Determinants of Exacerbation Frequency in COPD

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I, Jennifer Kathleen Quint, confirm that the work presented in this thesis is my own. Where information has been derived form other sources, I confirm that this has been indicated in the thesis.

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I always promised myself this would be the last page I would write; having been over it so many times in my head, I can hardly believe this is finally coming to an end. This has been an enormous project, and without the help of those listed below, arguably unachievable.

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Abstract

Background: Chronic obstructive pulmonary disease (COPD) is a heterogeneous collection of conditions characterized by irreversible expiratory airflow limitation. The disease is interspersed with exacerbations; periods of acute symptomatic, physiological and functional deterioration. There are large differences in yearly exacerbation incidence rates between patients of similar COPD severity giving rise to the concept of two distinct phenotypes; frequent and infrequent exacerbators. This thesis hypothesizes that frequent exacerbators are a distinct phenotype of COPD, and identifies some of the factors that influence exacerbation frequency.

Method: 356 individuals from the London COPD cohort were included in the analyses in different subgroups. All patients completed daily diary cards and reported exacerbations to the study team for sampling and treatment. Blood and sputum were collected in the stable state and at exacerbation. Samples were processed for cytokines, genetic polymorphisms and viruses. A subset of patients also had endobronchial biopsies for epithelial cell work and immunohistochemistry.

Results: Patient reported exacerbation frequency can be used to accurately stratify patients into frequent and infrequent exacerbators groups in subsequent years. Frequent exacerbators were more depressed and more likely to be female then infrequent exacerbators. There was no difference in social contacts, HRV positivity or load in sputum, Vitamin D levels, or cytokine variability between frequent and infrequent exacerbators. No differences in genetic polymorphisms (ICAM-1, IL-6, IL-8, VDR, Taq1 α1 –antitrypsin) were identified between the two groups.

Conclusions: The frequent exacerbator phenotype exists. There is not one single determinant of exacerbation frequency, and determinants vary with underlying disease severity.

Abbreviations

1,25(OH)2D3	1,25-dihydroxycholecalciferol
α1AT	alpha one antitrypsin
A	adenine
A&E	Accident and Emergency
Ab	antibody
ANOVA	analysis of variance
APHEA	Air Pollution and Health: a European Approach
AU	arbitrary units
BMI	body mass index
bp	base pairs
C	cysteine
°C	degrees Celsius
Ca2+	calcium ions
CD	cluster of differentiation
cDNA	complementary DNA
CES-D	Center for Epidemiological Studies Depression Scale
CI	confidence interval
CO_2	carbon dioxide
COPD	Chronic obstructive pulmonary disease
CPE	cytopathic effect
CRP	C-reactive protein
CXCR	Chemokine, CXC Motif, Receptor
D	vitamin D
D3	vitamin D3
DAB	3,3'-Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DLCO	Diffusing Capacity of the Lung for Carbon Monoxide
DMSO	Dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DPBS	Dulbecco's phosphate buffered saline
DPX	1,3-diethyl-8-phenylxanthine

DSM	diagnostic and statistical manual of mental disorders
E	glutamic acid
EDTA	ethylenediaminetetraacetic acid
EEO	electroendosmosis
ELISA	Enzyme-Linked ImmunoSorbent Assay
ETT	endotracheal tube
FBS	foetal bovine serum
FEV_1	Forced expiratory volume in 1 second
Flu	influenza
FVC	Forced vital capacity
G	guanine
GM	growth medium
GOLD	global initiative for obstructive lung disease
GST	glutathione S-transferase
GWAS	Genome-wide association studies
H&E	hematoxylin and eosin stain
H_20_2	hydrogen peroxide
HADS	hospital anxiety and depression scale
HCU	health care utilisation
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HHIP	hedgehog-interacting protein
HLA	human leukocyte antigen
HMOX-1	heme oxygenase (decycling) 1
hMPV	human metapneumovirus
HRP	horseradish peroxidase
HRV	human rhinovirus
HWE	Hardy-Weinberg equilibrium
ICAM-1	Inter-Cellular Adhesion Molecule 1
ICS	inhaled corticosteroids
IFN-γ	Interferon gamma
Ig	immunoglobulin
IHC	immunohistochemistry
II	index of individuality
IL	interleukin

IP-10	Interferon gamma inducible protein 10
IQR	interquartile range
IRR	inter-rater reliability
ISOLDE	inhaled steroids in obstructive lung disease trial
I-TAC	Interferon-inducible T-cell alpha chemoattractant
ITU	intensive therapy unit
K	lysine
LABA	long-acting β-agonists
LTB	Leukotriene
LVRS	lung volume reduction surgery
M	molar
MEM	Minimum Essential Medium Eagle
mEPHX	Microsomal epoxide hydrolase
mg	milligrams
Mg2+	magnesium ions
$MgCl_2$	magnesium chloride
MIG	Monokine Induced by Interferon-gamma
ml	millilitre
MM	maintenance medium
MMP	Matrix Metalloproteinase
MOI	multiplicity of infection
MRC	medical research council
Na	sodium
NDS	normal donkey serum
NEAA	non-essential amino acid solution
NETT	National Emphysema Treatment Trial
NF- κB	nuclear factor κB
ng	nanograms
NH4+	ammonia
Ni	nickel
NICE	national institute for clinical excellence
NIV	non-invasive ventilation
NK	Natural Killer
nm	nanometres

NTC	no template controls
OH	hydroxyl
P/S	Penicillin / Streptomycin
PBS	phosphate buffered saline
PBST	PBS with TritonX
PCA	principle component analysis
PCR	polymerase chain reaction
PDV	Phocine Distemper Virus type I
PEF	peak expiratory flow
PFA	paraformaldehyde
pfu/ml	plaque forming units per millilitre
pg	pictograms
PM_{10}	Particulate matter
PPM	potentially pathogenic microorganism
PPMM	primer probe master mix
qRT-PCR	quantitative RT-PCR
RBC	red blood cell
RE	restriction enzyme
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
ROS	reactive oxygen species
RSV	respiratory syncytial virus
RT	reverse transcriptase
SD	standard deviation
SEM	standard error of the mean
sICAM-1	soluble ICAM-1
SGRQ	St. George's Respiratory Questionnaire
SNPs	single nucleotide polymorphisms
T	threonine
TAE	Tris Acetate EDTA
Taq	Taq polymerase
TB	tuberculosis
TBS	Tris Buffered Saline
TE	TrisEDTA

T_H	T helper
TIMP	Tissue Inhibitor of Metalloproteinases
TNF-α	Tumour necrosis factor alpha
TORCH	Towards a Revolution in COPD Health
UK	United Kingdom
UV	ultraviolet
V	voltage
VDBP	vitamin D binding protein
VDR	vitamin D receptor
WBC	white blood cell
xg	times gravity

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Introduction

"Next to avoiding a fatal issue, our efforts must be directed to prevent the case going on to chronic bronchitis, especially in those who have had previous attacks"
R Douglas Powell, London 1878

1.1 Chronic Obstructive Pulmonary Disease

Chronic obstructive pulmonary disease (COPD) is a complex, heterogeneous collection of conditions characterised by irreversible expiratory airflow limitation. The disease involves a multifaceted progressive inflammatory process leading to the development of mucus hyper-secretion, tissue destruction and disruption to the normal repair and defence mechanisms. The result is increased resistance to airflow in small conducting airways, change in lung compliance, and the premature collapse of airways during expiration that leads to air trapping. Characteristically, the airflow limitation is progressive and associated with an abnormal inflammatory response of the lung to noxious particles or gases (GOLD 2006).

COPD is both preventable and treatable, and is currently the 4th leading cause of death in the United States and Europe (GOLD 2006). Recorded deaths have risen in COPD by 14% in men and 185% in women from 1980 to 2000 (Pauwels 2001) and will continue to rise. Understanding the pathogenesis of this disease is paramount, particularly in our ageing society, if we are to prevent it from becoming an even greater health problem worldwide.

Due to the heterogenic nature of COPD, the concept of multiple underlying phenotypes has arisen. Thinking of COPD as a collection of diseases and trying to understand more about the individual phenotypes may better our monitoring and treatment of this disease. To appreciate the need for identifying these various phenotypes, first understanding the aetiology and pathogenesis of COPD is essential.

1.1.1 Aetiology

The concept of COPD as a disease has been around for centuries and the debate on its aetiology dominated by 3 main theories: the British hypothesis, the Dutch hypothesis and the protease - antiprotease theory (Fletcher 1976, Speizer and Tager 1979; Burrows 1990; Vestbo 1998). The "British hypothesis" proposed that infections, themselves strongly related to mucus hypersecretion, are a risk factor for impaired airway function. This hypothesis originates from the observation clinically that infectious exacerbations of COPD lead to disease progression (Reid 1958, Medical Research Council 1965, Tager 1975).

In 1961, during the first Bronchitis Symposium in the Netherlands, Orie and colleagues hypothesized that the various forms of airway obstruction; asthma, chronic bronchitis and emphysema, should be considered as different expressions of one disease entity (Orie 1961), with endogenous (*host*) and exogenous (*environmental*) factors playing roles in pathogenesis. This hypothesis was later termed the "Dutch hypothesis" by Fletcher (Orie 1970).

Broadly speaking, the British hypothesis can be thought of as an *environmental* hypothesis and the Dutch a *genetic* hypothesis. The protease- antiprotease theory is a combination of the two (Janoff 1988); arising from the observation of the genetic defect in the circulating levels of alpha-1 antitrypsin in emphysema and the observation that smoking increases the number of circulating elastase containing neutrophils (Gadek 1981, Kueppers 1974, Kueppers 1978, Morse 1978, Lieberman 1976). The British hypothesis was subsequently refuted when Fletcher (Fletcher 1976) and others (Howard 1967, Johnston 1976) did not find a relationship between bronchial infections and FEV_1 decline. Consequently the concept of two distinct diseases has emerged; that of chronic bronchitis and emphysema. However, as with many chronic diseases, these two spectrums rarely exist independently and often a

degree of overlap is observed. Today, with an increased understanding of the causation and pathogenesis of COPD, even more phenotypes have been proposed.

1.1.2 Causation

The causes of COPD are multifactoral. Undoubtedly the commonest risk factor in the developed world is cigarette smoke (Fletcher 1976) but other *environmental* factors causing COPD include air pollution, exposure to biomass fuel, poverty, nutritional factors and infection (Anthonisen 2005). There is now considerable epidemiological evidence supporting the relationship between increased levels of particulate air pollution and increased morbidity and mortality from respiratory diseases (Pope 1999). There is even a strong relationship between PM_{10} and exacerbations of chronic obstructive pulmonary disease (COPD) (Pope 2000). Biomass fuels which are extensively used for cooking and home heating in developing countries have adverse health effects (Viegi 2004). A significant fraction of COPD in these countries could be attributed to biomass (wood) burned in open stoves for cooking (and heating in the colder, higher altitudes) (Caballero 2008, Menezes 2005, Ramirez-Venegas 2006). There is a direct relationship between socioeconomic status and COPD (Prescott 1999). Lower socioeconomic status has adverse effects on healthcare; these individuals are more likely to smoke, be exposed to indoor (Dasgupta 2006) and outdoor pollutants, and have poorer nutritional status.

Pathological factors including *genetic* factors (e.g. α-1 antitrypsin deficiency) and changes in airway inflammation also play a role in the development of the disease. A number of single nucleotide polymorphisms (SNPs) have been reported in association with different COPD features although much of this data remains controversial. Genetic susceptibility studies have demonstrated that in addition to familial aggregation, tumour necrosis factor alpha (TNF-α) and interleukin (IL)-13 promoter polymorphisms, as well as Tissue Inhibitor of Metalloproteinases (TIMP)-2 polymorphisms, are significantly associated with the presence of smoking-related COPD. SNPs in Matrix Metalloproteinase (MMP)1 and MMP12, in the anti-oxidant genes glutathione S-transferase (GST)M1, GSTT1, GSTP1, heme oxygenase (decycling) 1 (HMOX-1), and Microsomal epoxide hydrolase (mEPHX) are associated with an accelerated decline of lung function in COPD (Molfino 2004a, 2004b, 2007a). Genome-wide association studies (GWAS) may be able to pinpoint genetic loci associated with COPD (Silverman

2006). Most recently 4 SNPs on chromosome 4 were found to be associated with a reduced FEV1/FVC ratio. The associated SNPs corresponded to a non-gene transcript area near the hedgehog-interacting protein (HHIP) gene (Pillai 2009). Two SNPs at the alpha-nicotinic acetylcholine receptor locus have also been shown to be associated with lung function (Wilk 2009). The inflammatory response observed in the lungs of patients with COPD demonstrates evidence of activation of both innate and acquired immune processes, and the pattern of this inflammatory disease is diverse with the accumulation of the inflammatory components contributing to lung injury in these patients serving as a self-perpetuating stimulus for further immune activation. Thus it is unsurprising that disease that ensues is complex and multi-rooted, resulting in various clinical phenotypes.

1.1.3 Pathogenesis

Although the focus of the inflammatory changes seen in COPD has primarily been the airways, it is now known that there are several systemic manifestations of the disease and consequences secondary to that systemic inflammation. However, the relationship between the airway and systemic inflammation is poorly understood.

1.1.3.1 Pulmonary Inflammation

The innate defence system of the lung involves mucociliary clearance apparatus, (Knowles 2002) the epithelial barrier (Simani 1974), and the coagulation and inflammatory cascades (Kumar 2005). In patients with COPD, cigarette smoke interferes with this innate defence by increasing mucus production, reducing mucociliary clearance, disrupting the epithelial cell barrier and stimulating the migration of polymorphonuclear neutrophils, monocytes and macrophages, cluster of differentiation (CD)4^+, $CD8^+$, B-cell lymphocytes, dendritic cells and natural killer (NK) cells to the damaged tissue (Hogg 2004, Kumar 2005). Tobacco smoke also stimulates the humoral and cellular components of the adaptive immune response and it is important to remember that inflammatory changes in the airways are seen in smokers without COPD too (Kim 2008). Cigarette smoking elicits airway inflammation in all of those who smoke, but in those that develop airflow obstruction these pathological abnormalities persist in spite of removal of the noxious stimuli (Kim 2008). This has led to the concept that an abnormal inflammatory response to cigarette smoke leads to the development of COPD in the susceptible individual. As the disease

progresses, there is accumulation of inflammatory mucus exudates in the lumens of the small airways and thickening of airway walls.

Many of the cytokines secreted in COPD are regulated by transcription factor nuclear factor κB (NF- κB) which is activated in both airway epithelial cells and macrophages (Caramori 2003). Bronchial biopsies in COPD show infiltration of T cells and neutrophils (Hogg 2003). In some individuals with COPD, airway epithelial cells also show pseudostratification secondary to the release of epithelial cell growth factors and irritants. There is also increased expression of mucin genes in COPD patient biopsies (Caramori 2004). In emphysema, protease mediated degradation of connective tissue elements such as elastin and apoptosis of type I pneumocytes and endothelial cells leads to alveolar wall destruction (Majo 2001, Taraseviciene-Stewart 2006). Thus differing clinical phenotypes may result from differing patterns of underlying inflammation.

Oxidative stress plays an important role in the pathogenesis of COPD through direct injury to the respiratory tract, as well as through exacerbation. It is hypothesised that abnormally high concentrations of reactive oxygen species (ROS) in the cells might lead to permanent changes in signalling transduction and gene expression (Rajendrasozhan 2008). Oxidative stress has been attributed a central role in the pathogenesis of COPD because in addition to causing direct injury to the respiratory tract, oxidative stress triggers and exacerbates inflammation, protease-antiprotease imbalance and apoptosis (MacNee 2005, Barnes 2003).

Neutrophils are increased in the sputum of COPD patients, levels of which increase with disease severity (Keatings 1996). This neutrophilia is related to an increase in IL-8 which acts on Chemokine, CXC Motif, Receptor (CXCR)2; expressed predominantly by neutrophils. Macrophages, derived from circulating monocytes migrate to the lungs in response to chemoattractiants and there is increasing evidence that these lung macrophages co-ordinate the inflammation characteristic of COPD through chemokine release in turn attracting more neutrophils, monocytes, T cells and releasing proteases.

The T helper (T_H)1 $CD4^+$ T cells that accumulate in the airways and express CXCR3 (Grumelli 2004) are attracted to the lungs by IFN-γ induced release of CXCR3 ligands such as Monokine Induced by Interferon-gamma (MIG), Interferon gamma inducible protein 10 (IP-10), and Interferon-inducible T-cell alpha chemoattractant (I-TAC) which are present in high levels in the airways of patients with COPD (Saetta 2002, Costa 2008). This inflammatory process is also self perpetuating. The immunological response that results and the exact mechanisms of regulation are unclear, and it is possible that viral or bacterial antigens secondary to colonisation or latent infection are important in this process.

1.1.3.2 Systemic Inflammation

Patients with COPD, particularly with increasing severity have evidence of systemic inflammation in the stable state. This is measured as (1) increased circulating cytokines; IL-6 (Bhowmik 2000), TNF-α (Di Francia 1994, Takabatake 2000, IL-1B (Broekhuizen 2005), (2) chemokines; IL-8, leptin (Takabatake 2000), (3) acute phase proteins; CRP (Dahl 2007, de Torres 2008) fibrinogen (Donaldson 2005), Serum amyloid A (Bozinovski 2008), surfactant protein D (Sin 2007), (4) abnormalities in circulating cells; monocytes (Barnes 2004, Traves 2004, Aldonyte 2003) neutrophils (Sparrow 1984), lymphocytes (de Jong 1997, Kim 2002, Hodge 2003, Domagala-Kulawik 2007) and NK cells (Prieto 2001, Fairclough 2008). Smoking itself may cause systemic inflammation, and in COPD patients the degree of systemic inflammation seen is even greater than in smoking controls. It is unclear whether these systemic markers of inflammation are due to (1) "overspill" from inflammation in the peripheral lung, (2) a parallel abnormality, or (3) related to a co-morbid disease that in turn affects the lung (Sevenoaks 2006, Fabbri 2007, Barnes 2009). Systemic inflammation appears to relate to an accelerated decline in lung function and is increased during exacerbations (Donaldson 2005, Hurst 2006).

Conventionally, COPD severity has been graded on the basis of FEV_1; however, there are a range of significant extra-pulmonary effects that may also contribute. Some of these are thought to be associated with this systemic inflammatory response, such as muscle weakness (Agusti 2008, Hopkinson 2007) cardiovascular disease (Sin 2005), malnutrition and osteoporosis (Jorgensen 2008). However, psychological factors including anxiety and depression are also important and a patient's perception of his /

her disease can have a profound effect on outcome and management. Indeed depression has been linked with systemic inflammation in some diseases (Kojima 2009, Frasure-Smith 2009).

1.1.4 Phenotypes

A phenotype is defined as "The physical form of an organism as determined by the interaction of its genetic make-up and its environment" (Collins Essential English Dictionary 2006). In this situation the individual contributes the genetic make up and the surroundings e.g socioeconomic status, nutrition or sunlight exposure provide the environment. So arguably an individual can have his/ her own phenotype, but can this concept be applied to a group of patients with a particular disease? Perhaps a disease phenotype is the genetic basis of clinical heterogeneity of a disease if in this situation we consider the individual to be the "environment". The concept of disease phenotypes has been suggested in many disorders. For example, individuals with rheumatoid arthritis typed with human leukocyte antigen (HLA)-DRB1*04+ show more aggressive and severe disease (Weyand 1995). It could be argued that this is a poor example and it is simply that the genotype itself is linked with disease severity. However it is arguably more complicated than this with multiple gene interactions affecting disease phenotype. How multiple genes interact is likely to differ from one individual to another. One of the strengths of the argument for a frequent exacerbator phenotype in COPD is that frequent exacerbators exist across all disease severities (Anzueto 2009). This is discussed further in ***Chapter 4***.

Not everyone with the same amount of biomass fuel or cigarette smoking history develops the same pattern or level of inflammation either in the lungs or systemically. In fact not everyone who smokes even goes on to develop COPD. Thus it is only logical that various clinical outcomes exist in different individuals under the disease umbrella of COPD. It has long been apparent that the traditional "emphysema" and "chronic bronchitis" phenotypes of COPD are insufficient models for categorisation of patients. A number of other phenotypes have subsequently been described; broadly classified as clinical, physiologic and radiographic (Friedlander 2007). Clinical phenotypes proposed include dyspnoea, with some individuals disproportionally dyspnoeic to the severity of lung function, frequent exacerbators, low body mass index (BMI), inhaled corticosteroid

(ICS)-responsive, depression and anxiety and non-smokers. Physiologic phenotypes described include airflow limitation, rapid decliner, bronchodilator responsiveness, airway hyper responsiveness, hypercapnia, impaired exercise tolerance, hyperinflation, low Diffusing Capacity of the Lung for Carbon Monoxide (DLCO), pulmonary hypertension and radiological phenotypes include emphysema and airways disease. Needless to say, there is some overlap between all three groups (Friedlander 2007), and it is arguably difficult to establish that all of these are disease phenotypes and not just features of disease. To do this, the phenotypic characteristic must occur independently of disease severity.

As COPD is so heterogeneous, recognising disease phenotypes is important in terms of management and tailoring treatment. For example, those with bronchodilator responsiveness commonly have eosinophilic sputum, increased exhaled nitric oxide and an increased response to corticosteroids (Papi 2000, Brightling 2005).
This thesis will concentrate on the frequent exacerbator phenotype. In order to identify this frequent exacerbator group however, it is essential to understand what constitutes an exacerbation.

1.2 Exacerbations

Exacerbations are an important feature of COPD and patients experience on average 1 -2 treated exacerbations per year but this ranges anywhere from 1 to 4 (and arguably higher) depending upon the population studied and the definition of exacerbation used (Donaldson 2002, Garcia-Aymerich 2003, Seemungal 1998, Miravitlles 2000, Greenberg 2000, Calverley 2007, Niewoehner 2007). Exacerbations do not appear to be random events (Hurst 2009); some individuals are more susceptible to developing exacerbations, and the frequency with which patients have exacerbations remains relatively stable from year to year (Ball 1995). In a study of 109 individuals in the London COPD cohort, the number of exacerbations a patient experienced over the first year was highly and positively correlated with the number suffered during the following year. For those patients with data for more than 2 years there was a positive correlation between the annual rates for years 2 and 3 and similarly between years 3 and 4 (Donaldson 2002).

Preventing exacerbations is important as they contribute to the morbidity, mortality and healthcare cost associated with COPD (Seemungal 1998). COPD exacerbations are now the most common cause of medical hospital admission in the UK; accounting for 15·9% of hospital admissions (British Thoracic Society 2006) and have a major impact for patients both at the time of the event and in the longer term. Thus there is rationale for identifying patients who exacerbate frequently.

There is controversy surrounding what actually constitutes an exacerbation of COPD, and different definitions will lead to differing exacerbation rates. A validated definition of exacerbation has been used for this thesis and is described in ***Chapter 3***.

1.2.1 Aetiology of exacerbations

The word exacerbation has a Latin root: *exacerbātus*; which means more at edge / harsh / sharp. The Oxford English Dictionary defines to exacerbate as “To increase the smart of (a pain), the virulence of (disease), the bitterness of (feeling, speech, etc.); to embitter, aggravate”. Even the meaning of the word is complex; so it is not surprising that patient recognition and interpretation of these events and physician diagnosis is variable. Is an exacerbation simply a worsening of the disease itself, or is it something

else entirely that irritates the already inflamed airway resulting in a process that is different from the underlying COPD?

1.2.2 Causation of exacerbations

Much as the causes of COPD are multifactoral, so are the causes of exacerbations. Douglas Powell in 1878 identified cold weather as the most important cause of bronchitis (Powell 1878). He also observed that "dusty employments……dusty winds (and) irritating fogs" also bring on attacks of bronchitis. He recognised the importance of colds in triggering exacerbations (pg 173 Powell 1878), and some of the most frequently cited causes of exacerbations include viral infections, bacterial infections, inhalation of environmental irritants, discontinuation of medications, poor nutritional status (Voelkel 2000) and dynamic hyperinflation. Most COPD exacerbations are caused by episodes of tracheobronchial infection or pollutants, with the apparent contributions of individual agents varying by the methodology used for detection, and the severity of the underlying COPD.

1.2.2.1 Infections

50–70% of exacerbations are thought to be due to respiratory infections (Ball 1995). The most common bacterial and viral pathogens isolated from patients with COPD exacerbations include; *Haemophilus influenza, Moraxella catarrhalis*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, human rhinovirus, coronavirus, influenza, parainfluenza, adenovirus, and respiratory syncytial virus. However, it is important to remember that the presence of an organism in sputum does not always imply causation of that exacerbation and improved methods of detection of viruses and bacteria over the years has influenced the way we interpret results.

Bacteria

The importance of bacteria as a cause of exacerbation is controversial, as airway bacterial colonisation in the stable state is associated with the same organisms that are isolated at exacerbation. More recently it has been reported that exacerbation may result from a change in the colonizing strain (Sethi 2007), although not all exacerbations are associated with strain change, and not all strain changes cause exacerbation. Further evidence that bacteria cause exacerbations may be drawn from the benefit observed in

trials of antibiotics (Sethi 2003) and the demonstration of new strain-specific local and systemic antibody responses to organisms acquired at the time of these events. In general, the bacterial load and the proportion of patients with detectable bacteria increase at exacerbation. In one study in patients with moderate to severe COPD, bacteria were found in 48.2% of patients in the stable state, whereas at exacerbation bacterial detection rose to 69.6%, with an associated rise in airway bacterial load (Wilkinson 2006). The development of molecular typing methods has allowed the detection of changes in bacterial strains rather than species (Sethi 2002). The role of atypical bacteria such as Chlamydia, Legionella and Mycoplasma, although implicated at exacerbation is unclear (Mogulkoc 1999, Blasi 2002, Seemungal 2002, Lieberman 2002). As with viral exacerbations, sampling technique influences detection and load.

Bronchoscopic sampling with the use of a protected specimen brush yields reliable specimens from the lower airways. A pooled analysis of studies relying on this technique revealed that bacteria were present in clinically significant concentrations in the airways of 4% of healthy adults, 29% of adults with stable COPD, and 54% of adults with exacerbated COPD (Rosell 2005). One study reported the presence of intracellular *Haemophilus influenzae* in bronchial mucosal biopsy specimens from 87% of patients who were intubated because of exacerbations as compared with 33% of patients with stable COPD and 0% of healthy controls (Bandi 2003). Purulent sputum during an exacerbation is highly correlated with the presence of bacteria in the lower respiratory tract, providing an additional line of evidence for the pathogenic role of bacteria (Soler 2007). In a study of the upper and lower airways in patients with severe exacerbations of chronic obstructive pulmonary disease (COPD) requiring mechanical ventilation (Soler 1998), quantitative cultures of tracheobronchial aspirates (TBAs), bronchoscopically retrieved protected specimen brush (PSB) and bronchoalveolar lavage fluid (BALF) at admission to the ICU and after 72 h, as well as serology for bacteria and respiratory viruses were performed. Potentially pathogenic microorganisms (PPMs) and/or a positive serology were present in 36 of 50 (72%) patients, including 12 (33%) polymicrobial cases. Microbial patterns corresponded to community-acquired pathogens (*Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis)* in 19 of 34 (56%) and to gram-negative enteric bacilli (GNEB), *Pseudomonas*, and *Stenotrophomonas* spp. in 15 of 34 (44%) of isolates. *Chlamydia pneumoniae* and respiratory viruses were found in 18% and 16% of investigations,

respectively. Repeated investigation after 72 h in 19 patients with PPMs in the initial investigation revealed eradication of virtually all isolates of community-acquired pathogens and GNEB but persistence of three of five *Pseudomonas* spp. and both *Stenotrophomonas* spp. as well as the emergence of new GNEB, *Pseudomonas* and *Stenotrophomonas* spp. Stable COPD patients often have lower airway bacterial colonisation which may be an important stimulus to airway inflammation and thereby modulate exacerbation frequency (Patel 2002). In this study by Patel *et al* fifteen of the 29 patients (51.7%) were colonised by a possible pathogen: *Haemophilus influenzae* (53.3%), *Streptococcus pneumoniae* (33.3%), *Haemophilus parainfluenzae* (20%), *Branhamella catarrhalis* (20%), *Pseudomonas aeruginosa* (20%). The presence of lower airway bacterial colonisation in the stable state was related to exacerbation frequency. Patients colonised by *H. influenzae* in the stable state reported more symptoms and increased sputum purulence at exacerbation than those not colonised suggesting that lower airway bacterial colonisation in the stable state modulates the character and frequency of COPD exacerbations.

Viruses

The more frequent isolation of respiratory viruses at exacerbation than in the stable state (Seemungal 2001, Papi 2006), the association of viruses at exacerbation with greater airway inflammation (Seemungal 2001, Wilkinson 2006), and the high prevalence of coryzal symptoms during exacerbations (Seemungal 2001) have been cited as evidence in support of the concept that respiratory viruses cause exacerbation. However, perhaps the greatest evidence in support of viruses causing exacerbations comes from experimental infection. Mallia *et al* (Mallia 2006) carried out a virus dose-escalating study to determine the lowest dose of virus that would induce colds in COPD patients, starting with an initial dose of 10 $TCID_{50}$ of RV16; which they found to be sufficient. They identified a clinical cold and exacerbation in all 4 subjects using predetermined criteria and a fall in PEF. They showed rises in IL-6 and IL-8 levels. This study also highlights is the importance of timing of viral sampling; only 2/4 (50%) of the subjects were positive by nasal lavage PCR for rhinoviruses on day 11. In a study by Seemungal *et al* (Seemungal 2001) 64% of exacerbations were preceded by colds but a virus was detected in only 39% again suggesting that the true association of respiratory virus infection and COPD exacerbations is likely to be even higher than

reported. This in turn suggests that COPD patients have increased susceptibility to virus infection, but this has not been proven.

Viruses have been detected in > 50% of exacerbations depending on diagnostic techniques used. In an analysis of 85 hospitalizations for exacerbation, 56% of episodes were associated with the isolation of a respiratory virus (Rohde 2003). These were more commonly rhinovirus (36%) but other viruses detected included influenza A (25%), respiratory syncytial virus (22%), parainfluenza-3 (10%), and influenza B (7%). Seemungal *et al* (Seemungal 2001) investigated the effects of respiratory viral infection on the time course of COPD exacerbation by monitoring changes in systemic inflammatory markers in stable COPD and at exacerbation. 64% of exacerbations were associated with a cold occurring up to 18 days before exacerbation. 77 viruses (39 (58.2%) human rhinovirus (HRV)) were detected in 66 (39.2%) of 168 COPD exacerbations. Viral exacerbations were associated with frequent exacerbators, and colds with increased dyspnoea, a higher total symptom count at presentation, a longer median symptom recovery period of 13 days, and a tendency toward higher plasma fibrinogen and serum IL-6 levels. In a study of 64 COPD patients (Papi 2006), viruses were detected in 48.4% of exacerbations (6.2% when stable, $p < 0.001$). In a study of patients on the intensive therapy unit (ITU) (Cameron 2006) with a primary diagnosis of COPD exacerbation requiring non-invasive ventilation (NIV) or ventilation via endotracheal tube (ETT), virus was identified in 46 cases (out of 107 episodes in 105 individuals) (43%), with virus being the sole organism in 35 cases (33%) and part of a mixed infection in 11 cases (10%). In a very recent study (De Serres 2009) one third of COPD exacerbations were associated with virus; 9% with influenza A, 7% RSV and 7% with PIV-3. Virtually no HRV was detected. Importantly, patients were included up to 10 days post the onset of exacerbation and this may be why levels were so low.

The mechanisms by which viruses cause the deterioration in symptoms and lung function that characterize exacerbations remain poorly understood. Potential mechanisms to explain how viral infection can result in deterioration of symptoms and lung function include direct infection of the lower respiratory tract (Papadopoulos 2000), neural reflex responses, and "cross-talk" between the upper and lower airway. The latter may occur by passage of mediators directly along the mucosal surface or via the blood stream. In addition, the virus-associated airway inflammation, with resultant

increase in bronchial wall thickness and luminal exudates, is likely to be partially responsible for the clinical features of exacerbation.

Further complicating the interactions of airway infection and inflammation is the situation in which respiratory viruses and bacteria may be isolated together. A greater systemic inflammatory response has been reported in those exacerbations associated with both *H. influenzae* and rhinovirus isolation, and when the isolation of *Haemophilus* was associated with new or worsening coryzal symptoms (a surrogate of viral infection), such infections were more severe as assessed by changes in symptoms and lung function at exacerbation onset (Wilkinson 2006). In a different study, patients with exacerbations with co infection had more marked lung function impairment and longer hospitalizations (Papi 2006). However other studies have not found co-infection to increase severity of the exacerbation (De Serres 2009, Cameron 2006).

Viral infections are more common in the winter months, when respiratory viral infections are more prevalent in the community, and lung function has been shown to fall significantly with reduction in outdoor temperatures (Donaldson 1999). Therefore, it may not just be viral infection that is important seasonally with development of COPD exacerbations; the environment may play an equally important role.

Viruses in stable COPD

Respiratory viruses have also been detected in stable COPD, suggesting that chronic viral infection may occur, however the exact impact of this on the course of disease and exacerbations has not yet been established, but may be important in exacerbation frequency. In stable COPD patients rhinovirus and coronaviruses have been detected but the most common is RSV. In some studies, more patients had RSV detected in the stable state than at exacerbation (Seemungal 2001). Chronic viral infection is thought to be important in CD8+ recruitment to the airways. This CD8+ T lymphocytosis driven inflammation can further damage lungs leading to COPD progression. COPD patients with repeated RSV in sputum over 2 years have faster lung function decline over that time (Wilkinson 2006). RSV may persist in many cases of COPD and may contribute to pathogenesis of stable disease in a similar manner to latent adenovirus. Patients with RSV found in more than 50% of sputum samples had a mean decline in FEV_1 of 101.4

ml per year as compared to 51.2 ml per year in patients with RSV in 50% of samples or less (Wilkinson 2006).

Latent adenoviral infections may also be important in COPD. E1A has the ability to initiate a cycle of viral replication that results in either cell lysis of the host cell with release of a large number of viral particles to infect other cells, or the shedding of viral particles from the surface of a living host cell (Hogg 2001). Low levels of viral replication allow the virus to establish a persistent infection which can be long-lasting after the virus has cleared. Viral persistence after an acute infection can also occur if viral DNA forms a plasmid within the host cell or integrates into the host cell genome. During a latent infection, viral proteins are produced without replication of a complete virus and there is evidence this type of infection can influence the inflammatory response to stimuli in COPD. These principles may be applied to other latent viral infections.

Viral detection

Historically, detection of viruses has been by culture and serology of the virus from respiratory secretions. This is difficult however, as samples need to contain live virus. Immunochemical techniques for detection of virus have lower sensitivity and specificity. Serological techniques are also limited, as they rely on the host immune response. Additionally, sensitivity of a technique is only useful if sound methods of sample collection have been used, and in the case of viral detection, sputum is more sensitive than nasal samples. Prevention of sample contamination is also important.

Polymerase Chain Reaction (PCR) has enabled a more detailed evaluation for the role of viruses, particularly rhinovirus (Seemungal 2001). Studies evaluating the use of reverse transcription and amplification of viral nucleic acid by PCR have shown superior sensitivity and specificity than culture or antigen detection methods (Freymuth 2006). PCR techniques are not uniformly sensitive however, as it depends on primers and probes used and the region of the virus to be detected. This may explain some of the disparity in prevalence of viruses (particularly RSV) found in similar populations (Seemungal 2001, Rohde 2003, Beckham 2005, Borg 2003). Reasons for disparity in virus detection in studies not only include variable sensitivity of techniques used, but geographical differences may also account for some of the variation given that viral

epidemics appear to be local rather than global (Anderson 1991). Both qualitative and quantitative PCR have been discussed in detail in ***Chapter 3*** **(section 3.12)**.

With PCR, up to 40% of COPD exacerbations have been shown to be associated with viral infection (Seemungal 2001). In one study using PCR, rhinovirus was detected in 39/66 (59%) viral exacerbations, coronavirus in 7/66 (10.6%), influenza A in 6/66 (9%) and B in 3/66 (4.5%) and parainfluenza and adenovirus each detected at one exacerbation (1.5%). RSV, although detected in 19/66 (28.8%) exacerbations was also seen in a significant number of patients in the stable state, unlike the other viruses detected at exacerbation (Seemungal 2001). PCR has also allowed multiple viruses to be detected more easily at a single exacerbation. In a study of 81 exacerbations, 88 viruses were identified (Beckham 2005). Although serologic methods identified 57% of these, RT-PCR assays identified an additional 38 viruses, with rhinovirus the most common infection and the most frequently identified only by RT-PCR. We have used a very sensitive cut-off for our quantitative HRV RT-PCR of 1pfu/ml. This has been discussed in further detail in ***Chapter 10.2***.

HRV and inflammation

Experimental HRV infection has been shown to increase sputum IL-6 and IL-8 in normal and asthmatic subjects (Fraenkel 1995, Fleming 1999). In COPD, exacerbations associated with the presence of rhinovirus in induced sputum had larger increases in sputum IL-6, (Seemungal 2001) IL-8 and myeloperoxidase (MPO) (Wilkinson 2006) levels compared to exacerbations where HRV was not detected. Viral infections have also been associated with increased oxidative stress at exacerbation (Rahman 1997). HRV can replicate in the lower airway, and activates NF-kB thus up-regulating pro-inflammatory mediators in the airway (Biagioli 1999). Through their chemotactic effect on neutrophils (via IL-8, ENA-78 and LTB4), lymphocytes and monocytes (RANTES) and the upregulation of adhesion molecules, these mediators increase inflammation that is characteristically seen at exacerbation.

Viral infections can also induce the expression of stress response genes, such as haem-oxygenase-1 and genes encoding antioxidant enzymes. These antioxidant enzymes may be important in protecting against virally mediated inflammation at exacerbation. Endothelin-1, an important vasoconstrictor which is pro-inflammatory and mucogenic,

has also been implicated in the pathogenesis of virally-mediated inflammation. Levels of ET-1 increase in the airway and systemically at exacerbation (Wedzicha 2004). Plasma fibrinogen and IL-6 increase at exacerbation (Wedzicha 2000) and plasma fibrinogen is higher in the presence of a cold and with detection of respiratory viral infection at exacerbation (Seemungal 2001, Wedzicha 2000). This suggests that viral exacerbations are associated with an increased systemic inflammatory response, and may predispose to increased vascular disease.

Whether COPD patients are more susceptible to viral infection compared to normal subjects is strongly debated. Factors thought to be important in susceptibility to viral infection include upper and lower airway inflammation, cigarette smoking, airway bacterial colonisation, possible viral persistence, co-infection and seasonality. One study suggests that infection with *Hemophilus influenzae* increases Intercellular Adhesion Molecule-1 (ICAM-1) and Toll like receptor 3 (TLR3) expression in airway epithelial cells, thus increasing the potential for rhinovirus binding and subsequent cytokine responses (Sajjan 2006).

Background on specific viruses

HRV

Rhinovirus, most commonly responsible for the common cold, is currently thought to be the most important trigger of COPD exacerbations. A member of the picornavirus group of RNA viruses, it has over 100 serotypes making detection by culture and serologic methods very difficult. It is spread from person to person by infected respiratory secretions. The major group of rhinovirus attaches to airway epithelium through Intercellular Adhesion Molecule-1 (ICAM-1), inducing its expression, thus promoting inflammatory cell recruitment and activation (Papi 1999). Latent expression of adenoviral E1A protein in alveolar epithelial cells may increase ICAM-1 expression, and this may be a potential mechanism for increased rhinoviral susceptibility in COPD (Retmales 2001).

Using viral culture and serology, 27% of COPD exacerbations have been shown to be associated with viral infection as opposed to 44% of acute respiratory illness in control subjects (Greenberg 2000). In the COPD population, 43% of the viral infection was attributable to rhinovirus, thus making it responsible for about 12% of the total

exacerbations. The lower percentage of virus seen in COPD patients compared to controls may be due to detection methods.

In other studies, rhinovirus has been detected in up to 23% of patients during exacerbations but in <1% of stable patients. Studies have illustrated (Wilkinson 2006) a synergistic effect of viral and bacterial infections in that inflammatory and lung function changes are more pronounced in proven rhinoviral infections, measured with sputum IL-6. The presence of virus may indirectly increase bacterial load in addition to direct viral effects.

In a pilot study, mild COPD patients were infected with the minimal amount of rhinovirus to induce clinical colds. All of the first 4 patients exposed to the lowest dose experienced cold symptoms and lower respiratory tract symptoms including shortness of breath, wheeze, cough and increased sputum production (Mallia 2006). This study showed that COPD patients developed colds and exacerbations with 100 to 1000 fold lower doses of viruses than used in previous studies on normal and asthmatics. Also, there was a 3-4 day gap between the peak of cold symptoms and the peak of lower respiratory symptoms.

Coronavirus

Coronaviruses are enveloped RNA viruses and are the second most frequent cause of the common cold (Makela 1998). They can also occasionally cause pneumonia in elderly and immunocompromised patients (van Elden 2004). There are 2 human strains: HCoVs 229E and OC43 in 2 antigenic groups.

Use of RT-PCR has improved Coronavirus detection. In a large Asian study of acute exacerbations of COPD, coronavirus OC43 was detected in 4.9% of exacerbations (Ko 2007). Another study has detected coronavirus at a similar level in 4.2% of exacerbations (Seemungal 2001).

Influenza and parainfluenza

Due to the introduction of influenza immunisation in patients with chronic lung disease, influenza has become a less common cause of exacerbations, but is still likely to be important in times of epidemics and one study (Rohde 2003) has shown influenza to be

associated with as many as 25% of COPD exacerbations. Patients, who have not been vaccinated against influenza, have twice the hospitalisation rate in the influenza season compared with the non-influenza season, and influenza vaccination is associated with a lower risk of death. (Nichol 1999).

Serological evidence of a past infection with influenza virus has been detected in 5–28% of patients following an exacerbation, but is present in only 6% of patients who have not had an exacerbation. The prevalent strain of influenza has some effect on exacerbation frequency and severity. An American study carried out over four consecutive winters noted that, when the dominant strain of influenza was H1N1, hospital admissions for influenza-related chest infections were low. In contrast, when H3N2 influenza was prevalent (a more virulent strain), admissions for chest infections and related deaths were significantly raised (Centres for Disease Control and Prevention 2005).

Parainfluenza viruses have been associated with a lower percentage of exacerbations at 3% in one study (Greenberg 2000) and is usually seen in the summer months.

Adenovirus

This is a double-stranded DNA virus, of which there are more than 40 known serotypes that are responsible for a wide range of human infection, including infection in the upper and lower airways. Types 4 (group E), 7 (group B) and 1,2,5 (group C) cause the most frequent respiratory disease in adults and have an incubation period of 5 to 8 days.

Adenovirus adheres to the cell surface through its fibre protein and penton base and is internalized by receptor-mediated endocytosis. After it has entered the cell, it is translocated to the nucleus and transcribed in early and late groups of genes. The first early gene to be expressed is the E1A gene which generally activates the cell thus allowing the virus to gradually take over host cell protein generation to manufacture viral particles and initiate production of other molecules that contribute to host cell defence. Viral E1A protein has been shown to continue to be expressed in lung epithelial cells long after the virus has stopped replicating and clinical signs of infection have cleared. The latently-infected cells continue to produce viral proteins without replicating a complete virus and the host response to them includes an increase in CD8+ T cells (Hogg 2001).

Infections usually occur in the first year of life and in adults with COPD is detected in 0.5 – 1.5% of exacerbations. In a study of 136 COPD patients seen at exacerbation, 10 exacerbated patients were positive for the adenovirus hexon gene (capsular protein) and only 2 positive for adenovirus 5 E1A DNA in epithelial cells (McManus 2006).

RSV

RSV, a negative-single-sense-stranded ribonucleic acid (RNA) virus of the Paramyxoviridae family, predominantly recognised as a paediatric pathogen, is now becoming recognized as an important adult pathogen. RSV is implicated as a cause of acute exacerbations and the normal seasonal variation of acute RSV is linked to frequencies of COPD hospitalisations (McManus 2007). RSV enters its host's respiratory epithelium by cell surface fusion and replication triggers an inflammatory response which may be modulated by the virus itself. The non-structural proteins antagonize IFN-α, IFN-β and IFN-λ responses, which may impair antiviral immunity and contribute to persistence of the virus. The virus can also avoid early termination of infection via inhibition of apoptosis in host cells. Serological studies have indicated that RSV infection has been associated with up to 6% of exacerbations of COPD, although studies have reported that 11.4% of hospital admissions for COPD could be accounted for by the presence of RSV when diagnosed using serology, reverse transcriptase-PCR, and viral culture (Falsey 2005). In addition to the interest in the role of RSV in acute exacerbations, recent studies have led to the suggestion that the virus may persist in some cases, and it may contribute to pathogenesis of stable disease (Ramaswary 2009).

Human metapneumovirus (hMPV)

In 2001, van den Hoogen et al (van den Hoogen 2001) reported the discovery of hMPV which has subsequently been described by many others worldwide. It is similar to other paramyxoviruses, has a seasonal distribution and has been predominantly identified in winter and early spring in North America. It has been isolated in adults with upper and lower respiratory tract infections and is associated with acute onset of wheeze. hMPV infection is most likely due to re-infection as most individuals are seropositive by the age of 5-10 years. As with RSV and parainfluenza the degree of protective immunity may decrease with time. hMPV isolates segregate into 2 distinct genotypes and it is

possible infection with one hMPV genotype may not confer complete protective immunity against other strains (Martinello 2006).

hMPV in one study was identified in 6 out of 50 hospitalised COPD exacerbations (12%) (van den Hoogen 2001). Patients with hMPV were more frequently febrile during hospitalisation and also had change in cough, sputum production and dyspnoea. 3/6 patients positive for hMPV had evidence of a lower respiratory tract infection as the presence of a new infiltrate on Chest X-ray, and did not test positive for bacterial infection. Other investigators have found hMPV in 5.5% patients having a COPD exacerbation when no other pathogen could be identified. Other studies have shown no evidence of MPV in 194 respiratory illnesses in 96 patients over a 4 year study period. This may be due to temporal or geographical factors.

The commonest respiratory virus detected at exacerbation and therefore the focus of this chapter is human rhinovirus (HRV). The site of initial HRV infection is thought to be the nasal epithelium, but HRV can be also readily found in sputum of COPD patients at exacerbation (Seemungal 2000b). Its present in the lower airways might explain the appearance of the major respiratory symptoms necessary for diagnosis of an exacerbation. Deposition, by aerosol or direct contact, occurs in the anterior nasal mucosa or eye (with subsequent passage down the nasolacrimal duct). Mucociliary action transports the virus posteriorly to establish initial infection in the nasopharynx. Here, after entry into the cell via the appropriate receptor, viral replication occurs. Up to 95% of subjects without strain-specific protective antibody become infected, although symptomatic colds develop in only 75% of these subjects. There is little evidence of a cytopathic effect, although there may be epithelial shedding, and symptoms are therefore predominantly due to the host immune response. In support of this, the concentration of IL-8 in nasal lavage fluid correlates with symptom severity during experimental rhinovirus colds (Turner 1998). The rhinorrhea and nasal obstruction arise from vasodilatation and increased vascular permeability of the nasal mucosa. Cholinergic stimulation results in increased mucus production and sneezing.

Environment

Up to 10% of exacerbations are attributed to environmental pollution (depending on season and geographical placement) (Sunyer 1993). Environmental factors contributing

include changes in composition, temperature and humidity of inspired air both indoors and outdoors. The role of pollutants in causing exacerbation has been difficult to assess, but data from six European cities in the APHEA (Air Pollution and Health: a European Approach) project reported an association between increased air pollution and a rise in hospital admissions for COPD (Anderson 1997). Particulate matter up to 10 μm in size (PM_{10}), largely produced by diesel exhaust, seems to be particularly important. In a separate study providing a potential mechanism, exposure of smokers with COPD to increasing PM_{10} concentration was associated with a reduction in FEV_1 (Pope 1993). It has also been suggested that pollutants can improve the transfer and infectivity of viruses, and this may be an important mechanism in COPD exacerbation (Hammond 1989; Jaspers 2005). Thus measures to improve air quality may reduce exacerbation frequency.

Discontinuation of medications

Withdrawal of inhaled corticosteroids in COPD patients established on this treatment has been shown to cause a persistent decline in lung function and dyspnoea and an increase in exacerbations (Wouters 2005). A low FEV_1 is a risk factor for exacerbations, and so discontinuing medications may contribute to exacerbation frequency (Seemungal 1998, Garcia-Aymerich 2000). In the ISOLDE study, patients with an $FEV_1 < 60\%$ predicted treated with inhaled corticosteroids had a reduction in exacerbation frequency of approximately 20% (Jones 2003) and studies investigating the effects of long-acting β-agonists (LABAs) (Calverley 2007) and the long acting anticholinergic tiotropium have shown a reduction in exacerbation rates (Niewoehner 2005). It has been suggested that the mechanism behind this may be anti-inflammatory; however, tiotropium does not have anti-inflammatory properties (Powrie 2007). More recently, carbocysteine has been reported to decrease exacerbation rate (Zheng 2008). There has even been a study suggesting that the addition of a proton pump inhibitor (Sasaki 2009) to therapy can reduce exacerbations in COPD.

Nutritional status

Loss of body weight secondary to decreased dietary intake is an independent risk factor for mortality in COPD. Patients hospitalised with COPD who are underweight at baseline or lose weight have an independent risk for future exacerbations (Hallin 2005). Patients who lose weight during an exacerbation related hospitalisation are at increased risk of

early non-elective readmission (Pouw 2000). No relationship has been shown between deficiency in energy intake and exacerbations. However, vitamin D deficiency has been hypothesised to influence exacerbation susceptibility but this may just be a sign of poor nutritional status in these patients. This is discussed further in ***Chapter 8***.

Vitamin D consists of a group of fat-soluble pro-hormones; vitamin D1 to D5; Vitamin D1: ergocalciferol with lumisterol 1:1, Vitamin D2: ergocalciferol, Vitamin D3: cholecalciferol made from 7-dehydrocholesterol in the skin, Vitamin D4: 22-dihydroergocalciferol, Vitamin D5: sitocalciferol made from 7-dehydrositosterol. The highest concentrations of 7-dehydrocholesterol are found in the epidermal layer of skin and the two most important factors governing the generation of pre-vitamin D3 are the intensity and appropriate wavelength of the ultraviolet (UV)B irradiation reaching the 7-dehydrocholesterol. Peak synthesis occurs between 295-297 nm and these wavelengths are present in sunlight daily during the spring and summer seasons in temperate regions. Adequate amounts of vitamin D3 can be made in the skin after only ten to fifteen minutes of sun exposure at least twice a week to the face, arms, hands, or back without sunscreen. With longer exposure to UVB rays, equilibrium is achieved in the skin, and the vitamin simply degrades as fast as it is generated. The presence and concentration of melanin is also critical and functions as a light filter in the skin.

Vitamin D3 once synthesized from 7-dehydrocholesterol is photolyzed by ultraviolet light to give pre-vitamin D3. Pre-vitamin D3 spontaneously isomerizes to Vitamin D3. D3 is then hydroxylated in the liver to 25-hydroxycholecalciferol by 25-hydroxylase and stored until it is needed. 25-hydroxycholecalciferol is further hydroxylated in the kidney by 1α-hydroxylase into two dihydroxylated metabolites there are the biologically active hormone 1,25-dihydroxycholecalciferol (1,25(OH)2D3 or calcitriol) and 24,25-dihydroxycholecalciferol. Vitamin D2 is derived from fungal and plant sources, and is not produced by the human body.

Serum concentrations of vitamin D vary with age, race, sex, season and geographic location and subclinical deficiency is common, particularly in temperate climates (Janssens 2009). In humans, D3 is as effective as D2 at increasing the levels of vitamin D hormone in circulation and both D2 and D3 are used for human nutritional supplementation.

Vitamin D regulates calcium and phosphorus levels in blood by promoting absorption from food in the intestine and re-absorption of calcium in the kidneys. It promotes bone formation and mineralisation (and at very high levels bone resorption), inhibits PTH secretion from the parathyroid gland and affects the immune system by promoting phagocytosis, anti-tumor activity, and immunomodulatory functions. Once in its physiologically active form vitamin D is released into the circulation, binds to a carrier protein in the plasma; vitamin D binding protein (VDBP) and is transported to various target organs. The hormonally active form of vitamin D mediates its biological effects by binding to the vitamin D receptor (VDR), which is principally located in the nuclei of target cells. This binding allows the VDR to act as a transcription factor that modulates the gene expression of transport proteins, which are involved in calcium absorption in the intestine. The VDR is constitutively expressed in monocytes, activated macrophages, dendritic cells, NK cells, T and B cells. Locally produced D3 acts on immune cells in an autocrine and paracrine manner. Activation has potent anti-proliferative, pro-differentiative and immunomodulatory functions both immune enhancing and immunosuppressive. T cell activation is suppressed, regulatory T cells induced, cytokine secretion patterns altered, dendritic cell function altered, activity of natural killer cells increased, phagocytic activity of macrophages enhanced and production of cathelicidin (an antimicrobial peptide produced in macrophages triggered by bacteria, viruses, and fungi) increased. (Mora 2008).

Vitamin D deficiency results from inadequate intake coupled with inadequate sunlight exposure, disorders that limit its absorption, conditions that impair conversion into active metabolites and hereditary disorders. Deficiency leads to rickets, osteomalacia, osteoporosis, and has been linked with colon cancer and breast cancer, higher risk of heart attack in men, and an increased risk of infections, e.g. influenza, TB and pneumonia. Some studies have also found a strong relationship between pulmonary function (FEV_1) and serum Vitamin D levels (Black 2005) with deficiency associated with lower FEV_1. Thus in COPD, deficiency of vitamin D may influence disease severity, affect FEV_1 decline, or underlie the mechanism behind increased susceptibility to exacerbation.

Genetic variants in the vitamin D pathway have been associated with COPD. A SNP in the VDBP was shown to be protective for COPD, the mechanism of which is unclear

(Schellenberg 1998). There are other SNPs in the VDBP that influence circulating vitamin D levels (Lauridsen 2005, Taes 2006). Many polymorphisms exist in the VDR gene and the influence of these polymorphisms on VDR protein function may influence immunomodulatory responses (Uitterlinden 2004). The most frequently studied are three adjacent RFLPs; *Bsm*I, (Morrison 1992), *Apa*I (Faraco 1989) and *Taq*I (Morrison 1994) in the 3' end of the VDR gene. A further RFLP exists affecting the start site (*Fok*I (Arai 1997)). Upon comparison of the original sequence of VDR cDNA (Baker 1988), two potential translation initiation start sites were observed and subsequent sequence comparisons have shown that a T to C polymorphism exists at the first potential start site (Gross 1996, Saijo 1991). No study has found a link with VDR polymorphisms and infection in COPD although they have been linked with TB. The *Fok*I common variants and *Bsm*I polymorphisms have been associated with muscle strength in COPD (Hopkinson 2008).

Dynamic hyperinflation

Not all exacerbations are associated with a rise in inflammatory markers. A subsequent analysis from the National Emphysema Treatment Trial (NETT) comparing patients who underwent lung volume reduction surgery (LVRS) with a medical treatment only group, showed that those who underwent surgery had a 30% lower exacerbation rate in the 3 year follow up period and a significantly longer time to first exacerbation (Washko 2008). However, the patients in this study all had severe COPD with predominantly upper lobe emphysema and the improvements in exacerbation rate correlated with FEV_1 after surgery. It is also important to note that the exacerbation rate in the NETT was much lower than in pharmacological studies such as TORCH (Calverley 2007). Nonetheless, this study appears to offer a mechanism other than inflammation that drives exacerbation. It is also possible that dynamic hyperinflation reflects symptoms, and not causation (Stevenson 2005).

Psychological

It is also possible that psychological factors such as anxiety and depression influence exacerbation. In a multicenter prospective cohort study in 491 patients with stable COPD in China, exacerbations were determined using both symptom based and event based definitions. Depression (HADS depression score > or = 11) was associated with an increased risk of symptom-based exacerbations, event-based exacerbations and

hospitalization compared with non-depression (score < or = 7) and the duration of event-based exacerbations was 1.92 (1.04-3.54) times longer for patients with probable anxiety (HADS anxiety score > or = 11) than those with no anxiety (score < or = 7) (Xu 2008). Other studies have also shown depression to be a risk factor for exacerbation (Jennings 2009). This is discussed further in ***Chapter 6***.

1.2.3 Pathogenesis

At an exacerbation, there are complex interactions between the host, respiratory viruses, airway bacteria, and environmental pollution, which sometimes lead to an increase in the inflammatory burden (Sapey 2006).

1.2.3.1 Pulmonary Inflammation

In spite of the fact exacerbations are usually associated with increased inflammation both in the sputum and systemically, no definition of exacerbation makes any reference to changes in inflammation. Sputum inflammatory markers that rise at exacerbation include neutrophils and cytokines such as IL-8 and IL-6 (Seemungal 2000, Perera 2007). Increased sputum IL-6 is associated with experimental HRV infection in healthy individuals and asthmatics (Mallia 2006, Grunberg 2001). Increased neutrophilic inflammation is characteristic of exacerbations and there is an increased concentration of neutrophil chemoattractants in the airways at the time of an exacerbation. Evidence suggests that LTB4 (Gompertz 2001, Crooks 2000), TNF-α, GM-CSF and neutrophil elastase (Fujimoto 2005) all increase at exacerbation with concentrations higher at the beginning of an exacerbation and falling with effective treatment. There is debate as to whether IL-8 increases in all exacerbations (Fujimoto 2005, Drost 2005) or remains unchanged except in the most severe episodes (Bhowmik 2000).There are increased numbers of neutrophils in bronchial biopsy specimens during COPD exacerbations (Seatta 1994, Zhu 2001).

1.2.3.2 Systemic Inflammation

Increases in blood CRP, IL-6, IL-8, sICAM-1(Hurst 2006), procalcitonin (Stolz 2007), and others occur at exacerbation (Hurst 2006). There is a significant relationship between neutrophilic lower airway inflammation and the systemic inflammatory response at exacerbation (Hurst 2006), even in patients with mild/moderate COPD

(Saetta 1994, Zhu 2001). Peripheral blood neutrophils increase during exacerbation, with levels correlating to the severity of the exacerbation (Papi 2006). The percentage of apoptotic blood neutrophils at COPD exacerbation is reduced, which is not related to serum and sputum concentrations of IL-6, IL-8 and TNF-α (Schmidt-Ioanas 2006). The resolution of neutrophilic inflammation at exacerbation usually occurs within 5 days after treatment, paralleling clinical recovery (Gompertz 2001).

1.2.4 Definition of Exacerbation

What actually constitutes an exacerbation is a highly debated topic. Ideally the definition used should account for the severity of the event and the impact of the exacerbation on health status and disease. Unfortunately there is no single marker that can identify an exacerbation, due to the heterogeneity of these events. Additionally, as they are primarily a patient reported event, not all exacerbations are reported to or witnessed by health care professionals.

In an attempt to clarify the concept of exacerbation and to guide research efforts, a number of groups have developed definitions of COPD exacerbations. A few examples of these are given below:

1. British Thoracic Society / NICE Guidelines 2004 (BTS 2004)

An exacerbation is a sustained worsening of the patient's symptoms from his or her usual stable state that is beyond normal day-to-day variations, and is acute in onset. Commonly reported symptoms are worsening breathlessness, cough, increased sputum production and change in sputum colour. The change in these symptoms often necessitates a change in medication.

2. ATS/ERS June 2004 (Celli 2004)

An event in the natural course of the disease characterised by a change in the patient's baseline dyspnoea, cough, and/or sputum beyond day-to-day variability sufficient to warrant a change in management.

3. GOLD 2006 (GOLD 2006)

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 in onset, and may warrant a change in regular medication in a patient with underlying disease.

These definitions are based on respiratory symptoms alone, or respiratory symptoms and the prescription of medication, unscheduled visits to a physician, increased self-medication +/- hospital admission. In simpler terms, they are "symptom" based, or "health care utilisation" based (Pauwels 2004). Increasingly, patient perception / perspective is taken into account (Leidy 2009). Qualitative data collection can be useful for improving understanding through patient descriptions of their experiences, the terminology they use, the manifestations and the attributes that define them and the actions they may or may not take when they occur. A consensus definition is needed with exacerbations increasingly used as an outcome measure in trials as different definitions may lead to different results (Effing 2009).

Symptom-based definition

There are advantages and disadvantages of using a symptom-based definition. Obviously symptoms are of fundamental importance and are a primary concern of the patient. Generally, it is the change in symptoms that prompts contact with healthcare professionals. Assessment of patient symptoms and subsequent improvement with therapy is therefore an important consideration for both patient and physician. However, patient symptomatology varies greatly between patients and an absolute level of dyspnoea or sputum volume cannot be described as diagnostic. Although most patients experience the same pattern of exacerbation symptoms each time they exacerbate (Kessler 2006), it is not known to what degree the clinical features of exacerbation change within an individual as the disease progresses. Equally symptoms have been shown to differ between isolated and initial of recurrent exacerbations (Hurst 2009), with symptoms more typical of viral infection (coryzal symptoms, sore throat and cough) more common in isolated events. Therefore a subjective assessment of 'worsening' is required which begs the question of who is best placed to make this judgment: the patient or the doctor?

When using a symptom-based definition, exacerbation rates tend to be higher as not all exacerbations are reported to a health care professional (Langstemo 2008, Donaldson 2002). These unreported events are not to be underestimated in terms of their impact on disease and will be discussed later on in this section. One of the first studies to

explicitly use symptom criteria for exacerbation was by McHardy (McHardy 1980) who felt the most important symptoms to be cough, sputum and wheeze. While these symptoms are commonly seen during an exacerbation, one study has shown that only 64% of exacerbations were associated with increased dyspnoea, 42% with increased sputum volume and 35% with purulent sputum (Seemungal 2000).

While some scales for symptom assessment do exist such as the MRC dyspnoea score, not all scales have been validated nor has the sensitivity to change been established, although investigations are ongoing (Leidy 2009) and it may be helpful if the symptoms in a symptom scale relate to the efficacy of a therapeutic intervention. For example, the Anthonisen study (Anthonisen 1994) established the need to fulfil three clinical criteria (increased dyspnoea, increased sputum volume and the presence of purulent sputum) for an antibiotic treatment to be effective.

The most common approach to capturing symptom change over time has been with the use of paper-based diary cards. This method has been used successfully by the London COPD cohort (Donaldson 2002, Powrie 2006, Seemungal 2008, Quint 2008, and Hurst 2009) and others (Vijayasaratha 2008, Tzanakis 2001, ZuWallack 2001, Langsetmo 2008) for years. However this approach has been criticised for possible poor adherence to protocol instructions and data validity issues arising from retrospective record entry (Stone 2002). However in this study by Stone, patients were asked to fill in a 20 question questionnaire 3 times a day for over 21 days. Perhaps in this study lack of compliance was related to the time involved in the task. The diary cards used in the COPD studies are much quicker to complete and act really as a prompt for symptom worsening. It is also important to remember that in every day clinical practise, doctors rely on patient recall of symptoms, albeit without daily symptom recording. One has to wonder what the patient has to gain from poor adherence or retrospective record entry and surely not all patients are dishonest? With the advent of modern technology however, the move to computerised devices such as a blackberry™ that can be monitored for daily symptom input may become increasingly popular.

Unreported exacerbations

The issue of underreporting of COPD exacerbations has been addressed by a series of articles (Donaldson 2002, Seemungal 2000, Seemungal 2001, Langsetmo 2008). Results

from the London COPD cohort suggest that almost half of all exacerbations remain unreported. The first description of unreported exacerbations arose from this cohort (Seemungal 1998). Although one would expect reporting to be influenced by severity and impact of symptoms, these studies found that reported and unreported exacerbations had very similar characteristics. In the study by Langsetmo (Langsetmo 2008), the consequences of unreported exacerbations on health status were addressed. They found that patient age, baseline spirometry, the total number of symptoms at onset and the need for rescue medication were most commonly associated with reporting an exacerbation and patients who reported their exacerbation were more likely to have worsening of their health status compared with patients not reporting or those with stable disease. The symptoms with the strongest association to reporting an exacerbation in this study were cough and sputum quantity, and the symptoms least associated with reporting were colds and wheeze. However, the definition of exacerbation used was different from that of the London COPD cohort, and they noted that 60% of the events with at least two symptoms and one key symptom (dyspnoea, sputum quantity, sputum colour) recorded in a daily diary card were not reported. Clearly, underreporting is a widespread phenomenon.

So why don't patients report their exacerbations? This may be due to a lack of understanding on the part of the patient in terms of the effect exacerbations have on the underlying disease (Rennard 2002, Okubadejo 1996, Wilkinson 2004). Other times patients self medicate, and then if that does not work, they seek medical advice. With emergency courses of antibiotics and steroids increasingly being issued to patients in an attempt to prevent hospital admissions, it is important to distinguish unreported from untreated exacerbations as not all unreported exacerbations are untreated. Patients may also sometimes ignore milder symptoms, expecting them to resolve spontaneously, only seeking healthcare advice when symptoms linger and they show no signs of improvement. Underlying lung disease may also affect clinical intervention required for exacerbation. Individuals with more mild disease may be able to tolerate greater compromise in their lung function. Psychological status may influence exacerbation reporting as may disease perception and social status. These factors will be investigated in this thesis.

Health care utilisation definition

Health care utilisation (HCU) definitions of exacerbation distinguish exacerbations in terms of the number of unplanned clinic visits, Accident & Emergency department visits or hospitalizations. Socioeconomic status affects both health status and access to health care. The increased need of those with lower socioeconomic status might be offset by lower access to health care. With increasing use of patient self management plans to encourage individuals to take control of their disease, the initiation of treatment alone with the use of antibiotics and/or oral steroids has also been used as a criterion for an exacerbation.

There are disadvantages to using event based definitions, namely that patients do not report all of their exacerbations (as discussed above) and the use of an event based definition underestimates exacerbation frequency. Also, hospital admission is dependent upon patient co-morbidities and social circumstances, and practise varies within different countries; certainly there is increasing effort in this country to keep individuals with COPD out of hospital. Patient social support may also allow avoidance of the healthcare system. However, a HCU definition is a set definition and so helps to standardise the definition of exacerbation in different areas.

HCU definitions have been increasingly used in an attempt to circumvent the problems associated with identifying and defining symptoms or groups of symptoms, and simply capture all patients whose condition has changed enough to require hospitalization or a change of treatment (generally a requirement for oral steroids or antibiotics). This method is straightforward, and is therefore widely used in clinical trials. HCU definitions do, however, require a sequence of decision-making involving both the patient and the doctor and is likely to select a distinct patient group. However, in the absence of definitive signs and symptoms on which to base a diagnosis, it could be argued that HCU definitions currently represent the most unambiguous and practical approach to clearly identifying episodes of exacerbation.

1.2.5 Grading Exacerbation severity

Anthonisen (Anthonisen 1994) provided one of the earlier definitions both identifying and classifying exacerbations when testing the benefits of antibiotic therapy, and his

exacerbation grading definitions are still in use today. His definition has been adapted by the London COPD cohort and forms the basis of the definition of exacerbation used in this thesis; the full definition of exacerbation used for this work is in the methodology section (***Chapter 3***). Anthonisen graded exacerbations into type 1 (all three symptoms of increased dyspnoea, sputum volume and sputum purulence), type 2 (two of the above symptoms), and type 3 (one of the above symptoms plus one of the following: (an upper respiratory tract infection in the past 5 days, fever without other cause, increased wheezing or cough, or an increase in heart rate or respiratory rate by 20% compared with baseline readings). Severity as defined by health care utilisation has been discussed above, and exacerbation severity can also be graded using this method; moderate for an unscheduled clinic visit (Rodriguez-Roisin 2000) or severe if requiring hospitalisation (Calverley 2005).

The NICE guidelines (Thorax 2004) also provide criteria for assessing the severity of an exacerbation. They state that some exacerbations are mild and self-limiting, frequently managed by patients at home without consulting healthcare professionals. Whereas other exacerbations are severe, carry a risk of death and require hospitalisation. They go on to describe the following features that may be present at a severe exacerbation: marked dyspnoea, tachypnoea, purse lip breathing, use of accessory muscles (sternomastoid and abdominal) at rest, acute confusion, new onset cyanosis, new onset peripheral oedema and marked reduction in activities of daily living.

1.2.6 Differential diagnosis of an exacerbation

There are a number of other conditions that may cause increased breathlessness or cough; two standard symptoms included widely in definitions of exacerbation of COPD, which are not always caused by an exacerbation *per se*, but may be due to other conditions such as a pneumothorax, pulmonary embolus, or cardiac failure (Hurst 2007). Co-morbidities may also confound the diagnosis of exacerbation and the management.

1.3 Exacerbation Frequency

There is no agreed definition of a frequent exacerbator, and depending upon the definition of exacerbation used the annual rate of acute exacerbation of COPD changes. Exacerbations are not normally distributed, defining their initiation and duration is complicated and this leads to various statistical issues (Keene 2009 (b), Suissa 2006). In several studies frequent exacerbators have been defined as those with yearly exacerbation rates greater than the median for the study; usually around three symptom-defined exacerbations per year or two per year if the exacerbation is defined using a HCU definition. In a study by Burge (Burge 2000), the median exacerbation rate was 0.99 -1.32 per person /year and in a study by Donaldson (Donaldson 2003), the median 2.52 per person / year. In a study by Miravitlles (Miravitlles 2000), 55% of patients were considered frequent exacerbators with more than 2 acute exacerbations in the previous year, whereas in a study by Cote (Cote 2007), 63% of a cohort was classified as frequent exacerbators defined as more than 1 exacerbation in the past year.

The difficulty with using median split as a method for determining exacerbation frequency is that the median is invariably not a whole number, and patients are split on partial numbers of exacerbations, which in reality does not happen. It also depends on how the denominator is set; if patients have number of days of exacerbation symptoms divided by number of days of diary card data and patients are away and well and have not recorded their diary cards every day but these days are not included, this artificially increases the exacerbation frequency. Whole numbers of exacerbations have been counted in this thesis, and full details of the methodology for determining exacerbation frequency can be found in ***Chapter 3***.

1.3.1 Frequent exacerbator phenotype

On the whole, exacerbations become more frequent and more severe as the severity of the underlying COPD increases (Burge 2003). However, there are large differences in yearly exacerbation incidence rates between patients of similar COPD severity (Seemungal 1998), and one of the clearest factors predictive of frequent exacerbations is a high number of exacerbations in the previous year (Seemungal 1998). So it appears that some patients with COPD are particularly susceptible to exacerbations, and this

susceptibility occurs across all disease severities (Anzueto 2009), however the basis of this susceptibility is not clear. Understanding the mechanisms underlying this is important in the development of new therapeutic strategies, and in the prevention of exacerbations and reduction in their severity.

In the same way that genes and environment influence stable COPD, it is logical to approach identifying determinants of exacerbation frequency in a similar fashion.

1.3.2 Characteristics of exacerbation frequency; who are the frequent exacerbators?

What we know…..

There are certain risk factors known to be associated with more frequent exacerbations; higher daily cough and sputum production (Burgel 2009), faster FEV_1 decline (Donaldson 2002, Kanner 2001), chronic dyspnoea and wheeze, chronic mucus hypersecretion (Zheng 2008) and bacterial colonisation (Patel 2002). Frequent exacerbators also have worse health - related quality of life (Seemungal 1998), a greater degree of airway inflammation with increased airway IL-6 and IL-8 levels (Bhowmik 2000), and more severe exacerbations associated with an increased risk of hospital admission. Frequent exacerbators have a faster decline in daily activity over time and are more likely to become housebound. Exacerbations are associated with a decline in outdoor activity for up to 5 weeks after the onset of symptoms (Donaldson 2005).
A multicenter study in France (Burgel 2009) of 433 COPD patients compared those with and without chronic cough and sputum production and found that subjects with chronic cough and sputum production had increased total mean numbers of exacerbations per patient per year (2.20 ± 2.20 vs 0.97 ± 1.19), moderate exacerbations (1.80 ± 2.07 vs 0.66 ± 0.85), and severe exacerbations requiring hospitalizations (0.43 ± 0.95 vs 0.22 ± 0.56). They also found that frequent exacerbations (two or more per patient per year) occurred in 55% vs 22% of subjects, respectively, with and without chronic cough and sputum production.

Donaldson *et al* monitored peak expiratory flow (PEF) and symptoms over a 4 year period in 109 patients with COPD. Patients were defined as frequent exacerbators if they had > 2.92 exacerbations per year and infrequent if they had < 2.92 per year.

Donaldson showed that patients with frequent exacerbations had a significantly faster decline in FEV_1 and peak expiratory flow (PEF) than infrequent exacerbators and that frequent exacerbators also had a greater decline in FEV_1 if allowance was made for smoking status suggesting that the frequency of exacerbations contributes to long term decline in lung function of patients with moderate to severe COPD (Donaldson 2002).

