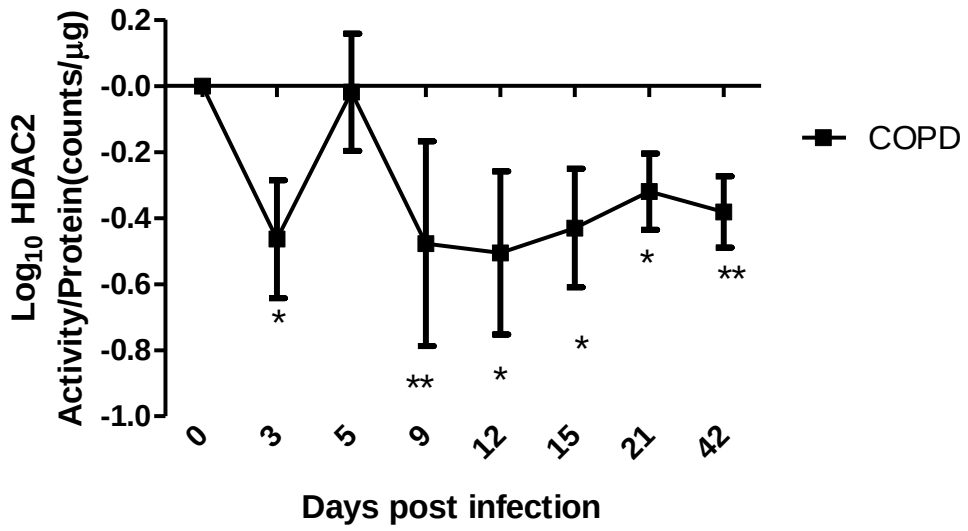


Figure 6.6c COPD subjects

Figure 6.6 Change from baseline in sputum macrophage HDAC2 activity after infection in (a) non-smoking controls, (b) smoking controls and (c) COPD subjects. Data presented mean +/- SEM. (Paired t test, *p<0.05, **p<0.01)

BAL macrophage HDAC2 activity assays

6.7 BAL HDAC2 activity at baseline in study groups

The results of the HDAC2 activity assay from BAL macrophages at baseline have been presented for all subjects recruited into the study in figure 6.7a, and for those later found to have been successfully infected, figure 6.7b. There is no difference in HDAC2 activity between the study groups at baseline, in either all subjects inoculated or in the smaller group of subjects that were successfully infected with RV16, p=0.81 and p=0.49 respectively measured by one way ANOVA analyses. These findings match those described in baseline sputum macrophages, although the magnitude of activity is approximately 2 fold higher in BAL samples.

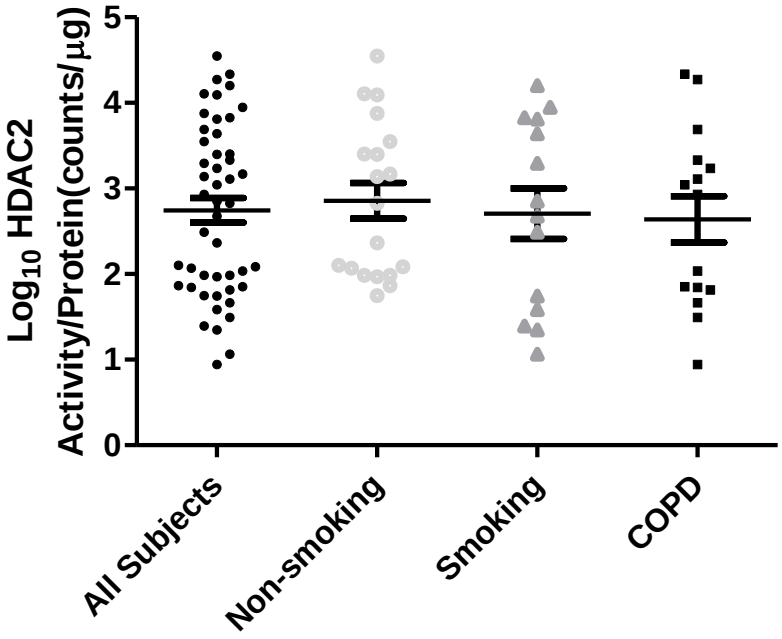


Figure 6.7a All subjects recruited into study

HDAC2 activity in baseline BAL macrophages from individual subject groups and all subjects combined. Data presented mean +/- SEM, (one way ANOVA, p=0.81)

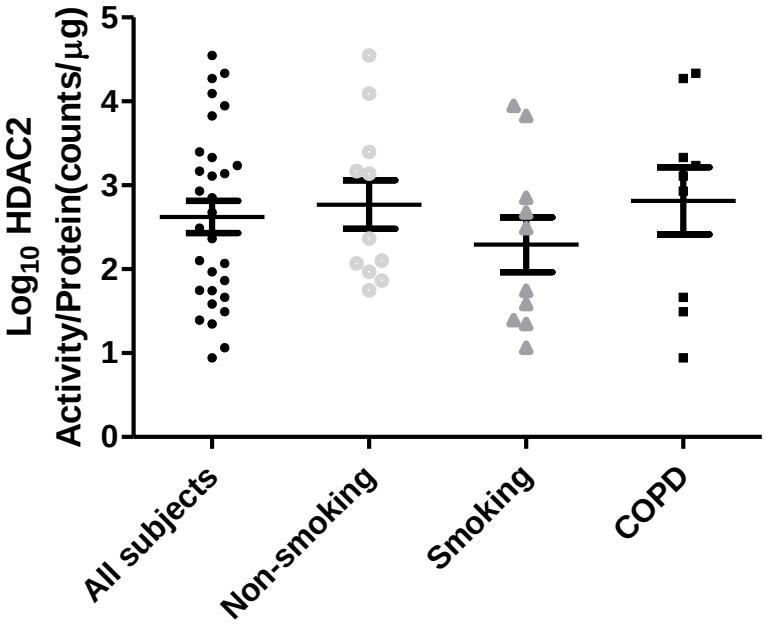


Figure 6.7b Successfully infected subjects only

HDAC2 activity in baseline BAL macrophages from individual subject groups and all subjects combined. Data presented mean +/- SEM, (one way ANOVA, p=0.499)

6.8 Pre and post-infection HDAC2 activity in BAL in all subjects

The HDAC2 activity from BAL macrophages in all infected study subjects combined at baseline and day 7 is shown, figure 6.8. There was a trend to reduced activity at day 7, but the standard error is large and this change is not significant.

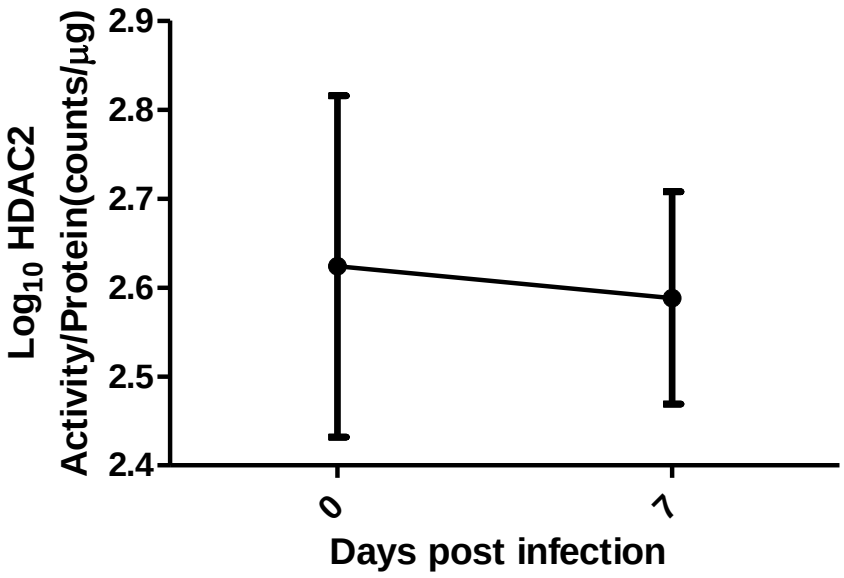


Figure 6.8 BAL macrophage HDAC2 activity in all study subjects at baseline and day 7 (paired t test, p=0.78)

6.9 Pre and post-infection HDAC2 activity in BAL in study groups

When the individual study groups were studied it was found that each group responded differently to RV infection, figure 6.9. There was no change in activity in non-smoking controls. In smoking controls there was a trend to increasing activity after infection, in COPD subjects however there was a trend to lower HDAC2 activity post infection. The fold difference (95% CI) in HDAC2 activity in BAL macrophages following infection for non-smokers, smokers and COPD subjects was 1.02 (0.22 to 4.80) p=0.982; 0.32 (0.04 to 2.30) p=0.294 and 4.52 (0.81 to 25.3) p=0.125 respectively.

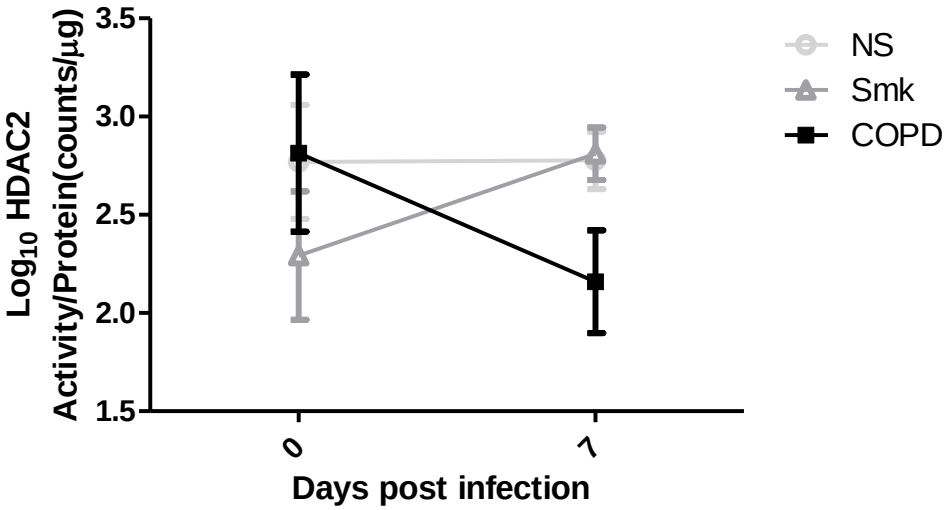


Figure 6.9 Time course of BAL macrophage HDAC2 activity in individual groups. Data presented mean +/-SEM

The time course of HDAC2 activity in BAL macrophages from individual study groups is shown, figure 6.10. There is no significant difference between the time points in any group. In non-smoking controls the levels are unchanged following infection, whereas in smoking controls there was a trend to higher levels of HDAC2 activity at day 7. The absolute levels of activity on day 7 are the same in both control groups. In COPD subjects there was a trend to reduced levels of HDAC2 activity following RV infection, displaying the opposite pattern to that seen in smoking controls. The post infection HDAC2 activity in COPD subjects has a trend to lower levels than in controls, (p=0.065, one way ANOVA).

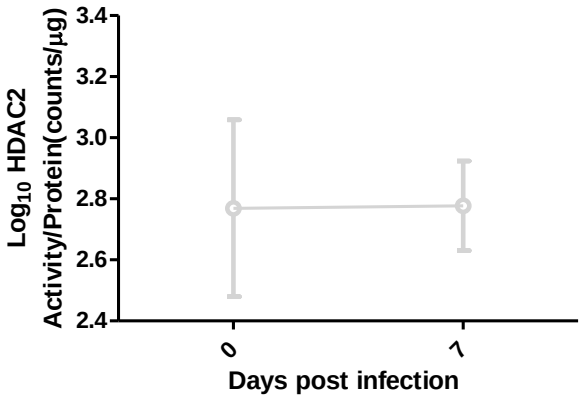


Figure 6.10a Non-smoking controls

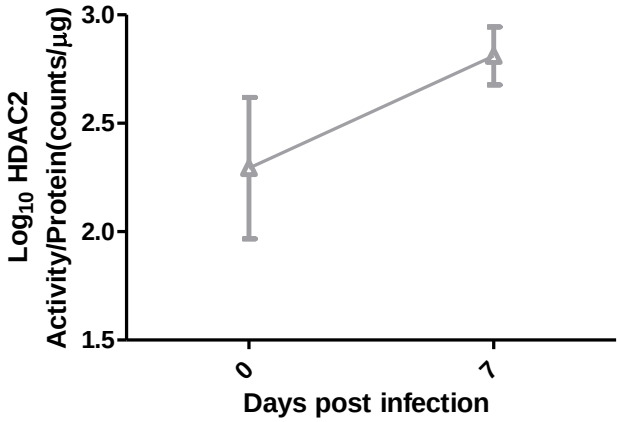


Figure 6.10b Smoking controls

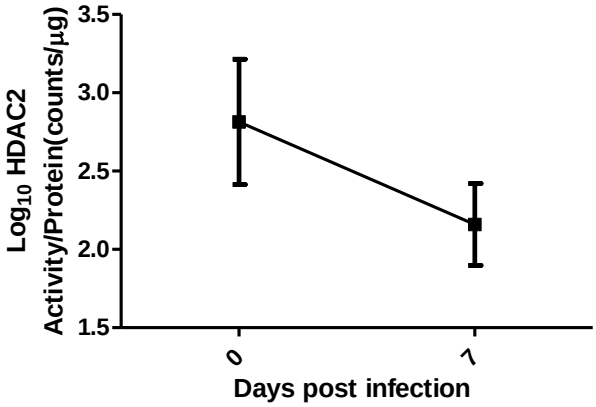


Figure 6.10c COPD group

Figure 6.10 Change from baseline in sputum HDAC2 activity after infection in (a) non-smoking controls, (b) smoking controls and (c) COPD subjects. Data presented mean +/- SEM

6.10 Relationship between sputum HDAC2 activity and airway inflammatory cytokines

To investigate the relationship between sputum macrophage HDAC2 activity and the concentration of inflammatory cytokines measured in corresponding sputum supernatant samples these parameters have been correlated. There was consistently a strong negative correlation only in the COPD group; data for TNF-α, GM-CSF and IL-8 are shown in the figures below to illustrate this followed by tabulation of correlation data for all cytokines measured using the MSD assay.

There was no significant correlation between sputum HDAC2 activity and TNF-α levels in either control group. However in COPD subjects there was a strong and highly significant negative correlation, (r= -0.53, p<0.001), illustrating that reduced HDAC2 activity was associated with increased sputum concentration of TNF-α only in COPD subjects, figure 6.11.

When sputum GM-CSF was correlated with HDAC2 activity there was again no significant relationship in control subjects, but in the COPD group there was a strong negative correlation, (r=-0.49, p<0.001), figure 6.12. The same pattern of results was found in sputum IL-8 correlations, where the COPD subjects were found to have a negative correlation, (r=-0.49, p<0.001), in contrast to controls, in whom there was no significant relationship identified, figure 6.13.

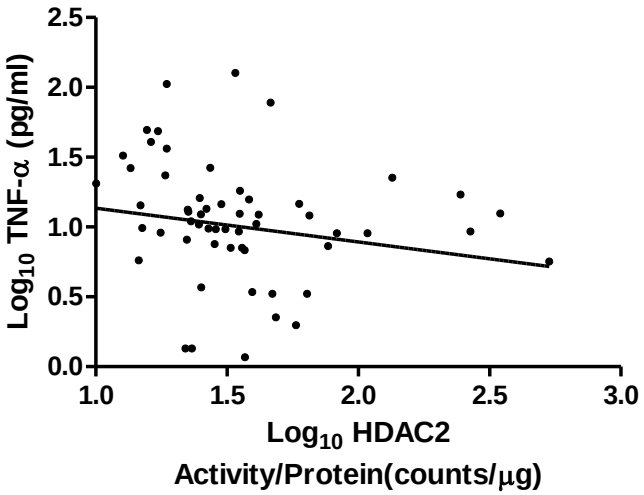


Figure 6.11a Non-smoking controls (r= -0.17, p=0.19)

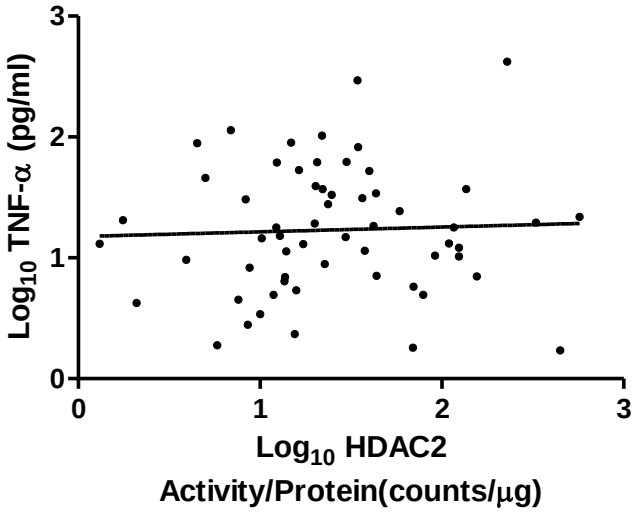


Figure 6.11b Smoking controls (r=0.04, p=0.75)

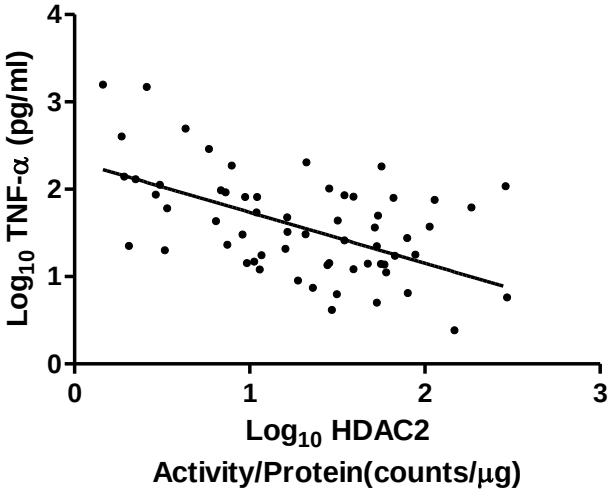


Figure 6.11c COPD subjects (r= -0.53, p<0.001)

Figure 6.11 Correlation of sputum macrophage HDAC2 activity and sputum TNF-α concentration in (a) non-smoking controls, (b) smoking controls and (c) COPD subjects

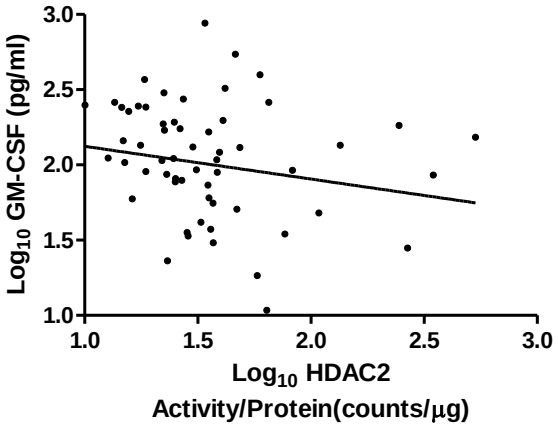


Figure 6.12a Non-smoking controls (r=-0.16, p=0.21)

