STUDIES OF COUGH IN IDIOPATHIC PULMONARY FIBROSIS

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Submitted in partial fulfilment of the Degree of Doctor of Medicine

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SUMMARY

A dry cough is a common symptom described in patients with IPF and impairs quality of life. The exact mechanisms causing cough in IPF remain unclear, however there is evidence that altered cough neurophysiology and sensitisation plays a role; IPF patients have an enhanced cough reflex sensitivity to the inhalation of capsaicin.

It was hypothesised that IPF patients have increased airway expression of the capsaicin receptor TRPV-1 and a co-expressed receptor TRPA-1. Bronchial biopsies were obtained in 16 IPF patients, 11 chronic cough patients and 8 controls. Quantitative PCR was used to detect TRPV-1 and TRPA-1 gene expression, with immunohistochemistry demonstrating protein expression. Mean TRPV-1 and TRPA-1 gene expression was higher in IPF patients compared with controls, but the difference did not reach statistical significance. Immunostaining supported these findings.

Gastroesophageal reflux is common in IPF patients and has also been implicated. An in-vitro study using cultured pulmonary epithelial cells was conducted to assess the expression of these receptors in a cell model of gastric reflux. TRPV-1 and TRPA-1 gene expression was demonstrated in pulmonary epithelial cells of bronchial and alveolar origin. No significant difference in receptor expression level was seen in either cell line when exposed to the major constituents of gastric refluxate.

This study suggests that a structural up-regulation of central airway TRP receptors is not the key mechanism for cough in IPF patients. Similarly, it does not support the role of the individual constituents of gastric refluxate resulting in cough hypersensitivity through a physical up-regulation of receptors in pulmonary epithelial cells. Overall this thesis outlines the complexity of the cough reflex. It is probable that cough in IPF results from the cumulative manifestation of various physiological changes and mechanisms.

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ABBREVIATIONS

6MWT	Six Minute Walk Test
ACE	Angiotensin-converting enzyme
ACE-i	Angiotensin-Converting Enzyme Inhibitor
AEC	Alveolar Epithelial Cells
AIP	Acute Interstitial Pneumonia
ANA	Anti-nuclear antibody
ANOVA	Analysis of Variance
ARS	American Thoracic Society
ASIC	Acid sensing Ion Channel
ATP	Adenosine Triphosphate
BAL	Broncho-alveolar Lavage
BDNF	Brain-derived neurotrophic factor
BIP	Bronchiolitis Obliterans with Interstitial Pneumonia
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
BTS	British Thoracic Society
CC	Chronic Cough
CD	Unconjugated chenodeoxycholic acid
cDNA	Complimentary DNA
CFA	Cryptogenic Fibrosis Alveolitis
CGRP	Calcitonin Gene-related Peptide
CNS	Central Nervous System
COP	Cryptogenic Organizing Pneumonia
COPD	Chronic Obstructive Pulmonary Disease

CPET	Cardiopulmonary Exercise Test
Cq	Cycle Quantity
CR1	Erythrocyte complement receptor 1
CRP	C-reactive protein
CT	Computerised tomography scanning
Ct	Crossing time
CXR	Chest X-Ray
DAB	3, 3-diaminobenzine tetrahydrochloride
DIP	Desquamative Interstitial Pneumonia
DMEM	Dulbecco's Modified Eagle's medium
DPLD	Diffuse Parenchymal Lung Disease
DRG	Dorsal Root Ganglion
EBV	Epstein-Barr virus
ECACC	European Collection of Cell Cultures
ECM	Extra-cellular matrix
ELISA	Enzyme-linked Immunosorbent Assay
EMT	Epithelial-Mesenchymal Transition
e-NANC	Excitatory NANC
ER	Endoplasmic Reticulum
ERS	European Respiratory Society
ESR	Erythrocyte sedimentation rate
FEV1	Forced expiratory volume in 1 second
FGF	Fibroblast Growth Factor
FIP	Familial Interstitial Pneumonia
FRC	Functional Residual Capacity

FVC	Forced Vital Capacity
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GD	Conjugated glycochenodeoxycholic acid
GIP	Giant Cell Interstitial Pneumonia
GOR	Gastro-oesophageal reflux
GWAS	Genome-wide Association Studies
HCl	Hydrochloric acid
HPRT	Hypoxanthine guanine phosphoribosyl transferase
HRCT	High-resolution Computed Tomographic Scanning
HRP	Horseradish peroxidase
ICD-9-CM	International Classification of Diseases, Ninth Revision, Clinical Modification
IgA	Immunoglobulin A
IHC	Immuno-histochemical
IIP	Idiopathic Interstitial Pneumonia
IL	Interleukin
ILD	Interstitial Lung Disease
i-NANC	Inhibitory NANC
IPF	Idiopathic Pulmonary Fibrosis
KCO	Transfer coefficient
LCQ	Leicester cough questionnaire
LDH	Lactate Dehydrogenase
LIP	Lymphoid Interstitial Pneumonia
LMP1	Latent Membrane Protein 1
LOD	Limit of Detection
MID	Minimal important difference

MIGET	Multiple inert gas Elimination Technique
MMP	Matrix Metalloproteinases
mRNA	Messenger RNA
MUC5B	Mucin 5B
NAC	N-acetylcysteine
NANC	Non-adrenergic, non-cholinergic
NC	Normal control
NCBI	National Centre for Biotechnology Information
NEB	Neuroepithelial bodies
NEP	Neutral endopeptidase
NGF	Nerve growth factor
NICE	The National Institute for Health and Care Excellence
NK	Neurokinin
NKA	Neurokinin A
NNOS	Neuronal isoform of NO synthase
NO	Nitric Oxide
NRQ	Normalized relative quantity
NSIP	Non-specific Interstitial Pneumonia
NTS	Nucleus Tractus Solitarius
OSA	Obstructive sleep apnoea
PAR2	Protease-activated 2 receptors
PEBC	Primary bronchial epithelial cell
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PDGF	Platelet-derived Growth Factor

PGE	Prostaglandin-E
PI	Propidium iodide
PKC	Protein Kinase C
PLC	Phospholipase C
PLG	Phase Lock Gel
PPFE	Idiopathic Pleuroparenchymal Fibroelastosis
PPI	Proton Pump Inhibitor
PS	Phosphatidyl- serine
QOL	Quality of Life
qPCR	Quantitative Reverse Transcription Polymerase Chain Reaction
RAR	Rapidy adapting receptors
RB-ILD	Respiratory Bronchiolitis-Associated Interstitial Lung Disease
RDCS	Research Design and Conduct Service
RIN	RNA integrity number
ROS	Reactive Oxygen Species
RT-PCR	Reverse transcription-PCR
RV	Residual Volume
SAR	Slowly adapting Receptors
SEM	Standard error of the mean
SFTP	Surfactant Protein
SP	Tachykinins substance P
TIFF	Tag Image File Format
TBP	TATA box binding protein
TBS-T	Tris-buffered saline and Tween 20
TERC	Telomerase RNA component

TERT	Telomerase reverse transcriptase
TGF	Transforming growth factor
THIN	The Health Improvement Network
TIMP	Tissue Inhibitors of Metalloproteinases
TLC	Total Lung Capacity
TLCO	Transfer factor for carbon monoxide
TMB	Tetramethylbenzidine
TNF	Tumour necrosis factor
TOLLIP	TOLL-Interacting Protein
TRP	Transient Receptor Potential
TRPA-1	Transient Receptor Potential Ankyrin-1
TRPM-8	Transient Receptor Potential Melastatin-8
TRPV-1	Transient Receptor Potential Vallinoid -1
TV	Tidal volume
UIP	‘Usual’ Interstitial Pneumonia
UK	United Kingdom
USA	United States of America
V/Q	Ventilation/Perfusion
VAS	Visual Analogue Score
VCA	Viral Capsid Antigen
VEGF	Vascular Endothelial Growth Factor
VIP	Vasoactive Intestinal Peptide
α-SMA	α-Smooth Muscle Actin

CHAPTER 1: INTRODUCTION

1.1 IDIOPATHIC PULMONARY FIBROSIS – BACKGROUND

Idiopathic pulmonary fibrosis (IPF) is the most common form of the idiopathic interstitial pneumonias and has been estimated to account for between 17- 37% of all interstitial lung disease cases (King et al. 2011; Nalysnyk et al. 2012) . IPF is a debilitating respiratory condition which is increasing globally (Hutchinson et al. 2015) and has no cure. There are currently very few treatments that are clinically and economically effective (Loveman et al. 2014), with supportive therapy the mainstay of treatment for most.

1.1.1 History and classification

IPF is one of the idiopathic interstitial pneumonias (IIPs), a group of diffuse parenchymal lung diseases (DPLDs), also known as the interstitial lung diseases. The IIPs are characterised by different patterns of inflammation and fibrosis.

Fibrosing lung diseases were initially recognised in the mid-19th century but it was Hamman and Rich who first published their clinical and pathological observations of rapidly fatal diffuse, idiopathic pulmonary fibrosis in four patients in 1935 (Hamman and Rich 1935; Homolka 1987). The term ‘Hamman-Rich Syndrome’ was subsequently applied, and despite the acute onset and rapid progression of disease observed in this series this is what many clinicians associated with idiopathic pulmonary fibrosis for several decades (Noble and Homer 2005). Over time it became recognised that there were a variety of potential aetiological factors or associations such as connective tissue diseases, environmental exposures and drugs, however there remained cases that appeared to be idiopathic. It was in the 1960’s that Scadding and Hinson proposed the term cryptogenic fibrosing alveolitis (CFA) for cases with defined clinical, pathological and radiological features but without clear aetiology (Scadding and Hinson 1967). This term was accepted throughout Europe however IPF was the preferred term in the USA, leading to the simultaneous use of these terms in reference to the same condition for a number of years.

In 1969 Liebow and Carrington proposed the first histopathological classification of idiopathic interstitial lung disease (Liebow 1969). Using experience of histological patterns they were able to divide interstitial pneumonias into five distinct groups; 'usual' interstitial pneumonia (UIP), desquamative interstitial pneumonia (DIP), bronchiolitis obliterans with interstitial pneumonia (BIP), lymphoid interstitial pneumonia (LIP), and giant cell interstitial pneumonia (GIP). At this time 'Hamman-Rich Syndrome' was considered an acute form of UIP known today as acute interstitial pneumonia (AIP). Liebow and Carrington believed that the histological characteristics were associated with clinical findings and also pathogenesis.

Other histological subtypes were described which resulted in a revised histopathological classification in 1998 by Katzenstein and Myers (Katzenstein and Myers 1998). Their classification includes five histopathologically distinct subgroups: UIP, which they felt was the 'characteristic pattern of IPF'; DIP now combined with respiratory bronchiolitis associated interstitial lung disease (RBILD), two smoking related diseases; AIP (formerly 'Hamman-Rich Syndrome') and non-specific interstitial pneumonia (NSIP). Katzenstein and Fiorelli described NSIP in 1994 after they had recognised a group of patients with a clinical course different from that of idiopathic pulmonary fibrosis (IPF) in which biopsies did not show features characteristic of UIP (Katzenstein and Fiorelli 1994).

Unfortunately, there are several problems with this histopathological classification. Firstly, histopathological patterns do not always correlate with the clinical or radiological picture. For example, the histological features of UIP are not unique to IPF but can be seen in patients with clinico-radiological features of other conditions such as drug or radiation induced disease, collagen vascular disease and asbestosis. Secondly it has been shown that surgical biopsies from different lobes of one lung may show different histological patterns; Flaherty et al. showed that 35% of patients with a histological pattern of UIP in any lobe had NSIP in other lobes but the prognosis was that of concordant UIP (Flaherty et al. 2001).

In 2002, in order to provide a distinction between the diagnostic terminologies of the disease entities e.g. IPF and the histological pattern (UIP) the American

Thoracic and European Respiratory Societies (ATS/ERS) issued a consensus classification. The ATS/ERS revised Katzenstein and Myers classification to emphasise the importance of an integrated clinical, radiological and pathological approach to the diagnosis of Idiopathic Interstitial Pneumonia (IIP). The Guideline group recommended that the term 'pattern' be added when referring to the lung biopsy pathologic pattern, to distinguish it from the clinico–radiologic–pathologic diagnosis (Agusti 2002).

In view of this universally accepted classification, in their 2008 update, the British Thoracic Society adopted the term IPF in favour of the historically preferred term CFA. It was felt that a true international consensus on terminology would not only improve communication between clinicians across the world but would also improve recruitment into large multinational studies and allow improved separation of conditions in terms of prognosis (Bradley et al. 2008).

More recently the ATS has provided an update to the 2002 ATS/ERS consensus classification (Travis et al. 2013). Although the main elements of the original classification are maintained, the major IIPs have now been distinguished from the rare and unclassifiable IIPs and have been sub-divided into chronic fibrosing, smoking-related and acute/sub-acute categories. Other major revisions include complete removal of the term CFA, acceptance of NSIP as a distinct clinical entity, thus removing its previous provisional status and the inclusion of idiopathic pleuroparenchymal fibroelastosis (PPFE); a rare IIP characterised by predominantly upper lobe fibrosis involving the pleura and sub-pleural lung parenchyma. Figure 1 outlines the current classification and its chronological development.

The ATS/ERS defines IPF as, '*a distinctive type of chronic fibrosing interstitial pneumonia of unknown cause limited to the lungs and associated with a surgical lung biopsy showing a histological pattern of UIP. Other causes must have been excluded and clinical findings must correlate*.' Consensus criteria for the diagnosis of IPF in the absence of a surgical lung biopsy have also been published by the ATS and are outlined later in this chapter. It is these definitions and criteria that will be employed throughout this thesis and used for the selection of patients in the studies (Agusti 2002).

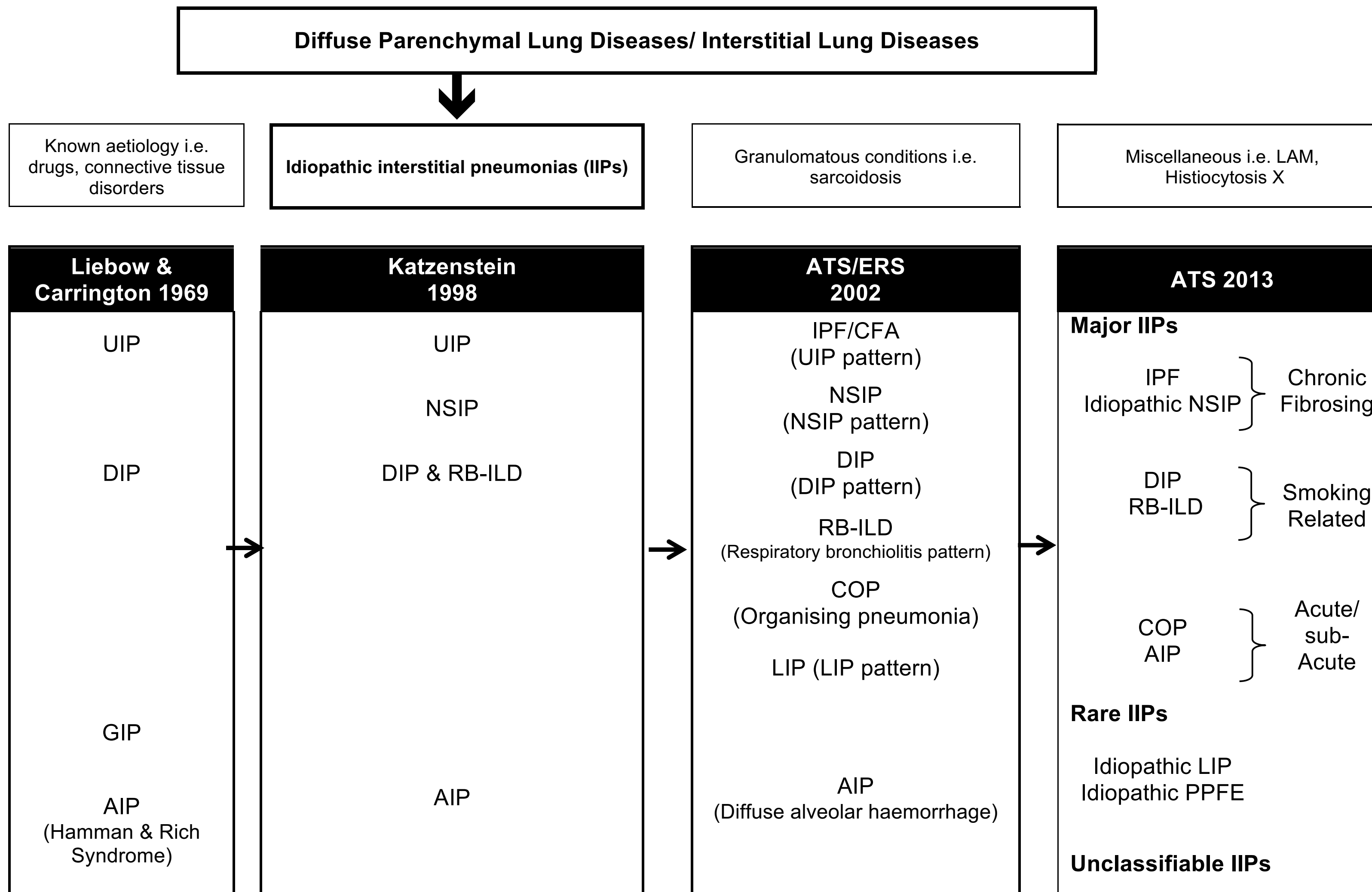


Figure 1 Classification of Diffuse Parenchymal Lung Diseases and chronological development of the sub-classification of the Idiopathic Interstitial Pneumonias. *Adapted from the ATS/ERS Guidelines* (Agusti 2002; Travis et al. 2013).