Kanner in 2001 analyzed Lung Health Study (LHS) data on 5887 individuals aged 35 to 60 and found that chronic bronchitis was associated with increased frequencies of lower respiratory illness and that smoking and lower respiratory illness had an interactive effect on FEV_1 in people with mild COPD.

In 2008, Zheng *et al* conducted a randomised, double-blind, placebo-controlled study of carbocysteine in 709 patients from 22 centres in China. Patients recruited had a history of at least 2 COPD exacerbations in the previous 2 years. They found that numbers of exacerbations per patient per year declined significantly in those patients receiving carbocisteine compared with the placebo group.

In 2002, Patel *et al* studied 29 COPD patients of whom 15 (51.7%) were colonised with a possible pathogen (*Haemophilus influenzae* (53.3%), *Streptococcus pneumoniae* (33.3%), *Haemophilus parainfluenzae* (20%), *Branhamella catarrhalis* (20%), *Pseudomonas aeruginosa* (20%)). The presence of lower airway bacterial colonisation in the stable state was related to exacerbation frequency.

In 1998 Seemungal *et al* using the median number of exacerbations as the cut-off point, classified patients as infrequent exacerbators (0 to 2) or frequent exacerbators (3 to 8) and showed that the SGRQ Total and component scores were significantly worse in the group that had frequent exacerbations: SGRQ Total score (mean difference = 14.8), Symptoms (23.1), Activities (12.2), Impacts (13.9). They also showed daily cough, cough and sputum and wheeze to be predictive of frequent exacerbations suggesting therefore that quality of life is related to COPD exacerbation frequency.

Frequent exacerbators have also been shown to have higher baseline sputum cytokine levels. In a study of induced sputum in 57 patients with moderate to severe COPD,

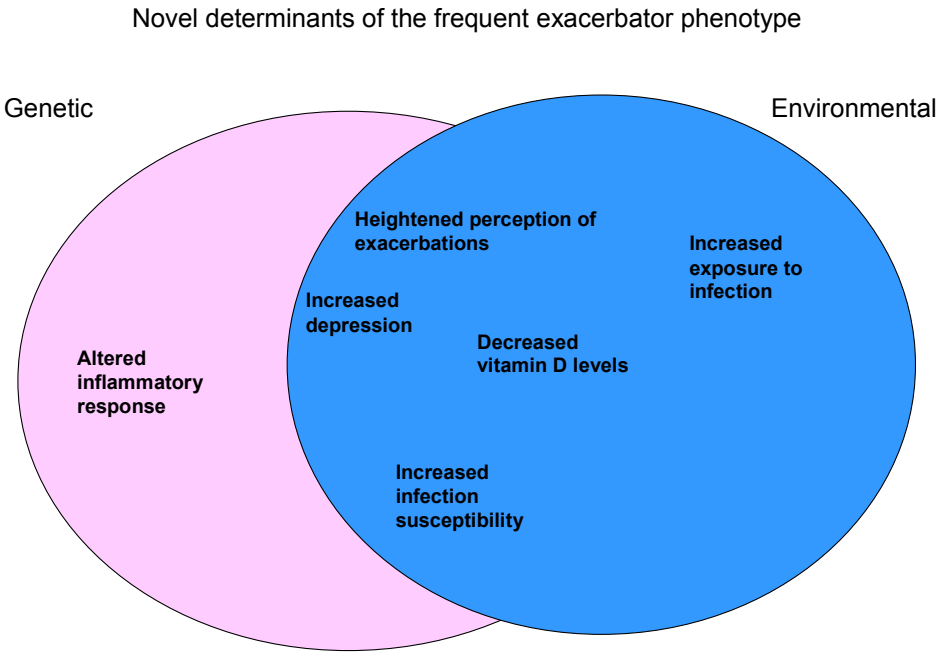
patients with ≥ 3 exacerbations per year were shown to have higher median stable sputum levels of IL-6 and IL-8 compared to those with ⩽2 exacerbations/year (Bhowmik 2000). Frequent exacerbators also have a smaller reduction in systemic inflammation between exacerbation onset and day 35 compared with infrequent exacerbators (Perera 2007), with non-recovery of symptoms at exacerbation associated with persistently heightened systemic inflammation. Perera *et al* also showed that a high CRP 14 days after an exacerbation can be used as a predictor of recurrent exacerbations within 50 days.

Donaldson *et al* in 2005 studied a rolling cohort of 147 patients over an 8-year period and found that time outdoors decreased by –0.16 hours/day per year. This decline was faster in frequent exacerbators. Subsequently frequent exacerbators have been found to be more depressed in the stable state, and this will be discussed in ***Chapter 6*** of this thesis.

What we don't know…..

A group of individuals exist across all disease severities in COPD who appear to be more susceptible to exacerbations. Although certain clinical characteristics have been associated with this group, clear risk factors have not been established. This thesis will cover some novel determinants that may be important in the development of the frequent exacerbator phenotype. A general introduction specific to each of these factors will be given in the relevant chapters, but an overview is given in **Figure 1.1**;

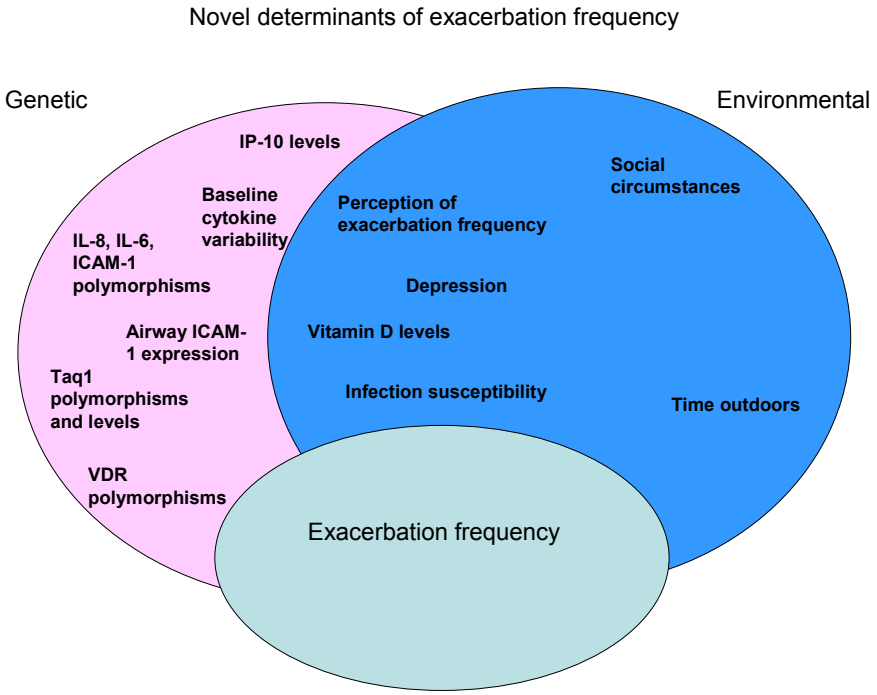
Figure 1.1:



As with any phenotype, there is likely to be complex interaction between genes and environment. For example, decreased time spent outdoors may result in decreased sunlight exposure which in turn results in decreased vitamin D levels thus increasing susceptibility to infection. Equally polymorphisms in the vitamin D receptor may influence vitamin D levels thus increasing risk of infection or the polymorphisms themselves via the immunomodulatory properties of vitamin D may increase risk.

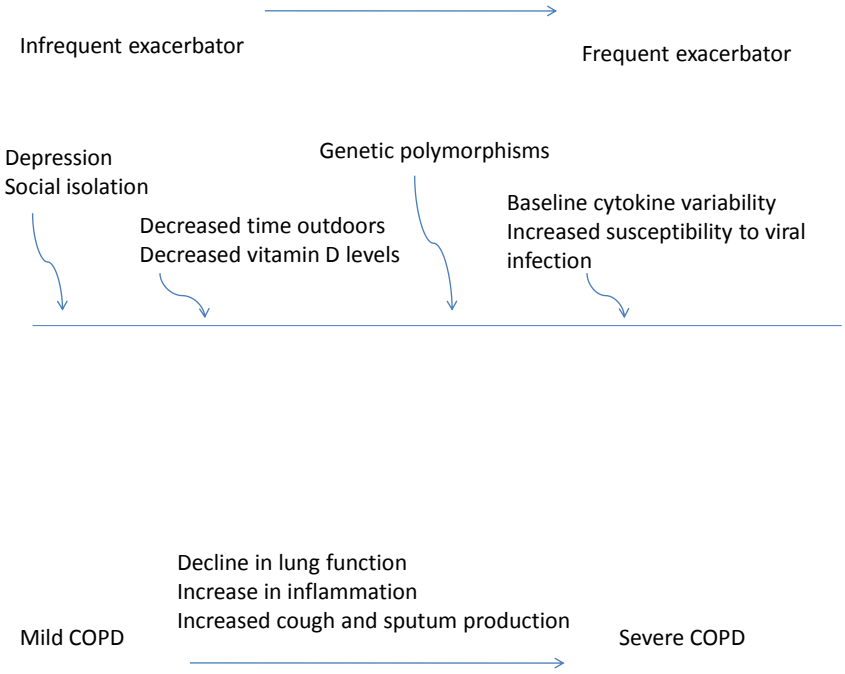
Each of the topics listed in the diagram above can be further subdivided and are likely to interact. This has been illustrated in **Figure 1.2.**

Figure 1.2:



Factors that influence exacerbation frequency are likely to vary depending upon disease severity as underlying inflammatory processes change. A possible scheme has been shown below in **Figure 1.3**:

Figure 1.3:



Chapter 2 provides the overlying hypothesis of this thesis and the specific aims.

2

Hypothesis & Aims

2.1 Hypothesis

This thesis tests the hypothesis that the frequent exacerbator is a distinct phenotype. This hypothesis is tested on an *environmental* level, patients with frequent exacerbations of COPD have better perception of their exacerbations, are more likely to be depressed, have decreased levels of vitamin D, increased contact with children and an increased incidence of HRV infection in the stable state and at exacerbation.

On a *genetic* level, frequent exacerbators are more susceptible to HRV infection and have higher viral loads, and this susceptibility is mediated through up regulation of ICAM-1 in the airway. Also, increased baseline cytokine variability decreases host defence, thus increasing susceptibility to infection, IP-10 levels (a marker of HRV infection) are higher in the airway and systemically, and there is a difference in specific genetic polymorphisms of ICAM-1, IL-6, IL-8, alpha one anti-trypsin and the vitamin D receptor between frequent and infrequent exacerbator groups.

2.2 Aims

1. To investigate whether patients with COPD when interviewed can accurately recall the number of exacerbations they have had in the past year, and this number used to stratify them into frequent and infrequent exacerbators groups. ***Chapter 5***

2. To determine if frequent exacerbators are more depressed in the stable state or at exacerbation than infrequent exacerbators and whether this depression increases symptom recognition and thus exacerbation frequency. ***Chapter 6***

3. To investigate whether contact with children effects acquisition of HRV in the stable state and whether this effects exacerbation frequency. ***Chapter 7***

4. To determine if vitamin D levels are lower in frequent exacerbators as a result of decreased time spent outdoors and decreased sunlight exposure, and whether these lower levels increase susceptibility to viral infection, thus increasing exacerbation frequency. To investigate whether vitamin D receptor polymorphisms influence exacerbation frequency or vitamin D levels. ***Chapter 8***

5. To investigate whether frequent exacerbators are more likely to have the rare TaqI α 11478G>A allele than infrequent exacerbators and are unable to upregulate α1AT acutely at exacerbation. ***Chapter 9.1***

6. To investigate whether the ICAM-1 K469E promoter polymorphism, the -251A>T and -781C>T IL-8 polymorphisms and the -174G>C, -597G>A or -572G>C IL-6 polymorphisms play a role in exacerbation frequency in COPD. ***Chapter 9.2***

7. To investigate baseline cytokine variability in sputum in IL-6, sICAM-1, IL-8 and in blood in CRP, sICAM-1, IL-8 and IL-6 between frequent and infrequent exacerbators. ***Chapter 9.3***

8. To determine if HRV is more prevalent in the stable state in frequent than infrequent exacerbators and to investigate differences in HRV load in the stable state and at exacerbation between frequent and infrequent exacerbators. ***Chapter 10.1***

9. To assess differences in serum and sputum IP-10 in frequent and infrequent exacerbators at baseline and at exacerbation. ***Chapter 10.2***

3

Materials and Methods

3.1 Patient selection and recruitment

COPD patients

The London COPD cohort is a rolling cohort of approximately 200 well characterised COPD patients who are followed up at regular intervals when stable, and also taught to report exacerbations to the study team. Patients are included in the cohort if they have COPD as defined by a forced expiratory volume in one second (FEV_1) of $\leq$ 80% and FEV_1 to forced vital capacity (FVC) ratio below 70% with β_2 agonist reversibility of less than 15% or 200ml. They are all past or present cigarette smokers and have had no antibiotics or oral steroids during the run-in period of six weeks prior to recruitment. Patients are excluded if they have a history of other significant respiratory disease.

Patients are also excluded if they are not able to complete daily diary cards, or they are housebound and not able or willing to attend for routine research clinic appointments. At enrolment into the study, data is collected on smoking status and history, daily respiratory symptoms; including sputum production, cough and nasal symptoms, co-morbidities, medications, and influenza vaccination. A detailed social history is taken including; marital status, number of occupants indoors, number of times out of the house per week (self reported hours outdoors each day), number of visitors, and frequency of contact with children under the age of 16 and the ages of the children. Details regarding occupational history are collected and family history, with particular reference to COPD.

Patients are also asked to recall the number of COPD exacerbations they have had in the preceding 12 months and the number of COPD hospitalisations in the past year. They are specifically asked about the dates of their last exacerbation and the treatment required.

Spirometry is performed (Vitalograph 2160, Maids Moreton, Buckingham, UK) and height and weight measured. Patients are asked to complete a Center for Epidemiological Studies Depression Scale (CES-D) (*Appendix 1*), a Medical Research Council (MRC) dyspnoea scale, and a health-related quality of life questionnaire; the St. George's Respiratory Questionnaire (SGRQ) (Jones 1992). Blood is collected for genetics and cytokine assay, and sputum is collected if spontaneously produced. A nasopharyngeal swab is also taken.

At recruitment patients are taught how to record on diary cards their daily peak flow, and any increase over their normal, stable condition symptoms of dyspnoea, sputum purulence or sputum volume and colds (nasal discharge/congestion), wheeze, sore throat, cough or fever. They are also asked to document any changes in therapy and hours spent outdoors each day. Patients are reviewed every three months in the study clinic over the first year and six monthly thereafter when stable to monitor compliance with data collection, and record changes in medication and baseline lung function. Daily diary cards are collected and checked for changes in symptoms, monitoring of morning peak flow treatment changes (if any). An example of a diary card is given in *Appendix 2*.

Information on time spent outdoors has been used in the depression (***Chapter 6***), social (***Chapter 7***) and vitamin D chapters (***Chapter 8***). In the depression chapter, the time outdoors for baseline and exacerbation visits is calculated as the average time spent outdoors from three days before the day the patient attended the clinic, to three days after. A seven day period is used to eliminate any variation due to specific days of the week. For the vitamin D chapter time outdoors is calculated as the average time spent outdoors in the 14 days preceding the clinic visit. For the social chapter, number of days out of the house in an average week was used.

Patients are trained to report exacerbations to the research team as soon as possible after onset of symptoms and before treatment is started. Exacerbations are usually treated with either antibiotics and/or oral corticosteroids according to the clinical severity.

At each routine baseline visit and at an exacerbation visit, details regarding symptoms and medication are recorded and spirometry is done. A nasopharyngeal swab and sputum sample are collected, and blood is taken for cytokine analysis.

Information was not recorded on social status but the majority of patients participating were retired.

Control subjects

Control subjects were recruited from a local primary care practice, and were similar in age to the COPD patients. Social status of the controls was not documented, and there was no attempt to match individuals for smoking history. Data were collected at recruitment as described above. All control subjects had an FEV_1 above 80% predicted, and an FEV_1/FVC ratio above 70% predicted. They were excluded if they had any other significant respiratory disease. Control subjects were asked to report colds to the study group as soon as possible after onset. At this time a nasopharyngeal swab was taken and blood collected for cytokine analysis.

Due to the nature of translational research, there is a slightly different cohort of individuals used in each chapter, and although the overall demographics of the cohort are discussed in ***Chapter 4***, different demographics applicable to each chapter are also given in the relevant sections.

3.2 Ethics and Consent

This study was approved by the Royal Free Hospital Research Ethics Committee and patients gave written informed consent.

3.3 Questionnaires

St. George's Respiratory Questionnaire (SGRQ)

The SGRQ is designed to measure health impairment in patients with COPD (Jones 1992). Scores are weighted according to Jones 1992, and a Total score is calculated which summarises the impact of the disease on overall health status, and three component scores are calculated; symptoms, activity and impact. Scores are expressed as a percentage of overall impairment where 100 represents worst possible health status and 0 indicates best possible health status.

Centre for Epidemiologic Studies Depression Scale (CES-D)

The CES-D is a 20 item questionnaire which assesses the frequency and duration of symptoms associated with depression (Radloff 1977). This score has been validated to measure change and improvements in depressive symptoms. A score of 16 or higher using this score classifies a person as having depressive symptoms, validated with DSM-IV criteria for clinical depression (Burnham 1988). This questionnaire has been used to investigate depressive symptoms in chronic diseases, including COPD (van Manen 2002). The maximum score attainable with this questionnaire is 60.

MRC Dyspnoea scale (Bestall 1999)

This scale has been in use for many years for grading the effect of breathlessness on daily activities (Fletcher 1960). It measures perceived respiratory disability, the WHO definition of disability being "any restriction or lack of ability to perform an activity in the manner or within the range considered normal for a human being". The MRC dyspnoea scale consists of five statements about perceived breathlessness: grade 1, "I only get breathless with strenuous exercise"; grade 2, "I get short of breath when hurrying on the level or up a slight hill"; grade 3, "I walk slower than people of the same age on the level because of breathlessness or have to stop for breath when walking at my own pace on the level"; grade 4, "I stop for breath after walking 100 yards or after a few minutes on the level"; grade 5, " I am too breathless to leave the house". Patients select the grade that applies to them.

3.4 Exacerbation definition

The definition of exacerbation and the use of diary cards for capturing symptom defined exacerbations has been discussed in ***Chapter 1***. The methodology for determining exacerbations in this thesis relies upon the use of daily symptom reporting on diary cards to first establish a baseline level of symptoms for the patient and then to detect any change, which is indicative of an exacerbation. However, if there is misunderstanding and daily symptoms are recorded, this baseline symptom does not automatically contribute towards the exacerbation symptom definition.

The London COPD cohort uses an established and validated definition of an exacerbation (Donaldson 2002, Powrie 2006, Seemungal 2008, Hurst 2009). An exacerbation is defined as two or more of three major symptoms; increase in dyspnoea, increase in sputum purulence and increase in sputum volume or any one major symptom together with anyone of the following minor symptoms; increase in nasal discharge, wheeze, sore throat, cough or fever. The symptoms must be recorded for at least two consecutive days. In addition, an exacerbation can be described if in the opinion of the clinician seeing the patient, the patient has an exacerbation. This is important in situations where patients attend the clinic and only have 1 symptom recorded on their diary card, but on direct questioning clearly have more symptoms, and require an increase in treatment. It also covers the situation where a patient has only recorded one or no symptoms but has been to see another clinician and been prescribed antibiotics +/- steroids or has self-medicated and recorded treatment only. Although it could be argued that in the case of self medication the patient may be treating events as exacerbations that physicians would not classically define as exacerbations, one has to wonder why they chose to increase their medication at that time. These are effectively "unreported" exacerbations and are important as discussed in ***Chapter 1***.

Under this definition, events are not called exacerbations if the patient has recorded 1 symptom but does not require an increase in treatment or visit to a clinician. All exacerbations are prospectively captured. Symptoms are binary coded and summed to give a daily symptom score. Exacerbation severity is based upon this symptom count as well as time outdoors, peak expiratory flow and FEV_1 change.

3.5 Determination of Exacerbation Frequency

As discussed in ***Chapter 1***, there is no agreed definition of a frequent exacerbator. For this research, exacerbations are symptom defined, and both reported and unreported events are captured on diary cards. Patients are defined as "frequent exacerbators" if they have three or more exacerbations in the year, and "infrequent exacerbators" if they have less than three exacerbations in the previous year. For those patients with 1 years worth of diary card data, the exacerbation frequency has been determined from counting the number of exacerbations recorded on diary cards over the 1 year period. This allows adjustment for seasonality. For those in less than 1 year (mostly in the genetics chapters; ***Chapter 8, 9.1 and 9.2***), exacerbation frequency has been determined from the number of exacerbations reported at study recruitment. Comparison of these methods and the stability of exacerbation frequency from one year to the next are discussed in detail in ***Chapter 5***. For those patients followed up for 2 years, the average number of exacerbations over the 2 year period has been calculated and those patients with ≥ 3 exacerbations per year classified as frequent and < 3 infrequent exacerbators. This ensures that a particular patient has the same exacerbation frequency used throughout the thesis.

3.6 Sample collection and processing

Blood

Venous blood samples were collected at recruitment for genetics analysis and at each baseline and exacerbation visit for cytokine analysis. For genetics analysis, 3x 6ml BD Vacutainer® Plus Plastic K2EDTA Tubes (Becton, Dickinson and Company) were collected and stored at -80^{o}C prior to DNA extraction. For cytokine analysis, 10mls of venous blood was collected into a 10.0 ml BD Vacutainer® SST II Plus plastic serum tube (Becton, Dickinson and Company) and centrifuged at 224xg for 10 minutes at 4°C within two hours of collection. The serum was then separated and stored at -80°C for later analysis.

5 millilitres of blood was collected into a 5.0 ml BD Vacutainer® SST II Plus plastic serum tube (Becton, Dickinson and Company) and sent directly to the Clinical Biochemistry Department at the Royal Free Hospital for CRP measurement.

CRP measurement

This was carried out using a Tina-quant C-Reactive protein (Latex) method (Roche/Hitachi) in the clinical biochemistry department at the Royal Free hospital. Briefly, this test uses a particle-enhanced immunoturbidimetric assay. Anti-CRP antibodies coupled to latex microparticles react with antigen in the sample to form an antigen/antibody complex. Following agglutination, this is measured turbidimetrically.

Vitamin D measurement

This was also carried out in the clinical biochemistry department at the Royal Free hospital. A 0.5ml frozen serum aliquot was defrosted and placed into a 5ml glass tube (Sarstedt). Samples were assayed using the LIAISON 25-OH Vitamin D TOTAL (DiaSorin, Italy), a fully automated antibody-based chemiluminescence assay. The assay recognises 100% 25-OH Vitamin D2 and 25-OH Vitamin D3 using magnetic micropeptide separation. The limit of detection is $\leq$ 4.0 ng/ml.

Sputum

Patients were instructed to blow their nose and rinse their mouth out with water before expectorating sputum into a sterile pot. This sample was then separated into two. Sputum samples were processed as soon as possible within two hours of collection. One sample was taken to the Microbiology department at the Royal Free Hospital for processing (see **section 3.11**). The other sample was separated from contaminating saliva, and the remainder processed using an adapted process from previously published methods (Bhowmik 1998). The weight of the total sample was recorded. The sputum was then separated from contaminating saliva by macroscopic examination using a pair of disposable plastic forceps. The selected portion of the sputum was placed in a pre-weighed 15ml polypropylene tube and the weight of the selected portion of the sputum recorded. The sputum was then mixed with nine times its weight of phosphate buffered saline (PBS) and agitated with silicanised 1-2mm diameter glass beads (332124G; VWR International Ltd, Poole, UK) to homogenise the sample. The tube was initially vortexed for 15 seconds and then rocked for 15 minutes. 0.5ml aliquots were stored at -80^{o}C until ready for virology processing.

The remainder of the sputum sample in PBS was filtered through 50 µm nylon gauze to remove mucus and debris without removing any of the cells and centrifuged at 790*g* (2000 rpm) for 10 minutes. This resulted in the formation of a cell pellet and a supernatant solution. The supernatant was decanted off and stored at -80°C for future cytokine analysis and the cell pellet discarded.

Upper airway samples

Patients were instructed to blow their nose prior to a swab being passed gently through the nose towards the posterior nasopharynx. The swab (APTACA sterile transport swab, Nuova, Italy) was then rotated on the nasopharyngeal membrane 5-6 times and allowed to remain in place for 10 seconds. The swab was then immediately placed in an eppendorf containing 0.5mls PBS and stored at -80°C until ribonucleic acid (RNA) was extracted.

Biopsy Collection

Biopsies were collected from routine bronchoscopies that COPD patients or non-COPD patients undewent for clinical indications (Patel 2003) according to BTS guidelines (2001).

Bronchoscopy was performed under light sedation with midazolam and local aneasthesia with lignocaine. Endobronchial biopsies were taken using endojaw/endotherapy disposable biopsy forceps (Olympus Medical systems corp model no FB241D) at the level of a segmental or sub-segmental carina on the side opposite to any radiological abnormality precipitating the bronchoscopy. Between 2 and 3 specimens were taken from the lower airway and immediately placed in ice-cold Medium 199 (Sigma, UK) containing Gentamicin (Cidomycin 80mg/2ml; 50µl per 20 ml, Aventis). Each biopsy specimen was processed for tissue culture within 30 minutes of collection.

3.7 Immunological Assays

Enzyme-Linked ImmunoSorbent Assay (ELISA)

Principles of ELISA

This is a technique used to detect the presence of an antibody or an antigen in a sample. An unknown amount of antigen is fixed to a surface and a specific antibody washed over the surface that can bind to the antigen. This antibody is linked to an enzyme, and in the final step a substance is added that the enzyme can convert to a detectable signal; usually fluorescence. When light of the appropriate wavelength is shone through the sample, any antigen/antibody complexes fluoresce so that the amount of antigen in the sample can be inferred through the magnitude of the fluorescence.

All of the ELISA kits used in this thesis involved a sandwich ELISA. A monoclonal antibody specific to the cytokine to be detected was pre-coated onto a microplate. Either standard or sample was then pipetted into the well and any cytokine present bound by the immobilized antibody. After washing away unbound substances, an enzyme linked polyclonal antibody specific for the cytokine was added to the well. Following washing to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and colour developed in proportion to the amount of cytokine bound in the initial step. Colour development was stopped and the intensity of colour measured.

Experimental protocols

A list of ELISAs performed is given in the table below (**Table 3.1**) and details specific to each ELISA in the subsequent sections.

Table 3.1: List of ELISA kits

ELISA	Kit	Company	Substance tested
Quantikine Human IL-6	D6050	R&D systems Abingdon, UK	Serum, sputum
Human sICAM-1	BBE 1B	R&D systems Abingdon, UK	Serum, sputum
Quantikine Human CXCL8/IL-8	D8000C	R&D systems Abingdon, UK	Serum, sputum
Quantikine Human CXCL10/IP-10	DIP100	R&D systems Abingdon, UK	Serum, sputum, cell supernatent
α_1 Antitrypsin ELISA kit	K6750	Immundiagnostik, Biosupply, UK	Serum

Validity of assays

On each ELISA plate all standard curve dilutions were run in duplicate and results pooled. Several samples were also run in duplicate on each plate to ensure reproducibility of the results. When multiple plates were required for a particular cytokine, 2 or 3 samples were run on several different plates to ensure reproducibility. Where publications using the kits for sputum were not available, validation experiments were done to optimise dilution and to ensure accuracy of results. Spiking experiments were also done for some of the sputum ELISAs to determine the optimum dilution needed to minimise inhibition. These experiments have been described in the appropriate cytokine sections below.

IL-6

Serum samples were not diluted and were added neat. Sputum samples were diluted 1:2 prior to ELISA by diluting with calibrator diluent. This assay has been used for both sputum and serum previously and various studies published using this methodology (Bhowmik 2000, Seemungal 2001).

Briefly, wash buffer was prepared by adding 20mL of wash buffer concentrate to 500mL of sterile water. IL-6 standard was reconstituted with 5.0mL Calibrator diluent RD5T for sputum sample ELISAs and calibrator diluent RD6F for serum ELISAs. This produced a stock of 300pg/ml. After sitting for 15 minutes with gentle agitation the following dilutions (**Table 3.2**) were made to produce the standard curve.

Table 3.2: Standard curve dilutions for IL-6 ELISA

Dilution	Volume calibrator diluent (µl)	Volume standard (µl)
100 pg/ml	667	333 of 300 pg/ml stock
50 pg/ml	500	500 of 100 pg/ml stock
25 pg/ml	500	500 0f 50pg/ml stock
12 pg/ml	500	500 of 25 pg/ml stock
6.25 pg/ml	500	500 of 12.5 pg/ml stock
3.12 pg/ml	500	500 of 6.25 pg/ml stock
0 pg/ml	Appropriate calibrator diluents	

100µl of assay diluent RD1W was added to each well. 100µl of standard, sample or control was then added to each well and the plate covered with the adhesive strip and

left at room temperature for 2 hours. After 2 hours each well was aspirated and washed, repeating the process 3 times for a total of 4 washes. The plate was then inverted and blotted dry. 200µl of IL-6 conjugate was added to each well; the plate covered with an adhesive strip and left 2 hours at room temperature. The aspiration / wash step was repeated. 200µl of substrate solution was added to each well (equal volumes of colour reagents A and B mixed together within 15 minutes of use). The plate covered with an adhesive strip and left 20 minutes at room temperature in the dark. 50µl stop solution was then added to each well with the colour changing from blue to yellow. The optical density was then determined using a microplate reader (EL 340 Biokinetics reader) set to 450nm.

Samples with concentrations that were too high to be read were diluted with the appropriate calibrator diluent and read again. The standard curve was also diluted by the same amount to determine the exact dilution factor. This is not the preferred method; ideally samples should be repeated on a new ELISA and diluted prior to use, however, in some cases samples were limited and there was insufficient to repeat the analysis.

Calculation of results

The duplicate readings for each standard were averaged and the average zero standard optical density subtracted. A standard curve was plotted by plotting the $\log_{10}$ of the optical density the y axis and the $\log_{10}$ IL-6 concentration on the x axis and a best fit line drawn. Any diluted factors were then multiplied by the appropriate dilution factor. The limit of detection was 0.70 pg/ml. An example of a serum and sputum standard curve is shown below in **Figure 3.1** and **3.2.**

Figure 3.1: IL-6 Serum ELISA standard curve (13.08.08)

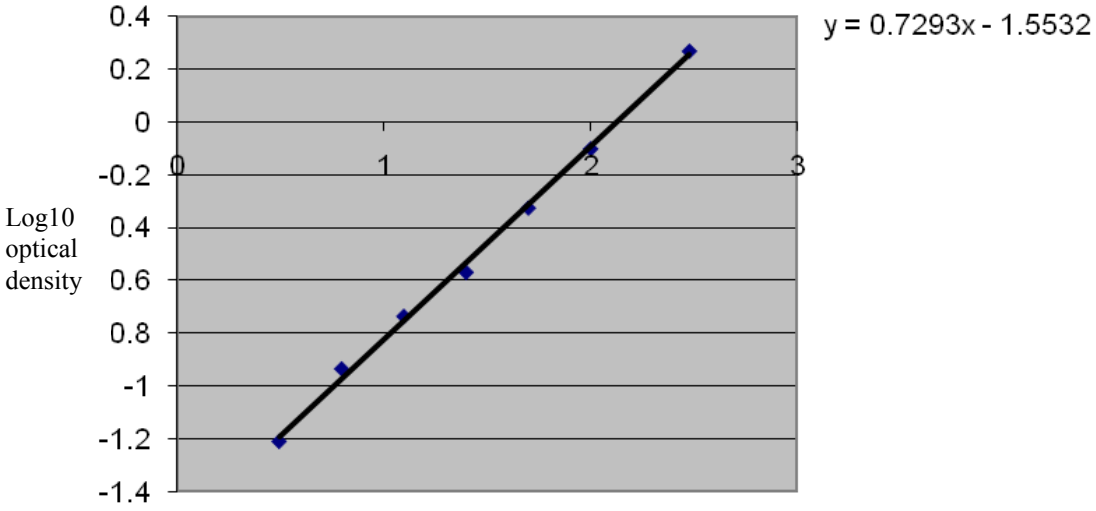
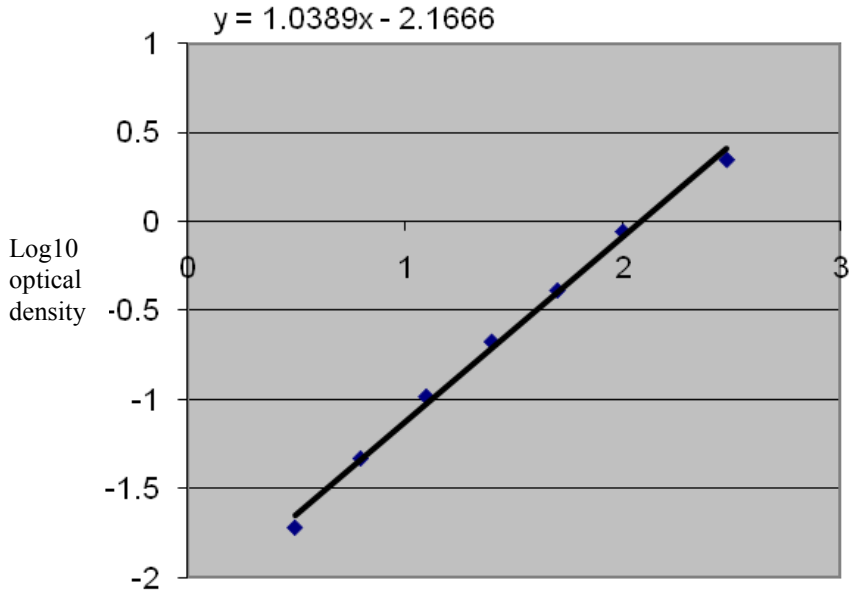


Figure 3.2: IL-6 Sputum ELISA standard curve (25.06.08)



soluble ICAM-1

This kit has been validated for serum but not sputum. Before use on sputum, the following validation experiment was carried out according to the manufacturer instructions. A 10x spiking stock solution was made by adding 100µl of sterile water to the highest concentration standard provided (standard 5). Standards were then made up

in the usual way by adding 1.0ml of sterile water to each standard provided; 1 to 5. The concentrations were stated on each vial and are below in **Table 3.3;**

Table 3.3: soluble ICAM-1 standard curve dilutions

Standard	Concentration ng/ml
0	0
1	2.23
2	9.29
3	17.61
4	32.49
5	45.26

Samples were made up as in **Table 3.4;**

Table 3.4: Samples for sICAM-1 ELISA validation protocol

Sample type	**1:2 dilution (A)**	**1:4 dilution (B)**	**1:8 dilution (C)**
1 ml neat sputum (1)	0.5ml sample (1) + 0.5 ml sample diluents	0.5ml A1 + 0.5 ml sample diluents	0.5ml B1 + 0.5 ml sample diluents
980ul sputum + 20ul spiking stock (2)	0.5ml sample (2) + 0.5 ml sample diluents	0.5ml A2 + 0.5 ml sample diluents	0.5ml B2 + 0.5 ml sample diluents
980 ul sample diluent + 20ul spiking stock (3)	0.5ml sample (3) + 0.5 ml sample diluents	0.5ml A3 + 0.5 ml sample diluents	0.5ml B3 + 0.5 ml sample diluents

The other reagents were prepared as follows. 20mL wash buffer was added to 500mL sterile water. 250µl of sICAM-1 conjugate was added to the conjugate diluent bottle and the bottle gently inverted. The ELISA control was reconstituted with 500µl sterile water and allowed to sit 10 minutes at room temperature. This was then diluted 20 fold (15µl control + 285µl sample diluent) prior to assay. The ELISA was carried out following the protocol as described below.

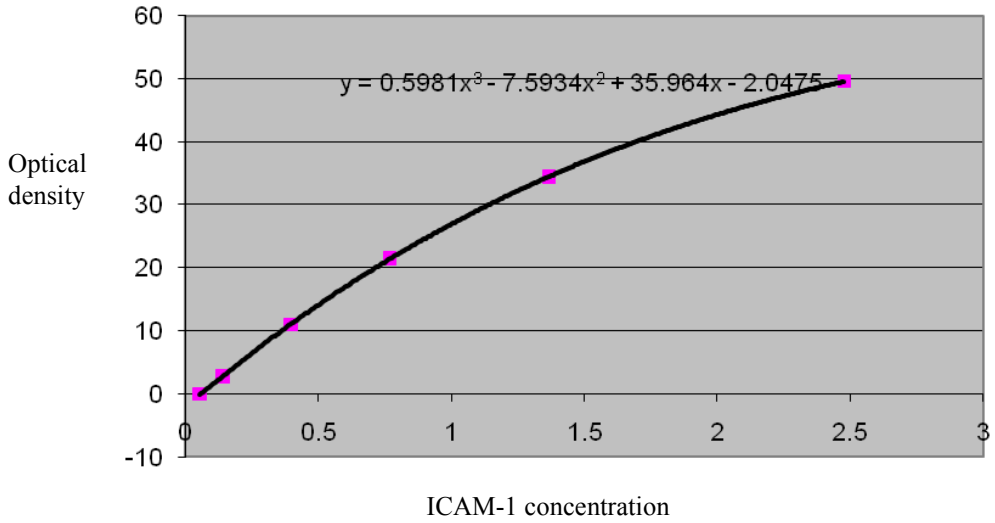
100µl of diluted conjugate was added to each well. 100µl of standard, sample or control was then added to each well with sufficient force to ensure mixing. The plate was

covered and incubated at room temperature 1.5 hours. Each well was aspirated and washed 5 times for a total of 6 washes. After the last wash the plate was inverted and tapped on clean paper towel to ensure all liquid was removed. 100µl substrate was then added to each well, the plate sealed and incubated at room temperature for 30 minutes. 100µl stop solution was then added to each well and the optical density determined using the microplate reader at 450 nm.

Calculation of results

The duplicate readings for each standard were averaged and the average zero standard optical density subtracted. A polynomial 3 curve was plotted in EXCEL (Windows 1997, Microsoft) with the optical density on the y axis and sICAM concentration on the x axis. The standard curve for the validation experiment is shown below (**Figure 3.3**).

Figure 3.3: sICAM-1 validation experiment standard curve (sputum)



Using the equation from the standard curve the concentrations for each sample were calculated. For validation purposes the following were then calculated:

1. The recovery of the spike / neat sample which should be in the range of 80 – 120% and was calculated as follows;

$$\% \text{ recovery} = \frac{\text{observed} - \text{neat}}{\text{expected}} \times 100$$

where; observed = spiked sample value
neat = unspiked sample value
expected = amount spiked into each sample

The recovery was calculated to be 80%.

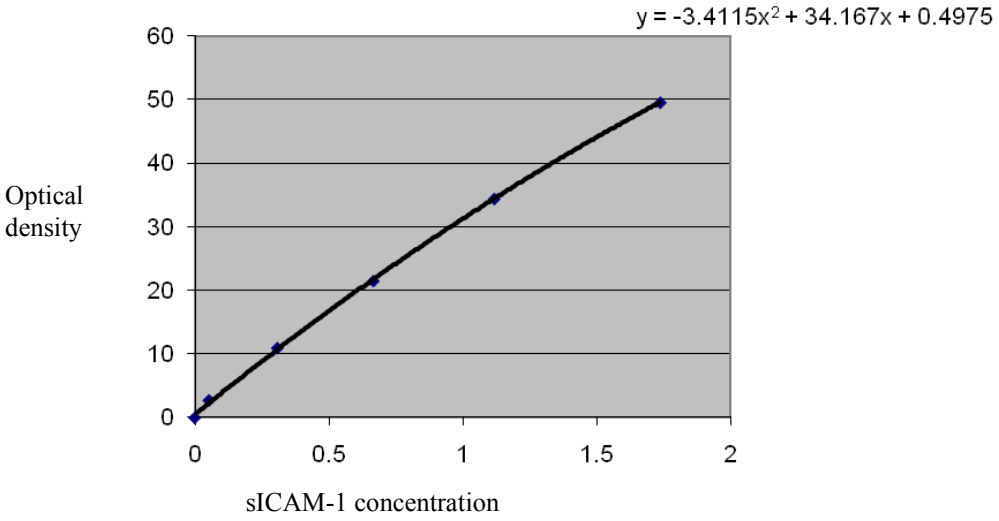
2. The Linearity was calculated to test for interference. This occurs when some component of a sample prevents the appropriate binding taking place, and can be a particular problem with sputum. Percent recovery is the measurement used and is calculated as the amount of material measured (observed) divided by the concentration expected. This was done by spiking the sample and assay diluent as described above. Recovery is calculated for each dilution using the equation above and the % recovery should be in the range of 80 to 120%.

- For the 1:2 dilution, recovery = 106%
- For the 1:4 dilution, recovery = 117%
- For the 1:8 dilution, recovery = 124%

With the recovery adequate for both parts of the validation experiment, the remaining sputum samples were run undiluted.

Serum samples were diluted 1 in 20 according to the manufacturer instructions; 15µl sample + 285µl sample diluent. The protocol was as described above. Below is an example of a standard curve (**Figure 3.4**). The limit of detection was 0.35ng/ml.

Figure 3.4: sICAM-1 serum ELISA standard curve



IL-8

Serum samples were not diluted and were added neat. Sputum samples were diluted 1:10 prior to ELISA by diluting with calibrator diluent RD5P (1x). This assay has been used for both sputum and serum previously and the methodology published (Bhowmik 2000, Wilkinson 2006,).

Briefly, wash buffer was prepared by adding 20mL of wash buffer concentrate to 500mL of sterile water. Calibrator diluent RD5P (1x) was made by adding calibrator diluent RD5P concentrate (5x) to 80ml sterile water to yield 100ml calibrator diluent (1x). IL-8 standard was reconstituted with 5.0mL Calibrator diluent RD5P (1x) for sputum sample ELISAs and calibrator diluent RD6Z for serum ELISAs. This produced a stock solution of 2000pg/ml. After sitting for 15 minutes with gentle agitation the following dilutions were made to produce the standard curve (**Table 3.5**).

Table 3.5: IL-8 standard curve dilutions

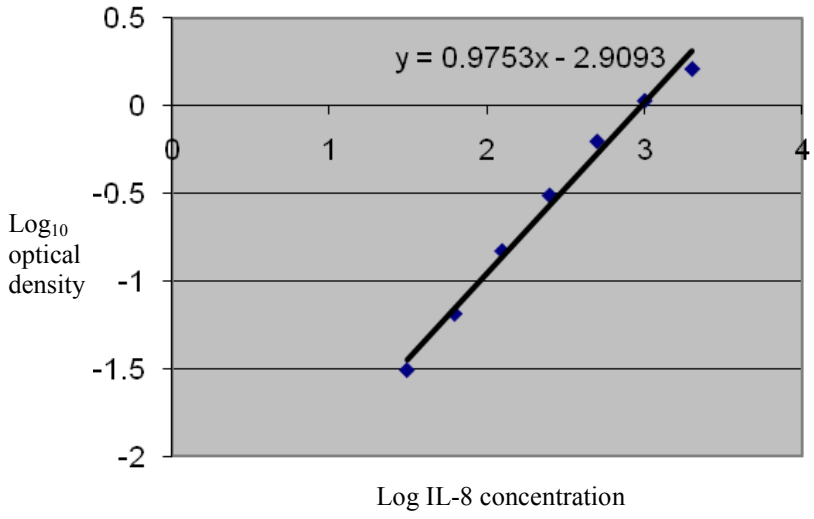
Dilution	Volume calibrator diluent (µl)	Volume standard (µl)
1000 pg/ml	500	500 of 2000 pg/ml stock
500 pg/ml	500	500 of 1000 pg/ml stock
250 pg/ml	500	500 of 500 pg/ml stock
125 pg/ml	500	500 of 250 pg/ml stock
62.5 pg/ml	500	500 of 125 pg/ml stock
31.2 pg/ml	500	500 of 62.5 pg/ml stock
0 pg/ml	Appropriate calibrator diluents	

100µl of assay diluent RD1-85 was added to each well. 50µl of standard, sample or control was then added to each well and the plate covered with the adhesive strip and left at room temperature for 2 hours. After 2 hours each well was aspirated and washed, repeating the process 3 times for a total of 4 washes. The plate was then inverted and blotted dry. 100µl of IL-8 conjugate was added to each well; the plate covered with an adhesive strip and left 1 hour at room temperature. The aspiration / wash step was repeated. 200µl of substrate solution was added to each well (equal volumes of colour reagents A and B mixed together within 15 minutes of use). The plate covered with an adhesive strip and left 30 minutes at room temperature in the dark. 50µl stop solution was then added to each well with the colour changing from blue to yellow. The optical density was then determined using the microplate reader set to 450nm. Samples with concentrations that were too high to be read were diluted with the appropriate calibrator diluent and read again. The standard curve was also diluted by the same amount to determine the exact dilution factor.

Calculation of results

The duplicate readings for each standard were averaged and the average zero standard optical density subtracted. A standard curve was plotted by plotting the $\log_{10}$ of the optical density the y axis and the $\log_{10}$ IL-8 concentration on the x axis and a best fit line drawn. Any diluted factors were then multiplied by the appropriate dilution factor. The limit of detection was 3.5pg/ml. An example of a standard curve is shown below (**Figure 3.5**).

Figure 3.5: Sputum IL-8 ELISA standard curve (24.06.08)



IP-10

This kit has been validated for serum but not sputum. Before use on sputum, a validation experiment was carried out as described above for sICAM-1. A 10x spiking stock solution was made by adding 100µl of sterile water to the IP-10 standard to provide a solution of 50ng/ml. Standards were then made up as per the ELISA protocol as below (**Table 3.6**). To make the 5000pg/ml stock the spiking solution was diluted 1:10.

Table 3.6: IP-10 standard curve dilutions

Dilution	Volume calibrator diluent (µl)	Volume standard (µl)
500 pg/ml	900	100 of 5000 pg/ml stock
250 pg/ml	500	500 of 5000 pg/ml stock
125 pg/ml	500	500 of 250 pg/ml stock
62.5 pg/ml	500	500 of 125 pg/ml stock
31.2 pg/ml	500	500 of 62.5 pg/ml stock
15.6 pg/ml	500	500 of 31.2 pg/ml stock
7.8 pg/ml	500	500 of 15.6pg/ml stock
0 pg/ml	Appropriate calibrator diluents	

Samples were made up as follows (**Table 3.7**);

Table 3.7: Samples for IP-10 ELISA validation protocol

Sample type	1:2 dilution (A)	1:4 dilution (B)	1:8 dilution (C)
1 ml neat sputum (1)	0.5ml sample (1) + 0.5 ml sample diluents	0.5ml A1 + 0.5 ml sample diluents	0.5ml B1 + 0.5 ml sample diluent
980ul sputum + 20ul spiking stock (2)	0.5ml sample (2) + 0.5 ml sample diluents	0.5ml A2 + 0.5 ml sample diluents	0.5ml B2 + 0.5 ml sample diluent
980 ul sample diluent + 20ul spiking stock (3)	0.5ml sample (3) + 0.5 ml sample diluents	0.5ml A3 + 0.5 ml sample diluents	0.5ml B3 + 0.5 ml sample diluent

The other reagents were prepared as follows. 20mL wash buffer was added to 500mL sterile water. Calibrator diluent RD5K was used for cell culture supernatant and sputum

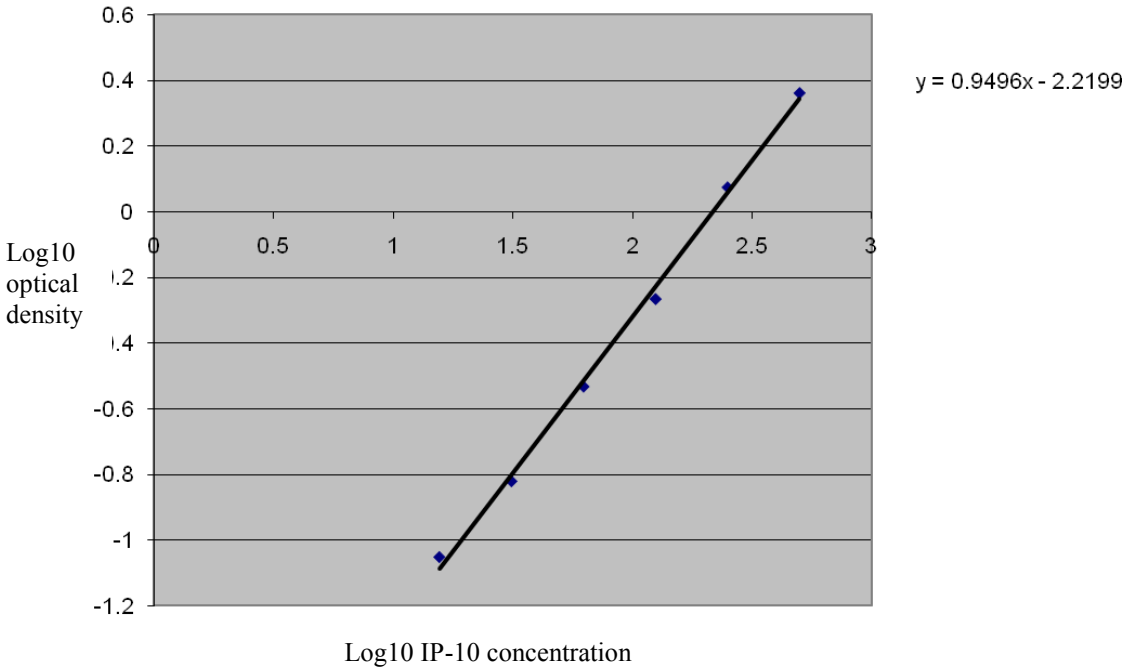
sample ELISAs and calibrator diluent RD6Q for serum ELISAs. The ELISA was then done following the protocol as described below.

For cell culture supernatant or sputum samples 150µl assay diluent RD1-56 was added to each well. For serum samples 75µl assay diluent was added. For cell culture / sputum samples 100µl of standard / control or sample was added to each well. For serum samples, 75µl standard, control or sample was added to each well. The plate was covered and incubated at room temperature for 2 hrs. Each well was aspirated and washed 3 times for a total of 4 washes. After the last wash the plate was inverted and tapped on clean paper towel to ensure all liquid was removed. 200µl of IP-10 conjugate was added to each well, the plate covered and incubated at room temperature for 2 hours. The wash step was repeated and then 200µl substrate solution added to each well. The plate was incubated at room temperature in the dark for 30 minutes. 50µl stop solution was then added to each well and the optical density determined using a microplate reader at 450 nm.

Calculation of results

The duplicate readings for each standard were averaged and the average zero standard optical density subtracted. A standard curve was plotted by plotting the $\log_{10}$ of the optical density the y axis and the $\log_{10}$ IP-10 concentration on the x axis and a best fit line drawn. Any diluted factors were then multiplied by the appropriate dilution factor. The limit of detection was 1.67pg/ml. The standard curve for the validation experiment is shown below (**Figure 3.6**)

Figure 3.6: IP-10 validation experiment standard curve



Using the equation from the standard curve the concentrations for each sample were calculated. Using the calculations described above for IP-10 the recovery and linearity were determined. The recovery for the spike / neat was 105%. For the linearity results:

- 1:2 dilution, recovery = 106%
- 1:4 dilution, recovery = 106%
- 1:8 dilution, recovery = 105%

With the recovery adequate for both parts of the validation experiment, the remaining sputum samples were run undiluted.

Cell supernatants were diluted 1:10. Serum samples were not diluted. Sputum samples were diluted 1:30. The protocol was as described above. Below is an example of a standard curve. The limit of detection was 1.67pg/ml.

α-1 antitrypsin

Wash buffer was made up by adding 100ml of wash buffer concentrate to 900ml sterile water. All samples were diluted 1:250,000 by diluting 10µl sample into 990µl wash buffer, then 10µl of this into 990µl wash buffer and 20µl this into 480µl wash buffer. A proportion of samples were run in duplicate and on separate plates. Each well on the

plate was first washed by dispensing 250µl wash buffer into each well and aspirating. This was repeated for a total of 5 washes. The plate was then inverted and tapped to remove any excess liquid. 100ul of standard, sample or control was added to each well. The standards were provided ready to use in concentrations of 0, 3.3, 10, 30 and 90µg/l. The plate was covered and left at room temperature on a horizontal mixer for 1 hour. The contents were then discarded and the plate aspirated / washed 5x with wash buffer. The plate was inverted and tapped to remove excess liquid. 100µl conjugate diluted 1:100 (50µl in 4950µl wash buffer) was added to each well. The plate was covered and left on a horizontal mixer at room temperature for 1 hour. The contents were discarded and the plate washed / aspirated 5x and excess liquid removed by inversion and tapping. 100µl substrate was added to each well and the plate incubated approximately 10 minutes in the dark at room temperature. As the colour change is temperature sensitive, the plate was monitored closely and the reaction stopped once good differentiation was observed. 50µl of stop solution was added to each well and the optical density determined using the microplate reader at 450 nm.

Calculation of results

The duplicate readings for each standard were averaged and the average zero standard optical density subtracted. A standard curve was plotted by plotting the $\log_{10}$ of the optical density the y axis and the $\log_{10}$ alpha one concentration on the x axis and a best fit line drawn. The sample results were multiplied by 250,000. For each of the ELISA plates the control sample result was in the range specified of 11.64 – 19.40 µg/l. The limit of detection was 1.8 mg/dl. A standard curve is shown in **Figure 3.7** and log transformed for analysis in **Figure 3.8**.

Figure 3.7: Standard curve for α-1 antitrypsin

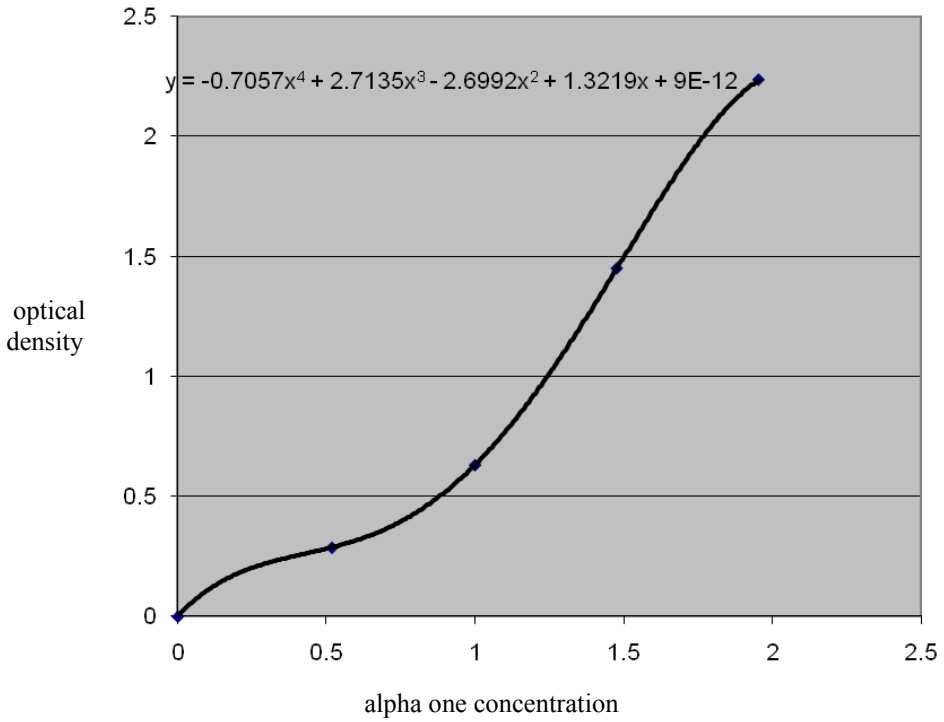
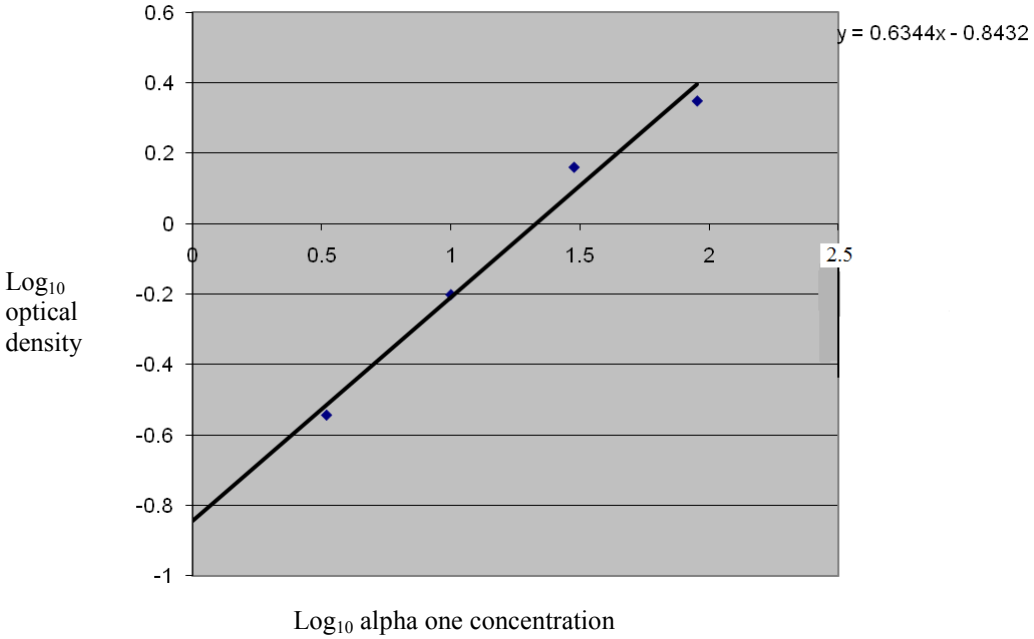


Figure 3.8: Standard curve log transformed



3.8 Cell Culture

Principles of cell culture

Cell culture is the process by which cells are grown under controlled conditions and has been described in various forms since the late 1800s. In 1885 Wilhelm Roux removed a portion of the medullary plate of an embryonic chicken and maintained it in a warm saline solution for several days, establishing the principle of tissue culture (Molecular Biology of the Cell 2007). Cells can be isolated from tissues for *ex vivo* culture in several ways; white cells can be purified from blood, mononuclear cells released from soft tissues by enzymatic digestion, or pieces of tissue placed in growth media and the cells that grow out cultured. This method is known as explant culture and has been used in this thesis.

Cells cultured directly from a subject are known as primary cells. With the exception of some derived from tumors, most primary cell cultures have limited lifespan. However, an immortalised cell line; one that has acquired the ability to proliferate indefinitely; is a group of morphologically uniform cells that can be propagated *in vitro* through many cycles of division and not become senescent. Immortalization can occur spontaneously during passage of a cell strain or can be induced by treatment with chemical mutagens, infection with tumerogenic viruses or transfection with oncogenes. Immortalization is accompanied by genetic changes; cells become aneuploid, contain abnormalities in the number and structure of chromosomes relative to the parent species and they do not all necessarily display the same karyotype. For these reasons stocks of cell lines are frozen which can be recovered if the properties disappear during culture. The following continuous cell lines were used for this work; HeLa Ohio, BEAS-2B, Hep2, and Vero.

Cells are grown and maintained at an appropriate temperature and gas mixture (typically, 37°C, 5% CO_2 95% air) in a cell incubator. Culture conditions vary for each cell type, and variation of conditions for a particular cell type can result in different phenotypes being expressed. Growth medium required also varies; in pH, glucose concentration, growth factors, and the presence of other nutrients. Growth factors used to supplement media are often derived from animal blood, such as calf serum. This has been used in this work. Cell culture systems are handled using a sterile technique to

avoid contamination with bacteria, yeast, or other cell lines, manipulations carried out in a biosafety hood and antibiotics added to the growth media.

Passaging cells (i.e. splitting cells) involves transferring a small number of cells into a new vessel. Cells can be cultured for a longer time if they are split regularly, as it avoids the senescence associated with prolonged high cell density. For adherent cultures (as used in this thesis), cells first need to be detached; this is commonly done with a mixture of trypsin-EDTA and a small number of detached cells used to seed a new culture.

Cell media and supplements

Below is an alphabetical list of the standard cell media and supplements used for the growth and maintenance of cell lines. All abbreviations are in the abbreviations list.

Antibiotic/antimycotic solution; (A5955 Sigma); 10,000 units penicillin G, 10mg streptomycin and 25mcg amphotericin B per ml.

Bovine pancreatic insulin; (I6634 Sigma). This is used as a medium supplement for primary epithelial cell culture. Insulin regulates the cellular uptake, utilisation and storage of glucose, amino acids and fatty acids and inhibits the breakdown of glycogen, protein and fat.

DMSO; Dimethyl sulfoxide; (D2650 Sigma); ≥99.7%, Hybri-Max™. This is a highly polar organic reagent that has exceptional solvent properties for organic and inorganic chemicals. It widely utilized in the storage of human and animal cell lines and bacteriophage, as a cryoprotective agent.

Dulbecco's phosphate buffered saline; (DPBS; Sigma; D8537). This is a balanced salt solution used for the handling and culturing of mammalian cells, used for irrigation, washing and dilution. The phosphate buffering maintains the pH in the physiological range. Tissue culture media contains calcium (Ca^{2+}) and magnesium (Mg^{2+}) ions and foetal calf serum contains proteins that are trypsin inhibitors, and both Mg^{2+}/Ca^{2+} inhibit trypsin. This PBS does not contain any Ca^{2+}/Mg^{2+} and is used to wash the cells

prior to trypsinisation to reduce the concentration of Divalent cations and proteins that inhibit trypsin action.

Epidermal growth factor; (354001 BD Biosciences, 20ng/ml) This is important for cell growth, proliferation and differentiation and is reconstituted in sterile water.

Foetal Bovine Serum (FBS); (F0926 Sigma); USDA approved, non-USA origin, sterile-filtered, cell culture tested. FBS is commonly used as an essential supplement to cell culture media. It is a cocktail of most of the factors required for cell attachment, growth, and proliferation *in vitro* (Gstraunthaler, 2003). FBS is the portion of plasma remaining after coagulation of blood and comes from the blood drawn from the unborn bovine fetus via a closed system. It is the most widely used serum due to being low in antibodies and containing more growth factors, allowing for versatility in many different applications.

Human transferrin; (T-1147 Sigma, apo-transferrin (1)); Transferrin is a glycoprotein with homologous N-terminal and C-terminal iron-binding domains and is related to several other iron-binding proteins. Transferrin is the iron-transport protein of vertebrate serum and donates iron to cells through interaction with a specific membrane receptor, CD71. It is referred to frequently as a growth factor because, in analogy to other growth factor-receptor interactions, proliferating cells express high numbers of transferrin receptors, and the binding of transferrin to their receptors is needed for cells to initiate and maintain their DNA synthesis. Apart from its role as an iron transport protein transferrin acts as a cytokine and has functions that may not be related to its iron-carrying capacity.

Hydrocortisone; (H0888 Sigma); this promotes cell attachment and cell proliferation and under certain circumstances cell differentiation.

L-glutamine; (G7513 Sigma); (200 mM solution). L-glutamine is an essential amino acid that is a crucial component of culture media and serves as a major energy source for cells in culture. L-Glutamine, is the uncharged and amidated analog of L-glutamic acid, and is an important amino acid for the incorporation of NH4 + into biomolecules. It is biosynthesized from NH4 + and glutamate via the enzyme glutamate synthetase. In

turn, degradation of glutamine to free the ammonia moiety is mediated by glutaminase. Glutamine also participates in acid-base regulation *in vivo*.

Medium 199; (M2154 Sigma); with Earle's salts and sodium bicarbonate, without L-glutamine. This media was originally developed as a completely defined media formulation for the culture of primary explants. It has broad applicability, particularly for non-transformed cells. Many early tissue culture media were predominantly formulated from animal products and/or tissue extracts. In 1950, Morgan (Morgan 1956) and his co-workers reported their efforts to produce a totally defined nutritional source for cell cultures. Their experiments, conducted with various combinations of vitamins, amino acids, and other factors revealed that growth of explanted tissue could be measured in what has become known as Medium 199. However, it was found that long-term cultivation of cells required addition of a serum supplement to the culture medium. When properly supplemented, Medium 199 has broad species applicability, particularly for cultivation of non-transformed cells. It is widely used in virology, and *in vitro* cultivation of primary explants of epithelial tissue.

Minimum Essential Medium Eagle (MEM); (M7278 Sigma); HEPES Modification (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), with Earle's salts, 25mM HEPES and sodium bicarbonate, without L-glutamine. Minimum Essential Medium (MEM), developed by Harry Eagle, is one of the most widely used of all synthetic cell culture media. MEM incorporates modifications to Eagle's Basal Medium, including higher concentrations of amino acids so the medium more closely approximates the protein composition of cultured mammalian cells. MEM has been used for cultivation of a wide variety of cells grown in monolayers. MEM Modified with HEPES contains 25 mM HEPES to provide additional buffering capacity to the medium. HEPES is a zwitterionic buffer, that has a pKa of 7.3 at 37 °C, which is more compatible with most culture systems than that of sodium bicarbonate, which is usually 6.2 under similar conditions. HEPES will reduce sudden, drastic pH shifts, but as with other buffers, it will not prevent pH shifts entirely.

MEM non-essential amino acid solution; (NEAA); (M7145 Sigma); The addition of amino acid supplements to media stimulates growth and prolongs the viability of cells in culture.

Nu-Serum IV; (355504 2.5% BD Biosciences). This is a growth medium supplement which provides a low-protein alternative to fetal bovine serum.

Penicillin / Streptomycin (P/S); (15140-122 Invitrogen); This contains 10,000 units of penicillin (base) and 10,000µg of Streptomycin (base)/ml utilising penicillin G (sodium salt) and streptomycin sulphate in 0.85% saline. It has good gram positive and negative bacterial cover.

Trypsin-EDTA solution (1x); (T3924 Sigma). This is 1 ×, 0.5 g porcine trypsin and 0.2 g EDTA (ethylenediaminetetraacetic acid), 4 sodium (Na) per litre of Hanks' Balanced Salt Solution with phenol red. It is used to detach cells. Trypsin cuts the adhesion proteins in cell-cell and cell-matrix interactions, and EDTA is a calcium chelator, which is required by integrins to interact with other proteins for cell adhesion. EDTA will mop up remaining divalent cations. If trypsin is in contact with the cells for too long, cell viability is reduced. This reaction is stopped by the addition of FBS.

3.8.1 Continuous Cell Lines

Cell Growth

All cells were grown in growth medium (GM). This is made by adding 50% serum i.e. 50mls FBS, 5mls L-glutamine, 5mls P/S and 5mls NEAA to 500mls of MEM. Cells were grown in a 37°C humidified incubator (Galaxy R CO_2 incubator, Wolf Laboratories) with 5% CO_2 in 95% air.

HeLaOhio cells

HeLaOhio cells are an established line of human epithelial cells derived from a cervical carcinoma (said to be from Henrietta Lacks). They provide a good environment for HRV growth. HeLaOhio cells (gift from Dr. D Alber) were stored in liquid nitrogen. To grow up the cell line for use, an aliquot was removed from liquid nitrogen, quickly thawed at 37°C and poured into a sterile 75cm^2 tissue culture flask (Falcon) containing 40mls of GM (pre-warmed to 37°C). The flask was then laid flat in the incubator and the cells left for 48 hours to grow. Cells were passaged every 3 to 4 days depending upon rate of cell growth. Cells were viewed under the microscope to check confluence and health prior to each use.

BEAS 2B cells

BEAS 2B cells; cultured human lung epithelial cells were a gift from Dr. D Alber. To grow up the cell line for use an aliquot was removed from liquid nitrogen, quickly defrosted at 37°C and poured into a sterile 75cm^2 tissue culture flask (Falcon) containing 40mls of GM that had been warmed in the incubator. The flask was then laid flat in the incubator and the cells allowed left for 48 hours to grow. Cells were passaged every 3 to 4 days depending upon cell growth. Cells were viewed under the microscope to check confluence and health prior to each use.

Hep2 cells

Hep2 cells, a cell line established from human laryngeal carcinoma in 56 year old Caucasian male, were obtained from Arrow Therapeutics (UK) and an aliquot stored in liquid nitrogen. To grow up the cell line for use, an aliquot (labelled LN4) was removed from liquid nitrogen, quickly thawed at 37°C and poured into a sterile 75cm^2 tissue culture flask (Falcon) containing 40mls of GM that had been warmed in a 37°C humidified incubator (Galaxy R CO_2 incubator, Wolf Laboratories) with 5% CO_2 95% air. The flask was then laid flat in the incubator so that the cells were covered with medium, with the lid left slightly open to allow CO_2 to enter the flask. The cells were left for 48 hours to grow. Cells were passaged every 3 to 4 days depending upon cell growth. Cells were viewed under the microscope to check confluence and health prior to each use.

Vero cells

The *Vero* lineage was isolated from kidney epithelial cells extracted from an African green monkey (*Cercopithecus aethiops*). The lineage was developed on 27th March 1962, by Yasumura and Kawakita at the Chiba University in Chiba, Japan. Vero cells provide a good environment for growing virus as the Vero cell lineage is continuous and aneuploid.

Vero cells were obtained from Arrow Therapeutics (UK) and an aliquot stored in liquid nitrogen. To prepare the cell line for use, an aliquot (labelled LN5) was removed from liquid nitrogen, quickly thawed at 37°C and pipetted into a sterile 75cm^2 tissue culture flask (Falcon) containing 40mls of GM that had been warmed in in a 37°C humidified incubator (Galaxy R CO_2 incubator, Wolf Laboratories) with 5% CO_2 95% air. The flask was then laid flat in the incubator and the cells allowed to grow for 48 hours. Cells

were then passaged every 3 to 4 days depending upon cell growth. Cells were viewed under the microscope to check confluence and health prior to each use. Vero cells were infected with RSV RSS to make a working stock (**section 3.9**).

Passaging cells for continual growth

The flask of cells to be passaged is removed from the incubator and the GM poured away. 10mls of DPBS was added, swirled around the flask to remove serum from the cells and then poured out. 3mls of trypsin EDTA was added to ensure cell sheet is covered. After careful mixing around the flask, 2mls was discarded, the flask was placed on its side (so the trypsin covered the cell sheet) and then in the incubator at 37^{o}C for 5 minutes. On removal from the incubator the flask was tapped so all the cells were in the trypsin at the bottom of the flask. The volume of GM then added depended upon the dilution needed. A 1:8 dilution required the addition of 7mls of GM. 1ml of this was then added to a new flask containing 20mls of GM. This flask was labelled with the cell type, passage number (i.e. one number on from previously) and the date. They were then left to grow until confluent and used to grow up virus or for viral titration or they were further passaged for future use.

Cryopreservation of cells

The flask of cells to be used was removed from the incubator and cell growth and health checked by looking at the cells under the microscope. The cell sheet was at least 70% confluent before use. The GM was carefully poured off before 10mls of PBS was added in a swirling motion and discarded. 3mls of 1% trypsin EDTA was then added to cover the cell sheet and 2mls removed. The flask was then placed on its side and left in the incubator for 5 minutes. On removal from the incubator the flask was tapped so all the cells were in the trypsin at the bottom of the flask. 20mls of GM was then added to the flask and the medium transferred to a 50ml Falcon polypropylene tube and centrifuged at 1000rpm for 7 minutes. The medium was poured off and the pellet resuspended in 3mls 10% DMSO and FBS. 1ml of resuspended pellet was added to each cryogentube (1.5ml polypropylene sterile cryogenic vile, Nunc), the tube labelled with the passage number, date, cell type and LN number and placed in a Nalgene labtop -80°C cooler (Nunc) to allow the cells to freeze at a rate of 1°C per minute. Once frozen the vial was transferred to liquid nitrogen for storage.

Primary Epithelial Cells

Biopsies from bronchial tissue were obtained and grown as explant cultures to obtain primary epithelial cells as described by Devalia and colleagues (Devalia 1990). The epithelium from each biopsy was dissected away from the underlying tissue under a dissecting microscope and cut further into smaller sections approximately 1-2mm^3 in size. All sections were then washed 3 times in pre-warmed medium 199 containing 1% (v/v) antibiotic/antimycotic solution (Sigma chemicals) and then 1-2 sections explanted onto 60mm diameter Falcon Primaria culture dishes (Becton Dickinson Ltd) in 1.5ml of complete culture medium and incubated at 37^oC in 5% CO_2 in air atmosphere (Wolf incubator). The medium in the dishes was left for 5-7 days until epithelial cells could be seen to grow away form the explants. After one week in culture the complete culture medium was replaced with a maintenance medium which was changed every 2 days. Culture normally took 3-4 weeks to grow to confluence. At confluence, the explants were removed from the culture dish and the epithelial cells cultures washed gently with 2mls of medium 199 containing antibiotics only and left a number of hours ready for experimenting with.