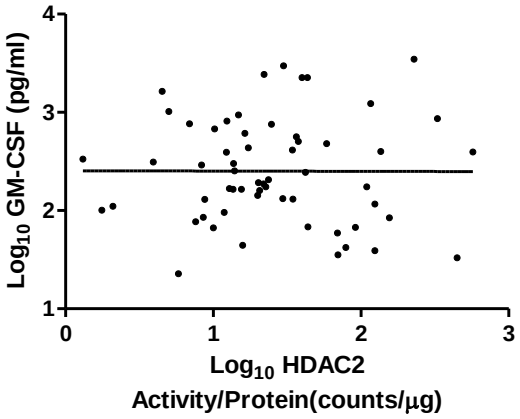


Figure 6.12b Smoking controls (r=-0.003, p=0.98)

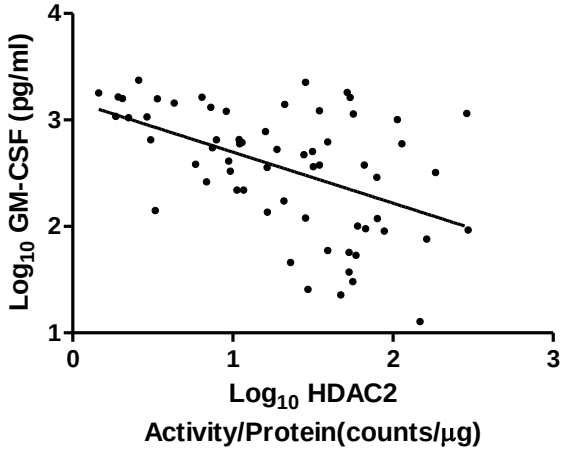


Figure 6.12c COPD subjects (r=-0.49, p<0.0001)

Figure 6.12 Correlation of sputum macrophage HDAC2 activity and sputum GM-CSF concentration in (a) non-smoking controls, (b) smoking controls and (c) COPD subjects

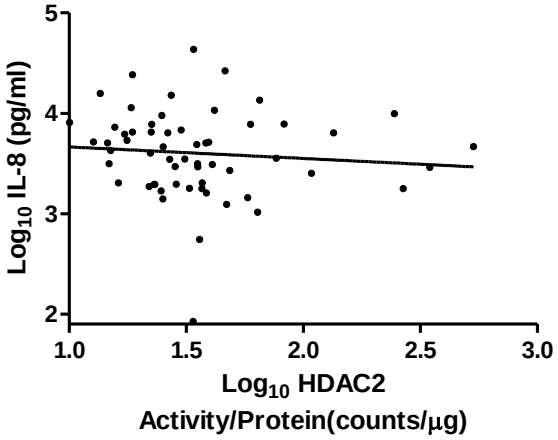


Figure 6.13a Non-smoking controls(r=-0.09, p=0.48)

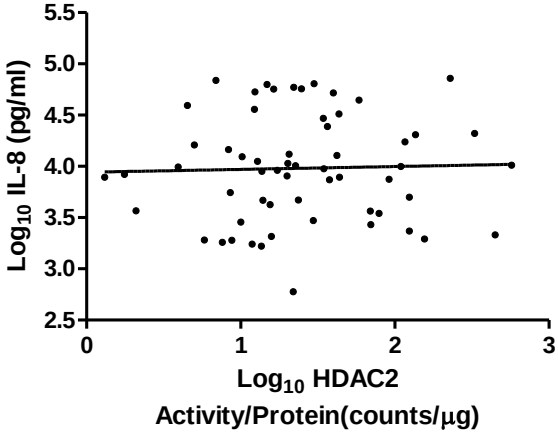


Figure 6.13b Smoking controls(r=0.03, p=0.82)

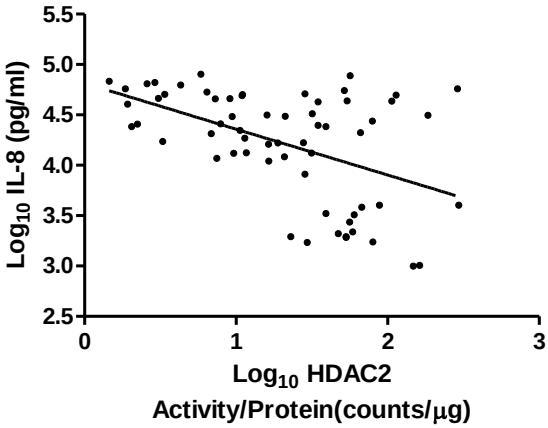


Figure 6.13c COPD subjects (r=-0.49, p<0.0001)Figure 6.13 Correlation of sputum macrophage HDAC2 activity and sputum IL-8 concentration in (a) non-smoking controls, (b) smoking controls and (c) COPD subjects

6.11 Relationship between sputum HDAC2 activity and airway anti-inflammatory cytokine IL-10

Concentration of the anti-inflammatory cytokine IL-10 in sputum also only correlated inversely with sputum HDAC2 activity in COPD subjects (r=-0.47, p<0.001), with no significant relationship between the two parameters in controls, figure 6.14.

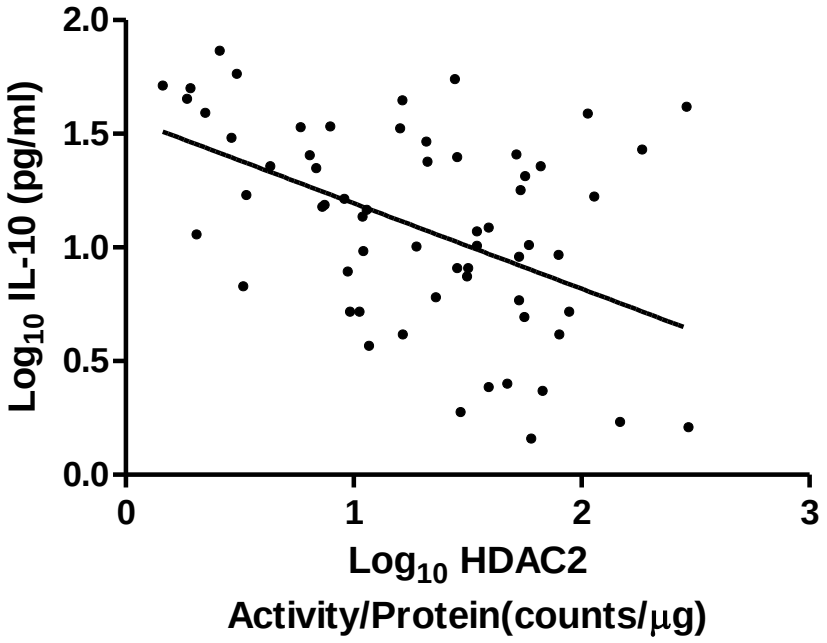


Figure 6.14 Correlation of sputum macrophage HDAC2 activity and sputum IL-10 concentration in COPD subjects (r=-0.47, p<0.001)

The results of the correlation between HDAC2 activity and cytokines measured in sputum using the MSD assay in the study is shown, table 6.1. This illustrates the finding that the correlation coefficients were stronger and highly significant in the COPD group compared to the controls for all the mediators presented. In both control groups the correlations were not statistically significant for any of the mediators measured.

	Non-smoking controls		Smoking controls		COPD	
	Coefficient (r)	p-value	Coefficient (r)	p-value	Coefficient (r)	p-value
TNF-α	-0.17	0.195	0.04	0.750	-0.53	<0.001
GM-CSF	-0.16	0.213	-0.00	0.981	-0.49	<0.001
IL-8	-0.09	0.480	0.03	0.820	-0.49	<0.001
IL-6	-0.20	0.127	-0.24	0.069	-0.48	<0.001
IL-10	-0.25	0.059	0.05	0.731	-0.47	<0.001
IL-12	-0.15	0.259	-0.04	0.740	-0.45	<0.001
IL-2	-0.06	0.653	0.04	0.761	-0.45	<0.001
IL-1β	-0.16	0.237	0.14	0.276	-0.41	<0.001
IFN-γ	-0.03	0.834	0.10	0.436	-0.35	0.005

Table 6.1 Pearson's correlation coefficient for sputum HDAC2 activity and cytokines in the three study groups

6.12 Relationship between sputum HDAC2 activity and airway inflammatory chemokines

The results of the correlation between sputum HDAC2 activity and chemokines measured in the sputum are displayed in this section. Only the COPD group had significant correlations between the two parameters, and the coefficients were negative for all of these. In COPD subjects the correlations were weaker for all chemokines compared with those observed with cytokines, described above. These data suggest HDAC2 regulates pro-inflammatory cytokines more tightly than it does chemokines.

The correlation between IP-10 and HDAC2 activity was not significant in the COPD group, (r=-0.15, p=0.25), figure 6.15. This contrasts with the relationships observed between IP-10 and virus load and symptoms scores which both correlated strongly with IP-10. These data suggest that HDAC2 does not play an important role in regulating increases in IP-10 following RV infection.

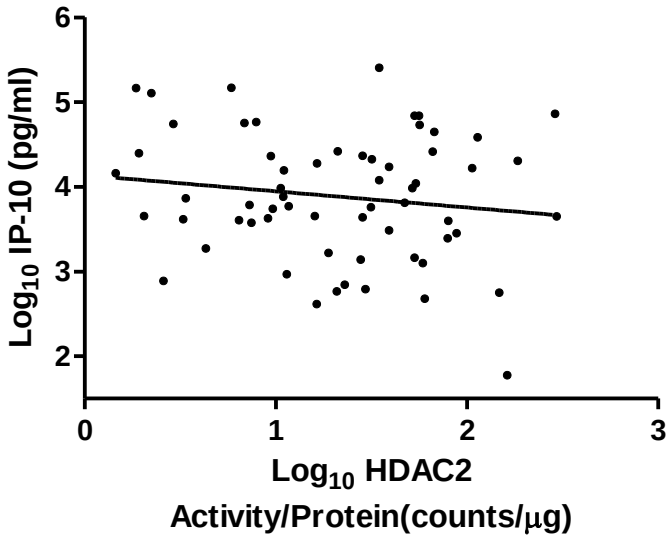


Figure 6.15 Correlation of sputum macrophage HDAC2 activity and sputum IP-10 concentration in COPD subjects (r=-0.15, p=0.25)

Results of the correlation coefficient for all chemokines measured using the MSD assay and HDAC2 activity are shown, table 6.2. There was no significant correlation between the two parameters in either control group. In COPD subjects the correlation was only significant for MIP-1β, eotaxin-3 and MCP-4.

	Non-smoking controls		Smoking controls		COPD	
	Coefficient (r)	p-value	Coefficient (r)	p-value	Coefficient (r)	p-value
MIP-1β	0.01	0.970	-0.20	0.125	-0.34	0.006
Eotaxin-3	0.04	0.739	0.13	0.313	-0.34	0.007
MCP-4	0.02	0.904	0.02	0.865	-0.33	0.008
MCP-1	-0.02	0.879	-0.18	0.166	-0.23	0.066
MDC	-0.04	0.748	0.04	0.739	-0.16	0.206
IP-10	-0.00	0.988	0.09	0.489	-0.15	0.250
Eotaxin	0.17	0.195	0.09	0.517	-0.02	0.872
TARC	0.10	0.476	0.17	0.203	0.17	0.181

Table 6.2 Pearson's correlation coefficient for sputum HDAC2 activity and chemokines in the three study groups

6.13 Relationship between sputum HDAC2 activity and clinical measurements

To investigate potential associations between HDAC2 activity and physiological and clinical measurements, sputum macrophage HDAC2 activity has been correlated with sputum virus load and total cell count; LRT symptom scores and clinic spirometry measurements in the three study groups, table 6.3.

	Non-smoking controls		Smoking controls		COPD	
	Coefficient (r)	p-value	Coefficient (r)	p-value	Coefficient (r)	p-value
Virus Load	0.24	0.062	-0.00	0.986	-0.19	0.148
Sputum TCC	-0.29	0.020	-0.24	0.056	-0.42	0.001
LRT scores	-0.05	0.689	0.22	0.168	-0.22	0.075
FEV_1	0.01	0.942	-0.09	0.535	0.19	0.133
% pred FEV_1	0.23	0.077	-0.01	0.952	0.11	0.417
FVC	-0.04	0.745	-0.09	0.537	0.18	0.170
% pred FVC	0.19	0.161	-0.15	0.296	0.14	0.291
FEV_1/FVC	0.23	0.077	-0.04	0.809	0.07	0.603
PEF	0.06	0.628	0.01	0.940	0.26	0.044

Table 6.3 Pearson's correlation coefficient for sputum HDAC2 activity and sputum virus load and total cell count, LRT symptom scores and clinic spirometry in the three study groups

When all successfully infected subjects were analysed, the correlation of HDAC2 activity with four spirometric measurements showed a weak positive correlation, these were % predicted FEV_1 (r=0.22, p=0.004), % predicted FVC (r=0.17, p=0.024), PEF (r=0.16, p=0.037) and airflow obstruction, FEV_1/FVC, (r=0.21, p=0.005). In individual study groups; no parameters shown in table 6.3 correlated with HDAC2 activity in smoking controls. In non-smoking controls and COPD subjects sputum TCC demonstrated a significant negative correlated with HDAC2 activity. In COPD subjects only there was a weak positive correlation between PEF and HDAC2 activity.

Chapter discussion

6.14 Sputum macrophage HDAC2 activity

The hypothesis tested in this chapter is that HDAC activity is reduced in COPD subjects compared to controls prior to RV infection and that this falls further following

successful experimental inoculation. The data shown in this chapter did not demonstrate a difference in HDAC2 activity in sputum or BAL macrophages between the three study groups at baseline. The trend to a small increase in HDAC2 activity in sputum macrophages from COPD subjects was the opposite finding to that expected. The reasons for this result are not known. It may reflect the relatively mild severity of COPD subjects enrolled in the study, in whom HDAC activity had increased appropriately in response to inflammation. Although published studies have demonstrated reduced HDAC activity in GOLD stage II COPD subjects, the population in this study were not receiving any medications including steroids and had relatively mild baseline symptoms. There are likely to be many disease phenotypes within the diagnostic umbrella of COPD and differences in HDAC activity within these groups may exist. Within this study and published work the total number of COPD subjects and controls is relatively small, so the true differences in HDAC activity within COPD phenotypes may not be apparent. Finally although subject numbers are greater in BAL when non-infected subjects are included, the total number of COPD subjects in this study is relatively small. The comparison between this data and published literature is described below.

Following RV challenge the activity of HDAC2 falls when the successfully infected study population in entirety is considered, with a peak and significant drop on day 9. When individual groups were considered the hypothesis was proved correct as only in COPD subjects did HDAC activity fall compared to baseline levels. Interestingly the activity of HDAC was the opposite in non-smoking controls, with a significant increase from baseline levels, in smoking controls HDAC activity was largely unchanged following infection. There was not a significant difference in absolute HDAC2 activity between the groups or the area under the curve for the entire study period, but this is likely to reflect (a) the small sample size, (b) the wide variation in results within groups and (c) the relatively mild severity of the COPD group. However a change in activity from baseline in the same subject group repeatedly sampled provided robust evidence for differences in HDAC activity between groups in response to RV infection.

This data therefore provides interesting results, suggesting that the normal response to RV induced inflammation is to increase HDAC activity to counter act this. In smoke exposed subjects the normal response is blunted and in COPD it is lost completely. The data described in this chapter does not provide a mechanism for this, but the central hypothesis of the thesis is that cigarette exposure and additional

oxidative stress in COPD subjects drives, at least in part this loss of HDAC activity. Although the data provided here does not confirm this mechanism, it has been shown that COPD subjects experience greater symptoms, RV load and inflammatory cytokines compared to smoking non-obstructed control subjects. In the following chapter, evidence is presented for increased oxidative and nitrative stress in the COPD population, further supporting this mechanism.

6.15 BAL macrophage HDAC2 activity

Although none of the changes in HDAC activity in BAL macrophages were significant, there was a trend to reduced activity after RV infection in the COPD group. An unexpected finding was a trend to increased activity in the smoking control group following infection; this is the opposite finding to that seen in sputum macrophages, the reasons for this are not clear. If subsequently shown to be a significant finding, HDAC activity in sputum and BAL macrophages may differ due to specific environmental variations in local lung compartments. For example sputum macrophages from large airways are directly exposed to a greater burden of cigarette smoke and replicating RV than those in smaller airways and the alveolar compartment. This could lead to abnormal activity in sputum macrophages while function is maintained in those from the lower airway.

The alternative hypothesis is that retention of a normal response in BAL macrophage HDAC activity to RV infection is a factor in protecting this group from exaggerated inflammation and symptoms of an exacerbation identified in the COPD group. Finally, the development of airway obstruction is a dynamic process. Smoking controls that presently have normal spirometry may progress to airway obstruction in association with worsening airway inflammation and a loss of HDAC activity in both BAL and sputum macrophages.

6.16 Correlation of HDAC2 activity with cytokines and chemokines

The known action of HDAC enzymes to reduce transcription of inflammatory genes lead to the hypothesis that reduced HDAC2 activity will correlate with increased concentrations of inflammatory cytokines[351]. Data has been published to support this hypothesis at a single time point[125], but not during the time course of a COPD exacerbation. Although only demonstrating an association, the data displayed in this chapter supports the concept that reduced HDAC activity is associated with increased levels of inflammatory cytokines in sputum. Interestingly only the COPD group demonstrate significant correlations, which were strong for several key

mediators recognised to be important in the pathogenesis of exacerbations, particularly TNF-α, GM-CSF and IL-8. The lack of association in the control groups suggests that only in COPD subjects is HDAC2 deficiency resulting in the production of cytokines and subsequent cellular inflammation. Overall the data in this chapter strongly supports the rationale that a reduction in HDAC activity has a central role in the pathogenesis of RV induced COPD exacerbations. Following RV infection only the COPD group were found to have a significant fall in HDAC2 activity, this was associated with statistically significant increases in inflammatory cytokine concentrations only in the sputum of COPD subjects, for example IL-1β, IL-6, IL-8 and TNF-α results of which are presented in the previous chapter. Additionally both virus load and clinical symptoms representative of an exacerbation have been shown to correlate with inflammatory cytokines only in the disease group. Therefore although these correlations have not been able to attribute direct causation, they do support the hypothesis that exaggerated symptoms in COPD subjects following RV infection are related to reductions in HDAC activity. Performing further studies to identify potential mechanisms for this process would also be desirable.

A strong correlation between reduced HDAC2 activity and increasing concentrations of the anti-inflammatory cytokine IL-10 was also seen. A mechanism for this association cannot be concluded from this data, but IL-10 may not be controlled directly by the activity of HDAC2. Therefore the increase in inflammatory cytokines, particularly GM-CSF and TNF-α which is mediated by reductions in HDAC2, may be responsible for the secondary increase in IL-10. Many of the other mediators which have been measured in the study may not be influenced at a transcriptional level by HDAC2. This may explain the weaker correlation seen between HDAC activity and the chemokines, for example if the transcriptional regions of DNA for these mediators were not tightly controlled by histone binding.

6.17 Comparison with published studies

No published study to date has provided longitudinal data on HDAC activity during an exacerbation of COPD; the particular strength of the information presented in this chapter is the presence of pre-exacerbation HDAC activity results. This baseline data enables comparison of enzymatic activity in paired samples pre-, peri- and post exacerbation. Studies of HDAC activity in COPD patients have also included both smoking and non-smoking subjects but all sampled at a single time point[125]. Macrophages from COPD patients were shown to have reduced HDAC activity when

compared to smokers and non-smokers and this correlated with disease severity[125]. The data presented in this chapter however did not support these findings from the published literature. Unexpectedly the HDAC2 activity was similar between subjects groups at baseline in both sputum and BAL macrophages in this study. Previous reports of diminished HDAC activity in smokers compared to non-smokers[352] were also not supported by data in this chapter. A brief discussion of the possible explanations for this in sputum and BAL samples follows.