1.1.2 Epidemiology

Many of the studies investigating the epidemiology of IPF were carried out before the diagnostic criteria detailed in the 2002 ATS/ERS consensus statement, therefore data on prevalence and incidence is potentially inaccurate. Estimates also vary between studies as a result of variation in the definition of IPF used, the diagnostic criteria and study design (Nalysnyk et al. 2012; Kaunisto et al. 2013; Caminati et al. 2015; Hutchinson et al. 2015).

The incidence of IPF in the United Kingdom has most recently been estimated to be 8.65 (95% CI 8.40–8.90) per 100,000 person-years (Maher et al. 2013), and 7-16 per 100,000 person-years in the USA (Raghu et al. 2014). A study by Navaratnam et al. estimated a 5% annual increase in the incidence of IPF (Navaratnam et al. 2011). In general, European and Asian prevalence and incidence estimates appear to be lower relative to those reported in the US populations, although it remains unclear whether these are true differences or as a result of different methodologies (Caminati et al. 2015). A recent systematic review has however demonstrated an overall global increase in IPF incidence, with the rates in different countries starting to come together (Hutchinson et al. 2015).

UK prevalence data from computerised general practice records pre-dates the ATS/ERS criteria however estimates the prevalence to be 15–18 per 100,000 person-years (Bradley et al. 2008). The estimated prevalence in the USA is between 14 and 43 per 100,000 person-years (Raghu et al. 2014).

IPF incidence increases with age (Navaratnam et al. 2011) and is more common in male patients with a male to female ratio of 1.5-2.0:1 (Bradley et al. 2008). A reduced median survival is associated with older age at presentation and male sex (Gribbin et al. 2006; Navaratnam et al. 2011). IPF is a progressive condition which is ultimately fatal with a median survival of less than 4 years. Cause of death in most patients occurs from progression of lung fibrosis and respiratory failure rather than from commonly occurring co-morbid conditions as illustrated in Figure 2 (Ley et al. 2011).

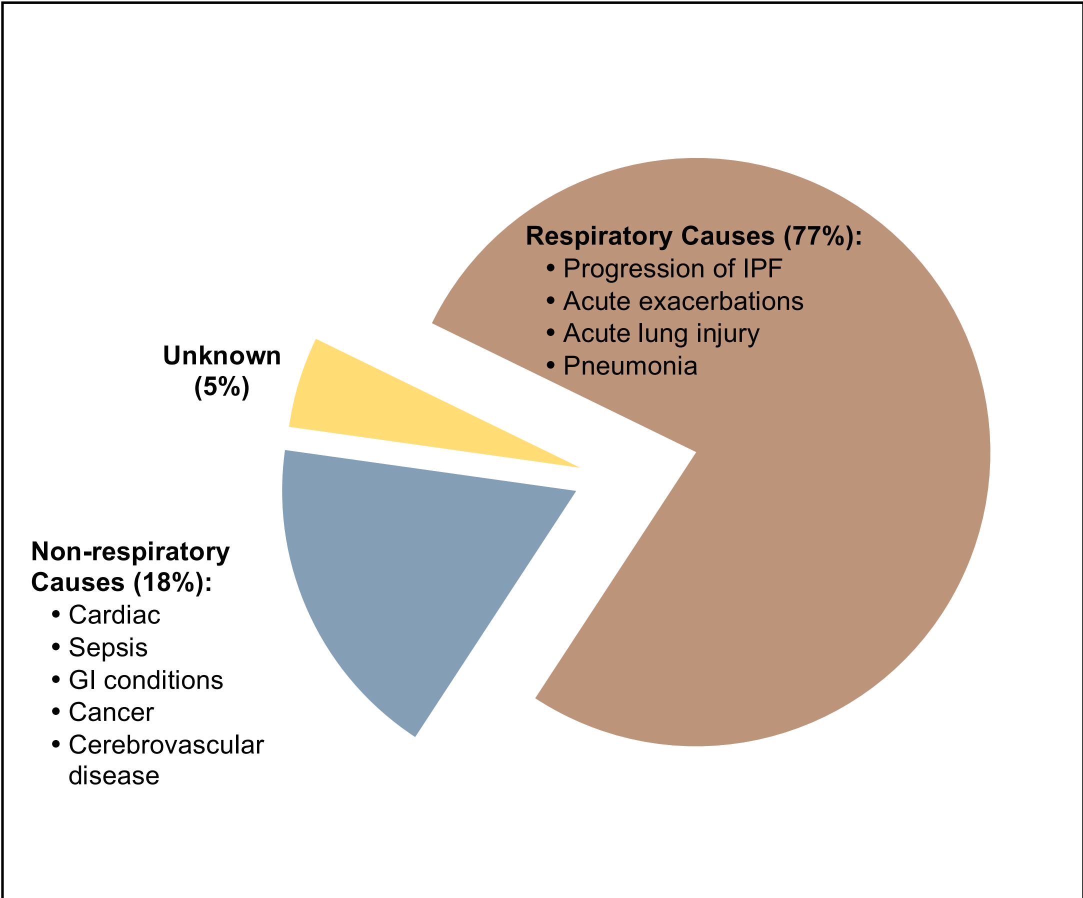


Figure 2 Causes of death in patients with IPF
Adapted from (Ley et al. 2011)

1.1.3 Aetiology

Idiopathic pulmonary fibrosis by definition is a condition of unknown aetiology, however a number of potential risk factors have been described. These include environmental exposures, genetic factors, and microbial agents as outlined below.

Environmental factors

Evidence suggests desquamative interstitial pneumonia (DIP) and respiratory bronchiolitis-associated interstitial lung disease (RB-ILD) are caused by cigarette smoking in susceptible individuals and smoking appears to be a risk factor for the development of idiopathic pulmonary fibrosis (Ryu et al. 2001). Two large case-control studies have examined this association. Hubbard et al. were first to report a statistically significant association between cigarette smoking and the development of IPF, with an odds ratio of 1.57 (Hubbard et al. 1996). An American multicentre study confirmed these results. The investigators found that 72% of patients with IPF had a history of smoking compared with 63% of case controls. The odds ratios were 1.6 (95% CI: 1.1-2.4) for ever-smokers, 1.9 (95% CI: 1.3-2.9) for former smokers and 2.3 (95% CI: 1.3-3.8) for smokers with a 21-40 pack-year history, suggesting a possible dose-dependent risk of developing IPF (Baumgartner et al. 1997).

Initial evidence suggested that current smokers had a survival advantage compared to former smokers and never smokers (King et al. 2001b). More recently however Antoniou et al reported that life-long non-smokers have a better outcome than former smokers as well as former smokers and current smokers combined. They found that adjusting baseline severity highlighted the survival advantage of non-smokers and concluded that the observation of higher survival in IPF among current smokers is likely to be the result of 'a healthy smoker effect' (Antoniou et al. 2008)

As well as cigarette smoking a meta-analysis of case-studies investigating the occupational and environmental risk factors for IPF identified that exposures to agriculture/farming (OR: 1.65), livestock (OR: 2.17), wood dust (OR: 1.94), metal

dust (OR: 2.44) and Stone dust (OR: 1.97) showed an increased risk of developing IPF (Taskar and Coultas 2006), with evidence of a significant exposure-response effect (Hubbard et al. 1996).

Genetic factors

Evidence suggests that genetic factors predispose to the development of IPF, with familial cases presenting the most convincing evidence for a genetic predisposition, with numerous reports of familial clustering and disease in monozygotic twins raised in different environments (Peabody and Hayes 1950; Hughes 1964; Hodgson et al. 2002). The mode of genetic transmission of susceptibility to pulmonary fibrosis in familial cases is likely to be autosomal-dominant with incomplete penetrance (Musk et al. 1986; Steele et al. 2005). The exact prevalence of familial pulmonary fibrosis is unknown; however studies suggest that familial disease accounts for between 0.5-3.7% of all IPF cases (Marshall et al. 2000; Hodgson et al. 2002).

Several gene mutations have been studied in patients with familial pulmonary fibrosis and IPF including surfactant proteins C and A2 (SFTPC and SFTPA2, respectively). These genes are expressed by type II alveolar epithelial cells in the lung, and the molecular mechanism thought to result from these mutations involves failure in the processing and/ or secretion of surfactant protein C and increased endoplasmic reticulum (ER) stress for SFTPC and SFTA2 mutations respectively, which results in epithelial cell injury (Nogee et al. 2001; Lawson et al. 2004; Lawson and Loyd 2006; Wang et al. 2009). Mutations in telomerase re-verse transcriptase (TERT), and telomerase RNA component (TERC), which are genes involved in telomere length maintenance have also been studied. Dyskeratosis congenita is a disease characterised by telomerase dysfunction and telomere shortening, and it was the association between this condition and pulmonary fibrosis that first led to the discovery that telomerase dysfunction may play a role in pulmonary fibrosis (Garcia 2011). Several researchers have since reported mutations of the gene encoding the protein component of telomerase (TERT) and gene encoding the RNA component (TERC) as being associated with pulmonary fibrosis, with up to 15% of patients with familial pulmonary fibrosis

and 3% of IPF patients having a mutation present (Armanios et al. 2007; Tsakiri et al. 2007; Cronkhite et al. 2008).

As well as studying patients with familial pulmonary fibrosis to gain insights into the genetic factors predisposing to IPF, several investigators have also studied a number of genes, identifying genetic polymorphisms. A recent link between a polymorphism in the promoter of the gene encoding a mucin (*MUC5B*) and IPF has been established (Cronkhite et al. 2008; Seibold et al. 2011; Zhang et al. 2011). The prevalence of this polymorphism was found to be high in familial (34%) and idiopathic (38%) pulmonary fibrosis with IPF patients significantly more likely to have at least one copy of the polymorphism compared with controls, and MUC5B expression in the lung was 14 times higher in these patients (Seibold et al. 2011). This susceptibility polymorphism has since been confirmed in two large genome-wide association studies (GWAS) (Fingerlin et al. 2013; Noth et al. 2013). The role of MUC5B in the development of IPF remains unclear, however as mucins provide barrier protection and are important in innate immunity it is believed that immune dysregulation or abnormal mucin expression may play a role (Parker and Prince 2011; Plantier et al. 2011). Despite the association of this polymorphism with increased susceptibility, a number of researchers have identified that its presence is also associated with a more favourable IPF phenotype (Yang et al. 2013), an improved survival (Peljto et al. 2013), and a trend to a slower decline in FVC (Stock et al. 2013). Interestingly another polymorphism in the gene for TOLLIP (Toll-interacting protein), has been found to be protective against the development of IPF but if the condition develops despite this protective allele it confers an increased risk of disease progression and mortality (Noth et al. 2013).

The genetics of IPF are complex and are in an early phase of development but have already improved our understanding of IPF susceptibility and pathogenesis. It is likely that combinations of polymorphisms and environmental exposures result in different gene expression profiles which may influence susceptibility and clinical phenotype. Future research may enable a better understanding of the molecular mechanisms and allow the development of therapies for prevention or treatment of IPF (Spagnolo et al. 2014). A more detailed review is provided in the

publications by Spagnolo et al and Mathai et al (Mathai et al. 2014; Spagnolo et al. 2014).

Microbial agents

The discovery of polymorphisms in genes controlling innate immunity has increased interest in the potential role of bacteria in the pathogenesis and progression of IPF. Recent studies have characterised the 'microbiome' of IPF and found an increased bacterial burden (Molyneaux et al. 2014), which may be associated with disease progression (Han et al. 2014).

Extensive research has also been performed to investigate a possible viral aetiology of IPF, as recently summarised by Moore et al (Moore and Moore 2015). The human herpes viruses; specifically Epstein-Barr virus (EBV) has been implicated. Several studies have reported an increased rate of detection of the virus in patients with IPF (Vergnon et al. 1984; Stewart et al. 1999; Manika et al. 2007). Vergnon and colleagues first made the association in a study which identified raised serum Immunoglobulin A (IgA) levels against viral capsid antigen (VCA) in IPF patients compared with controls (Vergnon et al. 1984). Egan et al. later demonstrated EBV replication within type II alveolar epithelial cells of patients with IPF (Egan et al. 1995), and this data was supported by evidence of EBV DNA in lung tissue of patients with IPF using PCR analysis (Stewart et al. 1999).

Despite accumulating evidence showing an association, there remains no clear causal link. However, a recent study looking at asymptomatic first-degree relatives of patients with familial interstitial pneumonia (FIP) has provided supportive evidence. Kropski et al. found increased herpesvirus DNA and antigen expression in alveolar epithelial cells in family members at risk, and this correlated with evidence of endoplasmic reticulum stress (Kropski et al. 2015). Tsukamoto and colleagues have also found that EBV can be associated with a poorer prognosis in IPF patients. They found that patients positive for Latent membrane protein 1 (LMP1), an EBV associated protein that is expressed on the surface of EBV infected cells, died more frequently from respiratory failure than LMP1 negative patients (Tsukamoto et al. 2000).

Other viral infections such as hepatitis C and adenovirus have also been implicated in the aetiology of IPF, however the observations have been inconclusive (Egan et al. 1997).

Gastro-oesophageal reflux

Gastro-oesophageal reflux (GOR) and micro-aspiration has been linked with a number of respiratory conditions and several studies have identified GOR as a possible risk factor for the development of IPF (El-Serag and Sonnenberg 1997). Patients with IPF have been found to have a high prevalence of GOR and hiatus hernia, and are often asymptomatic (Tobin et al. 1998; Patti et al. 2005; Raghu et al. 2006a; Sweet et al. 2007; Noth et al. 2012). Despite evidence of an association between GOR and IPF, a causal relationship has yet to be established (Hershcovici et al. 2011).

A UK study found that 12% (108/920) of patients diagnosed with IPF identified via The Health Improvement Network primary care database (THIN) had a prior diagnosis of GOR and were more likely to be taking antacids (OR 1.71, 95% CI 1.44-2.02) or histamine-2 receptor antagonist therapy/proton pump inhibitor (OR 2.22, 95% CI 1.89-2.60) (Gribbin et al. 2009). This study, although case-controlled, was not prospective and therefore relied on general practitioner diagnoses and accuracy of data entry.

Given that IPF results in reduced lung compliance it is possible that a reduced intra-thoracic pressure during inspiration results in lower oesophageal sphincter dysfunction and, or oesophageal dysmotility leading to GOR; therefore being a consequence of IPF rather than the cause. However, the study by Savarino et al. did not find a significant correlation between the degree of fibrosis and the number of reflux episodes in non-IPF patients with pulmonary fibrosis as it did in IPF patients, which provides evidence to suggest that GOR in IPF patients may not be the consequence of pulmonary stiffness and reduced compliance (Savarino et al. 2013).

Human and animal studies have shown that fibrosis can occur following aspiration of gastric contents (Moran 1955; Sladen et al. 1971). More recently in

vitro studies have shown that exposing bronchial epithelial cells to the bile salt chenodeoxycholic acid; a constituent of gastric refluxate, results in Transforming growth factor- β (TGF-β) induced fibrosis (Perng et al. 2007; Perng et al. 2008) With preliminary work demonstrating epithelial-mesenchymal transformation (a process involved in the pathogenesis of IPF) in airway epithelial cells upon exposure to pepsin (Ahmad et al. 2009). Studies have also shown that fibrosis and inflammation is independent of the acidity of the gastric fluid (Downing et al. 2008; Mertens et al. 2010) and may be related to pepsin and bile salts (Samareh Fekri et al. 2013). Interestingly, an association has been demonstrated between IPF and non-acid GOR as well as acid reflux (Savarino et al. 2013). Savarino and colleagues found that when compared with non-IPF ILD patients and normal controls, IPF patients had increased weakly acid episodes of GOR in the distal and proximal oesophagus as well as increased acid episodes. They also identified that IPF patients were at higher risk of aspiration as IPF patients were more likely to have bile acids and pepsin in saliva and bronchoalveolar lavage fluid than patients in the two control groups. The number of proximal and distal reflux events, concentration of pepsin and bile salts in BAL fluid also correlated with the degree of pulmonary fibrosis based on HRCT score, strengthening the evidence for an association between IPF and GOR (Savarino et al. 2013). A further study has confirmed this high prevalence of non-acid reflux in IPF patients (Kilduff et al. 2014).