Culture medium

Complete culture medium was prepared in 500ml aliquots. A mixture of 12.5ml foetal bovine serum (Sigma) and 5mls of each of the following: bovine pancreatic insulin (2.5µg/ml), human transferring (2.5µg/ml), hydrocortisone (0.36µg/ml), L-glutamine (0.02mg/ml), 150µl epidermal growth factor (20ng/ml) and 7.5 ml of antibiotic/antimycotic solution made up to a final volume of 500ml with Medium 199. The complete medium was then filtered through a sterile 0.2µm syringe filter (Elkay lab supplies Ltd.) and this medium was then used for culturing human epithelial cells for the first 7 days. 2ml of maintenance medium used to sustain the cells after the first 7 days and changed 3 times weekly thereafter. The maintenance medium was prepared in the same way as described above, except foetal calf serum was replaced with Nu-Serum IV (2.5% Becton Dickenson Ltd).

Cell culture experiments

Confluence was confirmed by light microscopy prior to experimentation. The explant was removed and incubated a further 24 hours in medium 199 and antibiotic/antimycotic

solution with no serum prior to experimentation. Immediately prior to use, cells were washed twice in medium 199 only.

Collection of cell supernatant for IP-10

Cell supernatant was collected in 500µl aliquots at 24 and 48 hours and stored at -80^{o}C for cytokine (IP-10) analysis.

TNF-α stimulation experiments

Primary epithelial cells from COPD patients and controls were stimulated with TNF-α in the following manner. Cells were prepared as described above and after washing in medium 199, the cells covered with maintenance medium containing 50ng/ml TNF-α. This dose was chosen based on previous experiments in the department (Patel 2003). Supernatant was removed at time 0 and 24 hours and stored at -80^{o}C for cytokine (IP-10) analysis.

Virus stimulation experiments

Primary epithelial cells from COPD patients and controls were infected with HRV at moi of 0.5 and 10 (described in **section 3.9**) in medium 199 for 2 hours at 33^{o}C in 5%CO_2 95%air. After 2 hours medium containing virus was removed and replaced with medium 199 alone. Aliquots of supernatant were collected at 24 hours and stored at -80^{o}C for cytokine (IP-10) analysis.

3.9 Virus culture and titration

Principles of virus culture and titration

There are several methods available for growing virus, depending upon the quantity of virus required, the environment in which the virus best grows, and the ease of the procedure. HRV and RSV were used in subsequent chapters of this thesis, both of which infect and can be grown and titrated in cell lines. To grow the virus, confluent monolayers of the appropriate cell line are exposed to virus and diluted to infect a fraction of the cells. The progress of the infection is monitored by light microscopy, observing the development of the cytopathic effect (CPE).

CPE consists of morphologic changes induced in individual cells or groups of cells by virus infection which are recognised under a light microscope and inclusion bodies, which are subtle alterations to the intracellular architecture of individual cells. A number of cell phenomena including rounding, shrinkage, increased refractility, fusion, aggregation, loss of adherence and lysis can be induced by viral infection, and the character of CPE can change during the course of infection. However, morphologic changes for a given virus are reproducible. Equally, inclusion bodies are highly specific for a particular virus type.

Two major types of quantitative assays exist for virus; physical and biological. Physical assays such as electron microscopic particle counts quantify only the presence of virus particles and not whether the particles are infectious. Biological assays, such as plaque assays measure only the presence of infectivity and may not count all particles present in a preparation even that may be infectious. This is because this technique relies on light microscopic identification of characteristics of cellular infection and individual virus particles are too small to be identified. Plaque assay has been used for this work. This method was originally developed for bacteriophage study in the early 1900s but was adapted to animal viruses by Dulbecco in 1953 (Dulbecco 1953). This technique is based on the ability of a single infectious virus particle to give rise to a macroscopic area of cytopathology on an otherwise normal monolayer of cultured cells. If a single cell in a monolayer is infected with a single virus particle, new virus resulting form the initial infection can infect surrounding cells, which in turn produce virus that infects additional surrounding cells. Over a period of time (dependent upon the particular

virus), the initial infection gives rise through these multiple rounds of infection to an area of infection, i.e. a plaque.

Quantification of a virus by plaque assay occurs as follows. A sample of virus of unknown concentration is serially diluted in an appropriate medium, and measured aliquots of each dilution are seeded onto confluent monolayers of cultured cells. Infected cells are overlayed with a semisolid nutrient medium consisting of GM and agar. This semisolid medium prevents the formation of secondary plaques through diffusion of virus from the original site of infection to new sites, ensuring that each plaque that develops in the assay originated from a single infectious particle in the starting inoculum. After an appropriate period of incubation, the monolayer is stained so the plaques can be visualised. For this work methylene blue was used, and plaques visualised as holes in the monolayer. Ideally 20 to 100 plaques should be identified on a single dish; large enough to be statistically significant but the plaques small enough to be individually identified and counted. Very low dilutions will result in only dead cells or too many plaques to count, and very high dilutions too few plaques. Once dishes containing the appropriate number of plaques have been counted, the concentration of the infectious virus in the original sample can be calculated taking into account the serial dilution. The resulting volume is a titre and expressed in plaque forming units per millilitre (pfu/ml).

As plaque assay only measures infectivity, only a fraction of the particles present may actually be counted. This is known as the particle to infectivity ratio and to determine this, ideally the virus must first be purified to determine the concentration of physical particles and then subjected to plaque assay. It is possible also that not all infectious particles will form plaques in a given plaque assay as infectious virus may require cells be in a particular stage of the cell cycle to infect.

Multiplicity of infection, (MOI) measures the average amount of virus added per cell in an infection and can be expressed as pfu/cell.

Virus stocks

For this work, virus obtained from a “master stock” was made into a “submaster” which was then used to make a “working stock”. The media used are listed below;

Growth medium;(GM); MEM (Sigma M7278) supplemented with 10% FBS, P/S (Invitrogen 15140-122), 200mM L-glutamine (invitrogen 25030-024) and NEAA (Invitrogen 11140-035).

Maintenance medium;(MM); MEM (Sigma M7278) supplemented with 2% FBS, P/S (Invitrogen 15140-122), 200mM L-glutamine (invitrogen 25030-024) and NEAA (Invitrogen 11140-035).

Overlay medium; (OM) consisted of 32mls of MM and 8mls of 3% agarose (Seakem LE Agarose; 500000; BMA products, ME, USA; 20% v/v of 3% w/v). 400µl $MgCl_2$ was added for HRV titration only. MM was warmed to 37^{o}C, the agarose heated in the microwave before adding it to the MM and the OM pipetted onto the cells prior to the agarose setting.

All of the following work was done under sterile conditions in a biosafety hood.

HRV

HRV working stock

The HRV "master" was a gift from Arrow Therapeutics Ltd (UK). To make the submaster and then working stock, HRV was grown up in HeLaOhio cells. A flask of confluent HeLaOhio cells was removed from the incubator. One vial of working HRV stock (FS28) which was stored at -80°C was held by hand to defrost. 3mls of MM was poured into a 15ml Falcon polypropylene tube and the medium poured off the flask containing the HelaOhio cells. 10µl of HRV was added to the Falcon tube containing maintenance medium (MOI 0.01). This was then poured into the flask with HeLaOhio cells, the cell sheet covered in the medium and left on a tube rocker in the incubator (5% CO_2) at 33°C for 2 hours to allow the virus to infect the cells. After 2 hours the medium containing virus was poured off carefully into Virkon solution (to deactivate the virus). The cells were washed with 13mls of MM and the medium poured away. 200µl of 2M magnesium chloride was added to 19.8mls of MM in a falcon tube. 8mls was added to the flask and flask returned to the tube rocker in the incubator (5% CO_2) at 33°C and left for 4 days.

After 4 days a good infection of the HeLaOhio cells was seen (i.e. CPE was observed as described above) with cells looking rounded in shape and no longer confluent under the light microscope (Olympus) at x200 magnification. To remove the adhered cells from the flask into the medium, cells were scraped off the side of the flask into the medium using a cell scraper (32 mm Nunc). The medium was then pipetted into a falcon tube, the tube frozen at -80^{o}C for 30 minutes, quickly thawed by hand and centrifuged at 2500rpm for 10mins at 4°C to produce a cell pellet and supernatant containing the virus. Although predominantly an extracellular virus, this method allowed release of HRV from cells thus maximising virus available. The supernatant was aliquoted in 300µl aliquots into cryogentubes (1ml polypropylene sterile cryogenic vile, Nunc) and frozen at -80◦C. This produced working stock FS29 HRV.

In total 30 vials of FS29 HRV were frozen. Once titrated to determine the concentration of the virus, virus was suitable for the various experiments that follow.

<u>HRV titration by plaque assay</u>

Cell sheet preparation

HeLaOhio cells were passaged 1:10 as described in **section 3.8.1**. Instead of placing the cells back in the incubator to grow in the tissue flask, cells were seeded in 6 well tissue culture plates (Sarstedt) at a concentration 8 x10^5 in 3mls of MM in preparation for viral titration. To determine the cell concentration, 10µl of GM containing cells was added to 90µl trypan blue stain (Sigma). A coverslip was adhered to a Haemocytometer (depth 0.1mm) and the counting chamber filled with the trypan blue stain/cell suspension using a P200 pipette to transfer this mixture. The cell suspension was allowed to run out of the pipette and was drawn under the coverslip by capillary action. The cells were viewed at a magnification of x400 and the number of cells seen in 2 sets of 16 squares (i.e within 2 squares within the 1mm^2 central square) counted and the counting value averaged. This was then multiplied by 10^5 to determine the number of cells / ml.

The following calculation was used to determine cell concentration;

1. $\frac{8 \times 10^5 \times \text{number of wells needed}}{\text{Number of cells / ml}}$ = volume of cells needed.

2. Total volume needed – volume of cells needed = volume of MM to be added
Cells were left to grow at 37°C in a 5% CO_2 / 95% air atmosphere and were confluent by 24 hours.

Virus titration
Using duplicate wells each concentration of compound was screened. Tubes were labelled -2, -3, -4, -5, -6, -7, -8 and cell control. 3mls of MM was added to the tube labelled -2 and 2.7mls to the tubes labelled -3 to -8. 5mls was added to the cell control. 300µl of MM was removed from the -2 tube and 300µl HRV stock FS29 added. The tube was vortexed and then 300µl from -2 added to -3, and vortexed. This was repeated until 10^{-8} dilution was reached. The 6 well plates were removed from the incubator and the cell sheet checked under the microscope at x200 to ensure cell confluence. The MM was gently aspirated off by tilting the plate, without disturbing the monolayer. 200µl of virus at concentration of -4 to -8 or cell control (cc) was added to each well. This was done in duplicate.

Format of cell 6 well plate;

-4	-5	-6
-4	-5	-6

-7	-8	cc
-7	-8	cc

The virus was left to absorb into the cells for 1 hour at 33°C on a tube rocker. After an hour 6mls of overlay medium (with 400µl $MgCl_2$) was added per well and the agarose allowed to set. The plates were incubated at 33°C in 5% CO_2 95% air for 3 days. On day 3, 3mls of 10% formalin/PBS was added to each well and left at room temperature for at least 3 hours. The agar plugs were removed from the wells using a spatula, taking care not to damage the monolayer. The monolayers were stained with 1ml per well of methylene blue (M9140 Sigma 3,7-bis(Dimethylamino)phenazathionium chloride, Basic Blue 9, Tetramethylthionine chloride) in water (w/v) for 15minutes at room temperature, washed with water and allowed to dry. The plaques were then counted under the microscope at x200 magnification. An example of the titration plates is given in **Figure 3.9 (a) and (b).**

Figure 3.9(a): HRV titration plates; 10^{-2}, 10^{-3} and 10^{-4}

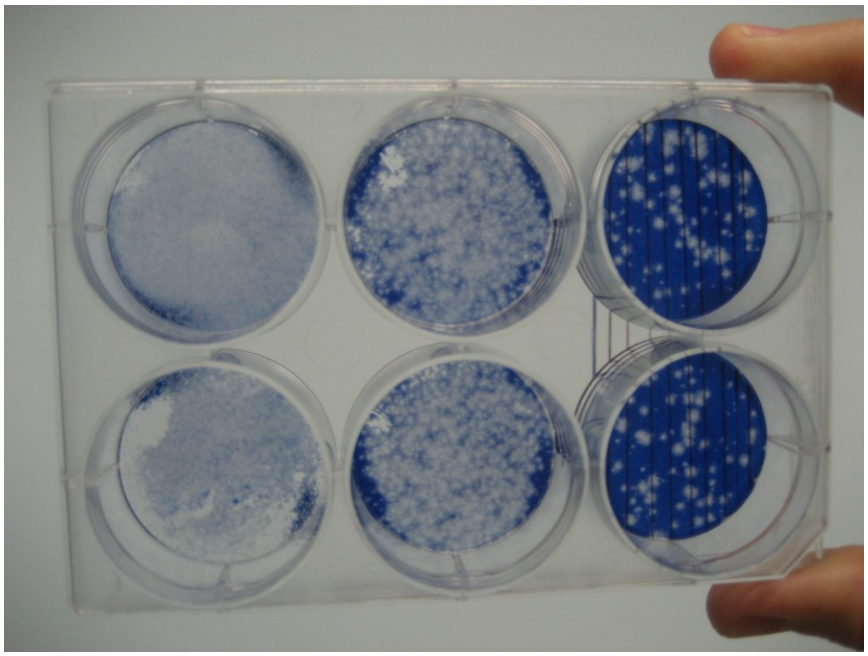
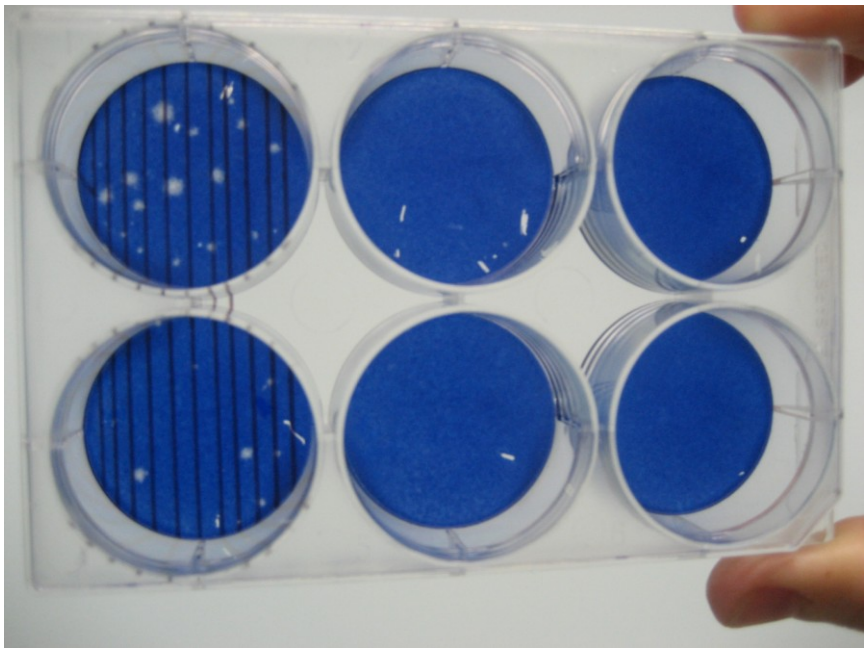


Figure 3.9(b): HRV titration plates; 10^{-5}, 10^{-6}, cell control



RSV

RSV working stock – Long and RSS

The RSV "master" was a gift from Arrow Therapeutics Ltd (UK). RSV was grown up in Vero cells (**section 3.8.1**). A flask of Vero cells that were 70% confluent was removed from the incubator. One vial of working stock (FS24 RSS) which was stored at -80°C was thawed quickly by warming with hands. 3mls of 6% FCS / MM was poured

into a 15ml Falcon tube. The medium then was poured off the flask containing the Vero cells. 200µl of RSV RSS was added to the Falcon tube containing 6% FCS / MM. This was poured into the flask containing Vero cells and the cell sheet covered in the medium. It was left in the incubator at 37^{o}C, 5% CO_2 95% air for 1 ½ hours to allow the virus to infect the cells. Every 20 minutes the flask was removed from the incubator and rocked to aid absorption. After 1 ½ hours the medium containing virus was poured carefully into Virkon solution (to deactivate the virus). 10mls of 6% FCS/MM was added to the flask and flask returned to the incubator for 6 days. This method was repeated for RSV Long (FS22).

After 6 days if there was good infection of the Vero cells and they looked circular under the microscope at x200 magnification and showed signs of CPE, the cells were scraped off the side of the flask into the medium using a cell scraper (32mm Nunc). The medium was then pipetted into a falcon tube, the tube frozen at -80^{o}C for 30 minutes, quickly thawed by hand and centrifuged at 2500rpm for 10mins at 4°C to produce a cell pellet and supernatant containing the virus. This was particularly important for RSV as it is predominantly intracellular. The supernatant was aliquoted in 300µl aliquots and frozen at -80°C in cryogentubes. These methods produced working stock FS33 for RSV RSS and FS34 for RSV Long.

A total of 41 vials of FS33 and FS34 were frozen. Once titrated to determine the concentration of the virus, the virus was stored at -80^{o}C until use in the experiments that follow.

RSV working stock was also made by infecting BEAS-2B cells. The method used was as described above, and in this case 30 vials of FS35 (RSV RSS) and 30 vials of FS36 (RSV Long) were produced.

<u>RSV titration to determine concentration by plaque assay</u>

This was carried out in both Hep2 and BEAS-2B cells using the same methodology. The assay was set up in 6 well plates (Sarstadt). Duplicate wells were used for each dilution of virus. Hep2 cells were seeded at 5-6x10^{5} cells/well in 3ml GM per well. Cells are grown at 37^{o}C in a 5% CO_2 95% air atmosphere and were checked for confluence at 24 hours. Tubes were labelled -2, -3, -4, -5, -6 and cell control. 3mls of

MM was added to the tube labelled -2 and 2.7mls to tubes -3 to -6. 5mls was added to the cell control. 300µls of MM was removed from -2 tube and 300µl virus added. The tube was then vortexed. 300µl was added from -2 to -3, vortexing in between. This was repeated until the 10^{-6} dilution was reached. The 6 well plate containing the cell sheet was removed from the incubator and the media gently aspirated by tilting the plate. 200µl of appropriate virus dilution or cell control was pipetted into each well. The virus was left to infect the cells for 2 hours at 37^{o}C in 5% CO_2 95% air incubator, mixing carefully every 15 minutes. 6mls of OM was added to each well and once the agarose had set the plate was incubated for 5 days. At day 5 3mls of 10% formalin/PBS was added to each well and the plate left at room temperature at least 3 hours. The agar plugs were removed; the monolayers stained with methylene blue for 15mins at room temperature, washed and allowed to dry. Plaques were counted at x200 magnification. Below are example titration plates (**Figure 3.10 (a) and (b)**).

Figure 3.10 (a): RSV titration plates; 10^{-2},10^{-3},10^{-4}

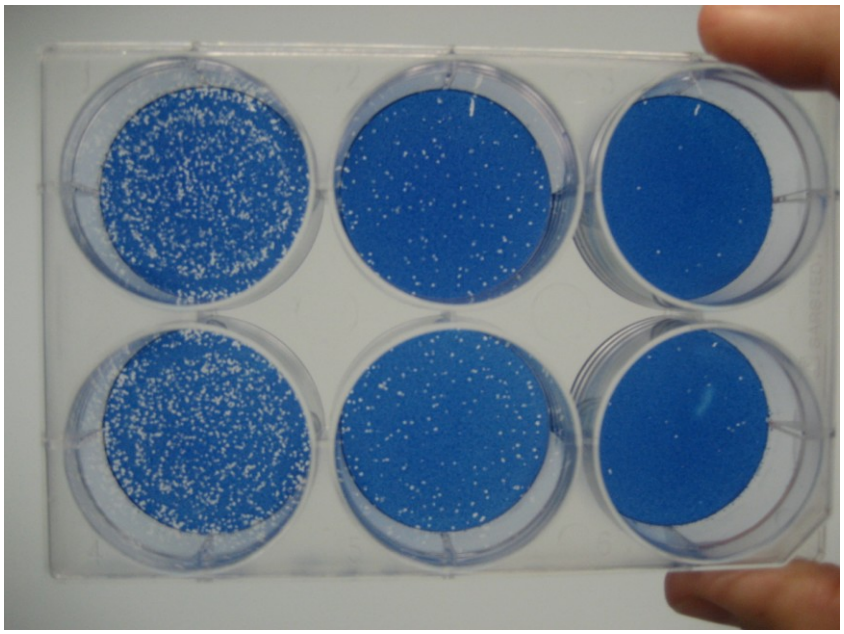
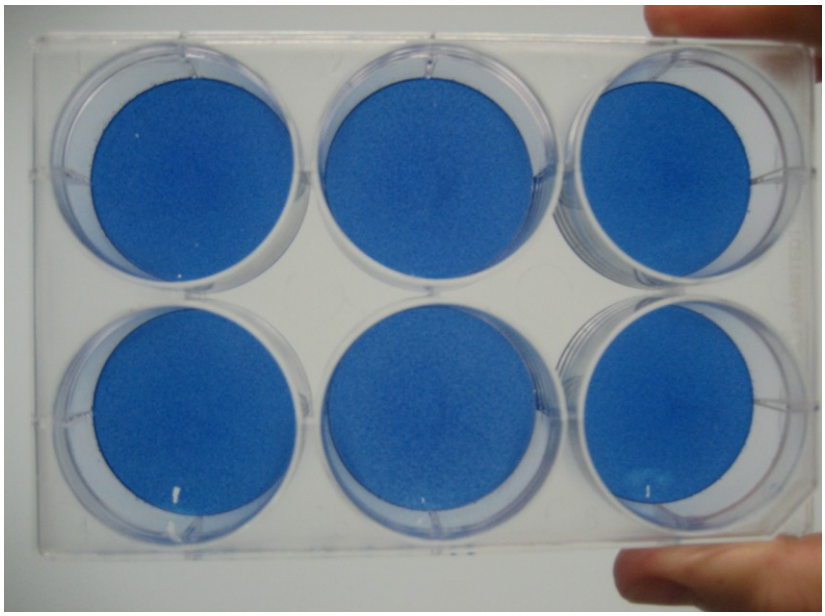


Figure 3.10 (b): RSV titration plates; 10^{-5},10^{-6},cell control



Viral experiments on cell lines

BEAS-2B cells and HeLaOhio cells once grown to confluence were infected with HRV at moi 0.5 and 10 in MM and left for 2 hours at 33^{o}C in 5%CO_2 95%/air. After 2 hours the medium was carefully pipetted off into Virkon, the cells were washed and medium replaced with MM. Aliquots of cell supernatant were collected at time 0, 2, 4, 12, 20, 28,36,44,62 and 140 hours post HRV infection. Supernatant was stored at -80^{o}C until cytokine analysis.

3.10 Bacterial Culture

Principles of bacterial culture

Microbiological culture is a method by which microbial organisms multiply in predetermined culture media under controlled laboratory conditions. Cultures are used to determine the type of organism and its abundance in the sample. This is usually done by streaking a sample on to agar plates.

Agar plates may be formulated as permissive; with the intent of allowing the growth of whatever organisms are present, or restrictive; with the intent of only selecting for growth a particular subset of those organisms. This may take the form of a nutritional requirement, for instance providing a particular compound such as lactose as the only source of carbon for energy and material and thereby selecting only organisms which can metabolize that compound, or by including a particular antibiotic or other substance in order to select only organisms which are resistant to that substance. Agar plates may also be indicator plates, where the organisms are not selected on the basis of growth, but a compound in the agar is altered by an enzyme or similar in some colonies so as to change color and identify them from those lacking the enzyme. Common agar plates used include;

1. Columbia agar with horse blood; for growth of non-fastidious aerobic organisms.

2. MaConkey agar; contains bile salts and is a good growth media for gram negative bacteria although *Staphlococcus* and *Enterococcus* species can still grow. These plates can also be used to grow Gram-negative bacteria and stain them for lactose fermentation thus allowing differentiation between different Gram negative bacteria and inhibition of growth of Gram positive bacteria. The addition of bile salts and crystal violet to the agar inhibits the growth of most Gram positive bacteria. Lactose and neutral red are added to differentiate lactose fermenters (which form pink colonies), from lactose non-fermenters (which form clear colonies). Lactose positive bacteria such as *Escherichia coli*, *Enterobacter* and *Klebsiella* will produce acid, thus producing pink colonies.

3. Chocolate agar; This is a type of blood agar plate in which the blood cells have been lysed by heating the cells to 56 °C allowing easier access to nutrients. Chocolate agar

is used for growing fastidious respiratory bacteria, such as *Haemophilus influenzae*. These bacteria need growth factors, like NAD and hematin, which are inside erythrocytes.

4. COBA ; blood agar plates containing colistin and oxylinic acid. These plates inhibit gram negative growth and select for streptococcus.

Growth of potentially pathogenic respiratory bacteria

The potentially pathogenic bacteria of interest for this thesis were; *Haemophilus influenzae, Moraxella catarrhalis, Staphylococcus aureus, Streptococcus pneumoniae and Pseudomonas aeruginosa*.

Haemophilus influenzae is a gram negative rod. Bacterial culture is performed on Chocolate agar, with added X(Hemin) & V(NAD) factors at 37°C in an enriched CO_2 incubator. Blood agar growth is only achieved as a satellite phenomenon around other bacteria. Colonies of *H. influenzae* appear as convex, smooth, pale, grey or transparent colonies. Gram-stained and microscopic observation of a specimen of *H. influenzae* will show Gram-negative, coccobacilli, with no specific arrangement. The cultured organism can be further characterized using catalase and oxidase tests, both of which should be positive. Further serological is necessary to distinguish the capsular polysaccharide and differentiate between *H. influenzae* b and non-encapsulated species. Although highly specific, bacterial culture of *H. influenzae* lacks in sensitivity. Use of antibiotics prior to sample collection greatly reduces the isolation rate by killing the bacteria before identification is possible. *H. influenzae* will grow in the hemolytic zone of *Staphylococcus aureus* on Blood Agar plates. The hemolysis of cells by *Staph aureus* releases nutrients vital to the growth of *H. influenzae. H. influenzae* will not grow outside the hemolytic zone of *Staph aureus* due to the lack of nutrients in these areas. An example of *H. Influenzae* plate is given in **Figure 3.11**.

Figure 3.11: *H. Influenzae* on agar plate. Photographed with digital camera.

Moraxella catarrhalis is a Gram-negative, aerobic, oxidase-positive diplococcus that may both colonize and cause respiratory tract-associated infection in humans. It grows on chocolate agar plates where colonies appear white (creamy). *H. influenzae* and *Moraxella* are commonly confused and a simple visible test to differentiate is to use a loop gently to 'push' the colony. If the colony smudges its *H.influenzae*, if it moves without smudging its *Moraxella*. An example of a plate is given below (**Figure 3.12**).

Figure 3.12: *Moraxella catarrhalis.* Photographed with digital camera

S. aureus is a facultatively anaerobic, Gram-positive coccus, which appears as grape-like clusters microscopically and on blood agar plates has large, round, golden-yellow colonies, often with hemolysis. *S. aureus* is catalase positive thus catalase test is useful to distinguish staphylococci from enterococci and streptococci. A small percentage of *S. aureus* can be differentiated from most other staphylococci by the coagulase test: *S. aureus* is primarily coagulase-positive (meaning that it can produce "coagulase", a protein product, which is an enzyme) that causes clot formation while most other *Staphylococcus* species are coagulase-negative.

Streptococcus pneumoniae cells are Gram-positive, lancet-shaped cocci, usually, seen as pairs of cocci (diplococci), but they may also occur singly and in short chains. When cultured on blood agar, they are alpha haemolytic. Individual cells are between 0.5 and 1.25 micrometers in diameter. They do not form spores, and they are nonmotile. They lack catalase and ferment glucose to lactic acid. *Streptococcus pneumoniae* is a fastidious bacterium, growing best in 5% carbon dioxide. Growth requires a source of catalase (e.g. blood) to neutralize the large amount of hydrogen peroxide produced by the bacteria. On blood agar, colonies characteristically produce a zone of alpha (green) haemolysis, which differentiates *S. pneumoniae* from the group A (beta hemolytic) streptococcus, but not from commensal alpha haemolytic (viridans) streptococci which are co-inhabitants of the upper respiratory tract. Optochin (an antibiotic) sensitivity is routinely employed to differentiate the pneumococcus from *Streptococcus viridans.* An example of *S. Pneumonia* is shown in **Figure 3.13.**

Figure 3.13: *S. pneumonia* on agar plate. Photographed with digital camera

Pseudomonas aeruginosa is a gram negative aerobic bacterium and from respiratory samples usually has a mucoid appearance, which is attributed to the production of alginate slime. It grows well on most laboratory media and commonly is isolated on blood agar or eosin-methylthionine blue agar. It is identified on the basis of its Gram morphology, inability to ferment lactose, a positive oxidase reaction, its fruity odour, and its ability to grow at 42°C.

On agar, pneumococci grow as glistening colonies, about 1 mm in diameter. The minimum criteria for identification and distinction of pneumococci from other streptococci are bile or optochin sensitivity, Gram-positive staining, and hemolytic activity. Pneumococci cause alpha hemolysis on agar containing horse blood. Under anaerobic conditions they switch to beta hemolysis caused by an oxygen-labile hemolysin. Typically, pneumococci form a 16-mm zone of inhibition around a 5 mg optochin disc, and undergo lysis by bile salts.

Experimental protocol

Samples were processed as soon as possible and within 48 hours of collection in a Category 3 laboratory in the Clinical Microbiology Department at the Royal Free Hospital. Samples were processed as routine clinical samples.

Differential stain

Initially a portion of the sputum was smeared onto a slide using a loop for differential stain to judge quality by counting the number of epithelial cells and white blood cells. This was done by light microscopy at x100 magnification. Briefly, a swab was dipped into the sputum pot and then smeared on to a slide. This was left to air dry for 10 minutes prior to staining. The slide was fixed in methanol and then stained with eosin (pro diff stain 1, 22001 Braidwood labs); by immersion 5 times, followed by buffered methylthianion (pro diff stain 2, 22002 Braidwood labs); by immersion 10 times and finally in buffer at pH 6.8 by immersion 5 times. If more than 25 epithelial cells were visible per field the sample was not processed further.

If the quality of the sputum sample was sufficient, an equal amount of sputasol (7.5ml aliquot of sputasol (SR0233A oxoid) is added to 92.5mls sterile water) was added to the remainder of the sample which was left to stand for10 minutes. Samples were streaked

onto the following plates in the following order (all plates oxoid): Columbia agar with horse blood, MaConkey agar, Chocolate, COBA. Plates were innocutlated in 2 halves; ½ streaked neat and the other half with diluent. The neat half was streaked first and then 10µl of sample (1 loop) was put into Ringers solution ¼ strength (BR0052 oxoid) and the other half of the plate streaked, giving a 10^{-5} sample dilution. The neat half provided a general impression of what was present but only if there were > 20 colonies in the diluent half was the organism considered to be present in significant numbers. All plates were left 18 – 24 hours to grow; Columbia agar and MaConkey agar in oxygen (aerobically), and chocolate and COBA plates in 5% CO2 (anaerobically).

The following plates were disced; chocolate agar disc of bacitracin (10mcg) (*haemophilus* resistant) and COBA has optochin added; (*streptococcus pneumoniae* sensitive).

Reading the plates and antibiotic sensitivities

24 hours later purity plates are checked and the Phoenix report collected. The Phoenix was a method of automated antibiotic selectivity (Becton Dickenson Phoenix 100) with the ability to identify 55 anaerobic and 57 aerobic organisms.

If an organism had grown, it was re-plated out for sensitivities, purity plates set up and the sample rerun on the Phoenix. Common antibiotic panels (oxoid) are given below:
1.Streptococcus pneumonia antibiotic bx panel; oxacillin 1µg, clartithromycin 15µg, co-trimoxazole 25µg, vancomycin 30µg, tetracycline 30µg and moxifloxacin 5µg.

2. Haemophilus panel; grown with factor X, V and XV. The antibiotic plate contained; tetracycline 30µg, ampicillin 10µg, co-trimoxazole 25µg, clarithromycin 15µg, augmentin 30µg and ceftriaxone 30µg.

3.11 Molecular Techniques

Nucleic Acid Extraction

DNA extraction

Principles of DNA extraction

DNA extraction allows collection of DNA for subsequent molecular analysis and involves 4 basic steps; cell lysis (to expose the DNA), removal of membrane lipids with detergent, removal of proteins with a protease and precipitation of DNA with alcohol. For this work, cells were lysed with an anionic detergent in the presence of a DNA stabilizer. The DNA stabilizer limited the activity of intracellular DNases and DNases found elsewhere in the environment. RNA was removed by treatment with an RNA digesting enzyme and other contaminants, such as proteins, removed by salt precipitation. Finally, the genomic DNA was recovered by precipitation with alcohol and dissolved in a buffered solution containing a DNA stabilizer.

Experimental protocol

Venous blood samples (10-20ml) were taken in EDTA tubes at the initial visit for all patients and control subjects and stored at -80^{o}C prior to DNA extraction. DNA extraction was performed using a Gentra® systems Puregene® genomic DNA purification kit (Qiagen Cat no. 158389) following the Whole-Blood-Enhanced Productivity protocol supplied by the manufacturer. This method had four stages and yielded between 100 and 300µg of DNA per sample.

Stage 1 - RBC Lysis

6mls of whole blood was added to a 15ml polypropylene Falcon tube containing 18ml RBC Lysis Solution. This was then inverted to mix and incubated for 5 minutes at room temperature. The sample was inverted again at least once during the incubation. The tube was then centrifuged at 1800 x g for 2 minutes. The supernatant was carefully poured off leaving behind a white blood cell pellet and ~200µl of residual liquid.

Stage 2 - Cell Lysis & Protein Precipitation

The tube was vortexed until the white cells were resuspended in the residual liquid to facilitate cell lysis.2mls of Cell Lysis Solution followed by 6ml of Protein Precipitation

Solution was pipetted into the sample. This was then vortexed at high speed for 20 seconds to completely lyse the WBC's and precipitate the proteins. The sample was further centrifuged (1800 x g) for 10 minutes and the precipitated proteins formed a tight, dark brown pellet.

Stage 3 - DNA Precipitation and Wash

The supernatant containing the DNA (leaving behind the precipitated protein pellet) was poured into a 15ml tube containing 6ml 100% Isopropanol (2-propanol) (59304 Sigma). The sample was mixed by inverting gently 50 times to precipitate the DNA. It was then centrifuged (1800 x g) for 5 minutes. At this point the DNA was visible as small white pellet. The supernatant was poured off and the tube drained briefly on clean absorbent paper. 6mls of 70% Ethanol (E7023 Sigma) was then added and the tube centrifuged (1800 x g) for 1 minute. The ethanol was poured away and the tube drained for 1 minute on absorbent paper.

Stage 4 - DNA Hydration

500µl of DNA Hydration Solution was then added and the DNA rehydrated by incubating at 65^0C for 1hour and then left overnight at room temperature. The sample was then centrifuged briefly and transferred to storage tubes. The DNA was stored at -20^0C.

RNA extraction

Principles of RNA extraction

RNA extraction involves the purification of RNA from biological samples but is complicated by the ubiquitous presence of ribonuclease enzymes in cells and tissues, which can rapidly degrade RNA. Several different methodologies were used for RNA extraction in this thesis depending upon the type of sample studied (sputum versus NPS) and whether the RT-PCR subsequent to the extraction was qualitative or quantitative.

Nuclisens easyMAG (bioMérieux)

Principles of easyMAG

This method was used to extract RNA from sputum and NPS samples that were qualitatively screened for the presence of viruses in the Virology department at the

Royal Free Hospital. The viruses investigated were respiratory syncytial virus (RSV), parainfluenza 1, 2 and 3, adenovirus, human metapneumovirus, enterovirus, rhinovirus and influenza A and B.

Essentially this is a semi automated platform that is based on a generic method for binding nucleic acids from complex biological samples to magnetic silica. The specimen was mixed with lysis buffer containing a chaotropic agent (GuSCN). Any cellular or viral material in the specimen is disrupted and the nucleic acids are released. This isolation process is initiated by adding magnetic silica to the lysed specimen utilising the magnetic property for separation. Nucleic acids present in the lysate bind to the silica under the high salt conditions. The magnetic silica is then washed several times using 2 buffers (wash buffers 1 and 2). Next the nucleic acids are eluted from the magnetic silica and concentrated in a specific volume of the elution buffer. This process is accelerated by flushing the magnetic silica in the elution buffer at an elevated temperature. The magnetic silica are separated from the concentrated nucleic acid elute.

Experimental protocol

2ml of lysis buffer was added to a 5ml capped tube. 110ul of sample (processed sputum or nps; see **section 3.6**) was added to its corresponding lysis tube having been vortexed prior to the addition for 5 seconds. Samples were then left for 10 minutes before loading on the machine. The samples were loaded by placing strips specific to the machine into the metal strip holder and pipetting the lysis mixture into its corresponding well. All the reagents needed were already loaded on the machine (Extraction buffer 1 -3). The magnetic silica was added to the run using a multichannel pipette. Once the machine finished its run, the corresponding extract from the sample strip was removed and placed into a lidded tube.

<u>High Pure Viral RNA kit</u>

This kit (Roche Cat. No. 11 858 874 001) was used for RNA extraction from processed NPS samples for quantitative RT-PCR.

Principles of extraction

Virus lysis is accomplished by incubation of the sample in a special Lysis/ Binding buffer in the presence of Proteinase K. Subsequently, nucleic acids bind specifically to

the surface of glass fibres in the presence of a chaotropic salt. The binding reaction occurs within seconds due to the disruption of the organized structure of water molecules and the interaction of nucleic acids with the glass fibres surface. The glass fibre fleece is contained in the high pure spin filter tubes. Thus, adsorption to the glass fibre fleece is favoured. Since the binding process is specific for nucleic acids, the bound nucleic acids are purified from salts, proteins and other impurities by a washing step and are eluted in low salt buffer or water.

Experimental protocol

200µl of PBS from the eppendorf containing the NPS was added to a nuclease-free 1.5 ml microcentrifuge tube. 200µl of freshly prepared working solution (carrier RNA-supplemented Binding Buffer) was added. 50µl of Proteinase K solution was added, the tube mixed immediately and incubated for 10 minutes at 72°C.100µl of Binding Buffer was added, the tube mixed and the sample transferred to a High Filter Tube in a Collection Tube. The entire sample was pipetted into the upper reservoir of the Filter Tube and the entire High Pure Filter Tube assembly centrifuged for 1 minute at 8,000 × *g*. After centrifugation the Filter Tube was removed from the Collection Tube, and the collection tube and elution discarded. The Filter Tube was combined with a new Collection Tube and 500µl Inhibitor Removal Buffer added to the upper reservoir of the Filter Tube and centrifuged for 1 minute at 8,000 × *g*. After centrifugation the Filter Tube was removed from the Collection Tube, and the collection tube and elution liquid discarded. The filter tube was then combined with a new Collection Tube. 450µl of Wash Buffer was added to the upper reservoir of the Filter Tube and centrifuged for 1 minute at 8,000 × *g*. The elution was then discarded. After this wash, the Filter Tube was removed from the Collection Tube, and the elution and the Collection Tube discarded. The Filter Tube was combined with a new Collection Tube, 450µl Wash Buffer added to the upper reservoir of the Filter Tube and this was centrifuged for 1 minute at 8,000 × *g*. The elution was discarded and the Filter Tube-Collection Tube assembly left in the centrifuge and spun for 10 seconds at maximum speed (approx. 13,000 × *g*) to remove any residual Wash Buffer. The collection Tube was discarded and the Filter Tube inserted into a nuclease free, sterile 1.5 ml microcentrifuge tube. 50µl of Elution Buffer was added to the upper reservoir of the Filter Tube and the tube assembly centrifuged for 1 minute at 8,000 × *g*. The eluted viral nucleic acids were then stored at –80°C or used immediately.

TRI LS reagent (T3934 Sigma).

This method was used to extract RNA from processed sputum samples for quantitative RT-PCR.

Principles of extraction

TRI Reagent LS is a mixture of guanidine thiocyanate and phenol in a monophase solution. When a biological sample is homogenized or lysed with it, and chloroform or 1-bromo-3-chloropropane is added, the mixture separates into 3 phases: an aqueous phase containing RNA, the interphase containing DNA, and an organic phase containing proteins. Each component can then be isolated after separating the phases. The procedure is very effective for isolating RNA molecules of all types from 0.1–15 kb in length. The resulting RNA is intact with little or no contaminating DNA or protein.

Experimental protocol

250µl of processed sputum was added to 750µl of Tri-reagents and vortexed for 2 minutes. 200µl chloroform was added after samples were left to stand for 5 minutes. Samples were then vortexed 2 minutes, left to stand on ice 10 minutes and then centrifuged at 12,000 g for 15 minutes. The top layer was transferred to a new tube, 500µl isopropanol added and the sample left for 10 minutes before centrifuging at 12,000g for 20 minutes. Pellets were then washed in 80% ethanol, dried and resuspended in 30µl RNase free water.

PCR

Principles of PCR

Polymerase chain reaction (PCR) is an *in vitro* method used to amplify specific regions of DNA (usually gene sequences) to be investigated. This process involves designing a pair of small oligonucleotide primers which anneal to the forward and reverse strands of the DNA template since the primers are complementary to the two strands. They act as the beginning and end points for allowing *Taq* DNA polymerase to bind and begin synthesizing a new complementary DNA strand. The PCR reaction contains a DNA Template, which contains the DNA sequence to be amplified, two primers; to determine the start and end of the region to be amplified, *Taq* Polymerase, a DNA polymerase that copies region to be amplified, Deoxynucleotide triphosphates (dNTPs) which are used

by *Taq* to build the new DNA, Buffer Solution to provide suitable environment for the *Taq* and Mg^{2+} to help *Taq* polymerase to associate with the annealed primer.

PCR has three major steps that are repeated for 20-40 cycles. (1) Denaturation of DNA at 94-96^{0}C causing double-stranded DNA to separate forming two single strands. (2) Annealing at 45-65^{0}C allowing the primer to bind to single-stranded DNA. (3) Elongation at 72^{0}C allowing *Taq* to copy/elongate the complementary strand using dNTPs. The PCR process is carried out on a thermal cycling machine which heats and cools the reaction mixture for a short period of time. Since the process is repeated many times there is an exponential growth in the number of copies of the target sequence/gene.

Reagents (in alphabetical order)

Agarose; (A-7431 Sigma). Agarose is a purified linear galactan hydrocolloid isolated from agar or agar-bearing marine algae. Structurally, it is a linear polymer consisting of alternating D-galactose and 3,6-anhydro-L-galactose units. As a gelling agent, agarose is used to separate nucleic acids electrophoretically because its gels have larger pore sizes than polyacrylamide gels at low concentrations. Anionic groups in an agarose gel are affixed to the matrix and cannot move, but dissociable cations can migrate toward the cathode in the electrophoresis tank, giving rise to electroendosmosis (EEO) – a movement of liquid through the gel. Since electrophoretic movement of biopolymers is usually toward the anode, EEO can disrupt separations because of internal convection.

Ethidium Bromide (E1510 Sigma; 3,8-Diamino-5-ethyl-6-phenylphenanthridinium bromide). This is used for staining a gel for electrophoresis. Ethidium bromide was incorporated into the gel and visualized immediately after electrophoresis on a UV light box (254 nm wavelength).

Loading dye (G2526 Sigma). The Gel Loading Buffer contains bromphenol blue, which serves as tracking dye, and sucrose to add density and facilitate sample loading. EDTA has been included to terminate the action of enzymes that require divalent cations. Sodium lauryl sulfate has been added to help dissociate DNA-protein complexes which can interfere with electrophoresis. Suitable for non-denaturing polyacrylamide and

agarose gel electrophoresis of nucleic acids. This was added to the PCR low ladder for visualisation.

PCR low ladder (100bp P1473 Sigma). This contains 10 bands from 100 to 1000 base pairs (bp) in exact 100bp spaced recombinant repeats. It is in solution in 10mM Tris-HCl, pH 8.0, 1mM EDTA. It is designed for use on agarose gels and is diluted with gel loading buffer as per instructions.

PCR master mix (M7143 Promega goTaq colourless master mix containing loading dye). This is a premixed ready to use solution containing *Taq* DNA polymerase, dNTPs, $MgCl_2$ and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR. This master mix has been optimised for use in routine PCR for amplifying DNA templates in the range of 0.2 – 2kb.

1xTAE (Tris Acetate EDTA 10x Sigma T9650). This product is a 10x concentrate containing 0.4 M Tris acetate (pH approximately 8.3) and 0.01M EDTA. It is prepared with 18 megohm water and is 0.2 µm filtered. This product is suitable for agarose gel electrophoresis after dilution to the working concentration. The 1x TAE buffer is used both in the agarose gel and as a running buffer. Applied voltages of < 5 V/cm (the distance between the electrodes of the unit) are recommended for maximum resolution.

Experimental protocols

Multiple optimisation experiments were performed altering Mg^{2+} concentrations and PCR cycling times and conditions. The final conditions used for each reaction are given below. PCR was carried out using the Techne TC-412 (Scientific Laboratories).

All primers were ordered from Operon (eurofins/mwg Operon) and made up to 100µM solutions by adding the appropriate volume of 1xTE (Tris EDTA) as per the data sheet according to the molarities provided. Primers were then diluted to 20µM (20µl of 100µM primer + 80µl TE) for use in experiments that follow. The primers are listed in **Table 3.8** below (5' to 3').

Table 3.8: Primers for PCR

PCR	Forward primer	Reverse primer
ICAM-1	CTTGAGGGCACCTACCTCTG	AGGATACAACAGGCGGTGAG
IL-6 For sequencing	TGGCAAAAAGGAGTCACACA	TCCTGGAGGGGAGATAGAGC
IL-6 For RFLP	AAGTTAAGGAAGAGTGGTTC	GATAAATCTTTGTTGGAGGG
IL-8 (-251A>T)	TGCCATTAAAAGAAAATCATCC	TCCACAATTTGGTGAATTATTAA
IL-8 (781 C>T)	CTCTAACTCTTTATATAGGAATT	TGGAGACTATGGAAGGCATCA
VDR *Fok*I	AGCTGGCCCTGGCACTGACTCTGCTCT	ATGGAAACACCTTGCTTCTTCTCCCTC
VDR *Taq*I	GGGACGATGAGGGATGGACAGAGC	GGAAAGGGGTTAGGTTGGACAGGA
VDR *Bsm*I	AACTTGCATGAGGAGGAGCATGTC	GGAGAGGAGCCTCTGTCCCATTTG

ICAM-1 PCR

The reaction consisted of 12.5µl PCR master mix, 0.5µl Forward primer, 0.5µl Reverse primer, 9µl water and 2.5µl DNA. PCR machine conditions; denaturation 94°C for 5 minutes, then 40 cycles of denaturation 94°C for 30 seconds, annealing 58°C 1 minute, extension 72°C 1 minute. The final extension was at 72°C for 15 minutes.

IL-6 PCR (for sequencing)

The reaction consisted of 12.5µl PCR master mix, 0.5µl Forward primer, 0.5µl Reverse primer, 1.5µl Mg^{2+}, 7.5µl water and 2.5µl DNA. PCR machine conditions; denaturation 94°C for 5 minutes, then 40 cycles of denaturation 94°C for 30 seconds, annealing 55°C 1 minute, extension 72°C 1 minute 10 seconds. The final extension was at 72°C for 15 minutes.

IL-6 PCR (for RFLP)

The reaction consisted of 12.5µl PCR master mix, 1.0µl Forward primer, 1.0µl Reverse primer, 8.0µl water and 2.5µl DNA. PCR machine conditions; denaturation 94°C for 5 minutes, then 35 cycles of denaturation 94°C for 30 seconds, annealing 54°C 1 minute, extension 72°C 1 minute. The final extension was at 72°C for 7 minutes.

IL-8 PCR (-251A>T)

The reaction consisted of 12.5µl PCR master mix, 1.0µl Forward primer, 1.0µl Reverse primer, 1.5µl Mg^{++}, 6.5µl water and 2.5µl DNA. PCR machine conditions; denaturation 94°C for 5 minutes, then 40 cycles of denaturation 94°C for 30 seconds, annealing 55°C 1 minute, extension 72°C 1 minute 10 seconds. The final extension was at 72°C for 15 minutes.

IL-8PCR (781 C>T)

The reaction consisted of 12.5µl PCR master mix, 1.0µl Forward primer, 1.0µl Reverse primer, 1.5µl Mg^{++}, 6.5µl water and 2.5µl DNA. PCR machine conditions; denaturation 94°C for 5 minutes, then 40 cycles of denaturation 94°C for 30 seconds, annealing 54°C 1 minute, extension 72°C 1 minute 40 seconds. The final extension was at 72°C for 20 minutes.

Vitamin D Receptor (VDR) polymorphism PCR

The primers and RFLP enzymes were chosen from previously published methodology (Wilkinson 2000). The conditions for both the PCR and RFLP were optimised and final protocols are given below.

*Fok*I PCR

The reaction consisted of 12.5µl PCR master mix, 1.0µl Forward primer, 1.0µl Reverse primer, 8.0µl water and 2.5µl DNA. PCR machine conditions; denaturation 94°C for 5 minutes, then 40 cycles of denaturation 94°C for 30 seconds, annealing 56°C 30seconds, extension 72°C 30 seconds. The final extension was at 72°C for 5 minutes.

*Taq*1α PCR

The reaction consisted of 12.5µl PCR master mix, 1.0µl Forward primer, 1.0µl Reverse primer, 8.0µl water and 2.5µl DNA. PCR machine conditions; denaturation 94°C for 5 minutes, then 40 cycles of denaturation 94°C for 30 seconds, annealing 66°C 30seconds, extension 72°C 30 seconds. The final extension was at 72°C for 5 minutes.

*Bsm*I PCR

The reaction consisted of 12.5µl PCR master mix, 1.0µl Forward primer, 1.0µl Reverse primer, 8.0µl water and 2.5µl DNA. PCR machine conditions; denaturation 94°C for 5

minutes, then 30 cycles of denaturation 94°C for 30 seconds, annealing 56°C 30seconds, extension 72°C 30 seconds. The final extension was at 72°C for 5 minutes.

All PCR products were run on a 2% agarose gel with the pcr low ladder. The agarose gel was made up as follows; for a 2% gel 2gm of agarose was added to 100ml 1xTAE buffer, heated until boiling and 3µl Ethidium Bromide added. Once cooled but still liquid, the agarose was poured into the running gel mould, a sample comb was placed at the top and the gel left to set fully. Once set the gel was covered in 1xTAE buffer and 4µl of sample was added to each well and 2µl loading dye and 4µl ladder at either end of the electrophoresis chamber. The gel was run at 100mV for 15 minutes (Horizon 58, Biometra). An example of a gel from each PCR has been given below (**Figures 3.14 – 3.21**).

Figure 3.14: ICAM-1 PCR product and ladder

PCR product size 154bp. The lower band represents primer dimer.

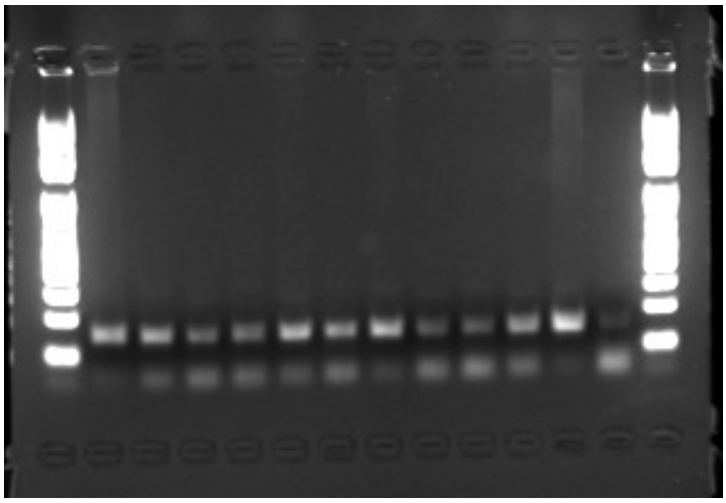


Figure 3.15: IL-6 PCR clean up product sent for sequencing

Product 716bp.

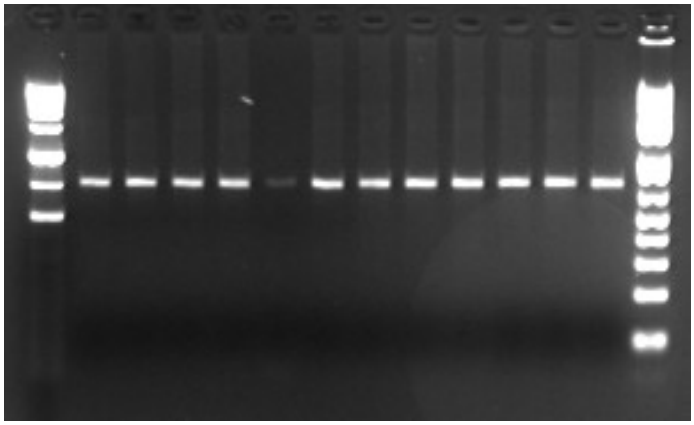


Figure 3.16: IL-6 PCR product for RFLP

Product 178 bp.

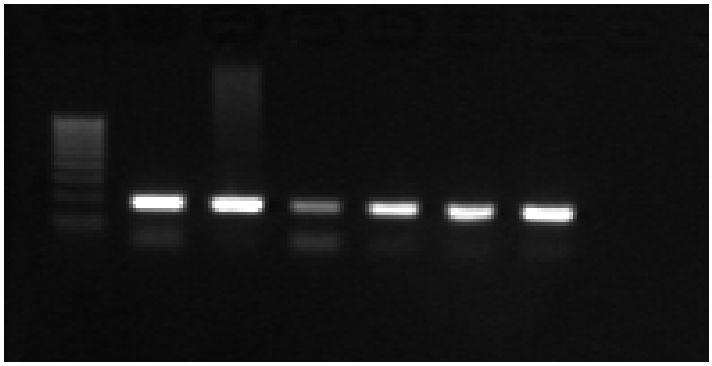


Figure 3.17: IL-8 -251A>T PCR product and ladder

PCR product 132 bp. Negative water at the end.

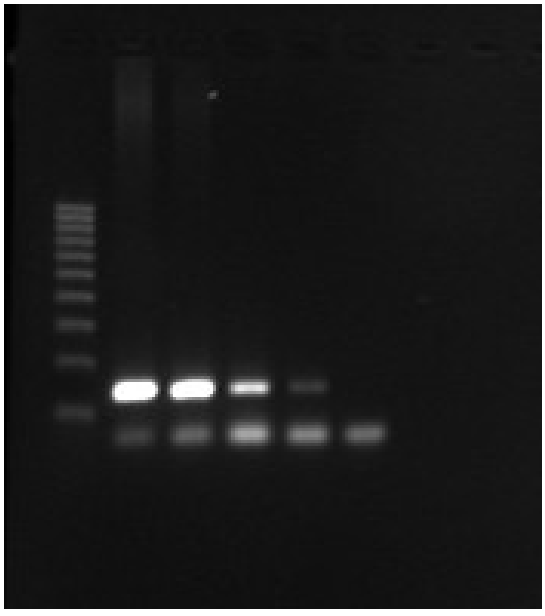


Figure 3.18: IL-8 781 PCR product and 1kb ladder

PCR product 139bp.

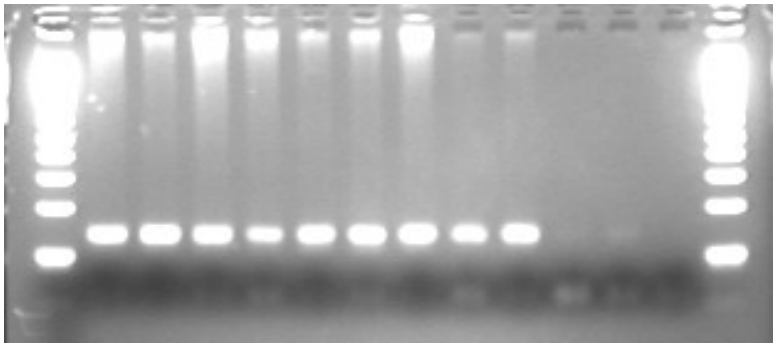


Figure 3.19: VDR *Fok*I PCR product

PCR product 265bp

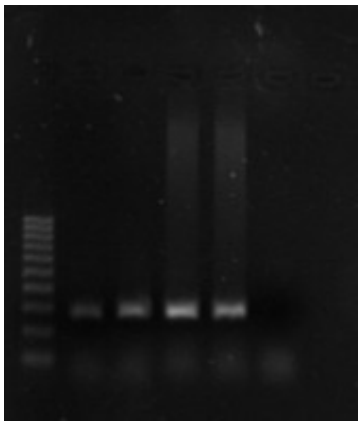


Figure 3.20: VDR *Taq*Iα PCR product

PCR product 495bp

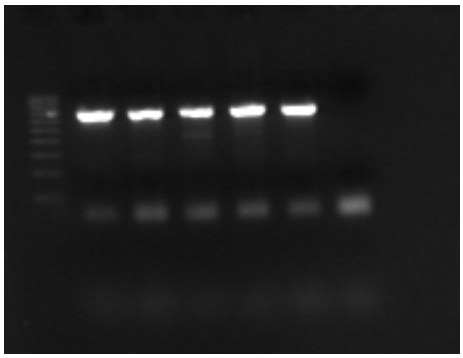
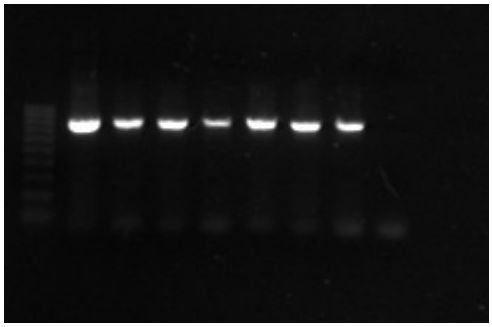


Figure 3.21: VDR *Bsm*I PCR product

PCR product 813bp



Real time RT-PCR and real time Quantitative RT-PCR

Principles of RT-PCR and quantification

Real time PCR is the continuous collection of a fluorescent signal from one or more polymerase chain reactions over a range of cycles. The real-time PCR relies upon the dynamic detection of a signal given by one of a number of fluorescent signals during a PCR reaction. This reporter dye is chemically bound to the 5' end of a 20-30bp "Taqman" DNA probe to which is also bound a quencher dye at the 3' end. Whilst these are attached to the probe, and thus in close proximity, any fluorescent excitation energy is passed to the quencher dye which does not emit light in the spectrum of the reporter dye. The probes are designed to have a ~ 10 degree Celsius higher melting temperature than the PCR primers and thus bind to any target before the primers. During polymerisation both dyes are released and the reporter dye is free to produce a signal upon excitation and is measured at the end of each PCR cycle. This fluorescent signal is in direct proportion to the amount of DNA amplified. The point at which the fluorescence data curve crosses a threshold set to eliminate background signal is known as the cycle threshold or ct. The ct is in direct relation to the amount of DNA product and thus the amount of target in the original sample. Quantitative real time PCR allows the conversion of the fluorescent signals from each reaction into a numerical value for each sample. RNA quantification begins with making cDNA (complementary DNA) by reverse transcriptase.

cDNA for real time RT-PCR

cDNA was synthesized from purified RNA using the Qiagen Sensicript reverse transcription kit (QIAGEN, Cat NO. 205213). This cDNA was used as template in

several multiplex real-time PCRs: (Influenza A, influenza B), (Respiratory Syncytial Virus), (Parainfluenza types 1-3), (Adenovirus and Enterovirus), (Metapneumovirus) and (Rhinovirus). This kit contained: 10X RT Buffer, DNase, RNase free water, dNTP's, Reverse Transcription enzyme (RT). Other reagents needed included RNAse Inhibitor (Qiagen Cat NO. N2615) and Random Hexamers at 100uM (Amersham Biosciences, Pd(N)6 Random Hexamer, Cat No 27-2166-01). One reaction consisted of 4µl 10xRT buffer, 4µl dNTPs, 4µl 100µM hexamer primer, 0.5µl RNase inhibitor, 2µl reverse transcriptase, 6.5µl RNase free water. The RT reaction was carried out as follows; RT 1 hour at 37°C, RT inactivation for 5mins at 95°C, Hold at 4°C. 5µl of RT product is added to each well for each of the multiplex mixes.

cDNA for quantitative real time RT-PCR

cDNA was prepared using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems 4368814). Prior to preparing the reaction plate the 2x reverse transcription (RT) master mix was prepared. All preparation was carried out on ice. The 2x RT solution contained the following per 20µl reaction; 2µl 10x RT buffer, 0.8µl 25x dNTP Mix (100mM), 2.0µl 10× RT Random Primers, 1µl MultiScribe™ Reverse Transcriptase, 1µl RNase Inhibitor, 3.2 µl Nuclease-free water.

10µl of 2xRT master mix was added to each well of a 96 well plate and 10µl of RNA sample added to each well, pipetting up and down to mix. The plate was sealed and loaded into the PCR machine. The programme was set to RT50; 10 min 25°C initial, 2 hours 37°C, 5 minutes 85°C inactivates. 3 controls were run on each plate. The positive control was known virus, the negative control water and the template control RT master mix with no RT enzyme to ensure no cDNA contaminant was present.

RT-PCR

Several real time RT-PCR assays have been developed to allow rapid, sensitive and simultaneous detection of viruses in clinical samples. One primer pair and probe designed to amplify a DNA sequence present in all human cells, the K-Ras oncogene was used to demonstrates adequate nucleic acid extraction and excludes false-negative results resulting from a lack of extracted nucleic acid from cellular material.

Below is a list of Oligonucleotides and probes for the real-time PCR reactions (**Table 3.9**).

Table 3.9: Primers and probes for qualitative virology RT-PCR

ID	sequence 5'---3' (F,R,Probe)	Region	Acc.No	Length	Working Conc (nm)
FluAFwd	CAAGACCAATCCTGTCACCTCTG	MP	U49119	151-257	900
FluARvr	TGCATTTTGGACAAAGCGTCTAC				900
FluAprobe	[6-FAM]AGTCCTCGCTCACTGGGCACGGT[BHQ1a-6FAM]				200
FluBFwd	GGCAACACCTGTTTCCATATTAAG	NP	AY60946	1094-1215	900
FluBRvr	GCCTGTTAATGCAGACAAAACTC				900
FluBprobe	[AminoC6+JOE]TCATAGGCAGCTCCGAAGCAAGACATGA[BHQ1a-JOE]				200
RSVMFW	GGAAACATACGTGAACAAGCTTCA	MP	AF067125		250
RSVMRVRA	CATCGTCTTTTTCTAAGACATTGTATTGA				250
RSVMRVRB	TCATCATCTTTTTCTAGAACATTGTACTGA				100
RSVprobe	[6-FAM] TGTGTATGTGGAGCCTT[MGBBNFQ]				100
Para1Fwd	ATTCAGACAGGATGGAACCGTTAA	NP	D01070	659-757	900
Para1Rvr	GATACTAAGCTTTGTTGTGACCTCAT				900
Para1probe	[AminoC6+JOE]ACCAATGCCTTCAACTGTGTCTCCCGTG[BHQ1a-JOE]				200
Para2Fwd	AGAGATAACAGGGTTTGAGAATAATTCAT	NP	M55320	462-599	900
Para2Rvr	CAAATGGAGTTTGGTGATTAAGGGTA				900
Para2probe	[6-FAM]TCCAGATGCTCGATCAACTATGTCCAGAGG[BHQ1a-6FAM]				200
Para3Fwd	CGATTAGAGGCTTTCAGACAAGATG	NP	M14552	636-735	900
Para3Rvr	CTGTTGAGACCGCATGATTGAC				900
Para3probe	[Cy5]CCACTGTGTCACCGCTCAATACCAGCC[BHQ3a-Cy5]				200
EnteroFwd	CCCCTGAATGCGGCTAATCC	5'UTL	AY343035	17-160	900
EnteroRvr	GTCACCATAAGCAGCCAATATAAGAA				900
Enteroprob	[AminoC6+JOE]AACACGGACACCCAAAGTAGTCGGTTCC[BHQ1a-JOE]				200
AdFwd	GCCCCAGTGGTCTTACATGCACATC	Hexon	J01917	249-376 Hiem etal 2004, modified	900
AdRvr	GCCACGGTGGGGTTTCTAAACTT				900
Adprobe	[6-FAM]TGCACCAGACCCGGGCTCAGGTACTCCGA[BHQ1a-6FAM]				200
RhinFwd	TGTGCTCRCTTTGAGTCCTC	5'UTL	X02316	419-555	900
RhinoFwdA	TGTGCTCAGTGTGCTTCCTC				900
RhinRvr	TGAAACACGGACACCCAAAGTA				900
Rhinprobe	[Cy5]CCCTGAATGYGGCTAACCTTAAMCCTGC[BHQ3a-Cy5]				200
MetaFwd	CATATAAGCATGCTATATTAAAAGAGTCTC	NP	AY145276	34-198 Maertzdorf et al-2004	500
MetaRvr	CCTATTTCTGCAGCATATTTGTAATCAG				250
Metaprobe	[6-FAM]TGYAATGATGAGGGTGTCACTGCGGTTG[BHQ1a-6FAM]				500
K-RasFwd	GCCTGCTGAAAATGACTGAATATAAAC	K-Ras	AF285779	443-534	600
K-RasRvr	TGATTCTGAATTAGCTGTATCGTCAAG				600
K-RasProbe	[AminoC6+JOE]TGCCTACGCCACAAGCTCCAACTACCA[BHQ1a-JOE]				100
DPVFwd	CGGGTGCCTTTTACAAGAAC				900
DPVRvr	TTCTTTCCTCAACCTCGTCC				900
PDVprobe	[AminoC6+JOE]ATGCAAGGGCCAATTCTTCCAAGTT[BHQ1a-JOE]				200

Primers and probes were delivered as freeze dried pellets and reconstituted to 100µM concentrations using 1xTE (Tris/EDTA Buffer Sigma 9285) prior to use except for the RSV probe which was diluted to 100nM at working strength. 45µl of each PPMM was added to the TaqMan plate and the plate stored at 4°C until inoculated. Samples were diluted 1:4 by adding 110µl of sample to 330µl of water (pharmacy grade). All samples were treated with proteinase K ; 44ul of proteinase K (Proteinase K lyophilized 100mg use at 10mg/ml (Merk Chemicals Ltd Cat No. 70663) added to each sample (10mg/ml, 0.5% SDS, 20 mM Tris-HCl, pH 8.3.), 50µl of PDV (pre-aliquoted) was added per sample. Samples were incubated at 55°C for 15 minutes in a water bath and vortexed every 5 minutes. All primers and probes were ordered from Operon (www.operon.com). The RSV MGB probe was ordered from Applied Biosystems (www.appliedbiosystems.com) and TaqMan® universal mastermix no AMPErase UNG (Applied Biosystems Cat NO. 4324018). The following table shows the reaction mixes for each PCR (**Table 3.10**).

Table 3.10: Sample preparation for qualitative virology RT-PCR

Primer/ Probe(PP) ID	PP µl/250 samples	Total PP µl	Total TE buffer µl	Total
FluA1	**112.5**			
FluA2	**112.5**	**250**	**250**	500ul
FluAprobe FAM	**25**			
FluB1	**112.5**			
FluB2	**112.5**	**250**	**250**	500ul
FluBprobe JOE	**25**			
Para1F	**112.5**			
Par1R	**112.5**	**250**	**250**	500ul
Par1probe JOE	**25**			
Par2F	**112.5**			
Par2R	**112.5**	**250**	**250**	500ul
Par2probe FAM	**25**			
Par3F	**112.5**			
Par3R	**112.5**	**250**	**250**	500ul
Par3probe CY5	**25**			
Entero/1	**112.5**			
Entero/2	**112.5**	**250**	**250**	500ul
Enteroprob JOE	**25**			
Ad1	**112.5**			
Ad2	**112.5**	**250**	**250**	500ul
Adprobe FAM	**25**			
Rhin1	**112.5**			
Rino1A	**112.5**	**362.5**	**137.5**	500ul
Rhin2	**112.5**			
Rhinprobe CY5	**25**			
DPVFwd	**112.5**			
DPVRvr	**112.5**	**250**	**250**	500ul
DPVProbe	**25**			
Meta1	**62.5**			
Meta2	**31.25**	**156.25**	**343.75**	500ul
Metaprobe FAM	**62.5**			
K-Ras1	**75**			
K-Ras2	**75**	**162.5**	**337.5**	500ul
K-RasProbe JOE	**12.5**			

500µl of monoplex forward, reverse primers and probe was mixed according to table 2. Working strength monoplex/ multiplex mix was made up from the initial monoplex primer/probe mixes. In each well for 1 reaction; 15µl working strength monoplex/multiplex, 25µl Universal Mastermix , 5µl H_2O.

Preparation of Controls

Controls were treated in exactly the same way as the samples. Each run contained a positive and negative control. K-Ras, an oncogene of the human genome, was already present in the samples and acted as an internal control to show that a sufficient amount of epithelial cells have been extracted. Therefore, if K-Ras is negative there were insufficient cells in the original sample. Phocine Distemper Virus type I (PDVI) is a control to test if there were any inhibitory substances in the reaction originated from the sample extraction. If PDV is negative then there were inhibitory substances in the original sample. PDV virus can be grown in Vero cells. PDV is generally used at a dilution of 1/1000.

The PCR procedure is susceptible to false positive results due to contamination. Only a small amount of target contamination need be present to give a positive result. For this reason each PCR run includes a negative water control which is extracted at the same time as the samples. The RT reaction contains 5µl of water instead of sample. This controls the extraction, RT step and the TaqMan detection. The positive control is a dilution of plasmid of the different respiratory viruses. It is DNA and therefore, it does not needs to be included in the RT run but in rtPCR detection. It is only a control for the TaqMan step. A RNA control is added before extraction for testing extraction, RT and PCR reaction. The RNA control is PDV. The thermal cycler conditions are 50°C for 2 mins then 95°C for 10 mins. This is then followed by 45 cycles of 15 seconds 95°C, 1min 60°C. 45µl of primer probe master mix is added per well with 5µl of cDNA per well. Water controls and NTC and positive controls are added on the plate.

It was ensured that all positive controls displayed amplification curves and had a ct between 25 and 35 cycles. If any positive control failed in an assay then the PCR for that virus was repeated from the DNA/RNA extracts. The amplification of the internal control (K-Ras) was demonstrated for all samples. If there is no amplification curve for

the K-Ras gene then this indicated inadequate nucleic acid in the sample or the presence of inhibitors in the sample. All water samples showed no amplification. If a positive result was obtained, the entire test was disregarded and repeat extraction process and analysis as contamination has occurred. Internal positive control should show a sigmoid curve during the amplification reaction. If internal control (PDV) does not come out positive, the test has to be repeated from the samples extraction step.

Calculation of PCR results

This was carried out on the computer connected to the PCR machine. The baseline and threshold were set and the run analysed. All plots used gave typical sigmoid curves. It was ensured that the water controls give no amplification, the NTC (no template controls) give no amplification, the positive control has amplified and standards have come up at the right levels.

Quantitative PCR for HRV and RSV

The primers that were used in this investigation for PCR were designed using widely available free software programs found on the internet. These packages (Primer3) were able to design the correct primer sequence needed for the polymorphisms to be located within the required gene sequence. They also determined the size of the PCR product that will be generated by working out the distance between the forward and reverse primer. The PCR product size varies, but is usually smaller for genotyping and can be slightly longer for sequencing especially if more than one polymorphism is located within the PCR product. The primers used were manufactured by Invitrogen™. The primers were diluted down to a working stock of 20μM which correlates to a working concentration of 0.2μM. The primers and probes were designed in our lab by Dr. Dagmar Alber using Primer Express software was used to design the primers for RSV A and B. For HRV; forward primer; 5'-TGADTCCTCCGGCCCCT-3' and reverse primer; 5'-AAAGTAGTYGGTCCCRTCC-3'. The probe was a minor groove binding probe; 6-FAM-AATGYGGCTAACCT-MGB. For RSVA: forward primer; 5'-CAAAGATCAACTTCTGTCATCCAGCAA and reverse primer; 5'-TGTGTTTCTGCACATCATAATTAGGA. The probe contained a 6-FAM reporter (6-carboxyfluorescein) and a 3' TAMRA quencher (6-carboxytetramethylrhodamine); ACACCATCCAACGGAGCACAGGAGA. For RSVB; forward primer, 5'-

AGGTACACCAAGAAACCAGGATCT and reverse primer 5'-CCTCCAATTCTTCTGCTGTTAAGTC and probe; 6-FAM-ATGCAGCCAAAGCATATGCAGAGCAAC-TAMRA.