6.18 Reduced HDAC activity in COPD subjects

The only published study of HDAC activity in stable COPD has reported reduced activity when compared to controls[125]. However this finding was not reproduced in the data presented in this chapter, where baseline samples in sputum and BAL did not demonstrate between group differences. When a greater number of subjects, including those who were not successfully infected, were considered a small trend to reduced activity in BAL macrophages from COPD subjects was observed, but this was not statistically significant. Although the COPD subjects appear well matched with this and in the study by Ito *et al* (mean FEV_1 69% and 65% predicted, age 60 and 63 years respectively) the pack year history was greater in Ito's cohort compared to this study (58pyh vs 39pyh) and no comment on the severity of symptoms was made. The subjects may therefore represent different extremes of COPD phenotypes, with greater cigarette exposure, symptoms and subsequent reduced HDAC activity. It has been shown that HDAC2 activity is reduced in COPD subjects as a result of posttranslational and oxidative modifications[353, 354], and so burden of total cigarette exposure may contribute considerably to differences between the studies. In both studies the subject numbers were small, and in a heterogeneous disease state this is likely to explain the differences identified. Also differences in sample collection and laboratory analysis exist between the studies. BAL samples were used by Ito *et al* compared to sputum in this study and different commercially available HDAC activity assays were also used.

A study by Yasuo *et al* examined total HDAC2 protein expression, rather than activity, in lung samples from COPD subjects and controls undergoing surgical procedures. In this study GOLD stage I & II COPD patients had the same expression of HDAC2 as patients with normal lung function, but significantly reduced protein expression was reported in GOLD stage III & IV patients[355]. This data supports the concept of a correlation between loss of HDAC and disease severity in COPD. It also adds evidence to the proposal that subjects in the RV challenge study had

sufficiently mild disease that differences in HDAC activity between COPD subjects and controls might not be identifiable at baseline.

6.19 Reduced HDAC activity in smoke exposed subjects

Cigarette smoke has been shown to reduce HDAC activity both *in vitro[356, 357]* and *in vivo*[125, 131, 352]. A study of BAL macrophages from cigarette smoking subjects demonstrated reduced HDAC2 activity when compared to samples from non-smokers. This study also identified an inverse correlation between HDAC2 activity and IL-8 release[352]. The reduction of HDAC2 protein following cigarette exposure was subsequently found to be due to phosphorylation and ubiquitination of the HDAC enzyme[358]. In this chapter the data presented did not show significantly reduced HDAC activity in sputum or BAL macrophages in smoking controls compared to non-smoking controls at baseline. Although this does not support published work, there are methodological differences between the two studies. Ito *et al* recruited subjects who were younger and may have had more intense cigarette exposure (mean age 29 years, >20 pack year history) than the smoking controls in this study (mean age 53 years, 32 pack year history). Additionally radio-labelled histones were used to measure HDAC2 activity, utilising an alternative and potentially more sensitive method than the fluorometric assay used in this RV challenge study. Finally in Ito's study subject numbers in whom BAL HDAC activity was measured were comparatively small with only 6 subjects in each of the smoking and non-smoking groups.

Following RV infection HDAC2 activity was reduced in smoking controls compared to non-smoking controls in sputum macrophages but not in BAL. There are no studies of HDAC2 activity in smoking subjects following RV infection, but as RV infection induces inflammation and oxidative stress it may impair HDAC activity in a similar way to that seen in COPD subjects in published studies.

6.20 HDAC activity and inflammatory cytokines

There are contradictions between published studies describing the effect of HDAC activity on inflammation, with some reporting an increase, and some a decrease, in inflammatory cytokine levels following inhibition of HDACs. The majority of studies which have examined the relationship in the lungs suggest that HDAC inhibition or reductions in activity have a predominantly pro-inflammatory effect.

A correlation between TNF-α release and reduced HDAC activity in human macrophages have been shown[352], additionally this effect was augmented by inhibition of HDAC activity using TSA. Similarly enhanced expression of IL-8 and reduced HDAC activity was found to correlate. Therefore data from my study is supported by others which have identified an association between increased inflammation and reductions in HDAC2 activity. Yang *et al* reported an increase in NFκB following TSA administration, and postulated that this was the mechanism of increased IL-8 release following cigarette exposure[356]. Several studies have identified a correlation between IL-8 release and histone acetylation[359-361]. Moodie *et al* performed the study using an epithelial cell line which demonstrated reduced HDAC activity after exposure to both cigarette smoke and oxidative stress (H_2O_2), however the effect of these mediators was lost when cells were pre-treatment with the antioxidant NAC, reducing subsequent HDAC degradation and IL-8 release[362]. Collectively these studies provide *in vitro* evidence that the loss of effectively function HDAC enzymes has a pro-inflammatory effect. There is also evidence that provides a mechanism for oxidative stress induced inflammation.

However the studies described above appear to contradict the findings of several reports in which HDAC inhibition leads to reduced cytokine release. An *in vivo* rodent study demonstrated an anti-inflammatory effect following HDAC inhibition[133]. In this study LPS was used to stimulate the release of IL-6, TNF-α and MIP-2α, however pre-treatment with TSA significantly reduced induction of these cytokines by LPS. Other experimental models have also demonstrated an anti-inflammatory effect associated with a reduction in cytokine levels and HDAC inhibition. Amongst these includes a rodent model of asthma in which TSA attenuated airway hyperresponsiveness in association with reductions in inflammatory cells and cytokines[363]; an experimental model of sepsis in which TNF-α and IL-1β release from PBMC was reduced by HDAC inhibition[364] and an arthritis model in which IL-6 and IL-1β were reduced following administration of HDAC inhibitors to rodents[365].

The majority of studies performed *in vitro* using pulmonary cells identify HDAC as repressors of inflammation while whole organ or *in vivo* studies identify a pro-inflammatory role for HDAC activity. This concept is supported by data in the study by Dombrowsky *et al* in which a discrepancy between results obtained following TSA exposure was found, specifically an up regulation of inflammatory genes in tissue

culture studies and a repression of cytokine release following LPS stimulation *in vivo* and perfused lung studies. Finally there is evidence that the degree of histone acetylation correlates poorly with gene expression[133].

The conflicting nature of this data illustrates the complexities of studies in this field. It is likely that changes in gene transcription are dynamic, with the response to external stimuli modulated by the activity of HDAC. A multitude of factors influence HDAC activity and it is likely that different responses will be seen in different disease states.

Studies of HDAC activity following smoke exposure and in COPD have consistently identified an increase in inflammation associated with reduced HDAC2 activity. The data presented in this chapter is supported by these studies; my data demonstrated a strong negative correlation between HDAC2 activity and inflammatory cytokines only in COPD subjects. Importantly this is the first study in which RV infection modulates HDAC2 activity, and this is performed in human subjects, providing evidence that the discrepancy described above is not related to differences between *in vitro* and *in vivo* studies. Although the data described here does not provide sufficient evidence to draw firm conclusions, it appears likely that the effects of HDAC activity are related to the specific tissue types or disease phenotypes.

6.21 Bacterial induced HDAC suppression

The finding of reduced HDAC2 activity only in COPD subjects in the study suggests an association with clinical features of an exacerbation. However the mechanism of action following RV infection has not been studied specifically. In addition to RV infection the COPD subjects had greater levels of bacterial detection in their sputum than control subjects. An *in vitro* study using bacterial infection identified reduced HDAC activity in BEAS2B cell line[366]. This suggests that secondary bacterial infection could be causing or contributing to the reduction in HDAC activity seen in the COPD group. It is possible that an infective process by either viruses or bacteria can lead to posttranslational reductions in HDAC activity, and both could contribute to this through induction of oxidative stress. Additionally exacerbations of COPD where co-infection with both bacteria and viruses have been shown to result in excess inflammation and clinical illness compared to those in which only a single infective agent is identified[1]. Therefore greater reductions of HDAC activity in co-infection may be responsible for this and further studies with larger numbers of subjects are justified to examine this hypothesis.

Legionella pneumophila was found to cause histone acetylation and pro-inflammatory gene transcription following cellular infection in an *in vitro* study[367]. The study demonstrated that these changes were induced by *L pneumophila* flagellin specifically, as studies with flagellin deleted mutants did not induce IL-8 release or histone acetylation. Interestingly the addition of TSA to *L pneumophila* infected cells further increased IL-8 protein expression, demonstrating that loss of HDAC activity contributes to excessive cytokine release in bacterial infection *in vitro*. It was therefore postulated that *in vivo* inflammation induced by lung infections might be mediated through HDAC activity and that this is exaggerated in obstructive lung disease[367].

M catarrhalis has been associated with colonisation and exacerbations in COPD has been shown to decreased HDAC activity and expression following infection *in vitro*[366]. In this study using BEAS2B cells, *M catarrhalis* induced IL-8 and GM-CSF production and the release of these mediators was augmented by addition of TSA, suggesting that HDAC enzymes have a role in limiting inflammatory mediator release. Following *M catarrhalis* infection the expression of HDAC1 and HDAC2 was found to be reduced by 25% and 50% respectively compared to pre-infection levels.

Collectively these data suggest that bacterial infection can lead to increased inflammation through HDAC suppression. The importance of this in COPD exacerbations has not been investigated, but this provides a possible mechanism for excessive inflammation associated with further reductions in HDAC activity in airways disease.

6.22 Virus infection and HDAC activity

It is possible that virus infection leads to changes in HDAC activity in a similar way to that described for bacteria. However no published studies have investigated the relationship between respiratory virus, or specifically RV, infection and HDAC activity.

The majority of studies it the literature focusing on HDACs and viruses have aimed to investigate either mechanisms of chronic virus infection or study the potential carcinogenic role of viruses. A study intended to identify cellular causes of increased resistance to CMV infection showed an association with increased HDAC activity, particularly HDAC3 and inhibition of CMV infection[368]. Here the permissiveness of cells for CMV infection was linked to the degree of histone acetylation. It is believed that part of the host response to virus infection is activation of HDACs to silence

transcription from the virus genome. To counteract this defence, it is postulated that viruses may directly inhibit HDACs. Data supporting this was shown in an *in vitro* study of varicella zoster infection, which identified virus related phosphorylation of HDAC1 & 2 following infection[369]. Finally a study using simian virus supported this concept, as it was demonstrated that inhibition of HDAC enzymes resulted in increased virus infection[370].

As virus infection is a major cause of respiratory tract illness and chronic airway diseases are typically associated with increased inflammation, further studies on the role of HDACs in this disease area are important.

6.23 HDAC activity and immune responses

The interaction between immunological mediators and the activity of HDACs have recently become an area of research interest. The potential ability of this class of enzymes to modulate immune responses may have an impact on airway diseases. This includes COPD which has been regarded by some authors as an autoimmune disease[371, 372]. Aberrant host defence mechanisms therefore may have a role in airway inflammation. In a study designed to investigate potential mechanisms of immune tolerance, IL-10, which has an anti-inflammatory role, was shown to be suppressed by overexpression of HDAC11[373]. However this finding appeared to be contradicted by a study using a malignant cell line, in which HDACi which are already in clinical use they were found to suppress IL-10 production[374].

A study on HDACi in cell lines and mice identified increased susceptibility to infection and failure in host defence mechanisms. There was a reduced host response to microbial pathogens and impairment of innate immune responses[135]. A recent review of the role of HDAC enzymes in innate and adaptive immune pathways suggested that they act as regulators of immunity as well as inflammation. Furthermore it was stated that their inhibition can compromise host defence[375]. This data hints at a potential mechanism of increased infections in COPD in the setting of reduced HDAC activity. Therefore although specific studies and data are not yet available, the potential impact of abnormal HDAC activity in COPD may extend beyond airway inflammation and into direct impairments of host immune function.

Of relevance to the data presented in this study; it might be postulated that RV infection leads to reductions in HDAC activity, which in turn impairs host defence

mechanisms as illustrated by studies discussed here and leading to increased rates of secondary bacterial infection identified in the COPD subjects in this study.

6.24 Mechanisms of HDAC reduction in COPD

The precise role of impaired HDAC performance in COPD is not known. Although disease severity is associated with a progressive reduction in HDAC activity, it is unclear if this is a consequence or a mechanism of the disease process. Evidence supporting both concepts exists and *in vivo* it is possible that the two situations coexist, leading to positive feedback and disease progression. A recent study in rats and a human pulmonary vascular cell line investigated the relationship between reduced HDAC activity and induction of emphysema. The data demonstrated an association between HDAC inhibition (using TSA and siRNA) and lung tissue destruction[376]. This provides intriguing evidence that fully functioning HDAC is required for the maintenance of normal lung structure, and that at least part of the inflammation and tissue destruction seen in COPD is driven by reduced HDAC expression and activity. However, the pulmonary environment in COPD is characterised by inflammation and excess oxidative stress. This leads to a loss of HDAC enzymes through a variety of mechanism. There is evidence of posttranslational modifications[353] and subsequent breakdown by ubiquitination of HDAC protein[358, 377]. Reductions in HDAC expression and activity as a result of oxidative and nitrative stress have also been demonstrated in cell culture and human *ex vivo* samples[377].

6.25 Conclusion

The data presented in both sputum and BAL supports the hypothesis that increased inflammation found in RV induced exacerbations is mediated by a fall in HDAC2 activity.

No other published studies have collected longitudinal data on HDAC2 activity both before and after RV infection in COPD and control populations. Published data of HDAC activity in COPD has focused on the stable state or at a single time point post exacerbation. The pattern of change in HDAC activity during the acute phase of an exacerbation has therefore not been examined until now.

The changes from baseline in individual study groups represent the most intriguing findings, particularly as HDAC2 activity behaves differently in the three study groups following RV infection. The significant falls from baseline only in COPD subjects offers the strongest evidence to support the hypothesis.

Within this chapter there are many interesting findings presented, particularly the correlation with HDAC2 activity and cytokines. However cautious interpretation of this data is required, due to limited sensitivity of the HDAC2 assay in sputum macrophages and detection levels close to the lower limit. Further work is required to investigate fully the associations with inflammatory and clinical markers and the findings of reduced HDAC2 activity in COPD subjects.

Chapter 7: Oxidative and nitrative Stress

7.1 Introduction

The importance of oxidative and nitrative stress in the pathogenesis of COPD is becoming increasingly evident despite the difficulties in accurate measurement of these parameters. Patients with COPD have been shown to have an excess of oxidative and nitrative stress which is compounded by a diminished antioxidant capacity; this in turn leads to tissue damage from an excess burden of harmful radicals. It is known that RV infection leads to induction of both oxidative and nitrative stress following infection in both healthy and diseased individuals. Here I measure nitrative stress and antioxidant potential following RV infection using the Griess assay and the Potential of Antioxidant (PAO) assay to explore the hypothesis that COPD subjects experience a greater burden of reactive oxygen species following RV infection when compared to non-obstructed controls and that this, in turn is associated with adverse inflammatory and clinical parameters.

Results

7.2 Oxidative and nitrative stress assays

The Griess assay was used to measure levels of organic nitrite and hence indirectly measure nitrative stress in BAL, EBC and sputum supernatant. However BAL and EBC samples were found to be too dilute for reliable measurement, with the majority of values falling below the lower detection limit of the standard curve. No attempts were made to concentrate BAL or EBC owing to concerns over the reliability of nitrite levels obtained this way; these results are therefore not discussed further in this chapter.

7.3 Nitrative stress at baseline

To investigate differences in nitrative stress between the three study groups prior to RV infection the results of the Griess assay from baseline sputum samples is presented, figure 7.1. At baseline there was a trend to lower levels of nitrite detected in the sputum supernatant of smoking controls. The highest level was detected in non-smoking controls. However there was no significant difference in the mean nitrite levels measured between the three study groups; $\log_{10}$ mean (SEM) concentrations in non-smoking controls, smoking controls and COPD was 0.7 (0.1), 0.5 (0.2) and 0.6 (0.1) respectively, (p=0.281, one way ANOVA).

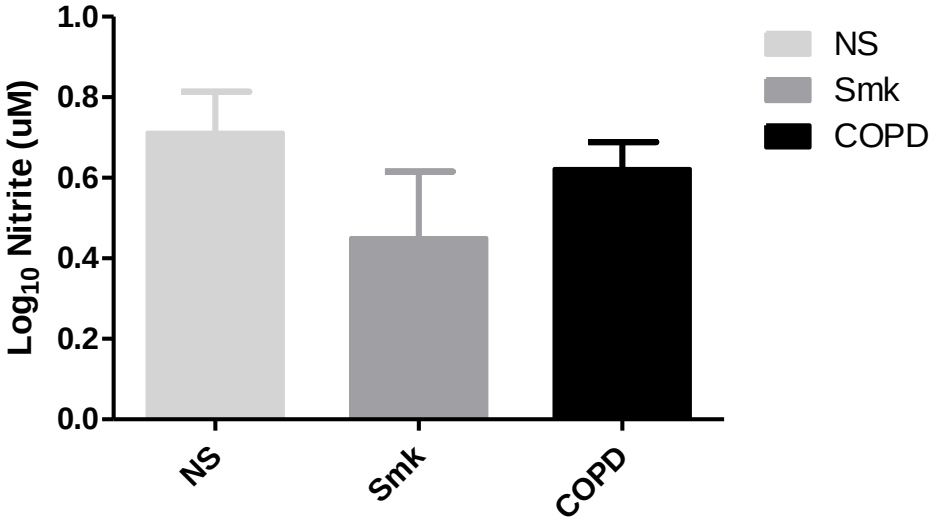


Figure 7.1 Baseline sputum nitrite levels in individual study groups, Data presented mean +/- SEM, (one way ANOVA, p=0.281)

7.4 Nitrative stress following RV infection

To assess the effect of RV infection on the whole study population the level of nitrite in sputum supernatant from all subjects who were successfully infected is shown over the time course of the study in figure 7.2. When compared to baseline using the paired t test and each time point there was a significant increase in nitrite levels on all days following infection, with a peak on day 9. The levels had still not returned to pre-infection concentrations by day 42.

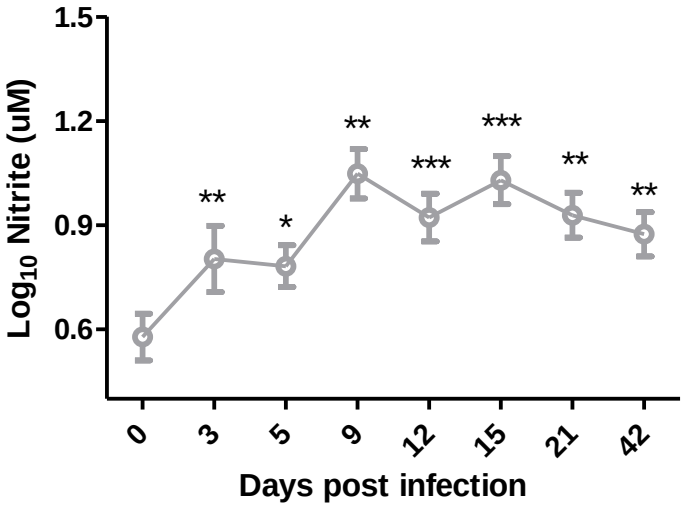


Figure 7.2 Time course of sputum nitrite levels in all RV infected subjects. Data presented mean +/- SEM, (paired t test, *p<0.05, **p<0.01, ***p<0.001)

To investigate differences between the study groups, the results of sputum nitrite concentrations are shown over the time course of the study in figure 7.3. This demonstrated that COPD subjects had statistically higher nitrite levels when compared to both control groups on day 15 (p=0.008, one way ANOVA) and on day 12 there was a higher level in COPD and non-smoking subjects when compared to smoking controls (p=0.003, one way ANOVA). In addition the mean nitrite level was non-significantly higher in COPD subjects during the study. The mean (SEM) total sputum nitrite levels for non-smokers, smokers and COPD subjects over the time course measured using the area under the curve (AUC) was 55.6 (5.7), 72.8 (19.0) and 102.1 (15.8) respectively. There was a significant difference in AUC between the non-smokers and COPD groups (mean difference 46.4, 95% CI 12.3 to 80.6, p= 0.010, one way ANOVA) with no difference between any other groups.