Recent studies have also shown that GOR and micro-aspiration may also play a role in acute exacerbations of IPF. Lee and colleagues detected pepsin, a previously identified biomarker for GOR in bronchoalveolar lavage (BAL) fluid obtained from IPF patients. They studied 24 cases with acute exacerbations and 30 stable controls; finding pepsin levels to be significantly higher ($p = 0.04$) in fluid from patients during an acute exacerbation. BAL pepsin levels were not predictive of survival, but this study supports the hypothesis that micro-aspiration and reflux of gastric juice into the lung parenchyma is involved in IPF pathogenesis (Lee et al. 2012; Raghu and Meyer 2012). A study by Tcherakian et al. supports this having recently found that patients with asymmetrical disease were more likely to have GOR and suffer exacerbations compared with patients with symmetrical disease. In the patients with asymmetric disease the right side was predominantly

affected with the majority of patients having a preference for sleeping on the worst affected side (Tcherakian et al. 2011).
A number of studies have reported an association between treatment of GOR and clinical outcomes in IPF, however the results are conflicting. A small case series by Raghu and colleagues first reported IPF patients treated with a proton pump inhibitor (PPI) and/or anti-reflux surgery (Nissen fundoplication) had stable or improved pulmonary function tests over a 4 year period (Raghu et al. 2006b). A larger study of 204 patients, identified anti-reflux medication use in IPF as an independent predictor of longer survival as well as being associated with a lower radiological fibrosis score on high resolution computed tomography (Lee et al. 2011), and a subsequent study demonstrated patients who received antacid therapy had a significant reduction in lung function deterioration compared to those that did not receive treatment (Lee et al. 2013a). The most recent study, which was a post-hoc analysis of IPF patients from the placebo groups of three clinical trials (CAPACITY 004, CAPACITY 006, and ASCEND) using pirfenidone (discussed further in section 1.1.7), did not support the previous findings. This study of 624 patients did not demonstrate improved outcomes in IPF patients who had antacid treatment but did show that in patients with ‘advanced IPF’ (FVC < 70%), antacid treatment was associated with increased infection rates (Kreuter 2016). Long-term, randomised-controlled trials are needed to investigate this further.

Present guidelines advise that some but not all patients with IPF and asymptomatic GOR need anti-reflux medication and that clinical trials looking at treatment for GOR are required (Raghu et al. 2011). However, a recent study has shown that aggressive acid suppression therapy may actually increase non-acid reflux (Kilduff et al. 2014). Some authors suggest that anti-reflux surgery should be considered early for patients with IPF (Allaix et al. 2014).

1.1.4 Clinical features

Exertional dyspnoea of insidious onset is the most common presenting feature of IPF and although, typically progressive, the rate of progression in individual patients can be variable (Turner-Warwick et al. 1980; Panos et al. 1990). An irritating dry cough frequently accompanies dyspnoea at presentation, and has

been found to be prevalent in 73-84% of patients (Turner-Warwick et al. 1980; Ryerson et al. 2011). Both symptoms can be debilitating and impair quality of life (French et al. 1998; Swigris et al. 2005). The exact mechanism causing cough in IPF and its link to the pathogenesis remain unclear. Constitutional symptoms such as lethargy and weight loss can be associated with IPF, however other causes should usually be excluded if these symptoms are a prominent feature (Schwarz and King 2010).

Patients typically present over the age of 60 years (Raghu et al. 2011). Clinical examination usually reveals fine, high-pitched crackles at the end of inspiration on chest auscultation. Crackles are said to be characteristic of IPF and are present early in the course of disease; typically audible at the bases of the lungs and extending to the mid and upper zones with disease progression (Baughman et al. 1991; Cottin and Cordier 2012). Finger clubbing, defined as '*the painless, uniform swelling of the soft tissues of the terminal phalanx*', is also a common feature, being present in up to 50% of cases (Shneerson 1981; Meltzer and Noble 2008). Former or current smokers are more likely to have clubbing as a presenting feature (Schwarz and King 2010). Patients may appear dyspnoeic at rest and cyanosed, however this is usually a late sign indicative of advanced disease (Schwarz and King 2010). Abnormalities on cardiac examination such as a loud P2 and right ventricular heave may also be present indicating pulmonary hypertension; common in advanced IPF and which significantly reduces survival (Hunninghake et al. 2013).

1.1.5 Investigations to confirm the diagnosis of IPF

The possible findings on investigation are outlined below. Current guidelines recommend a systematic, multi-disciplinary approach to the investigation of patients with suspected IPF (Raghu et al. 2011; Raghu et al. 2015).

Laboratory tests

Routine laboratory studies are nonspecific for a diagnosis of IPF. Hypoxaemia on pulse oximetry or blood gas testing is a common finding in IPF patients and is usually monitored at regular intervals to assess the need for supplemental

oxygen. Unlike other conditions causing chronic hypoxia, erythrocytosis is infrequent in IPF patients, with studies suggesting ineffective erythropoiesis (Tsantes et al. 2003).

Early studies suggested that patients with IPF had high levels of auto-antibodies and serological abnormalities that are usually associated with connective-tissue disorders; however patient selection in these studies may hamper interpretation of the data as they were undertaken prior to the currently appreciated classification of IPF (Crystal et al. 1976; Turner-Warwick et al. 1980). Recent studies show the frequency of circulating auto-antibodies to be similar in IPF patients and controls and low titre positive rheumatoid factor and/or ANA may occur in the absence of connective tissue disease (Lee et al. 2013b).

Serum lactate dehydrogenase (LDH) activity and surfactant protein A and D (SP-A and SP-D) levels have been shown to be raised in IPF patients, and to some extent are predictive of prognosis/mortality, however have not been validated sufficiently, and are therefore not used routinely (Matusiewicz et al. 1993; Greene et al. 2002; Kinder et al. 2009). CRP and erythrocyte sedimentation rate (ESR) may also be non-specifically raised as part of an acute phase response.

Recent studies have investigated the possible link between the coagulation cascade and the pathogenesis and or treatment of IPF. A large population based study in the UK identified that IPF patients were significantly more likely to have a pro-coagulant state compared with controls and those with two or more coagulation defects had an increased risk of death (Navaratnam et al. 2014) A study by Bargagli et al showed that this pro-coagulant state is more evident in those with an acute exacerbation compared to patients with stable disease (Bargagli et al. 2014). The significance of these results is yet to be fully understood and testing of these factors remains for research purposes only at present.

Lung function testing

Spirometric expiratory flow rates; forced expiratory volume in 1 second (FEV1) and forced vital capacity (FVC) are often reduced in IPF due to the reduction in

lung volumes, however due to increased elastic recoil maintaining the static airway dimensions flow rates are often higher than expected for the given lung volume. As the FEV1 and FVC are reduced in proportion to the other, the FEV1/FVC ratio is maintained, resulting in a restrictive picture as illustrated in Figure 3 (Schwarz and King 2010). This is not the case in current smokers in whom the FEV1 tends to be reduced relative to the FVC (Schwartz et al. 1991).

Lung volumes may be within normal limits early in the disease, however due to the fibrotic process the total lung capacity (TLC), functional residual capacity (FRC) and residual volume (RV) often become reduced proportionally. The pressure-volume curve is shown in Figure 4 and illustrates the higher trans-pulmonary pressure at any given volume in IPF patients compared with controls. This is a result of increased elastic recoil in fibrotic lung; the lung becomes stiff and non-compliant (Schwarz and King 2010; West 2013). Airway resistance is normal or decreased due to fibrosis causing increased radial traction on the airways at an often lower lung volume resulting in larger airway calibre (West 2013).

Gas exchange is abnormal in IPF patients with a reduced arterial PO_2 and an elevated alveolar-arterial oxygen tension gradient ($P_{(A\text{-}a)} O_2$). PH and Pco_2 are usually normal; however Pco_2 can be reduced as a consequence of alveolar hyperventilation. In terminal respiratory failure the Pco_2 may rise causing a reduction in pH.

The gas transfer factor (TLCO) is often reduced in IPF with partial correction when adjusted for alveolar volume (KCO) (Agusti et al. 1991). The reduced Po_2 and TLCO is predominantly a consequence of inequality of ventilation and blood flow within the lung. Ventilation-perfusion abnormalities (V/Q Ratios) have been identified using the multiple inert gas elimination technique (MIGET) (Wagner et al. 1976). The diffusion impairment between the alveolus and capillary also plays a role. This is a consequence of thickening of the alveolar-capillary membrane, a reduction in pulmonary capillary blood flow and a reduction in the average alveolar surface area as a result of the destructive, fibrotic process (Weibel 1973; Agusti and Barbera 1994; West 2013).

Baseline spirometry may be helpful in predicting prognosis; however serial measurements have been more reliably associated with mortality. Several studies have shown an association between a decline in FVC and reduced survival. A decline in FVC of 10% over a 6 month period has been regarded as clinically significant (Latsi et al. 2003; Egan et al. 2005) Baseline TLCO of < 40% predicted is an independent predictor of survival, with this cut-off suggested for the definition of advanced disease. A decline in TLCO of more than 15% over a 6-12 month period also predicts a worse survival, although this has not been found consistently (Mogulkoc et al. 2001; Flaherty et al. 2003a; Egan et al. 2005; King et al. 2005). Current British guidelines suggest appropriate patients who meet these criteria be referred to a transplant centre (Bradley et al. 2008).

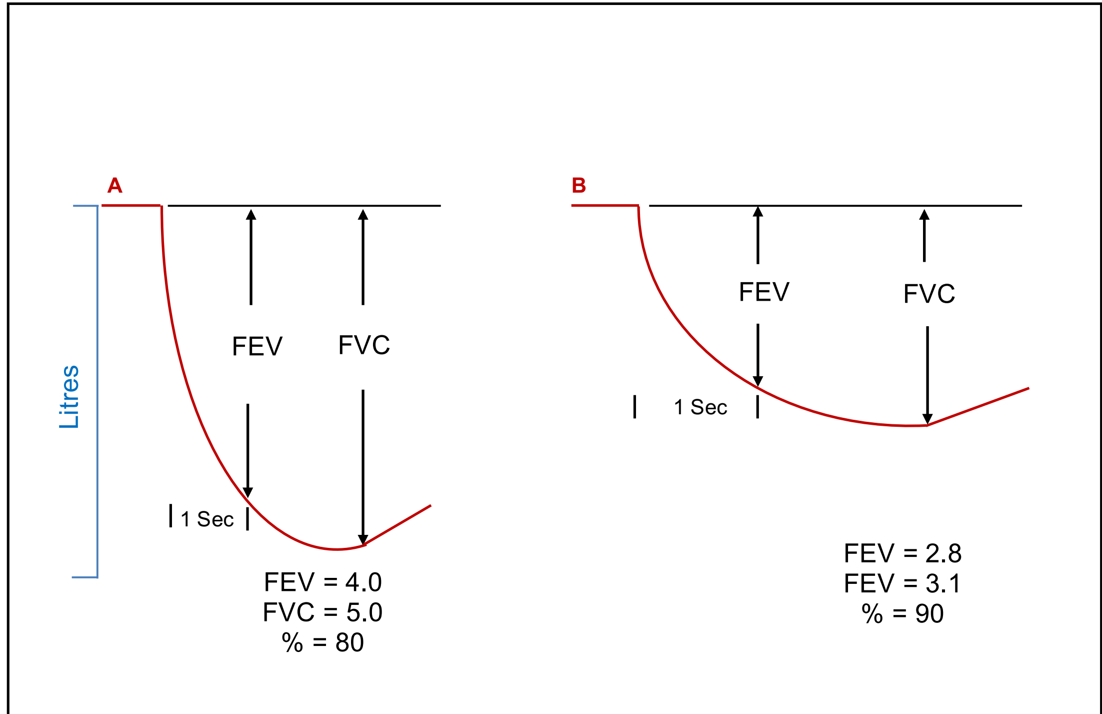


Figure 3 **Spirometric Picture in IPF**
Normal (A) and restrictive (B) patterns of a forced expiration.
Adapted from Pulmonary Pathophysiology (8th), (West 2013)

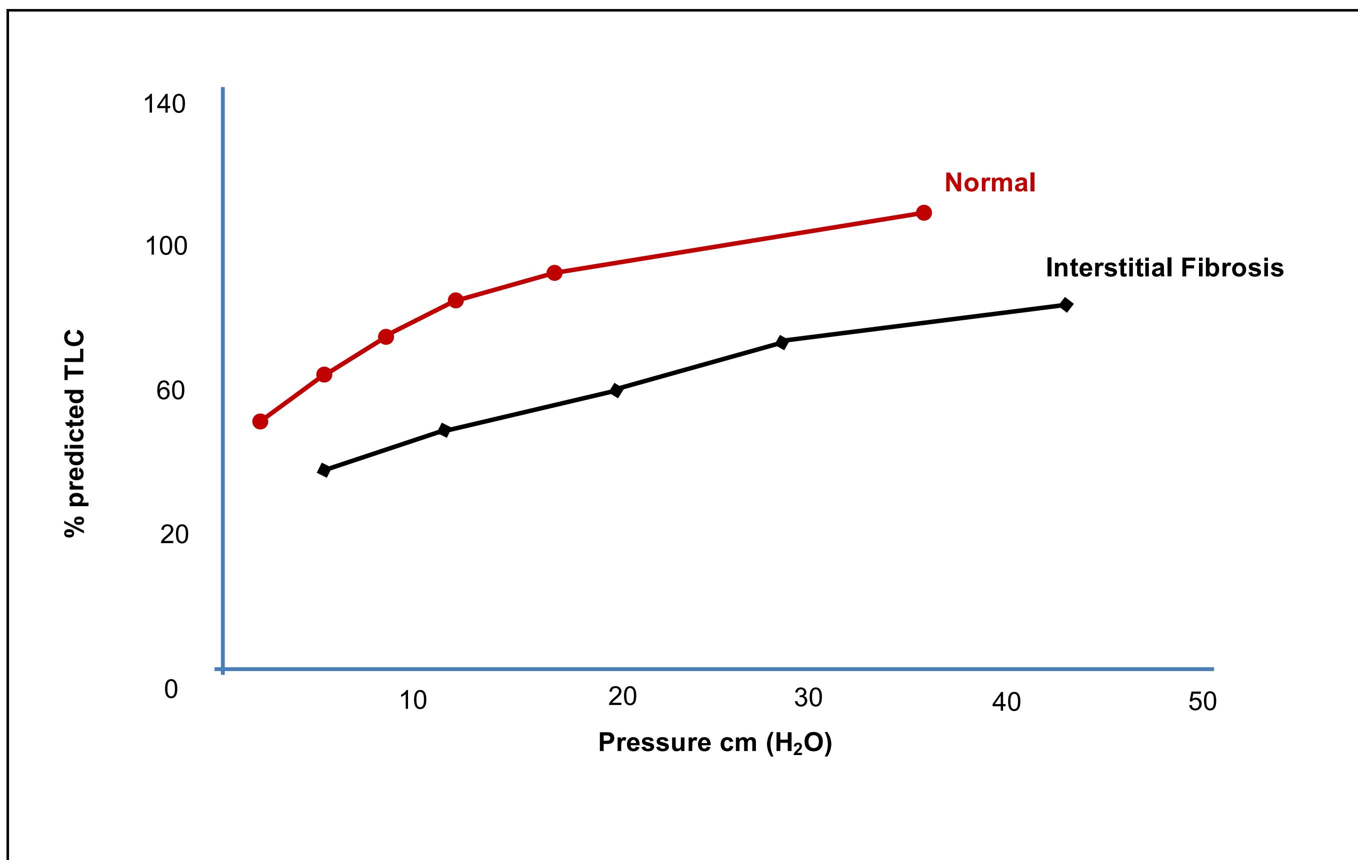


Figure 4 **Pressure-volume curve in IPF**
Pressure-volume curves of the Lung. Note the flattened curve for interstitial fibrosis
Adapted from Pulmonary Pathophysiology (8th), (West 2013)

Exercise testing

Lung function abnormalities are more evident during exercise than at rest even in patients with mild IPF (Lama and Martinez 2004). With exercise the ($P_{(A-a)} O_2$) widens, with an associated reduction in the arterial Po_2. Rather than increasing ventilation during exercise by increasing tidal volume (TV) as usually occurs in normal subjects, IPF patients typically have an increased respiratory rate, which results in a reduced Pco_2. The gas exchange abnormality during exercise in IPF has been shown to be associated with an increase in diffusion impairment. Using the MIGET, Agusti and colleagues identified at rest 80% of arterial hypoxaemia was due to V/Q mismatch with 20% due to a diffusion impairment of oxygen; however, during exercise these figures were 60% and 40% respectively (Agusti et al. 1991; West 2013). Other factors contributing to an impaired ventilatory response to exercise include a failure to decrease ventilatory dead space; a rapid, shallow breathing pattern and low mixed venous O_2 (Hansen and Wasserman 1996).

Several studies have examined gas exchange in IPF using the 6-minute-walk test (6MWT) and cardiopulmonary exercise tests (CPET). Some studies have shown that oxygen desaturation below 88% during 6MWT and a maximal oxygen uptake during CPET (VO_2max) of less than 8.3 ml/kg/min at baseline predicts mortality in patients with IPF (Enright 2003; Lama et al. 2003; Fell et al. 2009).