Real-time PCR was performed using the ABI Prism 7500 Real Time PCR System (Applied Biosystems). For HRV 25µl reaction volumes were set up (12.5µl QuantiTect Probe PCR Master Mix (ROX reference dye, Qiagen), 1µl forward and reverse primers (20µM), 0.35µl probe (20µM), 2.5µl template and 7.65µl RNase free water. PCR conditions: 95°C 15 min, 40 cycles of 95°C 15 sec and 58°C 80 sec. For RSV a 25µl reaction consisted of: 12.5µl QuantiTect Probe PCR Master Mix, 0.5µl of forward and reverse primers (20 µM), 0.25µl probe (20µM), 2.5µl template and 8.75µl RNase free water. PCR conditions were as follows: For RSVA: 95°C, 15 min followed by 40 cycles of 95°C 15 sec and 58°C 70 sec. RSVB: 95°C, 15 min followed by 40 cycles of 95°C 15 sec and 60°C 1 min. Samples were initially run as singles and any positive samples were repeated in duplicates and data pooled. If the two runs varied, the sample was re-run in triplicate.

Controls and standards for real-time RT PCR

The following controls were included. At the RNA extraction stage at least 1 positive and 1 negative control were included per 16 samples. Further template controls were included at the RT and PCR amplification stage. Standards were prepared for RSVA (Long strain) and RSVB, as follows: Vero cell monolayers were infected at multiplicity of infection (moi) of approximately 3 and incubated at 37°C in a humidified 5%CO_2 95% air incubator until almost complete cytopathic effect (CPE) was observed. Cells were scraped off the flasks and supernatant and cells were briefly vortexed and then centrifuged to remove cell debris. Virus was aliquoted and stored at -80°C. For HRV, HeLa-Ohio cell monolayers at 90% confluence were infected with HRV 16 at 1 moi and incubated at 33°C, 5% CO_2 95% air on a shaking platform in the presence of 2mM $MgCl_2$ for 24 hours until CPE was observed. Cells were scraped off the flasks and supernatant and cells were briefly vortexed and then centrifuged to remove cell debris. The supernatant was aliquoted and stored at -80°C. Virus was titrated by plaque assay (Chapman 2007) and used as standards for real-time RT-PCR. 2mM $MgCl_2$ was added to the overlay for HRV.

As additional standards a 92bp and 106bp region of the N-gene of RSVA and RSVB or a 137 bp region of the 5'UTR HRV containing the amplicon of the real-time RT-PCR was amplified, cloned into - pCRII-TOPO vector and transformed into either TOPO10 or Mach1 bacterial strain (Invitrogen). Clones were PCR verified and sequenced. Purified plasmids (Qiaprep Miniprep spin kit, Qiagen) were linearised with BamHI, DNAse treated, phenol chloroform extracted and *in vitro* transcribed using the Riboprobe *in vitro* transcription system (Promega). Samples were again phenol chloroform extracted and the size of the *in-vitro* transcript verified on a denaturing RNA agarose gel. Aliquots of the *in-vitro* transcripts were frozen at -80^0C prior to use as a standard for qRT-PCR.

A standard curve prepared from tissue culture grown virus extracted in the same manner as the clinical samples was set up at the RT stage using 6 10-fold dilutions. A second separate standard curve was included at the PCR stage. Results were only accepted if both standard curves were comparable, had an r^2>0.98 and a slope between -3.1 to -3.6. In addition a reverse transcribed standard curve of the RNA transcript was included in duplicate on the quantification plates. A cycle threshold (ct)-value of 37 was taken as a cut-off point for RSV positivity. Both RSVA and B real-time RT-PCR did not amplify any human rhinovirus or influenza A. RSVA positive samples did not produce a ct-value <31 when amplified using the RSVB real-time RT-PCR and vice versa. Only sputum was tested for RSV. For HRV a sputum ct value of 38 was taken as a cut off for positivity (1 pfu/ml) and for NPS a ct value of 39.5 (1 pfu/ml) was used. For RSV A a ct value of 35 and for RSV B a ct value of 37 was taken as the cut off for positivity. The following calculation was used to determine the pfu/ml value; pfu/ml = 960x(10^(-0.3156x(ct value)+9.1179)). This calculation was based on the standard curve derived and the results from the virus titration experiments.

Restriction Fragment Length Polymorphism (RFLP)

Principles of RFLP

In RFLP analysis a DNA sample is broken into pieces (digested) by restriction enzymes and the resulting restriction fragments separated according to their lengths by gel electrophoresis. The basic technique for detecting RFLPs involves fragmenting a sample of DNA by a restriction enzyme, which can recognize and cut DNA wherever a specific short sequence occurs. The resulting DNA fragments are then separated by

length by agarose gel electrophoresis, with the smaller fragments migrating farther than the larger fragments.

The ICAM-1 K469E, IL-8 -251T>A, IL-8 781C>T, IL-6 -174G>C and VDR polymorphisms were genotyped by forced restriction endonuclease digest of PCR products (forced-RFLP). Specific enzyme recognition sites were introduced into the PCR product by forcing sequence changes in the design of the PCR primers.

ICAM-1

To genotype the samples for the K469E polymorphism (rs5498), a forced RFLP analysis was carried out by using the *Bst*UI restriction endonuclease enzyme (R0518L New England BioLabs) which recognised the following site;

5′...CG▼CG...3′
3′...GC▲GC...5′

This resulted in the following bands; KK 154bp, KE 154, 101, 53bp, EE 101 and 53bp, shown in **Figure 3.22**.

Figure 3.22: ICAM-1 RFLP and 1Kb ladder either side

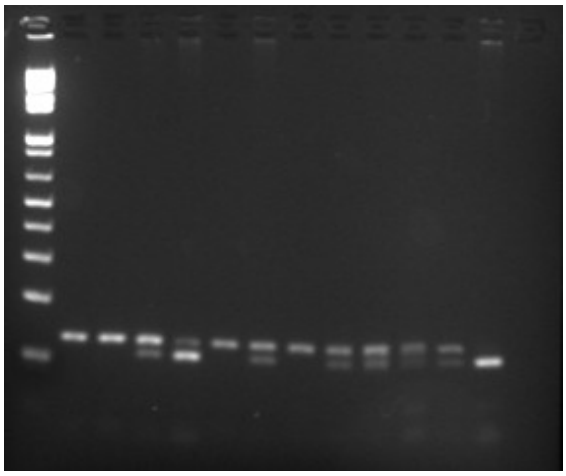
IL-8 -251T>A and IL-8 781C>T

To genotype the samples for the IL-8 -251T>A polymorphism (rs4073), a forced RFLP analysis was carried out by using the *Ase*I restriction endonuclease enzyme (R0526L New England BioLabs) which recognised the following site;

5′. . . A T T A A T . . . 3′
3′. . . T A A T T A . . . 5′

This resulted in the following bands; AA 132bp, AT 132, 113, 19bp, TT 113 and 19bp (**Figure 3.23**). The 19bp band is not visible.

Figure 3.23: IL-8 -251A>T RFLP and 1kb ladder

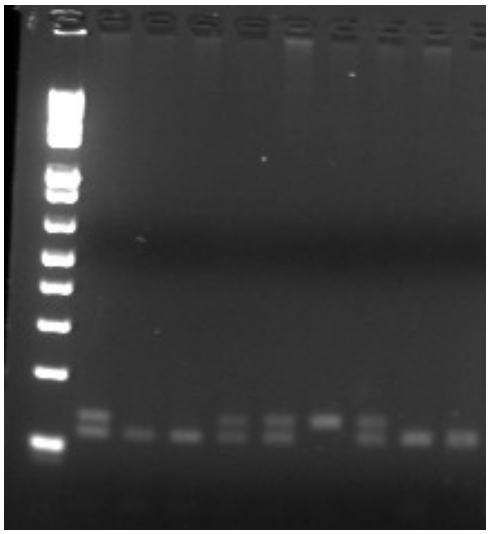


To genotype the samples for the IL-8 781C>T polymorphism (rs2227306), a forced RFLP analysis was carried out by using the *Eco*RI restriction endonuclease enzyme (R0101L New England BioLabs) which recognised the following site;

5′. . . G A A T T C . . . 3′
3′. . . C T T A A G . . . 5′

This resulted in the following bands; CT 139bp, CC 139, 120, 19bp, TT 120, 19bp (**Figure 3.24**). The 19bp product is not visible.

Figure 3.24: IL-8 -781C>T RFLP



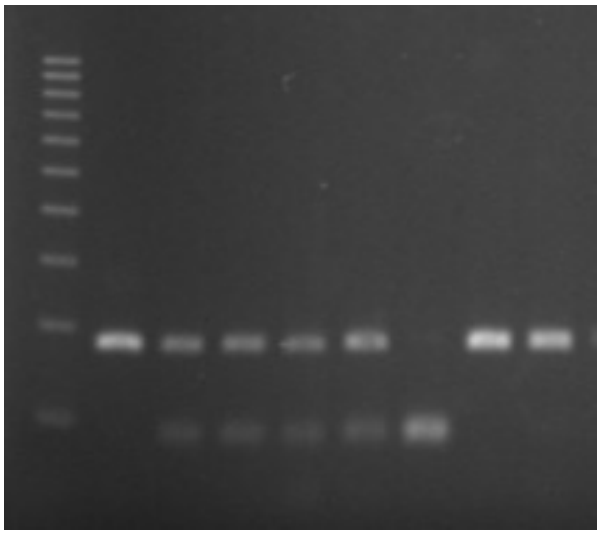
IL-6 -174G>C

To genotype the samples for the IL-6 -174G>C polymorphism (rs1800795), a forced RFLP analysis was carried out by using the *Nla*III restriction endonuclease enzyme (R0125S New England BioLabs) which recognised the following site;

5′...CATG▼...3′
3′...▲GTAC...5′

This resulted in the following bands; GG 178bp, GC 178, 95, 83bp, CC 95 and 83bp (**Figure 3.25**).

Figure 3.25: IL-6 RFLP product



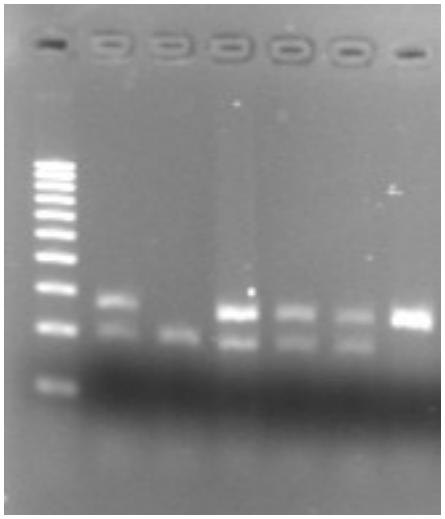
VDR *Fok*I RFLP

To genotype the samples for the *Fok*I polymorphism (rs2228570), a RFLP analysis was carried out by using the *Fok*I restriction endonuclease enzyme (R0109S New England BioLabs) which recognised the following site;

5′. . . G G A T G $(N)_9$ ▼. . . 3′
3′. . . C C T A C $(N)_{13}$ ▲. . . 5′

This resulted in the following bands; FF 265bp, Ff 265, 196 and 69bp, ff 196 and 69bp (**Figure 3.26**)

Figure 3.26; VDR *Fok*I RFLP



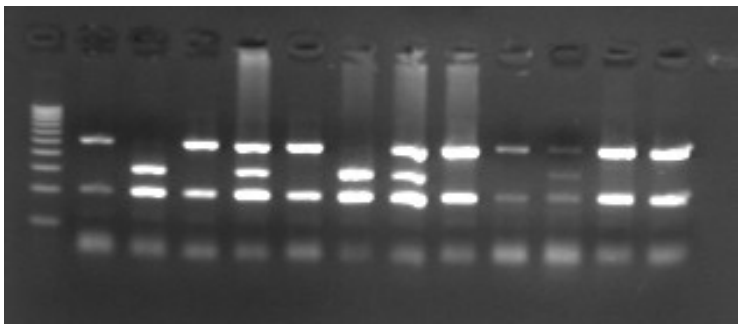
VDR *Taq*Iα RFLP

To genotype the samples for the *Taq*Iα polymorphism (rs731236), a RFLP analysis was carried out by using the *Taq*Iα restriction endonuclease enzyme (R0149T New England BioLabs) which recognised the following site;

5′...T▼CGA...3′
3′...AGC▲T...5′

This resulted in the following bands; TT 495bp, Tt 495, 290 and 205bp, tt 290 and 205bp (**Figure 3.27**).

Figure 3.27: VDR *Taq*Iα RFLP



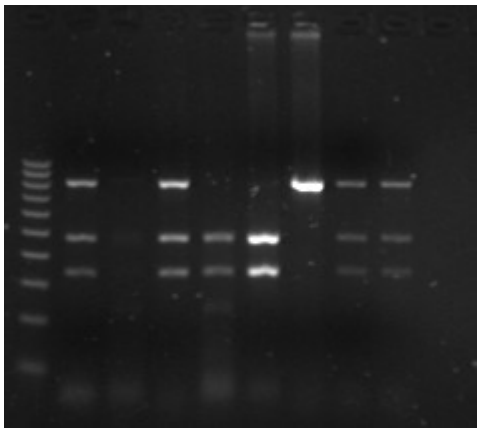
VDR *Bsm*I RFLP

To genotype the samples for the *Bsm*I polymorphism (rs1544410), a RFLP analysis was carried out by using the *Bsm*I restriction endonuclease enzyme (R0134S New England BioLabs) which recognised the following site;

5′...GAATGCN▼...3′
3′...CTTACG▲N...5′

This resulted in the following bands; BB 813bp, Bb 813, 670 and 145bp, bb 670 and 145bp (**Figure 3.28**).

Figure 3.28: VDR *Bsm*I RFLP



Experimental protocols

RFLP for ICAM-1

The reaction consisted of; 2µl 10x buffer, 12.5µl dH_2O, 0.5µl RE (*Bst*U1) and 5µl PCR template. The reaction digested at 60^{o}C overnight on PCR machine. The product was run on a 3% agarose gel.

RFLP for IL-8 (-251 A>T)

The reaction consisted of; 2µl 10x buffer, 12.5µl dH_2O, 0.5µl RE (*Ase*I) and 5µl PCR template. The reaction digested at 37^{o}C for 3 hours on PCR machine. The product was run on a 3% agarose gel.

RFLP for IL-8 (781 C>T)

The reaction consisted of; 2µl buffer, 12.7µl dH_2O, 0.3µl RE (*Eco*RI) and 5µl PCR template. The reaction digested at 37^{o}C for 2 hours on PCR machine. The product was run on a 3% agarose gel.

RFLP for IL-6 -174G>C

The reaction consisted of; 2µl buffer, 0.2µl BSA, 12.7µl dH_2O, 0.1µl RE (*Nla*III) and 5µl PCR template. The reaction digested at 37^{o}C for 3 hours on PCR machine. The product was run on a 3% agarose gel.

RFLP for VDR FokI

The reaction consisted of; 2µl buffer, 7.5µl dH_2O, 0.5µl RE (*Fok*I) and 10µl PCR template. The reaction digested at 37^{o}C for 3 hours on PCR machine. The product was run on a 3% agarose gel.

RFLP for VDR BsmI

The reaction consisted of; 2µl buffer, 7.75µl dH_2O, 0.25µl RE (*Bsm*I) and 10µl PCR template. The reaction digested at 65^{o}C for 3 hours on PCR machine. The product was run on a 3% agarose gel.

RFLP for VDR TaqIα

The reaction consisted of; 2µl buffer, 0.2µl BSA, 7.7µl dH_2O, 0.1µl RE (*Taq*Iα) and 10µl PCR template. The reaction digested at 65^{o}C for 3 hours on PCR machine. The product was run on a 3% agarose gel.

Sequencing

The IL-6 -597G>A, and -572G>C polymorphisms (rs1800797 and rs1800796 respectively) were genotyped by sequencing a 716bp PCR product.

Principles of sequencing

Prior to samples being sent for sequencing they are cleaned up as described below. PCR clean up (Qiagen; QIAquick PCR Purification Kit 28106) QIAquick Kits contain a silica membrane assembly for binding of DNA in high-salt buffer and elution with low-salt buffer or water. The purification procedure removes primers, nucleotides, enzymes, mineral oil, salts, agarose, ethidium bromide, and other impurities from DNA samples.Silica-membrane technology eliminates the problems and inconvenience associated with loose resins and slurries. Specialized binding buffers are optimized for specific applications and promote selective adsorption of DNA molecules within particular size ranges. The QIAquick system uses a simple bind-wash-elute procedure. Binding buffer is added directly to the PCR sample or other enzymatic reaction, and the mixture is applied to the QIAquick spin column. The binding buffer contains a pH indicator, allowing easy determination of the optimal pH for DNA binding. Nucleic acids adsorb to the silica-gel membrane in the high-salt conditions provided by the

buffer. Impurities are washed away and pure DNA is eluted with a small volume of low-salt buffer provided or water, ready to use in all subsequent applications.

Experimental protocol

125µl buffer PBI was added to 25µl PCR product, ensure the mixture was yellow. This was then aliquoted into QIAquick spin columns in provided 2ml tubes and centrifuged at 14,000 rpm for 1 minute. The elution was discarded, the column placed back in same tube, 750 µl of buffer PE added and the column and tube further centrifuged 1 minute. Again the elution was discarded, the system re-centrifuge for 1 minute and the QIAquick column placed into clean 1.5ml tube. 50 µl dH_20 was added, the column centrifuged and the product collected into an eppindorf. The product was run on a 2% agarose gel with quantification ladder to ensure adequate product and no contamination.

Sequencing reaction

This was carried out at the Wolfson Institute (UCL) using AB BigDye 3.1 chemistry and samples were analysed using a AB3730xl 96-capillary sequencer. Read lengths of between 800 and 1000 bases were produced.

10µl of template was required per sequencing reaction in 1.5ml eppendorf tubes at a concentration of 1ng/µl per 100bp for PCR product. Samples were quantified using a Nanodrop. Primers were required at a concentration of 2-5pmoles/µl, allowing 6µl per reaction.

Region of interest sequenced:

Agccacgcgg**tggcaaaaaggagtcacaca**ctccacctggagacgccttgaagtaactgcacgaaatttgagg**a**tggcc
aggcagttctacaacagcc**g**ctcacagggagagccagaacacagaagaactcagatgactggtagtattaccttcttcataat
cccaggcttgggggctgcgatggagtcagaggaaactcagttcagaacatctttggtttttacaaatacaaattaactggaac
gctaaattctagcctgttaatctggtcactgaaaaaaaatttttttttttttcaaaaaacatagctttagcttatttttttctctttgtaaaac
ttcgtgcatgacttcagctttactctttgtcaagacatgccaaagtgctgagtcactaataaaagaaaaaaagaaagtaaaggaa
gagtggttctgcttcttagcgctagcctcaatgacgacctaagctgcacttttccccctagttgtgtcttgccatgctaaaggacgt
cacattgcacaatcttaataaggtttccaatcagccccacccgctctggccccaccctcaccctccaacaaagatttatcaaatgt
gggattttcccatgagtctcaatattagagtctcaacccccaataaatataggactggagatgtctgaggctcattctgccctcga
gcccaccgggaacgaaagagaa**gctctatctcccctccagga**gcccagctatgaactccttctccacaagtaagtgcagga
aatccttagccctggaactgccagcggcggtcgagccctgtgtgagggaggggtgtgtggcccagggagggctggcgggc
ggccagcagcagaggcaggctcccagctgtgctgtcagctcacccctgcgctcgc

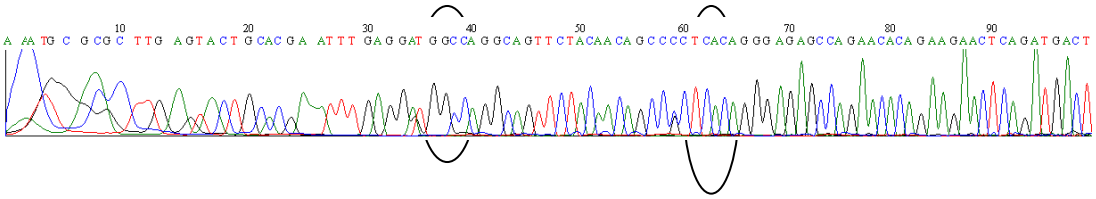
IL-6 Forward primer, shown in red
IL-6 reverse primer, shown in purple
572 G>C
597 G>A

Complications with sequencing

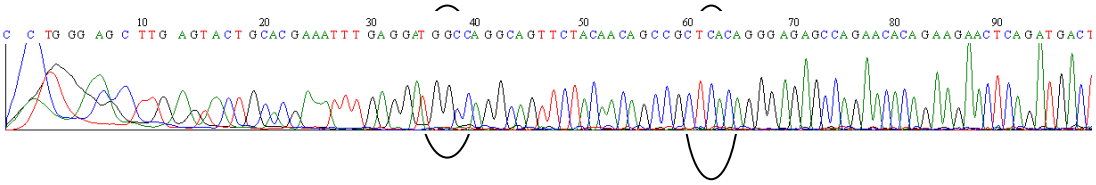
6 samples were genotyped in duplicate and sequences were read by a second person. Sequencing was only done one way as reactions in the forward direction generated a good read up until bases 270 and 280 respectively after which they generated multiple signals. This may have occurred for the following reasons; an insertion or deletion mutation in some of the template would result in reaction products that before the mutation are identical in sequence, and after the mutation differ, producing mixed signal. This effect can be seen with a difference of just one base. Secondly, the homopolymer A and T region prior to the mixed signal may have caused polymerase slippage due to a frame shift mutation. This is a phenomenon that is associated with runs of bases, where the polymerase 'stutters' along the template and leads to mixed signal. This may be remedied by designing a special primer that anneals to the repetitive region, but may be difficult in this case as the A and T runs are quite short. Examples of the IL-6 sequencing product are shown in **Figure 3.29**.

Figure 3.29: Examples of IL-6 sequencing product. The areas of interest are highlighted by ovals. The597G>A polymorphism occurs first and the -572G>C is further on the right.

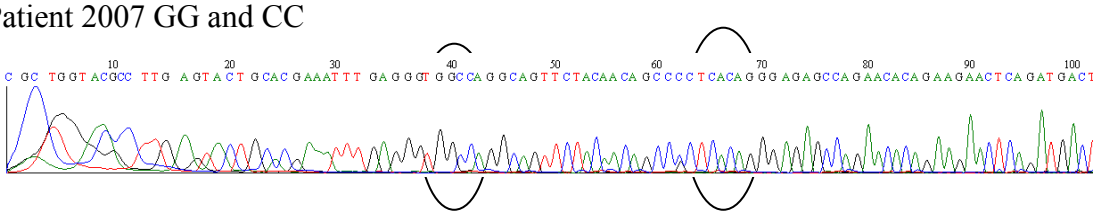
(a) Patient 2311 AG and CG



(b) Patient 2106 AA and GG



(c) Patient 2007 GG and CC



Taq1 genotyping

2µl aliquots of DNA at a concentration of 100ng/µl were put into polypropylene PCR plates, dried overnight at room temperature covered with paper towel. These plates were taken to Prof Phillippa Talmud at the Cardiovascular Genetics Institute, UCL for sequencing using primers designed in her lab. The SNPs were also read in her department and the genotyping results sent directly to us.

3.12 Statistical analysis

The majority of data were analysed using the following standard tests using SPSS version 15 or STATA version 8.2. The Kolmogorov-Smirnov test of normality was applied. Normally distributed data were expressed as mean and standard deviation (SD), skewed data as median and interquartile range (IQR). For some analyses skewed data were log transformed to obtain a normal distribution. Pearson correlation was used to assess parametric data, and Spearman rank was used to assess non-parametric correlations. Comparisons between baseline and exacerbation and between frequent and infrequent exacerbators were made by paired and unpaired t test as appropriate. Binary characteristics such as sex and current smoking status were compared using chi-squared test. The Mann Whitney U test was used for non-parametric exacerbation frequency analysis. For all statistical tests, $p \leq 0.05$ was taken as significant. Specific statistical analyses used for specific chapters are described below.

COPD exacerbation analysis (*Chapter 5*)

Exacerbations are recurrent events and so the data can be organised as a counting outcome; patients can have zero or 1 or several exacerbations over a given time period. Typically this leads to skewed data with a large proportion of values at zero and a small number of patients with a large number of exacerbations thus normality cannot be assumed. Therefore the data can be transformed to induce normality, analysed using nonparametric methods or a Poisson or negative binomial distribution can be assumed. The Poisson distribution was used in this thesis for analysing recalled and actual exacerbation frequencies. This model assumes that exacerbations occur randomly and independently of each other. Failure to determine independence of events may artificially inflate the rate of exacerbations in the group. We used a 5 day no symptom rule between each exacerbation for this work. We calculated rates of exacerbations per person per year and from this determined exacerbation frequency.

Determination of exacerbation rates can be adjusted according to follow up time accounting for time spent in the trial and this was used here with whole years as the denominator. Whole years were used as an unweighted mean rate overestimates exacerbations occurring in patients who drop out early.

Comparisons between exacerbation frequencies determined in the 3 ways were made by Poisson regression (Keene 2008) and using a weighted Kappa score with results reported as a summary Kappa statistic. The weighting adjusts for the importance / measurement of the agreement for each estimate. A perfect match is 1, with the smallest weight for the biggest disagreement. These data were analysed using STATA, which gives weights for agreement, rather than weights for disagreement. Kappa scores were calculated by Gavin Donaldson. Incident rate ratios were reported and can be interpreted as how many times one set of frequencies exceeds another. One way ANOVA was used to compare the SGRQ score by exacerbation frequency determined in the 3 different ways. The Wilcoxon signed rank test was used to compare the difference between actual and recruitment exacerbations and actual and recalled exacerbations for those in the cohort ≥ 3 years. Patients were categorised into frequent and infrequent exacerbator groups according to how the exacerbation was defined and the Sensitivity (% true positives) and Specificity (% true negatives) of dividing patients into these groups using the different methods were calculated. The Positive Predictive value was also recorded. A Bland-Altman plot was drawn to illustrate differences in recruitment and actual exacerbation frequencies and recalled and actual exacerbation frequencies.

Depression analysis (*Chapter 6*)

With regards to the depression analysis, the Chi-Square test was used to assess the relationship between exacerbation frequency and depression. A one way ANOVA was used to look at the relationship between MRC score and hours outdoors. Linear regression analysis was used to look at confounding factors affecting depression scores. To identify the features of depression that changed at exacerbation, we used baseline depression scores and principal component analysis (SPSS 14.0; varimax rotation with Kaiser normalization) to identify five factors (or summary groupings) that explained the pattern of correlations within the 20 question dataset. The five factors explained 61% of the variance. Questions whose rotated component were >0.5 were considered sufficiently similar to be grouped together. Changes in patient scores between baseline and exacerbation, in these five groups, were averaged and compared by paired t-test. We termed these groups: “depressive feelings” (questions 3,6,10,17,18), “activity” (questions 2,7,11,20), “feelings of self-hatred” (questions 15,19), “reflective feelings”

(questions 1,13), and "feelings of positivity" (questions 4,8,12,16)". The principal component analysis was done by Gavin Donaldson.

Vitamin D analysis (*Chapter 8)*

For the Vitamin D analysis FEV_1 decline was analysed using a generalised least squares random effects model. Adjustment for seasonality and investigation of exacerbation and baseline vitamin D levels was done using year period sine and cosine terms. Results in this chapter were therefore adjusted for repeated measures.

Genetics analysis (*Chapter 9.2*)

Hardy Weinberg equilibrium was calculated for all the genotype work. This applies the principle that gene frequencies and genotype ratios in a randomly breeding population remain constant from generation to generation. This fails to apply if the mutation of one allele occurs at a greater frequency than another allele, if there is alteration in gene flow, genetic drift or non-random mating (e.g. inbreeding). The equilibrium can also fail to apply as a result of a sampling effect (small population size). χ^2 tables were used to compare the observed numbers of genotype with those expected for a population in the Hardy-Weinberg equilibrium, and to compare genotype frequencies between the COPD population and a control group. Comparison also occurred within the COPD population comparing genotypes to exacerbation frequency. Allele frequencies between the different groups were compared using chi-squared analysis.

Baseline cytokine analysis (*Chapter 9.3*)

For baseline cytokine analysis, the coefficient of variation was calculated and expressed as a percentage: standard deviation / mean x 100. We also compared the average-area-under-the-curve-minus-baseline changes in cytokine levels in the two years following baseline to take into account the different numbers of observations stemming from each individual, the mean cytokine levels over time using a t-test or non-parametric test depending on the distribution, and a generalized linear model with generalized estimating equations. This gave a mean difference in cytokine levels between patients with an exacerbation and those without an exacerbation, averaged over all time points.

IP-10 and HRV analysis (*Chapter 10.2*)

The use of IP-10 in confirming the presence of HRV at exacerbation was investigated by receiver operating characteristic curve (ROC) analysis. Results are expressed as an area under the curve (AUC) with 95% confidence intervals (CI). The accepted standard of AUC is ≥ 0.8 (18).

4

Baseline Demographics

4.1 Introduction

The COPD patients and control subjects participating in the London COPD cohort differ as described in ***Chapter 3***. Some studies have shown that exacerbation frequency increases with increasing disease severity (Anzueto 2009), and it is important to show that frequent exacerbators exist in GOLD stage1 and infrequent exacerbators exist in GOLD stage 4, even if this is in differing proportions.

As different COPD patient / control subsets have been used in each of the subsequent results chapters, the demographics of each subgroup will be briefly presented in each chapter. Different patient subsets arose as analysis of varying chapters took place over several years and not all individuals remained in the cohort throughout the same time period.

4.2 Aims

1. To describe the baseline characteristics of the COPD patients and control subjects.

2. To describe the baseline characteristics in the frequent and infrequent exacerbators.

4.3 Methods

Age, pack years smoked, BMI, FEV_1, FVC, FEV_1/FVC ratio, FEV_1 % predicted and oxygen saturation measurements recorded at recruitment have been compared between COPD patients and control subjects. Patient selection and recruitment is described in ***Chapter 3*** **section 3.1**. The statistical methodology is described in **section 3.12**.

4.4 Results

COPD patients and controls

356 COPD patients and 93 control subjects have been included in the subsequent chapters. The baseline demographics of the COPD patients and controls (i.e individuals of similar age but normal lung function) are shown in **Table 4.1** below.

Table 4.1: Baseline demographics of all COPD patients and control subjects included in this thesis

	COPD patients n = 356 Mean (SD)	**Control subjects n = 93 Mean (SD)**	**p values**
Age	69.6 (10.0)	66.7 (7.5)	**0.01**
Smoking pack years	50.8 (35.9)	18.3 (21.1)	**< 0.001**
BMI	25.3 (5.5)	25.4 (5.5)	0.95
FEV1	1.15 (0.50)	2.55 (0.77)	**<0.001**
FVC	2.50 (0.88)	3.32 (1.05)	**<0.001**
% predicted FEV1	46.9 (19.1)	96.1 (21.3)	**<0.001**
Oxygen saturations	94 (2)	96 (1)	**<0.001**
Male Gender	224	40	**<0.001**

Frequent and Infrequent exacerbators

226 infrequent and 130 frequent exacerbators have been included in this thesis. The baseline characteristics are shown in the **Table 4.2**.

Table 4.2: Baseline characteristics of the frequent and infrequent exacerbators

	Frequent exacerbators n = 130 Mean (SD)	**Infrequent exacerbators n = 226 Mean (SD)**	**p values**
Age	68.9 (10.9)	70.0 (9.5)	NS
Smoking pack years	47.6 (27.6)	52.4 (39.8)	NS
BMI	25.5 (5.1)	25.4 (5.5)	NS
FEV_1	1.13 (0.50)	1.16 (0.51)	NS
FVC	2.47 (0.82)	2.51 (0.92)	NS
% predicted FEV1	46.6 (19.4)	47.0 (19.1)	NS
Oxygen saturations	94 (2)	95 (2)	NS
Male Gender	73	151	0.03

The proportion of current smokers was similar in both groups and is shown in **Table 4.3.**

Table 4.3: Smoking status in frequent and infrequent exacerbators.

	Not current smoker	***Current smoker***
Infrequent exacerbators	69.4%	30.6%
Frequent exacerbators	77.8%	22.2%

Exacerbation frequency and disease severity

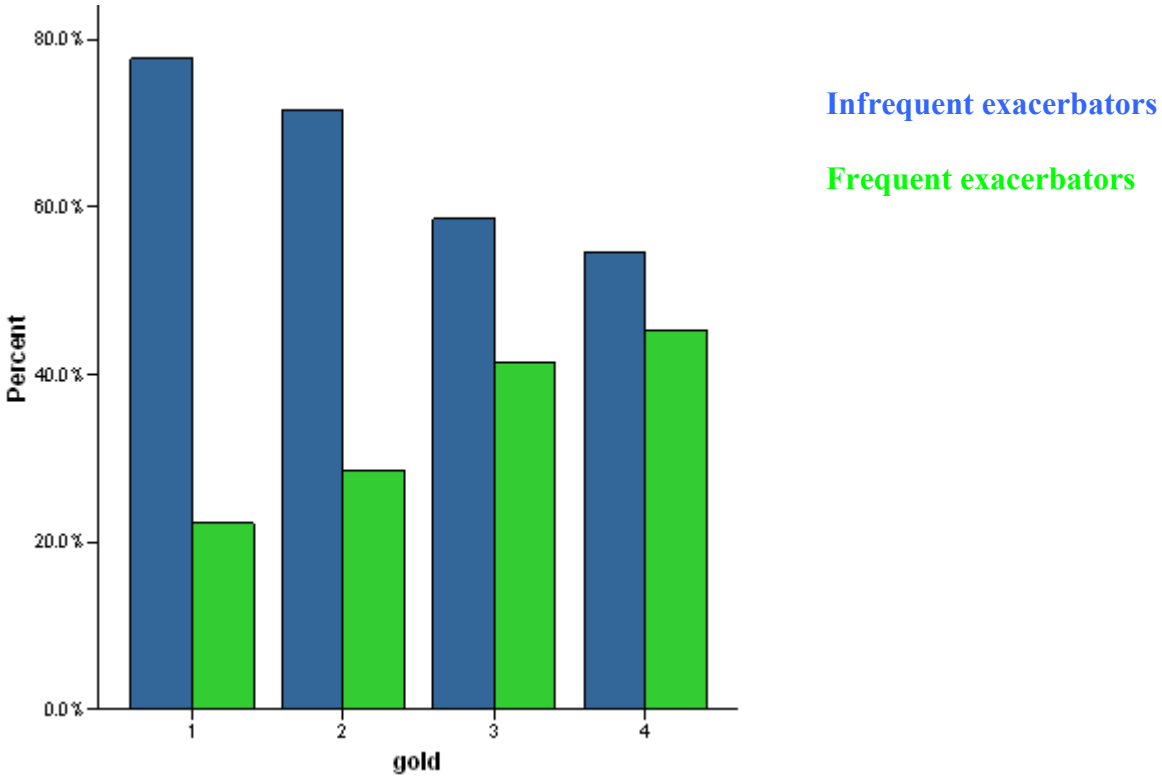
Frequent and infrequent exacerbators existed across all GOLD stages in similar proportions and this is illustrated in **Figures 4.1. Table 4.4** below shows the mean number of exacerbations / year across GOLD groupings. The calculation of exacerbations per year was by dairy card data over at least 1 year of study and is described in **section 3.5**. Only whole years of study were used. Although exacerbation

numbers are not normally distributed (**section 3.12**), the medians were too similar to show any variation and so the mean and SD is given for illustrative purposes.

Table 4.4: Mean number of exacerbations per year across GOLD groupings

GOLD stage	*Number of patients*	*Mean exacerbations / year (SD)*
1	25	2.00 (3.08)
2	124	2.16 (1.96)
3	127	2.52 (2.29)
4	80	2.73 (2.44)

Figure 4.1: The percentage of frequent and infrequent exacerbators in each GOLD stage as a proportion of each GOLD stage.



4.5 Discussion

There were significant differences between the control subjects and COPD patients for all demographics except BMI. The differences in lung function were by definition expected, however the age, smoking history and sex differences will be taken into account in comparisons in subsequent chapters by adjusting for differing factors in statistical analysis. The controls were younger than the COPD patients and more likely to be female.

There were no differences in the baseline characteristics between the frequent and infrequent exacerbators however the proportion of infrequent exacerbators who were male was greater than the proportion of frequent exacerbators. The difference in gender may be an important factor of exacerbation frequency and will be explored in **Chapter 7**.

Figure 4.1 illustrated that frequent and infrequent exacerbators exist across all GOLD stages, with the proportion of frequent exacerbators increasing and infrequent exacerbators decreasing with increasing disease severity. This is consistent with other studies (Montes de Oca 2009, Donaldson 2003). In the study by Donaldson *et al* (Donaldson 2003) patients in GOLD 4 had an annual exacerbation frequency of 3.43 exacerbations per year compared to 2.68 for those in GOLD 2. These rates are higher than those seen with this cohort; however the trend is the same. Rates may be lower now due to the differences in treatment of exacerbations; patients have easier access to out of hours healthcare and are more likely to have rescue courses of medication at home. Equally with increasing education of COPD exacerbation management they may be more likely to treat exacerbations early and reduce the risk of recurrence. They also may be less likely to take part in this type of research if they exacerbate frequently and have easy access to treatment. Distance to travel to the research clinic also affects exacerbation reporting (Rudd 2009) and although this should be accounted for by the use of diary cards to capture unreported exacerbations, distance to travel may influence participation in the first place.

The definition of exacerbation used will influence GOLD exacerbation distribution. ECLIPSE is the largest study to have used the same definition across all GOLD stages

and these data are also consistent with findings from the ECLIPSE study (Anzueto 2009). Obviously the use of exacerbation frequency in this thesis rather than actual numbers of exacerbations will influence distribution, so it is important to have shown the same pattern is attained with the definition used in this work.

In the PLATINO study, exacerbation perception was shown to increase with increasing disease severity (Montes de Oca 2009). These were self reported exacerbations, defined using a standardised question asking about deterioration in breathing symptoms such that usual daily activities were affected. Factors associated with a self reported exacerbation included dyspnoea, prior asthma diagnosis, receiving medications and increased disease severity. There may have been some overlap of asthma and COPD in this study but this should not confound the finding of increasing exacerbation number with increasing disease. However one difficulty with this study is that dyspnoea, although an exacerbation symptom is also a symptom of disease. It is possible that the increased exacerbation perception is a result of an increased awareness of disease symptoms which are likely to increase with increasing disease severity. Further questions establishing the severity of the exacerbation as measured by physician visit and hospitalisation are therefore helpful. It is important to ensure those with GOLD 4 disease are not simply reporting chronic disease symptoms but that these really are exacerbations and the phrasing of the question used may make it difficult to differentiate the two in the PLATINO study. Only 18.2% of the COPD PLATINO study population ever exacerbated. This is a very small proportion and the mean rate of exacerbations was 0.58 exacerbations per year; much lower than other studies.

Although we have used a symptom based definition for this research, our definition has been validated, is associated with an acute change in symptom severity, and is unlikely to simply represent disease symptoms as by definition symptoms must be worse than normal day to day variation. This has been discussed in detail in ***Chapter 1***.

In mild patients (FEV1 > 60% predicted), Miravitlles (Miravitlles 2000) found a rate of 1.6 exacerbations per year compared to 2.3 in those with an FEV1 < 40% predicted. If symptom perception is more acute with increasing disease severity, patients with more severe disease are more likely to notice worsening of symptoms and have an exacerbation. It is possible that the frequent exacerbators in GOLD 1 are the more

anxious patients, or a group of milder patients with better perception of their disease. Patient perception will be further studied in ***Chapter 5***.

The majority of patients in this research cohort have GOLD stage 2 or 3 disease. Milder patients may be less likely to volunteer for research as they do not require frequent health care intervention, and may feel less inclined to participate as they have less to benefit. Also not included in this research are patients who are too unwell to attend hospital outpatient appointments or those that are repeatedly admitted to hospital with exacerbations. Those with GOLD stage 4 may find it more difficult to take part if they are generally more symptomatic.

4.6 Conclusions

This chapter has shown that the control subjects are younger and more likely to be female than the COPD patients studied in this thesis. Frequent and infrequent exacerbators exist across all disease severities and exacerbation numbers increase with disease severity. Frequent exacerbators are more likely to be female, and perception of symptoms may influence exacerbation frequency.

5

Perception of Exacerbation Frequency

5. 1 Introduction

Psychology has been shown to influence disease outcome in several chronic diseases. An individual's state of mind affects not only their perception of disease, but also receptiveness to treatment, interventions and self-management. COPD is often under diagnosed, undertreated and under-recognised by patients (Montes de Oca 2009) and this is also true of exacerbations. Studies have shown that some patients do not even understand the term exacerbation (Kessler 2006) and patients perceive their disease severity and symptoms poorly (Rennard 2002).

The PLATINO (Montes de Oca 2009) study confirmed previous findings (by Rennard 2002), that patients with worse health status had increased frequency of respiratory symptoms. There is increasing evidence to suggest that there is disparity between a patient's perception of disease severity and the impact of COPD symptoms (particularly dyspnoea) and the clinician's evaluation of the COPD severity, indicating that it is not just FEV_1 that is important in determining disease severity. If a patient is able to perceive his / her exacerbation frequency correctly, that may help both the patient and the clinician to guide healthcare. Equally, if perception of disease plays a role in exacerbation frequency than this is an important feature to address.

For example, perception of symptoms when using a symptom based exacerbation definition can influence exacerbation frequency. This is not to say that patients are only frequent exacerbators because they are depressed or anxious or perceive themselves to be frequent exacerbators. What it does mean though is that a holistic approach to management may influence exacerbation frequency. This has also been discussed in ***Chapter 4***.

The frequency with which patients have exacerbations remains relatively stable from year to year (Donaldson 2003) but there are large differences in yearly exacerbation rates between patients of similar COPD severity (Seemungal 1998). One of the clearest factors predictive of frequent exacerbations is a high number of exacerbations in the previous year (Seemungal 1998, Ball 1995). Knowledge of exacerbation frequency is important for assessment of clinical risk and stratification into trials, especially where exacerbation interventions are being evaluated (Powrie 2007, Seemungal 2008). It is essential that the patient population is generalizable and that it is divided equally in terms of exacerbation frequency between drug and placebo groups. Shorter term studies examining interventions at exacerbation must also be cost effective by recruiting individuals who will exacerbate in the follow up period (Wedzicha 2008, Calverley 2003). However, in order to determine an accurate exacerbation frequency, patients ideally need to be prospectively followed for at least 12 months to allow for effects of seasonality on exacerbations (Donaldson 1999). In most situations when recruiting for clinical trials this is not practical. Thus, at an initial clinical assessment or study recruitment, patient recall of the number of exacerbation events is the standard method for assessing exacerbation risk and so determining exacerbation frequency (Wedzicha 2008, Calverley 2003). It is not known how accurately this patient recall relates to the actual number of events.

This chapter was also undertaken to assess whether patients who remained in the cohort under 1 year could be accurately stratified into frequent and infrequent exacerbators groups to be used in the genetics chapters to increase power.

5.2 Aims

1. To investigate whether patients with COPD when interviewed can accurately recall the number of exacerbations they have had in the preceding year, and to investigate whether this varies between frequent and infrequent exacerbators.

2. To investigate whether exacerbation frequency stratification remains unchanged over two years by comparing the recruitment exacerbation frequency with the actual exacerbation frequency over the subsequent year from diary card data.

3. To validate the use of patient recall by comparing patient recall of the number of exacerbation events at the end of the SAME one year time period of daily diary card data collection.

5. 3 Methods

Details regarding patient recruitment can be found in ***Chapter 3*** **section 3.1** and details regarding the definition of exacerbation used in **section 3.4**.

Two hundred and sixty seven patients were studied between 1st January 1995 and 31st March 2008. Patients were selected for this analysis from the London COPD cohort if they remained in this study for at least 1 year and had less than 30 days (not sequential) of missing diary card data over the period of study.

At the recruitment visit, patients were asked how many lower respiratory tract infections / exacerbations they had had in the preceding year (**recruitment exacerbations).** All exacerbations detected via diary cards, regardless of whether they were treated or reported to a healthcare professional were termed '**actual exacerbations**'.

Validation sub-study

A subset of 100 patients from the main cohort who had no missing diary card data between 1st May 2007 and 30th April 2008 were studied in more detail. These patients were called within 6 weeks of completion of the study (30th April 2008) after diary cards had been collected and asked a standardised scripted question:

"How many exacerbations have you had in the last year? By this I mean infections, bad attacks of your chest / worsening of symptoms?"

The number of exacerbations they recalled was recorded. If a patient gave a range e.g. 4 or 5, then the lower number was taken. An exacerbation detected in this way was termed a '**recalled exacerbation**'. This question deliberately avoided asking about treatment and so allowed us to capture information on treated and untreated exacerbations.

Diary cards were further evaluated in these patients; not only was the 'actual exacerbation' number recorded, exacerbations that were treated with antibiotics and/or a course of oral steroids were separately recorded and termed '**treated exacerbations**'. If the patient was not seen by a health care professional at the time of an exacerbation

(usually a member of the study team but also their own General Practitioner or in an Emergency Department) this was considered to be an unreported exacerbation. All of this data was obtained from the diary cards. The end of an exacerbation was taken as the last day of recorded lower airway symptoms. Patients had to be at least 5 days symptom free before a new exacerbation onset was defined. Patients had no input into the definition of exacerbation and no education on classification of exacerbations and as such this could not impact on their predictive accuracy and influence results. These patients also completed a St. George's Respiratory Questionnaire (SGRQ); a disease specific measure of health status during this one year time period (Jones 1992).

Exacerbation frequency

Exacerbation frequency was determined in 3 ways depending upon analysis;
(1) Using patient estimates of exacerbation number in the year before they entered the cohort. This allowed us to assess recall prior to diary card intervention.
(2) Exacerbations were counted from diary cards.
(3) Using patient estimates of exacerbation number at the end of the first year of study. When using this method to determine exacerbation frequency grouping, grouping did not change for any patient by taking the lower number recalled, i.e. no patient gave an answer of 2 or 3.

Patients were defined as 'frequent exacerbators' if they had three or more exacerbations, or 'infrequent exacerbators' if they had less than three exacerbations.

Statistical analysis is described in **section 3.12**.

5. 4 Results

Baseline patient characteristics

Two hundred and sixty seven patients were studied; 179 male and 88 female. 165 were classified as infrequent exacerbators (<3) and 102 as frequent exacerbators (≥3) using diary card data collected over the first year of participation in the London COPD cohort. The baseline characteristics of the cohort are reported in **Table 5.1(a)**. The patients had a mean FEV_1 of 1.14 l or 45.4% predicted.

Table 5.1(a): Baseline Characteristics of 267 patients

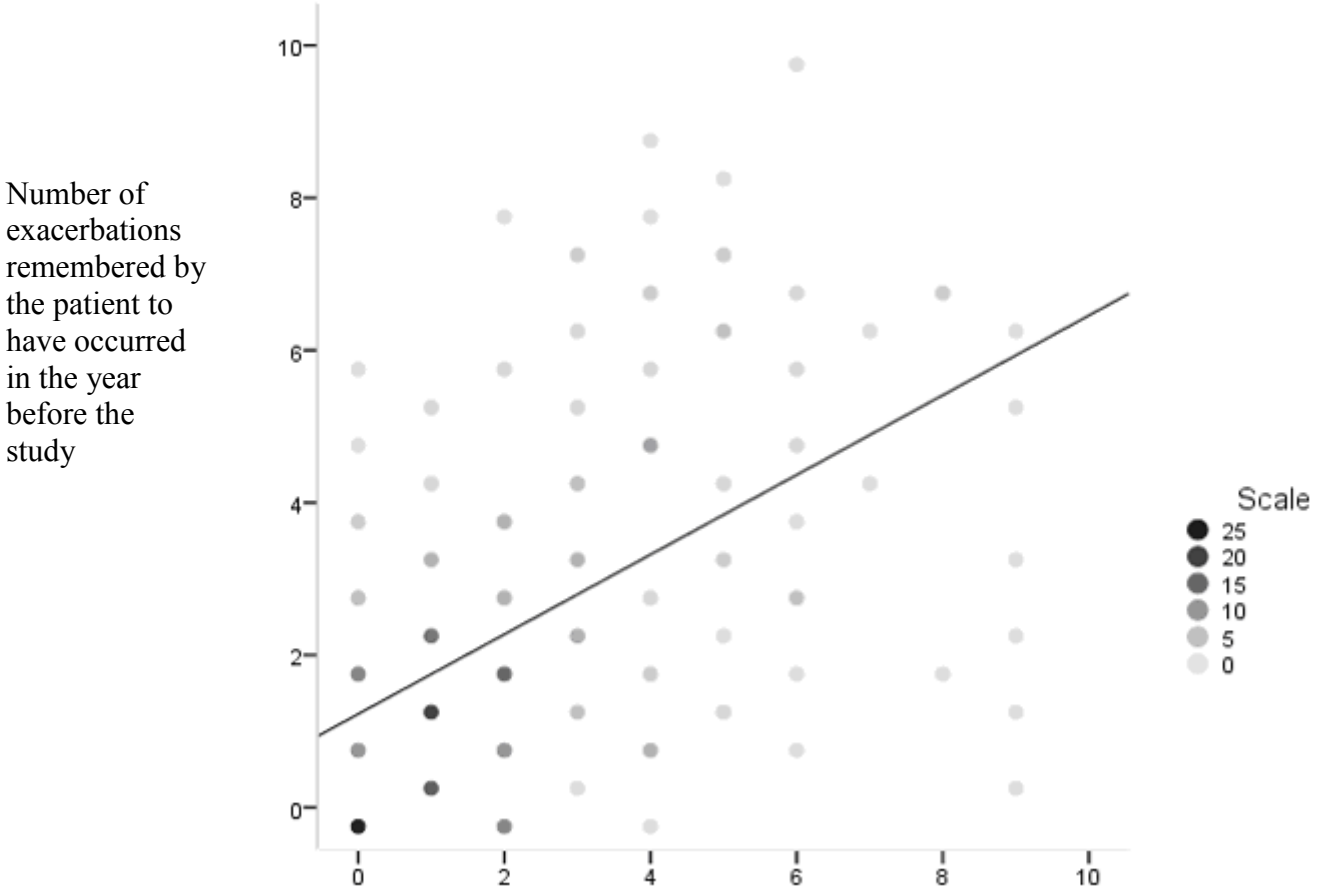
	Mean	**SD**
Age (years)	69.1	8.2
FEV1 (litre)	1.14	0.50
FEV1 (% predicted)	45.4	18.2
FVC (litre)	2.49	0.83
BMI (kgm^{-2})	25.2	5.8
Pack years smoking	49.2	33.4
SaO2 (%)	94	2

Comparison of recruitment and actual exacerbations

654 exacerbations were recorded on diary cards over the 1st year of study. Recruitment exacerbation number accurately predicted actual exacerbation number over the coming year. There was no significant difference between the number of recruitment exacerbations and actual exacerbations in the first year of the study; medians for both groups 2.0 (IQR 1 – 4.0), and means 2.34 (2.17) and 2.45 (2.2) respectively, Poisson IRR = 0.96 (95% CI 0.86 – 1.07) (**Figure 5.1**). 91 patients had more exacerbations in the year prior to recruitment, 106 had fewer and 67 had the same number.

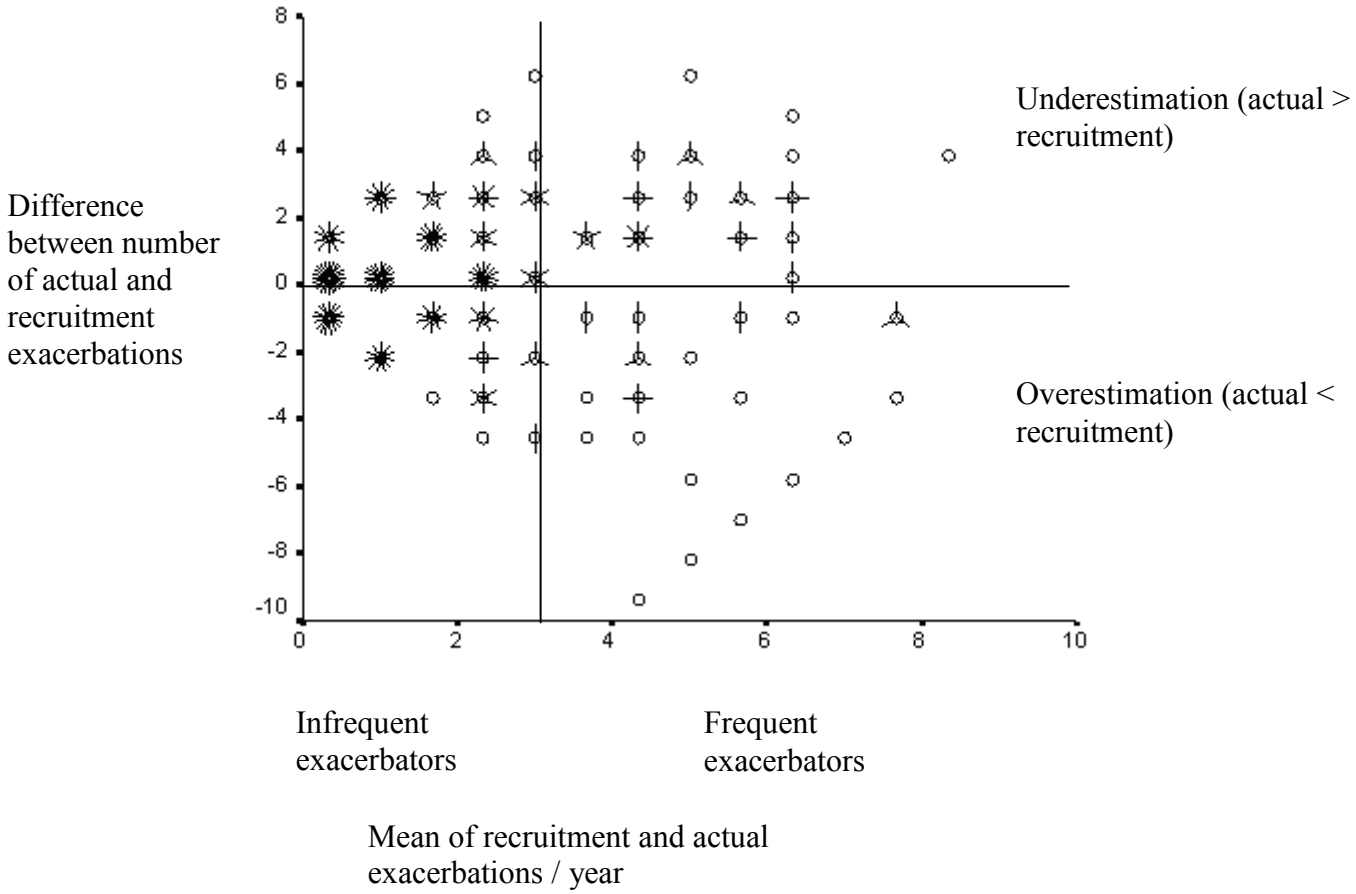
If patient estimates and actual exacerbation number were random, we would expect 76.4% agreement. In fact, they agreed by 84.6%; $\kappa = 0.3469$. This amount of agreement indicates that we can reject the hypothesis that patient estimates of their exacerbation number in the year prior to recruitment and actual exacerbation number was determined randomly.

Figure 5.1: Distribution of actual and recruitment exacerbations.



The relationship between the number of actual and recruitment exacerbations by frequent and infrequent exacerbators is illustrated in **Figure 5.2**. When examining the frequent exacerbator group alone, there was a difference between the number of recruitment and actual exacerbations in the first year of study. Frequent exacerbators *underestimate* the number of exacerbations they have a year by 1.23 exacerbations; Poisson IRR = 0.74 (95% CI 0.65 – 0.85; p <0.001). Similarly, when analysing the infrequent exacerbator group alone there was a difference between recruitment and actual exacerbations. However, infrequent exacerbators *overestimate* their exacerbations by 0.59 exacerbations per year; Poisson IRR = 1.59 (95% CI 1.31 – 1.93; p<0.001).

Figure 5.2: Sunflower Bland-Altman plot showing the relationship between actual and recruitment exacerbations. One patient is represented by a circle and multiple patients by petals. Infrequent exacerbators overestimate their exacerbations by 0.59 exacerbations per patient per year and frequent exacerbators underestimate their exacerbations by 1.23 exacerbations per patient per year.



68 out of 103 frequent exacerbators correctly perceived themselves to be frequent exacerbators (sensitivity 66.0%) and 133 out of 164 infrequent exacerbators correctly perceived themselves as infrequent exacerbators (specificity 81.1%). The Positive Predictive value; that is the probability that a patient who thinks he is a frequent exacerbator really is a frequent exacerbator is 68.7%

Validation sub-study

The baseline characteristics of the 100 patients studied from May 2007 to April 2008 who were telephoned at the end of the study are representative of the main cohort and shown in **Table 5.1b**.

Table 5.1(b): Baseline characteristics of 100 patients (in validation study)

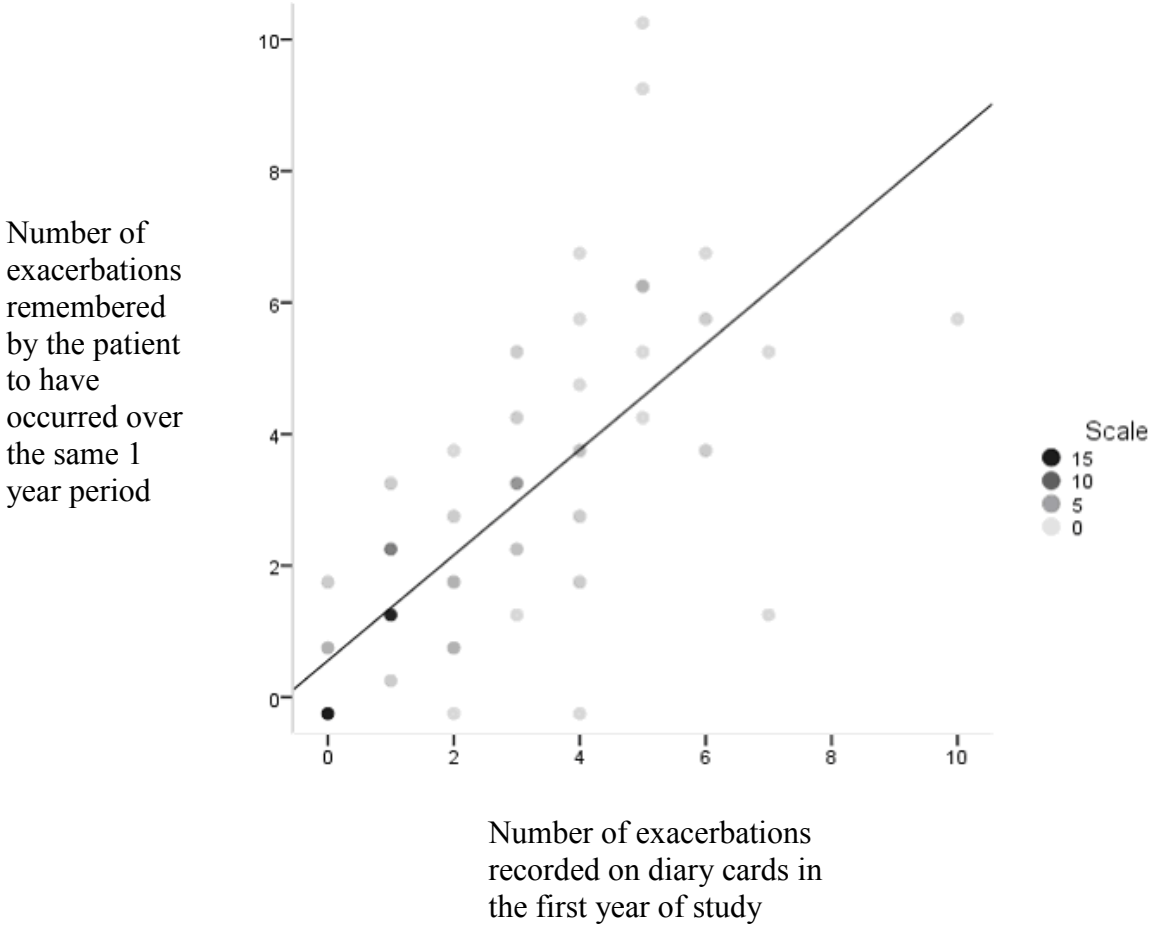
	Mean	**SD**
Age (years)	71.4	8.3
FEV1 (litre)	1.14	0.51
FEV1 (% predicted)	46.4	17.1
FVC (litre)	2.52	0.85
BMI (kgm^{-2})	26.4	5.0
Pack years smoking	48.9	36.0
SaO2 (%)	95	2
SGRQ Total Score	49.1	17.1
Activity	66.1	21.5
Impact	35.8	19.2
Symptoms	60.2	19.9
	Median	**IQR**
MRC Dyspnoea score	3.0	2 – 4

Actual and recalled exacerbations

Patients' actual exacerbations over the one year time period of diary card data studied were compared to their recall for that period when asked at the end of the study. The distribution of actual and recalled exacerbations is shown in **Figure 5.3** and the differences within a patient in **Figure 5.5**. Patients were accurately able to report their actual number of exacerbations. There was no significant difference between the number of actual and recalled exacerbations over the one year time period; median 2.0 (IQR 1.0 – 4.0) both groups and mean 2.4 (SD 2.2) and 2.3 (SD 2.1) respectively. Recalled exacerbation frequencies were 0.96 times (95% CI; 0.80 – 1.14) times actual exacerbation frequencies. 33 patients recalled fewer exacerbations than they actually had, 22 recalled more and 45 recalled the same number as on the diary cards.

If patient estimates of exacerbation number and actual exacerbation number over this same time period were random, we would expect 74.9% agreement. In fact, they agreed by 93.3%; $\kappa = 0.6146$. This amount of agreement indicates that we can reject the hypothesis that there is a difference between patient estimate of their exacerbation number and actual exacerbation number over the same 1 year time period.

Figure 5.3: Distribution of actual and patient recalled exacerbations.



In the frequent exacerbator group there was no difference between the number of actual exacerbations and recalled exacerbations. 19 patients recalled fewer exacerbations than they actually had, 7 recalled more and 12 recalled the same number. Poisson IRR = 0.88 (95% CI 0.71 – 1.09). However, as with the recruitment exacerbations, frequent exacerbators *underestimate* the number of exacerbations they have a year by 0.55 exacerbations. There was no difference between the number of actual and recalled exacerbations in the infrequent exacerbator group; 14 patients recalled fewer exacerbations than they actually had, 15 recalled more and 33 recalled the same

number. Poisson IRR = 1.18 (95% CI 0.84 – 1.65). Again, overall, infrequent exacerbators *overestimate* their exacerbations by 0.18 exacerbations per year.

33 out of 38 frequent exacerbators correctly defined themselves as frequent exacerbators and 54 infrequent exacerbators out of 62 correctly defined themselves as infrequent exacerbators, giving a sensitivity of 86.8% and specificity 87.1%. The Positive predictive value was 0.81. This is shown in **Figure 5.4**.

Figure 5.4: Patient recalled and actual exacerbation frequency

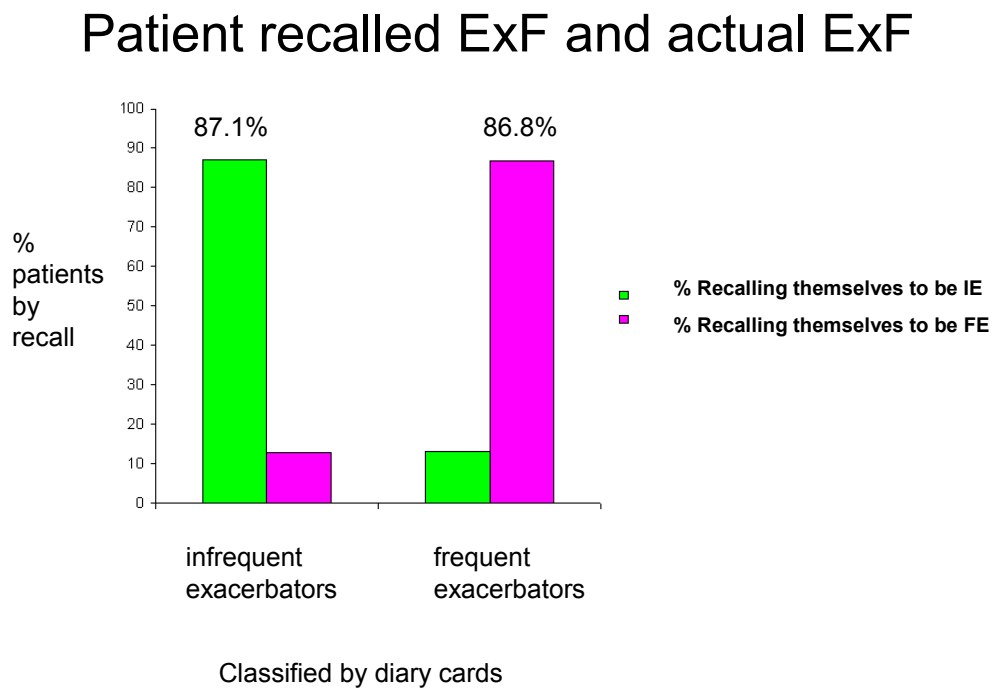
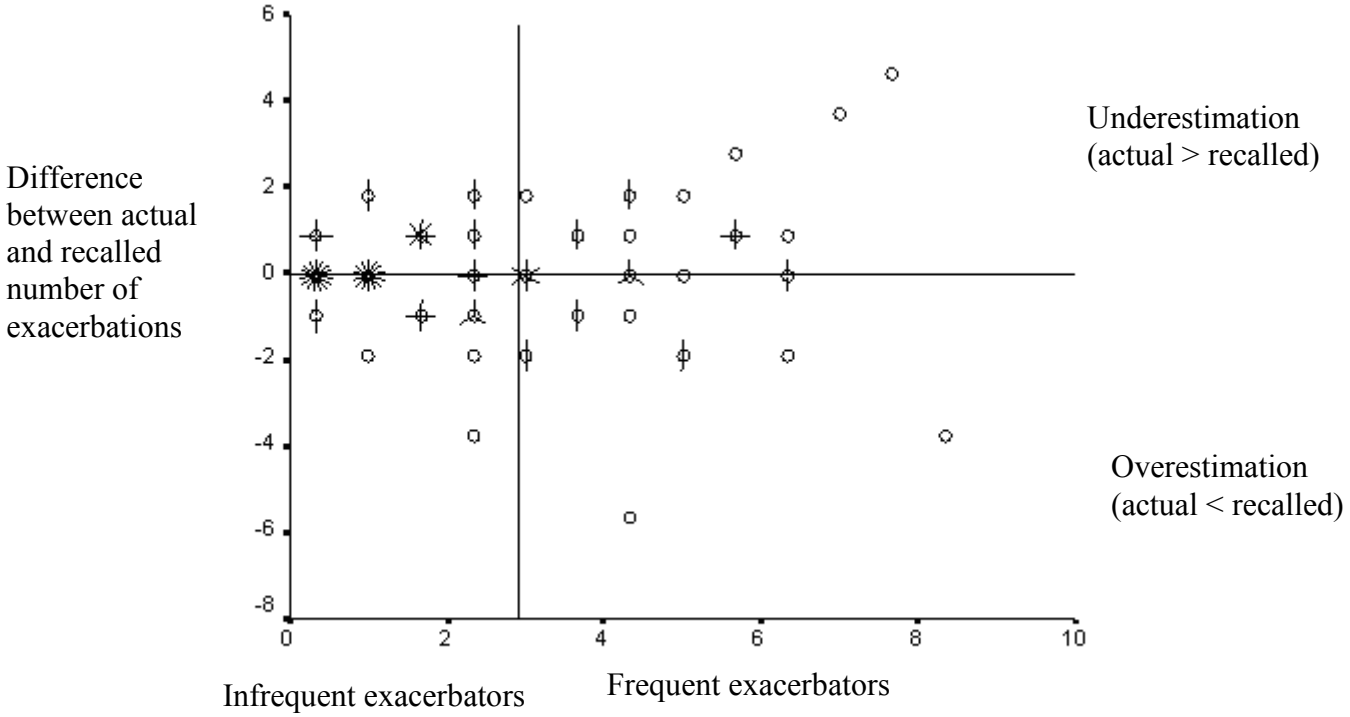


Figure 5.5: Sunflower Bland-Altman plot showing the relationship between actual and recalled exacerbations. One patient is represented by a circle and multiple patients by petals. Infrequent exacerbators overestimate their exacerbations by 0.18 exacerbations per patient per year and frequent exacerbators underestimate their exacerbations by 0.55 exacerbations per patient per year.

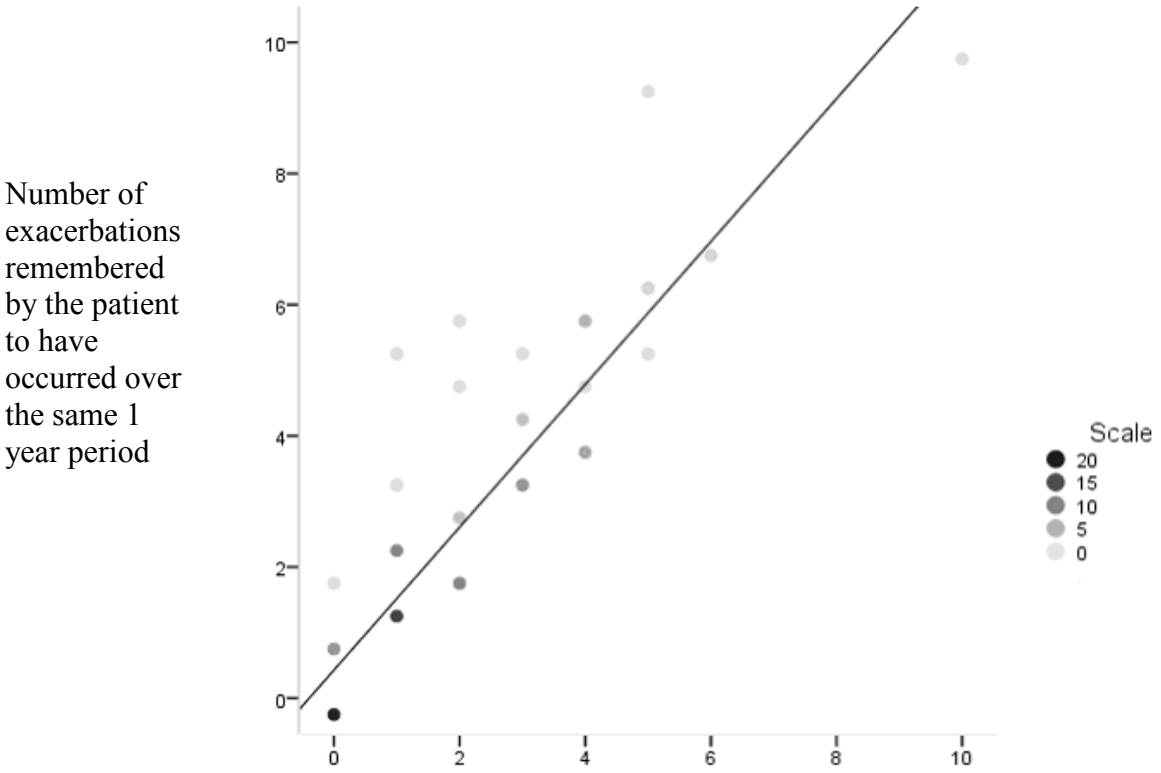


Treated and recalled exacerbations

240 exacerbations were recorded on diary cards over this 1 year period; 181 of those treated. We confirmed previous findings that patients do not report all of their exacerbations to health care professionals. There was a statistically significant difference between the number of treated exacerbations and the number of recalled exacerbations, (**Figure 5.6**); medians 1.0 (0.0 – 3.0) and 2.0 (1.0 – 4.0) respectively and means 1.8 (1.8) and 2.3 (2.1) respectively, IRR = 0.79 (95% 0.65 – 0.96; p =0.02. 16 patients recalled fewer exacerbations than they had treated, 40 recalled more and 44 recalled the same number.

If patient estimates of their exacerbation number and the number of treated exacerbations were random, we would expect 74.1% agreement. In fact, they agreed by 88.6%; κ = 0.5605.

Figure 5.6: Distribution of treated and patient recalled exacerbations.