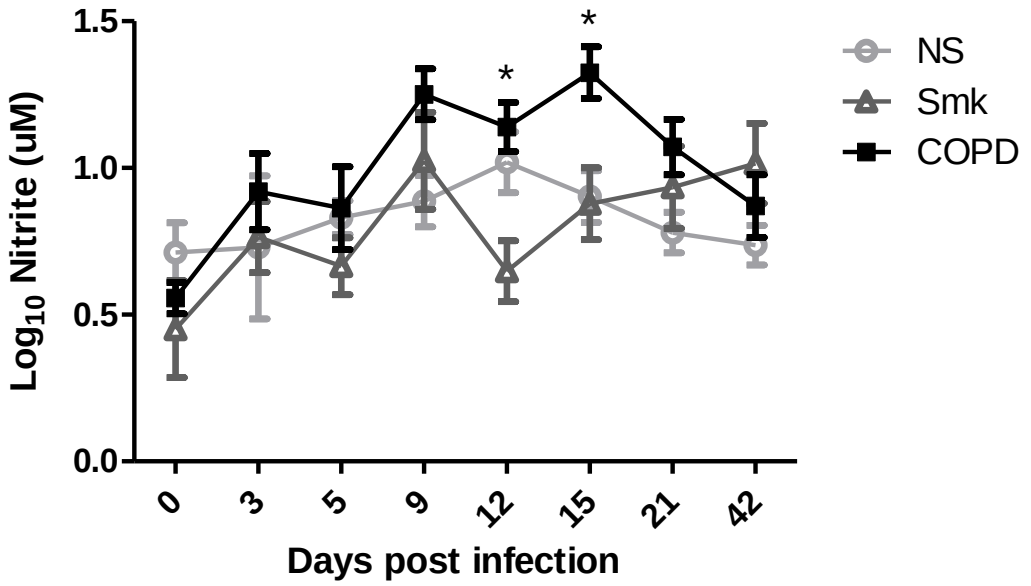


Figure 7.3 Time course of sputum nitrite levels in individual groups. Data presented mean +/- SEM, (one way ANOVA, *p<0.05; Tukey analysis, **Day 12 COPD vs Smk and **Day 15 COPD vs NS and **COPD vs Smk)

When the change from baseline in sputum nitrite concentration in individual groups was analysed using the paired t test from study time points, the increase following infection was found to be significant only in the non-smoking controls and COPD subjects, figure 7.4. In non-smoking controls there was a significant increase from baseline on days 3 and 15 with a peak on day 12. In smoking controls there was a

trend to increased nitrite levels but this was not significant on any day post infection. The peak change occurred on day 9 and a trend to increasing levels was seen from day 12 to day 42. Only the COPD group had a significant increase from baseline at all time points following infection with the peak increase identified on day 15. The magnitude of the increase in nitrite from baseline was greatest in the COPD subjects and least in the non-smoking control group.

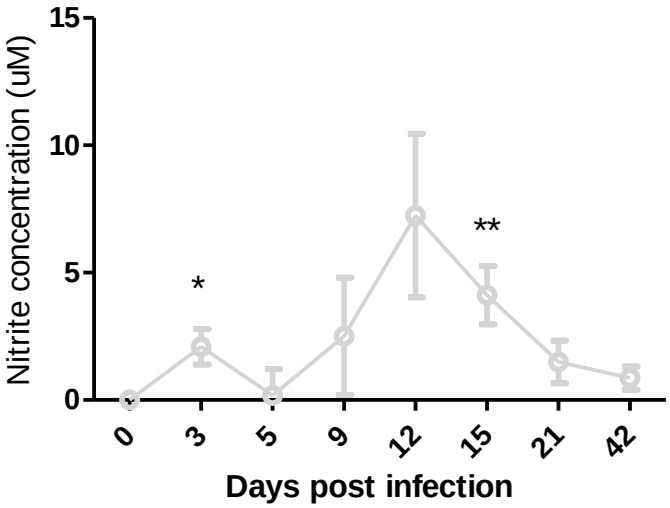


Figure 7.4a Non-smoking group

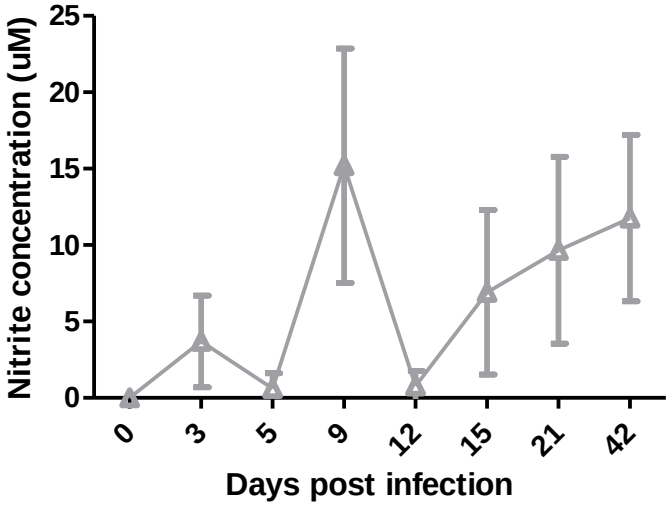


Figure 7.4b Smoking group

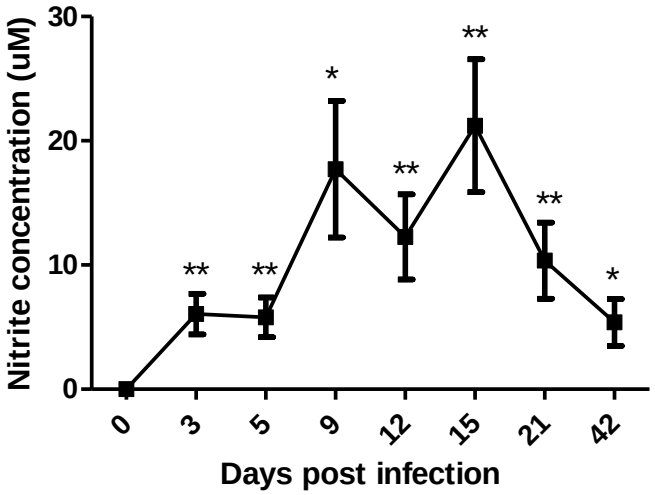


Figure 7.4c COPD group

Figure 7.4 Change from baseline in sputum nitrite levels after infection in (a) non-smoking controls, (b) smoking controls and (c) COPD subjects. Data presented mean +/-SEM, (paired t test from baseline at study time points, *p<0.05, **p<0.01)

7.5 Assay of antioxidant potential at baseline

The PAO assay was performed in BAL and sputum supernatant. However levels in BAL fell below the lower limit of detection for the assay, and so this will not be discussed further in this chapter. At baseline there was no significant difference between the PAO levels in sputum supernatant in the three study groups; mean (SEM) non-smoking controls 125.1 (9.4), smoking controls 180.2 (33.2) and COPD subjects 101.9 (27.7) p=0.097, one way ANOVA. Although not significant; this data illustrates a trend to higher levels in smoking controls and reduced levels in COPD subjects, figure 7.5.

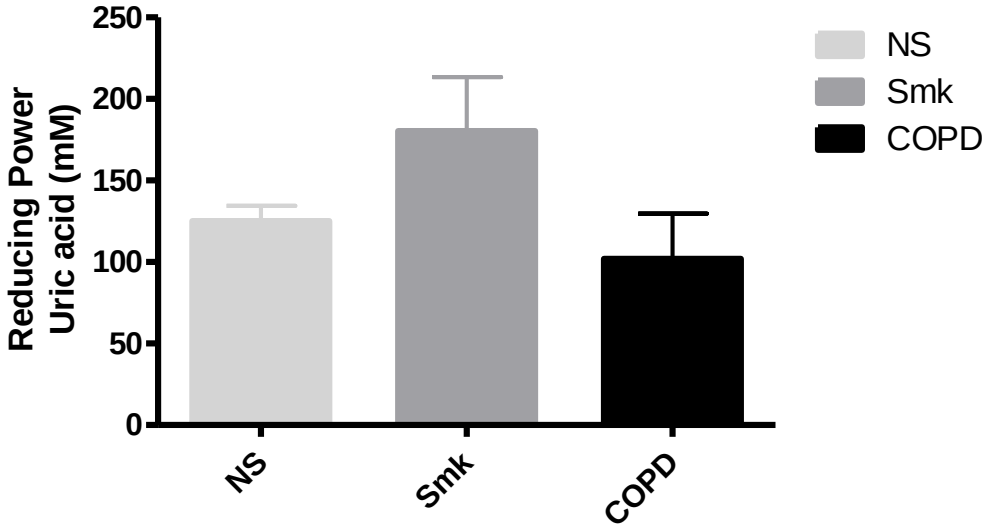


Figure 7.5 Results of PAO assay at baseline from sputum supernatant in study groups. Data presented mean +/-SEM, (p=0.097, one way ANOVA)

7.6 Assay of antioxidant potential in study groups

Investigation of the effects of RV16 infection on antioxidant potential in all study subjects is presented in figure 7.6. The pattern was of a decline in PAO activity from baseline at all post infection time points until day 42. However the variance of the data was large and this was not statistically significant when compared to baseline and analysed with a paired t test at any time point.

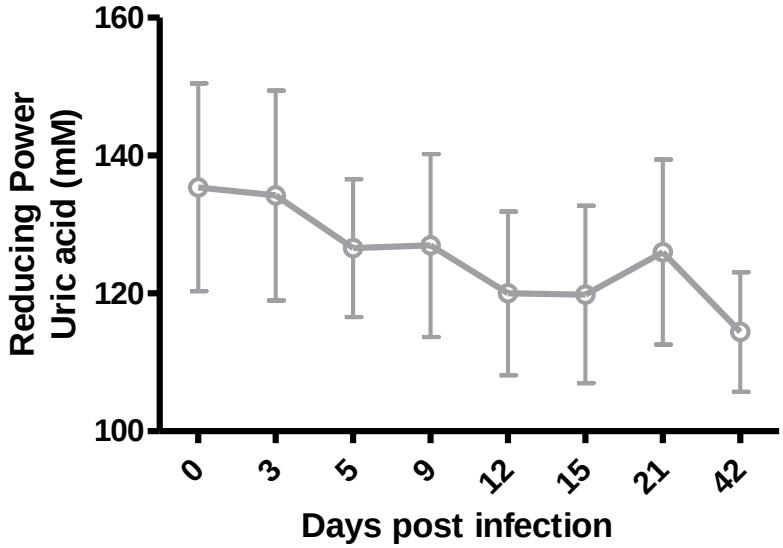


Figure 7.6 Time course of sputum PAO levels in RV infected subjects. Data presented mean +/-SEM, (paired t test, p>0.05)

When the results from the PAO assay in individual study groups was analysed across the whole time course of the study a complex pattern was identified, figure 7.7.

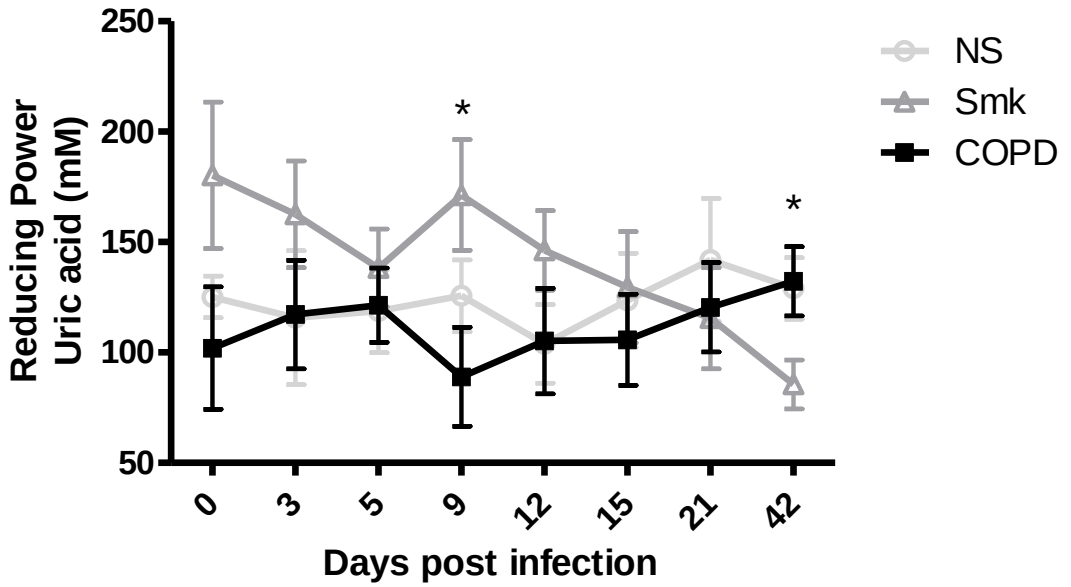


Figure 7.7 Time course of PAO level in sputum supernatant by study group. Data presented mean +/-SEM (*p<0.05, one way ANOVA)

Initially PAO levels increased in COPD subjects until day 5, with a fall at day 9, then a consistent rise until day 42. The overall trend was to lower levels in COPD subjects when compared to control groups across the whole study.

In smoking controls the concentration fell until day 5, increased on day 9 and then consistently fell until day 42. The overall trend in smokers was to higher levels compared to the other two groups across the whole study time course. In non-smoking controls there was little variation in PAO levels following RV16 infection.

There was a statistically significant difference between the means of the three study groups on day 9 and day 42, (one way ANOVA p=0.042 and p=0.036 respectively). Tukey's analysis demonstrated that the difference on day 9 between smoking controls and COPD subjects was significant, p=0.033. In summary the results of the

PAO assay following RV16 infection in COPD subjects has an inverse trend to that found in smoking controls, with significant differences between these two groups on day 9.

Because of the complex pattern in changes over time within each group an analysis of the area under the curve (AUC) for total antioxidant potential in each group over all study visits was performed. Overall the AUC values for the PAO time course demonstrated no significant difference between the groups with mean (SEM) results of 785.2 (50.1), 938.4 (81.5) and 690.3 (102.7) in non-smoking controls, smoking controls and COPD subjects respectively, ($p=0.102$, one way ANOVA). Despite the non-significant p value, this illustrates that there was a trend to a higher levels of PAO activity in control groups, with smoking subjects exceeding those of non-smokers, when compared to COPD subjects across the whole time course of the study.

7.7 Assay of antioxidant potential change from baseline

To evaluate the effect of RV infection on oxidant levels in each group, the change from baseline in PAO assay activity was calculated, figure 7.8. In non-smoking controls the antioxidant potential remained constant during the study time course with no change from baseline at any time point, (paired t test from study time points and baseline, $p>0.05$). In smoking controls there was a trend to falling levels of antioxidant activity compared to baseline following infection. This was greatest and significant on day 42, (paired t test, $p=0.039$). In contrast, in COPD subjects there was no significant change from baseline at any time point measured, $p>0.05$, paired t test. There was a small trend to increasing activity from day 15 until the final study time point.

The magnitude of the peak change from baseline was fivefold greater in smoking controls compared to the non-smoking and COPD groups, mean peak change from baseline for non-smoking controls, smoking controls and COPD subjects was 19.3, -99.3 and 26.5 respectively. The peak change from baseline occurred on day 21 in non-smokers and day 42 in smoking and COPD groups, figure 7.8.

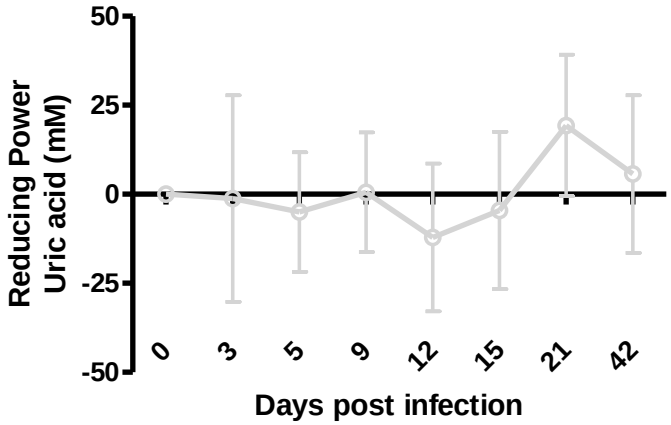


Figure 7.8a Non-smoking group

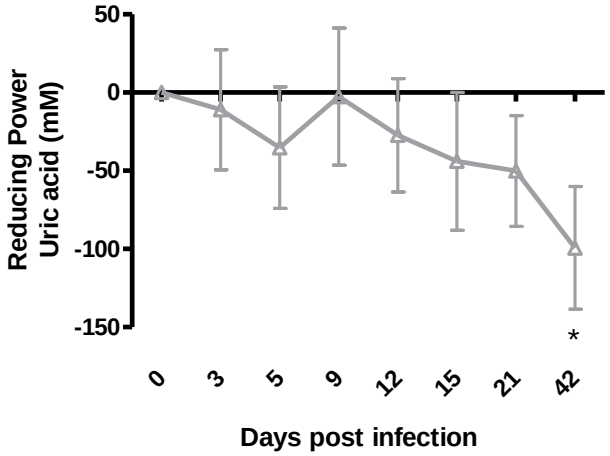


Figure 7.8b Smoking group

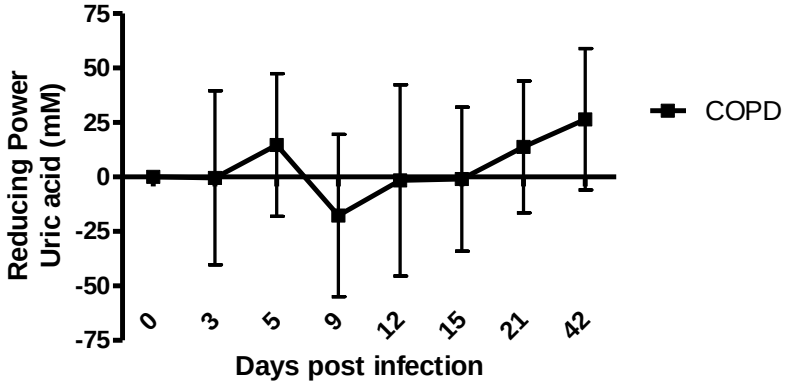


Figure 7.8c COPD group

Figure 7.8 Change in sputum PAO levels from baseline after infection in (a) non-smoking controls, (b) smoking controls and (c) COPD subjects. Data presented mean +/-SEM, (paired t test, *p<0.05)

7.8 Correlation of nitrative stress and antioxidant potential

The trend in all successfully infected subjects was an increase in nitrite concentration and reduction in the antioxidant potential over the whole study time course. However the mean changes in individual study groups for the results of the PAO assay did not follow this pattern. Therefore to investigate the relationship between the nitrite and PAO level in all subjects combined the two parameters have been correlated, figure 7.9. This identified a significant negative correlation suggesting that increased nitrative stress was associated with a reduction in antioxidant potential, (Spearman's coefficient rs=-0.31, p<0.001).