Radiological findings

A chest radiograph may be normal in early IPF, however it is abnormal in the majority of patients with interstitial lung disease (Epler et al. 1978). An abnormal chest X-ray may be an incidental finding in an asymptomatic patient or the first investigation alerting the physician to the possibility of a diagnosis of IPF in a patient presenting with dyspnoea or cough (Baughman and Du Bois 2004). The classical radiographic finding in patients with IPF is bilateral reticular shadowing; linear or curvilinear densities which form a net-like pattern (figure 5). Less commonly, nodular opacities are seen and the pattern is described as reticulo-nodular (McLoud et al. 1983). The distribution is typically diffuse with a sub-pleural and basal predominance. The reticular lines vary in diameter and the

patterns produce differing textures. Honeycombing, seen on CXR as a group of ring shadows, are formed by a cluster or row of cysts and are often seen late in disease (Crystal et al. 1976; Lynch and Newell 2000; Hansell et al. 2008). These radiographic changes are often associated with evidence of reduced lung volumes, however if chronic obstructive pulmonary disease (COPD) is also present the lung volumes may appear normal or hyper inflated (Lynch and Newell 2000). Chest radiography can be used sequentially to gauge disease progression and assess for complications of IPF such as; pneumothorax, lung cancer, heart failure and infective exacerbation (Bradley et al. 2008).

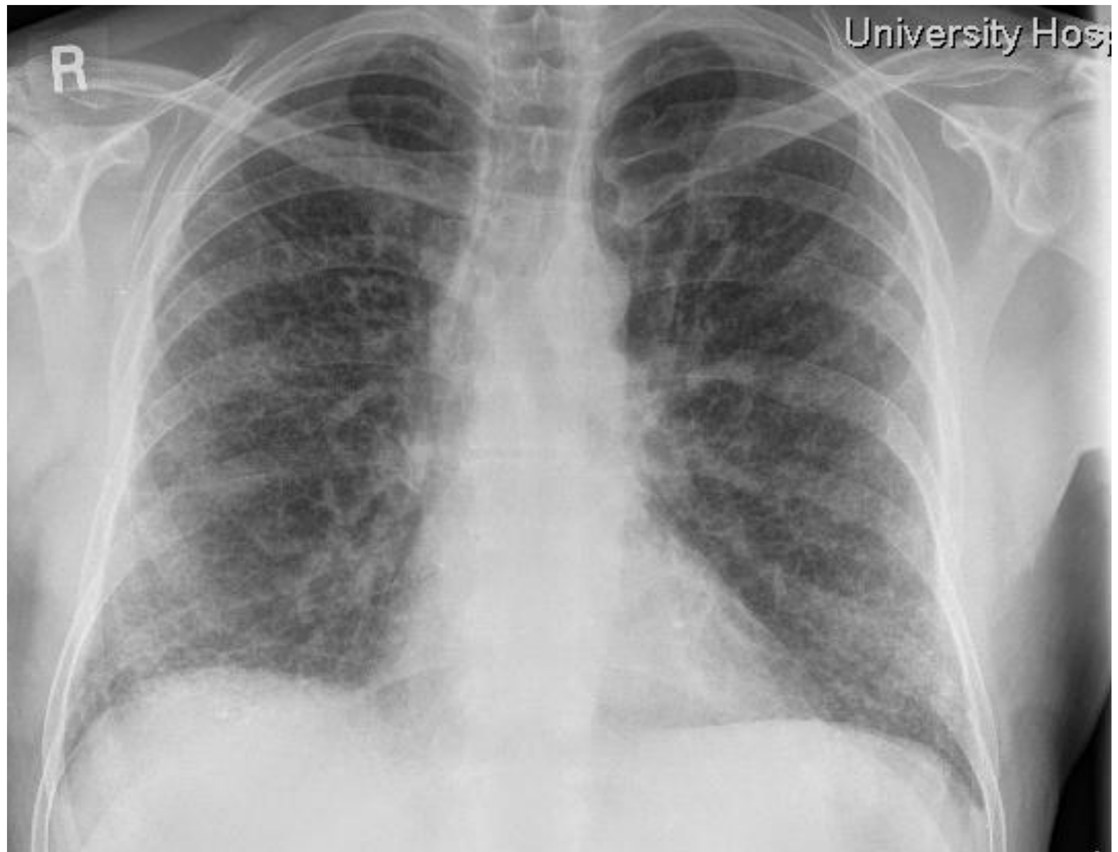


Figure 5 Chest radiograph from a study patient with IPF demonstrating bilateral reticular nodular shadowing predominantly at the bases.

High-resolution computerized tomographic scanning (HRCT) is an integral part of the investigation and diagnosis of a patient with suspected IPF. HRCT provides an assessment of the nature and extent of parenchymal abnormalities and allows a confident non-invasive diagnosis in a considerable proportion of patients (Raghu et al. 2011). A normal HRCT excludes IPF in the majority of cases and is more sensitive than CXR alone (Mathieson et al. 1989; Orens et al. 1995).

The typical HRCT features of the UIP pattern diagnostic of IPF are summarised in Figure 6. Characteristically on HRCT there is a sub-pleural, basal reticular pattern which is often patchy and can be asymmetrical (Tcherakian et al. 2011). Traction bronchiectasis and or bronchioloectasis (dilatation of bronchi or bronchioles respectively) are common and are a result of architectural distortion caused by the fibrotic process (Hansell et al. 2010). Honeycombing is a specific feature of the UIP pattern on HRCT, which correlates with histological findings of established fibrosis (figure 7). The appearance on HRCT is of clustered cystic air spaces, which have well-defined walls which vary in size from 3mm to 2.5cm (Meziane et al. 1988; Hansell et al. 2008).

Ground glass opacification seen as a ‘hazy increased opacity of the lung’ on HRCT is common but is usually in combination with the reticular pattern and is less prominent than the latter (Hansell et al. 2008; Hansell et al. 2010). If ground glass is seen in isolation or is the predominant abnormality, an alternative diagnosis should be considered.

Small volume (< 15mm) lymphadenopathy is seen in more than half of IPF patients on HRCT scanning. The most frequently affected nodal stations vary between studies. No correlation has been found between the presence of lymphadenopathy and the pattern or extent of disease on HRCT (Bergin and Castellino 1990; Souza et al. 2006).

Definite UIP pattern

- Reticular pattern
- Sub-pleural, lower lung predominance
- Honeycombing
- Traction bronchiectasis
- Absence of features inconsistent with a UIP pattern
    - Extensive ground-glass change
    - Upper or mid-lung predominance
    - Extensive upper lobe micro-nodular change
    - Discrete cystic change
    - Diffuse mosaic change (in more than 3 lobes)
    - Segmental/ lobar collapse

Possible UIP pattern

- Reticular pattern
- Sub-pleural, lower lung predominance
- Absence of features inconsistent with a UIP pattern as outlined above

Figure 6 HRCT criteria for UIP pattern in IPF
Adapted from (Raghu et al. 2011). The current guidelines have set criteria for a definite UIP pattern, possible UIP pattern and described a pattern inconsistent with UIP. All patients without a definite UIP pattern should proceed to surgical lung biopsy if appropriate.

HRCT also allows the diagnosis and appreciation of the extent of co-existing COPD more accurately than CXR (Wiggins et al. 1990). This is important to recognise as Akira et al identified that because emphysematous change can sometimes mimic honeycombing, concurrent emphysema on scanning can influence the distinction between NSIP and UIP and therefore could result in misdiagnosis (Akira et al. 2009).

HRCT scanning for the diagnosis of IPF has a specificity approaching 100% when interpreted by an experienced radiologist and with the characteristic features of UIP present. However, these features are not always present, and have been shown to be associated with advanced disease (Mathieson et al. 1989; Johkoh et al. 1999; Flaherty et al. 2003b). Current guidelines recommend proceeding to surgical lung biopsy if a definite UIP pattern (figure 6) is not present on HRCT (Raghu et al. 2011).

Several studies have shown that over time the extent of honeycombing and reticulation increase and ground glass opacification on imaging may regress initially but typically progresses eventually (Akira et al. 1993; Hartman et al. 1996; Wells et al. 1997). Serial HRCT has been used for research purposes to assess the response to treatment in IPF patients, however use in routine follow-up is not currently recommended.

As well as its diagnostic role in IPF, several studies have shown that the extent of fibrosis and pattern on HRCT scanning provides an indication of prognosis and survival (Wells et al. 1993; Flaherty et al. 2003b; Jeong et al. 2005; Lynch et al. 2005). Visual and digital fibrosis scoring methods have been designed to quantify the extent and severity of parenchymal disease on HRCT scans. Several studies have shown that scoring methods correlate with physiological variables, quality of life scores and surgical lung biopsy results (Gay et al. 1998; Lynch et al. 2005; Papiris et al. 2005; Lynch 2007). They have also proven to be an independent predictor of mortality; however are currently not used in routine practice (Gay et al. 1998; Best et al. 2008).

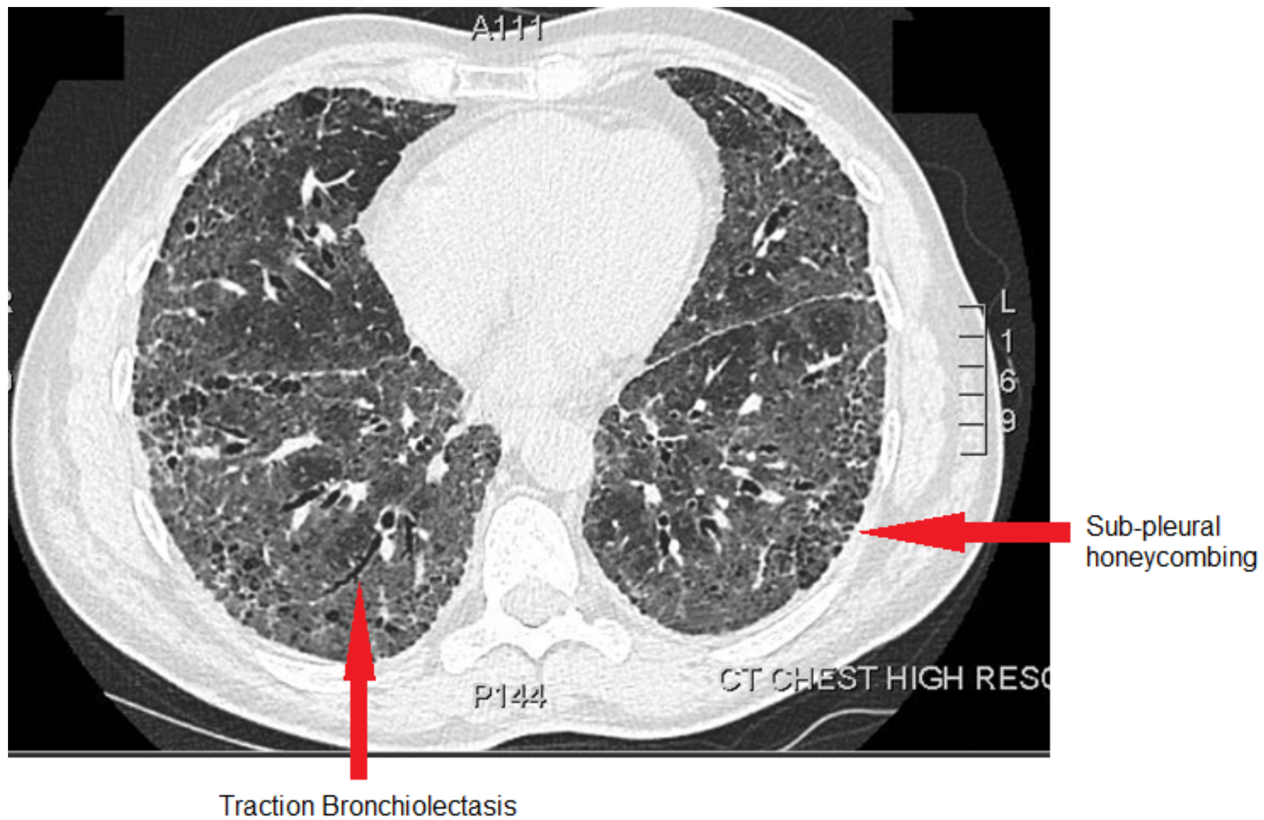


Figure 7 High resolution computed tomography image of a study patient with IPF showing traction bronchiolectasis and sub-pleural reticular change with honeycombing.

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) is a valuable research tool in IPF and is a useful adjunct in the diagnostic evaluation of patients without a typical UIP pattern on HRCT who are not suitable for surgical lung biopsy (Meyer et al. 2012). The technique allows sampling of cellular and acellular components at the alveolar level.

BAL is a minimally invasive procedure and has been shown to be well tolerated in most patients, including those who are critically unwell (Hertz et al. 1991). Fever has been found to occur more commonly with BAL than bronchoscopy alone and exacerbation of IPF has been observed, with frequency varying between case series (Hiwatari et al. 1994; Honeybourne et al. 2001; Kim et al. 2006a). One series reported 3 exacerbations in 124 patients that underwent BAL (Hiwatari et al. 1994). BAL cellular analysis in patients suspected of IPF but without characteristic HRCT findings may help narrow the differential diagnosis (Raghu et al. 2011). The cellular analysis in IPF characteristically shows an increase in total cell count with neutrophils > 5% and eosinophils > 2 % (Welker et al. 2004; Ryu et al. 2007; Kinder et al. 2008). Neutrophilia is found in more than 50% of patients with IPF and eosinophilia in 40-60%, however this pattern can also be seen in asbestosis, sarcoidosis or pulmonary fibrosis associated with rheumatological disease so is therefore not diagnostic of IPF (Pesci et al. 2010; Wells 2010).

Studies investigating the prognostic implications of BAL cellular analysis in IPF have shown conflicting outcomes. Initial studies found neutrophilia and eosinophilia denoted a poorer outcome in patients with IPF (Haslam et al. 1980; Watters et al. 1987). These studies were performed prior to the introduction of diagnostic guidelines however, and a subsequent study showed no diagnostic or prognostic role for BAL in IPF (Veeraraghavan et al. 2003). A further larger study also showed that neutrophilia had no prognostic role in IPF (Ryu et al. 2007). Kinder et al. have however since found that an increased neutrophil BAL cell percentage was an independent predictor of mortality in IPF patients (Kinder et al. 2008).

Lymphocytosis > 15% is not a feature of UIP. Historically however BAL lymphocytosis was thought to have been a good prognostic factor in UIP. Following the re-classification and description of NSIP, it is thought that misclassification occurred and BAL lymphocytosis is more likely suggestive of NSIP rather than UIP. Veeraraghavan and colleagues argued against this as they failed to identify any difference in BAL cell counts in patients with IPF and NSIP (Veeraraghavan et al. 2003). A subsequent larger study however, found a significantly higher BAL lymphocyte count in patients with surgically proven NSIP compared with UIP, and those with NSIP and lymphocytosis had a significantly longer survival period as previously identified (Watters et al. 1987; Ryu et al. 2007).

Acellular BAL biomarkers such as pulmonary surfactant phospholipids have been identified and shown to be abnormal in IPF. These are largely used for research purposes only and have been described in detail elsewhere (Haslam and Baughman 1999; Schwarz and King 2010).

One of the most important applications of BAL currently is to identify patients in whom treatment with immuno-suppressants may be of benefit: patients with NSIP and more importantly hypersensitivity pneumonitis (lymphocytes typically > 40 %.) A recent study showed that BAL improved the accuracy of diagnosis in patients with suspected IPF (Ohshimo et al. 2009; Raghu et al. 2011). Current guidelines recommend that the use of BAL in suspected IPF should be at the discretion of the treating physician (Raghu et al. 2011).

Histopathology

Gross inspection of the lung in IPF shows a honeycomb pattern, which as seen on imaging, predominantly affects the peripheries and lung bases (figure. 8). The pleural surface is typically nodular as a result of interlobular and alveolar septal fibrosis; this causes septal thickening and scarring which causes retraction from the surface (Schwarz and King 2010).

Microscopically at low magnification the characteristic UIP appearance is a ‘patchwork’ pattern with areas of normal lung interspersed with areas of chronic

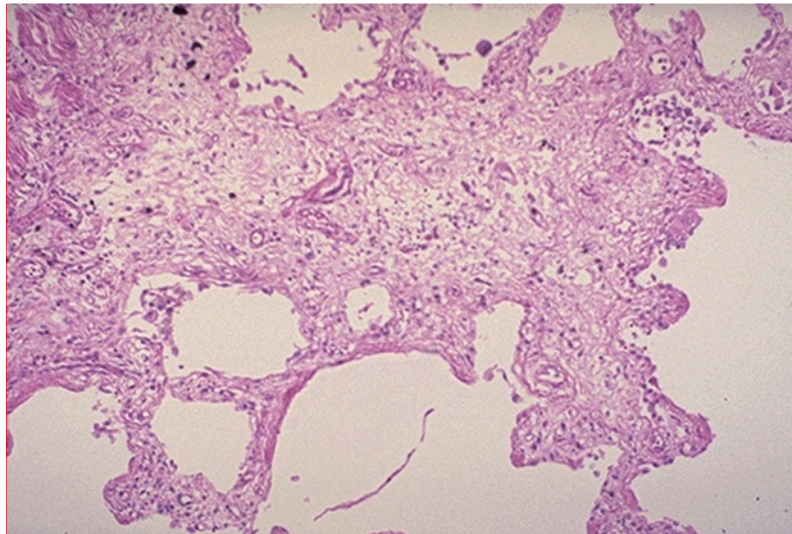
scarring and architectural distortion in the form of honeycomb change, interstitial inflammation and more active scarring with fibroblastic foci (figures. 9a and b) (Agusti 2002; Katzenstein et al. 2002).