In the frequent exacerbator group only, 8 recalled fewer than were treated, 18 more than were treated and 12 the same number; Poisson IRR = 0.87 (95% CI 0.70 – 1.10). However, infrequent exacerbators had fewer exacerbations treated than they recalled. 8 patients recalled fewer exacerbations than they had treated, 22 remembered more than were actually treated and 32 recalled the same number; Poisson IRR = 0.59 (95% CI 0.40 -0.86; p=0.006). Frequent and infrequent exacerbators have fewer exacerbations treated then they remember over the same time period by 1.14 exacerbations (31.4%; p <0.001) and 0.49 exacerbations (71.0%; p <0.001) per year respectively.

Relationship between SGRQ and actual, recall and recruitment exacerbation frequency

SGRQ data was collected on all 100 sub-study patients during the year of follow up. There was no significant difference in any of the baseline characteristics reported in **Table 5.1(b)** between the frequent and infrequent exacerbators except for Total SGRQ score, and all subcomponents of the SGRQ which is consistent with our previous work (Seemungal 1998). **Table 5.2** shows that these differences were similar when frequent and infrequent exacerbators were classified by actual, recall and recruitment exacerbations. Comparison of Total SGRQ score and all subgroups (Activity, Impact and Symptoms) in frequent exacerbators calculated in the 3 different ways was studied. Similarly for the infrequent exacerbators there was no difference.

Table 5.2: SGRQ Scores in Frequent and Infrequent exacerbators using (a) actual, (b) recall and (c) recruitment determined exacerbation frequencies.

(a)

ACTUAL	Frequent exacerbators n = 38	Infrequent exacerbators n = 62	P value
SGRQ Total	55.6 (16.0)	45.4 (16.1)	0.003
Activities	73.1 (18.2)	62.8 (20.6)	0.015
Impact	41.5 (19.2)	31.7 (19.0)	0.011
Symptoms	68.4 (16.3)	56.6 (21.0)	0.010

(b)

RECALL	Frequent exacerbators n = 40	Infrequent exacerbators n = 60	P value
SGRQ Total	56.0 (15.6)	44.6 (16.0)	0.001
Activities	73.2 (18.0)	62.2 (20.6)	0.009
Impact	41.4 (19.7)	31.3 (18.6)	0.008
Symptoms	70.5 (14.8)	54.5 (20.8)	<0.001

(c)

RECRUITMENT	Frequent exacerbators n = 32	Infrequent exacerbators n = 68	P value
SGRQ Total	55.8 (14.8)	46.1 (16.8)	0.008
Activities	73.3 (15.8)	63.6 (21.4)	0.04
Impact	40.7 (19.2)	33.0 (19.4)	0.04
Symptoms	71.5 (15.4)	56.2 (20.3)	0.001

Predictive accuracy of exacerbation recall

There were no factors i.e. $FEV_{1,}$ smoking history, gender, co-morbidities or social contacts, (living alone, living with spouse, and contact with children) that were predictive of an individual having poor recall of their number of exacerbations. There was no difference in Total SGRQ score or any subgroups between those that recalled the same number of actual exacerbations and those with poor recall.

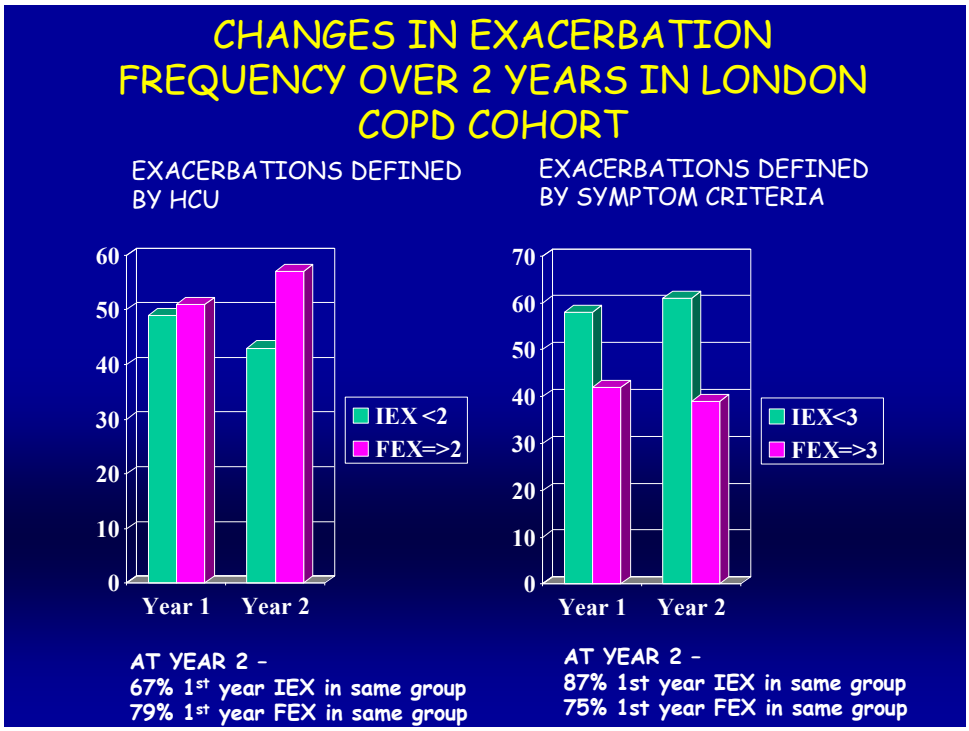
Patient recall and length of time in the cohort

We also investigated whether length of time in the cohort (i.e. diary card intervention) affected patient recall of exacerbations. Patients' recall of their exacerbations was not more accurate with increased length of time in the cohort. In 19 patients from the 100 patient sub-set, the time between the first year of diary card data and the 2007 – 2008 follow up was greater than 3 years. Three years was chosen as the cut off as we felt that if there was a difference with diary card intervention, it would be seen with patients in the London cohort after this amount of time. In these patients there was no difference in the difference between actual exacerbation number in the first year of study and recruitment exacerbation number, and the difference between recall exacerbation number and actual exacerbation number; mean difference -0.05 (1.99) and -0.32 (2.08).

Changes in exacerbation frequency over two years of study

Using a symptom-based definition, 87% of infrequent exacerbators remained infrequent exacerbators from year 1 to year 2 and 75% of frequent exacerbators remained in the same group. The results were similar using a health care utilisation definition (**Figure 5.7**).

Figure 5.7: Changes in exacerbation frequency over the two years of study



5.5 Discussion

This is the first study to prospectively show that COPD patients reliably recall the number of exacerbations they have had in the preceding year and that this patient reported exacerbation frequency can be used to accurately stratify patients into frequent and infrequent exacerbator groups in subsequent years. This study is also unique in that we were able to capture information on both treated and untreated exacerbations. Our findings are important for a number of reasons. Firstly, from the patient perspective; frequent exacerbators have reduced quality of life (Seemungal 1998), increased mortality (Soler-Cataluna 2005), faster decline in lung function (Donaldson 2002, Kanner 2001), reduced physical activity (Donaldson 2005) and increased depression (Quint 2008). This study shows that patients can recognise themselves to be frequent exacerbators and these individuals may benefit from self-management plans or increased patient education (Bourbeau 2003, Gadoury 2005, Monninkhof 2003). Secondly, for clinicians, as frequent exacerbators have more frequent hospitalisations (Soler-Cataluna 2005), increased health care costs and identification of these patients early on may affect management. Patients with frequent exacerbations may be specifically targeted for more aggressive therapy and exacerbation prevention to help prevent disease progression (Zheng 2008, Celli 2008) and improve quality of life (Calverley 2007), and our data suggest that simply asking patients about their exacerbations is sufficient to categorise them. Thirdly, as exacerbation frequency is often controlled for or used as a criterion for selection (Wedzicha 2008, Calverley 2003), stratification into frequent and infrequent exacerbator groups may be necessary at the time of recruitment in trials. Incorrectly defining patients into these two groups may lead to a marked effect on trial outcome.

We found that overall, the actual number of exacerbations in the first year of participation in the London COPD cohort accurately compared to the number of exacerbations in the year preceding recruitment. There was a difference between frequent and infrequent exacerbators in their ability to recall their exacerbations. Perception became less accurate at the extremes of numbers of exacerbations with the majority of patients, who have between 1 and 3 exacerbations per year the most accurate. Frequent exacerbators tend to underestimate and infrequent exacerbators overestimate the number of their exacerbations. It is possible that as frequent

exacerbators are symptomatic more of the time, multiple exacerbations overlap into one, and so they recall fewer, whereas in infrequent exacerbators who are well more of the time, symptom deteriorations are more acute and thus memorable events. Although this difference in perception does exist, in terms of recruitment to clinical trials, the underestimation of exacerbations in frequent exacerbators and overestimation in infrequent exacerbators at the extremes of exacerbation number would not affect exacerbation frequency grouping but instead affirms their position in the correct group. This is unlikely to be a regression to the mean effect as exacerbations are not random events (Hurst 2009).

We validated our finding at recruitment by comparing patient recall of exacerbations over a one year time period in which we had collected daily diary card data. Again we found that for each patient, the actual number of exacerbations they had experienced (both treated and untreated) accurately compared to recall after the follow up period. Once more, frequent exacerbators underestimated and infrequent exacerbators overestimated the number of exacerbations experienced.

Our cohort is unique in that we are able to study untreated and unreported exacerbations (Seemungal 1998, Donaldson 2002, Donaldson 2003) as most studies lack consistent information on these events. Previous studies have used treatment definitions to predict exacerbations in the next year (Niewoehner 2007). However, we and others have shown that unreported exacerbations have an important impact on health status in COPD (Seemungal 1998, Langstemo 2008, Vijayasaratha 2008). Although as stated above, patient recall accurately matched the actual number of exacerbations, patient recall did *not* match the number of treated exacerbations during the year either when considering the group as a whole or when considering the infrequent exacerbators as a subgroup alone. Infrequent exacerbators recalled more exacerbations then they actually had treated. Therefore, it appears that patients count unreported exacerbations as important events, even if they do not seek treatment for these episodes. They may not seek treatment due to difficulty in obtaining access to health care providers (Garcia-Aymerich 2007), or more likely they feel the episode is self-limiting and they will improve without therapy. This finding is also important from a trial recruitment perspective. Recruiting patients using a health care utilisation definition as an outcome could lead to over recruitment of patients with a lower rate of health care utilisation

defined exacerbations. This in turn may affect trial outcome. There was no difference between the number of actual exacerbations and treated exacerbations for the frequent exacerbators. As frequent exacerbators have worse quality of life (Seemungal 1998) and by definition spend a greater proportion of their time symptomatic with exacerbations it is likely they recognise these events and seek treatment. It is possible that frequent exacerbators are more likely to have courses of emergency treatment (antibiotics and steroids) at home and so are more likely to have treated exacerbations.

We have previously shown that there are significant differences in health status between frequent and infrequent exacerbators (Seemungal 1998) and this has been confirmed in this study. We have also shown similar differences in both exacerbator groups regardless of which definition of exacerbation were used in the classification analysis. This again supports our data that patients are able to accurately classify themselves into frequent and infrequent exacerbator groups.

Exacerbation frequency is stable in individual patients from year to year (Donaldson 2003). We have now shown that patient recall can be used to stratify patients as frequent or infrequent exacerbators in the next year. We appreciate that not all COPD patients fill out diary cards. We therefore compared patient estimates of exacerbation number prior to recruitment to the London COPD cohort with estimates after recruitment and showed that daily diary card monitoring does not make patients more aware of exacerbations as patient recall does not improve with participation in a longitudinal study. We found that patients who had been in the cohort ≥ 3 years did not have a change in their ability to recall their exacerbations suggesting that filling out diary cards does not provide reinforcement or more accurate recall of exacerbations later on. The fact that patient recall did not become more accurate with increased time spent in the cohort implies that cohort patients have a good understanding of their disease prior to commencement in studies. This emphasises the importance of patient education about the nature of COPD exacerbations. We investigated factors that could affect patient recall of their exacerbations, and found no factors that predicted poor recall. Specifically, there was no relationship with disease severity, exacerbation frequency or quality of life.

An important strength of this study is the accurate data collection and uniqueness in ability to detect all types of exacerbations. We used a validated and consistent definition

of exacerbation (Seemungal 1998, Donaldson 2002, Donaldson 2005, Bhowmik 2005) with the same individuals asking the questions. We made consistent attempts to obtain information on all exacerbations, including information on untreated events.

The stability of exacerbation frequency grouping form one year to the next is important and the decrease in frequent exacerbators from 1 to 2 years in the symptom defined group is likely to be due to optimisation of medication on joining the cohort as medication (tiotropium, inhaled corticosteriods) will effect exacerbation frequency.

5.6 Conclusions

This is the first study to prospectively show that COPD patients reliably recall the number of exacerbations they have had in the preceding year and that this patient reported exacerbation frequency can be used to accurately stratify patients into frequent and infrequent exacerbator groups in subsequent years. We found that overall, the actual number of exacerbations in the first year of participation in the London COPD cohort accurately compared to the number of exacerbations in the year preceding recruitment. Perception became less accurate at the extremes of numbers of exacerbations with the majority of patients, who have between 1 and 3 exacerbations per year the most accurate. Frequent exacerbators tend to underestimate and infrequent exacerbators overestimate the number of their exacerbations. We found that patients who had been in the cohort ≥ 3 years did not have a change in their ability to recall their exacerbations suggesting that filling out diary cards does not provide reinforcement or more accurate recall of exacerbations later on. This finding has important implications for the performance of clinical trials in COPD patients and also in clinical practice when stratification of COPD patients at risk of exacerbation is required.

6

Depression and Exacerbation Frequency

6. 1 Introduction

Anxiety and depression are associated with many chronic diseases, including COPD (van Manen 2002, Di Marco 2006, Kunik 2005) and commonly occur together (Kunik 2005, Laurin 2007, Brenes 2003). Some studies have suggested depression is more common in COPD than in a control population (Omachi 2009), with the prevalence increasing with increasing severity of disease from 19.6% in mild to moderate disease, to 25% in severe disease (van Manen 2002). Other studies have found a much lower prevalence of depression, similar to that of the general population (van Ede 1999). Recognition of depression is important as it affects quality of life, and identification and intervention in these cases with antidepressants may improve functional capacity and quality of life (Eiser 2005). However, there is a need for better understanding of the factors associated with depression. Depression affects how individuals utilise health care, and comply with and respond to treatment (Eiser 2005, Wilkinson 2004).

Depressed patients may be less likely to present early for treatment, or to report their exacerbations at all, thus it is possible that frequent exacerbators are more depressed than infrequent exacerbators. Psychological factors have been found to be related to the reporting of respiratory symptoms (cough, wheeze, sputum, and dyspnea) (Dales 1989).

6.2 Aims

1. To assess whether depression was related to exacerbation frequency, and to assess the relationship between depression and systemic inflammation, various social factors and the associations of any increases in depressive symptoms at exacerbation

2.To predict which patients become more depressed at the time of an exacerbation.

6.3 Methods

Patient recruitment, follow up sampling, questionnaires and statistical analysis have been described in **chapter 3 (sections 3.1, 3.3, 3.6, 3.7 and 3.12)**. The Center for Epidemiological Studies Depression Score (CES-D) was used. Principal component analysis (PCA; described in **section 3.12**) was used to tease out differences between disease and health status influencing depression.

6.4 Results

Baseline patient characteristics

One hundred and sixty nine patients were studied; 95 male and 74 female. The baseline characteristics of the cohort are reported in **Table 6.1**, with further subdivision by exacerbation frequency in **Table 6.2**. Patients had a mean FEV_1 of 1.13 l or 47% predicted. The mean CES-D score for all patients was 14.9 (SD 11.1). 15 patients in the cohort (8.9%) had a clinical diagnosis of depression, 13 of whom were on antidepressants, mean CES-D 18.2 (12.4). The mean CES-D for the two patients not on antidepressants was 34.0 (12.7).

Table 6.1: Baseline Characteristics, SGRQ and depression scores of 169 patients studied.

	Mean	**SD**
Age (years)	70.9	8.6
Hours outdoors	3.4	2.3
FEV1 (litre)	1.1	0.5
FEV1 % predicted	47.0	18.9
FVC (litre)	2.4	0.9
BMI (kgm^{-2})	26.3	5.5
Pack years smoking	48.5	33.7
SGRQ total	51.0	17.6
Activity	67.1	22.1
Impact	38.1	18.7
Symptoms	62.1	20.1
MRC Dyspnoea score	2.9	1.2
Depression score	14.9	11.1

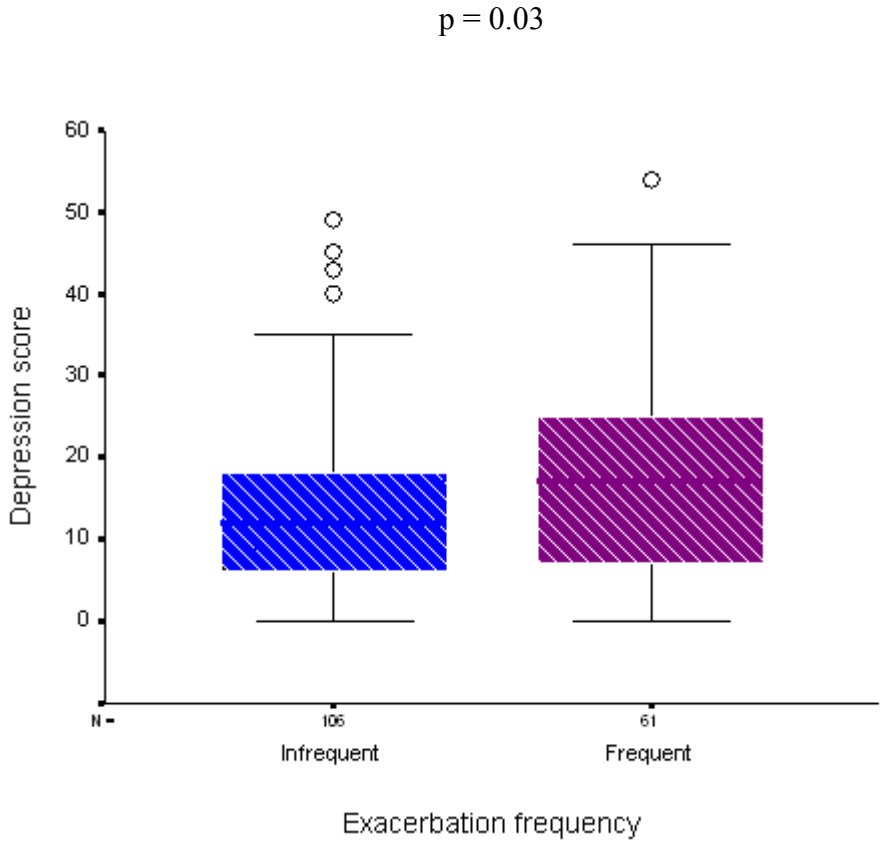
Table 6.2 :Baseline Characteristics of Frequent and Infrequent Exacerbators

	Frequent exacerbators n = 61	**Infrequent exacerbators n= 106**	**P value**
Age (years)	70.7 (8.5)	71.1 (8.5)	NS
Hours outdoors	3.4 (1.9)	3.5 (2.5)	NS
FEV1 (litre)	1.0 (0.4)	1.2 (0.5)	0.005
FEV1 % predicted	41.7 (15.3)	49.7 (20.0)	0.005
FVC (litre)	2.2 (0.8)	2.53 (0.9)	0.03
BMI (kgm^{-2})	25.9 (4.7)	26.5 (6.0)	NS
Pack years smoking	49.2 (28.3)	48.4 (36.1)	NS
SGRQ total	59.8 (14.5)	46.0 (17.2)	<0.001
Activity	75.9 (16.4)	62.3 (23.2)	<0.001
Impact	46.7 (18.0)	33.2 (17.3)	<0.001
Symptoms	71.9 (15.2)	56.6 (20.4)	<0.001
MRC Dyspnoea score	3.2 (0.9)	2.8 (1.2)	0.016
Depression score	17.7 (12.4)	13.6 (10.0)	0.03

Exacerbation frequency and depression

Sixty one patients were classified as frequent exacerbators, and 106 as infrequent exacerbators. 2 patients did not have their exacerbation frequency calculated due to insufficient diary card data. Frequent exacerbators had a significantly higher baseline depression score than infrequent exacerbators (17.7 (12.4) and 13.6 (10.0); p = 0.03. (**Figure 6.1**) When analysing our data using the cut off of clinical depression as a score of 16, we still found exacerbation frequency to be related to CES-D (p = 0.01). 35 % of the infrequent exacerbators scored > 16 at baseline compared to 54% of the frequent exacerbators.

Figure 6.1: Baseline depression scores in infrequent and frequent exacerbators.



Multiple linear regression analysis confirmed differences observed in CES-D scores between frequent and infrequent exacerbators were not due to confounding factors such as smoking (p = 0.03).

Systemic inflammation and depression

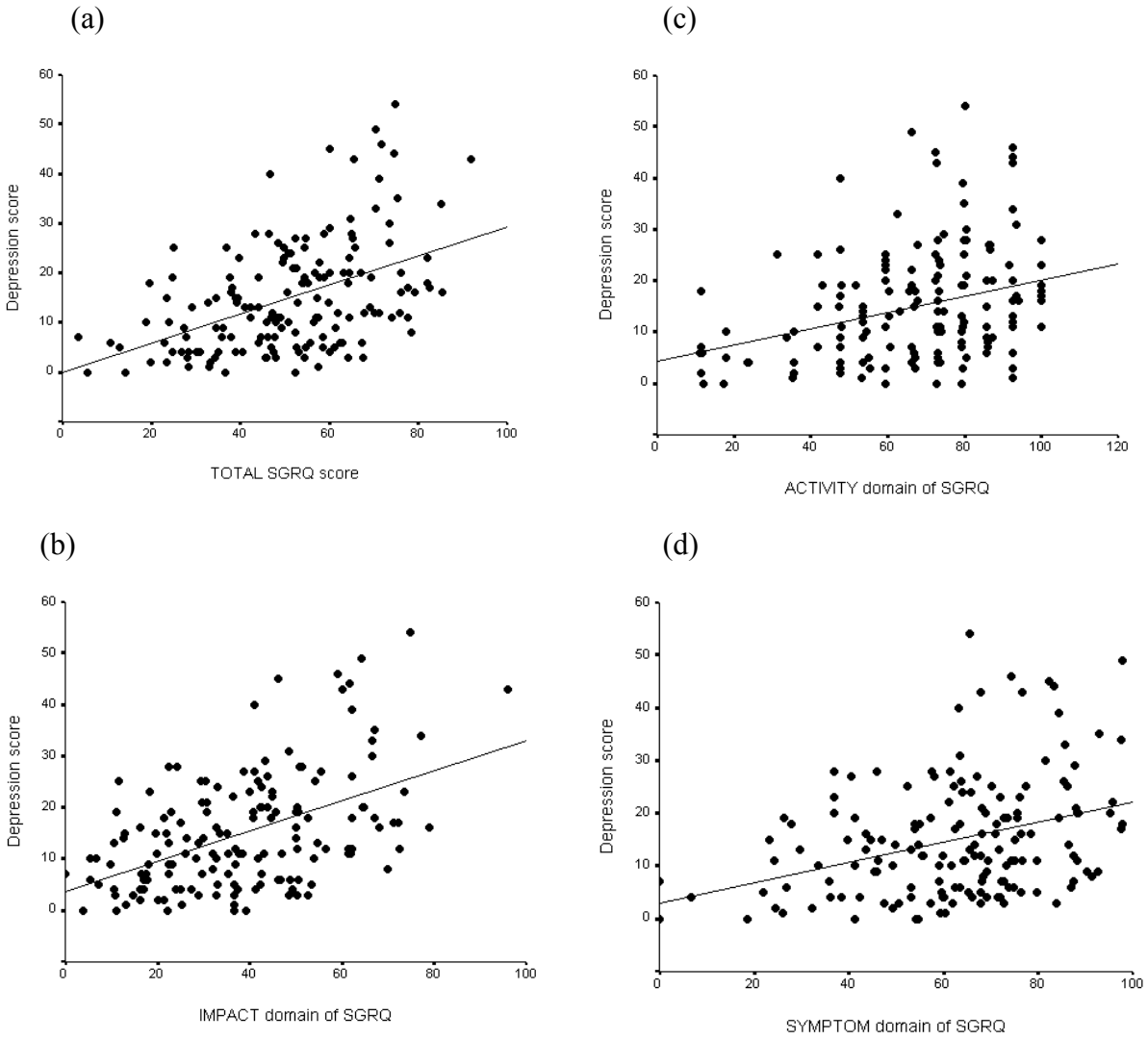
One hundred and forty two patients had CRP measured in the stable state and 155 had plasma IL-6 measured. There was no relationship between baseline CRP or baseline serum IL-6 and depression score. However, baseline CRP and serum IL-6 were significantly related (r =0.20, p = 0.02).

SGRQ and Social variables

One hundred and sixty patients completed the SGRQ when stable. There was a significant relationship between worse (higher) Total SGRQ scores and greater CES-D; r = 0.47, p < 0.001. The three domains of the SGRQ were also strongly related to CES-D; Activity; r = 0.32, p < 0.001, Impact; r = 0.49, p < 0.001 and Symptoms; r = 0.35, p

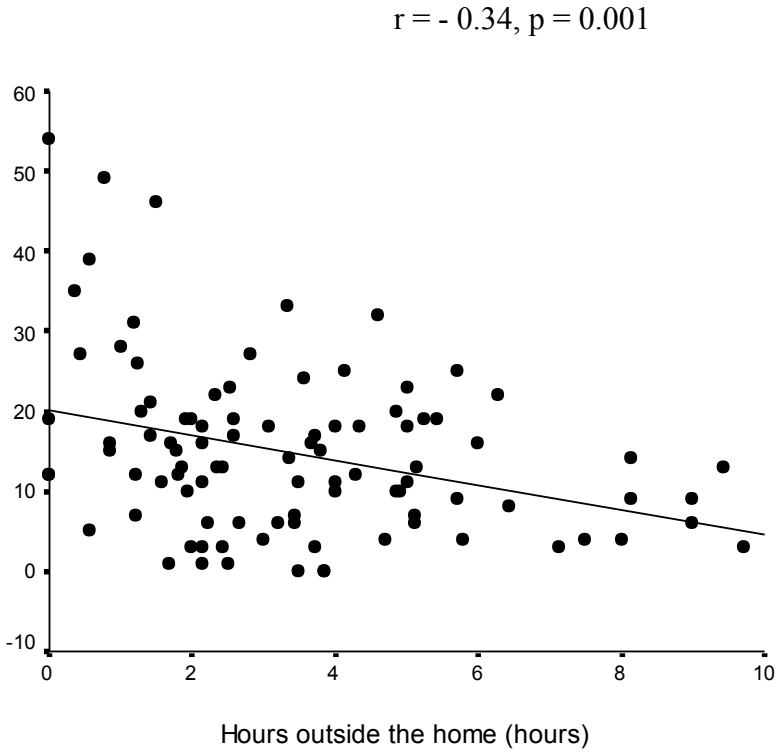
< 0.001 (**Figure 6.2**). CES-D also associated strongly with the MRC dyspnoea scores; r = 0.31, p < 0.001. CES-D increased by 1.2 points with a 4 unit measure in Total SGRQ. Multiple linear regression allowing for exacerbation frequency, found SGRQ was independently significantly associated with depression (p<0.001), with a 2.4 unit change in CES-D corresponded to a 4 unit change in SGRQ (r^2 =0.3).

Figure 6.2: Relationship between SGRQ and depression. a) Worse quality of life (higher Total SGRQ scores) is related to depression; r = 0.47, p < 0.001. All subgroups of the SGRQ; b) impact, c) activity and d) symptom domains are all strongly related to depression; ; r = 0.49, p < 0.001, r = 0.32, p < 0.001, and r = 0.35, p < 0.001 respectively.



Data on time outdoors was collected on 96 patients at baseline and 45 at an exacerbation visit. Patients who spend less time outdoors were more depressed; r = - 0.34, p = 0.001 (**Figure 6.3**) and this was related to worse quality of life as measured by the SGRQ; r = - 0.22, p = 0.03.

Figure 6.3: Relationship between depression and time spent outdoors.



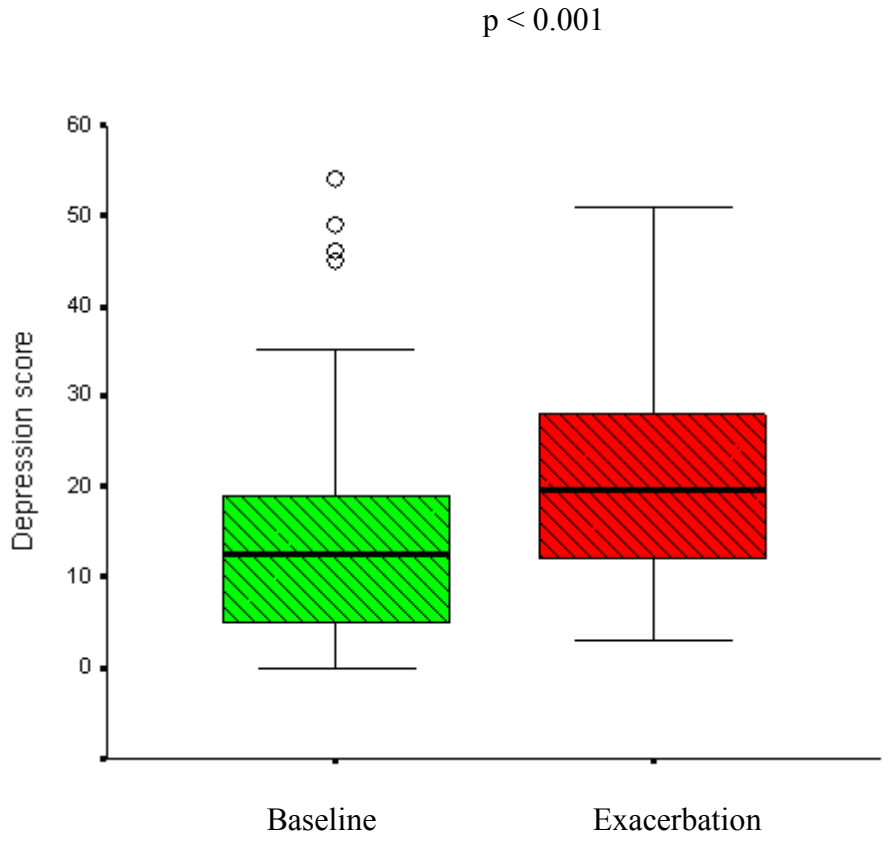
Women were more depressed than men with a mean CES-D of 18.3 (12.6) compared to 12.2 (9.0) for men; p = 0.001. Depression scores were higher in those who lived alone compared to those co-habiting; 18.9 (12.6) and 11.6(8.6) respectively; p < 0.001. CES-D also related to the number of people indoors, with individuals being less depressed the more people they live with; r = - 0.21, p = 0.007, and specifically less depressed if they live with a spouse; p < 0.001. Sex, exacerbation frequency, living alone and MRC dyspnoea score were all independently related to CES-D (p < 0.001, p = 0.009, p = 0.001 and p = 0.005 respectively).

Depression scores were similar in current and ex-smokers; (53 current smokers and 116 ex-smokers). CES-D was not related to pack years smoked.

Exacerbation characteristics

39 frequent and 31 infrequent exacerbators completed the CES-D at an exacerbation visit. The baseline characteristics of these patients are in Table 3. The CES-D increased significantly from baseline to exacerbation; mean CES-D 14.6 (11.8) and 20.3 (10.4) respectively; $p < 0.001$ (**Figure 6.4**). In the frequent exacerbators the baseline CES-D score increased on average by 5.0 (9.4) points at exacerbation, and in the infrequent exacerbators the CES-D increased on average by 6.7 (9.4) at exacerbation. 42% of the cohort scored above 16 on the CES-D at baseline, and 60% at exacerbation.

Figure 6.4: Depression score at baseline and exacerbation.



Exacerbation inflammatory markers and depression

CRP and IL-6 was measured in 35 patients at an exacerbation visit. CRP and IL-6 increased from baseline to exacerbation; $p = 0.001$ and $p = 0.07$ respectively. The increase in CRP correlated with the increase in IL-6; $r = 0.62$; $p < 0.001$ but not to the

change in CES-D. Exacerbation CRP and IL-6 levels were not related to the CES-D exacerbation score.

The mean duration of exacerbation symptoms was 13.2 days (9.6). There was no relationship between the change in depression score from baseline to exacerbation and the length of the exacerbation, the time taken to present for treatment of the exacerbation, symptom count, or individual symptoms at exacerbation. There was also no relationship between exacerbation depression score and time to the next exacerbation. Only 2 patients were hospitalised at exacerbation in the cohort and so we were unable to study the effect of hospitalisation and depression.

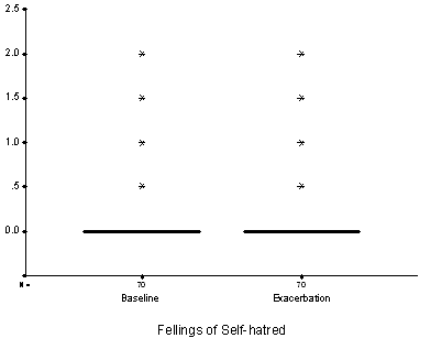
No baseline factors e.g. FEV_1, FVC, age, inflammatory markers, predicted the change in CES-D from baseline to exacerbation. There were no specific symptoms at exacerbation or pattern of symptoms at presentation of exacerbation associated with greater risk of depressive symptoms.

Principal component analysis groupings

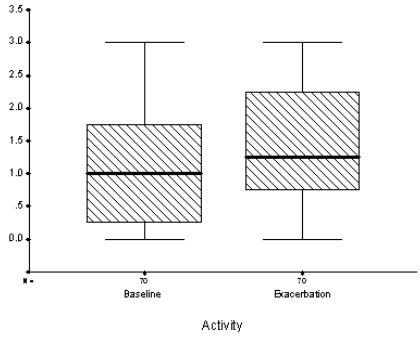
Scores in all five subgroups of the PCA changed significantly from baseline to exacerbation except for feelings of self-hatred. Feelings of "positivity" and "activity" increased at exacerbation (difference of - 0.30, $p = 0.02$ and difference of - 0.41, $p = 0.001$ respectively). "Reflective feelings", and "depressive feelings" increased (difference of - 0.48, $p = <0.001$, and difference of - 0.25, $p = 0.06$ respectively). The largest change between baseline and exacerbation was seen in reflective feelings (**Figure 6.5**).

Figure 6.5: PCA groupings at baseline and exacerbation. The x axis represents the PCA score.

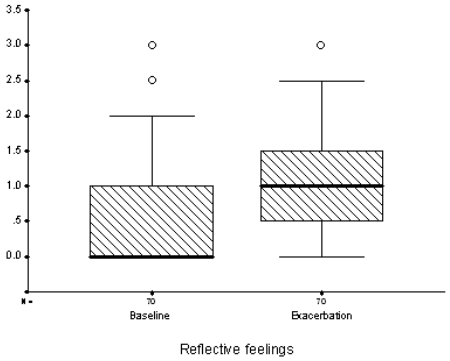
Depressive feelings



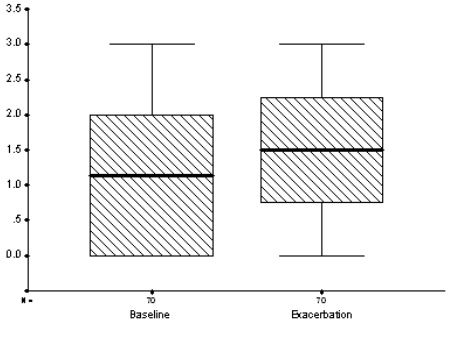
Fellings of Self-hatred



Activity



Reflective feelings



Feelings of Positivity

6. 5 Discussion

This is the first study to show a relationship between depression and exacerbation frequency in patients with COPD. The finding that frequent exacerbators are more depressed than infrequent exacerbators is crucial, as exacerbation frequency is an important outcome measure in COPD. Patients prone to frequent exacerbations have faster decline in lung function (Kanner 2001, Donaldson 2002) reduced physical activity (Donaldson 2005), impaired health status (Seemungal 1998) and faster disease progression. Frequent exacerbators also have increased mortality (Soler-Cataluna 2005), more frequent hospitalisations and thus increased health care costs.

Depression affects how individuals utilise health care, and comply with and respond to treatment. Therefore, identifying and treating depression may help to maximise patient outcome and quality of life (Eiser 2005). Early presentation to health care professionals can affect the outcome of an exacerbation and reduce hospitalisation (Wilkinson 2004). Depressed patients may be less likely to present early for treatment, or to report their exacerbations at all.

Although most COPD exacerbations are treated in the community, they are an important cause of hospitalisation and are responsible for around 10% of all acute medical admissions (Hospital Episode Statistics , 2003). Depression is a strong independent predictor of mortality in COPD patients admitted to hospital for an acute exacerbation (Almagro 2002), and depression may be a risk factor for frequent admissions (Dahlen 2002, Yohannes 2000) as well as being a frequent exacerbator (Garcia-Aymerich 2003). We were not able to study the effect of depression on hospitalisation in our cohort as so few patients were hospitalised during the study.

We found that depression is related to worse quality of life, higher MRC score, less time spent outdoors, female sex (Di Marco 2006), and social isolation (van Manen 2002). Increased MRC dyspnoea score which relates to perceived symptoms of dyspnoea were strongly related to depressive symptoms, and less time spent outdoors. Previous studies have shown that awareness of dyspnoea is higher in those who have symptoms of anxiety and depression (Neuman 2006, Ferguson 2006). Higher depression scores may lead more patients to feel breathless, or to perceive changes in their symptoms more

readily. As depression was closely related to MRC breathlessness score, it is likely patients underestimate their abilities and do not go out when they are depressed. Current lack of recognition and treatment of depression may have implications for uptake and completion of pulmonary rehabilitation, self management plans or use of community services. Patients who score MRC 5 have less significant improvement in pulmonary rehabilitation and a higher dropout rate (Garrod 2006, Wedzicha 1998) and this may be due to unrecognised depression. This data suggests we should be routinely screening and treating frequent exacerbators for depression if appropriate, and addressing psychological aspects prior to rehabilitation. Awareness of social isolation may also partly explain the benefits of pulmonary rehabilitation.

Depression increased acutely at the time of an exacerbation. Factors occurring at exacerbation which raise the CES-D score above 16 are likely to be of clinical relevance. Therefore we believe that increased depression at exacerbation is clinically important. Whether the increase in CES-D at the time of exacerbation was due purely to psychological factors, or to physical limitations imposed by the exacerbation is difficult to ascertain. Nonetheless, the difference between the frequent and infrequent exacerbators indicates the psychological impact of an exacerbation is greater on those who are less depressed, i.e. the infrequent exacerbators. Thus it is essential not to overlook this group, in whom psychological intervention at exacerbation may particularly be of benefit.

Detailed analysis of groups of specific questions in the CES-D showed that the increased depression seen at exacerbation was mostly due to introversion and reflection. Patients were “bothered by things that do not usually bother them”, and “talk less than usual.” There is often a negative emotional balance associated with COPD; patients feel guilty they did not give up smoking, they feel that there is a stigma attached with the disease and often have feelings of frustration, fear and hopelessness (Kessler 2006). They also have a fear of dying and the increased depression seen at exacerbation may be patients reflecting about death at this time.

There was no relationship between markers of systemic inflammation and depression in COPD patients. Depression has been shown to be linked with systemic inflammation in other diseases such as cancer (Jehn 2006), suggesting that the mechanism driving

depression in COPD does not have an inflammatory basis. This is consistent with the observation that depression was not linked to severity of disease as defined by GOLD staging. Systemic inflammation has been shown to be linked to GOLD staging (Broekhuizen 2006), and CRP has also been shown to be related to SGRQ (Broekhuizen 2006). Previous studies have reported depression to be more strongly related to functional status than COPD severity and other studies have shown depression to be more common in severe disease (van Ede 1999).

This study was conducted in a well characterised cohort of COPD patients, with robust data for exacerbation frequency. It is possible our cohort was less depressed than other populations studied as they volunteered to attend frequent outpatient appointments and fill in daily diary cards. However the prospective nature of the study, accurate calculations of exacerbations frequencies over time from completion of daily diary cards and prompt presentation at exacerbations add to the validity, strength and uniqueness of the depression data in this study. This study was not designed to look at clinically meaningful changes in depression, but we estimated that a change in 1.2 points in the CES-D would be associated with a 4 point change in SGRQ.

Subsequent to this work, other studies have also shown a relationship between depression and exacerbations of COPD. Jennings et al (Jennings 2009) studied 194 COPD patients who had completed pulmonary rehabilitation and found that patients with depressive symptoms were more likely to exacerbate in the following year and were 2.8 times more likely to have ever exacerbated before. Equally, depression has been linked with hospitalisation for exacerbation in COPD (Xu 2008). Depression has also been linked to all cause mortality in COPD (de Voogd 2009).

6.6 Conclusion

This study has shown for the first time that frequent exacerbators are more depressed than infrequent exacerbators. Depression was more common in women, those with little social contact and negatively affected quality of life. The increased depressive symptoms seen at exacerbation reflect patients' increased introversion and contemplation. Understanding the nature of depressive symptoms and treating them is important if we are to improve a patient's quality of life, maximise healthcare utilisation and treatment outcomes.

7

Social circumstances influencing exacerbation frequency

7.1 Introduction

Positive emotions have been associated with lower rates of morbidity and mortality (Pressman 2005) and with the expression of signs and symptoms of illness. The effect of depression on exacerbation frequency has been discussed in ***Chapter 6***. Good social support can influence health status by buffering an individual's response to stress and by increasing positive emotions, which in turn have been shown to decrease symptom burden (Cohen 1997). However, the effect of social support on infection susceptibility is less clear. Certainly close social contact can increase infection susceptibility (Badger 1953) and studies undertaken as early as 1926 have shown that children are particularly at risk of developing viral respiratory infections (Sydenstricker 1926, van Volkenburgh 1933) and are good transmitters of disease.

Viruses are increasingly thought to be an important cause of exacerbations and high exacerbation frequency has been shown to be associated with increased frequency of acquiring the common cold (Hurst 2005). Hurst *et al* showed that frequent exacerbators experienced significantly more colds, and significantly more exacerbations associated

with a cold than infrequent exacerbators (1.73 *versus* 0.94·yr^{-1}, p = 0.003; and 0.95 versus 0.45·yr^{-1}, p<0.0001, respectively), and that patients with frequent colds experienced significantly more exacerbations than those with infrequent colds (3.23 *versus* 2.10·yr^{-1}, p<0.001).

Social circumstances such as contact with children, living with a spouse and frequency of visitors into the home may therefore be important influences on exacerbation frequency. Social isolation and a decreased social support network may increase symptom burden, and regular contact with children may increase viral acquisition. Virus acquisition in the stable state may increase exacerbation susceptibility and thus exacerbation frequency.

7.2 Aims

1.To investigate whether contact with children affects acquisition of HRV in the stable state and whether this affects exacerbation frequency.

2. To determine if vitamin D levels are lower in frequent exacerbators as a result of decreased time spent outdoors and decreased sunlight exposure, and whether these lower levels increase susceptibility to viral infection, thus increasing exacerbation frequency.

7.3 Methods

All patients had been in our London Cohort for more than one year. Recruitment, monitoring, data collection, sampling and sample processing has been described in ***Chapter 3*** **(sections 3.1, 3.6, 3.9, 3.12)**.

7.4 Results

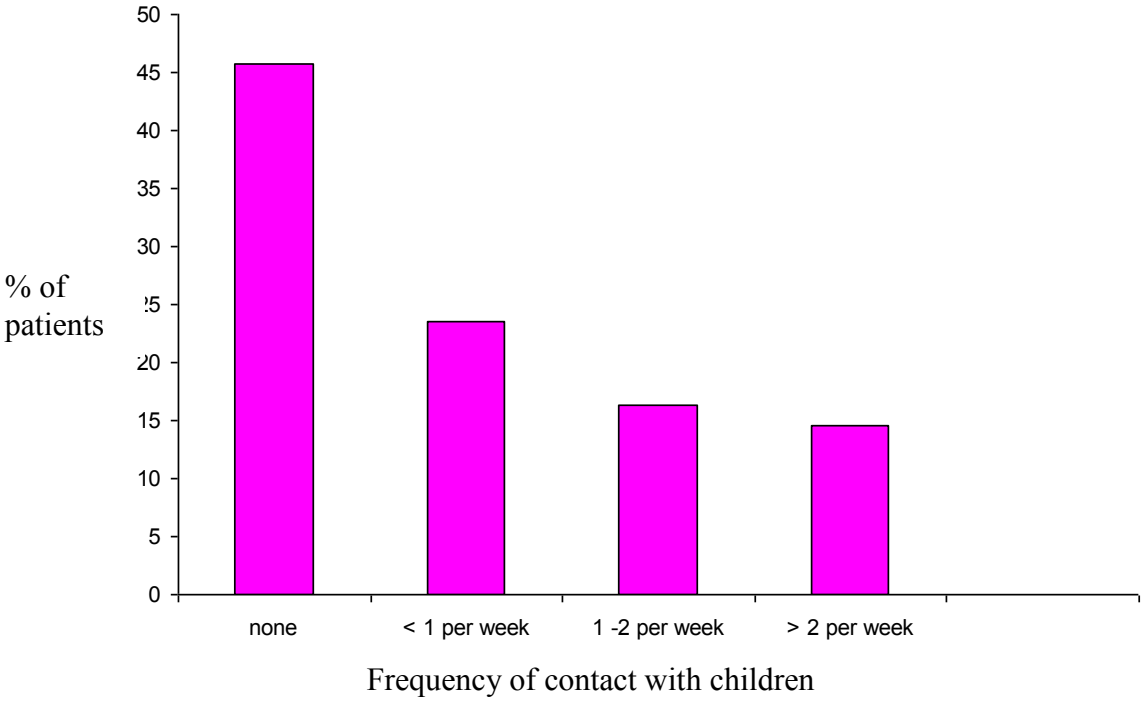
The baseline demographics of the 166 COPD patients included in the analysis are given in **Table 7.1**.

Table 7.1: Baseline characteristics of the 166 patients

	Mean	**SD**
Age (years)	71.3	8.5
FEV1 (litre)	1.2	0.5
FEV1 % predicted	49.7	18.7
FVC (litre)	2.54	0.94
BMI (kgm^{-2})	25.9	5.5
Pack years smoking	52.1	37.3

55 patients were frequent exacerbators (33.1%). 100 patients were male (60.2%), 68 lived alone (41.0%) and 78 (47.0%) lived with a spouse. 24 patients (14.5%) had contact more than twice weekly with children under the age of 16, 27 (16.3%) had contact 1-2 times / week, and 39 (23.5%) less than once a week. 76 patients (45.8%) had no contact with children under the age of 16 (**Figure 7.1**). The mean age of the youngest child patients had contact with was 4.2 years (SD 4.0). There was no data available on social class but the majority of patients were retired.

Figure 7.1: Percentage of patients according to frequency of contact with children.



There was no relationship between exacerbation frequency and living with a spouse, ever having contact with children under the age of 16, the frequency of contact with children under the age of 16 or the age of the child. There was also no relationship between exacerbation frequency and the number of times visitors entered into the patient's home during the week or the number of days a week the patient left the house.

Social factors, seasonality and HRV detection and load at baseline

In a subset of 89 patients, sputum samples were collected at least once in the stable state and tested for the presence of HRV. Patients who had any contact with a child under the age of 16 were more likely to ever have HRV detected in sputum at a baseline visit; 70% vs. 30%; p = 0.02 (**Table 7.2**).

Table 7.2: Percentage of patients positive for HRV in sputum and frequency of contact with children

	No contact	*Contact*	**Total**
Never positive sputum Baseline	21%	15%	**36%**
At least one positive HRV baseline	18%	35%	**53%**
Total	**39%**	**50%**	**89%**

There was a trend towards increasing likelihood of ever being positive for HRV in sputum and an increased frequency of contact with children at baseline; p = 0.08 (**Table 7.3**) but no relationship with the age of the child.

Table 7.3: Frequency of contact with children and HRV detection

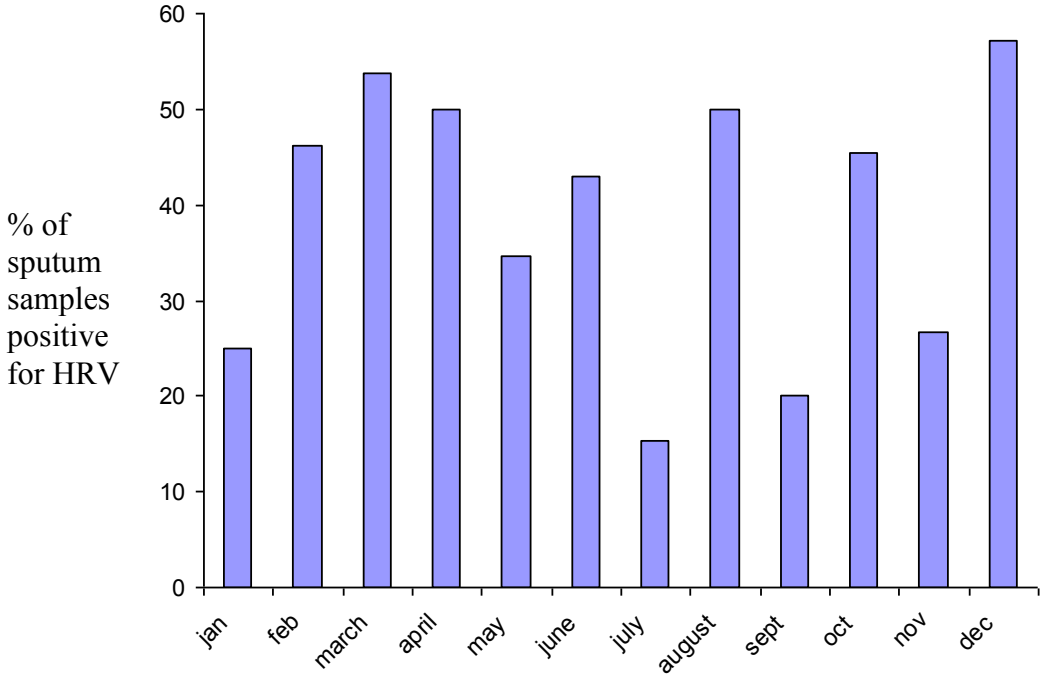
Frequency of contact with children	***Number of patients ever positive at baseline (%)***	**Total number of patients**
Never	18 (46.0)	39
Less than once / week	16 (72.7)	22
1 – 2 per week	12 (66.7)	18
> 2x per week	7 (70.0)	10

There was no relationship between the number of times visitors visited a patient's home and positivity for HRV in sputum at baseline. There was no relationship between the number of adult occupants in the patient's home and the likelihood of ever being positive for HRV in sputum at baseline. There was no relationship between the number of hours spent outside the home and positivity for HRV detected by sputum. There was no difference in the proportion of men and women positive for HRV in sputum.

Seasonality of HRV detection

There was no difference in the proportion of samples positive for HRV by sputum over the course of the year (**Figure 7.2**).

Figure 7.2: Graph showing the percentage of samples positive for HRV by month of the year



7.5 Discussion

We did not find a relationship between exacerbation frequency and social contacts; living with a spouse, contact with children, frequency of visitors to the home or number of days out of the house during the week. There was no relationship between HRV positivity in sputum at baseline and co-habitants, frequency of visitors into the home or the number of days out of the house during a week. However, we did find a relationship between HRV positivity in sputum at baseline and contact with children, with a trend towards an increased likelihood of positivity with increasing frequency of contact.

Several studies have investigated social circumstances with regard to susceptibility to infection and results regarding social support versus exposure to illness are mixed. One of the earliest studies investigating social circumstances and infection was the Cleveland family study undertaken in the 1940s and 50s (Dingle 1953). This study, focusing on the co-habiting family unit, found the frequency of respiratory illness to be highest in young children, with respiratory illnesses decreasing with age. Respiratory infections were more common in females; particularly in women who did not work outside the home. Other factors associated with increased risk included lower household income, increased numbers of individuals sharing a bedroom and an increased risk of developing an illness if another family member was ill. Fathers were the least likely introducers of illness to the household, with mothers, school age children, preschool-age children at home, and preschool-age children in day care or pre-school being the next most likely introducers.

Therefore it follows that in patients with COPD, who are arguably at increased risk of acquiring respiratory viral infection (Monto 1978, Wald 1995) compared to control populations, contact with children and acquisition of HRV is linked. The Cleveland family study only identified influenza and traditional bacterial infections. We were specifically interested in HRV as it is the commonest virus detected at exacerbation of COPD (Seemungal 2001, Rohde 2003) and is a cause of the common cold. It is possible that other viruses or bacteria are also important contributors to exacerbation frequency, which we did not investigate here. Certainly bacterial colonisation in the stable state has been shown to effect exacerbation frequency (Patel 2002).

In a sub analysis in the Hurst *et al* paper (Hurst 2005), there were no significant relationships between cold frequency and the number of people living at home, the number of visitors into the home, or the number of trips made outside the home. Our results concur with regard to acquisition of HRV. Only contact with children was found to be related to acquisition of HRV in this work. As those with frequent contact with children were not found to be the frequent exacerbators, it is likely that factors other than susceptibility to HRV drive exacerbation frequency. It is also likely that the individuals in the cohort are educated about exacerbations enough not to have contact with children when they are ill.

We used a very low cut of detection for HRV (1 pfu/ml) in this work. The significance of low levels of HRV in COPD is not clear and requires further study. It is possible that very low levels near this sensitive cut off are too low to trigger an exacerbation or an inflammatory response and thus symptoms. These low levels do not appear to increase exacerbation frequency and this has been discussed further in ***Chapter 10.2***. Not all colds lead to exacerbation and perhaps the load of HRV acquired is more important in terms of exacerbation frequency. We only used lower airway loads in this analysis, not upper airway. Also, our HRV RT-PCR was designed to detect as many serotypes of HRV as possible, and it is known that not all serotypes are pathogenic.

Other studies have linked viral infection with gender and co-habitants; however, we did not find any relationship. In a study investigating subjective socioeconomic status (SES) and the common cold, increased subjective SES was associated with a decreased risk for developing a cold with both HRV and influenza (Cohen 2008). Several ecological studies suggest that socioeconomic status may be important in determining respiratory admissions (Hawker 2003, Watson 1996, Gilthorpe 1998) but evidence is conflicting (Crocetti 2001, Puig-Barbera 2004, Puig-Barbara 1997, Garcia-Aymerich 2003, Garcia-Aymerich 2001, Prescott 1999(b)). It is possible that social contacts are more important with regard to severity of exacerbation rather than frequency. We did not investigate exacerbation severity in this group. A study of pneumonia admissions in the UK showed that deficient social support i.e. not being married was a significant risk factor for admission, however crowding in the home was not (Farr 2000). A Canadian study investigating factors for readmission of exacerbation for COPD found that those who

were single, were at increased risk of re-admission; (OR 4.18, 95% CI 1.03 to 17.02; P<0.046) (Wong 2008). A UK study establishing factors associated with winter hospital admissions among older people presenting with acute respiratory disease found that social isolation (odds ratio [OR] 4.5; 95% confidence interval [CI] = 1.3 to 15.8) resulted in an increased risk of admission with COPD as the most important independent risk factor (COPD; OR 4.0; 95% CI = 1.4 to 11.4). They also found that being housebound was associated with an increased independent risk; (OR 2.2; 95% CI = 1.0 to 4.8) (Jordan 2008). In this study living alone had no effect on admission; however patients without regular visits from friends or family were at higher risk. In a study by Smith (Smith 2001) in asthmatics, social support did not independently influence susceptibility to colds. However negative life events were associated with increased episodes of colds when levels of social support were low. Social support may act as a buffer to against the effects of stress thus influencing infection. Under low levels of stress, high levels of social support have been associated with decreased risk of infection (Turner-Cobb 1996). Social support may be more relevant to health care utilisation and not individual symptoms in this group. As we did not study exacerbations associated with hospitalisation, and most of our cohort are not usually hospitalised with their exacerbations, our findings are arguably in keeping with these studies.

Certain viral outbreaks are related to children returning to school (Monto 2002). We investigated the seasonality of HRV in our cohort. We found no difference in the percentage of HRV positive sputum samples by season. Seasonality was examined in the Cleveland study where a sharp increase in illnesses occurred in September and continued through March. In the Greenberg study however (Greenberg 2000) viral respiratory tract infections were detected in every month of the year, with an increase in total respiratory illnesses and virus-associated illnesses during the autumn and winter months. In the COPD subjects, a rise in reported respiratory illnesses began in September and remained through May. We did not see the same pattern here. This may be due to different assays and detection of different serotypes. It also may be related to our very sensitive level of detection; when not causing an outbreak viruses are likely to still be present the community in low levels and if COPD patients are more susceptible than others to acquisition, this may explain the low levels seen here. Pollution and meteorological factors associated with viral infection are complex and have not been

fully elucidated and may be important. In children, HRV has been associated with relative humidity (du Prel 2009).

Although a large proportion of patients have regular contact with young children, they may stay away when children / family / visitors are unwell as they are aware of the consequences of exacerbations. If this is the case, it suggests that something other than viral infection is driving exacerbation frequency. This has been explored further in ***Chapter 10.1***.

It may even be simpler than virus acquisition; social circumstances may influence exacerbation frequency through adherence to medication or psychological outlook. Individuals with COPD who adhere to their medication have been shown to experience better survival and lower risk of hospital admission due to exacerbations than those who do not adhere (Vestbo 2009), thus suggesting that frequent exacerbators do not adhere to treatment. Equally, social class and education may be important.

7.6 Conclusion

We did not find a relationship between exacerbation frequency and social contacts; living with a spouse, contact with children, frequency of visitors to the home or number of days out of the house during the week. There was no relationship between HRV positivity in sputum at baseline and co-habitants, frequency of visitors into the home or the number of days out of the house during a week. However, we did find a relationship between HRV positivity in sputum at baseline and contact with children, with a trend towards an increased likelihood of positivity with increasing frequency of contact. Although a large proportion of patients have regular contact with young children, they may stay away when children / family / visitors are unwell as they are aware of the consequences of exacerbations. We found no difference in the percentage of HRV positive sputum samples at baseline by season.

8

Vitamin D deficiency, receptor polymorphisms and exacerbation frequency

8.1 Introduction

Exacerbations are approximately 50% more likely in the winter, and are associated with colder outdoor temperature (Donaldson 1999), possibly via improved respiratory virus survival in colder weather, crowding together of people indoors over the winter and a reduced immunological response (Eccles 2002). Data are available suggesting that the seasonality of influenza (and possibly other viruses) is related to sunlight exposure and consequently vitamin D levels. Hope-Simpson (Hope-Simpson 1981) postulated that humans have a physiological system dependent on solar radiation that improves innate immunity in the summer but impairs it in the winter and that with vitamin D synthesis being so dependent on sunlight exposure, falling winter levels may trigger immune deficiencies. We hypothesised that with exacerbations peaking in the winter / early spring when vitamin D levels are at their lowest, the decrease in adaptive immunity associated with deficiency in vitamin D may increase exacerbation risk and therefore contribute to exacerbation frequency.

8.2 Aims

1. To investigate whether frequent exacerbators have lower vitamin D levels in the stable state than infrequent exacerbators.

3. To investigate whether patients deficient in vitamin D are more likely to be HRV positive at exacerbation and if vitamin D levels correlate with HRV load.

4. To investigate whether vitamin D receptor polymorphisms influence exacerbation frequency or vitamin D levels.

8. 3 Methods

Recruitment and sampling have been described in ***Chapter 3*** **sections 3.1 and 3.6**. Ninety seven COPD patients and 33 controls were studied between 1st April 2006 and 30th March 2009. Detail regarding patient recruitment and monitoring can be found in section 3. Summer blood samples were collected for vitamin D assay in June, July or August, and winter samples in January, February or March. Both samples were collected in the same calendar year.

At an exacerbation visit information was collected on symptom type and duration. Spirometry was performed and blood taken for vitamin D assay. Sputum was collected if spontaneously produced and a nasopharyngeal swab (NPS) taken and tested for HRV. Exacerbation visits were not limited to the summer or winter periods above and results were adjusted for seasonality. Vitamin D measurement and HRV detection were described in ***Chapter 3***.

Time outdoors and hours of sunlight

The time outdoors for baseline and exacerbation visits was calculated as the average time spent outdoors in the 14 days preceding the clinic visit. Hours of sunlight were obtained from Meteorological Office data (at Heathrow Airport, London) on the day of the visit to clinic. Further details are provided in ***Chapter 3*****, section 3.6**. Hours of sunlight were obtained from the meteorological office.

8.4 Results

Baseline patient characteristics

Ninety seven COPD patients were studied; 61 male and 36 female. The patients had a mean FEV_1 of 1.19 l or 50.3% predicted. 33 control subjects were studied; 16 male and 17 female. The control subjects had a mean FEV_1 of 2.87 l or 107.9% predicted. There were significant differences in age, smoking history and oxygen saturations between the control subjects and COPD patients (all $p < 0.001$) but not BMI. The baseline characteristics are reported in **Table 8.1**.

2 control subjects and 10 COPD patients were on vitamin D supplementation (Calcichew), and were not included in the following analysis unless otherwise stated.

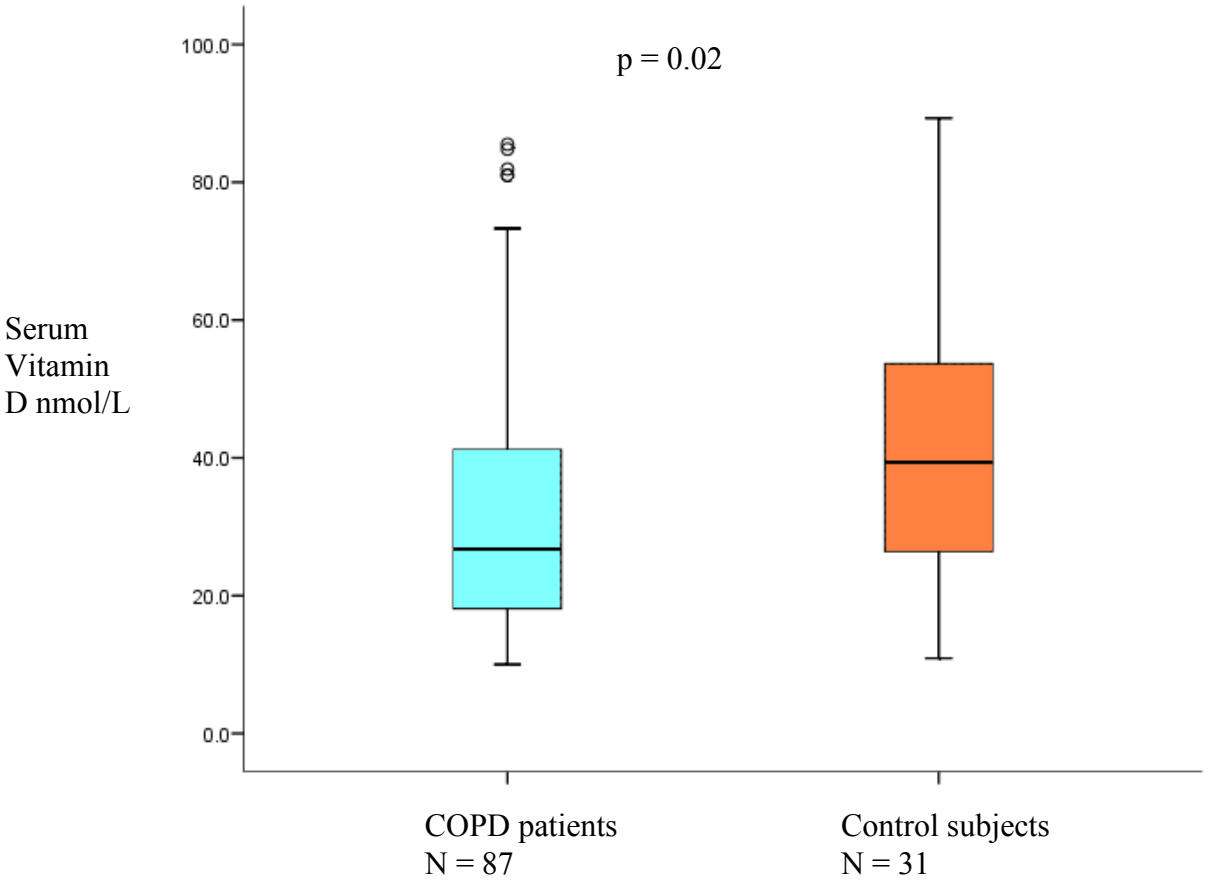
Table 8.1: Baseline Characteristics of 97 patients: 61 male (62.9%), 28 Frequent exacerbators (28.9%), 25 current smokers (25.8%) and 33 controls: 16 male (48.5%), 5 current smokers (15.2%), 15 never smokers.

	COPD patients Mean (SD)	**Control subjects Mean (SD)**
Age (years)	71.8 (8.8)	65.5 (6.7)
FEV1 (litre)	1.19 (0.54)	2.9 (0.73)
FEV1 (% predicted)	50.3 (19.7)	107.9 (16.4)
FVC (litre)	2.5 (0.84)	3.70 (1.04)
BMI (kgm^{-2})	27.0 (6.0)	26.0 (3.7)
Pack years smoking	50.7 (34.2)	12.0 (19.5)
SpO_2 (%) on air	95 (2)	96 (1)
Vitamin D nmol/L	**Median (IQR)**	**Median**
Summer	41.3 (26.8 – 64.8)	
Winter	27.8 (19.4 – 44.4)	39.6 (26.4 – 59.4)

Vitamin D levels in COPD patients and controls

Figure 8.1 shows that in the winter, 87 COPD patients not on Calcichew had significantly lower vitamin D levels than the 31controls not on Calcichew; medians 26.7 nmol/L (IQR 17.8 – 41.2) and 39.3 nmol/L (26.3 – 58.0) respectively; p = 0.02. 42 (48.3%) COPD patients were deficient in vitamin D (9 had levels < 10 nmol/L), 40 (46.0%) had insufficient levels and 5 (5.7%) had sufficient levels. Of the controls, 8 (25.8%) were deficient in vitamin D, 20 (64.5%) were insufficient and 3 (9.7%) were sufficient. There was no difference in the proportions of those sufficient, insufficient or deficient in the two populations.

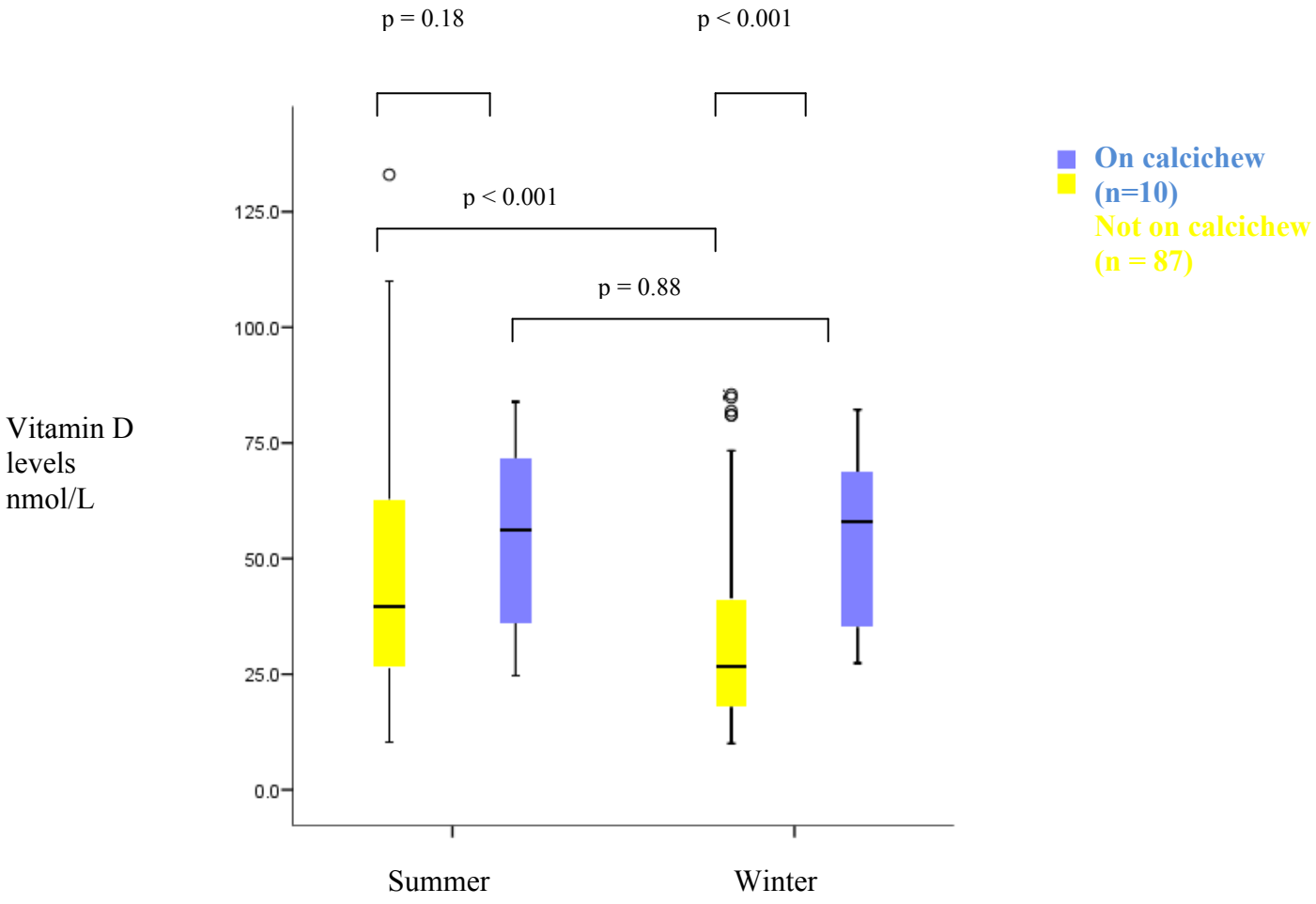
Figure 8.1: Winter vitamin D concentration in COPD patients and controls not on Calcichew



Seasonal variation and vitamin D levels in COPD

COPD patients not on supplementation had lower vitamin D levels in winter compared to summer; medians 26.7nmol/L (IQR 17.8 – 41.2) and 39.6nmol/L (26.4 – 62.9); p<0.001. Summer and winter vitamin D levels varied by 10% monthly and by 50% between summer and winter, with summer levels being higher. Patients taking Calcichew did not show the same significant seasonal variation in vitamin D levels; winter median 58.1nmol/L (35.2 – 69.6), summer median 56.2nmol/L (33.9 – 73.1). The seasonal difference in vitamin D levels between these patients is illustrated in **Figure 8.2**.