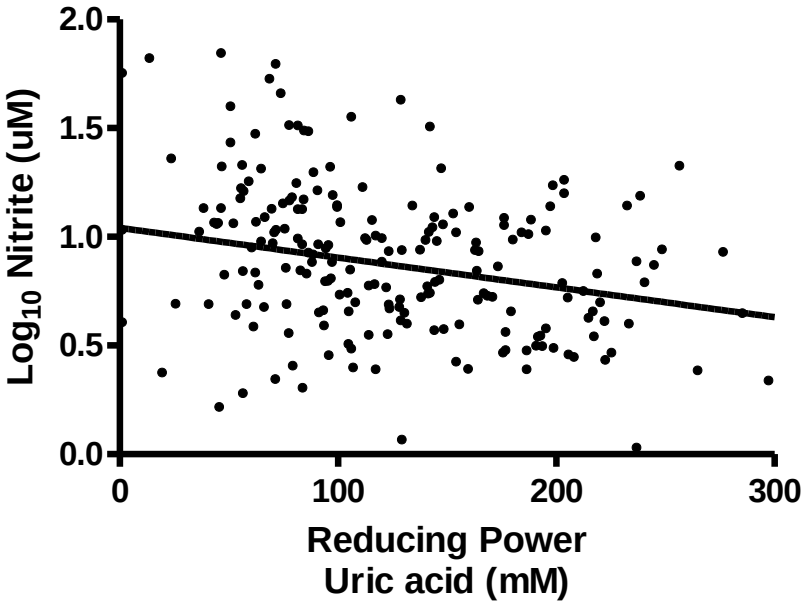


Figure 7.9 Correlation of sputum supernatant nitrite and antioxidant levels in all infected subjects, (rs= -0.31, p=<0.001)

The association between sputum nitrite concentration and PAO results was then investigated in individual study groups, figure 7.10. There was a significant negative correlation only in smoking controls and COPD subjects, (rs=-0.44, p<0.001 and r=-0.36, p=0.002 respectively). In non-smoking controls there was no relationship, (rs=0.17, p=0.162).

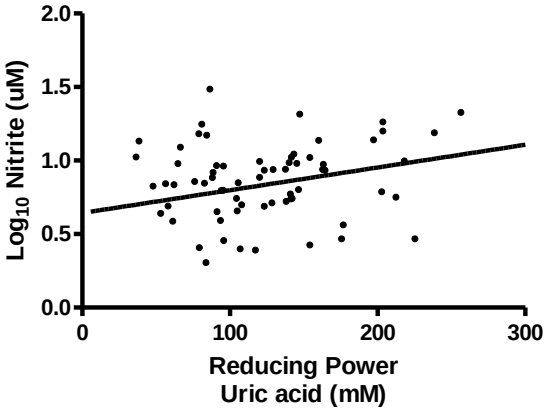


Figure 7.10a Non-smoking group, (rs=0.17, p=0.162)

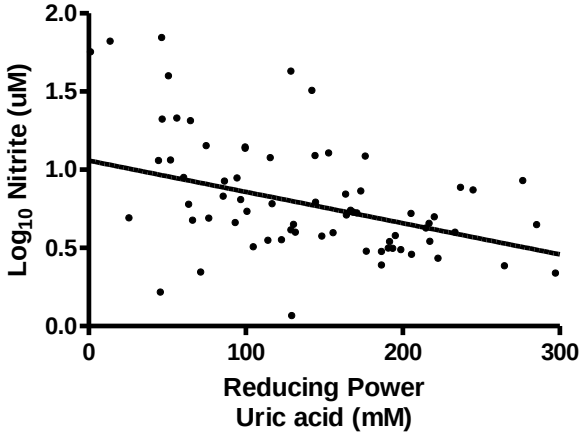


Figure 7.10b Smoking group, (rs= -0.44, p<0.001)

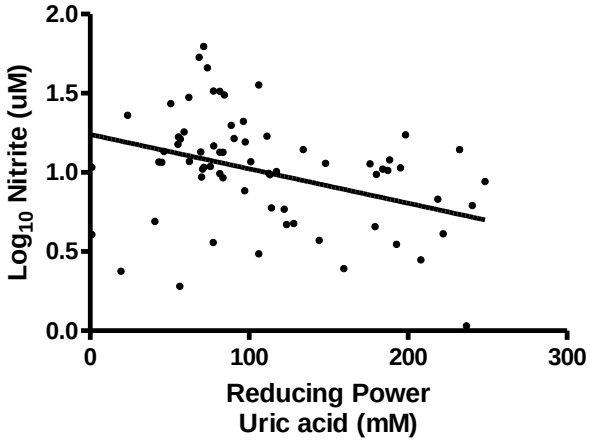


Figure 7.10c COPD group, (rs= -0.36, p=0.002)

Figure 7.10 Correlation of sputum nitrite and antioxidant levels in RV infected (a) non-smoking controls (b) smoking controls (c) COPD subjects

7.9 Ratio of Griess to PAO assay

To further investigate the degree of redox stress in the three study groups the ratio of the Griess to PAO assay has been calculated. At baseline there were no differences between the three study groups when this nonparametric data was analysed using the Kruskal-Wallis test, (p=0.235), figure 7.11.

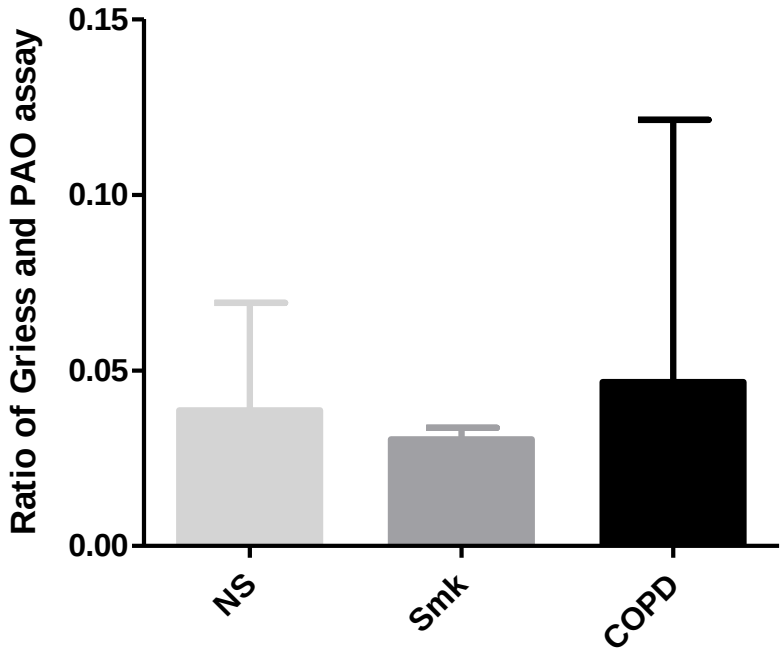


Figure 7.11 Baseline ratio of Griess to PAO assay in individual study groups, data presented as median (interquartile range). Kruskal-Wallis, p=0.235

To investigate the effect of RV16 infection on the Griess to PAO ratio the time course from each study group is plotted in figure 7.12. When the median values between the three study groups were analysed on each time point using the Kruskal-Wallis analysis, there was found to be significant differences on days 9, 12 and 15 (p=0.008, p=0.015 and p=0.011 respectively). This was due to differences between smoking controls and COPD subjects, figure 7.12.

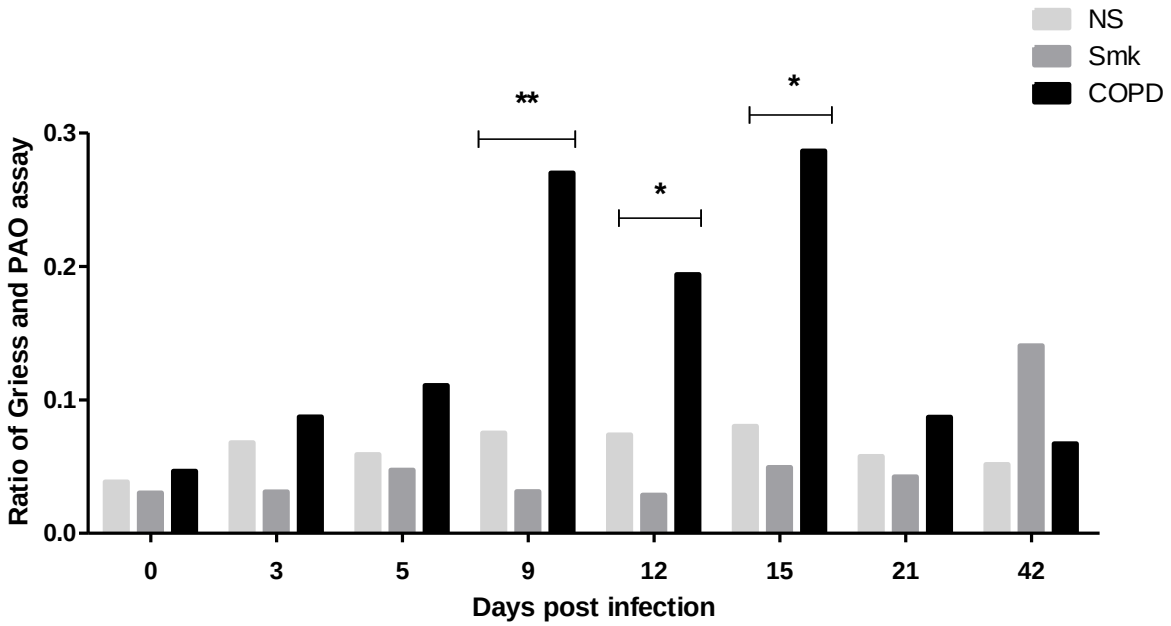


Figure 7.12 Time course of ratio of Griess to PAO assay in individual study groups, data presented as median. Kruskal-Wallis, *p<0.05 **p<0.01 between study groups

7.10 Correlation of virus load and nitrative stress

To investigate the relationship between RV infection and nitrative stress the sputum virus load and nitrite concentration have been correlated, figure 7.13. In all subjects there was a positive correlation between the two parameters, r=0.32 (p<0.001), demonstrating that subjects with a greater virus load had higher nitrative stress levels.

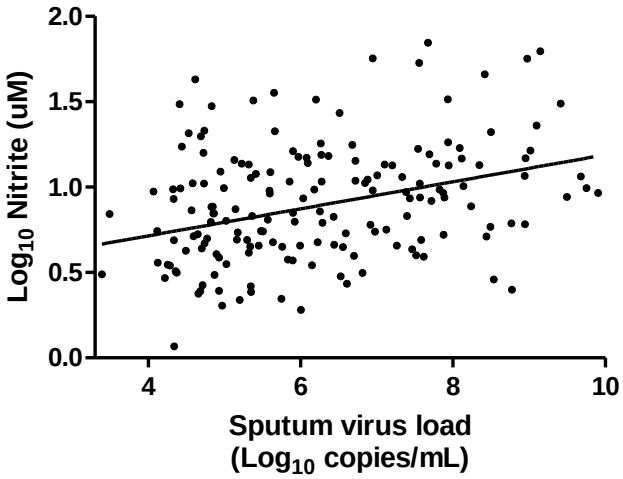


Figure 7.13 Correlation of sputum virus load with nitrite concentration in all successfully infected subjects, (r=0.32, p<0.001)

When the relationship between sputum nitrite and virus load in study groups was analysed, a positive correlation was found in smoking controls and COPD subjects, figure 7.14. In non-smoking controls there was no association demonstrated, r=0.08 (p=0.548). The correlation was stronger in COPD subjects r=0.39 (p=0.004) compared to smoking controls, r=0.28 (p=0.03).

Neither of the control groups had a significant increase in both nitrite and virus load on the same day following infection. However the COPD group had a significant increase in both parameters on days 3, 5, 9 and 12. The stronger positive correlation in this group suggests that the increase in virus load may be responsible for the rise in nitrite concentration to a greater extent than in control groups.

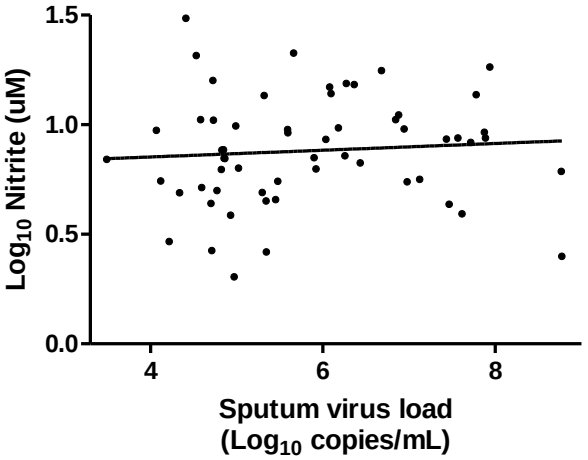


Figure 7.14a Non-smoking group, (r=0.08, p=0.548)

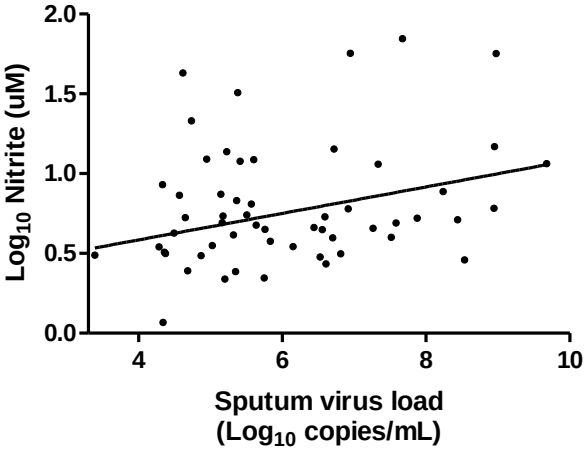


Figure 7.14b Smoking group, (r=0.28, p=0.035)

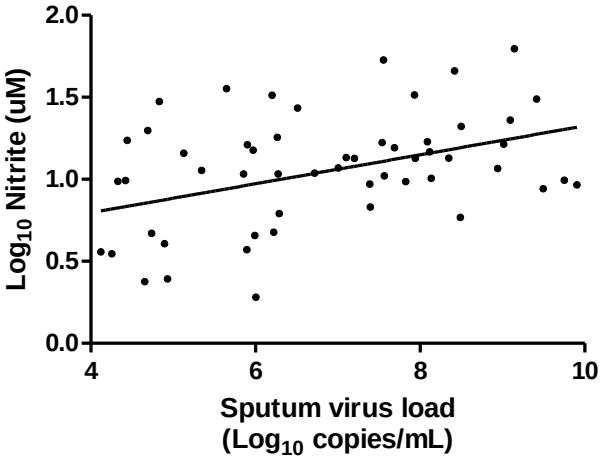


Figure 7.14c COPD group, (r=0.39, p=0.004)

Figure 7.14 Correlation of sputum nitrite and virus load in (a) non-smoking controls (b) smoking controls (c) COPD subjects

7.11 Correlation of virus load and antioxidant potential

The sputum virus load and PAO assay results from all subjects was correlated to investigate their relationship, figure 7.15. There was a significant but weak negative association with a Spearman's correlation of rs=-0.15, (p=0.048) identified. This suggests that an increased virus load was associated with reduced antioxidant potential.

However when the results of the PAO assay and virus load in individual groups was studied, no significant correlation was identified for any group. Non-smoking controls rs=0.06 (p=0.672), smoking controls -0.11 (p=0.404) and COPD subjects -0.08 (p=0.581).

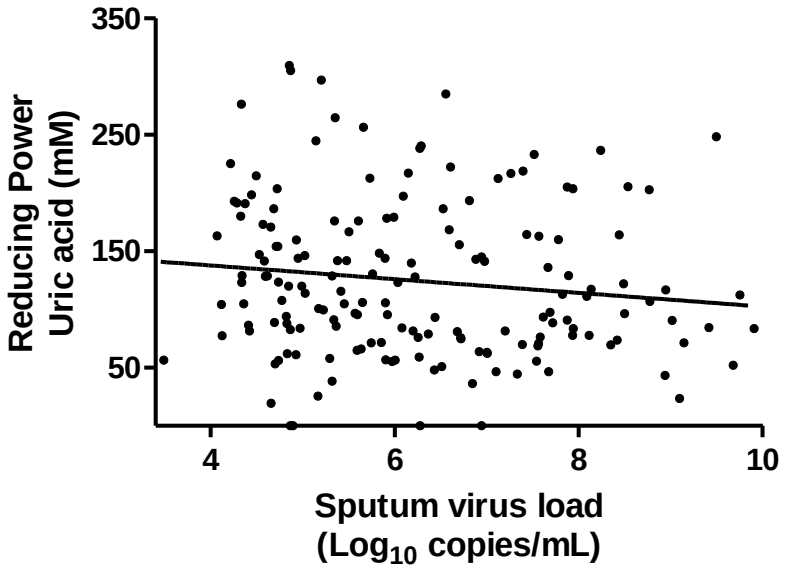


Figure 7.15 Correlation of sputum virus load with antioxidant potential in all successfully infected subjects, (rs=-0.15, p=0.048)

7.12 Association of nitrative stress and antioxidant potential with airway inflammation

To study the relationship between markers of redox stress and airway inflammation, the sputum total cell count (TCC) and sputum supernatant level of IL-8 were correlated with the results of the Griess and PAO assay. The sputum TCC had a strong correlation with nitrite concentration when all subjects were considered in combination, r=0.65 (p<0.001), figure 7.16. There was a weaker but significant negative correlation between the TCC and antioxidant capacity, r=-0.18 (p=0.008), figure 7.17.

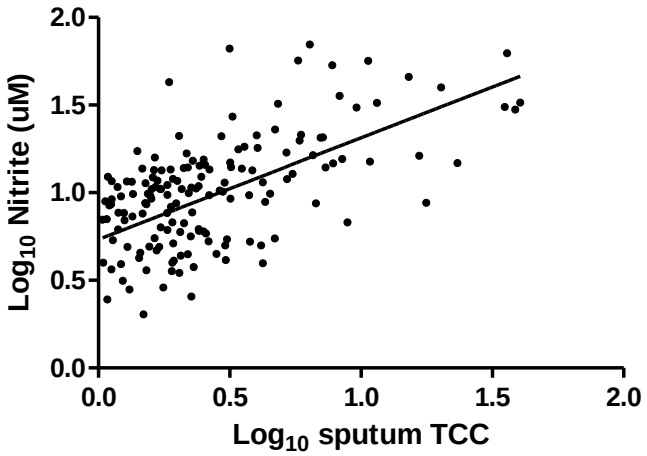


Figure 7.16 Correlation of sputum TCC with nitrite concentration, (r=0.65, p<0.001)

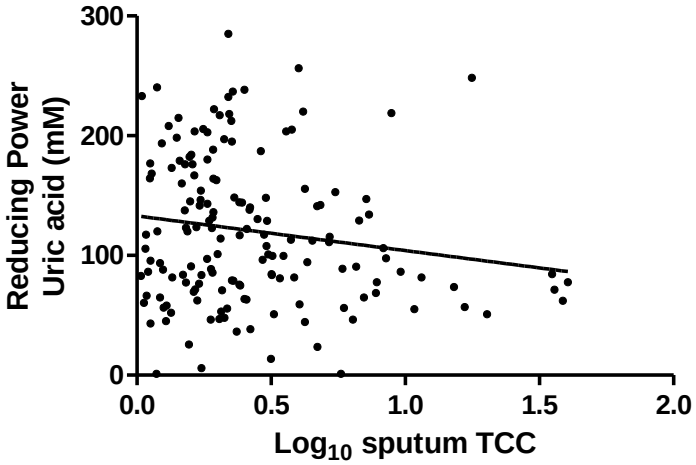


Figure 7.17 Correlation of sputum TCC with antioxidant potential, (r= -0.18, p=0.008)

There was a strong positive correlation between sputum nitrite and IL-8 levels in all infected subjects r=0.70 (p<0.001), figure 7.18, and a significant but weak negative correlation between the PAO assay and sputum IL-8 levels in all subjects r=-0.15 (p=0.036), figure 7.19.