At higher magnification fibrosis is seen to be the predominant feature, which is composed largely of dense acellular collagen and interspersed with fibroblastic foci, which are areas of fibroblasts, myofibroblasts and newly formed collagen. These areas of organising connective tissue are often seen between areas of normal and abnormal, fibrotic lung, and were thought to be discrete areas until 3-dimensional reconstruction showed that they can be interconnected; forming the leading edge of a complex reticulum which extends between the pleura and underlying parenchyma (Agusti 2002; Cool et al. 2006). Fibroblastic foci are characteristic of UIP but are not specific to the diagnosis (Leslie 2012). A prospective study found that a greater extent of fibroblastic foci present on lung biopsy predicted shorter survival in IPF, however this study was performed prior to the description of NSIP and before the current classification of IIPs came into use (King et al. 2001a). Honeycomb change is characterised by dilated cystic airspaces, which are often lined by columnar respiratory epithelium and is often associated with fibrotic scar tissue and areas of smooth muscle hypertrophy. Lymphocytes, plasma cells, and hyperplastic, hypertrophied type II alveolar cells and bronchiolar epithelial cells makeup the patchy alveolar infiltrate, often described as interstitial inflammation. Inflammatory cell infiltrate in UIP is minimal, and is overshadowed by the fibrosis (Raghu et al. 2011).

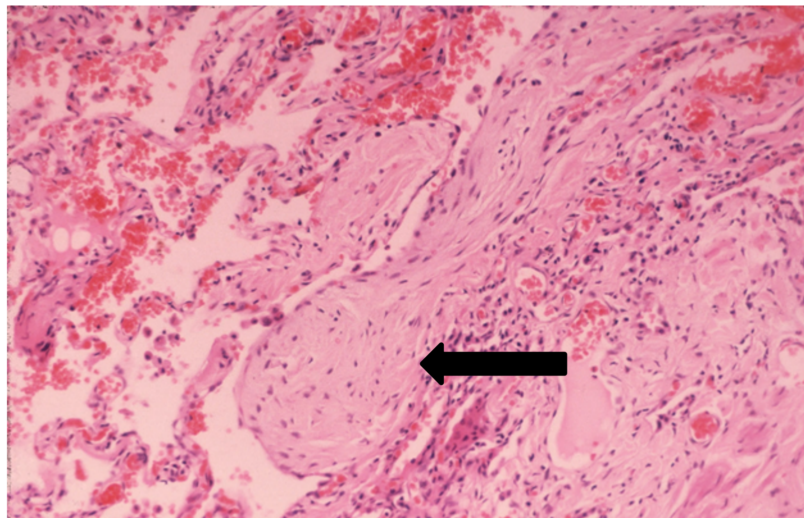
Current guidelines recommend tissue sampling from multiples lobes if possible, as histo-pathological variability has been found in biopsies from individual patients, and has prognostic implications (Flaherty et al. 2001; Monaghan et al. 2004). Current guidelines also provide criteria for a definite, probable and possible UIP pattern on histopathological examination (figure 10). These definitions are used to improve diagnostic accuracy as illustrated below in the diagnosis section of this thesis (Raghu et al. 2011).

Figure 8 Macroscopic appearance of IPF lung at post-mortem
Reproduced with the kind permission from Dr Ben Hope-Gill.

a.



b.



Figures 9a and b Microscopic appearance of lung in IPF

Figure 9a is a haematoxylin and eosin section from a surgical lung biopsy of a patient with IPF which demonstrates loss of normal lung architecture with increased inflammatory cell infiltrate and extracellular matrix deposition.

Figure 9b is a higher power image demonstrating a fibroblastic foci (arrow). Images reproduced with kind the kind permission of Dr Ben Hope-Gill.

Definite UIP pattern

- Predominantly sub-pleural/ para-septal marked fibrosis and/or honeycombing
- Patchy parenchymal involvement
- Fibroblastic foci
- Absence of features against UIP or suggesting an alternative diagnosis
    - Hyaline membranes or organising pneumonia (unless associated with an acute exacerbation)
    - Granulomas (unless isolated or occasional only)
    - Marked inflammatory cell infiltrate
    - Predominant airway changes
    - Features of an alternative diagnosis

Probable UIP pattern

- Marked fibrosis and/or honeycombing
- Absence of patchy parenchymal involvement or fibroblastic foci (not both)
- Absence of features against UIP or suggesting an alternative diagnosis as outlined above

or

- Honeycomb changes only (i.e. sampling bias in end-stage disease)

Possible UIP pattern

- Patchy or diffuse fibrosis, with or without interstitial inflammation
- Absence of other definite UIP criteria
- Absence of features against UIP or suggesting an alternative diagnosis as outlined above

Figure 10 Histopathological criteria for UIP pattern in IPF
Adapted from (Raghu et al. 2011). The current guidelines have defined criteria for UIP pattern on surgical lung biopsy. These criteria can be used in a diagnostic algorithm as outlined below.

1.1.6 Diagnosis

The most recent ATS/ERS consensus statement on the diagnosis and management of IPF has emphasised the importance of a multidisciplinary discussion between respiratory physicians, radiologists, and pathologists experienced in the diagnosis of ILD in order to achieve an accurate diagnosis (Raghu et al. 2011). The diagnoses ‘definite IPF’, ‘probable IPF’ and ‘possible IPF’ are described in the guideline depending on the level of diagnostic certainty of UIP pattern using HRCT and histological examination, with the importance of multidisciplinary discussion emphasised in the ‘probable’ or ‘possible’ cases.

The guideline requirements for the diagnosis of IPF are outlined below and shown diagrammatically in Figure 11:

1. Exclusion of any known causes of interstitial lung disease.
2. All characteristic features of UIP pattern on HRCT as shown in figure 6.
3. A combination of HRCT and Histological pattern as shown in figure 10.

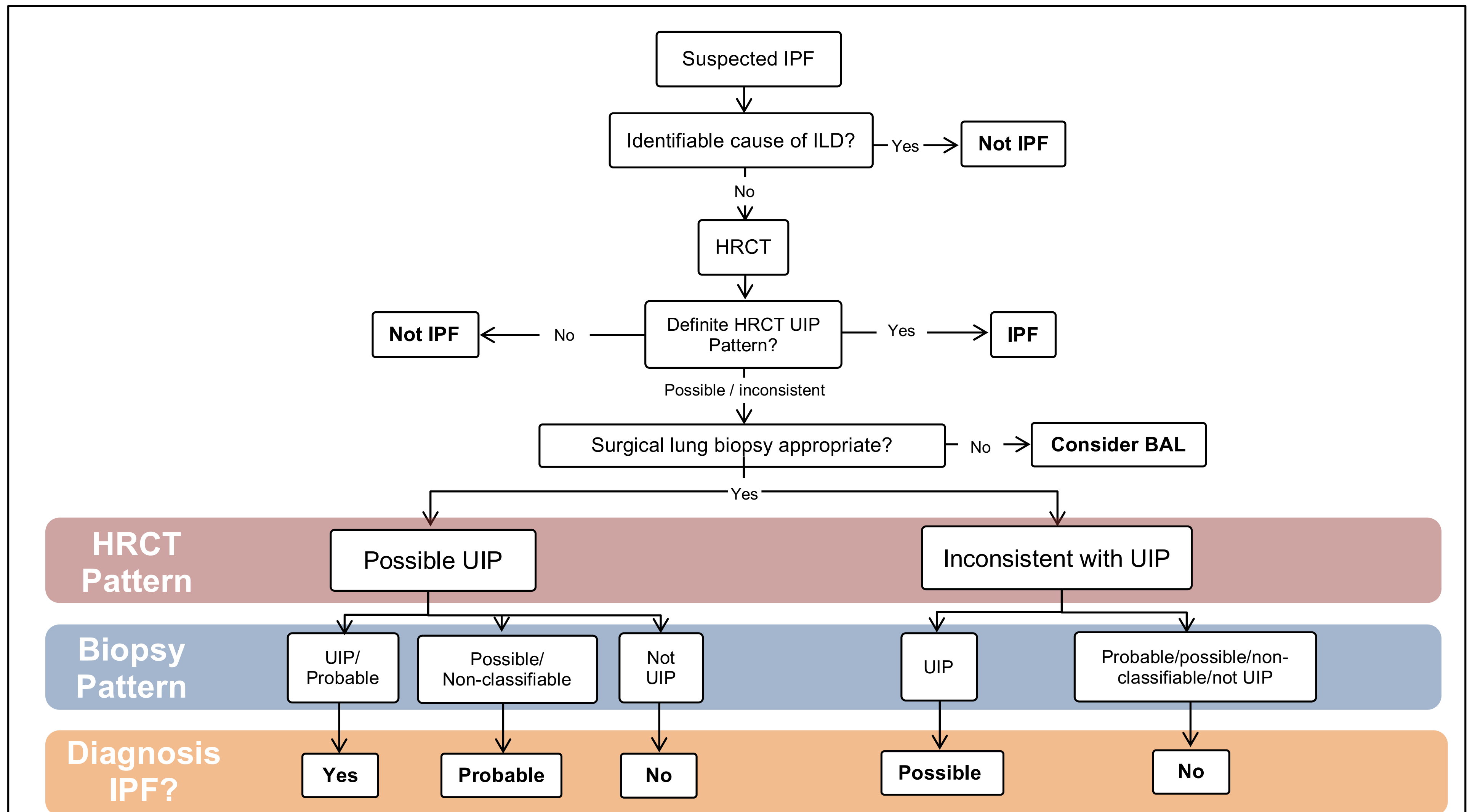


Figure 11 Diagnostic algorithm for patients with suspected IPF. Adapted from (Raghu et al. 2011)

1.1.7 Therapeutic Strategies

Due to the progressive nature of IPF, it is recommended that best supportive care in the form of specialist nurse support, symptom relief, co-morbidity management, withdrawal of futile therapies and end-of-life care be considered at the time of diagnosis and be tailored specifically to the needs of the patient (NICE 2013). There has not been a prospective study investigating the use of oxygen in IPF however its use is recommended when there is resting hypoxia, as evidence for the use of oxygen has been extrapolated from studies showing a significant survival benefit in COPD patients using long-term oxygen therapy (Raghu et al. 2011; NICE 2013). Pulmonary rehabilitation has also been recommended by current guidelines (Raghu et al. 2011; NICE 2013), as several small studies have demonstrated an improvement in exercise capacity and quality of life (Holland et al. 2008; Nishiyama et al. 2008; Ferreira et al. 2009; Ozalevli et al. 2010).

The evidence for disease modifying treatments has changed considerably over recent years as the understanding of the pathogenesis of IPF has improved, with a thorough review published by Rafii et al (Rafii et al. 2013). Therapeutic strategies originally targeted the process of inflammation, whereas current strategies are primarily focused on the fibrotic process.

Since April 2013, Pirfenidone (Esbriet, InterMune) has been endorsed by the National Institute for Health and Care Excellence (NICE) for the treatment of IPF in patients with a FVC between 50-80% provided they have yearly follow-ups and no evidence of disease progression (reduction in FVC by 10%) whilst on treatment. The exact mechanism of action is unclear, but it is believed to be an anti-fibrotic agent (NICE 2013). Four large phase III randomised controlled trials, three of which were multi-national, demonstrated a reduction in lung function decline, all-cause mortality at one year and an improved progression-free survival (Taniguchi et al. 2010; Noble et al. 2011; King et al. 2014). More recent studies suggest that it is well-tolerated, with gastrointestinal upset and photosensitivity being the most commonly reported side effects (Cottin and Cordier 2012; Cottin and Maher 2015; Kim and Keating 2015).

Another new agent was also recently endorsed by NICE in the same patient group (NICE 2016). Nintedinib, is an intracellular inhibitor which targets a number of tyrosine kinases that have been implicated in the pathogenesis of IPF, namely vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) (Hilberg et al. 2008) and is thought to block signalling pathways involved in the fibrotic process (NICE 2016). Results from the two phase III randomised, double-blind, placebo controlled trials INPULSIS I and INPULSIS II demonstrated a significant reduction in FVC in patients with IPF compared to placebo (Richeldi et al. 2011; Richeldi et al. 2014), which was in keeping with previous findings and may suggest a slowing of disease progression (Richeldi et al. 2011). The two studies had conflicting results with regards quality of life and time to first acute exacerbation however, with INPULSIS I failing to show an increased time to first exacerbation or a slowing of deterioration of quality of life. Also although the authors report the drug as having an acceptable tolerability profile, they report diarrhoea in over 60% of patients (Richeldi et al. 2014). Both drugs are only recommended if the manufacturer provides the discount agreed in the patient access scheme (NICE 2016).

Despite the promising advances made in drug therapy over recent years, lung transplantation should be considered in IPF patients with no contraindications as it is the only treatment with a clear long term survival advantage (Christie et al. 2012). Unfortunately few people are eligible as a result of co-morbidity and the number of lung transplantations performed are limited by organ availability (Kistler et al. 2014).

1.1.7 Prognosis

As discussed in the previous sections a number of individual predictors of survival have been identified. The most effective method for staging IPF has yet to be established. Despite new therapeutic strategies the prognosis for the majority of patients with IPF remains poor with a median survival of less than 4 years. In clinical practice the rate of decline varies between individuals with some experiencing a gradual deterioration, some showing a rapid deterioration and others showing a step-wise deteriorating pattern with periods of stability (Figure 12) (Ley et al. 2011).

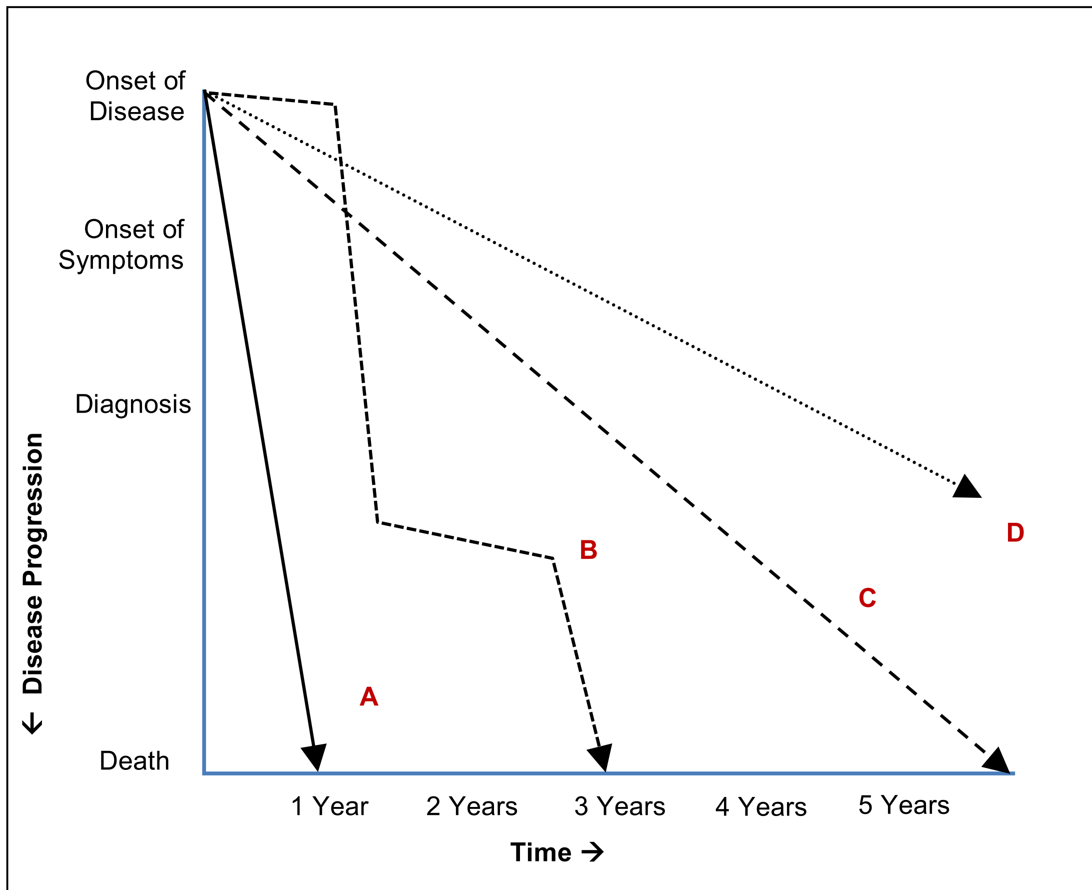


Figure 12 Clinical Course in patients with IPF
Progression to death can be rapid (A), slow (C & D), or mixed with periods of relative stability punctuated with episodes of acute decline (B).
Adapted from (Ley et al. 2011)

1.2 IDIOPATHIC PULMONARY FIBROSIS – PATHOGENESIS

As described in the previous section, UIP pattern is the characteristic histological finding in patients with IPF, with alternating areas of fibrosis, honeycombing, interstitial inflammation and normal lung. Despite the consistently observed features of epithelial disruption, collagen deposition and presence of fibrotic foci the patho-physiological basis of IPF is not fully understood. The theories of pathogenesis in IPF are discussed below.