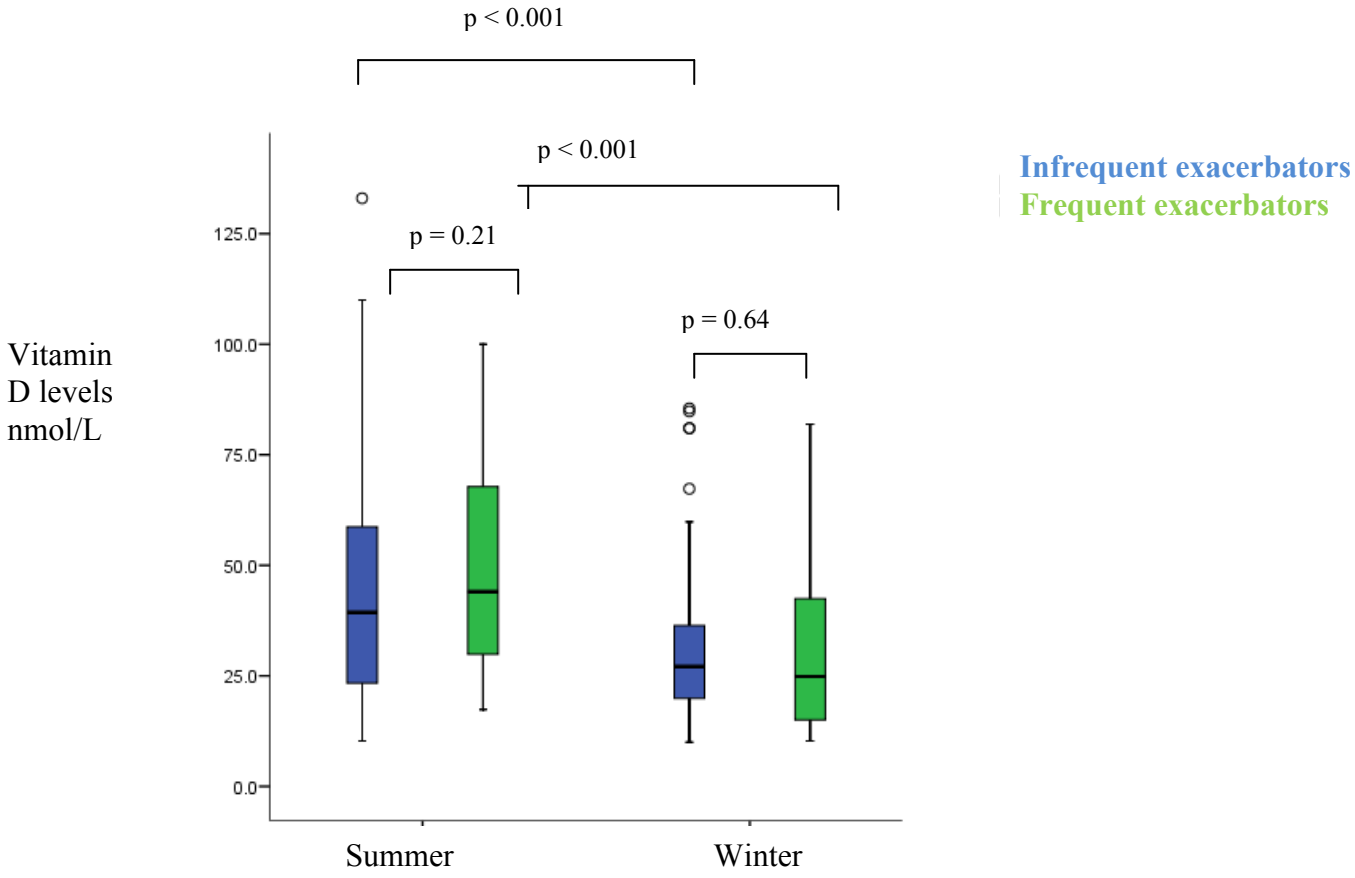
Figure 8.2: Vitamin D levels in COPD patients in summer and winter in those on and not on Calcichew



Exacerbation frequency and vitamin D levels

There was no difference in Vitamin D levels between frequent and infrequent exacerbators in the summer; medians 44.1nmol/L (29.1 – 68.0) and 39.4nmol/L (22.3 – 59.2) or winter; medians 24.9nmol/L (14.3 – 43.1) and 27.1nmol/L (19.9 – 37.6) (**Figure 8.3**)

Figure 8.3: Vitamin D levels in summer and winter in frequent and infrequent exacerbators



The proportion of patients' deficient, insufficient and sufficient in vitamin D was the same in both frequent and infrequent exacerbators groups.

Exacerbation frequency pre and post calcichew

Exacerbation history was available in a subset of 10 patients before and after treatment with calcichew (**Table 8.2**).

Table 8.2: Exacerbation number pre and post calcichew

patient number	*Number of exacerbations in the year preceding calcichew*	*Number of exacerbations in the first year on calcichew*
1	0	0
2	2	4
3	1	3
4	0	0
5	0	0
6	0	0
7	2	3
8	0	0
9	1	1
10	3	1

There was no difference in actual exacerbation number from year 1 to 2, or in exacerbation frequency from year 1 to 2.

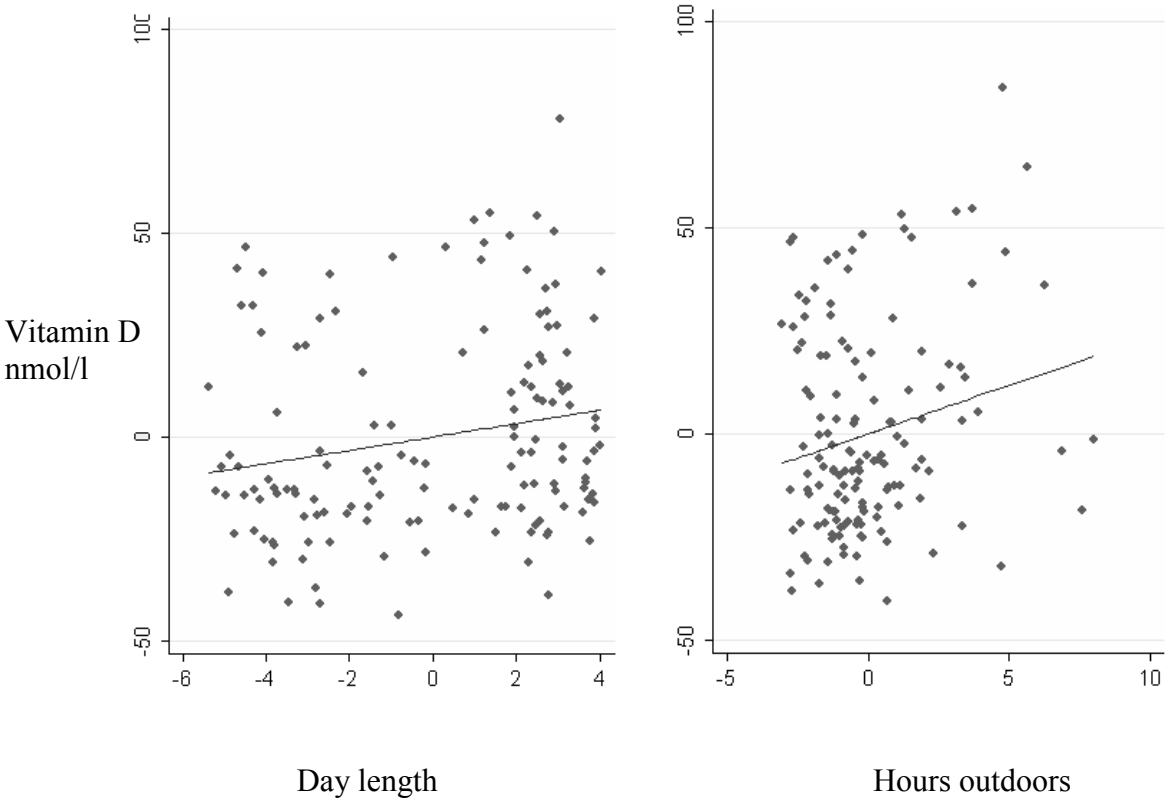
Smoking status and vitamin D levels

There was no difference in summer or winter vitamin D levels between current smokers and ex-smokers; summer medians; 45.5nmol / L (27.4 – 63.3) and 40.3 nmol/L (26.7 – 66.3) and winter medians; 27.0 nmol/L (14.6 – 47.7) and 28.0 nmol/L (19.9 – 43.7).

Time outdoors and Vitamin D levels

Figure 8.4 shows that fewer hours of daylight on the 14 days preceding sampling was associated with lower levels of vitamin D ($p = 0.02$). Patients who spent less time outdoors in the 14 days prior to sampling also had lower vitamin D levels ($p = 0.02$). This was independent of hours of day length.

Figure 8.4: Relationship between vitamin D levels and (1) day length, and (2) hours outdoors. The graphs shown below are relative to each other.



Vitamin D levels and exacerbations of COPD

58 exacerbations were tested for vitamin D; 1 per patient in the study period. Adjusting for seasonality, there was no difference in vitamin D levels between baseline and exacerbation.

Vitamin D and HRV exacerbations

46 exacerbations were tested for the presence of HRV in sputum or NPS. HRV positive exacerbations (n = 12, viral load > 175pfu/ml; the rationale for this cut off for exacerbation is explained in ***chapter 10.2***) were not associated with lower vitamin D levels at exacerbation than exacerbations that did not test positive for HRV; medians 30.0nmol/L (20.4 – 57.8) and 30.6nmol/L (19.4 – 48.7). HRV load in sputum or NPS at exacerbation did not correlate with exacerbation vitamin D levels. When including all exacerbations per patient taken over the study period tested for HRV, patients deficient in vitamin D at baseline did not have an increased proportion of HRV positive exacerbations (**Table 8.3**).

Table 8.3: Vitamin D status and positivity for HRV at exacerbation

Vitamin D status	Ever positive for HRV at exacerbation		Total
	No	Yes	
Deficient	4 (25.0%)	7 (23.3%)	11
Insufficient	10 (62.5%)	19 (63.3%)	29
Sufficient	2 (12.5%)	4 (13.3%)	6
Total	16	30	46

29 sputum samples were tested for the presence of a potentially pathogenic micro-organism (PPM) at exacerbation; 11 were positive and 18 negative. There was no difference in vitamin D levels between PPM positive and negative exacerbations; medians 46.0nmol/L (30.0 – 69.3) and 34.1nmol/L (19.8 – 48.8).

Exacerbation length and severity

There was no relationship with Vitamin D levels either at that exacerbation or the baseline preceding that exacerbation and the length of the exacerbation or time to the next exacerbation.

VDR polymorphisms

All genotypes were within Hardy Weinberg equilibrium. The *Fok*I polymorphism was not related to *Taq*I or *Bsm*I, however the *Bsm*I and *Taq*I polymorphisms were linked ($p < 0.001$). There was no relationship with genotyping and exacerbation frequency for any of the polymorphisms; **Table 8.4**.

Table 8.4: VDR polymorphisms and exacerbation frequency

SNP	Genotype	Frequent exacerbators (n=28)	Infrequent exacerbators (n=68)	Chi squared P value
Rs BsmI	BB	3 (10.7%)	15 (22.1%)	
	Bb	12 (42.9%)	26 (38.2%)	0.43
	Bb	13 (46.4%)	27 (39.7%)	
HWE p value		0.93	0.08	
		(n= 26)	(n = 66)	
Rs TaqI	TT	10 (38.5%)	24 (36.4%)	
	Tt	13 (50%)	29 (43.9%)	0.64
	Tt	3 (11.5%)	13 (19.7%)	
HWE p value		0.69	0.43	
		(n=28)	(n=68)	
Rs FokI	FF	10 (35.7%)	21 (30.9%)	
	Ff	14 (50.0%)	38 (55.9%)	0.87
	Ff	4 (14.3%)	9 (13.2%)	
HWE p value		0.80	0.21	

There was no difference in summer or winter vitamin D levels between genotypes for any of the polymorphisms; **Table 8.5**. *Bsm*I p = 0.53 and 0.89, *Taq*I p = 0.49 and 0.96 and *Fok*I.

Table 8.5: Seasonal vitamin D levels by genotype

SNP	Genotype	Summer vitamin D nmol/L	p value	Winter vitamin D nmol/L	p value
	BB	42.2 (34.6 – 70.8)		25.1 (20.7 – 37.9)	
BsmI	Bb	45.4 (22.5 – 66.2)	0.53	27.8 (15.6 – 54.5)	0.89
	bb	36.3 (26.7 – 63.5)		28.0 (19.9 – 40.4)	
	TT	34.5 (25.6 – 64.6)		27.9 (18.4 – 40.7)	
TaqI	Tt	47.5 (25.7 – 64.2)	0.49	29.5 (17.7 – 55.4)	0.96
	tt	41.2 (34.1 – 75.5)		25.1 (20.4 – 41.2)	
	FF	35.1 (25.8 – 58.8)		26.9 (19.0 – 45.4)	
FokI	Ff	49.1 (30.6 – 66.8)	0.30	29.5 (20.4 – 56.6)	0.44
	ff	36.4 (20.8 – 64.0)		20.9 (16.4 – 35.4)	

8.5 Discussion

We found that COPD patients have lower vitamin D levels in the winter months than control subjects (even when adjusting for age) and that COPD patients show the same seasonal variation in Vitamin D levels as the general population. This seasonal variation loses statistical significance in those on Calcichew, and Calcichew slows the rate of decline of vitamin D levels. In an age-adjusted healthy population, vitamin D levels have been shown to decrease over time. This is thought to be linked to nutritional status, increase in BMI, dietary changes and the increased use of sun protection (Looker 2008, Ginde 2009).It is possible that the decline in vitamin D over time is faster in COPD patients compared to controls but we did not have enough vitamin D readings in the controls to investigate this. It is estimated that up to 100% of the elderly population in the USA and Europe are vitamin D deficient (Chapuy 1997) and those with COPD are at particularly high risk due to reduced food intake, decreased vitamin D synthesis, skin aging, less time outdoors (Janssens 2009). Perhaps vitamin D deficiency in the COPD group is simply a marker of poor general health status (as it is related to low BMI) rather than having particular importance in the disease itself.

Smoking status did not influence vitamin D levels and there was no difference in vitamin D status between the frequent and infrequent exacerbators. Patients deficient in vitamin D in the stable state (< 25nmol/L), were not more likely to be frequent exacerbators and did not have a shorter time to their next exacerbation than those insufficient or sufficient in vitamin D.

It is difficult to establish the cause and effect of low vitamin D and FEV_1 within the COPD population. A relationship was found between vitamin D levels and FEV_1 and FVC (Black 2005) in a cross sectional US population after adjusting for confounding factors. However, no significant association was found with airway obstruction. Maternal vitamin D in pregnancy has been associated with asthma symptoms in childhood (Camargo 2007, Hyppönen 2004), and prenatal vitamin D deficiency is though to affect foetal lung and immune system development. Thus it is possible that vitamin D deficiency predisposes individuals to a lower FEV_1. This is consistent with our finding that COPD patients had lower vitamin D levels than control subjects, and that initial FEV_1 measurements were lower in those COPD patients with vitamin D

levels below the group median. Studies in asthma linking low vitamin D levels with disease severity have postulated the relationship may be secondary to diet and time spent indoors which in themselves are influenced by a diagnosis of asthma (Brehm 2009). This may be similar in COPD. We found that patients with lower vitamin D levels spent less time outdoors in the 14 days preceding sampling, and lower levels were associated with shorter day length. We also know that patients with more severe COPD spend less time outdoors (Donaldson 2005). However, it appears that in COPD factors other than vitamin D influence FEV_1 decline and it is unclear whether vitamin D supplementation would influence disease progression. We did not have sufficient power to investigate the effect of calcichew on starting FEV_1 or to assess the rate of decline in these individuals.

Vitamin D levels were unchanged at exacerbation in COPD when adjustments were made for seasonality and we did not find a relationship between vitamin D deficiency and exacerbation severity, or susceptibility to HRV at exacerbation. Vitamin D may down-regulate the inflammatory immune response in the airways and boost the innate immune defence against micro-organisms. Vitamin D deficiency has been associated with self-reported upper respiratory tract infections (URTI) (Ginde 2009). However, this was a subjective assessment; individuals were asked if they had had a cough, cold or respiratory illness in the days preceding vitamin D testing, and there was not any objective measurement of viral infection. Nevertheless, a serum vitamin D level of < 10ng/ml was associated with a 2.26 higher odds of recent URTI in those with COPD compared to 1.27 in those without COPD. This was statistically significant. In a study of young men in Finland, those with vitamin D levels < 40nmol/L in the summer had significantly more days off with URTI in the next 6 months (Laaksi 2007). Again, in this study, there was no objective measurement of viral infection and URTI included otitis and pneumonia. Patient susceptibility to exacerbation therefore must be driven by some other means and it appears from these data that increasing vitamin D levels will not decrease exacerbation frequency, suggesting it is driven by some other means. Vitamin D supplementation may increase levels significantly but this has no effect on HRV load or susceptibility to HRV exacerbations. In a subset of patients with an exacerbation history before Calcichew and on Calcichew there was no difference in exacerbation number.

Viral host defence is complex, involving both innate and acquired immunity. Macrophages release cytokines into infected respiratory tissue and antimicrobial peptides prevent viral replication (Rogan 2006). Vitamin D modulates macrophage response (Helming 2005). Vitamin D also prevents macrophage maturation (Abu-Amer 1993). Multiple studies have linked vitamin D deficiency with Tb (Grange 1985, Nnoaham 2008, Gibney 2008) and supplementation studies have resulted in mixed results (Martineau 2007, Wejse 2009). However, increasing vitamin D levels into an optimal range in COPD may still be of benefit. What this optimal range is is debateable. It may reduce bacterial load, bacterial colonisation or even reduce susceptibility to bacterial exacerbations. We did not find any difference in vitamin D levels with our bacterial culture data at exacerbation. We did not investigate bacterial colonisation and vitamin D levels in the stable state. Further studies are warranted, as there were too few patients in our study to comment on exacerbation susceptibility or severity in this group. Ideally we would need 100 exacerbations.

Several studies have linked VDR polymorphisms and infection. A study in young Canadian children (Roth 2008) found the *Fok*I polymorphism to be strongly associated with acute lung injury, predominantly RSV bronchiolitis. We did not find a link with RSV and the *Fok*I polymorphism. This may be due to the assay we used or perhaps there is a different mechanism underlying infectivity in COPD population. Studies suggest (Jurutka 2000, Selvaraj 2004) that although the f allele encodes a less active VDR and may affect the host's ability to use vitamin D for antimicrobial activities or inflammatory regulation, higher circulating vitamin D levels could overcome the hypo functionality. *Taq*I polymorphisms have been linked to infectious diseases, particularly Tb (Wilkinson 2000).

There are several mechanisms by which activated vitamin D binding to the VDR could modulate viral lower respiratory tract disease. These include down regulation of the toll like receptor 4 (Sadeghi 2006) to which RSV binds (Openshaw 2005), suppression of T cell proliferation (Bhalla 1986), TNF α synthesis (Sadeghi 2006) or stimulation of the production of an antimicrobial host protein (Liu 2006).

Another reason we may not have found a relationship is that there is varying evidence in the literature as to what level constitutes vitamin D deficiency. In terms of calcemic

effects, levels below 50nmol/L are probably deficient (Holick 2007, Norman 2001) whereas levels may even need to be lower for parathyroid insufficiency (Lips 2001). In terms of the immunomodulatory mechanisms of vitamin D, it really is not clear as to what level is deficient. It has been suggested that levels > 100nmol/L may even be needed for optimal immune functioning.

In a more recent study, it has been proposed that low levels of vitamin D in autoimmune disorders (systemic lupus erythematosis, rheumatoid arthritis, scleroderma, sarcoidosis, Sjogren's syndrome, autoimmune thyroid disease, psoriasis, ankylosing spondylitis, Reiter's syndrome, type I and II diabetes mellitus, and uveitis) result from chronic infection with intracellular bacteria that dysregulate vitamin D metabolism by causing vitamin D receptor (VDR) dysfunction within phagocytes and that VDR dysfunction causes a decline in innate immune function that causes susceptibility to additional infections that contribute to disease progression (Waterhouse 2009).

8.6 Conclusion

We found that COPD patients have lower vitamin D levels in the winter months than control subjects (even when adjusting for age) and that COPD patients show the same seasonal variation in Vitamin D levels as the general population. We have also shown that low vitamin D levels are associated with a low $FEV_{1.}$ Vitamin D levels were unchanged at exacerbation in COPD when adjustments were made for seasonality and we did not find a relationship between vitamin D deficiency and exacerbation severity, or susceptibility to HRV at exacerbation. Vitamin D supplementation may increase levels significantly but this has no effect on HRV load or susceptibility to HRV exacerbations. We did not find any difference in vitamin D levels with our bacterial culture data at exacerbation. VDR polymorphisms do not appear to play a role in exacerbation frequency.

9.1

Taq1α polymorphisms and exacerbation frequency

9.1.1 Introduction

Alpha 1-antitrypsin (α1AT) is a 394 amino acid, 52-kDalton acute phase glycoprotein produced in the liver and by neutrophils, pulmonary alveolar cells and macrophages. α1AT is important in protecting the lungs from neutrophil elastase, an enzyme that disrupts connective tissue. Normal blood levels of α1AT are 1.5-3.5 g/l making it the most abundant endogenous serine protease inhibitor in the circulation and levels can increase four fold in inflammation (Carrell 1986, Massi 1994).

The gene for α1AT is located on chromosome 14 and contains over 100 variants. Differences in S, Z and M (1-4) phenotypes have been described in relation to COPD, with those with the ZZ phenotype are diseased and SZ phenotype at increased risk. None of the other phenotypes have increased risk of COPD despite differences in serum concentration, indicating there is a threshold below which COPD susceptibility increases. In individuals with SS, MZ and SZ phenotypes, blood levels of α1AT are reduced to between 40 and 60% of normal levels. In ZZ, levels can be as low as 10 – 15% of normal (De Serres 2002). This is usually sufficient to protect the lungs from the

effects of elastase in individuals who do not smoke. However, in COPD, in individuals with the ZZ phenotype, where α1AT levels are less than 15% of normal, patients are likely to develop panacinar emphysema at a young age.

Two RFLPs of the α1AT gene have been reported in association with COPD (Kalsheker 1987, Poller 1990, Kalsheker 1990), arising due to the loss of recognition sites for the restriction enzymes Taq1α and Hind III in the 3' flanking sequence of the α1AT. The first description of the Taq1α polymorphism was by Matteson *et al* in 1985. Individuals with TaqI α 11478G>A polymorphism have normal mass and activity, but an inability to up-regulate α1AT during an acute-phase response. The Taq1α variant has been associated with accelerated progression of atherosclerosis (Talmud 2003), a process associated with degradation of elastic fibres in arterial walls; analogous to the degradation of elastin in lung that results in emphysema. However, α1AT deficiency has not been associated with greater vascular risk, and the c36 fragment of α1AT is pro-inflammatory and has been shown to interact with lipids (Lobritto 1998).

We hypothesised that as exacerbations are acute phase events, individuals with the A allele may not be able to acutely up-regulate α1AT at exacerbation, may have increased elastin degradation in the lung and therefore greater FEV_1 decline and that these individuals are the frequent exacerbators. We also hypothesised that as the A allele disrupts a co-operative binding site that this may also result in suppressed IL-6 responses during acute events.

9.1.2 Aims

1. To investigate whether frequent exacerbators are more likely to have the rare TaqI α 11478G>A allele than infrequent exacerbators and are unable to upregulate α1AT acutely at exacerbation.

2. To investigate whether there is a relationship between IL-6 levels and α1AT levels in frequent and infrequent exacerbators or between genotypes.

9.1.3 Methods

204 patients were studied between 1st April 2006 and 31st March 2009. Sixty five smoking and non-smoking control subjects of similar age but without COPD were also genotyped. The recruitment, sampling and genotyping have been described in ***Chapter 3*****, sections 3.1, 3.6 and 3.12**.

9.1.4 Results

Baseline patient characteristics

Two hundred and four COPD patients were studied; 119 male and 85 female. 65 control subjects were studied; 21 male and 44 female. The patients had a mean FEV_1 of 1.17 l or 48.2 % predicted. The control subjects had a mean FEV_1 of 2.50 l or 99.4% predicted. The baseline characteristics of the cohort are reported in **Table 9.1.1**. There were significant differences in age, smoking history and oxygen saturations between the control subjects and COPD patients (all $p < 0.001$) but not BMI.

Table 9.1.1 Baseline Characteristics

	COPD (n=204)	Control (n=65)	p value
Age (years)	70.7 (11.1)	66.9 (6.6)	p<0.001
FEV1 (l)	1.17 (0.51)	2.50 (0.77)	
FEV1 %	48.2 (19.9)	99.4 (18.6)	
FVC (l)	2.50 (0.93)	3.22 (1.04)	
BMI (kgm-2)	26.0 (5.5)	26.3 (3.8)	p=0.34
Pack Years	51.6 (38.7)	17.4 (22.2)	p<0.001
SpO2 (air)	95 (2)	96 (1)	p<0.001
Male	119 (58%)	21 (32%)	p<0.001
Current Smoker	52 (26%)	12 (18%)	p=0.12
Frequent Exacerbator	69 (34%)	n/a	

Genotype frequencies

All genotype distributions were in Hardy-Weinberg equilibrium. There were no differences in the genotype frequency between the COPD patients and controls, (**Table 9.1.2**) suggesting that the Taq1α variant does not increase the risk of developing COPD. There were also no differences in genotype distribution between COPD frequent and infrequent exacerbators suggesting that the Taq1 variant does not increase susceptibility to exacerbation. These data are reported in Table **9.1.3**. Removing individuals with S

and Z variants made no difference to the analysis and therefore all subjects are included in all the analyses. None of the patients were homozygous for deficiency alleles.

Table 9.1.2 Genotype frequencies control subjects and COPD patients

	COPD (n = 204)	**Controls (n = 65)**	**Chi-squared p**
GG	169 (83%)	56 (86%)	0.35
GA	33 (16%)	8 (12%)	
AA	2 (1%)	1 (2%)	
HWE p value	0.78	0.28	

Table 9.1.3 Genotype frequencies in frequent and infrequent exacerbators

	COPD Frequent Exacerbators (n=69)	**COPD Infrequent Exacerbators (n=135)**	**chi square p=**
GG (wild type)	59 (86%)	110 (82%)	**0.32**
GA	9 (15%)	24 (18%)	
AA	1 (2%)	1 (1%)	
HWE p=	**0.36**	**0.80**	

Demographics and TaqIα genotypes in COPD

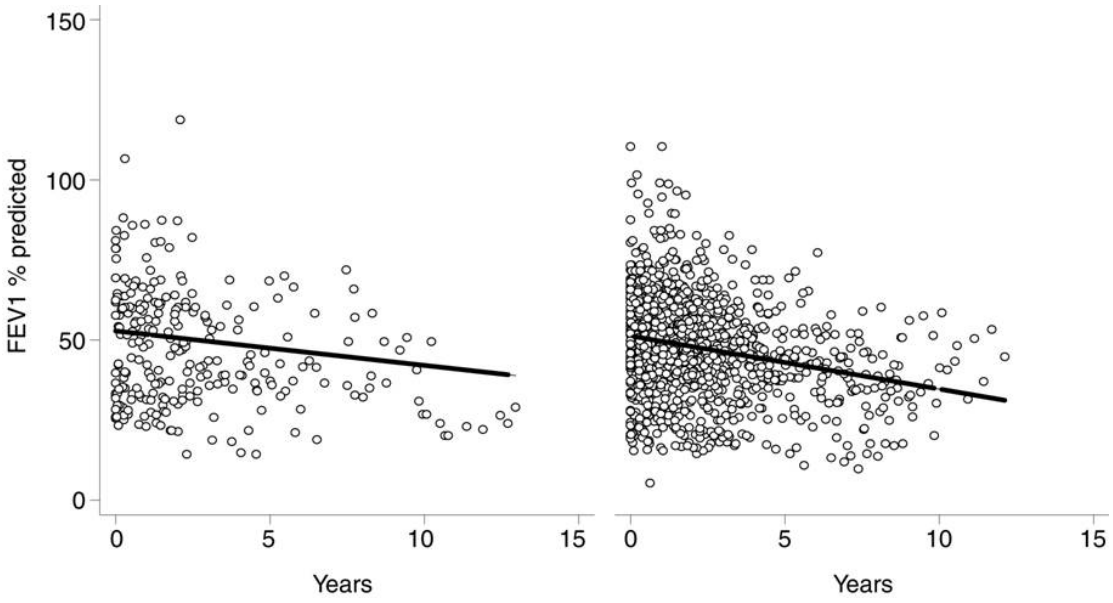
Baseline FEV_1 and FVC were lower in the GG group compared to those with an A allele; p = 0.024 and p = 0.029 respectively. There was no difference in any other baseline characteristics or baseline chronic symptoms between genotypes; all p> 0.05 (**Table 9.1.4**).

Table 9.1.4: Baseline patient characteristics and Taq1 genotype in COPD. Data are expressed as mean (SD) or %.

	GG	**GA**	**AA**	**p=**
FEV_1 (l)	1.14 (0.51)	1.33 (0.49)		0.024
FEV_1 (% predicted)	47.0 (19.5)	54.4 (20.9)		0.069
FVC (l)	2.43 (0.91)	2.82 (1.0)		0.029
FEV_1/FVC	48% (15)	48% (13)		0.974
Smoking (Pack Years)	52.3 (38.1)	49.6 (42.0)		0.457
BMI (kgm^{-2})	26.1 (5.5)	26.0 (5.2)		0.782
Current Smoker	26%	29%		0.735

In addition to having less impaired lung function in this cross-sectional analysis, COPD patients with variant (A) alleles had an attenuated rather than more rapid decline in FEV_1 over time. This is illustrated in **Figure 9.1** (GG (wild type) decline, right panel -1.655 %/year vs. GA/AA decline, left panel -1.066 %/year; p = 0.034). The analysis using absolute FEV_1 (uncorrected for age and height) did not achieve statistical significance (GG decline -38.2 ml/yr vs. GA/AA -26.6 ml/yr; p = 0.077). The model does not include smoking, as smoking status may change with time, but there were no differences in the number of active smokers between the GG and GA/AA groups (**Table 9.1.4**). The FEV_1 (% predicted) analysis is based on 1132 observations in 106 GG subjects, and 253 observations in 22 GA/AA subjects. All FEV_1 values were in the stable state, and not within 7 days of the start or 49 days of the recovery from an exacerbation. The mean length of time in the cohort was 4.5 years.

Figure 9.1.1: Lung function decline is more rapid in Taq1 wild-type (GG) than patients with A alleles (GG decline, right panel -1.655 %/year vs. GA/AA decline, left panel -1.066 %/year; =0.034).



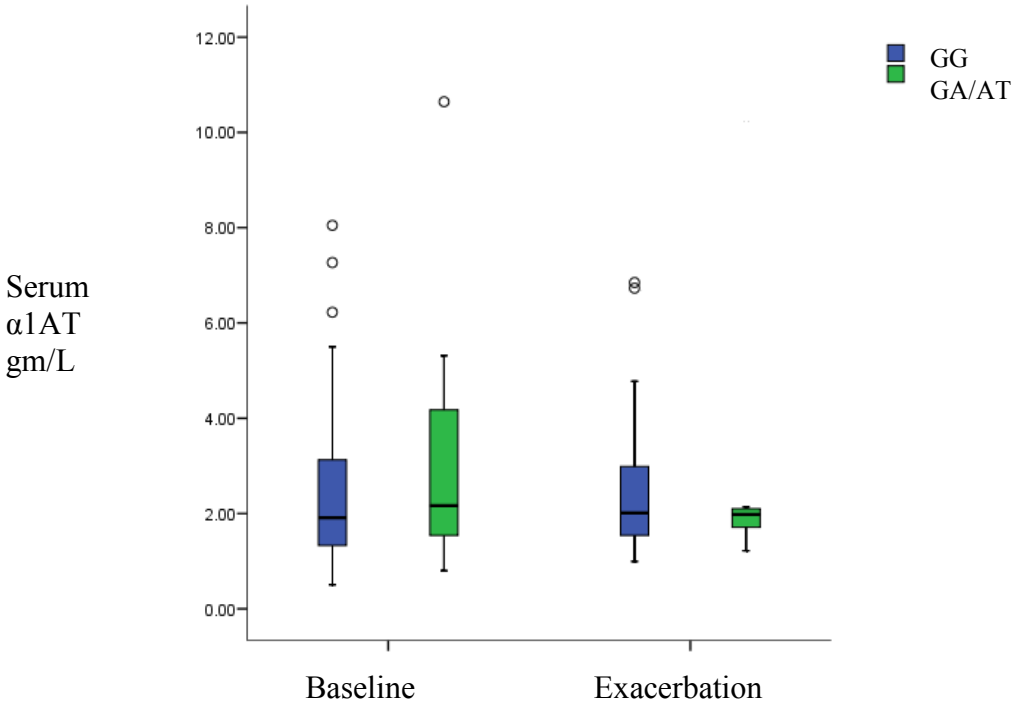
Serum α1AT levels and genotypes

There was no difference in serum α_1-antitrypsin concentration in the stable state, or at exacerbation onset, in patients with COPD by Taq1 genotype. These data are reported in **Table 9.1.5**. **Table 9.1.5** also reports that there was no detectable up-regulation of α_1-antitrypsin at the time of exacerbation in either genotype, and that any change in α_1-antitrypsin between baseline and exacerbation also did not vary by genotype. This is despite significant up-regulation of CRP between baseline and exacerbation in both genotypes, and IL-6 in the wild-type (GG) patients. An increase in IL-6 at exacerbation in patients the A allele did not reach statistical significance.

Table 9.1.5: Baseline and Exacerbation serum α_1-antitrypsin concentration by Taq1 genotype in COPD α_1-antitrypsin expressed as median (IQR) g/l, CRP as mg/l and IL-6 as pg/ml.

	GG	**GA / AA**	p= GG vs. GA/AA
Baseline α_1-antitrypsin	1.91 (1.33-3.21)	2.17 (1.54-4.31)	0.58
Exacerbation α_1-antitrypsin	2.01 (1.54-2.99)	1.98 (1.67-2.12)	0.75
p= (Baseline vs. Exacerbation)	0.87	0.92	
Change in α_1-antitrypsin	-0.07 (-1.24 – 1.17)	-0.09 (-0.60 – 1.00)	0.90
Baseline CRP	4.0 (2.0–7.0)	2.0 (1.0–4.8)	0.09
Exacerbation CRP	9.0 (4.0–26.5)	5.5 (1.3– 74.3)	0.54
p= (Baseline vs. Exacerbation)	<0.001	0.03	
Change in CRP	3.0 (0.0 – 17.5)	2.0 (0.0 – 79.8)	0.90
Baseline IL-6	3.14 (1.60–6.42)	3.03 (0.61–8.23)	0.52
Exacerbation IL-6	5.27 (2.23–13.5)	5.08 (0.28–55.3)	0.67
p= (Baseline vs. Exacerbation)	0.002	0.18	
Change in IL-6	2.03 (-1.2–6.9)	4.4 (-5.0 –58.0)	0.49

Figure 9.1.2: Baseline and exacerbation serum alpha one levels by genotype



Correlations between serum α1-anti-trypsin, IL-6 and CRP

The major stimulus to α_1-antitrypsin (and CRP) release is IL-6. We examined correlations between the baseline serum concentrations of α_1-antitrypsin, IL-6 and CRP by genotype with results that are reported in **Table 9.1.6**. As expected, whilst IL-6 was correlated with α_1-antitrypsin concentrations in the wild-type GG patients, this relationship was not present in those with the A allele Taq1 promoter variant. In both groups there was a significant relationship between CRP and α_1-antitrypsin (which was also present at exacerbation).

Table 9.1.6: Correlations between serum α1-anti-trypsin, IL-6 and CRP by Taq1 genotype in COPD.

	GG		**GA / AA**	
	r=	p=	r=	p=
IL-6 vs. α_1-antitrypsin	0.227	0.029	0.132	0.528
CRP vs. α_1-antitrypsin	0.172	0.050	0.479	0.021

Serum α1-anti-trypsin concentration by exacerbation frequency status in COPD

There were no differences in serum α_1-antitrypsin concentration in the stable state or at exacerbation between frequent and infrequent exacerbators; stable baseline median 2.00 (1.54–3.63) vs. 1.81 (1.32–2.94) g/l and exacerbation 1.94 (1.38–3.03) vs. 2.04 (1.67–2.88) g/l respectively.

There were no differences in symptom duration at exacerbation, change in FEV_1 from baseline to exacerbation, absolute FEV_1 levels at exacerbation, or time to the next exacerbation (TTNE) between the α_1-antitrypsin genotypes. These data are reported in **Table 9.1.7**.

Table 9.1.7: Clinical indices at exacerbation by Taq1 genotype in COPD. Data expressed as mean (SD) or median (IQR). (TTNE= Time to the Next Exacerbation).

	GG	**GA / AA**	**p=**
Exacerbation Onset FEV_1 (l)	1.09 (0.51)	1.19 (0.56)	0.37
Fall in FEV_1 at Exacerbation (l)	0.11 (0.33)	0.09 (0.59)	0.74
Exacerbation Length (days)	12.00 (7.00-17.00)	13.50 (9.25-24.00)	0.21
TTNE (days)	95.50 (41.75-221.00)	117.00 (60.50-322.00)	0.31

9.1.5 Discussion

This study was established to test the hypothesis that patients with the Taq1 11478G>A α_1-antitrypsin promoter variant may not sufficiently up-regulate serum α_1-antitrypsin at exacerbation of COPD and may therefore experience a more rapid decline in lung function due to the unopposed action of neutrophil elastase. We found no difference in Taq α1AT genotypes between the frequent and infrequent exacerbators, with an equal proportion of patients with the A allele in both groups. This suggests that the Taq1 α1AT polymorphism does not play a role in determining exacerbation frequency.

There is increasing evidence that the 'frequent exacerbator' may represent a distinct phenotype, and whilst there has been much interest in COPD susceptibility genes (Matteson 1985, Hodgson 1987), work examining genetic determinants of exacerbation frequency remains limited. It is plausible that deficiencies in innate host responses may underlie a susceptibility to exacerbation such that otherwise trivial infections result in clinically significant events. We have excluded the Taq1 variant as such a susceptibility gene. There was also no evidence that exacerbations were more severe in patients with the Taq1 variant. We hypothesised that carriers of the A allele would mount an insufficient acute-phase anti-protease response during exacerbations. We were not able to find a difference in α1AT levels between genotypes from baseline to exacerbation. We found no relationship with exacerbation severity, or time to the next exacerbation.

We found no difference in baseline and exacerbation α1AT levels between frequent or infrequent exacerbators or between genotypes. We had expected to find that those *without* the A allele would have increased α1AT levels at exacerbation, with little or no up regulation in those with the A allele. In 1979 Stockley *et al* studied 17 COPD patients at exacerbation and 14 at baseline, and found serum α1AT to be significantly higher at exacerbation ($p<0.05$). Most work relates to sputum-serum ratios (Stockley 1979) and these ratios when corrected for albumin show no difference in α1AT levels between baseline and exacerbation. It is known that patients with homozygous (PiZ) α1AT deficiency have low baseline serum α1AT levels and an attenuated acute phase response. These individuals are thought to be particularly susceptible to lung damage during bacterial exacerbations when there is a significant neutrophil influx (Hill 1999). Perhaps with increased power in this study, subdivision of the exacerbations by

causation would illustrate a difference with up-regulation in the different genotypes. Equally disease phenotype as being more emphysematous or chronic bronchitic may influence levels.

It has often been stated (Kohnlein 2008) that α_1-antitrypsin concentrations in serum are up-regulated during acute phase responses (such as exacerbations of COPD). It was not previously known whether the Taq I variant was associated with reduced α_1-antitrypsin at times of heightened inflammation. Our hypothesis was that patients would up-regulate α_1-antitrypsin at exacerbation, but that this response would be attenuated in patients with the Taq1 variant. Our data do not support this hypothesis and, indeed, we were not able to detect up-regulation of α_1-antitrypsin in wild-type patients. There are a number of possible explanations for this. We considered a problem with the α_1-antitrypsin assay but the assay standards performed as expected and our median values were within the expected physiological range for α_1-antitrypsin of 1.5-3.0g/l (Kohnlein 2008). We were able to demonstrate up-regulation of IL-6 and CRP at exacerbation. A significant relationship between CRP and α_1-antitrypsin has been reported previously (Borawski 2003) and the presence of this relationship in our data is supportive that there was indeed no problem with our samples or assay. Our finding that there was no up-regulation of α_1-antitrypsin at exacerbation of COPD therefore seems robust and we have reviewed previous reports in this area. In fact, there is very little published information on serum α_1-antitrypsin concentration at exacerbation of COPD. Sputum:serum α_1-antitrypsin ratio has been shown to fall with exacerbation treatment (Crooks 2000) but we have been unable to locate any reports of paired pre-exacerbation and exacerbation serum α_1-antitrypsin samples. There is data that serum α_1-antitrypsin concentrations may be higher at exacerbation than baseline in two small studies, one of which included patients with α_1-antitrypsin deficiency (Hill 1999), and one in which the samples were not paired (Stockley 1979). There is therefore minimal existing evidence that systemic α_1-antitrypsin concentration is generally up-regulated at exacerbation and our current results challenge the dogma that this is true. Indeed, original data suggesting that α_1-antitrypsin is an acute-phase reactant derives from post-operative patients (Dickson 1974) in whom the inflammatory stimulus may have been much greater than at exacerbation of COPD. It is possible that our exacerbations were too mild to result in up-regulation of serum α_1-antitrypsin. Although all exacerbations were judged to require therapy with antibiotics and/or corticosteroids by the attending

physician, the median increase in CRP was only between 2 and 3mg/l. Alternatively, by sampling exacerbations early in the course of the event, we may not have detected the peak of α_1-antitrypsin release and further work would be required to ascertain the time-course of such responses. A difference may occur due to exacerbation type.

Our data also have relevance to clinical practice in that it may not be necessary (Kohnlein 2008) to delay screening for α_1-antitrypsin deficiency using protein concentration until after exacerbation, and our data would not support a hypothesis that α_1-antitrypsin augmentation at exacerbation would be necessary for carriers of the Taq1 variant.

The history of the 11478G>A promoter variant itself is complex. First described in 1985 (Matheson 1985), a subsequent study of 24 patients suggested a higher prevalence in patients with emphysema compared to controls (Hodgson 1987). A higher prevalence in emphysema was also found in further studies (Kalsheker 1987, Poller 1990) one of which additionally (and paradoxically) reported that the polymorphism was not associated with differential systemic α_1-antitrypsin concentration or function (Kalsheker 1987). Kalsheker *et al* (Kalsheker 1990) found the Taqα polymorphism in 17% of unrelated individuals with COPD compared to 5% controls, with the estimated risk for individuals with the polymorphism developing COPD being 13 fold. It is possible that control subjects develop COPD at a younger age if they carry the A allele. Prevalence studies in COPD patients and controls (Hodgson 1987) screening 101 healthy controls and 24 emphysematous patients showed a disease prevalence of the rare allele of 20.8% compared to 5.0% in controls. However it seems more plausible that the genotype frequencies are the same in the control subjects and COPD patients as this Taqα polymorphism does not affect α1AT levels. Numbers in the previous studies are all small, and this may account for the conflicting results.

It is generally accepted that for an α_1-antitrypsin allele to results in clinical disease, serum levels must be less than 35% of normal values (Kohnlein 2008). We report the largest study to date of the Taq1 variant in COPD and find no increased risk of COPD in patients with the Taq1 mutation, in keeping with a variant that does not affect serum α_1-antitrypsin protein concentration (a finding confirmed in this analysis). That there is no increased risk of COPD in the Taq1 carriers is also in agreement with recent

genome-wide and subsequent meta-analysis of studies examining COPD susceptibility genes (Matteson 1985, Hodgson 1987).

In 1992, the polymorphism was sequenced as a G>A change, and found to occur in a region associated with other regulatory sequences (Morgan 1992). Further work confirmed this as a nuclear-factor binding region, with the mutation associated with decreased promotion of the downstream gene (Morgan 1993). The Taq1 variant was subsequently demonstrated to result in diminished IL-6 induced α_1-antitrypsin responses *in vitro* (Morgan 1997). We provide the first *in vivo* evidence of uncoupling between serum IL-6 and α_1-antitrypsin acute-phase responses in patients with the Taq1 α_1-antitrypsin variant.

There was no difference in serum IL-6 levels between genotypes or any relationship between serum IL-6 and α1AT. In *in vitro* work (Morgan 1997), this Taq1α polymorphism was shown to regulate α1AT response to IL-6. The mutation occurs at a binding site for the ubiquitous transcription factor octamer-1 (Oct-1) and results in a loss of co-operativity between Oct-1 and tissue specific transcription factor NF-IL6. This is a major pathway through which IL-6 mediates its effects, thus providing a mechanism for a decreased IL-6 response. The consequence of the mutation in the 3' enhancer is to blunt the IL-6 induced response and Morgan *et al* hypothesised that individuals with the mutation have a deficiency of α1AT relative to the load of neutrophil elastase and that this is not detectable in the stable state, only at an acute phase response such as exacerbation. IL-6 increases α1AT approximately 3 fold in tissue culture systems (Perlmutter 1989, Kalsheker 1990). More recently, Pott *et al* (2009) showed an enhanced stimulation of cytokine production in individuals with α1AT deficiency. On serial dilution of blood and the addition of cytokine stimulants exogenous α1AT inhibited IL-8, IL-6 and TNF α production. They concluded that α1AT in blood suppresses pro-inflammatory cytokine synthesis and in 9 patients with α1AT deficiency, those deficient had higher IL-6 levels than healthy controls. This supports the hypothesis that frequent exacerbators have decreased α1AT, as they are known to have higher IL-6 levels in the stable state. However we could not find a relationship in this study albeit in the stable state. Previous data has related serum α_1-antitrypsin concentration to other inflammatory markers (including CRP) in patients on haemodialysis (Borawski 2003). Interestingly, in patients with the variant allele, serum

CRP remained correlated with α_1-antitrypsin suggesting there may be additional mechanisms associated with α_1-antitrypsin production in these subjects that would required further study.

We found that COPD patients with the A allele had a higher FEV_1 and FVC in the stable state and a slower rate of FEV_1 decline. This was the antithesis to our hypothesis; that individuals with the A allele would have increased elastin degradation and a faster rate of decline. It appears that the process by which this Taq1 polymorphism influences atherosclerosis is not analogous to lung destruction. Or, perhaps this is related to the fact we did not find decreased α1AT levels in those with A alleles and rather than the genotype being influential in atherosclerosis, it is actually α1AT levels. Equally there may be local factors in the airway that counteract the elastin degredation. Perhaps underlying disease characteristics in terms of whether the process occurring is predominantly chronic bronchitis or emphysema.

Our primary hypothesis arose from the later observation that the 11478G>A polymorphism was associated with accelerated atherosclerosis (Talmud 2003). Atherosclerosis is associated with stiffening of arteries through degradation of elastin fibres in the arterial wall, in a process analogous to the degradation of elastin in airways resulting in emphysema and progressive airflow obstruction. Arterial stiffness is known to occur in COPD, independent of any effect on endothelial or fibrinolytic dysfunction (Matteson 1985, Kalsheker 1987) though the relationship between α_1-antitrypsin deficiency, blood pressure and cardiovascular risk is complex as homozygotes for deficiency alleles have lower blood pressure, and even heterozygotes may be protected from ischaemic events (Matteson 1985, Morgan 1993). Our data do not support the hypothesis that 11478G>A carriers experience more rapid decline in lung function; in fact the converse was true. This is an important finding, but one that requires replication in separate cohorts particularly in view of population data linking FEV_1 and cardiovascular risk (Matteson 1985, Morgan 1992). In addition to exacerbations, factors known to affect the rate of FEV_1 decline in COPD include smoking status (Fletcher 1977), and bacterial colonisation (Wilkinson 2003). We were not able to detect a difference in cigarette smoke exposure or smoking status between wild-type and variant genotypes excluding this as an explanation of our observation.

9.1.6 Conclusion

In conclusion, we were not able to confirm our hypothesis that the Taq1 α_1-antitrypsin promoter variant results in increased decline in lung function through an inability to up-regulate α_1-antitrypsin at exacerbation. Indeed, we found no evidence to support general up-regulation of α_1-antitrypsin during exacerbations, and patients carrying the mutant allele paradoxically had attenuated disease progression. The latter finding is provocative and requires confirmation in separate cohorts. We additionally provide data to show that the Taq1 variant does not increase susceptibility to COPD, or to exacerbation in COPD, and the first *in vivo* data demonstrating uncoupling of IL-6 and α_1-antitrypsin responses in patients with the Taq1 variant. We found no difference in Taq α1AT genotypes between the frequent and infrequent exacerbators, with an equal proportion of patients with the A allele in both groups. This suggests that the Taq1 α1AT polymorphism does not play a role in determining exacerbation frequency.

9.2

Genetic polymorphisms in ICAM-1, IL-6 and IL-8

9.2.1 Introduction

Genetic susceptibility is thought to play a role in development of COPD as only a small proportion of smokers develop COPD. A well known example of this is α1AT deficiency and there have been many subsequent candidate genes investigated as discussed in ***Chapter 1***. In the same way that genetic susceptibility is likely to play a role in disease susceptibility, it is possible that genetic polymorphisms are important in the susceptibility to exacerbations and therefore exacerbation frequency. Certain genetic polymorphisms may play a role in the inflammatory response or susceptibility to infection in COPD. For example, gene polymorphisms in ICAM-1 may affect viral load or susceptibility to viral infection and polymorphisms in the IL-8 and IL-6 genes may influence the inflammatory response in COPD and therefore contribute to frequent exacerbations. Variation in the phenotypic expression of these cytokines is likely to result from differences in the response to basic molecular-cellular interactions which themselves result from genetic variations in the inflammatory response to environmental stimulants.

ICAM-1 polymorphisms

ICAM-1, located on chromosome 19p13.3 is a transmembrane glycoprotein receptor which acts as a ligand for β2 integrin molecules on leukocytes. The gene has 7 exons; 1 signal sequence, 2 – 6 Ig like domains and a 7 – transmembrane region and short tail. Several polymorphisms have been described in the ICAM-1 gene including dinucleotide repeats in the 3' untranslated region and coding changes in the N-terminal domain (K29M), exon 4 (G241R) and 6 (K469E). Both K29M and K469E have a functional effect, however K29M has only been reported in an African population (Craig 2000, Iwao 2001). K469E has been associated with increased soluble (s)ICAM levels. The polymorphism results in a change from lysine (K) to glutamic acid (E) in Ig like domain 5. This affects the interaction between ICAM-1 and its receptor by altering the shape of the binding site. These polymorphisms have been shown to predispose individuals to cerebral malaria (Fernandez-Reyes 1997), increase risk for spontaneous cervical artery dissection (Longoni 2006), adult onset celiac disease (Abel 2006), asthma (Puthothu 2006) and increase susceptibility to diabetic nephropathy (Ma 2006). The K469E polymorphism has also been linked with increased risk of RSV infection (Krueger 2006). *H. influenzae* increases airway epithelial cell ICAM-1 expression and enhances HRV binding (Sajjan 2006). Therefore it is possible that the K469E ICAM-1 polymorphism plays a role in exacerbation frequency in COPD.

IL-8

The IL-8 gene is located on chromosome 4q12-q21 and consists of four exons. IL-8 is a major chemo-attractant and activating factor for neutrophils and has been shown to be upregulated in COPD (McCrea 1994, Keatings 1996), in intubated patients with severe exacerbations (Qui 2003), and frequent exacerbators of COPD have higher levels of IL-8 in their sputum compared to infrequent exacerbators (Bhowmik 2000). Several polymorphisms are described in the IL-8 gene, including a promoter polymorphism -251A>T (alanine to threonine) that increases risk of respiratory syncytial virus bronchiolitis and asthma (Hull 2000, Heinzmann 2004). The -251 has also been associated with nasopharyngeal carcinoma (Nasr 2007). The -251 polymorphism is relative to the transcription start site and modulates binding of transcription factors to the promoter region of IL-8. A second polymorphism at position -781 within the IL-8 promoter results in a C to T (cysteine to threonine) substitution which leads to enhanced binding of transcription factors that regulate IL-8 expression.

IL-6

The IL-6 gene is located on chromosome 7p21. IL-6 is a multifunctional cytokine with a key role in host defence including differentiation or activation of macrophages and T cells and growth and terminal differentiation of B cells. It is not constitutively expressed; rather it is highly inducible and produced in response to numerous inflammatory stimuli including TNF-α, bacterial products (endotoxin) and viral infection (Terry 2000). Many cell types produce IL-6 including monocytes and macrophages, and endothelial cells. Circulating levels of IL-6 are mostly regulated at the level of expression due to the rapid plasma clearance (Castell 1988). As IL-6 is a pro-inflammatory cytokine it has been postulated that certain polymorphisms in the IL-6 gene may contribute to COPD susceptibility.

IL-6 gene polymorphisms have been linked to several respiratory conditions including ARDS, RSV infection, bronchiolitis obliterans syndrome and non-small cell lung cancer. In addition to -174G>C, other functional promoter polymorphisms have also been reported including -597G>A, -572G>C and -373AnTn. Increased levels of IL-6 have been observed in the induced sputum from patients with stable COPD with further increases observed during exacerbations. There appears to be a relationship between the levels of sputum IL-6 and IL-8 and exacerbation frequency. The contribution of IL-6 polymorphisms affecting IL-6 expression to susceptibility and/or progression in COPD and their effect on HRV infection has not been investigated. IL-6 is a potent mediator of inflammation in COPD and levels are increased in serum, exhaled breath condensate and sputum both in the stable state and at exacerbation (Agusti 2003). There are also differences in sputum between frequent and infrequent exacerbators and increased levels predict a faster decline in FEV_1 (Donaldson 2005).

Polymorphisms in the IL-6 gene promoter region mentioned above are associated with higher plasma cytokine levels (Fishman 1998). In juvenile chronic arthritis the reduced frequency of the potentially protective CC genotype is thought to contribute to its pathogenesis. -174 G/C has been associated with increased cardiovascular events and CRP, fibrinogen and hypertension (Humphries 2001, Vickers 2002). -572 G/C has also been related to CRP levels (Ferrari 2003). In COPD, the -174 polymorphism is thought to influence cachexia (Broekhuizen 2005).

9.2.2 Aims

1. To investigate whether the ICAM-1 K469E promoter polymorphism plays a role in exacerbation frequency in COPD.

2. To investigate whether the -251A>T and -781C>T IL-8 polymorphisms play a role in exacerbation frequency in COPD.

3. To investigate whether the -174G>C, -597G>A or -572G>C IL-6 polymorphisms play a role in exacerbation frequency in COPD.

9.2.3 Methods

Detailed methodology can be found in ***Chapter 3*****, sections 3.1, 3.6, 3.7** and **3.12**. Cytokine values for baseline were used on the day of genetic sampling and the exacerbation values the first exacerbation sampled after the baseline.

9.2.4 Results

Baseline demographic data is shown below in **Table 9.2.1**. 293 individuals were studied; 209 COPD patients and 85 control subjects.

Table 9.2.1: (a) Baseline Characteristics of 209 COPD patients: 122 male (58.4%), 54 current smokers (25.8%), 69 frequent exacerbators (33.0%)

	Mean	SD
Age (years)	70.6	11.0
FEV1 (litre)	1.18	0.51
FEV1 (% predicted)	48.1	20.2
FVC (litre)	2.51	0.94
FEV1/FVC	0.48	0.14
BMI (kgm^{-2})	26.1	5.6
Pack years smoking	51.4	38.5
SpO_2 (%) on air	95	2

(b) Baseline characteristics of 85 control subjects: 34 male (40.0%), 18 current smokers (21.2%).

	Mean	SD
Age (years)	66.1	6.8
FEV1 (litre)	2.61	0.77
FEV1 (% predicted)	101.2	17.6
FVC (litre)	3.39	1.05
FEV1/FVC	0.77	0.06
BMI (kgm^{-2})	26.2	3.8
Pack years smoking	17.7	21.2
SpO_2 (%) on air	96	1

All individuals were included in the ICAM-1 analysis. For IL-8 -251A>T all COPD patients and 77 controls were included. The RFLP could not be read in the other controls. For IL-8 -781C>T 205 COPD patients and 78 control subjects were included. All individuals were included in the IL-6 -174C>G analysis but in 3 individuals (2

COPD and 1 control) the sequences for the other 2 IL-6 polymorphisms could not be read.

ICAM-1 polymorphisms

COPD compared to control population

Neither the control population nor the COPD population were within HWE for the ICAM K469E polymorphism (**Table 9.2.2**). Cross-referencing these data with the SNP500 Cancer database also showed failure to meet HWE in 280 control patients so it is likely both groups failed in these data due to small sample sizes therefore data must be interpreted with caution.

Table 9.2.2: ICAM-1 polymorphisms in COPD patients and control subjects

SNP	Genotype	COPD (n = 209)	Controls (n = 84)	**Chi squared P value**
rs5498 ICAM-1 K469E	**KK**	67 (32.1%)	15 (17.9%)	0.03
	KE	116 (55.5%)	60 (71.4%)	
	EE	26 (12.4%)	9 (10.7%)	
HWE p value		0.03	< 0.001	

Frequent and infrequent exacerbators

There was no difference in genotype frequency distributions between the frequent and infrequent exacerbators (**Table 9.2.3**).

Table 9.2.3: ICAM-1 polymorphisms in frequent and infrequent exacerbators

SNP	Genotype	Infrequent exacerbators	Frequent exacerbators	% Frequent Exacerbators	**Chi squared P value**
rs5498 ICAM-1 K469E	**KK**	46	21	31.3%	0.41
	KE	74	42	36.2%	
	EE	20	6	23.1%	
	Total	140	69		

Average cytokine values and polymorphisms

Cytokine values were log transformed and compared using a T test. Values expressed below are log value means and standard deviations. There were no differences in cytokine values in sputum or blood between genotypes (**Table 9.2.4**)

Table 9.2.4: ICAM-1 polymorphisms and cytokine levels.

Genotype	Baseline Sputum ICAM-1	Exacerbation Sputum ICAM-1	Baseline Blood ICAM-1	**Exacerbation Blood ICAM-1**
KK	1.03 (0.48)	1.08 (0.34)	2.80 (0.38)	2.91 (0.13)
KE	0.93 (0.45)	1.14 (0.40)	2.92(0.16)	2.93 (0.11)
EE	0.98 (0.47)	0.92 (0.74)	2.84 (0.22)	2.93 (0.11)
p value	0.70	0.47	0.08	0.86

ICAM-1 polymorphisms and HRV detection at baseline in sputum

93 COPD patients had baseline sputum samples tested for HRV. There were no differences in genotype frequency distribution between those that were HRV positive in sputum and those who were not (**Table 9.2.5**).

Table 9.2.5: ICAM-1 polymorphisms and HRV positivity

Genotype	Never positive	Ever positive	**P value**
KK	7	19	
KE	23	29	0.28
EE	7	8	

IL-8 polymorphisms

The COPD and control populations were within HWE for both IL-8 polymorphisms (**Table 9.2.6**).

Table 9.2.6: IL-8 polymorphisms in COPD patients and controls

SNP	Genotype	COPD (n = 209)	Controls (n = 77)	**Chi squared P value**
rs4073	**AA**	63 (30.1%)	22 (28.6%)	0.84
IL-8 -251A>T	**AT**	106 (50.7%)	42 (54.5%)	
	TT	40 (19.1%)	13 (16.9%)	
HWE p value		0.70	0.35	
		(n= 205)	(n = 78)	
Rs2227306	**CC**	38 (18.5%)	9 (11.5%)	0.34
IL-6 -597G>A	**CT**	109 (52.2%)	43 (55.1%)	
	TT	58 (28.3%)	26 (33.3%)	
HWE p value		0.29	0.16	

Frequent and infrequent exacerbators

There was no difference in genotype frequency distributions between the frequent and infrequent exacerbators (**Table 9.2.7**).

Table 9.2.7: IL-8 polymorphisms in frequent and infrequent exacerbators

SNP	Genotype	Infrequent Exacerbators	Frequent Exacerbators	% frequent Exacerbators	**Chi squared P value**
rs4073	**AA**	42	21	33.3	
IL-8 -251A>T	**AT**	71	35	33.0	0.99
	TT	27	13	32.5	
Totals		140	69		
Rs2227306	**CC**	26	12	31.6	
IL-6 -597G>A	**CT**	76	33	30.3	0.46
	TT	35	23	39.7	
Totals		137	68		

Cytokine values and IL-8 polymorphisms

Cytokine values were log transformed and compared using a T test. Values expressed below are log value means and standard deviations. There were no differences in cytokine values in sputum or blood between genotypes (**Table 9.2.8**).

Table 9.2.8: IL-8 polymorphisms and cytokine levels

Genotype	Baseline Sputum IL-8	Exacerbation Sputum IL-8	Baseline Blood IL-8	**Exacerbation Blood IL-8**
AA	3.36 (0.61)	3.56 (0.29)	-0.31 (0.41)	1.26 (0.28)
AT	3.51 (0.35)	3.56(0.38)	-0.31(0.31)	1.39(0.46)
TT	3.49 (0.45)	3.69 (0.41)	-0.33 (0.44)	1.35 (0.55)
p value	0.40	0.45	0.96	0.63
CC	3.52 (0.47)	3.65 (0.39)	-0.23 (0.29)	1.44 (0.54)
CT	3.49 (0.41)	3.54 (0.35)	-0.32 (0.35)	1.29 (0.46)
TT	3.42 (0.53)	3.66 (0.37)	-0.36 (0.45)	1.37 (0.36)
p value	0.73	0.41	0.42	0.63

IL-6 polymorphisms

All alleles for -597G>A and -572G>C were within HWE and the frequencies of these alleles are shown below. However, in the control group, the alleles for -174C>G were not. Some of the RFLP were checked against sequences where they could be read to ensure this was not due to inadequate digestion in the RFLP. The results are shown in **Table 9.2.9**.

Table 9.2.9: IL-6 polymorphisms in COPD patients and controls

SNP	Genotype	COPD (n = 209)	Controls (n = 85)	**Chi squared P value**
rs1800795 IL-6 -174 C>G	**GG**	82 (39.2%)	31 (36.5%)	0.03
	GC	104 (49.8%)	52 (61.2%)	
	CC	23 (11.0%)	2 (2.4%)	
HWE p value		0.24	0.0004	
			(n = 83)	
rs1800797 IL-6 -597G>A	**GG**	20 (9.6%)	10 (12.0%)	0.54
	GA	97 (46.4%)	39 (47.0%)	
	AA	91 (43.5%)	34 (41.0%)	
HWE p value		0.42	0.82	
rs1800796 IL-6 -572G>C	**GG**	173 (82.8%)	72 (86.7%)	0.80
	GC	31 (14.8%)	10 (12.0%)	
	CC	4 (1.9%)	1 (1.2%)	
HWE p value		0.08	0.35	

There was no difference in IL-6 levels between the COPD patients and control subjects.

COPD patients versus control subjects

There was no difference in the frequency of allele distribution between the COPD patients and the controls with the -597 and -572 polymorphisms. With regards to the -174 genotype, patients who were homozygous CC were far more likely to have COPD and the presence of a G allele appeared to confer a degree of protection against developing COPD.

IL-6 genotypes and exacerbation frequency

There was no difference in genotype distribution between frequent and infrequent exacerbators for any of the IL-6 polymorphisms (**Table 9.2.10**).

Table 9.2.10: IL-6 polymorphisms in frequent and infrequent exacerbators

SNP	Genotype	Infrequent Exacerbators	Frequent Exacerbators	% frequent Exacerbators	**Chi squared P value**
Rs1800796	**CC**	4	0	0	
572 G>C IL-6	**GC**	23	8	25.8	0.21
	GG	112	61	35.3	
Totals		139	69		
Rs1800797	**AA**	16	4	20	
IL-6 -597G>A	**AG**	63	34	35.1	0.42
	GG	4	31	88.6	
Totals		140	69		
Rs1800795	**CC**	17	6	26.0	
-174G>C	**GC**	68	36	34.6	0.73
	GG	55	27	32.9	
Totals		140	69		

Cytokine levels and genotype

There was no difference in sputum or serum IL-6 levels in the COPD patients between polymorphisms of each genotype (**Table 9.2.11**).

Table 9.2.11: IL-6 cytokine levels by genotype.

Genotype	Baseline Sputum IL-6	Exacerbation Sputum IL-6	Baseline Blood IL-6	**Exacerbation Blood IL-6**
-174 G>C				
CC	2.01 (0.88)	2.28 (0.55)	0.61 (0.46)	0.89 (0.68)
GC	2.21 (0.54)	2.28 (0.49)	0.56 (0.43)	0.73 (0.57)
GG	2.01 (0.70)	2.36 (0.50)	0.44 (0.45)	0.82 (0.78)
p value	0.39	0.84	0.24	0.75
-572				
CC	1.88 (0.34)	1.57 (0.50)	0.80 (0.56)	0.70 (0.69)
CG	2.17 (0.87)	2.34 (0.49)	0.40 (0.39)	0.73 (0.50)
GG	2.11 (0.64)	2.32 (0.49)	0.54 (0.45)	0.80 (0.70)
p value	0.79	0.32	0.34	0.92
-597				
AA	1.81 (0.92)	2.43 (0.57)	0.54 (0.53)	0.83 (0.71)
AG	2.19 (0.53)	2.19 (0.50)	0.57 (0.42)	0.69 (0.55)
GG	2.12 (0.69)	2.42 (0.46)	0.48 (0.44)	0.87 (0.76)
P value	0.21	0.19	0.54	0.53

Linkage of polymorphisms in COPD

The -572, -597 and -174 polymorphisms were all significantly related to each other within the COPD population. There was no link between the different polymorphisms in

the different genes, however within genes the presence of the polymorphisms were related; IL-8 $p < 0.001$ and IL-6 $p < 0.001$. This is consistent with published data in other disease.

9.2.5 Discussion

Genotyping studies are difficult to perform, particularly as large sample sizes are needed to confirm differences between polymorphisms and even in studies with thousands of individuals, the same polymorphisms have not always reached significance in all studies. Hence it is unsurprising in some respects that this chapter is negative. It is also complicated by the fact polymorphisms are sometimes linked, adding an increased level of complexity to gene interactions. Additionally, frequent exacerbators make up a relatively small proportion of the COPD population in the first place. Regardless of the sample selection throughout this thesis, they usually constituted 1/3 of the population.

Interpreting the cytokine data is also difficult; not only from a statistical power perspective; other studies have shown differences in cytokine levels with some of the polymorphisms, but even on a given day there is variability in cytokine values. They are influenced by co-morbidities, the presence of infection, and some even by diet or the time of day they are taken. The concept of variability of cytokine levels in the stable state has emerged and the need for multiple measurements to average out these factors will be discussed further in ***Chapter 9.3***. Average values were not used in this chapter as it would have reduced the statistical power even further. Exacerbation sampling adds another level of complexity as exacerbation cytokine levels vary depending upon the causation of the exacerbation; this will be discussed further in ***Chapter 10.2***.

We found no difference in the frequency of distribution of the K469E ICAM-1 polymorphism between the COPD patients and controls or frequent and infrequent exacerbators. There was also no relationship between ICAM-1 levels in sputum or blood between genotypes. The K469E polymorphism has been associated with asthma in one study (Puthothu 2007) and in this study a correlation was found with serum sICAM-1 levels; with those with the KK genotype having lower levels. This was a German population and they used an association analysis using Armitage's trend test in their analysis of the results. Studies regarding ICAM-1 genotypes and RSV infection are conflicting; in a study of German children, no relationship was found (Krueger 2006). We were unable to assess RSV infection on genotypes as the number of patients with RSV detected at baseline or exacerbation was too low, but we did investigate the

presence of HRV at baseline with the ICAM-1 K469E polymorphism and found no relationship.

We found no difference in the frequency of distribution of the IL-8 polymorphisms between the COPD patients and controls or frequent and infrequent exacerbators. There was also no relationship between IL-8 levels in sputum or blood between genotypes. In an Australian study of 1,232 participants, no association was found with the IL8 -251 polymorphism and lung function (Matheson 2006). Several polymorphisms in IL-8 including the one at position -251 of the IL-8 promoter have been shown to be associated with greater IL-8 expression (Hull 2000). A study of the IL8 -251 T >A polymorphism with risk of COPD (Arinir 2005) failed to detect an association or even an association between the polymorphism and respiratory symptoms or lung function.

Hull (2000) identified the IL8-251A allele to be significantly associated with disease severity of respiratory syncytial virus (RSV) bronchiolitis in affected children. Furthermore, the IL-8-251A allele tended to be associated with increased IL-8 production by lipopolysaccharide stimulated whole blood *in vitro*. Recently, Heinzmann (2004) reported an association of IL-*8* gene variations with asthma. They genotyped four polymorphisms (251 A/T, 781 C/T, 1633 C/T, 2767 A/T) in children with asthma, atopic children, and children with juvenile idiopathic arthritis. A significant association with asthma was found for all these polymorphisms. Because of the tight linkage disequilibrium of all four polymorphisms, the authors could not identify the responsible polymorphism for the observed association. A screening of the coding region of the IL-*8* gene did not identify any coding polymorphisms.

We found no difference in the frequency of distribution of the IL-6 polymorphism between the COPD patients and controls or frequent and infrequent exacerbators. There was also no relationship between IL-6 levels in sputum or blood between genotypes. Haplotype linkage indicates complex multiple polymorphisms in promoter region that interact and this could explain why we have not seen any relationship with these data. In other studies the -572C allele has been shown to confer a diminished risk of developing COPD but we did not find that in our population (Cordoba-Lanus 2008). In other disease populations, a relationship has been found with serum IL-6 and these promoter

polymorphisms. It is likely that the effect of genotype on IL-6 levels is lost in COPD as there are multiple factors influencing inflammation and production of IL-6.

Previous studies have identified a functional single nucleotide polymorphism (SNP), -174G/C, in the promoter region of IL-6 (Fishman 1998, He 2009). Only three studies have been published on associations of IL-6 SNPs with COPD. Seifart (2005) reported that there was no association of IL-6 -174 with COPD, Broekhuizen (2005) did not find an association between IL-6 -174 and a cachexia phenotype in subjects with COPD and Córdoba-Lanús (2008) recently reported that IL-6 -572 but not IL-6 -174 was associated with COPD. All three studies have relatively small sample sizes. He (2009) found an association between IL-6 SNPs with decline in FEV_1 but did not find any relationships between IL-6 serum levels and lung function decline.

Studies that have examined the effects of IL-6 SNPs on IL-6 mRNA and protein expression have led to conflicting results. The first reporter gene study demonstrated that a construct containing the -174G allele had higher reporter gene expression in HeLa cells, both under basal conditions and after LPS or IL1 stimulation (Fishman 1998) However, a second reporter gene study showed that a construct containing -174C had higher IL1-induced expression in HeLa cells than that of the -174G construct, although the difference did not reach statistical significance (Terry 2000). By comparison of the two different cell types, the authors concluded that there is a cell type-specific regulation of IL-6 expression. A recent meta-analysis of 5659 subjects from 17 studies concluded that the -174 IL-6 SNP was not associated with circulating IL-6 levels (Huth 2008). There are several explanations for the lack of consistent associations; IL-6 -174G/C polymorphism might not be a strong determinant of serum IL-6 levels, the serum half-life of IL-6 is short and IL-6 levels show marked diurnal variability (Sothern 1995). The blood samples for IL-6 measurement in most studies, including this one, were not taken at a specific time of the day. It is also possible that these SNPs are not actually functional, or functional in each disease.

It is possible that the association is seen between FEV_1 and IL-6 is driven via local pulmonary IL-6 expression or that it is driven by serum IL-6 levels. FEV_1 may reflect the average IL-6 levels and thus the degree of lung inflammation over the years of the study (He 2009). In addition, the SNPs could influence IL-6 levels and thus lung

inflammation during exacerbations but not the constitutive levels during stable periods. IL-6 is a pleiotropic cytokine which also modulates expression of many other genes including α1AT (***Chapter 9.1***) and it may be that it is the effect of the IL-6 variants on these genes that underlie the mechanism for the associations we observed.

Undoubtedly there are genes that play a role in exacerbation susceptibility (Takabatake 2006). However, the interaction between genes and genes and the environment is complex and a single genetic polymorphism is unlikely to hold the key to exacerbation frequency in COPD.

9.2.6 Conclusion

We found no difference in the frequency of distribution of the K469E ICAM-1 polymorphism between the COPD patients and controls or frequent and infrequent exacerbators. There was also no relationship between ICAM-1 levels in sputum or blood between genotypes. We found no difference in the frequency of distribution of the IL-8 polymorphisms between the COPD patients and controls or frequent and infrequent exacerbators. There was also no relationship between IL-8 levels in sputum or blood between genotypes. We found no difference in the frequency of distribution of the IL-6 polymorphism between the COPD patients and controls or frequent and infrequent exacerbators. There was also no relationship between IL-6 levels in sputum or blood between genotypes.

9.3

Baseline Cytokine Variability

9.3.1 Introduction

Frequent exacerbators have a heightened inflammatory response in the stable state compared to infrequent exacerbators. Sputum IL-8 and IL-6 at baseline are higher in frequent than infrequent exacerbators. These cytokine changes have not been consistently shown with blood inflammatory markers. There are also systemic and pulmonary changes in cytokines between baseline and exacerbation. This has been discussed in ***Chapter 1*** of this thesis. The multiple inflammatory changes associated with exacerbations in frequent exacerbators may lead them to have increased baseline sputum or systemic cytokine variability and this in itself may influence exacerbation frequency. This increased variability may also influence susceptibility to virus acquisition in the stable state.

Donaldson *et al* (2005) studied 148 COPD patients over a median of 2.91 years and showed that sputum IL-6 rises by 9pg/ml/year and that frequent exacerbators have a faster increase in sputum IL-6 over time; 29.5 pg/ml/yr. Sputum IL-8 and IL-6 levels are higher in frequent than in infrequent exacerbators in the stable state (Bhowmik 2000) and frequent exacerbators also have a faster rise in plasma fibrinogen (Donaldson 2005) over time. Patients with lower airway bacterial colonisation also have increased airway inflammatory markers and increased exacerbation frequency (Hill 1999, Patel 2002, Wilkinson 2003). Therefore we hypothesised that frequent exacerbators would

have greater baseline sputum and systemic cytokine variability than infrequent exacerbators.

The statistical technique of measuring variability has been used in other studies to assess inter relationships between inflammatory markers both between patients and within the same patient (Sapey 2008). Measurement of variability allows assessment of how "spread out" a group of values is, and can be investigated by several methods; simply by looking at the range of values (i.e. highest to lowest score), the interquartile range or by using more complicated statistical methodology such as variance. Variance is defined as the average squared difference from the scores of the mean and the standard deviation is the square root of the variance. For this work, we have measured variance by using the co-efficient of variation. This is a statistical measure of the dispersion of data points in a data series around the mean and is calculated as the standard deviation divided by the mean. It therefore represents a ratio, and is a useful statistic for comparing the degree of variation from one data series to another, even if the means they are drastically different from each other. This method is not useful when the mean value is near zero, and it cannot be used to construct confidence intervals for the mean.