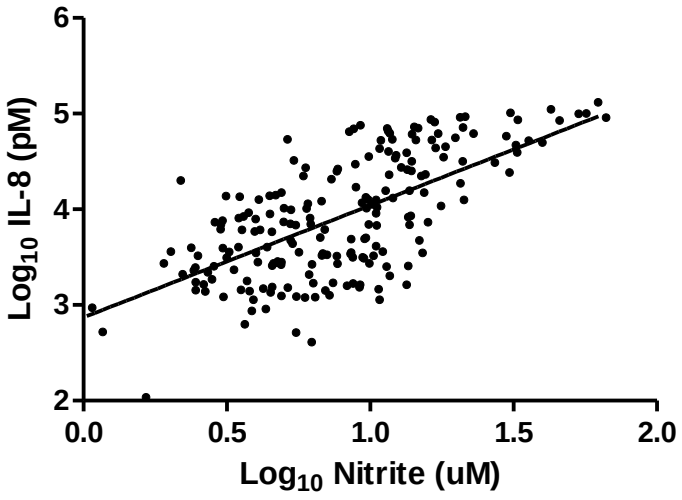


Figure 7.18 Correlation of sputum nitrite with IL-8 concentration in all infected subjects, (r=0.70, p<0.001)

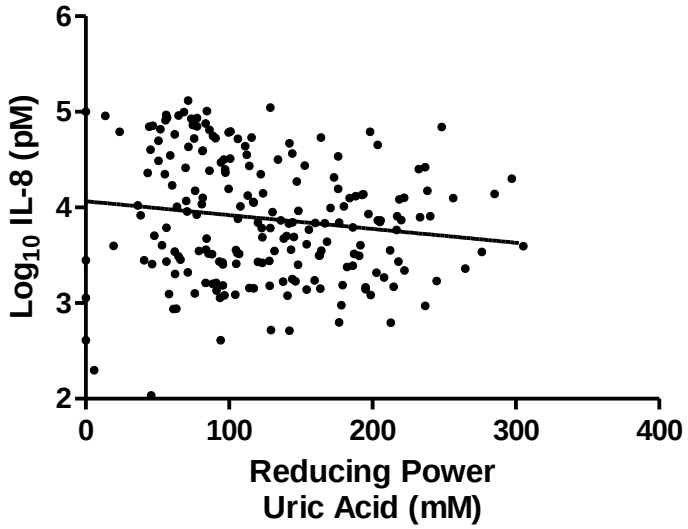


Figure 7.19 Correlation of sputum antioxidant capacity with IL-8 concentration in all subjects, (r=-0.15, p=0.036)

When individual study groups were examined there was a significant positive correlation between sputum nitrite and IL-8 in all groups, figure 7.20.

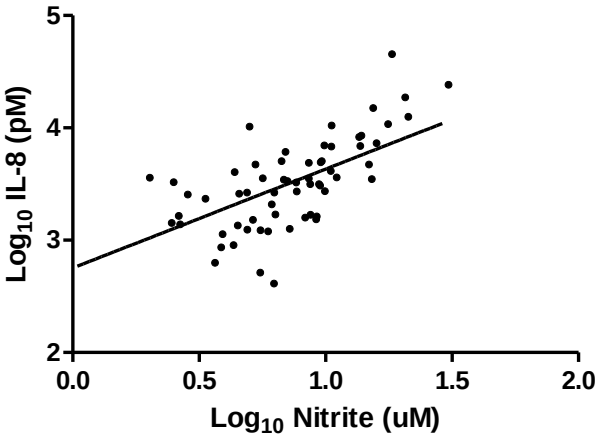


Figure 7.20a Non-smoking controls, (r=0.70, p<0.001)

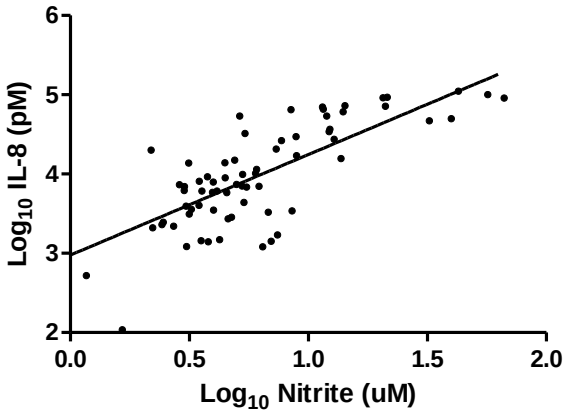


Figure 7.20b Smoking controls, (r=0.78, p<0.001)

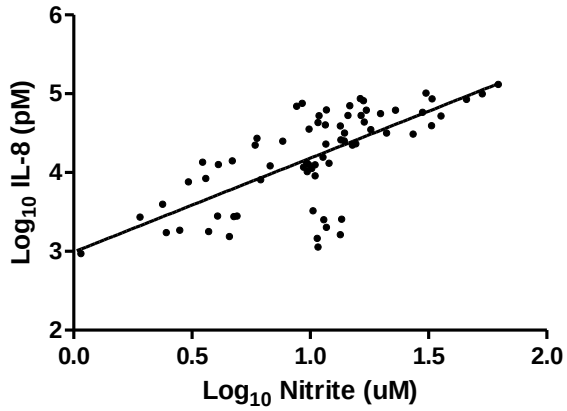


Figure 7.20c COPD group, (r=0.72, p<0.001)

Figure 7.20 Correlation of sputum nitrite with IL-8 concentration in (a) non-smoking, (b) smoking and (c) COPD subjects

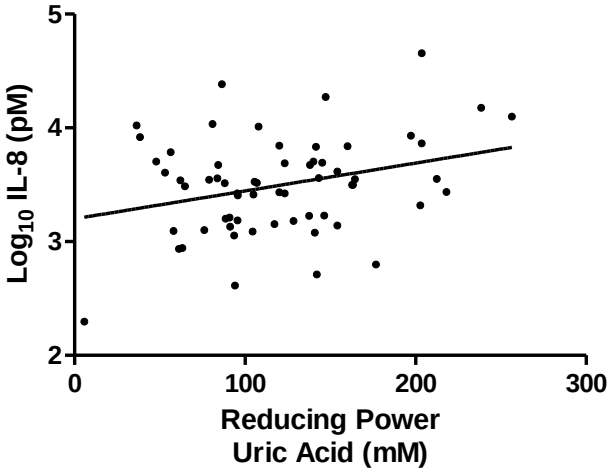


Figure 7.21a Non-smoking controls, (r=0.30, p=0.018)

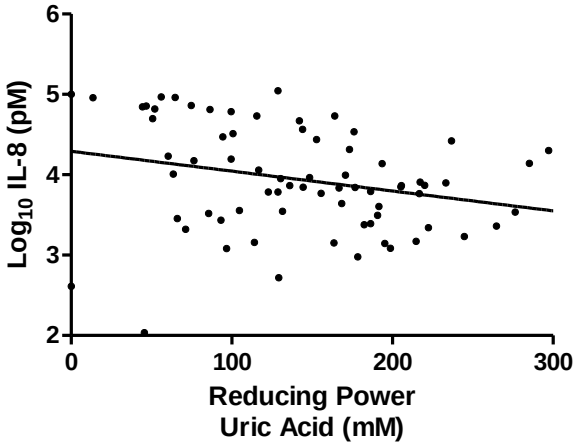


Figure 7.21b Smoking controls, (r=-0.27, p=0.021)

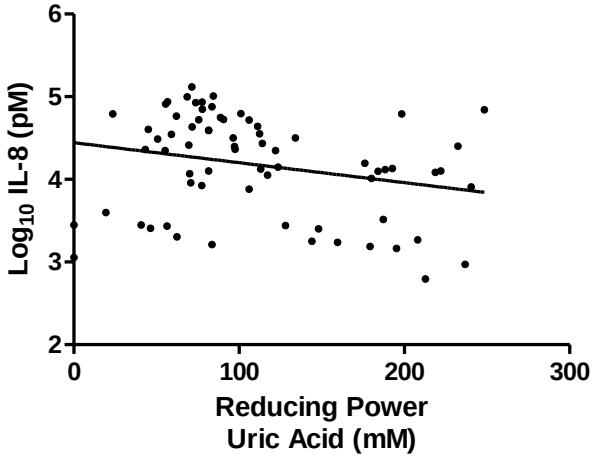


Figure 7.21c COPD group, (r=-0.25, p=0.038)

Figure 7.21 Correlation of sputum antioxidant capacity with IL-8 concentrations in (a) non-smoking, (b) smoking and (c) COPD subjects

The correlations between the antioxidant potential measured using the PAO assay and IL-8 concentrations in individual subject groups have been shown, figure 7.21. There was a significant negative correlation seen in smoking controls and the COPD group, r=-0.27 (p=0.021) and r=-0.25 (p=0.038) respectively. However in non-smoking controls there was a positive correlation r=0.30 (p=0.018).

7.13 Correlation of nitrative stress and antioxidant potential with HDAC2 activity

To investigate possible mechanisms for the relationships described above between IL-8 and oxidative and nitrative stress; the results of the HDAC2 activity assay have been correlated with the PAO and Griess assays. When all infected subjects were considered, figure 7.22, there was a weak but highly significant negative correlation between HDAC2 activity and nitrite concentration. This suggests that increased nitrative stress is associated with reductions in HDAC2 activity. When the three study groups were examined, figure 7.23, this relationship was found only to remain significant in COPD subjects, figure 7.23c with a negative correlation, (r=-0.47, p<0.001). In control groups there was no association, non-smoking controls, figure 7.23a, (r=-0.12, p=0.336) and smoking controls, figure 7.23b, (r=-0.11, p=0.396).

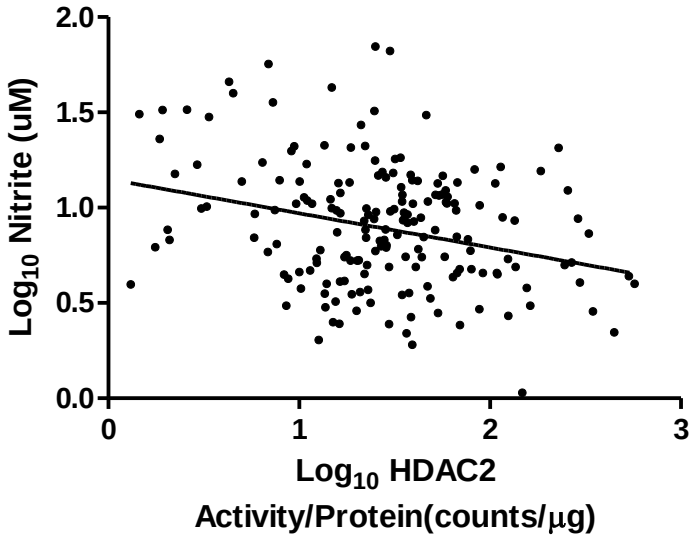


Figure 7.22 Correlation of sputum nitrite with HDAC2 activity in all successfully infected subjects, (r=-0.26, p<0.001)

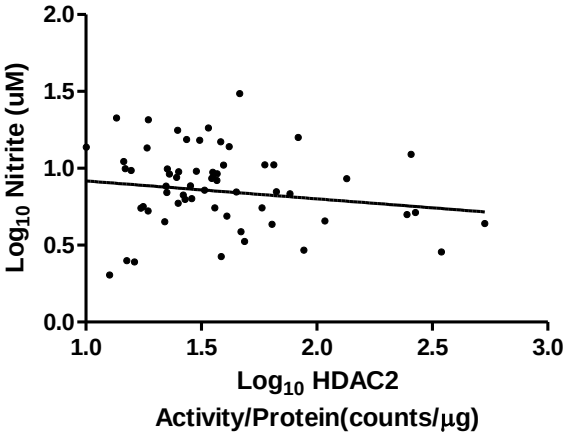


Figure 7.23a Non-smoking controls, (r=-0.12, p=0.336)

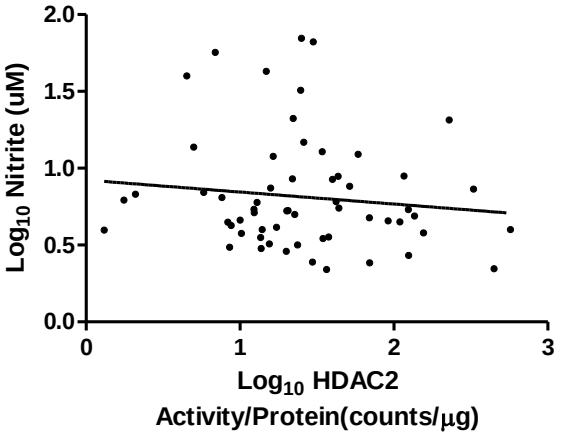


Figure 7.23b Smoking controls, (r=-0.11, p=0.396)

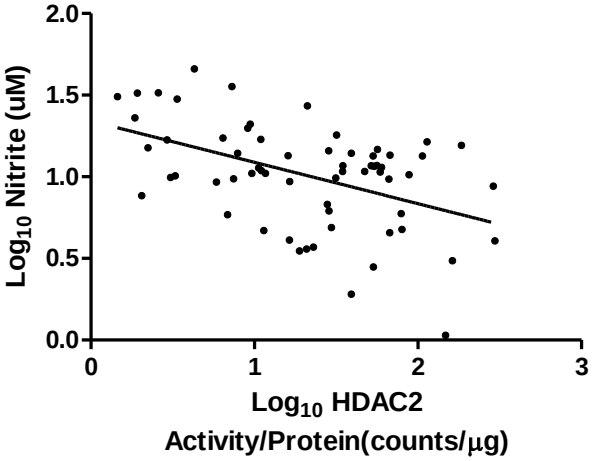


Figure 7.23c COPD group, (r=-0.47, p<0.001)

Figure 7.23 Correlation of sputum nitrite concentration with HDAC2 activity in (a) non-smoking, (b) smoking and (c) COPD subjects

When the relationship between HDAC2 activity and the PAO assay was examined, there was no correlation in all subjects, or in any of the three study groups individually figure 7.24.

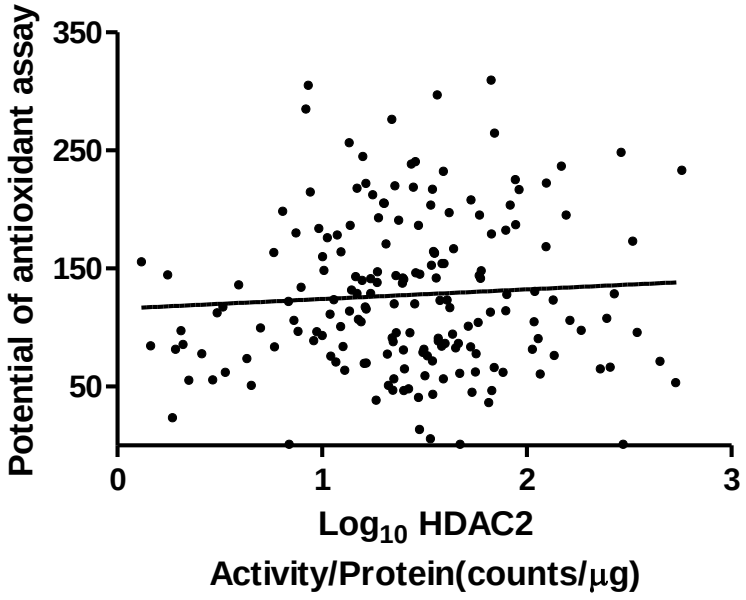


Figure 7.24 Correlation of sputum antioxidant capacity with HDAC2 activity (rs=0.03, p=0.706)

Chapter discussion

7.14 Baseline observations of nitrative stress

Data presented in this chapter examined the concentration of sputum nitrite levels in baseline samples from individual study groups prior to RV infection. No significant difference in nitrite levels was identified between the three study groups. There was a trend to lower levels in smoking controls and greatest in non-smoking controls. Published work on nitrative stress in COPD have been equivocal with some studies reporting elevated levels of nitrative stress while others have shown no differences between COPD and healthy subjects. These are discussed below.

7.15 Excess nitrative stress in COPD

A study by Ichinose *et al* compared three markers of reactive nitrogen species; exhaled NO and nitrotyrosine and iNOS levels from induced sputum, in healthy subjects and those with asthma and COPD. The level of nitrotyrosine was greater in

COPD subjects compared to both other groups, correlated with airway obstruction and iNOS was detected at higher levels in COPD. However there was no difference between COPD and healthy subjects in exhaled NO concentrations[378]. The 10 COPD subjects studied by Ichinose *et al* were all ex-smokers with a mean FEV_1 of 58% predicted[378]. Direct comparison between data presented in this chapter and the study by Ichinose *et al* is difficult because of the different methods of measuring nitrative stress. Although the COPD subjects are both GOLD stage II, those in my RV challenge study had a higher mean FEV_1 % predicted and the majority were current smokers. The potential influence of these factors on measured nitrative stress is not known.

A further study of nitrative stress in GOLD stage II COPD subjects measured peroxynitrite derivatives, nitrosothiols and exhaled NO in stable disease. Greater levels of nitrosothiols were found in induced sputum from COPD subjects but not exhaled NO when compared to healthy controls[379]. However the control subjects in this study were significantly younger than those with COPD (38 years versus 61 years) making the reliability of these results uncertain. Comparison between these results and those presented in this chapter is difficult because although the COPD subjects appear well matched nitrative stress was measured using different assays.

Finally, in an investigation of bronchial biopsies from stable patients Ricciardolo *et al* demonstrated increased markers of nitrative stress in severe COPD patients compared to moderate COPD subjects, healthy smokers and non-smokers. Here nitrotyrosine and MPO cell numbers were significantly greater in severe COPD; however there was no difference between these markers in moderate COPD subjects and non-obstructed controls. The concentration of iNOS was greater in both smoking controls and moderate COPD subjects when compared to non-smokers[380]. This data suggests that nitrative stress worsens with increasing severity of COPD. Although the results in this chapter cannot be compared directly with those reported by Ricciardolo *et al*, the moderate COPD group appeared well matched, and did not show markers of increased nitrative stress compared to smoking controls.

7.16 No difference in nitrative stress between COPD and controls

As described previously there are contradictory reports in the literature, regarding the role of nitrative stress in COPD compared to controls, where no differences in markers of nitrative stress were found between COPD subjects and healthy controls.