1.1.2 'Inflammation' Hypothesis

The theory of IPF pathogenesis, developed in the 1970-1980's suggested that IPF was the result of a chronic inflammatory response to an unknown stimulus that caused lung injury and progressive fibrosis. This theory was based upon several observations. Early studies of bronchoalveolar lavage fluid found IPF patients had increased number of inflammatory cells compared to patients with normal lung function (Merrill and Reynolds 1983; Reynolds et al. 2005). An increased number of neutrophils and activated macrophages were also observed in BAL fluid taken from asymptomatic members of families with autosomal dominant IPF, suggesting alveolar inflammation in the absence of fibrotic disease (Bitterman et al. 1986). Also, animal models provided support for this theory as lung injury resulted in inflammation and a subsequent fibrotic response (Snider 1986). It was believed that irrespective of the trigger, therapies should be targeted at the inflammatory process rather than the fibrosis (Crystal et al. 1976).

This theory of inflammation having a pivotal role in the fibrotic process seen in IPF has since been questioned. Several pieces of evidence suggested the observations that formed the basis of the theory, as outlined above, were invalid. The pathological classification of IPF in 1998 by Katzenstein, highlighted a paucity of inflammation in early disease, and yet was in association with areas of collagen deposition and honeycombing, suggesting underlying fibrosis (Katzenstein and Myers 1998). Several animal studies also showed that inflammation is not always a pre-requisite to fibrosis (Adamson et al. 1988; Xu et al. 2003). Finally, the failure of anti-inflammatory medications to alter the natural history of IPF also brought this theory into question. Until recently no randomised controlled trials had been conducted to investigate the effect of anti-inflammatory

medications such as corticosteroids in IPF. Uncontrolled studies, conducted prior to the re-classification in 2002 showed mixed results. Some patients were steroid responders but this effect was often short-term, while others failed to show any significant clinical advantage in the majority of those prescribed corticosteroids alone or in combination with immunosuppressive therapies (Turner-Warwick et al. 1980; Douglas et al. 2000; Davies et al. 2003). A recent controlled study that randomized 236 patients with mild-to-moderate IPF, found that patients treated with a combination of prednisolone, azathioprine and N-acetylcysteine (NAC) had increased all-cause mortality (Raghu et al. 2012). This arm of the study was discontinued prematurely following an interim analysis and current guidelines recommend against the use of corticosteroid monotherapy or immune modulating combination therapy (Raghu et al. 2011).

1.2.2 Epithelial Hypothesis

The 'epithelial' hypothesis has been proposed as an alternative theory of IPF pathogenesis. It is currently believed that the fibrotic process in IPF is a result of repeated micro-injury to the alveolar epithelium and a failure of normal wound healing (Gauldie et al. 2001; Selman et al. 2001).

In normal circumstances, alveolar epithelial injury results in a complex interaction of cellular, immune and coagulation pathways, which are co-ordinated by a variety of soluble mediators such as growth factors, chemokines, interleukins and prostaglandins. This interaction results in rapid restoration of the epithelial integrity via migration, differentiation and proliferation of epithelial cells allowing re-epithelialisation and re-endothelialisation. Extra-cellular matrix is deposited as part of the normal healing process, but is reabsorbed following removal of the injurious agent (Strieter 2008; Crosby and Waters 2010). Angiogenesis, a process of new blood vessel growth is also a feature of normal wound repair and healing. It is suggested that the fibrosis in IPF occurs as a consequence of failure of regulation at various stages of these normal repair processes.

1.2.3 Alveolar epithelial injury

As discussed in the previous section numerous factors such as environmental agents, genetics, viruses and GOR, have been implicated in the aetiology of IPF,

and may cause epithelial injury. However causation remains poorly defined. It is believed that repeated, low level injury causes epithelial damage and subsequent disruption to the alveolar-capillary basement membrane. This has been demonstrated using electron microscopy (Katzenstein 1985; Harrison et al. 1991).

Alveolar epithelium in the normal lung is comprised of predominantly type I alveolar epithelial cells (AECs), with a smaller number of type II AECs. Following alveolar epithelium injury, type II AECs proliferate and differentiate into type I AECs, thus repairing the alveolar epithelium (Crapo et al. 1982; Castranova et al. 1988). In IPF, loss and/or damage to type I cells and proliferation of type II cells are seen. There is inadequate repletion of type I cells however, and altered epithelial cell phenotypes are often present, resulting in failure to re-establish a normal epithelium (Selman et al. 2001). Programmed cell death of type II AECs may also play a role in the failure to repair the integrity of this region of the lung (Barbas-Filho et al. 2001).

The cause of this atypical response of AECs is not fully understood; it may result from the initial injury or be a consequence of accelerated cell proliferation in response to injury, which has been observed in honeycomb lesions of patients with IPF (Selman and Pardo 2006).

The epithelial injury and consequent damage to the alveolar- capillary basement membrane is described as a critical step in the irreversible fibrotic process. The basement membrane is a complex structure of type IV collagen, laminin, perlecan, and entactin. The cause of membrane disruption is not fully understood but loss of alveolar epithelium allows enzymes produced by a variety of lung cells to have contact with and degrade components of the membrane. The loss of basement membrane integrity results in its failure to provide structural support and act as a barrier, which regulates cellular processes. This may contribute to the failure of re-epithelialisation and allow migration of fibroblasts and myofibroblasts into the alveolar space where extra-cellular matrix is secreted (Selman et al. 2001; Strieter 2008). Alveolar epithelial repair processes in health and IPF are summarised in figure 13.

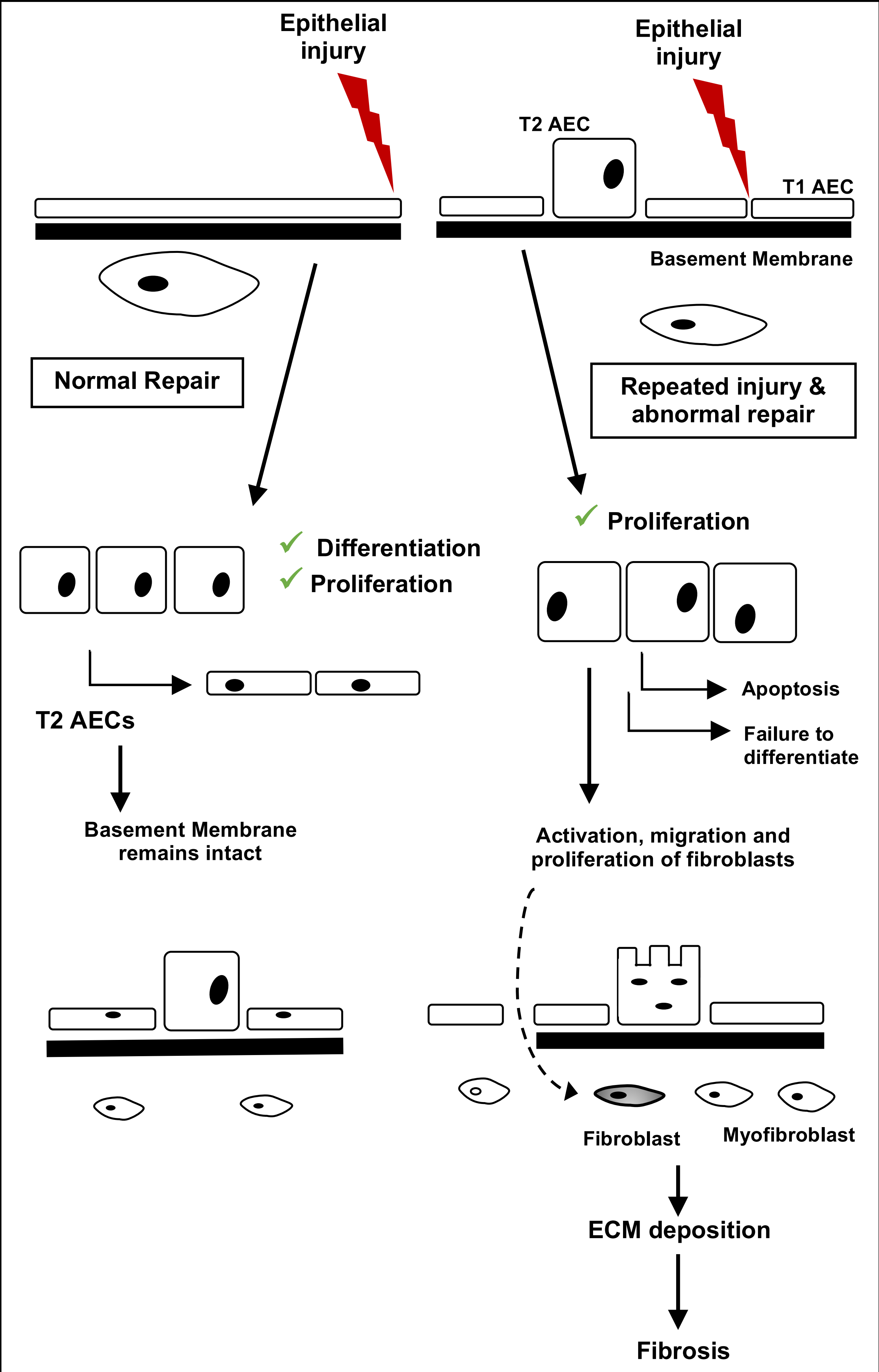


Figure 13 **A Schematic Representation of alveolar epithelial repair.** *Adapted from* (Uhal and Hang 2013)

1.2.4 Cellular Interactions

Epithelial cells

While epithelial cells are the target of the injurious processes, they also have a fibrogenic role. Following injury they become activated and secrete pro-fibrotic mediators, including platelet-derived growth factor (PDGF), transforming growth factor β (TGF-β), tumour necrosis factor α (TNF α), endothelin-1, connective tissue growth factor, and osteopontin (Antoniades et al. 1990; Nash et al. 1993; Pardo et al. 2005; Selman and Pardo 2006). Epithelial injury also activates the coagulation cascade which has several pro-fibrotic effects (Borensztajn et al. 2008). These processes result in the activation, migration and proliferation of fibroblasts.

Fibroblasts

Fibroblasts when activated migrate to the site of injury, and along with other mesenchymal cells including myofibroblasts become organised into fibroblastic foci. These foci represent active areas of fibrosis and are situated adjacent to areas on epithelial cell and basement membrane damage (Katzenstein and Myers 1998). In a study looking at three dimensional reconstruction of the alveoli Cool et al have shown that these fibroblastic foci are highly interconnected (Cool et al. 2006).

Several sub-populations of fibroblasts are present in IPF. The pro-fibrotic environment induced by the injured epithelial cells is thought to induce these changes; with the injured alveolar epithelium seen as the leading edge of active fibrosis (Fries et al. 1994; Selman et al. 2001; Sakai and Tager 2013). Phenotypes include migratory, proliferative and pro-fibrotic types and have been distinguished by the expression of varying surface membrane proteins (Akamine et al. 1992; Fries et al. 1994), and their genetic expression (Emblom-Callahan et al. 2010). The pro-fibrotic phenotype secretes extracellular matrix and collagen.

In addition to their role in the activation of fibroblasts, injured epithelial cells also promote the aberrant fibrotic response. Under normal conditions, epithelial cells have a role in suppressing fibroblast accumulation through the action of prostaglandin-E2 (PGE-2). In IPF epithelial cell PGE-2 synthesis is reduced and

as a consequence of down-regulation of the specific receptor that mediates the inhibitory effect of PGE-2 on fibroblasts, the fibroblasts also show resistance to its effect (Moore et al. 2005; Selman and Pardo 2006; Sakai and Tager 2013).

Myofibroblasts

Myofibroblasts are activated mesenchymal cells that have the combined properties of fibroblasts and smooth muscle cells. They secrete extra-cellular matrix but also have contractile ability through expression of α-smooth muscle actin (α-SMA) (Kuhn and McDonald 1991; Hinz et al. 2007). Via cell contraction the cells are aligned in parallel within the fibroblastic foci which is believed to contribute to the remodelling of the lung in IPF (Kuhn and McDonald 1991).

The origin of myofibroblasts has been the subject of much debate. One possibility is that pulmonary fibroblasts differentiate into myofibroblasts under the influence of the pro-fibrotic mediators secreted by injured AECs (Hong et al. 2007). Another theory proposes that myofibroblasts may be derived from circulating fibroblasts or other bone marrow-derived progenitor cells (Lama and Phan 2006). Recent evidence suggests a mechanism of epithelial-mesenchymal transition (EMT). EMT is a form of metaplasia where cells develop a phenotype depending on the stimulus received (Vancheri et al. 2010). Several studies have shown that under the influence of pro-fibrotic cytokines, epithelial cells can lose epithelial markers and acquire markers present in mesenchymal cells such as myofibroblasts (Kim et al. 2006b; Willis et al. 2006).

TGF- β has been shown to be increased in the lungs of IPF patients and is a key pro-fibrotic mediator in the interaction between AECs, fibroblasts and myofibroblasts (Khalil et al. 1991; Wuyts et al. 2013). This cytokine promotes the differentiation of fibroblasts into myofibroblasts, induces the secretion of extra-cellular matrix and has also been shown to inhibit collagen degradation and myofibroblast apoptosis, which leads to an accumulation and persistence of extracellular matrix (Zhang and Phan 1999; Hong et al. 2007; Wuyts et al. 2013).

Activated fibroblasts and myofibroblasts may also contribute to AEC injury; Angiotensin II, reactive oxygen species and their interaction with the pro-inflammatory cytokines secreted have been implicated (Wang et al. 1999;

Waghray et al. 2005). This coupled with myofibroblasts causing disruption to the basement membrane via the production of gelatinases A and B may therefore induce a vicious cycle of injury and fibrosis (Selman et al. 2001).

1.2.5 Extra-cellular matrix (ECM) deposition

In normal lung, collagen is the predominant type of protein in the extra-cellular matrix and has a constant turn-over which is tightly regulated to ensure the preservation of normal lung structure and function. Fibroblasts and myofibroblasts have a key role in ECM synthesis and re-modelling. Extracellular matrix is degraded via proteolysis by a family of matrix metalloproteinases (MMPs) which are secreted by fibroblasts, epithelial cells, neutrophils, and macrophages. Endogenous inhibitors known as tissue inhibitors of metalloproteinases (TIMPs), control the activity of matrix metalloproteinases (Birkedalhansen et al. 1993). Achieving the optimum degree of ECM deposition requires close regulation of processes of synthesis and degradation. In IPF there is an imbalance of these processes. Excessive ECM synthesis and deposition is coupled with a reduction in degradation as a result of increased expression of TIMPs (Selman et al. 2001). Consequently extensive structural disorganization is seen with progressive loss of alveolar units.

1.2.6 Angiogenesis

Neovascularisation following tissue injury is another process that is tightly regulated by stimulatory (angiogenic) and inhibitory (angiostatic) growth factors. Studies of this process in IPF have yielded conflicting results. There appears to be increased vascularity of non-fibrotic lung and reduced vascularity in fibrotic lung, with fibroblastic foci in particular being almost void of capillaries, suggesting that angiogenesis is not a requirement for fibrosis (Cosgrove et al. 2004; Ebina et al. 2004). The role of angiogenesis in IPF remains unclear (Hanumegowda et al. 2012).

The theory of epithelial injury and cell death has superseded the theory in which inflammation leads to injury and progressive fibrosis, however as outlined above there are complex interactions between many processes which ultimately lead to

the characteristic fibroblast proliferation, collagen deposition and architectural distortion. Current therapeutic strategies are focused on fibrotic processes.

Although our understanding of the basic mechanisms involved in the fibrotic process in IPF has greatly improved, the prognosis remains poor. Symptom control is a frequently adopted strategy. The pathogenesis of cough, a debilitating symptom in IPF remains unclear, meaning treatment options are limited. This symptom suggests that there is an abnormality of pulmonary innervation in IPF however this is currently poorly understood. The subsequent section outlines innervation in the normal lung.

1.3 PULMONARY INNERVATION

The human lung is an endoderm-derived organ that develops as a ventral outgrowth of the foregut during the embryonic stage. This diverticulum grows caudally and divides, forming the primitive trachea and lung buds (Laudy and Wladimiroff 2000). Lung neurons arise from neural crest cells which have migrated to the embryonic foregut, and subsequently to the developing lungs (Burns et al. 2008). This process occurs in close association with lung development in the early embryonic stage, with evidence of well-established innervation of the lungs by weeks 15-17 of foetal development (Mizukoshi 1953; Richardson 1982).