9.3.2 Aims

1. To investigate baseline cytokine variability in sputum in IL-6, sICAM-1, IL-8 and in blood in CRP, sICAM-1, IL-8 and IL-6 between frequent and infrequent exacerbators.

2. To relate any differences in this variability with HRV susceptibility.

9.3.3 Methods

Sputum IL-6, IL-8 and ICAM-1 and blood IL-6, IL-8, sICAM-1 and CRP were measured at 3 monthly baselines in our cohort in the first year and six monthly thereafter. Patients were included in this analysis if they had at least 3 readings 6 months apart in the stable state. Detailed methodology can be found in ***Chapter 3*****, sections 3.1, 3.6, and 3.7.** There is further statistical discussion surrounding this chapter in ***Chapter 3*****, section 3.12.**

9.3.4 Results

Baseline demographics

Baseline demographic data is shown below in **Table 9.3.1**. 136 COPD patients were included; 78 men and 58 women.

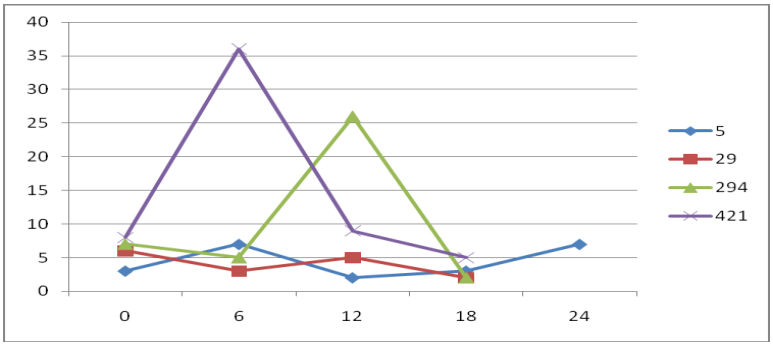
Table 9.3.1: Baseline Characteristics of 136 COPD patients: 78 male (60.94%), 32 current smokers (25.0%), 31 frequent exacerbators (24.2%)

	Mean	SD
Age (years)	71.7	8.3
FEV1 (litre)	1.17	0.51
FEV1 (% predicted)	49.0	18.8
FVC (litre)	2.49	0.90
FEV1/FVC	0.48	0.14
BMI (kgm^{-2})	25.9	5.3
Pack years smoking	51.5	39.7
SpO_2 (%) on air	95	2

Intra-patient cytokine variability

The following graphs (**Figures 9.3.1 to 9.3.5**) give examples of changes in baseline cytokine values over time in both frequent and infrequent exacerbators. Only a few representative examples are given for each cytokine for ease of interpretation. These graphs simply illustrate the complex nature and diversity of cytokine measurement and highlight that measurements even in the stable state are extremely variable.

Figure 9.3.1: Absolute CRP values over time. Subjects 5 and 421 are frequent exacerbators, 29 and 294 are infrequent exacerbators.



Time in months

Figure 9.3.2: Absolute sputum ICAM-1 values over time. Series 1 is a frequent exacerbator and 2 an infrequent exacerbator.

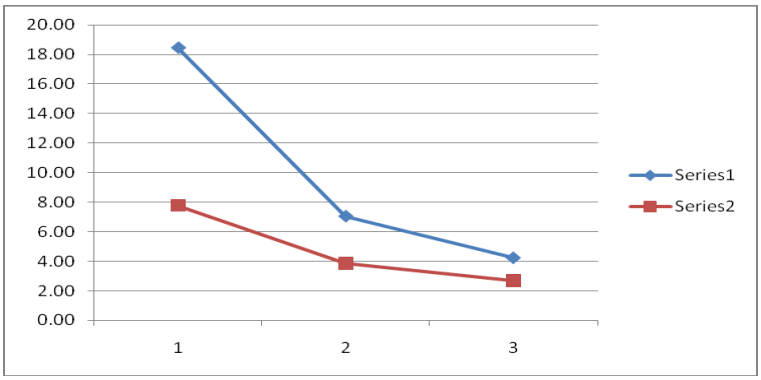


Figure 9.3.3: Absolute sputum IL-6 values over time. Subjects 421 and 3063 are frequent exacerbators and 294 and 678 infrequent exacerbators.

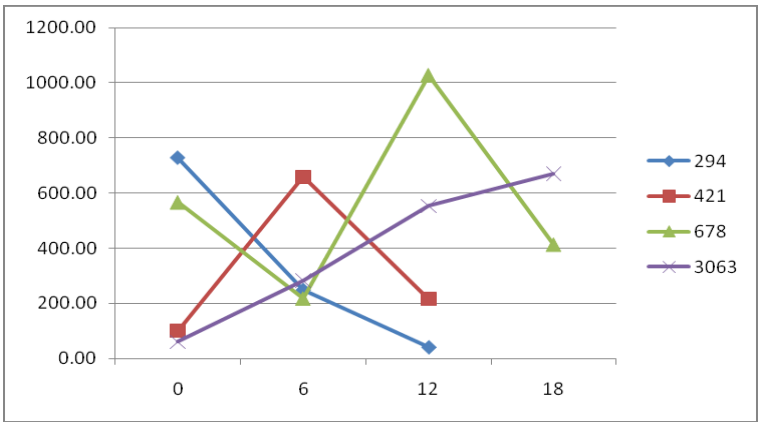


Figure 9.3.4: Absolute serum IL-6 values over time. Subjects 5 and 421 are frequent exacerbators and 29 and 294 infrequent exacerbators.

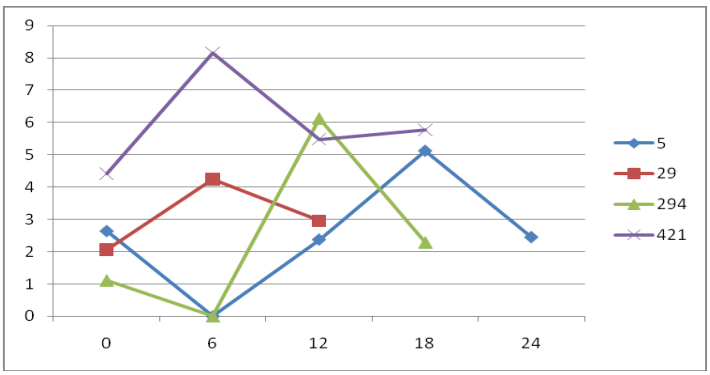
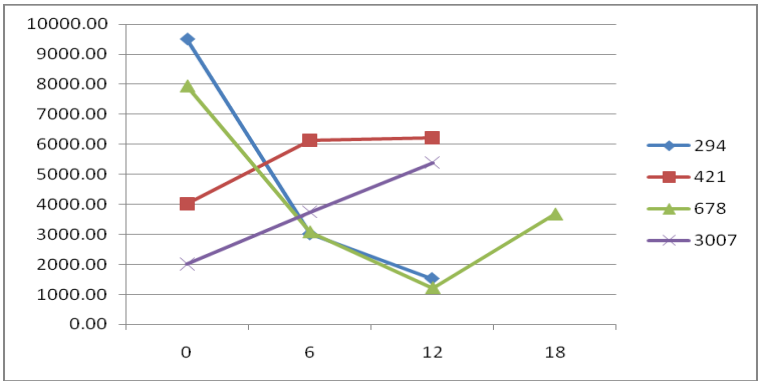


Figure 9.3.5: Absolute sputum IL-8 values over time, Subjects 421 and 3007 are frequent exacerbators and 294 and 678 infrequent exacerbators.

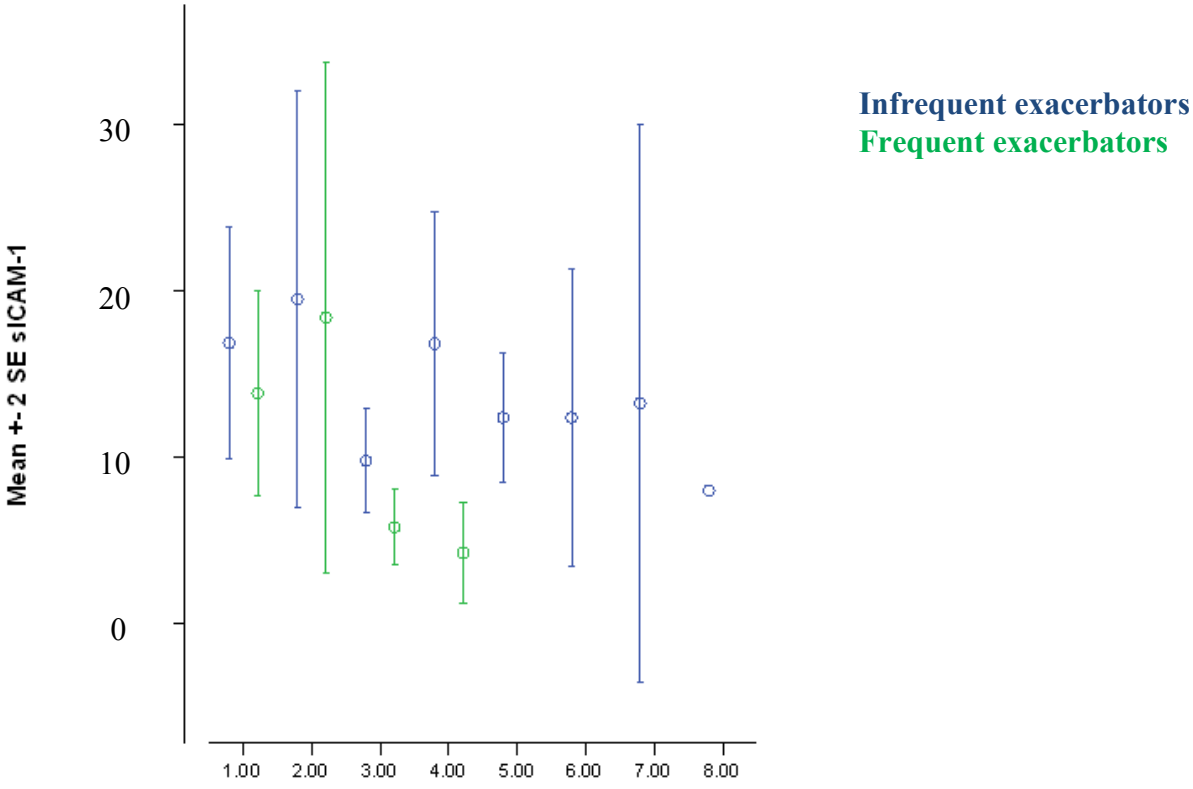
Sputum IL-8 pg/ml



Time in months

The following graphs (**Figures 9.3.6 to 9.3.12**) show the mean +/- 2SEM for each cytokine value at time points 6 months apart. Again, these graphs illustrate clearly the variable nature of the change in cytokine values over time in both frequent and infrequent exacerbators.

Figure 9.3.6: Sputum ICAM-1 mean +/- 2SEM over time in frequent and infrequent exacerbators



Time points at 6 month intervals

Figure 9.3.7: Sputum IL-6 mean +/- 2SEM over time in frequent and infrequent exacerbators

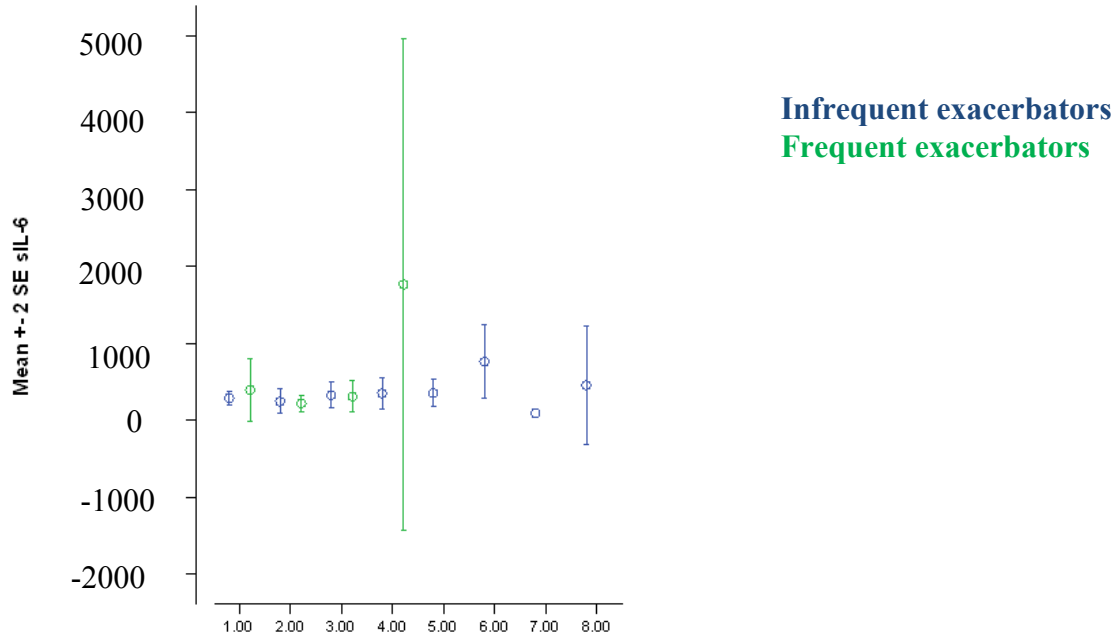


Figure 9.3.8: Sputum IL-8 mean +/- 2SEM over time in frequent and infrequent exacerbators

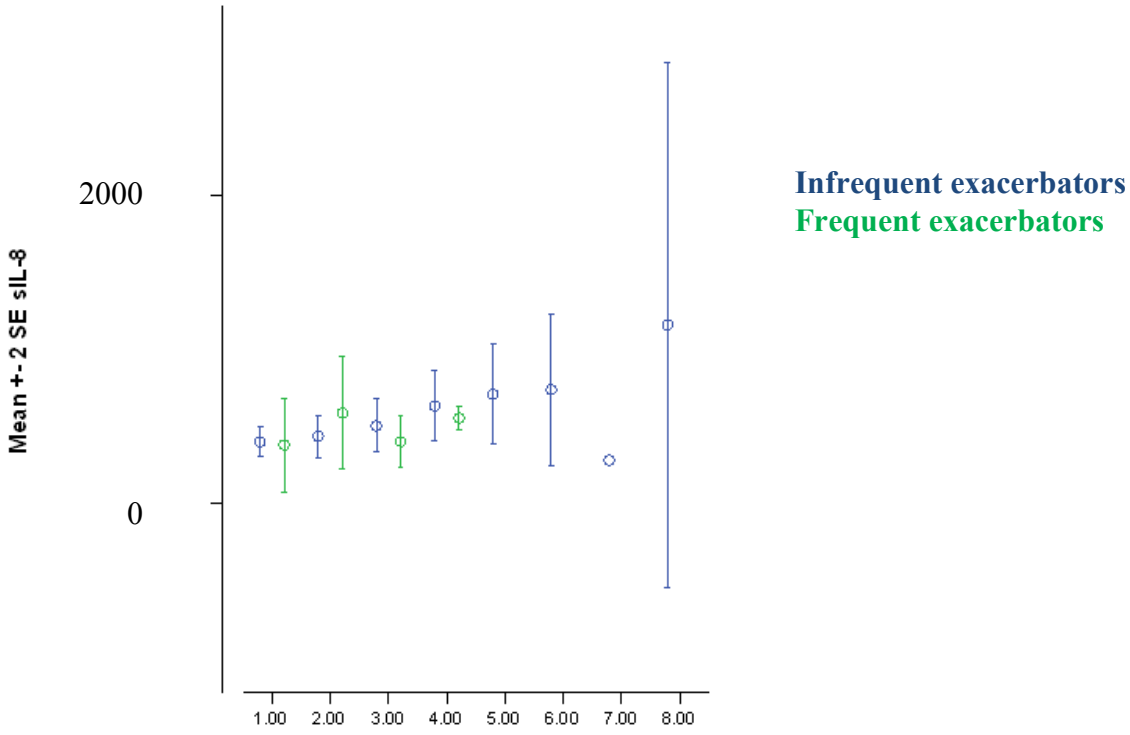


Figure 9.3.9: Serum sICAM-1 mean +/- 2SEM over time in frequent and infrequent exacerbators

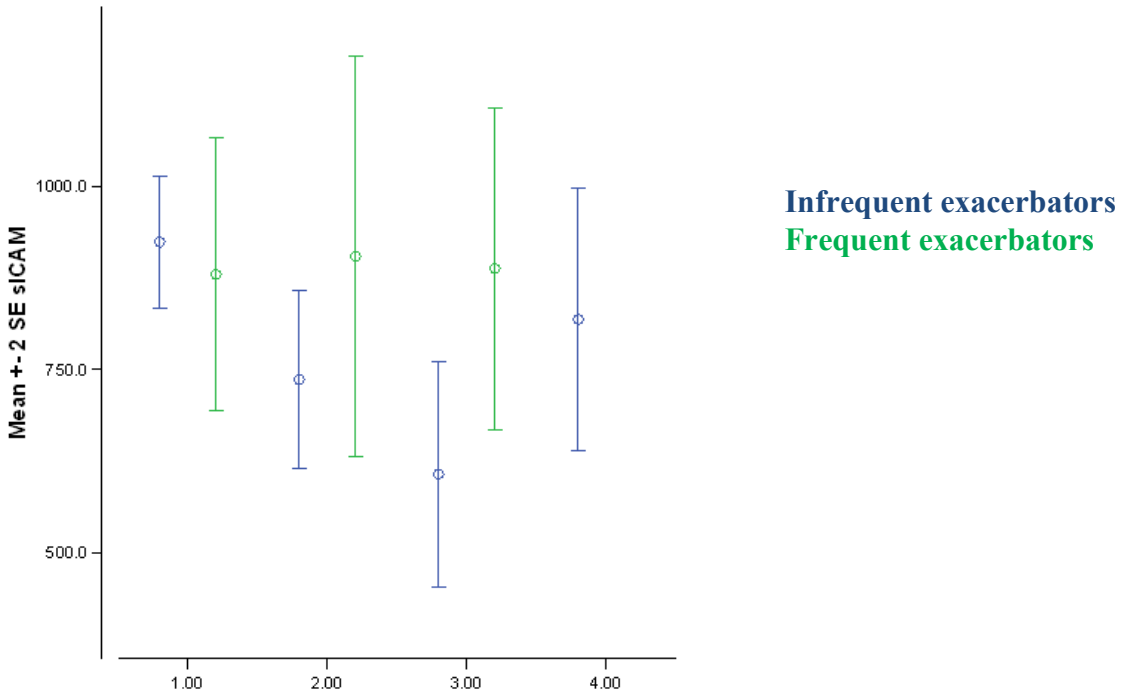


Figure 9.3.10: Serum IL-6 mean +/- 2SEM over time in frequent and infrequent exacerbators

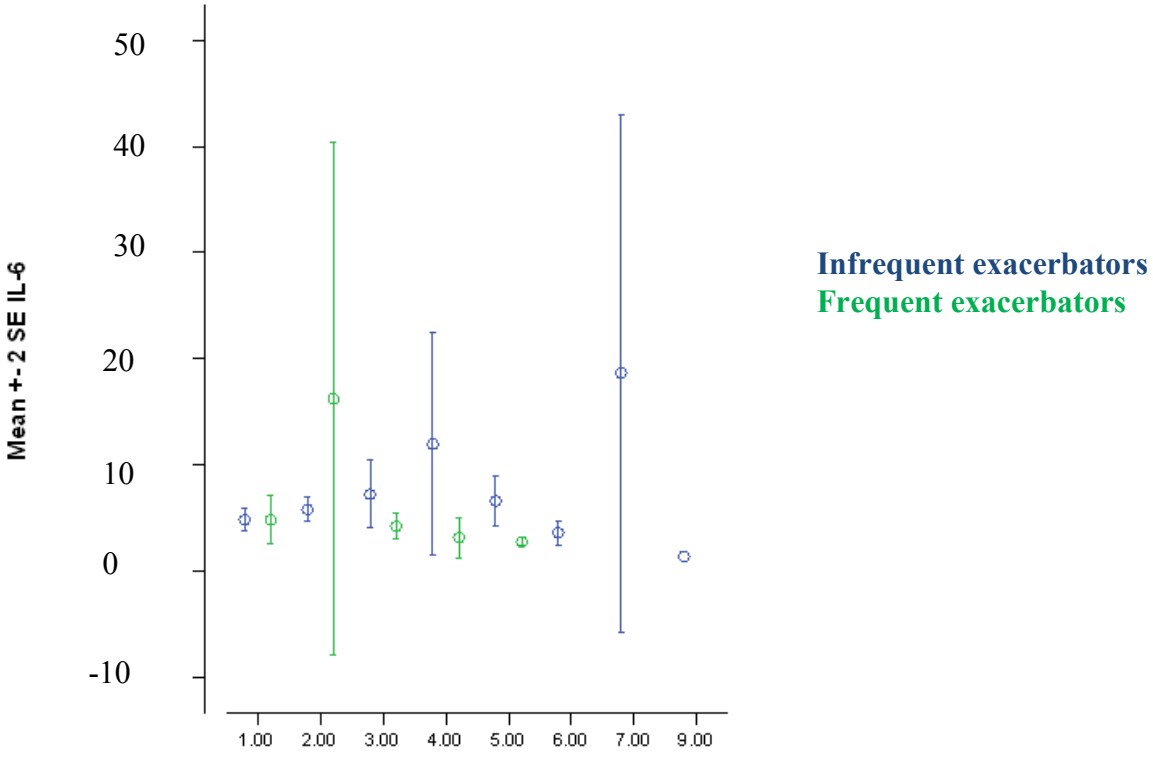
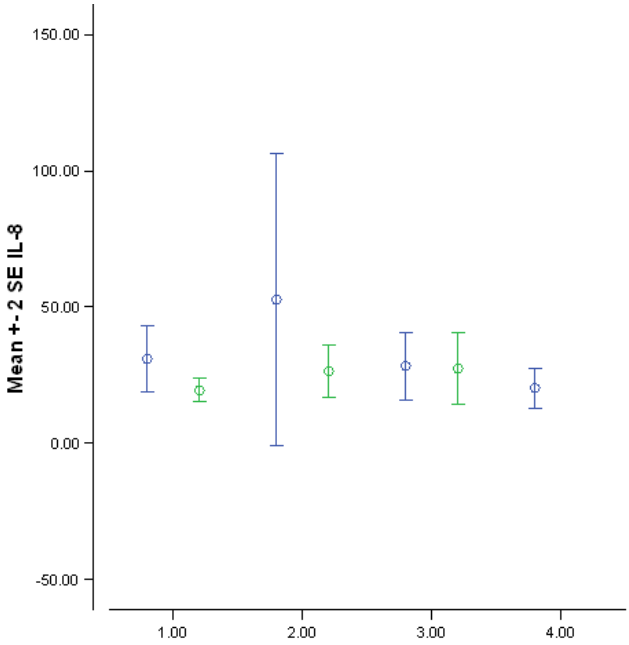
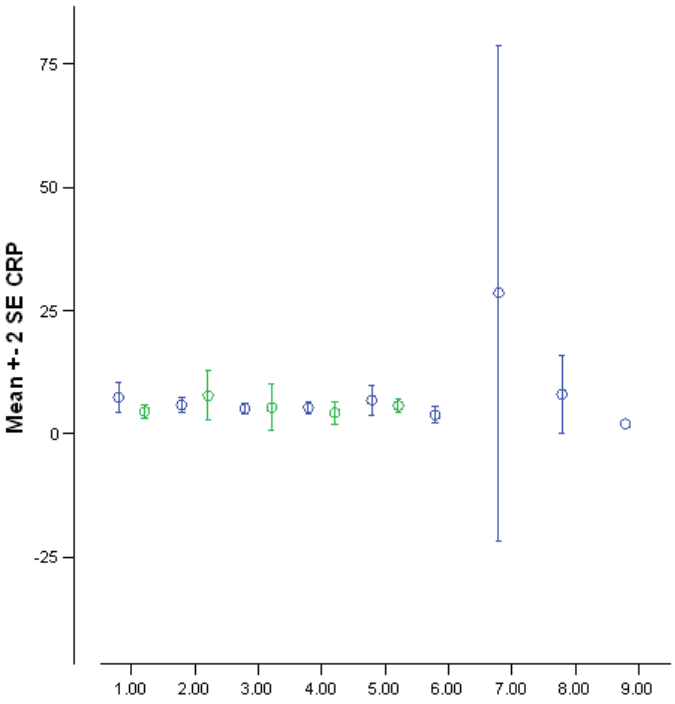


Figure 9.3.11: Serum IL-8 mean +/- 2SEM over time in frequent and infrequent exacerbators



Time in 6 month intervals

Figure 9.3.12: CRP mean +/- 2SEM over time in frequent and infrequent exacerbators



Time in 6 month intervals

Average cytokine values in frequent and infrequent exacerbators

The average log value of each cytokine for each group is given in the table below (**Table 9.3.2**) and the p value corresponds to the difference between frequent and infrequent exacerbators. The number of individuals included in each analysis is given in brackets for each variable.

Table 9.3.2: Average cytokine levels in frequent and infrequent exacerbators

Cytokine	Log average value Infrequent exacerbators	Log average value Frequent exacerbators	p value
Sputum ICAM-1 ng/ml	1.00 (0.32) (n = 48)	0.81 (0.37) (n = 15)	0.05
Sputum IL-6 pg/ml	2.15 (0.49) (n = 55)	2.07 (0.61) (n = 17)	0.58
Sputum IL-8 pg/ml	3.56 (0.49) (n = 53)	3.36 (0.49) (n = 17)	0.11
Blood IL-6 pg/ml	0.52 (0.46) (n = 96)	0.53 (0.37) (n = 32)	0.88
CRP mg/L	0.57 (0.40) (n = 96)	0.54 (0.33) (n = 31)	0.89

Cytokine variability in frequent and infrequent exacerbators

The following table (**Table 9.3.3**) illustrates the variability as measured by the co-efficient of variation for each cytokine for the frequent and infrequent exacerbators. A coefficient of variation was calculated in each patient for each cytokine and these values compared between the 2 groups to give an overall value. This is expressed as the coefficient of variation for each group and the p value represents the relationship between the variability in both groups.

Table 9.3.3: Cytokine co-efficient of variation in frequent and infrequent exacerbators

Cytokine	Infrequent exacerbators	Frequent exacerbators	p value
Sputum ICAM-1 (n = 63)	0.32 (0.24)	0.52 (0.83)	0.42
Sputum IL-6 (n = 72)	0.24 (0.17)	0.19 (0.21)	0.40
Sputum IL-8 (n = 70)	0.08 (0.05)	0.07 (0.04)	0.24
Blood IL-6 (n = 128)	0.53 (2.39)	0.17 (1.26)	0.44
CRP (n = 127)	0.62 (0.52)	0.80 (0.66)	0.26

At baseline individual sputum IL-8, ICAM-1 and IL-6 levels correlated with each other for a given day. None of the sputum markers correlated with the blood markers. Systemic variability was also linked; blood CRP variability followed that of IL-6. Variability of CRP was linked with sputum IL-8. Sputum variability was not linked between cytokines however average values were linked.

Variability and HRV acquisition

A multiple regression allowing for repeated measures (multiple samples per person) which was layered for baseline and exacerbation (data not shown) showed the following. Sputum ICAM-1 increased from baseline to exacerbation ($p = 0.04$) but there was no difference in sputum ICAM-1by HRV positivity. Sputum IL-6 increased from baseline to exacerbation; $p < 0.001$ but there was no difference in sputum IL-6 by HRV positivity. Sputum IL-8 increased from baseline to exacerbation; $p = 0.001$ but there was no difference in sputum IL-8 by HRV positivity. There was no difference in blood ICAM-1 between baseline and exacerbation and no difference by HRV positivity. Blood IL-6 increased from baseline to exacerbation; $p < 0.001$, but there was no difference by HRV positivity. There was no increase in blood IL-8 from baseline to exacerbation and no difference if HRV was detected.

9.3.5 Discussion

The concept of cytokine variability between frequent and infrequent exacerbators is difficult to address as there is so much intra patient variability. The graphs plotted showing the actual cytokine values in individual patients over an 18 to 24 month period illustrate the variable nature of these values even in the stable state. IL-6 concentrations for example, are linked with multiple diseases and concentrations are influenced by many factors including age (Ershler 1993), weight (Malavazos 2007), disease conditions (Cesari 2003, Thakore 2007, Pradhan 2001) and even food intake (Dugue 1998).

Sputum cytokines are likely to be more variable due to the nature of sputum as a medium; it is never expectorated in a constant volume, and can contain inhibitory factors that make processing it difficult. Therefore even the methodology of processing can influence variability; this was discussed in ***Chapter 3***, with particular regard to ELISA kit validation.

We found no difference in sputum or blood cytokine variability between the frequent and infrequent exacerbators groups. However, we did find differences in paired cytokine values between baseline and exacerbation in individual patients, consistent with previous work, suggesting that the power of this study is adequate but the issue of variability itself is difficult to prove. It is difficult to sample frequent exacerbators in the stable state at multiple baselines for obvious reasons.

Several other statistical methodologies were considered when analysing this work. We could have compared the average-area-under-the-curve-minus-baseline changes in cytokine levels over the two years of study - this would have taken into account the different numbers of observations stemming from each individual. Or, we could have compared the mean cytokine levels over time using a t-test or non-parametric test depending on the distribution of the data or performed a generalized linear model with generalized estimating equations. This would have given the mean difference in cytokine levels between patients with an exacerbation and those without an exacerbation, averaged over all time points. But of course exacerbations occur throughout the period of study, exacerbation frequency was prospectively determined

and this method would only have provided information on what was happening in an individual patient. Other options included using a random effects model (an extension of an ANOVA) with a fixed slope and to evaluate the variation from this slope, although this does not give a quantitative description of the difference in variation between the groups of interest. Another possibility would have been to fit a standard ANOVA on the two or three groups of interest, performing tests at 6-monthly intervals, (baseline, 6-months, 1-year, 18 months and at two years) considering a p-value less than 0.01 to be significant (Bonferonni correction). However, this technique does not provide a quantitative picture of the variation in each of the groups and so does not take into account the individual variation over time. We felt that the best approach to assess variability of cytokine at baseline would be to calculate the maximum and minimum values of each cytokine for each individual over the 2 year follow up period, subtract the minimum value from the maximum value and get the range of values for each individual, then to perform some simple non-parametric tests (i.e. Kruskal Wallis) on these changes/ranges (either between the three groups or between the two groups) to give a crude comparison of the variation over time. However as it did not account for different numbers of follow up visits from different patients we decided to measure the coefficient of variation.

Using the coefficient of variation to assess the variance has been used in other work (Sapey 2008). Sapey *et al* investigated interrelationships between inflammatory markers in patients with bronchitis and measured inter and intra patient variability and to minimise intra-patient variability used a 3 day rolling mean. They found marked variability in inflammatory biomarkers between patients from a single day. Using 3 day rolling means did not alter inter-patient variability suggesting it relates to individual differences in the degree of airway inflammation. This is in agreement with other studies (Biernacki 2003, Rutgers 2000). They also did not find a change in symptom scores even with daily fluctuations in cytokines. Sapey also showed that using mean results for each patient demonstrated clear relationships with inflammatory markers and markers of disease such as FEV_1.

The concept of index of individuality (II) to assess inter and intra variability of cytokines was introduced (Harris 1979) to assess variations in IL-6. This is the ratio of

the total within subject variation to between subject variation. Of course this method does not take changes over time into account. A low II (< 0.6) means the cytokine has marked individuality and a high II (>1.4) indicates little individuality. Ideally the analyte should have a high II making population based references useful in a clinical setting. There are few studies examining cytokine variability between individuals. Therefore even in well adults little is known about day to day variation, and when investigating the effect of a disease on top of that, single measurements are unlikely to be helpful. A paper in Cytokine (Picotte 2009) assessed inter and intra variability of plasma IL-6 concentration in an adult population. Picotte *et al* found significant inter variability but not intra variability. The index of individuality was 0.20 and standard error of the mean (SEM) 0.16 pg/ml. They stated that an II of 0.20 demonstrates a need to carefully evaluate changes in plasma IL-6 concentration instead of using population based reference norms. They argued that in an older adult population values could be considered normal until differences exceed 0.32 pg/ml. It is expected that variability between subjects should be greater than that within subjects. The study by Picotte concluded that evaluation of serial changes within an individual maybe more meaningful in detecting changes in health status than single values compared to population based reference ranges. In this study IL-6 was measured daily for 6 days of course this is not practical in the long term. In another study IL-6 was measured at 12 and 24 months and the interclass correlation coefficient was found to be 0.48. Cava *et al* (Cava 2000) studied IL-6 concentrations monthly for 6 months and although determined an II of 1.4, the reliability coefficient was low and they concluded more than one sample should be taken to determine an individual's true IL-6 level. This certainly provides thought for future studies in longitudinal cohorts and can likely be applied to the other inflammatory markers.

We did not find a link between cytokine variability and HRV acquisition. If an individual has a subclinical infection; with low levels of virus, it is possible cytokine patterns are minimally influenced at this time, if at all. Changes in medication throughout the study period and even changes in medication doses may have an effect on variability.

9.3.6 Conclusion

The concept of cytokine variability between frequent and infrequent exacerbators is difficult to address as there is so much intra patient variability. We found no difference in sputum or blood cytokine variability between the frequent and infrequent exacerbators groups. However, we did find differences in paired cytokine values between baseline and exacerbation in individual patients, consistent with previous work, suggesting that the power of this study is adequate but the issue of variability itself is difficult to prove. This certainly provides thought for future studies in longitudinal cohorts and can likely be applied to the other inflammatory markers. We did not find a link between cytokine variability and HRV acquisition.

10.1

HRV prevalence in the stable state and at exacerbation

10.1.1 Introduction

Approximately half of COPD exacerbations are associated with viral infections, the majority of which are due to rhinovirus (Hurst 2005, Rohde 2003, Seemungal 2000(b), Seemungal 2001). It is possible that airway and systemic responses to viral infections are greater in frequent exacerbators, and this may explain why they have more exacerbations. It is also possible they are more susceptible to virus in the stable state, and have a higher baseline inflammatory response at this time making them more susceptible to further infection. Patients with a history of frequent exacerbations have an increased chance of acquiring an upper airway cold (1.73 colds/year), compared to infrequent exacerbators (0.91 colds/year; p=0.003) (Hurst 2005). Frequent exacerbators may therefore be more susceptible particularly to HRV in the stable state and this may lower their threshold for development of exacerbations.

Viruses and exacerbations of COPD

As many as 78% of hospitalised COPD exacerbations have a virus and /or bacteria detected; 30% bacterial, 23% viral and 25% co-infection (Papi 2006). Exacerbations caused by viral infection are more likely to lead to hospitalisation and viral infection has been identified in up to 47% of COPD patients with very severe exacerbations requiring intubation and mechanical ventilation (Qui 2003). Viral exacerbations are more common in the winter months when temperatures are colder (Donaldson 1999) and when there are more respiratory viral infections present in the community. The major viruses associated with COPD exacerbations include HRV, coronavirus, influneza A and B, parainfluenza, adenovirus and respiratory syncytial virus (RSV). Human metapneumovirus has also been found at exacerbation in some studies. This has been discussed in more detail in ***Chapter 1***.

10.1.2 Aims

1.To investigate differences in virus prevalence in stable COPD between frequent and infrequent exacerbators.

2.To investigate differences in HRV load in the stable state and at exacerbation between frequent and infrequent exacerbators.

10.1.3 Methods

One hundred and sixty six patients were studied between 1st April 2006 and 31st May 2009. Seventy smoking and non-smoking control subjects of similar age but without COPD were recruited from a primary care practice. Detailed methodology can be found in ***Chapter 3*** **(sections 3.1, 3.6, 3.8, 3.9 and 3.12)**.

10.1. 4 Results

Qualitative Results

In preliminary qualitative experiments 204 patients have had samples analysed for viruses; 280 nasopharyngeal swabs and 147 sputum samples. 285 of the samples were collected at baseline, 128 at an exacerbation and 14 at the time of a cold. Of the kRas and PDV positive samples (internal controls) 34/ 383 were positive at PCR for rhinovirus, 3/423 for Flu A, 0/426 for Flu B, 2/424 for parainfluenzae virus, 0/424 for adenovirus, 4/422 for human metapneumovirus and 3/422 for RSV. 20 of the viruses were detected at baseline and 26 at exacerbation.

Analysis of the data using the chi squared test showed the following;

- In the stable state, there was no difference in the detection of viruses between frequent and infrequent exacerbators. 11/126 infrequent and 8/68 frequent exacerbators tested positive for a virus in the stable state.

- At exacerbation, any virus was detected in the sputum of 9/16 infrequent and 7/37 frequent exacerbators ($p = 0.007$) and by nasopharyngeal swab (NPS) in 5/21 infrequent and 4/48 frequent exacerbators.

- At exacerbation, human rhinovirus was detected in the sputum of 4/16 infrequent and 4/37 frequent exacerbators, and by NPS in 4/21 infrequent and 2/48 frequent exacerbators ($p = 0.04$)

In view of this we decided to focus specifically on HRV using HRV primers designed to detect as many serotypes of HRV as possible and with a sensitive PCR cut off.

Quantitative virology; HRV

Baseline patient characteristics

One hundred and sixty six COPD patients were studied; 100 male and 66 female. The baseline characteristics of the cohort are reported in **Table 10.1.1**. The patients had a mean FEV_1 of 1.23 l or 50.6 % predicted. 72 control subjects were studied; 27 male and 43 female. The control subjects had a mean FEV_1 of 2.66 l or 104.0% predicted. There were significant differences in age, lung function, smoking history and oxygen saturations between the control subjects and COPD patients (all $p < 0.001$).

Table 10.1.1: Baseline Characteristics of 166 COPD patients: 100 male (60.2%), 52 current smokers (31.3%), 55 frequent exacerbators (33.1%). 72 control subjects: 29 male (40.3%), 16 current smokers (22.2%).

	COPD patients Mean (SD)	**Control subjects Mean (SD)**
Age (years)	69.6 (8.6)	66.1 (6.8)
FEV1 (litre)	1.23 (0.48)	2.66 (0.76)
FEV1 (% predicted)	50.6 (17.8)	104.0 (16.0)
FVC (litre)	2.63 (0.88)	3.46 (1.05)
FEV1 / FVC	0.48 (0.13)	0.78 (0.06)

Baseline COPD patients and control subjects

A higher percentage of nasal swabs at baseline were positive in controls (29/86; 33.7%) than in COPD patients 48/237; 20.3% (p=0.01). However, there was no significant difference in viral load in control subjects; median 2.30 (1.21 – 7.09) compared to the COPD patients; median 1.84 (1.21 – 3.8); p = 0.35.

Comparison of sputum and NPS for viral detection and load

56/93 (60.2%) patients were positive at baseline for HRV in sputum at least once. 71/180 (39.4%) sputum samples were HRV positive at baseline (15 multiple positive). 46/166 (27.7%) patients were positive for HRV in a NPS sample at least once at baseline (total of 48/237(20.3%) positive samples). 6 patients were positive in NPS and sputum at the same time. Sputum and NPS viral load did not correlate with each other.

There was no relationship between ever being positive for HRV in sputum and ever being positive in NPS.

Baseline characteristics and HRV positivity and load at baseline

Patients were more likely to have HRV ever detected at baseline either in sputum or by NPS if they self reported sputum production ≥ most days a week (i.e. had a diagnosis of chronic bronchitis) ($p = 0.007$). 62.4% of chronic sputum producers had at least one baseline positive for HRV compared to 55% of those who did not chronically produce sputum (**Table 10.1.2**).

Table 10.1.2: Chronic bronchitis and HRV detection

	Not chronic sputum producer	Chronic sputum producer	Total
Never positive HRV baseline	36	32	68
At least one positive HRV Baseline	44	53	97
TOTAL	80	85	165

There was no relationship between baseline positivity in NPS or sputum and symptoms of chronic dyspnoea, wheeze, or cough. The proportion of patients with daily symptoms is given in Table **10.1.3**.

Table 10.1.3: Percentage of COPD patients with daily symptoms

Symptom:	*Number*	*Percentage*
Shortness of breath	62/164	37.8%
Wheeze	41/164	25%
Cough	85/165	51.5%
Sputum	88/165	53.3%

Table 10.1.4: Percentage of Control subjects with daily symptoms:

Symptom	*Number*	*Percentage*
Shortness of breath	3/72	4.2%
Wheeze	0/72	0%
Cough	6/72	8.3%
Sputum	3/72	4.2%

When detection of HRV was separated by NPS or sputum, HRV positivity was higher in ex-smokers in sputum: 67.2% vs. 46.9%; (p = 0.04) (**Table 10.1.5**)

Table 10.1.5: Smoking status and HRV detection

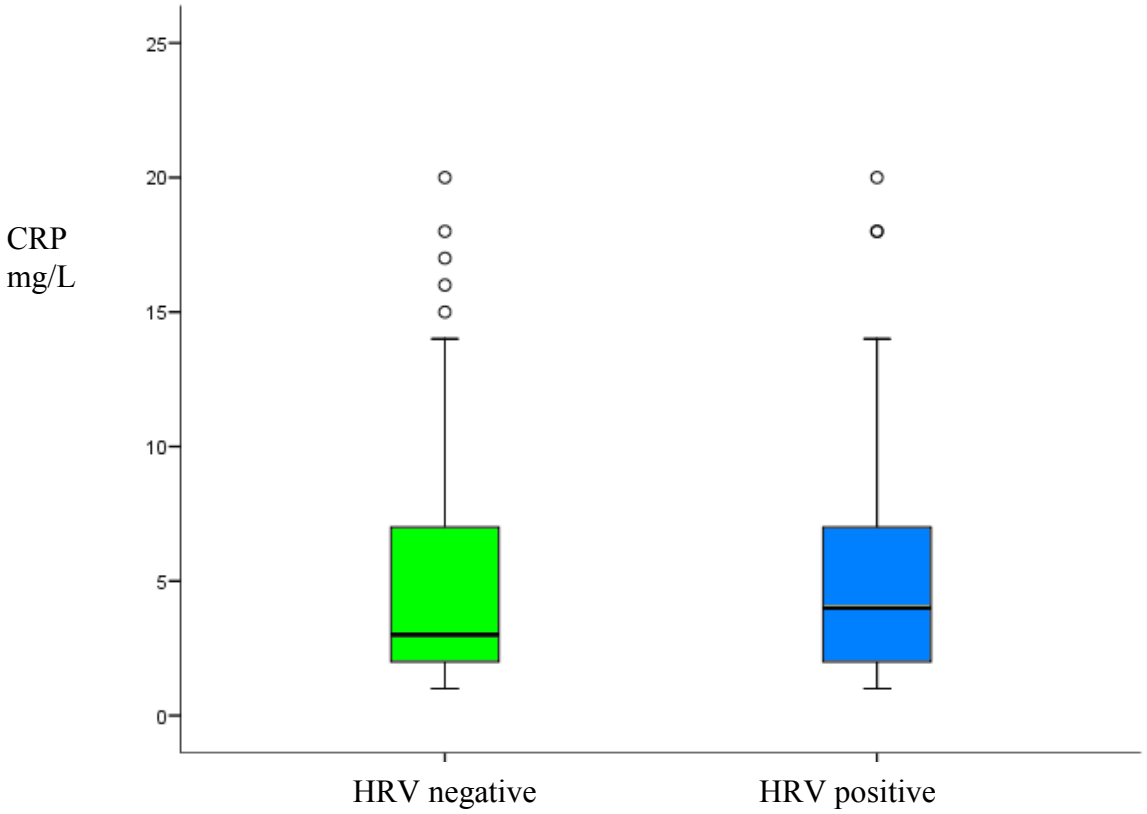
	Ex smoker	Current smoker	Total
Never positive HRV baseline In sputum	20	17	37
At least one positive HRV Baseline in sputum	41	15	56
Total	61	32	93

There was no relationship between viral load in NPS or sputum and the presence of nasal symptoms; runny nose, post nasal drip, blocked nose, sneezing, or anosmia.

Inflammatory markers and baseline positivity for HRV

There was no difference in CRP levels at baseline between those that were positive for HRV by NPS or sputum and those that were negative; p = 0.89. This is shown in **Figure 10.1.2.** There was also no relationship with viral load detected by NPS or sputum and CRP.

Figure 10.1.2: Graph showing serum CRP levels in those patients positive and negative for HRV in sputum at baseline

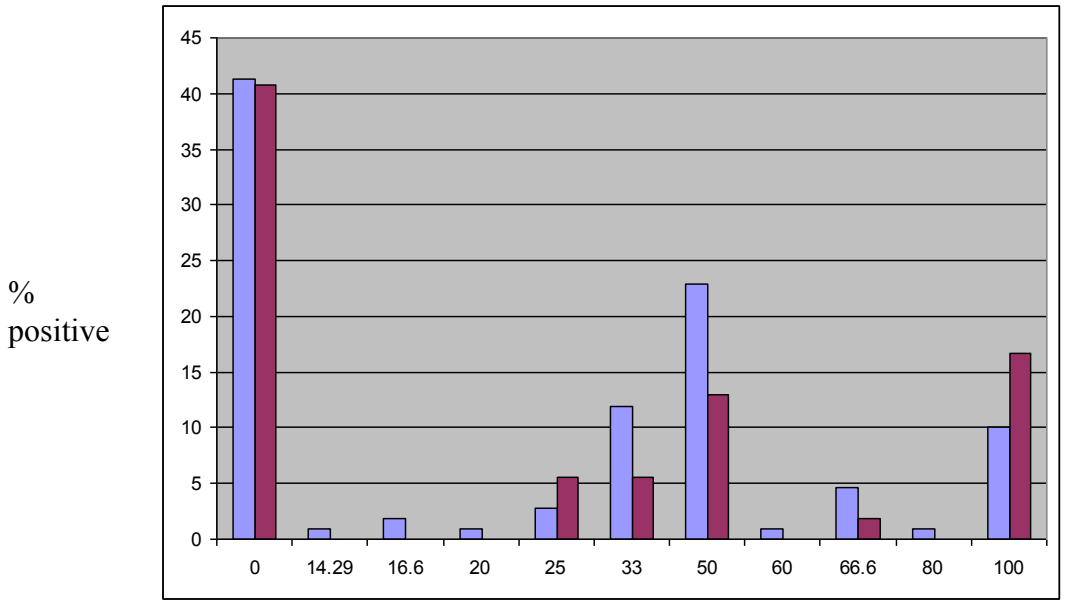


Exacerbation frequency and viral detection and load in COPD patients

Over the period of study, 97 of the 166 patients (58.4%) had at least one positive baseline for HRV (cut off of 1 pfu/ml). 65 of the infrequent exacerbators had at least 1 positive baseline (58.6%) and 32 of the frequent exacerbators (58.2%).

There was no relationship between HRV positivity and exacerbation frequency when splitting HRV detection by NPS and sputum separately. There was no relationship between the % of baseline samples that were positive for HRV and exacerbation frequency. This is illustrated graphically in **Figure 10.1.3**.

Figure 10.1.3: Graph showing the percentage of frequent and infrequent exacerbators and the percentage of HRV positive sputum samples at baseline



In a regression analysis with the number of positive baseline samples as the dependent and the total number of baseline samples as the independent variable, there was no relationship with exacerbation frequency.

HRV and bacterial detection at baseline

There was no relationship between positivity at baseline with HRV either in sputum or NPS and bacterial detection at exacerbation. However, only 7 exacerbations were positive for a PPM.

HRV detection at exacerbation and positivity at baseline

The detection of HRV at exacerbation was independent of the detection of HRV at baseline and exacerbation frequency. In a regression analysis with the number of HRV positive exacerbations as the dependent variable and the number of positive baseline samples and exacerbation frequency as the independent variables, there was no significant relationship. This is shown in **Table 10.1.6**.

Table 10.1.6: HRV positivity at baseline and exacerbation

	Never positive at exacerbation	Ever positive at exacerbation	Total
Never positive at baseline	11	17	28
Ever positive at baseline	20	36	56
Total	31	53	84

	Infrequent Exacerbators	Frequent exacerbator	Total
Never positive at baseline	44	23	67
Ever positive at baseline	65	31	96
Total	109	54	163

Persistent detection of HRV at baseline

There were 29 patients who were tested 3 or more times for the presence of HRV in their sputum over the course of the 2 years. Those individuals who tested positive for HRV in over 50% of their baseline samples were no more likely to be positive for HRV at exacerbation. There was no difference in exacerbation frequency, bacterial colonisation, or symptoms between the two groups. Those that were persistently positive for HRV were more likely to be ex-smokers 78.1% vs. 21.9% ($p = 0.02$). There was a trend towards a lower FEV_1 % predicted in those who were colonised ($p = 0.06$).

HRV load from baseline to exacerbation

HRV load increased significantly from baseline to exacerbation in both NPS and sputum samples ($p < 0.001$ for both) (**Figure 10.1.4 (a) and (b)**. These changes have been further categorised in the next chapter (***Chapter 10.2***).

Figure 10.1.4 (a): Graph showing the difference in sputum viral load between exacerbation and baseline

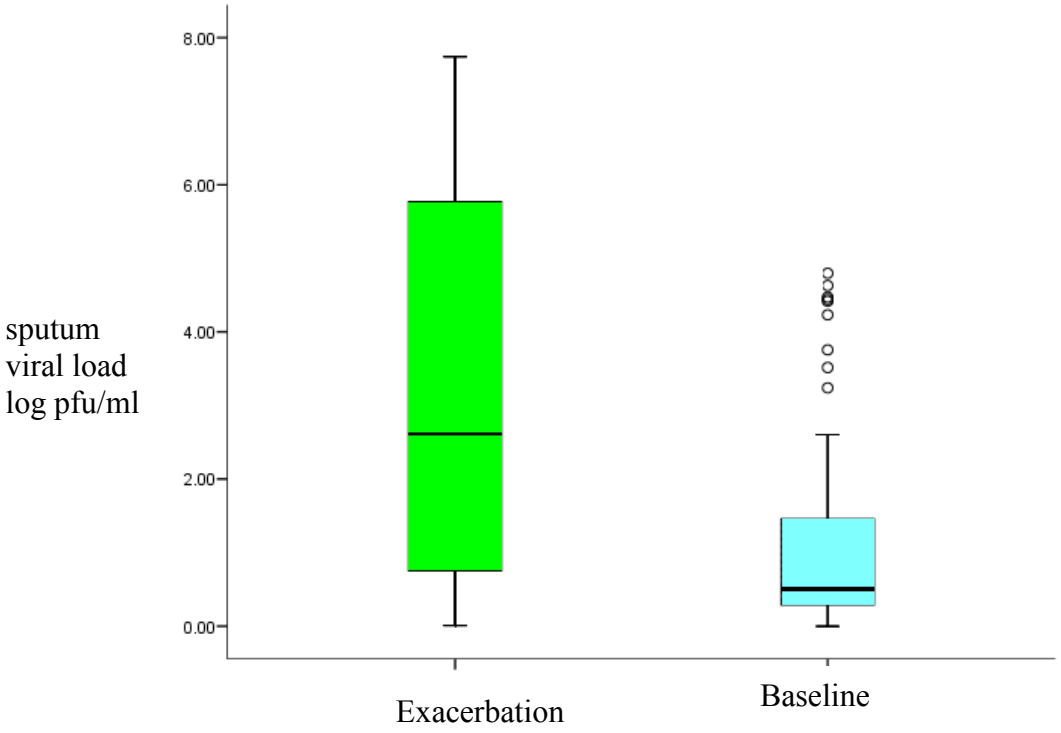
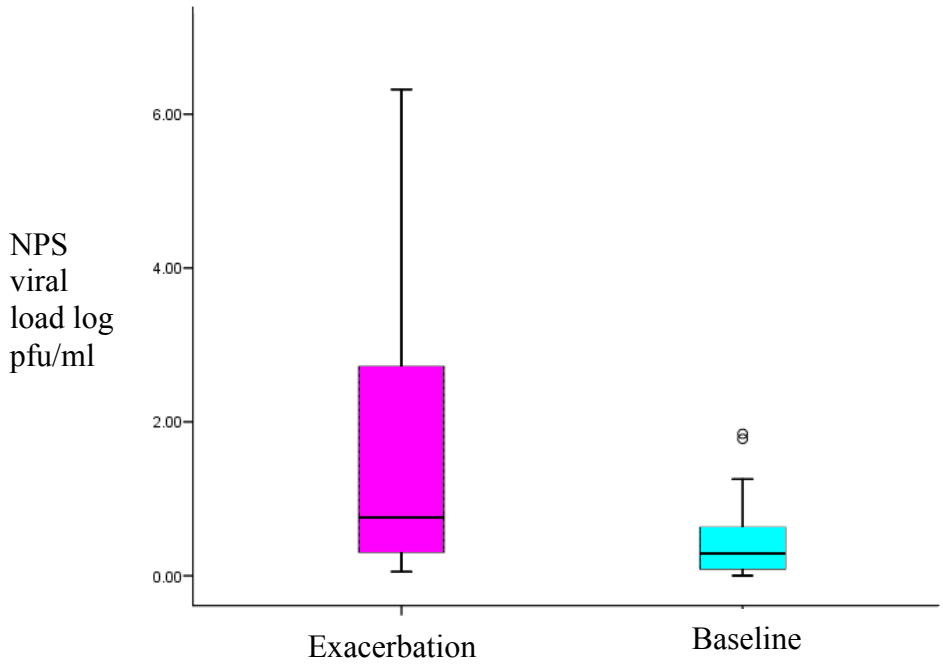


Figure 10.1.4 (b): Graph showing difference in NPS viral load between exacerbation and baseline



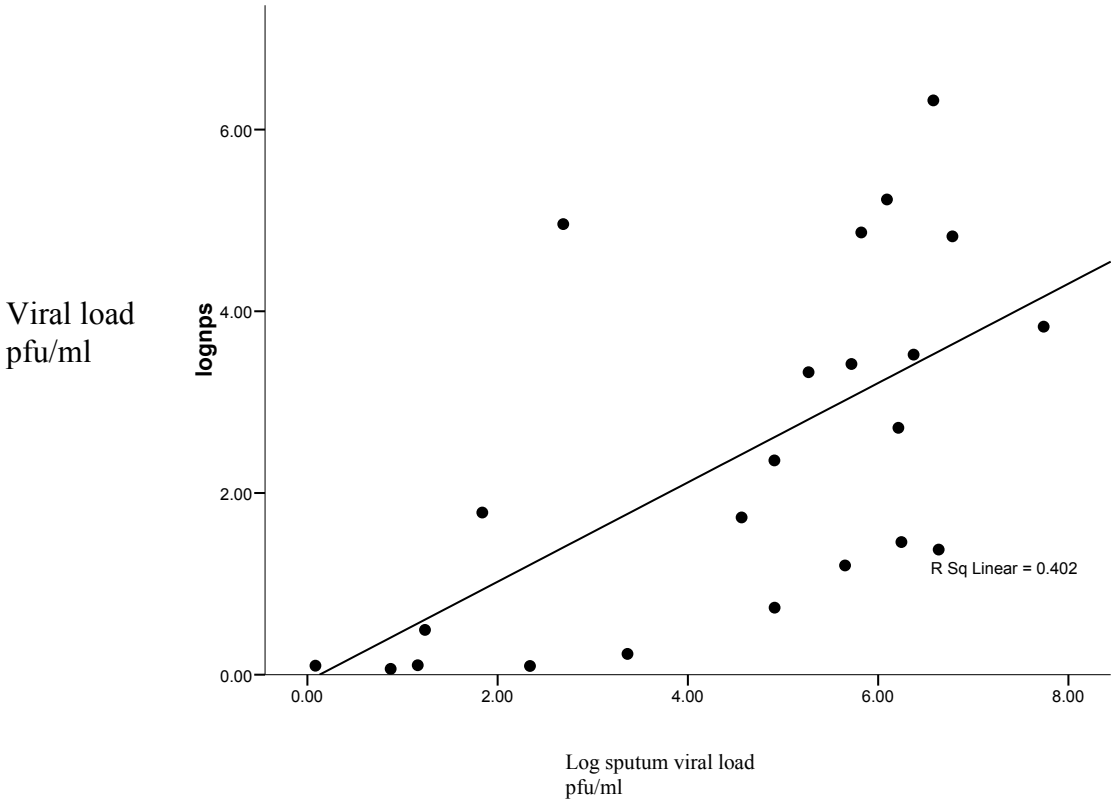
HRV detection at exacerbation and exacerbation frequency

There was no relationship between the proportion of exacerbations positive for HRV and exacerbation frequency.

HRV and exacerbations

210 exacerbations were sampled, 91 of which were positive for HRV in sputum or NPS (43.3%). 68/163 (41.7%) sputum samples were positive and 46/142 NPS (32.4%). Sputum and NPS viral loads correlated with each other at exacerbation (rho = 0.67 and $p < 0.001$). This is shown in **Figure 10.1.5.**

Figure 10.1.5: Graph showing the relationship between viral load in NPS and sputum



Exacerbation characteristics

Exacerbations were further categorised into those associated with HRV, i.e. including those with a low load of ≥ 1 pfu/ml, and those exacerbations that were likely to have

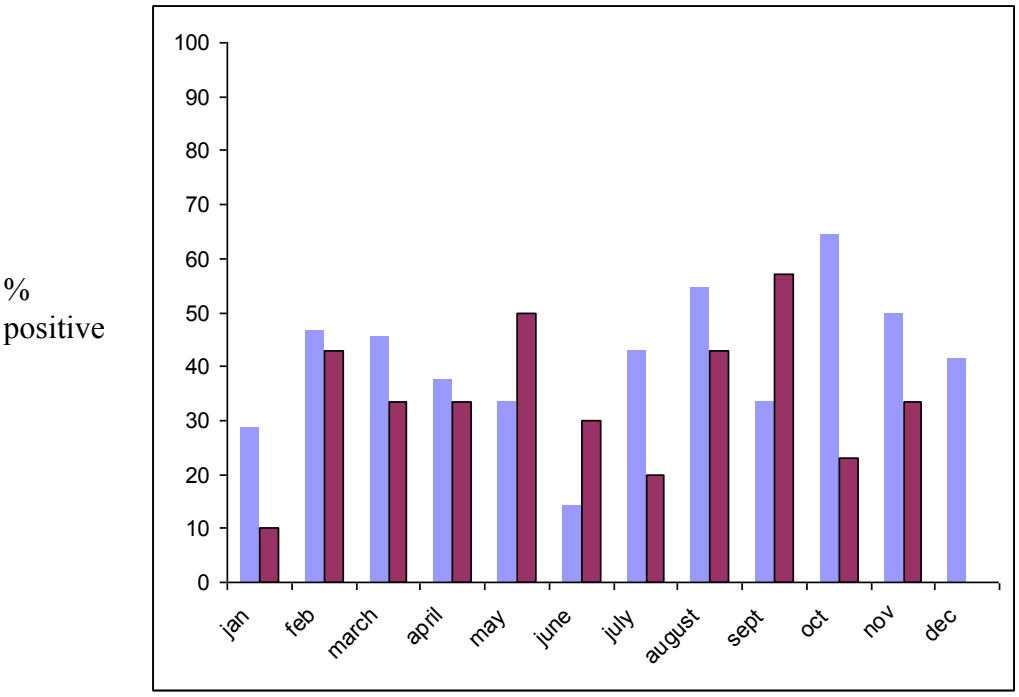
been caused by HRV and had a load of ≥ 175 pfu/ml. Justification for this cut off is detailed in ***Chapter 10.2***.

HRV associated exacerbations (positivity > 1pfu/ml sputum)
There was no difference in exacerbation reporting between HRV positive and negative exacerbations either to the GP or us and none of the exacerbations studied were reported to A&E. Only 2 exacerbations were self-treated.

Patients who had HRV detected in their sputum were less likely to complain of an increase in sputum volume at exacerbation (p=0.04). There was no difference in any other symptoms at exacerbation between those in which HRV was detected and not detected. With regard to nasal symptoms there was no difference in reporting of rhinorrhoea, PND, blocked nose, sneezing or anosmia between patients who had HRV detected in sputum and those who did not. With regards to NPS viral detection, there was a trend for those exacerbations associated with HRV to be more likely to have symptoms of blocked nose and sneezing (both p = 0.06). HRV associated exacerbations did not have a worse health burden and patients were no more likely to have had contact with children in the week preceding the exacerbation. A relationship between contact with children and HRV positivity at baseline was found and discussed in ***Chapter 7***.

There was no difference in increase in bronchodilator between exacerbations that had HRV detected in sputum and those that did not. These exacerbations were less likely to be treated with oral steroids (p = 0.01), but there was no difference in antibiotic use or steroid with antibiotics. There was no difference in inflammatory response as measured by CRP and WBC count between HRV associated and HRV not associated exacerbations. There was also no difference in time spent outdoors in the week preceding the exacerbation or in the delay in onset of exacerbation to reporting that exacerbation. The graph below (**Figure 10.1.6**) illustrates the detection of HRV in sputum and NPS by month of the year.

Figure 10.1.6: Presence of HRV in sputum and NPS at exacerbation according to the time of year. Sputum samples are shown in blue and NPS in pink.



HRV exacerbations (175 pfu/ml cut off)

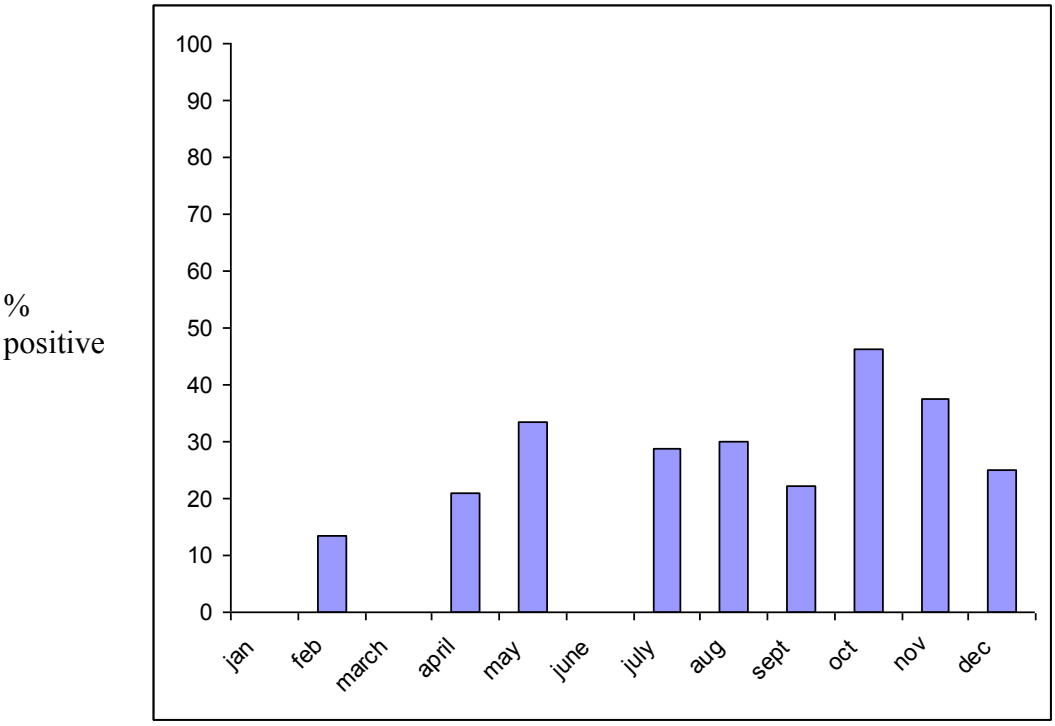
There was no difference in exacerbation reporting between HRV positive and negative exacerbations either to the GP or us and none of the exacerbations studied were reported to A&E. Only 2 exacerbations were self treated. There was no difference in the number of times out of the house in the week preceding the exacerbation, the number of visitors in, or the time spent outdoors.

There was no difference in exacerbation symptoms between those that had HRV exacerbations and those who did not. With regard to nasal symptoms there was no difference in reporting of rhinorrhoea, PND, blocked nose, sneezing or anosmia between patients who had HRV detected in sputum and those who did not. HRV associated exacerbations did not have a worse health burden and patients were no more likely to have had contact with children in the week preceding the exacerbation.

There was no difference in increase in bronchodilator between exacerbations that had HRV detected in sputum and those that did not. These exacerbations were less likely to be treated with oral steroids alone ($p = 0.01$), but there was no difference in antibiotic use or steroid with antibiotics. There was no difference in inflammatory response as

measured by CRP and WBC count between HRV positive and negative exacerbations. There was no difference in delay in onset of exacerbation to reporting that exacerbation. Exacerbations with a PPM detected were not more likely to be associated with HRV either in sputum or NPS. CRP was higher in PPM positive exacerbations (p = 0.03).

Figure 10.1.7: Seasonality of HRV detection at exacerbation



There was a significant difference in HRV causation of exacerbation according to the time of year (p = 0.003) with those exacerbations positive for HRV more likely in the winter months. Therefore, viral load was seasonality dependent.

Viral load at exacerbation

Higher NPS viral load was associated with a runny nose (p = 0.01) and sneezing(p = 0.05). Sputum viral load was not associated with any particular symptoms.

Sputum and NPS sampling at exacerbation

95 exacerbations were sampled for HRV in both sputum and NPS. Of these, 19 (20%) were positive in sputum but not NPS, 6 (6.3%) were positive in NPS but not sputum and 23 (24.2%) were positive in both, 47 (49.5%) were negative in both. The loads in both

NPS and sputum were higher in those exacerbations that had HRV detected in both; p = 0.08 and p = 0.004 respectively (**Table 10.1.7**).

Table 10.1.7: NPS and sputum positivity in viral load pfu/ml for HRV

	NPS median (IQR)	Sputum median (IQR)
Both tested and sputum positive only		10.3 (4.4 – 3434.3)
Both tested and NPS positive only	4.1 (2.0 - 8.3)	
Both tested and both positive	61.0 (3.1 – 6764.9)	185559.5 (218.9 – 1756868.4)

10.1.5 Discussion

In the preliminary qualitative experiments, the most common viruses detected were HRV, Flu B, hMPV and RSV. Although there was no difference in detection between the frequent and infrequent exacerbators in the stable state, this may be due to RNA extraction and PCR techniques used, including the limits chosen for detection. Also, mostly NPS samples were studied, not sputum, which again could account for the low numbers detected. Sputum was not used as it was not obtained in the stable state for this part of the analysis.

With regards to the quantitative virological data, a greater proportion of control subjects compared to COPD patients were positive for HRV in the stable state. This was not found in the subsequent chapter where even more controls were included. Most of the control subjects were sampled in the autumn and spring, when HRV is most prevalent so it is unsurprising such a large proportion were positive. Also, it is possible that people with underlying lung disease are more likely to become symptomatic even with low levels and therefore be classified as exacerbating. This suggests that viral infection and growth can occur without causing respiratory symptoms. Asymptomatic HRV infection has been well documented in otherwise healthy adults (Winther 1986). It is possible that no respiratory symptoms are generated until the inflammatory and immune response exceeds a certain threshold, or unless the virus replication rate exceeds a certain level.

Both in the stable state and at exacerbation sputum viral load was higher than NPS viral load and correlated better with symptoms. This study provided a direct comparison of sputum and nasal swab sampling for the detection and quantification of virus in COPD patients at baseline and at exacerbation. In general, the sensitivity of nasal swabs to detect viruses in sputum was poor, particularly at baseline, but their specificity was good. Viral loads were lower in nasal samples possibly because of dilution by nasal secretions which are more copious than sputum, alternatively, replication and shedding from the lower airways maybe greater because there is a larger surface area that can be infected. Another possible reason for the discrepancy between viral detection and load in the upper and lower respiratory tract may be the sampling procedure. Nasal swab

samples although less sensitive have the advantage that they can always be taken and are well tolerated by the patient, whereas some patients refuse nasal washing.

HRV positivity at baseline in the COPD patients was more common in chronic sputum producers, ex-smokers and those with a more obstructive lung function ratio. Either those with more severe disease are more susceptible to HRV, or, HRV influences disease severity. Establishing the cause and mechanism is important, as the development of antiviral agents may be of benefit in stable COPD in reducing lung function severity. Persistence at baseline was also not linked with exacerbation frequency but was again more likely in severe patients.

CRP was a very poor marker of HRV positivity. Further details of inflammatory markers with regard to HRV are discussed in ***Chapter 10.2***. Inflammation at exacerbation is discussed in relation to HRV is discussed further in ***Chapter 10.2***.

There was no difference in the prevalence of HRV at baseline between frequent and infrequent exacerbators indicating that HRV is not a driver of exacerbation frequency. There was a trend towards increased likelihood of HRV positivity with PPM positivity in sputum at baseline and this merits further investigation. Perhaps the two together will influence respective loads, symptoms and even exacerbation thresholds. Interestingly the positivity of HRV at baseline was not linked to positivity at exacerbation. Perhaps as with bacteria, acquisition of a new serotype is important rather than latency of virus and re-activation. The explanation of baseline viral levels deserves further investigation as to whether the viral material is fragmented and benign, or complete and capable of replication, i.e. represents persistent infection. We have previously argued that COPD patients may be colonised with RSV. The mechanisms which allow RSV to persist in the airways of COPD patients may be similar to how HRV can also remain but not cause clinical symptoms.

In the current study we chose to use quantitative RT-PCR to evaluate the viral load in sputum and nasal swab samples as this presents one of the most sensitive and specific methods for virus detection to date. There has been criticism of the use of real-time qRT-PCR because it does not provide an indication as to whether the virus is able to replicate and the instability of RNA is such that viral loads obtained may be lower. In

the present study we took the following effort to address these issues: Primers and probes were designed to a region within the 5'UTR which is almost identical in all HRV serotypes. Therefore this should allow the detection of potentially any HRV at the true viral load present within a sample. Although it is feasible that these primer/probe sets would potentially detect other enteroviruses, a separate PCR for enteroviruses carried out by the clinical virology laboratories within the Royal Free Hospital (London, UK) and did not detect any enteroviruses. Extensive experiments were carried out to assess the stability of RNA in sputum at various temperatures and after freeze-thawing and our qRT-PCR method was adapted accordingly. After minimizing these problems, we found viral RNA at lower concentrations at baseline and higher levels at exacerbation. Viral load was associated with exacerbation severity. In addition, rhinovirus-positive samples were significantly associated with nasal symptoms of a runny nose and sneezing. Exacerbation loads were much higher than baseline, as expected. This makes sense as the virus is likely to hang around at low levels between infections.

At exacerbation, HRV was not associated with increased severity in terms of treatment required. We did not have sufficient hospitalisations to look at hospital admissions. The fact that these exacerbations were far less likely to be treated with steroids alone, suggests they were more likely to be associated with increased sputum volume and sputum purulence. Traditionally sputum volume and purulence are associated with bacterial exacerbations and perhaps this is not always the case, or perhaps these viral exacerbations were associated with bacterial infection.

We also found that the load of HRV detected was higher in the winter months. The seasonality at baseline was discussed in ***Chapter 7***.

10.1.6 Conclusion

In the preliminary qualitative experiments, the most common viruses detected were HRV, Flu B, hMPV and RSV. Both in the stable state and at exacerbation sputum viral load was higher than NPS viral load and correlated better with symptoms. HRV positivity at baseline in the COPD patients was more common in chronic sputum producers, ex-smokers and those with a more obstructive lung function ratio. CRP was a very poor marker of HRV positivity. There was no difference in the prevalence of HRV at baseline between frequent and infrequent exacerbators. Exacerbation HRV loads were much higher than at baseline, and at exacerbation, HRV was not associated with increased severity in terms of treatment required. We also found that the load of HRV detected was higher in the winter months.

HRV is prevalent both in the stable state and at exacerbation in COPD. Lower airway loads of HRV are higher than upper airway viral loads.

10.2

IP-10

10.2.1 Introduction

One of the possible factors contributing to frequent exacerbations is an increased susceptibility to infection. Low levels of virus at baseline may predispose to bacterial infection, or conversely, bacterial colonisation in the stable state may increase susceptibility to acquisition of virus thus triggering an exacerbation. This has been discussed in ***Chapter 10.1***. Cytokine responses at exacerbation vary depending upon the pathogens detected at exacerbation (Wilkinson 2006, Rohde 2008) and this may also be true in the stable state. Cytokine differences in the stable state between frequent and infrequent exacerbators may also increase susceptibility to infection; if frequent exacerbators are more susceptible to virus, particularly HRV at baseline and / or at exacerbation, this may be reflected in the cytokine profile, specifically in IP-10 levels; a marker of HRV infectivity (Spurrell 2005).