Rutgers *et al* measured exhaled NO, iNOS in cells and nitrite and nitrate in supernatant from induced sputum to evaluate the degree of nitrative stress in stable COPD[107]. In this study, the mean FEV_1 was 63% predicted in the COPD group and half the subjects were active smokers. No difference was identified in exhaled NO between COPD and non-obstructed subjects. Interestingly in healthy controls NO was lower in smoking compared to non- or ex-smoking subjects but in the COPD group active smoking did not affect NO levels. Nitrite levels were measured using the Griess assay and the pattern of results in study groups matched that described for exhaled NO. Finally the number of iNOS positive staining cells was also not different between COPD and control subjects. The data from Rutgers *et al* supports the results presented in this chapter. Both studies used similar methods for measuring nitrite in induced sputum and although not statistically significant there was a trend to lower levels in smoking controls in the RV challenge study. The severity and age of COPD subjects was also similar allowing for accurate comparisons to be made.

A study designed to identify biomarkers in COPD measured NO in serum samples during the stable and exacerbated state. When compared to controls the stable COPD group did not have a significant difference in NO concentrations[381]. Interestingly the NO concentration showed a positive correlation with pack year history in COPD subjects, these subjects had a mean FEV_1 of 46% predicted. Although not the primary aim of the study by Karadag *et al*, this data provides further evidence that stable COPD subjects do not have increased nitrative stress compared to control subjects.

Another study has been published which reports the use of the Griess assay in COPD patients[382]. However in this Italian study nitrite levels were lower in the plasma of COPD patients compared to controls, which the authors suggested might be related to pulmonary hypertension. It is not possible to compare these results with those obtained in this study.

Collectively these studies illustrate the complexities of measuring nitrative stress in stable COPD. Overall the published literature supports the results presented in this chapter as the only study to use the Griess assay did not identify significant differences between stable COPD and healthy controls. The contradictions between studies are likely to represent differences in methods of measuring nitrative stress and the severity of COPD subjects included.

7.17 Baseline observations of oxidative stress

At baseline there was no significant difference in the results of the PAO assay between the three study groups. There was a trend to higher levels in smoking controls with the lowest detected in COPD subjects. The interpretation of these results raises interesting questions. There is evidence from previous studies of increased oxidative stress and reduced antioxidant levels in COPD subjects.

7.18 Increased oxidative stress in stable COPD

In an early study to investigate the relationship between oxidative stress and airflow obstruction the *in vivo* measurement of O_2^- was found to correlate with bronchial hyperactivity in patients with airflow obstruction[383]. Subsequently the Thiobarbituric acid reactive substances (TBARS) assay was used to measure markers of lipid peroxidation by several studies. In a study primarily designed to investigate lipid peroxidation in lung cancer, it was also found that the degree of airway obstruction was associated with markers of oxidative stress measured using the TBARS assay and that recent cigarette smoke exposure also increased these markers[384]. Greater levels of both H_2O_2 and TBARS mediators were measured in EBC from COPD subjects compared to non-smoking controls[100]. Active smoking had a trend to increase markers of lipid peroxidation but this was not significant. Although results from this study are of interest, 13 of the 44 COPD subjects had never smoked raising uncertainty about the aetiology of their lung disease.

An alternative measure of lipid peroxidation was studied by assessing cellular staining for the aldehyde 4-hydroxy-2-nonenal (4-HNE) which is a stable and specific end product of oxidation[385]. COPD subjects were found to have higher levels than smoking controls in bronchial and alveolar cells and this was inversely correlated with FEV_1. The mean FEV_1 % predicted in the COPD group was 66%, and although the control group was well matched, the majority of both groups were ex-smokers making comparison with active smokers less reliable. Additionally all tissue samples were obtained from subjects undergoing lung resection for malignancy, introducing a confounding factor.

Several studies have examined EBC for markers of oxidative stress in COPD. An early study identified increased levels of H_2O_2 in EBC from stable COPD subjects compared to non-smoking controls[101]. The COPD group had a mean FEV_1 of 51% predicted and age of 70 years. A further study measured both H_2O_2 and 8-isoprostane in EBC from stable COPD subjects and found increased levels of both

parameters compared to smoking controls[82]. In addition the concentration of H_2O_2 correlated significantly with FEV_1, neutrophil count and dyspnoea score in COPD subjects, however this correlation was not observed with 8-isoprostane. The study included three COPD groups; mild, moderate and severe, in whom H_2O_2 concentration was found to increase with disease severity. Individually mild, moderate and severe subjects were not reported to have significantly higher levels than controls. Finally a study investigating clinical features and their association with oxidative stress, identified increased EBC 8-isoprostane in stable COPD was associated with an increased BODE index, hypoxia and increased dynamic hyperinflation[386].

Collectively these reports provide significant evidence for increased oxidative stress in stable COPD, which worsens with disease severity. However there are several factors which make comparing these studies with the data presented in this chapter problematic; the severity of COPD subjects is similar but control groups typically haven't included non-smoking subjects. Additionally assay methods are different and none of these studies report results in sputum supernatant.

7.19 Antioxidant capacity in COPD

Excessive oxidative stress occurs either when antioxidant capacity is reduced or when redox burden is increased and several studies have identified reduced antioxidants as a possible disease mechanism in COPD. However research in this area is complicated by difficulties in defining a 'normal range' for antioxidant parameters and the considerable number of environmental and genetic interactions which exist, particularly in a dynamic system in which antioxidants respond to oxidant burden. Several studies have also reported increased antioxidant levels in COPD.

Rahman *et al* measured plasma antioxidant capacity using the Trolox equivalent antioxidant capacity (TEAC) assay which equates substrate antioxidant capacity to the vitamin E analogue Trolox. This study identified greater antioxidant levels in the serum of non-smokers compared to non-obstructed smokers and COPD subjects, but there was no difference in levels between these last two groups[387]. Studies in smokers have reported reduced antioxidants in plasma[388] and BAL despite dietary supplementation[389] compared to non-smokers. Additionally an epidemiological study reported reduced lung function in association with decreased dietary intake of antioxidant vitamins C and E[390].

However other studies have discovered increased levels of antioxidants in COPD. Beeh *et al* reported increased concentrations of the antioxidant glutathione in induced sputum samples[379], a finding that was associated with increases in other markers of both oxidative and nitrative stress. A study of antioxidants in skeletal muscle found that biopsy samples contained greater levels of several antioxidants in COPD subjects compared to controls[391].

It has been postulated that low antioxidant capacity in blood reflects excessive oxidant burden, but in sputum the opposite hypothesis has been suggested. Studies in this field contain contradictions or have limitations which indicate that further work is required. Direct comparison with the data presented in this chapter is problematic, particularly as different assays have been used. However where studies have been performed in sputum a reduction of antioxidants has been reported and a trend to this was seen in my COPD subjects prior to RV infection.

7.20 Does RV infection increase oxidative and nitrative stress?

Data presented in this chapter supports the hypothesis that nitrative stress is increased following RV infection and that this is elevated in COPD subjects compared to controls. When all subjects were combined nitrative stress significantly increased on all days post infection. This was greater in COPD subjects compared to controls and was significantly higher by day 15. The delay of two weeks from nasal inoculation is likely to reflect the initial virus incubation period and then the time taken before inflammation develops in the lower airway. The increase from baseline in nitrative stress in COPD subjects was greater compared to controls.

When all subjects were combined the antioxidant levels fell following RV infection, although this was not found to be statistically significant compared to baseline. The fall in antioxidant capacity is likely to occur as a result of RV induced oxidative stress; however the data presented in this chapter cannot refute or confirm this. When the three individual subjects groups were analysed, only the smoking controls had a significant change from baseline at any time point, day 42. The range of inter and intra subject results obtained using the PAO assay was large making detecting significant changes difficult to identify. This may have been due to wide variations in antioxidants levels in the samples tested, or could reflect poor sensitivity or reliability in the assay. Measuring oxidants directly is difficult due to their short lived state and reactive nature; therefore surrogate markers are often used. However the results of

the PAO assay presented in this chapter are likely to have been limited by the small subject numbers, particularly in the COPD group.

The interpretation of the results of the PAO assay following RV infection is complex. The trend in smoking controls is a fall from relatively high baseline levels following RV inoculation. A possible interpretation of this data is that non-obstructed smokers are able to mount an appropriate antioxidant response to chronic cigarette smoke exposure, however following RV infection the additional oxidative stress results in falls of antioxidants. COPD subjects have a trend to lower antioxidant levels at baseline and following RV infection these remain unchanged or fall further until day 21. This may reflect higher levels of oxidative stress in COPD subjects prior to infection, with an additional ROS burden following RV infection, resulting in a further non-significant drop in antioxidants on day 9. At this time point there was significantly higher antioxidant levels in smoking controls compared to COPD subjects. The results of the PAO assay are relatively unchanged following RV infection in non-smoking controls, with no significant changes from baseline.

There are both *in vitro*[279, 280] and *ex vivo*[134] studies which have demonstrated induction of oxidative stress by RV infection. However no longitudinal studies have investigated oxidative stress as a result of RV infection in humans. The data presented in this chapter therefore illustrates novel data.

7.21 Increased nitrative stress in AECOPD

There are reports of increased nitrative stress identified during exacerbations compared to stable state in COPD[144, 381]. The study by Tsoumakidou *et al* recruited 12 COPD subjects and found increased nitrotyrosine positive inflammatory cells in induced sputum samples and work by Karadag *et al* identified serum nitric oxide as a candidate biomarkers in AECOPD.

Increased H_2O_2 has also been reported in EBC in AECOPD[101]. However this study excluded patients with a clinically suspected upper airway infection because of concern over contamination of EBC with H_2O_2 derived from the upper airways. This limits comparisons with the data presented in this chapter, but does provide interesting evidence that acute exacerbations due to aetiologies other than respiratory viruses are associated with increased oxidants. A further study in AECOPD identified an increase in superoxide anion from blood[93].

Finally at least four studies have measured antioxidants during an AECOPD. However none have used the PAO assay or presented longitudinal data similar to that in this chapter. A study measuring markers in serum identified reductions in antioxidants during an AECOPD[93], furthermore this was found to return to normal levels with treatment[392].

A study which collected serum samples from COPD subjects during an exacerbation, with repeat samples during the stable state, was designed to investigate the activity of the antioxidant vitamins A, C and E[393]. Lower levels of vitamin A and E were identified during the acute illness, suggesting that oxidative stress was significantly involved in this. The authors suggest that supplementation with vitamin A and E may be beneficial to the treatment of acute exacerbations.

7.22 Is there an association between oxidative and nitrative stress?

There was a highly significant inverse correlation between the Griess and PAO assay when all subjects were studied. The link between oxidative and nitrative stress was stronger and consistent when smokers and COPD subjects were examined individually but non-smokers did not demonstrate any correlation. This data demonstrate that lower levels of nitrative stress are associated with higher levels of antioxidant in cigarette exposed subjects. It also supports the use of the PAO assay as an indirect measure of oxidative stress. The ratio between the Griess and PAO assay was also helpful in providing additional information in redox stress in the study.

7.23 Is there a correlation between oxidative and nitrative burden and inflammation?

The total non squamous cell count in sputum and IL-8 concentrations were found to correlate strongly with increasing nitrite concentrations, supporting an association between RV infection, nitrative stress and airway inflammation. COPD subjects were found to have a similar strength of correlation to control groups although their absolute markers of inflammation and nitrative stress were elevated compared to controls. A negative correlation was identified between the PAO assay and inflammatory markers in all subjects combined; providing supporting evidence for a role of oxidative stress in inflammation. When subjects were examined individually this pattern was repeated in smokers and COPD subjects with lower PAO levels associated with increased IL-8 but was reversed in non-smokers.

Several published studies have investigated the relationship between oxidative and nitrative stress and modifications to HDACs. There is evidence that inflammation induced by nitrative stress may act though loss of HDAC activity, specifically by nitration of tyrosine residues[377]. An *in vitro* study investigating mechanisms of renal disease, in which nitrative stress was measured using the Griess assay, identified a relationship between HDAC2 and activation of the iNOS gene[394]. The data presented in this chapter identified a significant negative correlation between HDAC2 activity and nitrite concentrations in COPD subjects only. This data suggests that this relationship is involved in the excess inflammation and symptoms identified in the RV induced COPD exacerbation. However the data presented here does not provide information of the mechanisms involved in this process, it cannot be determined if nitrative stress in impairing HDAC2 activity or if falls in HDAC2 activity are inducing nitrative stress.

A study by Rhaman *et al* illustrated a mechanism of oxidative induced inflammation which was exaggerated by inhibition of HDAC enzymes, illustrating a possible mechanism for exaggerated inflammation induced by oxidative stress in COPD (where HDACs have been shown to be deficient)[360]. Other studies have also demonstrated that oxidative stress reduces HDAC and this results in an increases in IL-8 levels[395]. Finally a possible mechanism for this was proposed by Marwick *et al*, in this study it was shown that oxidative modifications are responsible for the posttranslational destruction of HDAC2[353, 354].

7.24 Correlation of oxidative and nitrative stress with HDAC2 activity

There was found to be a significant negative correlation between HDAC2 activity and nitrite concentration only in the COPD subjects. There was no association demonstrated between the PAO assay and HDAC2 activity in any of the study groups.

It is not possible to identify a causative relationship for the correlation between nitrite and HDAC2 activity from the data presented in this chapter. However it is interesting to note that only in the COPD group are the two parameters significantly related. It has been demonstrated *in vitro*, that oxidative and nitrative stress may be responsible for inactivating HDAC enzymes[126, 395]. It is therefore postulated that increased nitrative stress is responsible for the reduction of HDAC2 activity demonstrated by this correlation. This supports the central hypothesis of the work presented in this chapter and provides a possible mechanism for the increased

inflammatory cytokines seen with increases in nitrite. This is the first study to identify a correlation in inflammation and nitrative stress *in vivo* following a RV induced COPD exacerbation. It was anticipated that increases in oxidative stress would be associated with reductions in HDAC2 activity. It is not known if there is a direct relationship between antioxidants and HDAC activity; no published studies have specifically addressed this question. However the data presented in this chapter did not demonstrate any correlation between antioxidants and HDAC2 activity. The reasons for this are not known. The PAO assay is not a direct measurement of oxidative stress and so it is not possible to comment on the role of oxidants specifically, but oxidative changes are mirrored *in vivo* by those in nitrative stress, suggesting that the correlation between the Griess assay and HDAC2 activity may be mirrored by oxidants. It could be postulated that changes in antioxidants are too slow to respond to RV infection and so are not detected in the two to three week period following infection when the changes in HDAC2 activity are most dynamic. Additionally if there is a lag period between changes in antioxidants and oxidative stress, then the former measure will not demonstrate a correlation with HDAC activity in corresponding sputum samples. An alternative method of analysing this data would therefore be to relate samples collected a different time points, for example day 5 HDAC activities with day 9 antioxidants or peak changes in each mediator. However this has not been performed on the data from this chapter as the changes from baseline in the PAO assay were not significant following RV infection.

In summary there are no published longitudinal studies in AECOPD which have examined HDAC activity with oxidative and nitrative stress. Therefore the data presented in this chapter is novel and interesting. The increase in nitrite in the COPD group following RV infection and the correlation between this and reduced HDAC2 activity and increased cytokine concentrations supports the hypothesis that this is a mechanism which induces inflammation specifically in an exacerbation.

7.25 Conclusion

The data shown has provided evidence of increases in oxidative and nitrative stress following RV infection and that these are exaggerated in COPD subjects compared to controls. There are also strong correlations between these and clinically relevant markers of inflammation. However although interesting trends have been identified many results were not significant. The wide range of variation in assay results and the small sample size are likely to be the major factors responsible. Further work with an increased sample size and possibly the use of more sensitive oxidative stress assays are likely to be helpful.

Chapter 8: Summary of results and future work

8.1 Introduction

This chapter includes a summary of the results presented and discussed in each of the proceeding results chapters and a comparison of these with the stated hypothesis for the thesis. Following this is a brief discussion of proposed future work which has arisen from the findings presented in this thesis.

8.2 Clinical findings

The results of clinical data presented in chapter 3 demonstrated that COPD subjects had greater symptomatic and spirometric changes when compared to controls and that these findings were consistent with those identified during naturally occurring exacerbations. Specifically the LRT symptoms scores were higher and took longer to return to baseline in COPD subjects, who were the only group in the study to have a significant fall in FEV_1 when compared to baseline following RV infection, therefore this data supports the hypothesis that experimental RV16 infection leads to features consistent with naturally occurring exacerbations.

This was the first RV challenge study in COPD subjects to include non-smoking controls. It was hypothesised that this group would experience the least LRT symptoms and changes in lung function compared to smoking controls and COPD subjects following experimental inoculation. The results showed in non-smoking controls there was a minimal reduction in FEV_1 compared to the two other study groups. In addition to this, non-smoking controls demonstrated lower LRT symptom scores compared to the COPD and smoking controls following RV infection. My findings are thus consistent with this hypothesis.

8.3 Virus load

Following RV infection there were increases in the virus load from nasal lavage, sputum and BAL samples in all successfully infected subjects. There was no difference in the virus load in nasal lavage between the three study groups, but in the sputum of COPD subjects it was determined that on days 12 and 15 to be elevated compared to the control groups. I have shown that the URT symptom scores correlated with virus load in nasal lavage and LRT symptom scores with sputum virus load in all infected subjects supporting the clinical findings described above. I have also found that there was an increased rate of bacteria detection following RV

infection. This was more common in COPD subjects where pathogenic bacteria were frequently isolated compared to controls.

8.4 Pro-inflammatory cytokines and chemokines

Following RV infection sputum inflammatory mediators were found to be increased in all subjects. There were higher levels in the COPD group compared to non-smoking and smoking controls for several of the mediators measured. In BAL the concentrations were higher in smoking controls whilst the COPD group were more likely to have had significant increases from baseline after RV infection. Interestingly correlations with symptoms and inflammatory mediators were only found in COPD subjects. This data demonstrated that the exaggerated inflammatory response following RV infection is only associated with increased symptoms in COPD subjects.

8.5 HDAC2 activity

The central aim of this study was to examine the role of HDAC2 activity in COPD and control subjects following RV infection. I have shown at baseline that there was no difference in HDAC2 activity between the three study groups. HDAC2 activity in sputum and BAL macrophages was found to fall only in COPD subjects following RV infection. In non-smoking control subjects there was an increase in sputum HDAC2 activity following RV infection, with little change in smoking controls. Only in the COPD subjects was there a significant negative correlation between HDAC2 activity and acute mediators of inflammation. This data demonstrates that HDAC2 activity is reduced following RV infection only in COPD subjects and supports the hypothesis that HDAC2 deficiency plays a central role in RV induced exacerbations.

8.6 Oxidative and nitrative stress

Measurements of nitrative stress were shown to increase following RV infection and there was evidence of a trend to reduced antioxidant capacity. Nitrite concentration was higher in COPD subjects at time points following RV infection and had greater increases from baseline compared to the control groups.. The results of the PAO assay suggested antioxidant capacity fell in COPD subjects compared to smoking controls. The Griess and PAO ratio as a surrogate marker of redox stress was elevated in COPD subjects compared to controls reflecting an overall increase in redox stress exclusive to COPD subjects. There was no difference between the groups at baseline, demonstrating that these changes in oxidative stress occurred as a result of RV infection.

My results have shown that mediators of inflammation correlated with nitrative stress. Reduced HDAC2 activity in COPD subjects was also negatively associated with markers of nitrative stress. These findings substantiate the hypothesis of my study.

8.7 Conclusion

My data supports the previous work performed with RV challenge in COPD, but it is strengthened significantly by the addition of a non-smoking control group. Many parameters have been measured in this work which demonstrates increased symptoms, inflammation and markers of disease burden in COPD subjects which are consistent with an exacerbation and were not identified in controls.