The airway is innervated throughout, down to the level of the alveolus (Fox et al. 1980; Hertweck and Hung 1980) The nervous system of the respiratory tract is more complex than originally thought. In addition to the sensory and autonomic (sympathetic and parasympathetic) pathways, otherwise known as the adrenergic and cholinergic pathways respectively, there is a non-adrenergic, non-cholinergic system, which remains poorly defined (Barnes 1984; Undem et al. 1999; Groneberg et al. 2004b). Figure 14 illustrates the afferent and efferent innervation of the lung, which is described in detail in the following text.

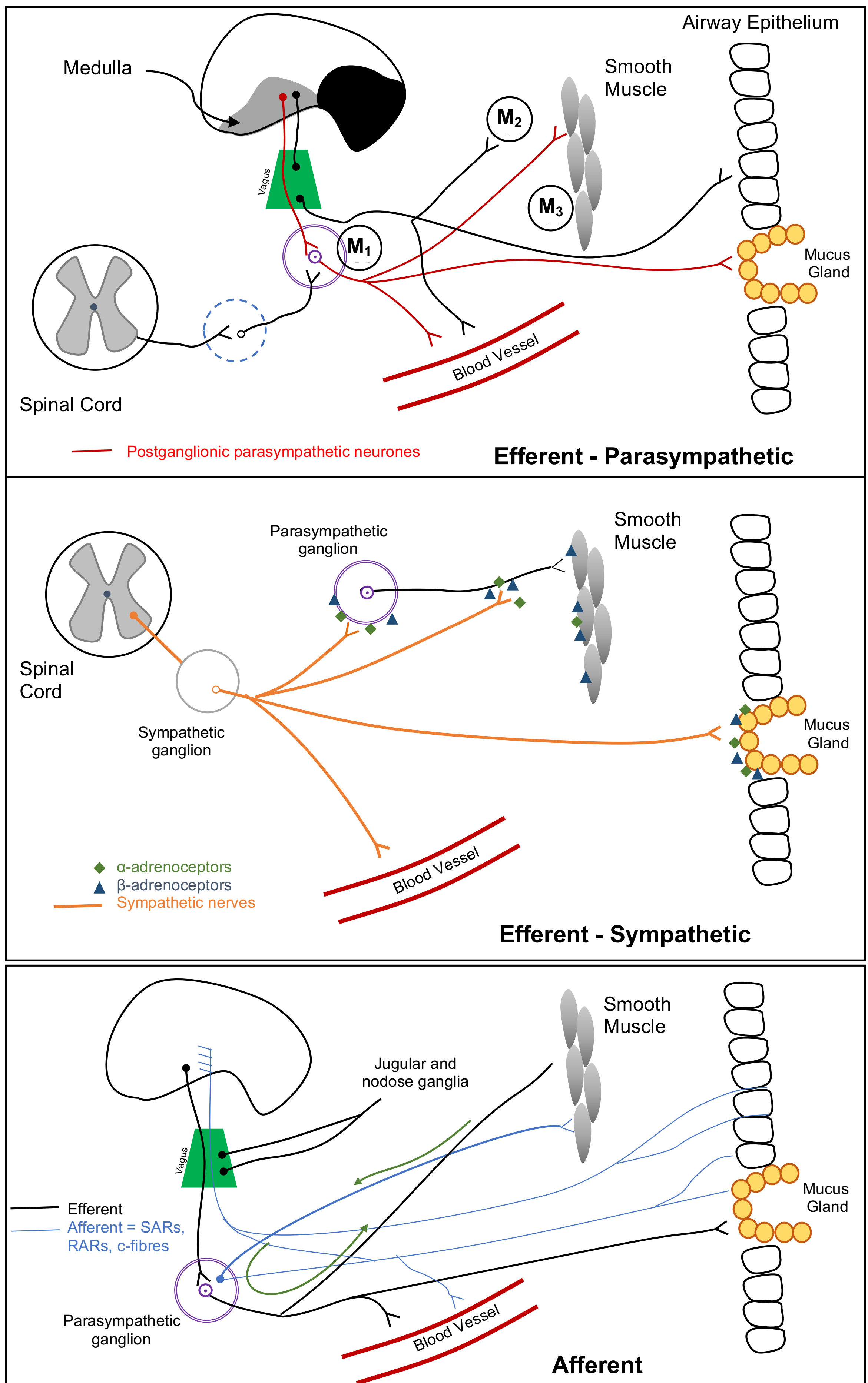


Figure 14 Efferent and afferent innervation of the lung.
Adapted from (Barnes 1990; Undem et al. 1999; Hope-Gill 2004)

1.3.1 The Efferent Innervation

Sympathetic (adrenergic) innervation

Sympathetic nerve fibres originate from the upper thoracic segments of the spinal cord and synapse at the paravertebral sympathetic ganglia. Postganglionic fibres travel from these ganglia and pass anteriorly to merge with vagal fibres within the pulmonary plexus, located anterior and posterior to the lung roots (Barnes 1990).

It was originally believed that the postganglionic sympathetic nerves released noradrenaline, which along with adrenaline released from the adrenal medulla, activated the alpha and beta-adrenoceptors in the periphery to inhibit broncho-constriction. It has however since been demonstrated that although adrenergic nerve fibres are found in close proximity to sub-mucosal glands and blood vessels they are sparse in airway smooth muscle and therefore have very little, if any influence on bronchial airway calibre (Barnes 1990; Undem et al. 1999). Adrenergic vaso-constriction mediated by alpha-adrenoceptors has been confirmed (Kummer 2011), however studies have failed to show an adrenergic stimulation of sub-mucosal glands (Baker et al. 1985; Undem et al. 1999) .

Although adrenergic fibres are poorly represented in the human lung, it is believed that they may have an indirect effect on these neural processes via modulation of the parasympathetic pathway, and circulating catecholamines may also stimulate alpha and beta- adrenoceptors irrespective of sympathetic innervation (Barnes 1990; Undem et al. 1999).

Parasympathetic (cholinergic) innervation

The preganglionic cholinergic fibres arise in discrete nuclei of the medulla and travel down the vagus nerve, which joins the pulmonary plexus. The fibres synapse with postganglionic fibres at ganglia situated along the vagal nerve and in the airways. The parasympathetic ganglia are situated in the larger airways and the postganglionic fibres supply bronchial smooth muscle, bronchial and pulmonary vasculature and mucous secreting glands of the airways (Barnes 1990; Myers 2001). The density of innervation does decrease from the trachea

to the terminal bronchioles however, with the airway epithelium and alveoli being devoid of cholinergic fibres (Barnes 1990).

Acetylcholine, released by cholinergic fibres binds to nicotinic acetylcholine receptors mediating ganglionic transmission in the airways. At least three subtypes of muscarinic receptors can then be activated by acetylcholine to cause an effect on the target cell. Stimulation of M3 receptors results in airway smooth muscle contraction, broncho-constriction, mucus secretion, and vasodilation of bronchial vasculature. Atropine has been found to inhibit the effects of broncho-constriction and mucus secretion, providing further evidence for this pathway. Pre-junctional M2 receptors are believed to serve to limit cholinergic broncho-constriction and when activated do so by inhibition of acetylcholine release. Muscarinic receptors present on preganglionic cell neurons facilitate parasympathetic neurotransmission through the ganglion. (Barnes 1990; Belvisi 2002).

This pathway is complicated by the presence of other neurotransmitter and neuro-active compounds at the pre- and postganglionic membranes in addition to acetylcholine that may affect the excitability of these membranes (Myers 2001).

Non-adrenergic, non-cholinergic innervation (NANC)

Despite knowledge of the embryological development of the lungs and the confirmed presence of this pathway in the gastro-intestinal tract, NANC was not described in the lung until the 1980’s (Richardson 1981; Barnes 1984). This is the only neurally-mediated bronchodilator pathway given the lack of functional sympathetic postganglionic fibres in the lung, as outlined above. In-vitro experiments allowed identification, by illustrating that bronchial smooth muscle was able to relax despite blockade of the adrenergic and cholinergic innervation (Barnes 1990).

The neurotransmitters facilitating this inhibitory effect are the vasoactive intestinal peptide (VIP), and nitric oxide (NO) synthesized from arginine by the neuronal isoform of NO synthase (Nnos), both of which have been localised to nerve fibres innervating airway smooth muscle and in parasympathetic ganglia innervating the

airways (Ward et al. 1995; Fischer and Hoffmann 1996). The exact role and contribution of these and possibly other neurotransmitters remains unclear. Initial studies suggested that this pathway was associated with the parasympathetic nerve supply to the lung; it was thought that VIP and NO were released from post-ganglionic parasympathetic nerves along with acetylcholine. Subsequent studies in guinea pigs indicate that the pathways are in fact distinct from each other (Barnes and Belvisi 1993; Undem et al. 1999).

This inhibitory- NANC (i-NANC) system is accompanied by an excitatory- NANC (e-NANC) pathway in rodents, however airway smooth muscle contraction in response to excitatory neurotransmitters has not been demonstrated in human studies (Barnes 2001).

1.3.2 Afferent innervation

The vagus nerve carry the majority of afferent innervation. A subset of fibres also originate from the dorsal root ganglion of the spinal cord in association with sympathetic fibres, but this is of uncertain significance (Undem et al. 1999). Afferent fibres arise from receptors within the airway epithelium, smooth muscle, sub-epithelial tissue, the large airway perichondrial area, alveolar tissue and pulmonary and bronchial blood vessels (Qutayba and Hamid 2005). The cell bodies of vagal afferent fibres are within the vagal sensory ganglia; the jugular (superior vagal) or nodose (inferior vagal) ganglia are situated on the cervical aspect of the vagus nerve and fibres project centrally to terminate in the brainstem, primarily the nucleus tractus solitarius (NTS). Fibres projected to the airways can be small un-myelinated C-fibres or larger myelinated A-fibres. This definition is based on action potential conduction; those with a conduction velocity of less than 2 ms^{-1} are classified as C fibres and those with velocities of greater than 3 ms^{-1} are classified as A fibres, of which there are two types: $A\delta$ and $A\beta$. The $A\delta$ fibres were originally believed to be unmyelinated due to their slower conduction velocity than $A\beta$ fibres, however have since been classified as thinly-myelinated fibres (Undem et al. 1999).

Airway afferent nerves can be further sub-classified based on their properties. Lack of specificity of the characteristics displayed by these fibres and differences

between species adds to the complexity of this classification. The three general categories of afferent fibres, classified on the basis of their functional properties are outlined below.

Slowly adapting (stretch) receptors

Slowly adapting receptors (SARs) are myelinated fibres, responsible for relaying information on lung volume and transpulmonary pressure to the respiratory centre. They were originally described following the observation that an increase in airway wall tension resulted in increased, regular receptor discharge but this increase in discharge slowly adapted as airway wall tension was maintained (Schelegle and Green 2001).

SARs regulate respiratory reflexes such as the Hering-Breuer reflex which inhibits sustained inspiration, and at high lung volumes prolongs expiration (Barnes 1990). In addition to responding to changes in airway wall tone, they are stimulated by histamine and acetylcholine and inhibited by CO_2. Stimulation results in reflex bronchodilatation by inhibition of vagal tone (Widdicombe 2001).

Histologically SARs resemble mechanoceptors and are anatomically located within airway smooth muscle, carefully organised in close proximity to individual muscle cells (Santambrogio 1982). There appears to be considerable variation in the distribution of SARs throughout the lungs between mammalian species, but generally they are considered to be confined to the airways, up to the terminal bronchioles with no association with the alveoli (Santambrogio 1982; Schelegle and Green 2001). In the trachea they are believed to be limited to the muscular posterior wall (Schelegle and Green 2001; Widdicombe 2001).

Rapidly adapting (irritant) receptors

Rapidly adapting receptors (RARs) are also myelinated fibres, but differ from SARs in that upon stimulation they fire irregularly and adapt quickly to a sustained mechanical stimulus such as is present at the end of inspiration. They are able to respond to airway mechanical changes and their activity during respiration depends upon the respiration rate and inspiratory volume (Canning 2006).

Stimulation of these irritant receptors causes bronchoconstriction and mucous secretion by a reflex increase in parasympathetic efferent activity.

In addition to their mechanoreceptor properties they are also chemoreceptors, stimulated by a variety of chemical stimuli, inflammatory and immunologic mediators, and inhaled particles (Santambrogio 1982; Widdicombe 2001). RARs however, are relatively insensitive to direct chemical stimuli so it is likely that increased RAR activity is a result of the mechanical consequences of bronchoconstriction and obstruction from excess mucus secretion evoked by substances such as capsaicin, the compound which gives chilli peppers their heat, and substance P. In support of this argument is the observation that RAR discharge can be inhibited by preventing mucous secretion and bronchoconstriction (Canning et al. 2006).

RARs were originally reported to be intra- and extra-pulmonary, with extra-pulmonary RARs having two subtypes; tracheal/bronchial RARs and intermediate receptors that had properties suggestive of both SARs as well as RARs (Widdicombe 1954a, b, c; Widdicombe 2001). Evidence from animal models suggests that the RARs were concentrated in the upper airways and in particular at branch points with terminations primarily in the intrapulmonary airways. Unlike SARs they were thought to be circumferentially distributed throughout the trachea (Santambrogio 1982). However, more recent studies using guinea pigs dispute these findings and suggest that the extra-pulmonary afferent neurones are a unique subset, not readily classifiable as RARs, SARs or C-fibres (Canning et al. 2004). The electrophysiological experiments by Canning et al. identified the presence of afferent neurones originating in the nodose ganglia that conducted action potentials in the Aδ range and were highly sensitive to mechanical stimuli and acid but insensitive to capsaicin and bradykinin. The authors identified differences between these Aδ fibres and the pulmonary RARs and SARs, suggesting that they may have originally been poorly classified (Canning and Chou 2009). In contrast to pulmonary RARs, the re-defined fibres were found to have a slower action potential conduction velocity, no response to lung distension, smooth muscle contraction or negative airways pressures and no activation in response to purines such as ATP or histamine (Canning et al. 2004). Findings by other authors supported these observations (Fox et al. 1993; Ho et

al. 2001); however the presence of RARs and SARs localised to the trachea in dogs and cats that did respond to lung distension and/or smooth muscle contraction suggest that this unique subset may not be present in all species (Schelegle and Green 2001; Widdicome 2003). Canning et al. suggest that these Aδ fibres rather than representing RARs represent the 'cough receptor', which will be discussed further in the next section.

C-fibre receptors

C-fibre receptors are non-myelinated afferent nerves, which are subdivided into two types; the bronchial C-fibres are located within the airway mucosa, and the pulmonary C-fibres, (previously termed J-receptors) are situated in the parenchyma of the lung. Experiments on dogs by Coleridge and Coleridge distinguished these subtypes through assessing the response of receptors to a variety of drugs injected into the left and right atrium respectively. Local probing to identify the fibre site was also used (Coleridge and Coleridge 1977).

As well as having differing locations within the airway these two groups of afferents were also found to have different sensitivities to chemical and mechanical stimuli. Both subtypes were found to be chemosensitive. Pulmonary C-fibre activation occurred on injection of capsaicin into the right atrium resulting in bradycardia and apnoea. Stimulation also occurred on exposure to prostaglandins (Coleridge and Coleridge 1977; Coleridge et al. 1978). Mechano-sensitivity was also a feature of pulmonary C-fibres but this occurs on large inflations of the chest only; they are quiescent during normal respiration. Bronchial C-fibres were not mechano-sensitive but were found to be more chemo-sensitive, with a reduced time to activation following the administration of histamine and prostaglandins and increased activity to bradykinin, a feature not present in pulmonary C-fibres (Kaufman et al. 1980). Studies show that broncho-pulmonary C fibres are chemosensitive to a number of other exogenous chemical substances and environments, but importantly are also sensitive to endogenously released mediators at physiological levels (Lee and Pisarri 2001). Murine studies have illustrated that various inflammatory mediators, such as prostaglandin E3 (PGE-3) released in the airways during local inflammatory reactions can stimulate C-fibres and enhance their sensitivity (Ho et al. 2000).

The locations of the receptors were distinguished by using intravenous drugs to map the vascular routes by which they were accessible. Pulmonary C-fibres were found to have their endings adjacent to pulmonary capillaries, whilst bronchial fibres were largely found in the central airways but were identified in bronchi as small as 1.0 millimetre in diameter (Coleridge and Coleridge 1977). The accuracy of this technique of localisation has been questioned due to the possibility that some nerve endings that could not be located may have been within the vasculature itself and the presence of systemic-pulmonary anastamoses may have also affected the interpretation (Santambrogio 1982).