The prevalence of HRV in the stable state in frequent and infrequent exacerbators has been discussed in ***Chapter 10.1***. Up to 64% of exacerbations are associated with colds (Seemungal 2001). The commonest respiratory virus detected in the airways at exacerbation is HRV, which using molecular techniques has been identified as present in up to 60% of respiratory virus associated exacerbations (Seemungal 2001, Wilkinson

2006). Cold symptoms at exacerbation increase the severity of the exacerbation, lengthen the recovery time (Seemungal 2000(b)), and when virus presence is confirmed by polymerase chain reaction (PCR), the exacerbation is associated with higher levels of airway inflammatory markers including Interleukin (IL)-6, (2) IL-8 and myeloperoxidase (MPO) (Wilkinson 2006). Exacerbations associated with a virus are more likely to lead to hospitalisation (Greenberg 2000, Rohde 2003) and viral infection has been identified in up to 47% of COPD patients with exacerbations requiring intubation and mechanical ventilation (Qui 2003). Serum IP-10 has previously been shown to rise in virus triggered acute asthma exacerbations (Wark 2007), if frequent exacerbators are more susceptible to HRV, then a marker of that susceptibility may be a useful to identify a patient for early intervention.

Interferon (IFN)-γ-induced protein 10 (IP-10), a chemokine secreted by bronchial epithelial cells, monocytes, lymphocytes and neutrophils in response to IFN-γ and (TNF)-α (Cassatella 1997, Sauty 1999) has been shown to be elevated by HRV infection (Spurrell 2005). HRV infects and replicates in bronchial epithelial cells (Papi 1999), triggering production of cytokines and chemokines including IP-10 (Spurrell 2005).

Expression of IP-10 and its receptor CXCR3 is higher in the airway in smokers with COPD (Grumelli 2004, Saetta 2002, Hardaker 2004), and IP-10 is present at higher levels in induced sputum in COPD patients compared to controls (Costa 2008) so before addressing differences in serum levels between frequent and infrequent exacerbators, it is important to ascertain differences between COPD and control subjects.

10.2.2 Aims

1. To assess differences in serum IP-10 levels between COPD patients at baseline and control subjects, both when positive and negative for HRV.

2. To assess differences in serum and sputum IP-10 in frequent and infrequent exacerbators at baseline and at exacerbation.

3. To assess the use of serum and sputum IP-10 as markers of airway HRV positivity at exacerbation. To assess the relationship between serum and sputum IP-10 and airway HRV load. This was further assessed with *in vitro* work.

5. To assess whether IP-10 levels are influenced by RSV at baseline or exacerbation.

6. To assess whether the presence of a potentially pathogenic microorganism in the stable state or at exacerbation influences IP-10 levels in sputum or serum.

10.2.3 Methods

The methodology relating to this chapter can be found in ***Chapter 3,*** **sections 3.1, 3.6, 3.7, 3.9, 3.11 and 3.12.** 136 patients and 70 controls were studied between 1st April 2006 and 31st May 2008.

10.2.4 Results

Baseline patient characteristics

One hundred and thirty six COPD patients were studied; 83 male and 53 female. The baseline characteristics of the cohort are reported in **Table 10.2.1**. The patients had a mean FEV_1 of 1.16 l or 53.9 % predicted. 70 control subjects were studied; 28 male and 42 female. The baseline characteristics are reported in **Table 10.2.1**. The control subjects had a mean FEV_1 of 2.63 l or 112.1% predicted. There were significant differences in age, smoking history and oxygen saturations between the control subjects and COPD patients (all $p < 0.001$) but not BMI.

Table 10.2.1: Baseline Characteristics of 136 COPD patients: 83 male (61.0%), 41 current smokers (30.1%); 40 frequent exacerbators (29.4%) and 70 control subjects: 28 male (40.0%), 12 current smokers (17.1%).

	COPD patients	**Controls**
	Mean (SD)	**Mean (SD)**
Age (years)	72.6 (8.4)	67.4 (8.7)
FEV1 (litre)	1.16 (0.46)	2.63 (0.80)
FEV1 (% predicted)	53.9 (18.7)	112.1 (28.3)
FVC (litre)	2.53 (0.86)	3.43 (1.08)
BMI (kgm^{-2})	26.3 (5.8)	26.0 (5.1)
Pack years smoking	51.1 (38.6)	18.4 (20.9)
SpO_2 (%) on air	95 (2)	96 (1)
	Median (IQR)	**Median (IQR)**
Serum IP-10 pg/ml	154.4 (107.0 – 220.6)	115.5 (81.2 – 181.6)
CRP mg/L (n=77)	4.0 (2.0 – 7.0)	2.0 (1.0 – 3.3)
IL-6 pg/ml (n=72)	3.2 (2.4 – 5.9)	3.3 (2.0 – 5.4)

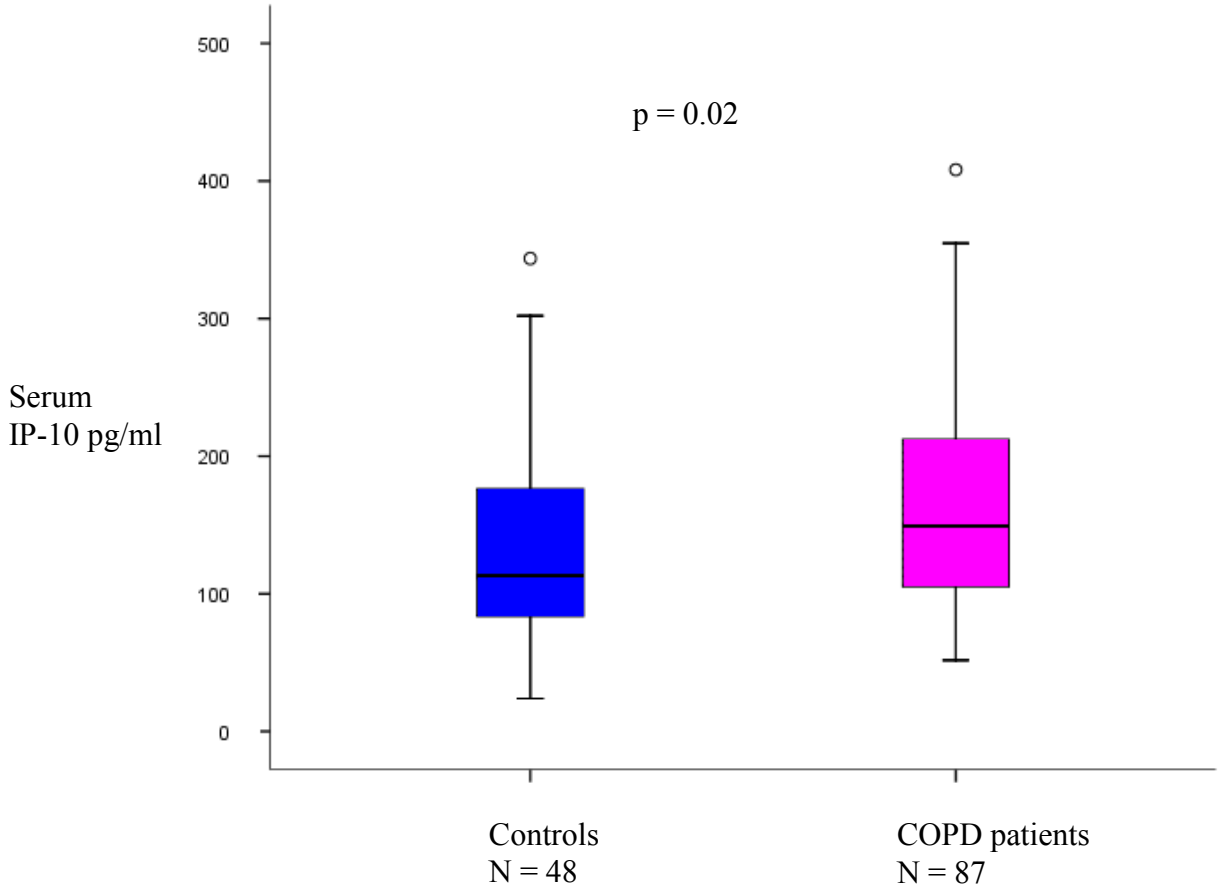
COPD patients and controls

Baseline serum IP-10, IL-6 and CRP in COPD and control subjects when HRV negative

87 COPD patients (64.0%) and 48 of the control subjects (68.6%) were negative for HRV when sampled by NPS and / or sputum at a baseline visit and had serum IP-10 measured. **Figure 10.2.1** shows that serum IP-10 levels were higher in COPD patients

than controls; medians 149.4 pg/ml (IQR 103.4 – 215.2) and 111.7 pg/ml (82.6 – 178.5) respectively (p = 0.02). In a regression analysis accounting for age, smoking status, sex and disease (COPD or control) there was still an effect of disease status on IP-10 (p = 0.01).

Figure 10.2.1: Median serum IP-10 in 48 controls and 87 COPD patients when HRV negative in the stable state. Circles represent extreme outliers.



CRP was measured in 77 COPD patients and 23 controls and IL-6 measured in 72 patients and 45 controls. CRP was higher in the COPD patients than the controls; medians 4.0 mg/L (2.0 – 7.0) and 2.0 mg/L (1.0 – 3.3) respectively (p = 0.006), but there was no difference in serum IL-6; medians 3.2 pg/ml (2.4 – 5.9) and 3.3 pg/ml (2.0 – 5.4) respectively.

Higher IP-10 correlated with increasing age in both the COPD patients and controls: rho = 0.24; p = 0.03 and rho = 0.34; p = 0.02 respectively. Serum IP-10 correlated with

smoking pack years in the COPD patients; rho = -0.30, p = 0.005. Serum IP-10 did not correlate with increased disease severity (lower FEV_1 % predicted) in the COPD patients.

Baseline IP-10 in COPD and control subjects when HRV positive

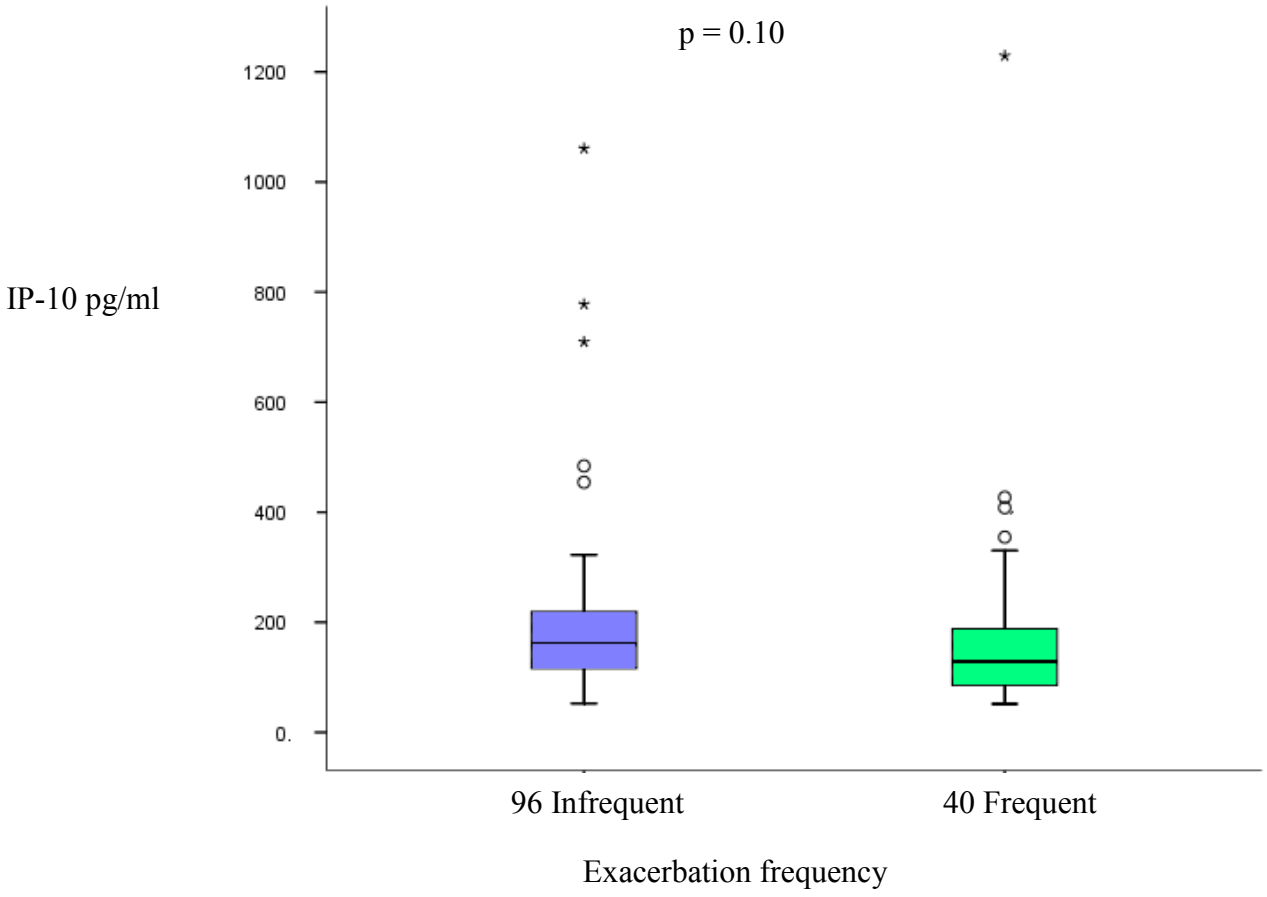
Forty nine COPD patients and 22 control patients when sampled in the stable state were found to be HRV positive at very low levels using a sensitive cut off of 1pfu/ml as positive. They were all asymptomatic at this time and the presence of HRV did not increase serum IP-10 levels in either the COPD patients or control subjects; medians 166.9 pg/ml (110.1 – 240.3) and 149.4 pg/ml (IQR 103.4 – 215.2) and medians 136.4 pg/ml (77.3 – 204.0) and 111.7 pg/ml (82.6 – 178.5). There was no correlation between serum IP-10 levels and HRV load in NPS in the controls or stable COPD patients or between serum IP-10 and sputum HRV load in COPD patients at a HRV positive baseline.

Exacerbation Frequency and IP-10

Serum IP-10

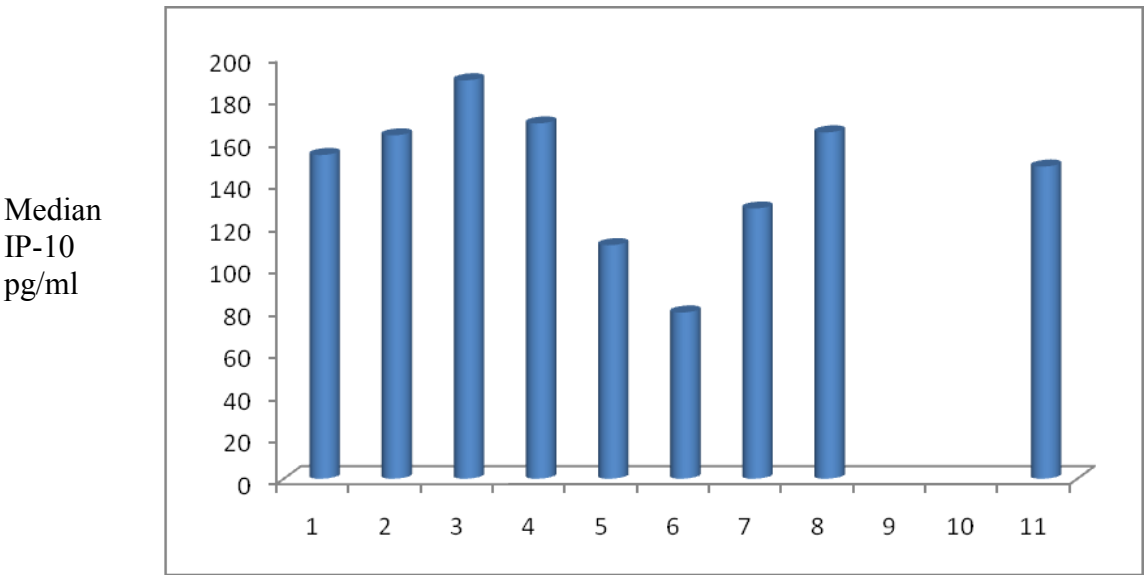
There was no difference in serum IP-10 levels between frequent and infrequent exacerbators at baseline when HRV negative; medians 128.2 pg/ml (84.6 – 198.6), and 160.1 pg/ml (115.4 – 219.7) respectively (**Figure 10.2.2**).

Figure 10.2.2: Serum IP-10 in frequent and infrequent exacerbators at baseline when HRV negative. The circles and stars represent extreme outliers.



It appears as though frequent exacerbators may be trending towards a lower IP-10 level in the stable state. Median IP-10 levels were therefore plotted for each exacerbation number and are shown below in **Figure 10.2.3**.

Figure 10.2.3: Median IP-10 level plotted by number of exacerbations.



Sputum IP-10 in frequent and infrequent exacerbators when HRV negative in the stable state

25 infrequent exacerbators and 12 frequent exacerbators had sputum tested for IP-10 when HRV negative at baseline. There was no difference in sputum IP-10 levels between the two groups; medians frequent exacerbators 424.6 pg/ml (98.8 – 984.7) and medians infrequent exacerbators; 147.5 pg/ml (81.1 – 275.7).

Exacerbations and IP-10

All exacerbations

72 exacerbations were studied. In 63 exacerbations paired with a baseline sample, serum IP-10 increased from baseline to exacerbation; median baseline IP-10 157.5 pg/ml (106.4 – 215.2) and exacerbation IP-10 199.1pg/ml (128.3 – 290.8) ($p = 0.004$). Serum IL-6 and CRP also increased from baseline to exacerbation; medians 2.96 pg/ml (1.8 – 4.8), 4.37 pg/ml (1.8 – 10.4); $p = 0.049$, medians 3.00 mg/l (1.3 – 7.0) and 7.00 mg/l (2.5 – 14.0) ($p < 0.001$).

In 27 paired baseline – exacerbation samples there was no difference in sputum IP-10 levels; medians 197.38 pg/ml (105.3 – 487.1) and 400.2 pg/ml (184.0 – 3280.0).

HRV positive exacerbations

52 of the 72 exacerbations were tested for the presence of HRV in sputum (n = 41) and/or NPS (n = 44). Of those tested, 31 (59.6%) were positive. **Figure 10.2.4(a)** shows that serum IP-10 increased significantly from baseline to exacerbation in the HRV positive exacerbations; medians; 154.9 pg/ml (114.0 – 195.1) and 207.5 pg/ml (142.1 – 333.5) (p = 0.009).

HRV negative exacerbations

Figure 10.2.4(b) shows that there was no change in IP-10 levels between baseline and exacerbation in HRV negative exacerbations; medians 168.3 pg/ml (94.3 – 249.8) and 175.6 pg/ml (107.2 – 290.4).

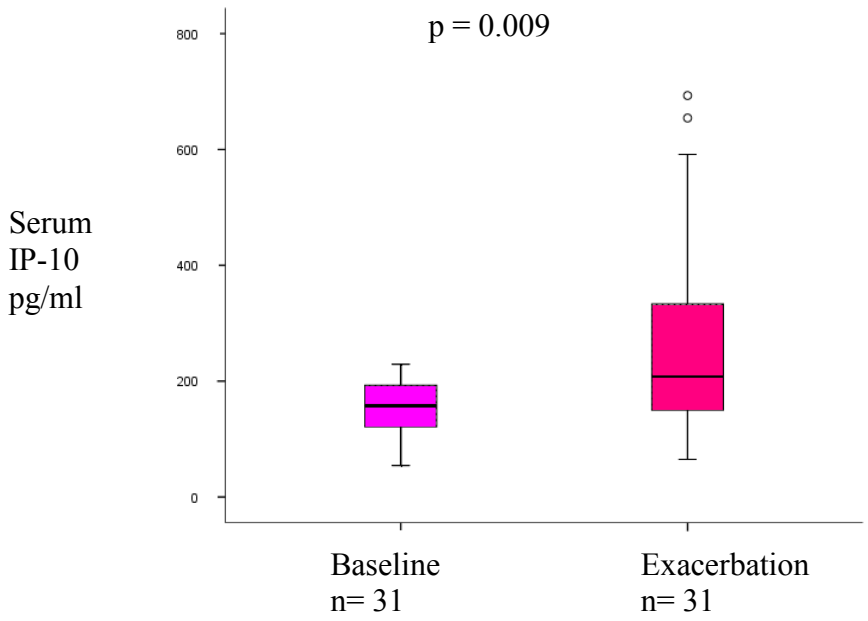
Sputum IP-10 and HRV

There was no difference in sputum IP-10 levels between the 14 HRV negative and 20 HRV positive exacerbations detected in sputum; medians 253.6 pg/ml (160.0 – 640.5) and 404.6 pg/ml (150.9 – 1712.4).

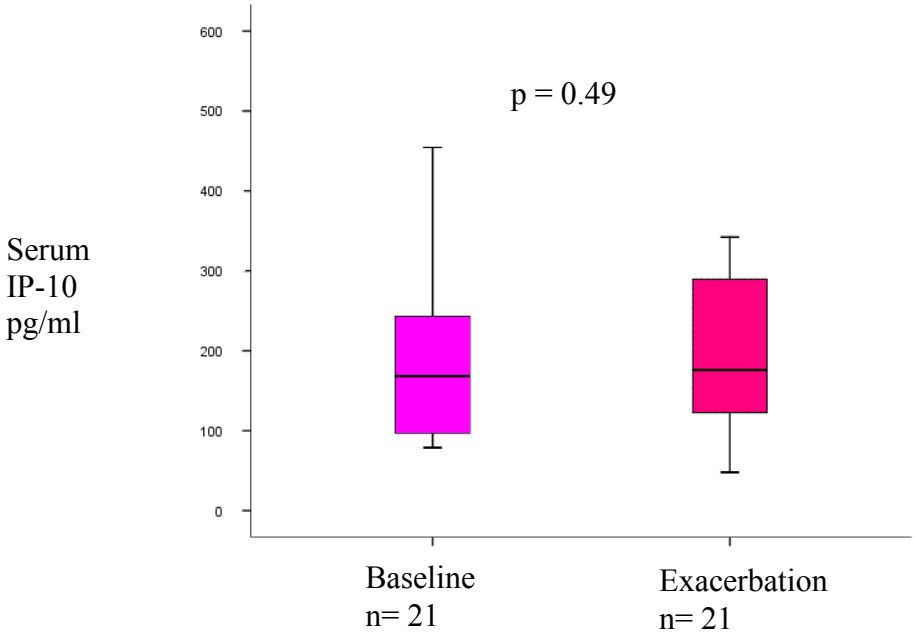
Figure 10.2.4: (a) Increase in IP-10 from baseline to exacerbation in 31 paired COPD exacerbations that were positive for HRV. (b) Median serum IP-10 at baseline and exacerbation in 21 paired baseline - exacerbations that were negative for HRV. Circles represent extreme outliers.

(a)

IP-10



(b)



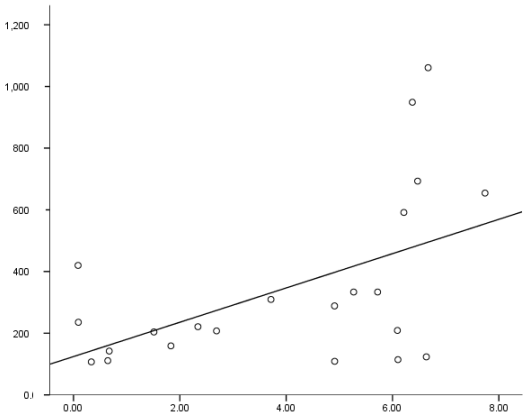
IP-10 and viral load

At exacerbation, serum IP-10 levels correlated with sputum viral load (**Figure 10.2.5a**) but not viral load measured from NPS. There was no relationship between CRP or IL-6 and viral loads in sputum (**Figure 10.2.5b and 5c**) or NPS. CRP and IL-6 did not correlate with IP-10. Sputum viral load did not correlate with sputum IP-10: rho = 0.5; (p = 0.06). Sputum and serum IP-10 at exacerbation correlated with each other: rho = 0.29; (p = 0.05).

Figure 10.2.5: (a) At exacerbation IP-10 levels correlated with sputum viral load. (b) IP-10 levels did not correlate with CRP or (c) IL-6.

a)

Serum IP-10 pg/ml

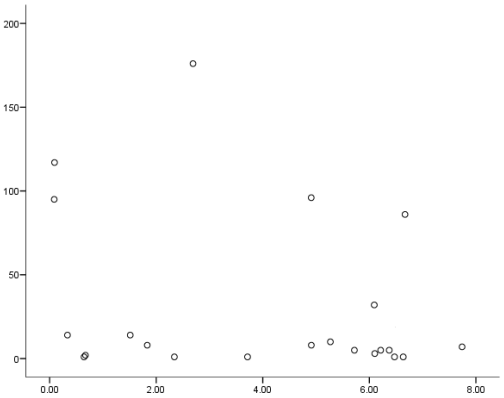


rho = 0.48 and p = 0.02

Sputum viral load log10 pfu/ml

b)

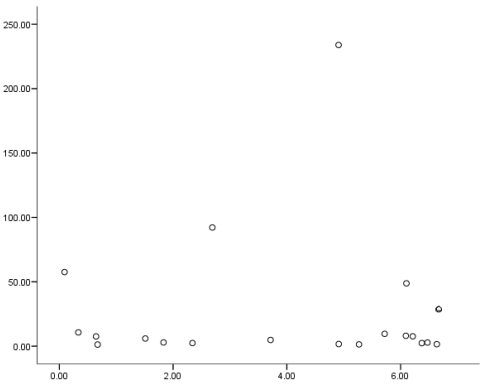
CRP ml/L



rho = - 0.26; p = 0.24

c) Sputum viral load log10 pfu/ml

IL-6 pg/ml



rho = 0.29; p = 0.22

Sputum viral load log10 pfu/ml

In vitro work

HRV load in BEAS-2B cells and Hela Ohio cells infected with HRV moi 0.5 is shown over time with IP-10 levels measured in corresponding cell supernatant samples. **Figure 10.2.6** shows the $\log_{10}$ HRV load in both cell lines over time.

Figure 10.2.6: Time course of HRV load

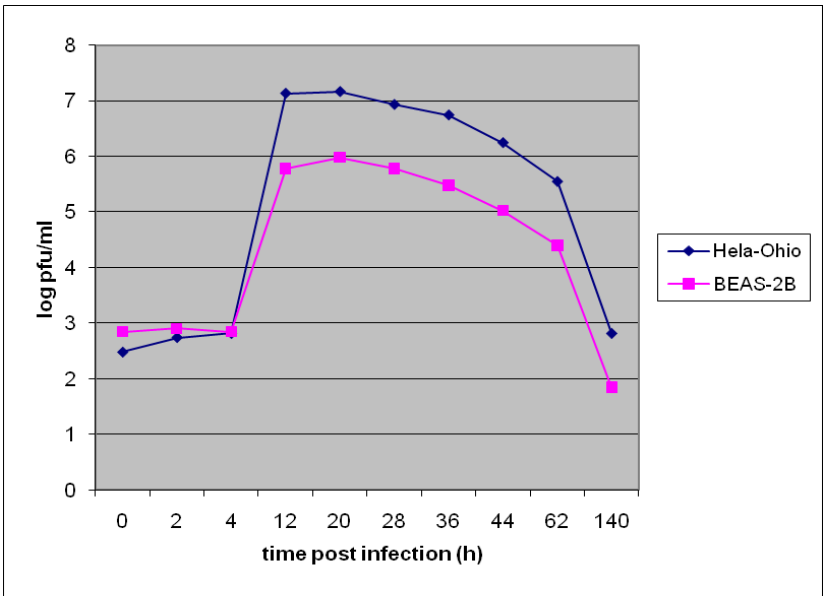
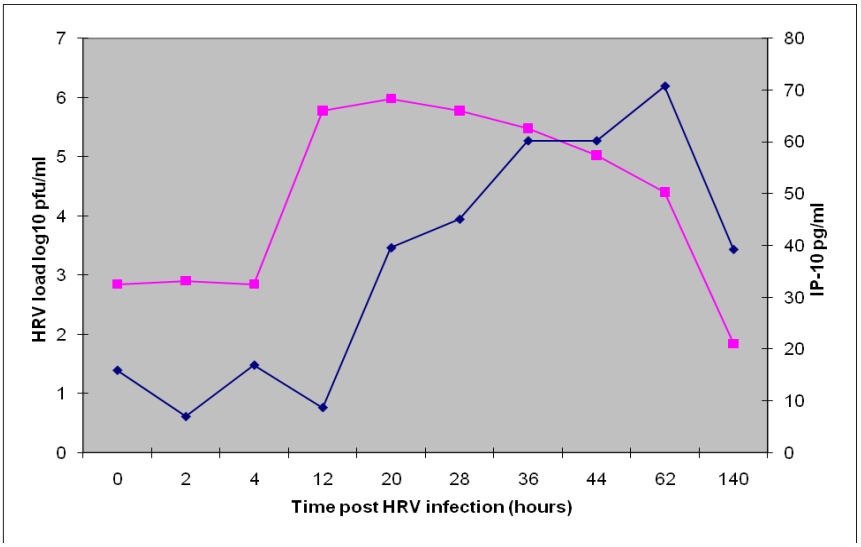


Figure 10.2.7 shows the log10 HRV load in BEAS-2B cells and the corresponding IP-10 values in the cell supernatant.

Figure 10.2.7: IP-10 and HRV load in cell culture



Preliminary experiments suggest that HRV infection of 1˚ECs upregulate IP-10 and this is multiplicity of infection (moi) dependent. No increase was seen after TNF-α stimulation (**Figure 10.2.8**).

Figure 10.2.8: IP-10 and HRV / TNFα stimulation in primary epithelial cells

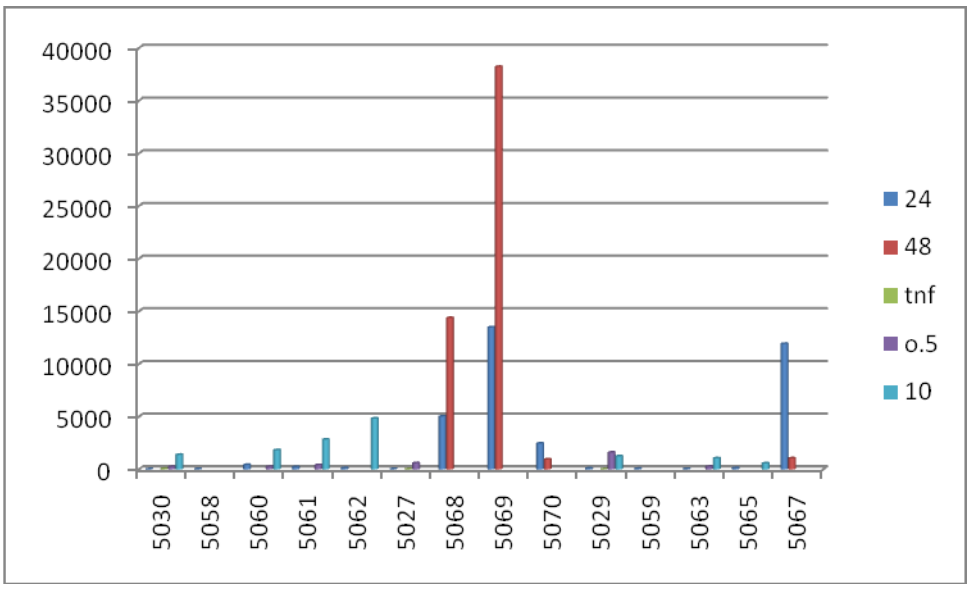
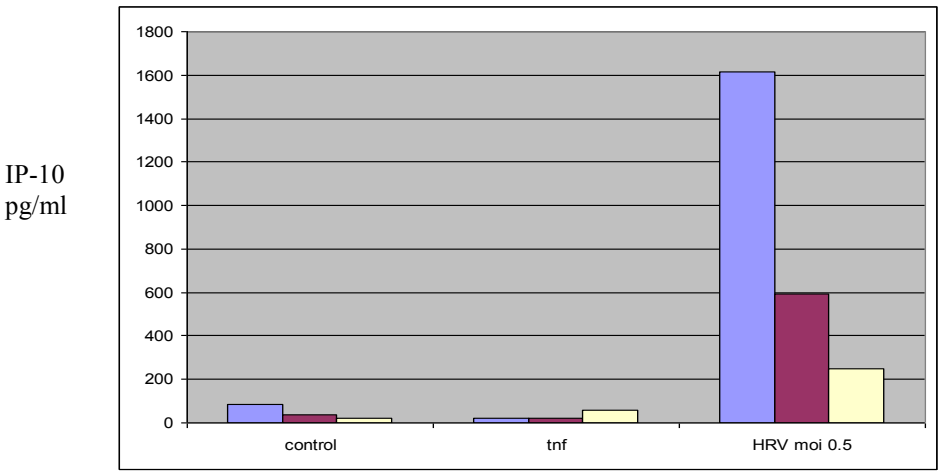


Figure 10.2.9: IP-10 levels



Cold symptoms at exacerbation and IP-10

At exacerbation 36 patients reported coryzal symptoms while 34 did not. In 2 patients data was not available. **Figure 10.2.10a** shows that patients reporting coryzal symptoms had significantly higher levels of serum IP-10 but not CRP or IL-6 at exacerbation; IP-

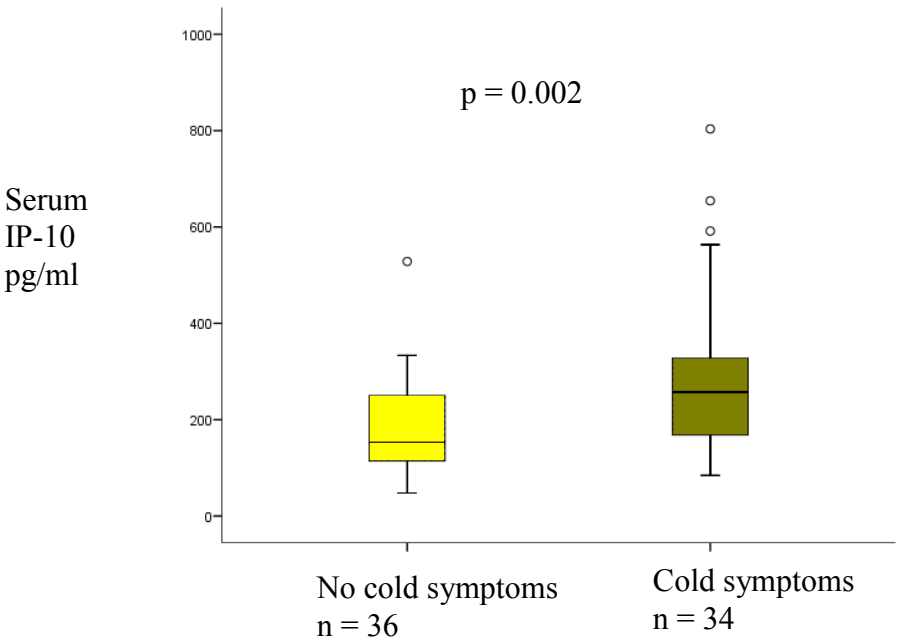
10 medians 257.0 pg/ml (167.6 – 330.3) and 150.8 pg/ml (113.5 – 252.5), (p = 0.002); CRP medians 7.0 mg/L (4.5 – 19.5) and 4.0 mg/L (1.8 – 11.0), and IL-6 medians 5.7 pg/ml (2.3 – 9.5) and 3.3 pg/ml (1.6 – 14.0).

Of the 21 patients who were tested for HRV and were negative, 11 reported coryzal symptoms. These patients did not have higher IP-10 levels than those who were HRV negative and did not report coryzal symptoms; medians 269.7 pg/ml (150.2 – 342.1) and 150.8 pg/ml (65.0 – 264.0), (p = 0.06) (**Figure 10.2.10b**). There was no difference in CRP or IL-6 in the two groups; medians 7.5 mg/L (4.0 – 32.3) and 8.0 mg/L (2.0 – 43.0), and medians 5.7 pg/ml (1.6 – 8.7) and 3.9 pg/ml (1.3 – 25.5).

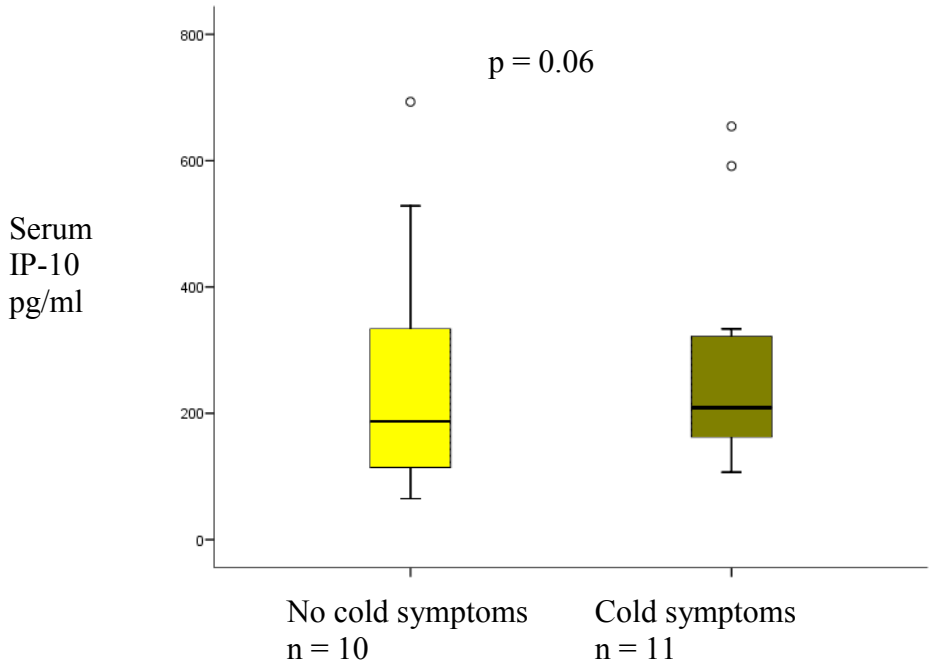
There was no difference in sputum IP-10 levels in those reporting or not reporting cold symptoms at exacerbation; medians 553.7 pg/ml (208.5 – 2628.6) and 269.8 pg/ml (160.0 – 2778.2).

Figure 10.2.10: (a) 34 patients reporting coryzal symptoms had significantly higher levels of IP-10 than 36 patients not reporting coryzal symptoms. Circles represent extreme outliers. (b) In 21 HRV negative exacerbations where 11 patients reported cold symptoms there was no difference in IP-10.

a)



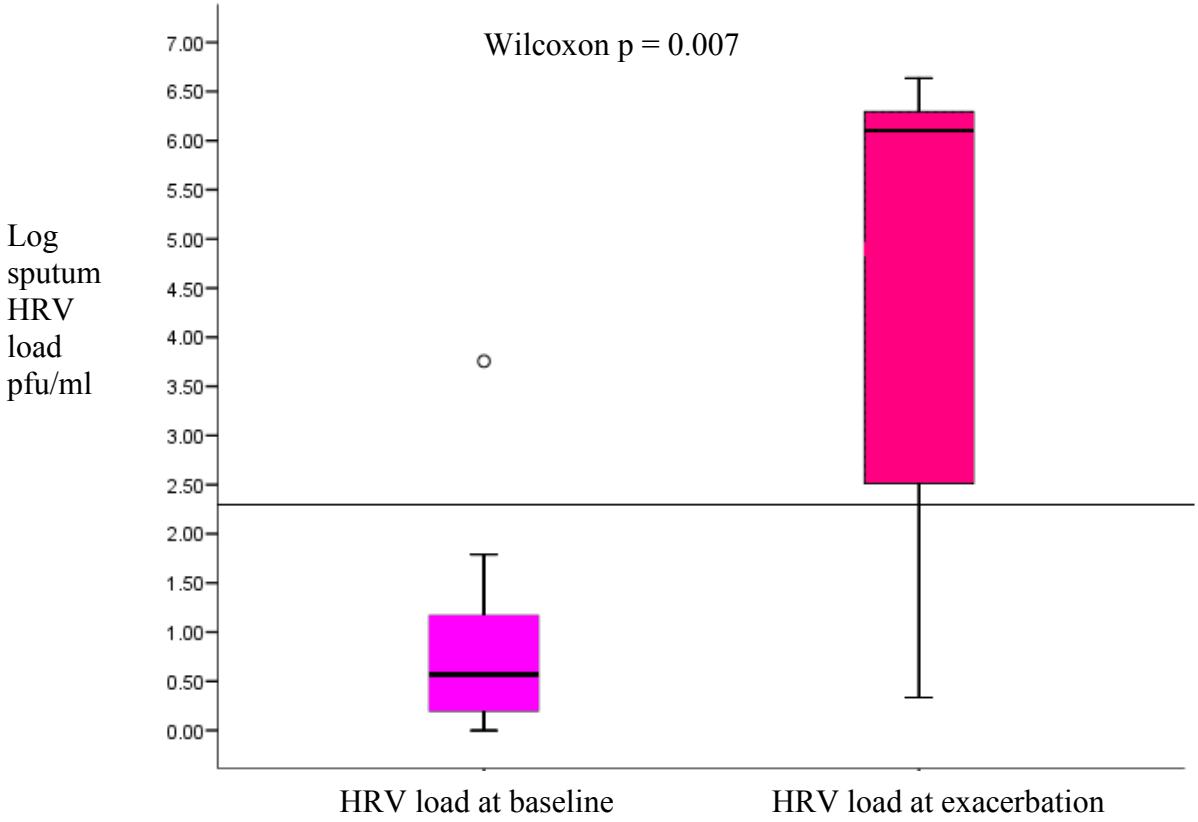
b)



HRV positivity at baseline and exacerbation

13 patients were positive for HRV both at baseline and exacerbation. **Figure 10.2.11** shows that sputum HRV loads were significantly higher at exacerbation than at a HRV positive baseline; medians 1236514 pfu/ml (218.90 – 2360930) at exacerbation and 3.73 pfu/ml (1.43 – 23.41) at baseline (p = 0.007). IP-10 was significantly higher at HRV positive exacerbation than HRV positive baseline; medians 204.06 pg/ml (123.26 – 333.46) at exacerbation and 162.68 pg/ml (122.99 – 194.89) at baseline (p = 0.04).

Figure 10.2.11: HRV load in 13 baseline – exacerbation pairs. The horizontal line represents the cut off chosen for positivity at exacerbation.



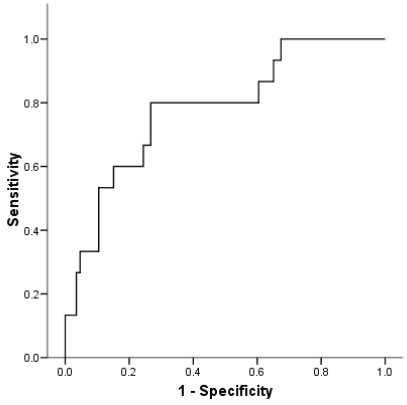
Use of serum IP-10 in the diagnosis of HRV at exacerbation in COPD

To apply these data to clinical practice, we set a cut off of HRV load associated with COPD exacerbation based upon the difference in HRV load between baseline and exacerbation (**Figure 10.2.12**). We chose a $\log_{10}$ HRV load value of 2.24 which corresponds to 175pfu/ml as this was higher than the upper limit of the inter-quartile range for the baseline samples but below that for the exacerbation samples. We would suggest that the presence of HRV at a load ≥ 175pfu/ml at exacerbation indicates that HRV is the cause of that exacerbation and anything below this level although HRV is present, does not cause symptoms of exacerbation or trigger much of an inflammatory response. We based our ROC analysis on this cut off using all sputum samples (baseline and exacerbation, HRV positive and negative) and the presence of cold symptoms on the day of visit. We used sputum samples only as NPS samples did not correlate with

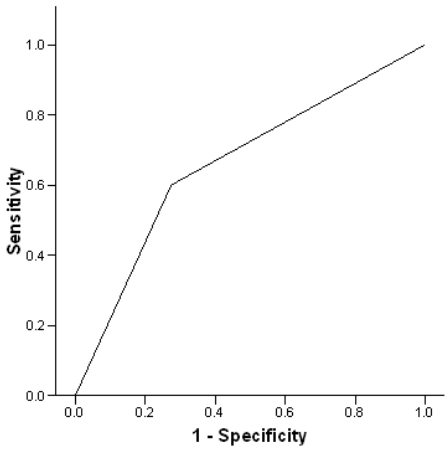
IP-10 or with the sputum samples. The area under the curve (AUC) for IP-10 alone was 0.78 (95% confidence interval 0.65 – 0.91). Using the presence of coryzal symptoms alone, the AUC was 0.66 (0.51 – 0.82). Using the combination of IP-10 and coryzal symptoms the AUC was 0.82 (0.74 – 0.90) (**Figure 10.2.12**). Thus by measuring IP-10 in the blood in the presence of a coryzal symptom at exacerbation, the likelihood of HRV infection can be determined. Applying a cut off of IP-10 in the blood of 260 pg/ml in the presence of cold symptoms would be 80% specific and 67% sensitive for the presence of HRV at ≥175 pfu/ml.

Figure 10.2.12: ROC curves. (a) Using IP-10 alone the AUC = 0.78 (95% confidence interval 0.65 – 0.91). (b) Using the presence of coryzal symptoms alone, AUC = 0.66 (0.51 – 0.82). (c) Using the combination of IP-10 and coryzal symptoms the AUC = 0.80 (0.66 – 0.94).

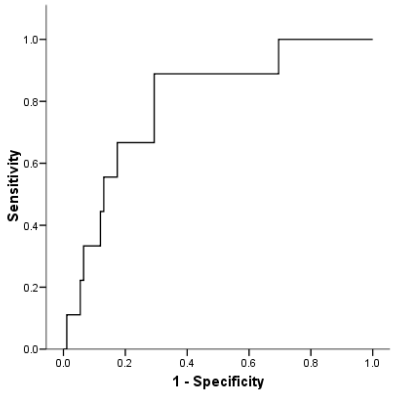
a)



b)



c)



RSV positive exacerbations

In 43 exacerbations sputum was tested for the presence of RSV A and B; 6 were positive. In a regression analysis; serum IP-10 was related to HRV (p = 0.02) but not RSV infection (p = 0.43); medians 167.1 (112.7 – 290.4) and 252.4 (196.5 – 487.3). Only 3 samples were RSV positive at baseline; too few for analysis. Sputum IP-10 was higher in those exacerbations that were RSV positive; medians; 268.1 pg/ml (141.6 – 677.0) and 6574.5 (1315.4 – 16035.4); (p= 0.02).

Detection of PPM at exacerbation

Thirty six exacerbations were tested for the presence of a PPM by routine bacterial culture of sputum. 7 were positive. There was no difference in symptom number or type (e.g. sputum purulence or coryzal symptoms) between PPM positive and negative exacerbations. IL-6 was significantly higher at exacerbation if a PPM was detected; medians 3.07 pg/ml (1.47 – 9.02) and 48.77 pg/ml (14.05 – 92.14), p = 0.02 but not IP-10; medians 207.49 pg/ml (114.33 – 288.41) and 244.31 pg/ml (132.68 – 376.64). Sputum IP-10 however was lower if a PPM was present; medians 658.4 (217.0 – 5896.4) and 225.2 (55.9 – 427.5); p = 0.04.

Detection of PPM at baseline

Too few samples were tested for a PPM at baseline on the same day as viral sampling. We investigated whether there was a difference in serum or sputum IP-10 in those colonised with a PPM (> or = 3 pos in that year). In serum, (no PPM n = 29) medians

139.6 (108.7 – 232.5) and PPM present (n = 25) 162.7 (92.4 – 298.9). In sputum (no PPM n = 13) medians 197.4 (93.4 – 472.5) and PPM (n = 9) 377.9 (120.0 – 697.7).

10.2.5 Discussion

We have shown for the first time that serum IP-10 increases from baseline to exacerbation in COPD specifically in exacerbations positive for human rhinovirus. Serum IP-10 has previously been shown to rise in virus triggered acute asthma exacerbations (Wark 2007), and we have now shown that in COPD exacerbations, sputum HRV load determined by quantitative RT-PCR correlates with IP-10 levels in the blood. Although HRV infection in most individuals is mild and self limiting, in COPD patients viral infection not only triggers exacerbations but increases the severity of the exacerbation, lengthens recovery time (Seemungal 2000 (b)), and increases the likelihood of hospitalisation (Rohde 2003). Thus serum IP-10 may be a useful biomarker to identify a patient for early intervention; either with standard exacerbation therapy (El Moussaoui 2008, Roede 2008) or future novel (Maugeri 2008) antiviral agents (Pevear 2005, Weinberger 2004).

This study shows that sputum HRV load correlated with serum IP-10 at exacerbation, and thus IP-10 may be useful in detecting the presence of HRV and monitoring HRV exacerbation length and response to treatment. Currently there are no good markers of exacerbation severity and HRV causes more severe exacerbations than virus negative exacerbations (Seemungal 2000(b)). Ease of sampling blood makes serum IP-10 a more practical marker than a sputum marker. Further work is required to examine the role of IP-10 as a marker of exacerbation recovery as there is increasing interest in this post exacerbation period; (Roede 2008, Hurst 2009) when morbidity is still significant.

We have not tested whether IP-10 rises at exacerbation in the presence of other viruses. In tissue culture IP-10 has been shown to rise in the presence of influenza virus (Veckman 2009) and RSV (Culley 2006), but this has not been confirmed *in vitro*. Although it is possible other viruses not tested for may influence IP-10, we did not find an increase in IP-10 in the presence of coryzal symptoms (a marker of viral infection) when HRV was not detected. We also did not find a relationship with serum IP-10 and RSV or the presence of a PPM. Sputum IP-10 was however higher in those with RSV. This study focused on the role of IP-10 as a marker of HRV infection and we have not defined the role of bacteria fully, though IP-10 is not known as a marker of bacterial infection.

In this study, neither serum CRP nor serum IL-6 correlated with HRV load with levels remaining low independent of high viral loads, thus indicating that they are poor markers of HRV positive COPD exacerbations. Previous studies have shown that in exacerbations of asthma, neither serum IL-6 nor IL-8 significantly differed between viral and non-viral exacerbations (Wark 2007) and our data confirmed these findings in COPD. In response to experimental infection with HRV in COPD, nasal lavage IL-8 increases significantly, but not IL-6 (Mallia 2006). The change in sputum IL-6 from baseline to exacerbation is greater if HRV is present (Seemungal 2000(b)), and absolute sputum IL-6 levels are higher at exacerbation if virus has been detected in nasal lavage (Rohde 2008). Thus although there are airway markers that may potentially indicate the presence of HRV at exacerbation, sputum is not readily available in all patients and serum IP-10 appears to be a novel and more appropriate marker.

The importance of the presence of HRV in the sputum at low concentrations in stable COPD was not associated with a heightened inflammatory response as measured by serum IP-10. In a study of adults using nasal wash, asymptomatic HRV positivity by PCR was found to be 22% (Wright 2007). Our assay detected HRV at a level of 1pfu/ml. None of the negative water controls were positive at this level however there was some cross reactivity with RSV and the standard curve was extrapolated to reach this point. These low levels were only detected at baseline, predominantly in NPS samples, and were not used in the exacerbation ROC analysis. This very sensitive cut off may account for the greater proportion of asymptomatic HRV positivity found at baseline in both the control population and COPD patients compared to other studies (Wright 2007). Other studies have used different primers and probes and have been designed to detect fewer strains of HRV. Both of these factors will lead to lower detection rates. Our HRV PCR was designed to detect as many different strains of HRV as possible and we sampled most of our baselines in the spring and autumn when HRV is most prevalent.

Viruses circulate all year round, and it is likely that COPD patients acquire virus frequently, possibly only at low levels, however only some strains of HRV demonstrate pathogenicity. The relevance of low levels of HRV in COPD is not clear and with increasingly sensitive methods of viral detection, the use of viral load in determining the significance of the presence of virus is increasingly important.

We used these data to suggest a cut off for viral load being significant at exacerbation, i.e. most likely causing the exacerbation. As symptoms are integral to the definition of a COPD exacerbation (Gold 2006), and coryzal symptoms a putative marker of virus positivity, it is reasonable to combine the presence of coryzal symptoms with IP-10 as a predictor of HRV positivity. The current data from the receiver operating characteristic (ROC) analysis presented indicate that serum IP-10 and the presence of a cold symptom could be used as a novel marker for HRV associated exacerbations. Cold illnesses are usually caused by respiratory viruses (Veckman 2009) and we found that cold associated exacerbations that were negative for HRV did not have increased serum IP-10 levels. This strengthens the case for IP-10 as a biomarker of HRV infection in the airway at exacerbation.

We have shown that COPD patients have higher serum IP-10 levels in the stable state than controls. IP-10 may also play a role in the inflammatory process of COPD. Expression of IP-10 and its receptor CXCR3 is higher in the airway in smokers with COPD (Grumelli 2004, Saetta 2002, Hardaker 2004), and IP-10 is present at higher levels in induced sputum in COPD patients compared to controls (Costa 2008). Our finding that IP-10 correlated negatively with pack years smoked indicates that local upregulation of IP-10 in the airways does not spill over in to the systemic circulation.

It is interesting that NPS load was not related to serum IP-10. We know that colds do not always lead to exacerbation (Hurst 2006) and the upper airway is difficult to sample.

We did not find a difference in IP-10 levels in frequent and infrequent exacerbators. We split frequent and infrequent exacerbators on 3 exacerbations detected on daily diary cards as our definition of an exacerbation includes both treated and unreported exacerbations. As approximately 50% of these exacerbations are associated with HCU, this reflects similar exacerbation rates to those observed in large clinical trials such as TORCH (Calverley 2007). Our finding of similar IP-10 levels in frequent and infrequent exacerbators may be explained by a number of factors. Firstly, frequent exacerbators are more likely to be colonised with bacteria in the stable state (Patel 2002). Our results indicate that IP-10 levels may actually be decreased in the presence of bacteria. Secondly, frequent exacerbators have a heightened inflammatory response at baseline; this may increase their susceptibility to HRV infection at this time. HRV

infection even at initial lower viral loads may trigger symptoms, replicate and thus trigger an exacerbation. Thirdly, perhaps complex cytokine interactions resulting from the already heightened inflammatory state in the frequent exacerbators at baseline have an inhibitory effect on IP-10 release.

10.2.6 Conclusion

We have shown for the first time that serum IP-10 increases from baseline to exacerbation in COPD specifically in exacerbations positive for human rhinovirus. In COPD exacerbations, sputum HRV load determined by quantitative RT-PCR correlates with IP-10 levels in the blood. IP-10 may be useful in detecting the presence of HRV and monitoring HRV exacerbation length and response to treatment. We did not find an increase in IP-10 in the presence of coryzal symptoms (a marker of viral infection) when HRV was not detected. We also did not find a relationship with serum IP-10 and RSV or the presence of a PPM. Sputum IP-10 was however higher in those with RSV or HRV positive exacerbations. In this study, neither serum CRP nor serum IL-6 correlated with HRV load with levels remaining low independent of high viral loads, thus indicating that they are poor markers of HRV positive COPD exacerbations.

In conclusion, we have shown that serum IP-10 can be used as a novel biomarker for HRV infection at exacerbation in COPD. Serum IP-10 is higher at baseline in COPD patients than controls and increases with age. Measurement of serum IP-10 may enable more rational therapy for COPD exacerbations and reduce morbidity from this disabling disease.

11

Conclusions and future work

11.1 Conclusions and future work

This thesis was undertaken to test the hypothesis that the frequent exacerbator is a distinct phenotype in COPD. This important subgroup of patients with COPD, who are known to have frequent exacerbations, with accelerated disease consequences, are often difficult to identify early. Ascertaining who these frequent exacerbators are early on allows modification of disease therapies to help modulate consequences of frequent exacerbations.

Prior to commencing this work it was known that frequent exacerbators have higher daily cough and sputum production (Burgel 2009), faster FEV_1 decline (Donaldson 2002, Kanner 2001), chronic dyspnoea and wheeze, chronic mucus hypersecretion (Zheng 2008), bacterial colonisation (Patel 2002), worse health - related quality of life (Seemungal 1998), a greater degree of airway inflammation with increased airway IL-6 and IL-8 levels (Bhowmik 2000) and more severe exacerbations associated with an increased risk of hospital admission.

This thesis has added to gaps in our knowledge allowing a greater understanding of this phenotype, which exists across all disease severities. Not only has this work provided further evidence for their existence, it has provided evidence that they exist as a phenotype; unrelated to severity of disease. We have shown that frequent exacerbators exist across all GOLD disease stages, with the proportion of the population as frequent exacerbators increasing with disease severity. It is unlikely that

there is one mechanism driving exacerbation frequency. Instead, this work provides a body of evidence that suggests the mechanisms differ between individuals and with different stages of disease. Patients with mild COPD are more likely to be frequent exacerbators due to poor social support and increased anxiety and depression, whereas those with more severe disease, with lower lung function reserve are likely to have a more inflammatory basis and infection susceptibility to their exacerbation frequency.

Interestingly, frequent exacerbators seem to know who they are, in that patients are able to identify and remember exacerbations, even over a time period of a year. And yet what is most striking, is that even though they know they have them, they do not always seek treatment for the events with both frequent and infrequent exacerbators remembering more exacerbations then they have treated, and both really actually having more exacerbations than they have treated. It seems that regardless of exacerbation frequency, there is a reluctance to seek clinician input every time. Clearly just the ability to recognise their exacerbation frequency status is not enough. More patient education, self management plans, and understanding of patient reluctance to seek clinical input is needed to minimise these events and maximise treatment in these phenotypes.

Frequent exacerbators are more likely to be women. Perhaps women are more open and honest about their symptoms, and understand the need for early detection and treatment of exacerbations. Or, perhaps they complain more, and have different expectations about quality of life compared to men. Whatever the underlying mechanism, this is an important finding in this study as the incidence of COPD is rising in women and they live longer than men.

Frequent exacerbators are more depressed than infrequent exacerbators and depression is more common in women and those with little social contact. Social support and health outlook are important in any chronic disease and appear to be important targets to address regarding exacerbation frequency in COPD. There is undoubtedly fear associated with exacerbation events, and this may include an element of denial and partly explain the underreporting of events seen when assessing patient recall. Whether it is that patients who are depressed notice their symptoms more and so have more exacerbations or that exacerbations get people down more and therefore cause them to be depressed is difficult to establish. What is important is that I think there is more than

just increased susceptibility to infection driving exacerbation frequency and a holistic approach to individual patients is paramount. How co-morbidities factor into all of this will be important to establish. Equally how depression and social factors influence health care utilisation and exacerbation treatment needs addressing.

Exacerbation frequency did not seem to be driven by social factors that may increase exposure to viral infections. We did not find a relationship between exacerbation frequency and social contacts; living with a spouse, contact with children, frequency of visitors to the home or number of days out of the house during the week. However, patients who had contact with children were more likely to have HRV in their sputum in the stable state. This suggests that patients (and/or their relatives) appreciate the importance of avoiding infection and avoid contact with individuals who have respiratory symptoms and obvious infection. Those patients with HRV in their sputum at baseline were not more likely to be frequent exacerbators suggesting that there is more than just susceptibility to infection driving exacerbation frequency. I suspect that mechanisms vary with disease severity, baseline inflammatory status, co-morbidities and perception and tolerability of symptoms.

Vitamin D deficiency appears to be a marker of disease severity and poor overall health and nutrition, rather than a driving factor for infection susceptibility and exacerbation frequency. COPD patients have lower vitamin D levels in the winter months than control subjects and show the same seasonal variation as the general population with lower levels associated with increasing disease severity. Vitamin D supplementation trials are needed to assess whether increasing vitamin D levels into the normal range will reduce exacerbation susceptibility, severity and even exacerbation frequency. The immunomodulatory effects and roles of vitamin D are clearly complicated but this study has shown that vitamin D receptor polymorphisms are not related to vitamin D levels, and do not play a role in exacerbation frequency in COPD. Whether or not the vitamin D binding proteins are important is yet to be established.

Genotyping studies are difficult to undertake, particularly to power adequately. One of the main difficulties with this work was recruiting sufficient numbers of patients on whom accurate exacerbation frequencies could be established for the genetic work. To date, this is the largest cohort of well characterised frequent and infrequent exacerbators

with COPD, and I think it is fair to conclude the negative findings in the genetics chapters are not due to lack of power, but rather the fact that too many other factors influence genetic polymorphisms in disease. Further work on these polymorphisms; Taq1 α1-antitrypsin promoter variant, K469E ICAM-1, IL-8 -251A>T and -781C>T, and IL-6 -174G>C, -597G>A and -572G>C is not warranted after these studies. None of the aforementioned polymorphisms were related to sputum or blood cytokine levels and again, this illustrates that there are multiple factors influencing disease in individual patients. Co-morbidities, timing of sampling, medications are just a few examples of factors that influence cytokine levels.

Equally the lack of a relationship between baseline cytokine variability and exacerbation frequency is likely to be related to the complex interactions in each individual patient associated with disease severity, co-morbidities, medications, and timing of sampling. Each individual has their own phenotype and therefore establishing a disease phenotype on top of this is challenging. Nonetheless, this work adequately concludes that baseline cytokine variability does not hold the key to exacerbation frequency and does not warrant any further study. This work has also shown that cytokine variability does not increase susceptibility to HRV infection.

Viruses are undoubtedly important pathogens in exacerbations to COPD. Susceptibility to viral infection, particularly HRV varies between individuals for a variety of reasons. The hypothesis that frequent exacerbators of COPD have increased susceptibility to HRV infection is logical, however is unlikely to be the driving factor in all patients. I suspect that in a subgroup of frequent exacerbators with COPD, HRV infectivity is important but more work is needed in this area to prove or refute the idea. HRV positivity at baseline in the COPD patients was more common in chronic sputum producers, ex-smokers and those with a more obstructive lung function ratio, indicating that this is important in a subgroup of patients. Studies investigating frequent exacerbators who are sputum producers or who have more severe disease are needed with regards to HRV. There may also be other viruses not yet detected that are important at this time. Further classification of the role of low levels of HRV; i.e. HRV associated versus HRV causation of exacerbations and the role of HRV at baseline; particularly with relevance to secondary bacterial infection and colonisation deserves

attention. Equally blood markers of viral infectivity such as IP-10 may prove useful screening tools in the future.

However it is unlikely there will ever be a single marker or predictor of exacerbation frequency. With an ever changing environment affecting viral growth and replication, it is possible that the viruses that are important now will not necessarily be as important in the future, and the interaction between pollution and meteorological factors associated with viral infection may provide further clues to environmental aspects of exacerbation frequency. It may be that changes in temperature or pollution rather than viruses themselves trigger exacerbations, or changes in levels of circulating viruses.

This thesis has provided evidence for the hypothesis that the frequent exacerbator is a distinct phenotype in COPD. It has illustrated the complex nature of exacerbation frequency and provides evidence that the mechanisms driving frequent exacerbations differ between individuals and with different stages of disease.

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NAME	
Study Number	

September 2006	NEXT APPOINTMENT
WORSENING SYMPTOMS? CALL US 07762 038662	/ / . am

THE LONDON COPD STUDY

DATE	1 fri	2 sat	3 sun	4 mon	5 tue	6 wed	7 thu	8 fri	9 sat	10 sun	11 mon
Peak Flow											
CHANGE in Symptoms											
CHANGE in Treatment											

DATE	12 tue	13 wed	14 thu	15 fri	16 sat	17 sun	18 mon	19 tue	20 wed	21 thu	22 fri
Peak Flow											
CHANGE in Symptoms											
CHANGE in Treatment											

DATE	23 sat	24 sun	25 mon	26 tue	27 wed	28 thu	29 fri	30 sat
Peak Flow								
CHANGE in Symptoms								
CHANGE in Treatment								

When there is no change or no reading – score through with a line or write nil. *Received by:* *Date:*

Instructions for filling in the DIARY CARDS

EVERY DAY…

1. After taking morning medications record the best of 3 attempts at the PEAK FLOW blowing test in the box on the sheet.

2. Please record any **CHANGE** to your usual treatment for as many days as it applies. Again, just put the appropriate letter in the box on the sheet.

Letter	Treatment
H	I am in Hospital.
I	I am taking more than usual INHALED STEROID (red / brown). **HOW MANY PUFFS?** eg. I2 for 2 puffs more than usual.
R	I needed to take extra RELIEVER (blue / green / grey / nebuliser). **HOW MANY PUFFS?** Write, eg 'R3' for 3 puffs, 'R2' for 2 etc
S	I am taking STEROID (Prednisolone) TABLETS. **HOW MANY TABLETS?** Write, eg 'S6' for 6 tablets, 'S5' for 5 etc
X	I am taking ANTIBIOTIC TABLETS. **PLEASE RECORD WHICH** (write the name on the diary card).

3. Please record any **WORSENING** of symptoms from your usual daily level. The symptoms we are interested in are listed below, just put the appropriate letter in the box on the sheet. Continue recording until the symptom has gone away or got back to the level you consider 'normal'.

Letter	Symptom
A	increased BREATHLESSNESS.
B1	increased SPUTUM COLOUR.
B2	increased SPUTUM AMOUNT.
C	a COLD (such as a runny or blocked nose).
D	increased WHEEZE or CHEST TIGHTNESS.
E1	SORE THROAT.
E2	increased COUGH.
F	FEVER.

If you experience a worsening in any one of these symptoms please phone us to arrange an assessment visit, and do this BEFORE starting any antibiotic or steroid tablets. The phone number is 07762 038662.

Jenni or James will have the phone and we can usually arrange to see you later the same day or the following morning. **It is best to phone first-thing in the morning.**

Center for Epidemiologic Studies Depression Scale (CES-D), NIMH

Below is a list of the ways you might have felt or behaved. Please tell me how often you have felt this way during the past week.

	During the Past Week			
	Rarely or none of the time (less than 1 day)	Some or a little of the time (1-2 days)	Occasionally or a moderate amount of time (3-4 days)	Most or all of the time (5-7 days)
1. I was bothered by things that usually don't bother me.	☐	☐	☐	☐
2. I did not feel like eating; my appetite was poor.	☐	☐	☐	☐
3. I felt that I could not shake off the blues even with help from my family or friends.	☐	☐	☐	☐
4. I felt I was just as good as other people.	☐	☐	☐	☐
5. I had trouble keeping my mind on what I was doing.	☐	☐	☐	☐
6. I felt depressed.	☐	☐	☐	☐
7. I felt that everything I did was an effort.	☐	☐	☐	☐
8. I felt hopeful about the future.	☐	☐	☐	☐
9. I thought my life had been a failure.	☐	☐	☐	☐
10. I felt fearful.	☐	☐	☐	☐
11. My sleep was restless.	☐	☐	☐	☐
12. I was happy.	☐	☐	☐	☐
13. I talked less than usual.	☐	☐	☐	☐
14. I felt lonely.	☐	☐	☐	☐
15. People were unfriendly.	☐	☐	☐	☐
16. I enjoyed life.	☐	☐	☐	☐
17. I had crying spells.	☐	☐	☐	☐
18. I felt sad.	☐	☐	☐	☐
19. I felt that people dislike me.	☐	☐	☐	☐
20. I could not get "going."	☐	☐	☐	☐

SCORING: zero for answers in the first column, 1 for answers in the second column, 2 for answers in the third column, 3 for answers in the fourth column. The scoring of positive items is reversed. Possible range of scores is zero to 60, with the higher scores indicating the presence of more symptomatology.

Appendix 3: Airway ICAM-1

Final protocols used for ICAM-1 and anti-cytokeratin staining

As a positive control, and to help identify problems with the protocol, a flask of HeLaOhio and a flask of BEAS 2B cells were grown to confluence (as described in **section 3.8** and stimulated with TNF-α (30ng/ml, overnight) to increase ICAM-1 expression. The cells were trypsanised and removed, made up to 10mls with GM and 30µl of this added to 570µl GM to make a 1:20 dilution. 75µl of this was added to a cryospin tube (enough to provide 10 slides for each cell type) and then spun at 450rpm for 6 minutes and left to dry. The remainder of the solution was spun at 1000rpm for 7 minutes to form a cell pellet. The supernatant was removed, the pellet resuspended in 1ml GM, spun again at 1000rpm for 5 minutes and the supernatant removed. The pellet was fixed in formalin for 2 hours and then dehydrated through graded alcohols and wax embedded as described previously in this section.

Immunofluorescence for cytospins

Protocol – anticytokeratin Ab

Cytospins were blocked in acetone/methanol or 4% PFA for 10 minutes and then washed 3 times in PBS for 5 minutes. They were then incubated in 10% NDS in PBS for 30 minutes and washed once for 5min in 1% NDS in PBS. Incubated in primary antibody (anticytokeratin) at room temperature in 1% NDS in PBS, washed twice for 5 minutes in PBS, then stained for 1 hour in a secondary antibody (Cy5) stained with DAPI at 1:10000 for 1 min and mounted in citiflour.

Protocol – ICAM-1

Cytospins were blocked in acetone/methanol or 4% PFA for 10 minutes, washed 3 times for 5minutes in TBS, incubated in 10% NDS in TBS 30 min and then washed once for 5 minutes in 1% NDS in TBS. They were then incubated for 1hr in primary Ab (ICAM-1; Leica) at room temperature in 1% NDS in TBS, washed twice for 5 minutes in TBS, then stained for 1 hour in secondary antibody (Cy5) stained with DAPI at 1:10000 for 1 min and mounted in citiflour.

Ni-DAB protocol

Samples:

a. HeLaOhio wax - anticytokeratin, negative, ICAM-1
b. BEAS2B wax - anticytokeratin, negative, ICAM-1
c. Biopsy wax - anticytokeratin, negative, ICAM-1
d. HeLaOhio cyto - anticytokeratin, negative, ICAM-1
e. BEAS2B cyto - anticytokeratin, negative, ICAM-1

Slides were dewaxed and rehydrated through reverse graded alcohols and left in distilled water for 5 minutes. Slides were placed in citrate buffer (1.05g citric acid in 500mls water-pH to 6 with NaOH; approx 3mls) and heated in the microwave on medium power for 10 minutes. The slides were allowed to cool in buffer for 20 minutes and the biopsies drawn around with an ImmEdge pen. Slides were washed 1x 5 min in TBS buffer (50mM Tris, 0.15M NaCl, pH 7.6), incubated in 10% normal donkey serum (in TBS) for 10 minutes and then in primary Ab (ICAM-1 Leica or anti-cytokeratin diluted 1:25 in 1% normal donkey serum in TBS for ICAM or PBS for anti-cytokeratin) for 1 hour at room temperature. Slides were washed 2x 5 min in TBS then incubated with peroxidise secondary antibody (1:200 in TBS) for 45 minutes. They were washed 3 times in TBS; once quickly, then for 2 minutes and 3 minutes. They were then incubated with Ni-DAB solution for 20 minutes, washed

twice with TBS quickly and then once for 5 minutes. Cells were dehydrated, mounted with DPX and coverslipped.

ICAM-1 biopsy analysis

Analysis of the biopsies was done using Adobe Photoshop CS4 adapting previously published methodology (Lehr 1997). A digital camera was attached to an Olympus microscope and images were transmitted to the computer. All images were obtained using x200 magnification with the camera on maximum zoom. The images were analysed using Adobe Photoshop CS4 (Adobe Systems; Mountain View, CA) for quantification. None of the images were altered in any way. The focus of the image was adjusted manually with the camera but there was no alteration in intensity of the image. Using the Crop tool in the Select menu of Photoshop, an area of epithelium was selected. Using the Similar command in the Select menu, all immunostained epithelium present on the picture was automatically selected. A colour plot of the selected area was generated using the Histogram tool in the Image menu. The mean staining intensity (in arbitrary units, AU) was recorded. Subsequently, the background was selected using the Inverse tool in the Select menu, and immunostaining was quantified using the Histogram tool in the Image menu. Immunostaining intensity was calculated as the difference between ICAM-1 immunostaining and background immunostaining and was designated immunocytochemical index with arbitrary units (AU).

Differences in staining intensity between COPD patients and controls and between frequent and infrequent exacerbators

There was no difference statistically in ICAM-1 expression between the COPD patients and controls; means 41.3 (13.6) and 26.7 (22.2) respectively; $p = 0.18$. There was no difference statistically in ICAM-1 expression between the infrequent and frequent exacerbators; means 34.0 (5.3) and 56.1 (13.7); $p = 0.24$.

Abstracts

1. **JK Quint**, DG Alber, JJP Goldring, GC Donaldson and JA Wedzicha. IP-10: A biomarker for cold associated COPD exacerbations. *ATS 2009*
2. **JK Quint**, DG Alber, JJP Goldring, GC Donaldson and JA Wedzicha. HRV load in stable COPD and controls *ATS 2009*
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