The incorporation of a non-smoking control group has enabled identification of modest increase in symptoms and airway inflammation compared to smoking controls. Cigarette smoke exposure is known to increase oxidative stress and airway inflammation. Although the smoking control group in this study have normal spirometry the extent of their future risk of developing COPD is unknown. It is possible that smoking subjects with a propensity to developing COPD also display features of airway obstruction following RV infection. In addition absolute falls in some measures of lung function were greatest in the smoking control subjects.

The overall conclusions from this work are that the results presented here support the study hypothesis. COPD subjects experienced features of an exacerbation, higher virus loads and greater airway inflammation compared to controls. This was associated with an excess of redox stress and falls in HDAC2 activity. Although specific mechanisms have not been studied, all of these parameters correlated more strongly in COPD subjects and were temporally augmented following RV infection.

Future work

Further assessment in this study group

8.8 Oxidative and nitrative stress

Further measurements of oxidative and nitrative stress could be performed to strengthen the findings of the assays described here. Specifically this would include measurement of 8-isoprostane in EBC, sputum and BAL supernatant. Assessment of protein damage due to oxidative stress, for example assessment of reactive carbonyl groups by mass spectrometry, would also provide further evidence of the role of oxidants in the pathogenesis of exacerbations. Measurement of samples

already collected and stored using the TBARS and TEAC assays may also prove interesting.

8.9 Antioxidants

Recent interest in the systemic manifestation of COPD has led to recognition of malnutrition and weight loss, predominantly due to reduced muscle mass. This relationship reflects a complex interaction of increased energy demand due to elevated metabolic rate and work of respiratory muscles, alongside a decreased intake of energy due to breathlessness and depression. Poor nutritional status may impact disease progression. One area of particular interest is vitamin B6 metabolism. Interest in COPD has been stimulated by the link between B6 status and cardiovascular disease. A recent study compared vitamin B6 status in a group of COPD outpatients to non-obstructed controls and found plasma levels of vitamin B6 to be significantly reduced in the COPD group[396]. This provides evidence for future work in this field both to assess their role as antioxidants and co-factors for effective immune function.

8.10 HDAC studies

Measurement of HDAC2 and other HDAC isoenzymes gene expression in mRNA from stored macrophages pellets would also support the data presently obtained. In addition immunohistochemistry staining for HDAC could be performed in bronchial biopsies, although this will not provide evidence of changes in HDACs functional activity.

8.11 Future RV challenge studies

Further RV challenge studies should be performed with several aims. Firstly to increase the numbers of successfully infected COPD subjects, as although this study demonstrates interesting results these could be statistically strengthen by increasing the subject numbers. Secondly future studies should include an ex-smoking non-obstructed control group to investigate the cofounding feature of active cigarette smoking. Although not specifically documented in the inclusion and exclusion criteria of my study, all subjects in the smoking control group and the majority of COPD subjects were active smokers during participation in the study. The extent to which results are due to active smoking during the study cannot be investigated without this additional control group in future studies.

Detailed studies of bacteria should be included in any future RV challenge study as this relationship with RV in exacerbations is important. These would include molecular phenotyping of samples at baseline in order to identify acquisition of new strains during an experimental exacerbation or overgrowth of a previous commensal species. Some work on immune mediators has already been performed in sputum supernatant from this study, and investigations of defensins and similar molecules may prove helpful. Additionally RV challenge could be performed with the addition of prophylactic antibiotics and using biomarkers of bacterial infection, including procalcitonin guided treatment[319].

Given the huge global disease burden resulting from COPD it is important that excellent future clinical studies are performed in collaboration with translational research which leads to greater understanding of disease mechanisms and potential new therapies.

Chapter 9: Appendix

9.1 List of abbreviations used in this thesis

6MWT	Six minute walk test
α1-AT	Alpha-1 antitrypsin
AECOPD	Acute exacerbation of COPD
AM	Alveolar macrophages
AM3	Immunomodulatory research drug (see reference 161)
ANOVA	Analysis of variance analysis
ARTP	Association for Respiratory Technology and Physiology
ATS	American Thoracic Society
AUC	Area under curve
BAL	Bronchoalveolar lavage
BCA	Bicinchoninic acid assay
BEAS2B	Bronchial epithelium and adenovirus SV40 cell line
BLF	British Lung Foundation
BMI	Body mass index
BODE	Body mass index, airflow obstruction, dyspnoea and exercise capacity score
BTS	British Thoracic Society
CC	Chemokine Cysteine-Cysteine (beta) chemokine
CCR2	CC motif receptor 2
CD4+	T helper cells
CD8+	Cytotoxic T cells
CFU	Colony forming units
ChIP	Chromatin immunoprecipitation
CLED agar	Cystine lactose electrolyte deficient medium
CMV	Cytomegalovirus
CO_2	Carbon dioxide
COPD	Chronic Obstructive Pulmonary Disease
CPE	Cytopathic effect
CRP	C reactive protein
CSE	Cigarette smoke extract
CT	Computed tomography scan
Ct	Cycle number
Cu^+	Copper (1) ion

Cu^{++}	Cupric ion
DLCO	Diffusing capacity
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsRNA	Double stranded ribonucleic acid
DTT	Dithiothreitol
DUOX	Dual oxidase enzymes
EBC	Exhaled breath condensate
ECP	Eosinophilic cationic protein
EDN	Eosinophil derived neurotoxin
ELISA	Enzyme-linked immunosorbent assay
ENA-78	Epithelial neutrophil activating protein 78
EPO	Eosinophil peroxidase
ERS	European Respiratory Society
FBC	Full blood count
FCS	Fetal calf serum
FEV_1	Forced expiratory volume in 1 second
FEV_1/FVC	Forced expiratory volume in 1 second and forced vital capacity ratio
FOB	Fibre optic bronchoscopy
FRC	Functional residual capacity
FRV	Filtered rhinovirus
FVC	Forced vital capacity
GM-CSF	Granulocyte macrophage-colony stimulating factor
GOLD	Global initiative for chronic obstructive lung disease
GRO-α	Growth related oncogene alpha
GSH	Glutathione
H_2O_2	Hydrogen peroxide
H_3PO_4	Phosphoric acid
HAT	Histone acetyltransferase
HDAC	Histone deacetylase enzymes
HDAC2	Histone deacetylase enzyme 2
HDACi	Histone deacetylase enzyme inhibitor
HeLa	Henrietta Lacks cell line
HIV	Human immunodeficiency virus
HMGB1	High mobility group box 1 protein
HOCl	Hypochlorous acid

ICAM	Intercellular adhesion molecule 1
IFN-γ	Interferon gamma
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-1β	Interleukin 1 beta
IL-2	Interleukin 2
IL-27	Interleukin 27
IL-6	Interleukin 6
IL-8	Interleukin 8
iNOS	Inducible nitric oxide synthase
IP	Immunoprecipitation
IP-10	Interferon gamma-induced protein 10
IRF	Interferon regulatory transcription factor
IS	Induced sputum
KCO	Lung diffusion capacity corrected for alveolar ventilation
LABA	Long acting beta agonist
LPS	Lipopolysaccharide
LRT	Lower respiratory tract
LTB_4	Leukotriene B4
MAPK	Mitogen-activated protein kinase
MBP	Major basic protein
MCP-1	Monocyte chemotactic protein 1
MCP-4	Monocyte chemotactic protein 4
MDC	Macrophage derived chemokine
MDI	Metered dose inhaler
MDM	Monocyte derived macrophages
MIP-1α	Macrophage inflammatory protein alpha
MIP-1β	Macrophage inflammatory protein beta
MMP-9	Matrix metalloproteinase-9
mRNA	Messenger ribonucleic acid
MSD	Meso scale discovery platform®
MUC5AC	Mucin 5AC gene/protein
MUC5B	Mucin 5B gene/protein
NAC	N-acetylcysteine
NADPH	Nicotinamide adenine dinucleotide phosphate oxidase
NE	Neutrophil elastase

NFκB	Nuclear factor kappa light chain enhancer of activated B cells
NHS	National Health Service
NK	Natural killer cells
NL	Nasal lavage
NO	Nitric oxide
NOS	Nitric oxide synthase
NS	Non smoker
O_2^-	Superoxide
OH^-	Hydroxyl
$O\text{-}O^{2-}$	Peroxide
PAMP	Pathogen-associated molecular patterns
PAO	Potential of antioxidant assay
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDI	Phosphodiesterase 4 inhibitor
PEF	Peak expiratory flow
PEFR	Peak expiratory flow rate
PHA	Phytohaemagglutinin
PiZZ	Homozygous deficiency of α1-AT
PKC	Protein kinase C
PU.1	Transcription factor PU.1
PYH	Pack year history
qPCR	Quantitative PCR
r	Pearson's correlation coefficient
RANTES	Regulated upon activation, normal T cell expressed and secreted
ResV	Residual volume
RIPA	Radioimmunoprecipitation assay
RLT	Ribonucleic acid lysis buffer
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPM	Revolutions per minute
rs	Spearman's correlation coefficient

RSV	Respiratory syncytial virus
RT	Room temperature
RV	Rhinovirus
RV16	Rhinovirus 16
SEM	Standard error of mean
SIRT1	Silent information regulators (sirtuins) (1)
Smk	Smoker
SOB	Shortness of breath
SOD	Super oxide dismutase
SpO_2	Peripheral oxygen saturation
TARC	Thymus and activation regulated chemokine
TBARS	Thiobarbituric acid reactive substances
TCC	Total cell count
$TCID_{50}$	Tissue infective dose 50%
TEAC	Trolox equivalent antioxidant capacity
Th1	T-helper cell type 1
Th2	T-helper cell type 2
TIMP	Tissue inhibitors of matrix metalloproteinases
TLC	Total lung capacity
TNF-α	Tumour necrosis factor alpha
TSA	Trichostatin-a
UK	United Kingdom
URT	Upper respiratory tract
USA	United States of America
V/Q	Ventilation perfusion
VEGF	Vascular endothelial growth factor
WHO	World Health Organisation

9.2 Patient information sheet

PARTICIPANT INFORMATION SHEET

MECHANISM OF EXACERBATION IN COPD FOLLOWING VIRAL INFECTION

We are inviting you to take part in a research study. We have put together some information here to tell you more about the research; why we are doing in and what is involved. You may take as much time as you would like to decide if you would like to take part in this study, please consider the information carefully and discuss with other people if you wish.

WHAT IS THE PURPOSE OF THE STUDY?

This research study is designed to find out about inflammation in the lungs after an infection with the common cold virus, called the human rhinovirus.

We want to try to find out why there is a different response to infection in normal healthy people, those who smoke and those with chronic bronchitis and emphysema (called Chronic Obstructive Pulmonary Disease – COPD).

It is not known why infection makes people with COPD more breathless and we aim to see if better treatments for this condition can be developed. There are many millions of people in the world who suffer from COPD. However there will not be an immediate benefit to your health from taking part in this study.

WHY HAVE I BEEN INVITED and DO I HAVE TO TAKE PART?

We would like to study a wide range of individuals. This means involving both people who smoke and those who do not. We also want to study people with and without lung disease. You do not have to take part in the study if you do not want to and can withdraw at any time.

WHAT WILL HAPPEN IF I TAKE PART?

The study will involve attending St Mary's Hospital for a screening visit, involving a brief medical examination and questions to find out if you are suitable to take part. You should not have had a cold in the last six weeks. We will perform some routine breathing tests, a skin test for allergy and a blood test. These are all done in the Outpatient Clinic. If these tests show no evidence of infection, or any other problems with your health, you can continue to the main part of the study. We ask you to have a Chest X-ray and an ECG – heart tracing test. No-one in previous studies like this developed any adverse events, but we will monitor you closely to ensure that any serious adverse events are detected. We will arrange for you to have prompt treatment at St Mary's Hospital in the unlikely event that this did occur. In the previous study around half of people with COPD developed mild bacterial bronchitis but they all improved with no additional treatment. The serious adverse events which are monitored include difficulty in breathing, wheeze, change in breathing tests or if you or the study doctor think that treatment is required.

MAIN STUDY

For the main part of the study you will need to attend the hospital ten times. On the second visit we will perform more breathing tests, washings from your nose and a sputum test. These are all common tests in a Chest Clinic. On the next visit we will perform a bronchoscopy; this is a telescope test into the lungs. This is repeated a second time when you come back to the hospital in 21 days. There are further details about bronchoscopy below.

On the visit following the first bronchoscopy (Day -14) we will infect volunteers with the human rhinovirus, common cold, to measure how this affects the lungs. Although this may sound alarming it has been done at St Mary's in other volunteers who have both asthma and COPD. You are given the cold by breathing in a fine mist containing the virus. We perform the sputum & breathing tests, nose washings and blood tests on subsequent visits. Specifically blood is taken on days 0, 7, 9, 12, 15 and 42. On each occasion about 15mls (three teaspoons) of blood are taken. Timings of each test are on the timetable given to volunteers when they join the main study.

WHERE DID THE VIRUS COME FROM?

The virus has been used in many previous studies, where it has been shown to be safe. It was prepared especially for research and has been stored carefully in our laboratory since that time. Unlike medicines there are no special rules for preparing viruses in this country, but it was made by experts following previously established procedures.

Typical symptoms of the common cold include a sore throat and runny nose which usually lasts for 3-4 days. Those with COPD may also notice a worsening of usual symptoms with increased sputum production, cough and possible bronchitis and wheeze. Symptoms may last for up to five weeks, but this does not occur in everyone.

We then ask you to return to the hospital for frequent visits. These are so we can sample your sputum, blood, washings from your nose, breath and breathing tests. We will not do every test on every visit. The fourth and consecutive visits will be three days later, five days, seven days, nine days, twelve days, fifteen days, twenty-one days and a final visit forty-two days later. Each visit will last about an hour. Although this may sound like a lot of trips to the hospital we will make sure you do not have to wait or make more trips than necessary. To collect your sputum we will ask you to inhale salty water, this is done with a nebuliser machine which makes a fine mist for you to breath. Rarely this can cause narrowing of the airways, but this will be monitored closely. We will telephone you frequently during the study to answer any questions and to follow your progress.

WHAT IS A BRONCHOSCOPY?

This is a test performed frequently in chest departments. It is usually done as an Outpatient and involves passing a small telescope tube (about the width of a biro) into the lungs. There is no need for a general anaesthetic but we use mild sedation and local anaesthetic. You are able to breath normally around the tube. This enables us to look into the lungs and take samples. We wash the airways and take small samples of cells and tissue. Specifically we collect six small tissue samples (biopsies) using forceps, four sets of cell samples using a brush and finally lung washings using up to 8 tubes of sterile salty water These are 30ml

tubes (about the size of a perfume bottle) and the water is sucked back into the telescope. These are all routine parts of many bronchoscopy tests.

You will need to come to the Endoscopy Unit with an empty stomach. We ask you to have nothing to eat for six hours before the test. We will put a small needle in your arm to give you medications. The test usually takes about twenty minutes. We ask you to stay in the department until the sedation has worn off. HOWEVER YOU SHOULD NOT DRIVE A CAR AFTER SEDATION. The test is performed with nursing staff and another research doctor or nurse present.

ARE THERE ANY SIDE EFFECTS FROM BRONCHOSCOPY?

There may be some discomfort during the test but this is reduced with local anaesthetic. The test may make you cough. This is a safe procedure but on rare occasions the airways can narrow, if this occurs medication can be given to open the airways. Some people have mild fever, and rarely infection, after the test but this can usually be treated with paracetamol, other treatments are not normally required. Occasionally after taking the tissue sample (biopsy) people can cough up small amounts of blood, this stops without further treatment. The test can be stopped at any time if you wish. In the unlikely event that you experienced any problems, again we would arrange for you to have prompt and appropriate treatment at St Mary's Hospital.

WHAT IF SOMETHING GOES WRONG?

Imperial College London holds insurance policies which apply to this study. If you experience harm or injury as a result of taking part in this study, you may be eligible to claim compensation without having to prove that Imperial College is at fault. This does not affect your legal rights to seek compensation.

If you are harmed due to someone's negligence, then you may have grounds for a legal action. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been treated during the course of this study then you should immediately inform the Investigator Professor Sebastian Johnston or Dr Joseph Footitt. The normal National Health Service complaint complaints mechanisms are also available to you. If you are still not satisfied with the response, you may contact the Imperial College Clinical Research Office.

AM I PAID FOR TAKING PART IN THIS STUDY?

We do not pay people specifically for taking part in the study. However we will cover any costs for your travel and make a donation to you to compensate you for your time and inconvenience while involved in the study.

IS EVERYTHING KEPT CONFIDENTIAL?

Yes. No personal information is taken out of the hospital. We use a code in the laboratory so you cannot be identified. We will write to your GP to let them know that you are taking part

in the study. The anonymous samples obtained in the study will be stored in a freezer in our laboratory.

CAN I WITHDRAW PERMSSION TO TAKE PART IN THIS STUDY?

Your participation in this study is voluntary. You are free to withdraw at any time and do not have to give a reason for this, even after you have agreed to take part. Being part of this study will not affect your normal medical care, either now or in the future.

The investigator may also withdraw you from the study at any time if considered appropriate. The investigator will have access to your hospital records but your confidentiality will be maintained. All records from the study are stored in the strictest confidence. Results from the study may be published in medical journals but you will not be identified. We will inform your GP that you have taken part in this study. Samples will be stored anonymously for future research, you can ask for these to be destroyed at any time in the next 10 years.

FURTHER INFORMATION

If you require further information about the study, please contact:

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Or

Professor Sebastian Johnston
Professor Respiratory Medicine

Telephone: 020 7594 3764
Email: s.johnston@imperial.ac.uk

This study has been approved by the St Mary's Research Ethics Committee (REC Ref 07/H0712/138)

9.3 REC confirmation letter

NHS

National Research Ethics Service

St Mary's REC
Royal Free Hospital
Research Ethics Committees
Pond Street
London
NW3 2QG

Telephone: 0207 794 0635

20 February 2008

Professor Sebastian Johnston
Professor of Respiratory Medicine
National Heart and Lung Institute
Wright Fleming Institute of Infection & Immunity &
Imperial College London
Norfolk Place, London
W2 1PG

Dear Professor Johnston

Full title of study: **Role of oxidative and nitrative stress and histone de-acetylation in rhinovirus induced acute exacerbations of Chronic Pulmonary Disease (COPD)**

REC reference number: **07/H0712/138**

Thank you for your letter of 24 January 2008, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information was considered at the meeting of the Sub-Committee of the REC held on 08 February 2008. Your project was reviewed by the Chairman, Barrie Newton and Dr Graham Taylor.

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

Please note the following comment:

The investigators attention is drawn to the following: The patient information describes a single biopsy and washings from the lungs during the bronchoscopy. Thus approval has been given only for these procedures. Should the investigators wish to take SIX bronchial biopsies, FOUR bronchial brushings and up to 8 x 30ml lavages this should be specifically stated in the patient information sheet (ideally with a brief description of what each entails for the patient) then the patient information sheet will need to be changed and the new version (along with amended consent form which will need to refer to the correct date and version number of the PIS) submitted formally as an amendment.

Ethical review of research sites
The favourable opinion applies to the research sites listed on the attached form.

Conditions of approval
The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

BN.

9.4 Telephone participant screening sheet

Telephone Participant Screening Sheet

Date: Address:

Name:

Age (40-75 years):

DoB:

Contact Number:

Smoking History (Yes - over 20pyh):

Treatments:

Illnesses: GP:

Allergies:

Suitable for screening visit: YES NO

Screening Visit Arranged:

Map & Reminder:

Chapter 10: Thesis references

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