Unmyelinated fibres identified in the epithelium of mammals, (believed to be synonymous with bronchial C-fibres) contain neuropeptides, in particular calcitonin gene-related peptide (CGRP) and the tachykinins substance P (SP) and neurokinin A (NKA). Retrograde tracing studies in guinea pigs have identified that neuropeptide-containing fibres usually arise from the jugular ganglion, whereas non-neuropeptide containing fibres arise from the nodose ganglia (Undem et al., 2004). Following synthesis of the neuropeptides they are transported from the jugular ganglion and stored in the peripheral fibre endings until C-fibres are stimulated in the respiratory tract which triggers their release (Lundberg and Saria 1987; Barnes 1990). The stimulation of these nerves endings can result in neuropeptide release by a local axon reflex, or a central reflex pathway (Lee and Pisarri 2001). Given the close proximity of these neuropeptide containing C-fibres to airway parasympathetic ganglia (Lundberg et al. 1984; Lundberg and Saria 1987) which contain receptors for tachykinins, it is believed that there may be a peripheral sensory autonomic reflex which controls cholinergic and NANC activity when C-fibres are activated (Undem et al. 1999).

The effector cells targeted by the neuropeptides are airway and vascular smooth muscle cells, cholinergic ganglia, inflammatory cells and mucous cells, which have been seen to result in broncho-constriction, increased mucous secretion and bronchial vasodilatation (Lundberg and Saria 1987).

Via the local axon reflexes and antidromic neuropeptide release, C-fibres have been associated with ‘’neurogenic inflammation’’ (Barnes 2001). This theory of sensory nerves amplifying inflammation in the airways by the release of

tachykinins has been described in a number of species, however the significance in humans continues to be debated (Barnes 1990).

Several studies have identified SP and CGRP containing fibres distributed in the epithelium, lamina propria, bronchial and vascular smooth muscle and surrounding glands in human airways (Lundberg et al. 1984; Ollerenshaw et al. 1991; Lamb and Sparrow 2002). Other studies failed to identify these fibres however (Laitinen et al. 1983; Howarth et al. 1991), which may be as a result of tissue fixation delays or as others have found may be related to age and smoking (Hislop et al. 1990; Lamb and Sparrow 2002). A more recent study using immunohistochemistry in smoking and non-smoking COPD patients identified that non-smoking COPD patients had increased SP immunoreactivity in the epithelium and around mucous glands but reduced immunoreactivity in the smooth muscle layer, identifying a possible difference of innervation in disease states and environmental exposures (Vatrella et al. 2010).

Neuroepithelial bodies

Neuroepithelial bodies (NEBs) are clusters of neuroendocrine cells seen throughout human airway epithelium. The study of these bodies has been limited by their low numbers and widespread distribution. The cells are more numerous in foetal lungs and act as modulators of foetal lung growth and differentiation, but their physiological role after this stage is less well understood (Barnes 1990). Electron microscopy has shown that neuroepithelial body cells contain numerous dense core vesicles which are believed to be storage sites for peptide neurotransmitters, and murine studies suggest that these bodies are innervated by various fibre types and may represent polymodal receptors (Cutz et al. 2013). Currently they are believed to be involved in a co-ordinated system with sensory afferents: studies in humans with chronic lung disease support this as immunohistological techniques have identified that NEBs form a dense network with numerous nerve fibres storing SP, CGRP, and VIP beneath the epithelium (Pilmane et al. 1995).

1.3.3 Innervation and the pathogenesis of IPF

The role of pulmonary nerves in the pathogenesis of IPF is currently unclear, but several observations imply that there may be an association.

Firstly, neural tissue has been found to be in close association with the cells implicated in the pathogenesis of pulmonary fibrosis, such as epithelial cells, fibroblasts and the extra-cellular matrix (Brewster et al. 1990; Lamb and Sparrow 2002). It is suggested that it is the interactions between these components that allow cellular function, including a co-ordinated response to inflammation and tissue repair (Evans et al. 1999).

Secondly, there is increasing evidence that neuronal dys-regulation is involved in the pathogenesis of asthma (Nockher and Renz 2006). Through the process of 'neurogenic inflammation,' sensory airway nerves amplify the immune response through the release of tachykinins such as substance P, which can induce capillary leak and a subsequent cascade of inflammatory responses (Barnes 2001). Increased numbers of SP containing nerves have been found in the airways of asthmatic patients (Ollerenshaw et al. 1991) as well as elevated levels of SP in sputum and BAL fluid of asthmatic patients when compared to controls (Nieber et al. 1992; Tomaki et al. 1995). There is also evidence that SP and neurokinin A influence fibroblast activity suggesting a role in connective tissue deposition and fibrogenesis (Harrison et al. 1995).

Finally the role of neurotrophins such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) in this neuro-immune interaction have also been implicated in asthma. Increased levels of NGF and BDNF have been found in serum, BAL fluid and bronchial biopsies in patients with asthma (Nieber et al. 1992; Bonini et al. 1996; Nockher and Renz 2006). Neurotrophins are growth factors that as well as controlling the development and function of neurons have been found to regulate inflammatory processes (Hoyle 2003) and have a fibrogenic effect on skin and lung fibroblasts (Micera et al. 2001). They can be expressed and released by a variety of cell types within the respiratory epithelium. Studies have shown that amongst other actions neurotrophins modulate the activity of sensory neurons and enhance the synthesis and release of

neuropeptides such as SP (Lindsay and Harmar 1989), resulting in further neural stimulation and sensitisation, immune cell activation and epithelial disruption. What remains unclear is whether the neural stimulation and epithelial disruption is a cause or consequence of increased neurotrophin levels. Murine studies have suggested that epithelial disturbance is independent of sensory hypersensitivity; however the underlying mechanisms have not yet been characterized (Nassenstein et al. 2007).

Given that an increase in SP (Takeyama et al. 1996) and neurotrophin (Hope-Gill et al. 2003) levels have been measured in the BAL fluid and induced sputum of patients with IPF respectively, it is possible that similar mechanisms operate in IPF. These findings may also provide further information with regards the pathogenesis of cough in IPF; however the exact processes remain unclear. The next section aims to outline the current understanding of the cough reflex in man.

1.4 COUGH REFLEX NEUROPHYSIOLOGY

Cough reflexes play an essential role in protecting the respiratory tract, maintaining airway function and gaseous exchange. Our understanding of the neurophysiology of cough is largely derived from animal studies with limited human data.

The vagal afferents are responsible for initiating the cough reflex. Cough can originate from any of the structures that are innervated by the vagus, including the lungs, external ear, oesophagus and abdominal organs (Widdicombe 1995). Sensory information from these structures is carried in the vagus to the brainstem, where the afferent fibres synapse with second order neurones in the nucleus tractus solitarius (NTS) (Chung and Widdicombe 2009). This 'cough centre' then coordinates the efferent response (Polverino et al. 2012). As well as this central cough reflex, interaction of sensory afferent nerves and local axon reflexes are also believed to mediate cough. Consciousness is not required for this protective mechanism, however a conscious awareness of the 'urge to cough' may be described, which occurs before or during the cough. This awareness can result in control of the brainstem motor cough pattern and result in voluntary cough or cough suppression (Davenport et al. 2007).

Current opinion based primarily on animal studies suggests that the rapidly acting receptors (RARs), C-fibres and a third vagal afferent nerve subtype that has not been classified as a RAR or a C-fibre may play the key roles in regulating cough. Whether studies in experimental animals can be directly translated to humans remains a subject of much debate.

1.4.1 Rapidly Acting Receptors

RARs have long been thought of as the principal afferent nerve fibres that mediate cough in the airways (Widdicombe 1995). The location of RARs in the extra-pulmonary airways (larynx, trachea and large bronchi) and their activation by chemical and mechanical stimulation provided the initial evidence for their involvement (Widdicombe 1954c). Vagal cooling studies in cats and dogs provided further evidence as cough was inhibited on cooling the vagus nerve

which inhibits conduction through RARs whilst allowing C-fibres to function as normal (Widdicombe 1954c; Anderson et al. 1990). Some authors questioned the primary role of RARs in cough mediation, however due to the following observations to the contrary. Firstly, it was noted that whilst RARs had shown activation to a number of chemical stimuli such as tachykinins, methacholine, and forced inspiration or expiration against a closed glottis, stimulation with these mediators often failed to result in cough (Widdicombe 1954c; Canning et al. 2004; Canning 2006) Secondly, animal studies had shown that RARs can be active throughout the respiratory cycle whereas cough occurs only intermittently, therefore doubt was raised as to their ability to induce cough upon stimulation by specific stimuli (Canning 2006). Finally, the classification of the afferent fibres in the extra-pulmonary airways has recently been questioned, with Canning and colleagues proposing the fibres originating from the large and upper airways being a fibre type distinct from RARs (Canning et al. 2004). The presence of intrapulmonary RARs has not been questioned, however rather than playing a key role in mediating cough it is believed that at most they may have a non-essential role in the modulation of cough (Canning and Chou 2009).

1.4.2 C-fibre receptors

The role of C-fibres in mediating cough has also been the subject of much controversy and still remains unclear. Their activation by capsaicin, bradykinin and citric acid provides supportive evidence as these stimulants have been shown to induce cough in animals and humans (Coleridge et al. 1965; Choudry et al. 1989; Midgren et al. 1992). The selectivity of the C-fibre for capsaicin is of particular importance, as animal studies have shown that Aδ fibres are largely insensitive to capsaicin (Fox et al. 1993; Canning et al. 2004). Studies in guinea pigs have also shown that the cough induced by capsaicin and citric acid can be inhibited by desensitisation with capsaicin whereas it failed to inhibit cough induced by mechanical irritation believed at that time to be mediated by RARs (Forsberg et al. 1988). This is believed to occur as a result of peptide (SP) depletion from the sensory nerve endings. The capsaicin antagonist, capsazepine and a bradykinin receptor antagonist were also found to inhibit cough understood to be induced by C-fibres in these animals (Lalloo et al. 1995; Fox et al. 1996).

The main argument against the role of C-fibres in cough results from the inability of the known C-fibre stimulants, capsaicin and bradykinin, to evoke cough in anaesthetised animals, whereas mechanical induction of cough remains possible (Canning et al. 2004). Evidence of ongoing cardiopulmonary reflexes elicited by C-fibres suggests that general anaesthesia does not inhibit the activation of C-fibres, but either results in inhibition of the neural pathway or the conscious perception of airways irritation (Canning 2006). This latter hypothesis is supported by the ability of normal volunteers to suppress cough induced by capsaicin inhalation, which suggests that C-fibre mediated cough may not strictly be a reflex response (Hutchings et al. 1993; Mazzone 2005). It has been postulated that the C-fibre reflex response may be inhibitory as shown in experiments on anaesthetised cats (Tatar et al. 1988), and when conscious this inhibitory reflex is voluntarily over-ridden (Mazzone 2005). This theory does not correlate with cough suppression following capsaicin inhalation however, and the experiments on guinea pigs by Canning et al. are in support of there being C-fibre subtypes with neuropeptide containing fibres that originate from the jugular ganglion which initiate coughing, whereas the nodose originating non-neuropeptide containing fibres inhibit cough (Canning et al. 2004; Canning and Chou 2009). Current opinion suggests that C-fibres do have a key role in regulating cough, however the exact mechanism and interactions between these and other afferent fibres remain poorly understood. The molecular targets of C-fibres have been identified as the transient receptor potential (TRP) channels and will be discussed in detail in a subsequent section.

1.4.3 The cough receptor?

As outlined briefly in the section on pulmonary innervation, Canning et al. have identified Aδ fibres in guinea pigs which they suggest rather than representing RARs represent the principal afferent fibre regulating cough, or the ‘cough receptor’ (Canning et al. 2004). As described above, these fibres have been identified in the larynx, trachea and large airways, and are stimulated by punctate mechanical stimuli and rapid changes in pH. Their location in the upper airways and between the epithelium and airway smooth muscle, where they tether to the adventitial side of the matrix, dictates their ability to evoke reflex defensive coughing and their exquisite sensitivity respectively (Canning et al. 2004). The

ability of this fibre type to evoke cough in both awake and anaesthetised animals provides further evidence in support of this as a cough receptor.

The main controversy regarding these cough receptors at present is whether the findings in guinea pigs can be applied directly to other species. The absence of this fibre type in mice and rats which do not cough is supportive; however in cats and dogs fibres responsive to airway distension and smooth muscle contraction, which are not features of the guinea pig cough receptor, are present in the trachea, suggesting that a comparable fibre type may not be present in these animals (Schelegle and Green 2001; Widdicome 2003). Recent preliminary data from Immunohistochemical studies on whole-mount preparations of tissue from patients suffering from chronic cough identified fibres that were consistent with the Aδ-fibres described in guinea pigs, suggesting that 'cough receptors' may be present in human airways (West et al. 2012).

In summary, current evidence suggests that C-fibre afferents and extra-pulmonary Aδ-fibres, (known in guinea pigs as cough receptors) have key roles in cough regulation. These two fibre types may represent different neuronal pathways, with the extra-pulmonary Aδ-fibres facilitating a basic defensive cough reflex and the C-fibres facilitating cough which is under a degree of voluntary control (Canning and Chou 2009; Woodcock et al. 2010). It is likely, that interactions exist between the two pathways and the pathways of other afferent nerves such as RARs and SARs, however these are not fully understood at present.

1.4.4 Endogenous cough mediators

A number of endogenous substances are involved in neuronal transmission and or activation as outlined in the previous section, and as a consequence also play a role in the cough reflex. These mediators may also be involved in the apparent increased sensitivity of the cough reflex observed in patients with chronic cough of various aetiologies. In such patients it appears that cough is evoked by stimuli that would not normally initiate a cough reflex. Increased sensitivity may result from functional and or structural changes in nerve tissue (Kollarik and Undem

2003). Evidence for the involvement of endogenous substances in this process is outlined below.

Tachykinins

As previously described, unmyelinated C-fibres have been found to contain several neuropeptides, including substance P (SP), neurokinin A (NKA), and calcitonin gene–related peptide (CGRP), which may be released from sensory nerves upon stimulation. Neuropeptide release occurs via a local axon reflex, or a central reflex pathway (Lee and Pisarri 2001). Neuropeptides have also been shown to have neuromodulatory and immunodulatory influences and a role in inflammation of the lung (Daniele et al. 1992). A peripheral sensory autonomic reflex is also present, by which the neuropeptides released mediate cholinergic and Nonadrenergic, noncholinergic (NANC) activity (Undem et al. 1999) Therefore as well as their local effects neuropeptides are believed to have effects in the lung distant to their origin.

SP and NKA are members of the tachykinin family, which act via the NK-1 and NK-2 receptors respectively. Neutral endopeptidase (NEP) is the major enzyme that degrades tachykinins. Located on the airway epithelium NEP cleaves and inactivates tachykinins that have been released following nerve stimulation (Sekizawa et al. 1996). As well as the role of tachykinins and in particular SP in 'neurogenic inflammation' as described previously, its role in cough has been extensively investigated. Animal studies have shown that there is a dose-dependent increase in cough following intra-peritoneal injection of a NEP inhibitor, which was inhibited by the use of neurokinin receptor antagonists (Ujiie et al. 1993) Human studies have also provided support for the role of SP in cough, with IPF patients amongst others having an increased cough sensitivity to inhaled SP and capsaicin, (Hope-Gill et al. 2003) and elevated SP levels in BAL fluid (Takeyama et al. 1996). More recently raised plasma levels of SP have been measured in patients with chronic cough (Otsuka et al. 2011).

There may also be a link between the role of SP in 'neurogenic inflammation' and cough; it is postulated that conditions resulting in a damaged airway epithelium allow better penetration of SP, resulting in neurone activation and increased

cough. This was demonstrated in experiments of SP inhalation in patients with common cold: SP induced cough in those with common cold, but failed to do so in control subjects (Katsumata et al. 1989). The inflammatory response may also result in local changes in bronchial smooth muscle contraction and mucous production which in turn may evoke cough via activation of the mechanically sensitive Aδ-fibres (Advenier and EmondsAlt 1996).

CGRP is co-localised with SP and NKA in airway C fibres. CGRP is a vasodilator of bronchial vessels in vitro and given the localisation of its receptors to airway blood vessels it is believed to play a role in airway hyperaemia that is commonly seen in asthmatic airways, however its role in cough is less clear (Mak and Barnes 1988).

Inflammatory mediators

Pro-inflammatory mediators such as bradykinin, histamine and prostaglandin-E2 (PGE-2) are produced and secreted in the lungs in response to a variety of inflammatory conditions. They are all known endogenous C-fibre stimulants in animals (Kaufman et al. 1980) and have been demonstrated to induce cough directly in normal and asthmatic patients (Choudry et al. 1989). Patients with chronic cough have also been shown to have increased levels of these pro-inflammatory mediators in induced sputum (Birring et al. 2004).

These mediators are also believed to increase the sensitivity of afferent nerve fibres (Carr 2004). They sensitise protein channels on afferent C-fibres via G protein-coupled receptor mechanisms, as discussed in more detail in the next section (Maher et al. 2011). Studies confirming this heightened sensitivity have been conducted in animals and humans. For example, bradykinin reduced the electrical threshold of citric acid induced cough in guinea pigs (Mazzone et al. 2005), and in man capsaicin induced cough was found to be increased after inhalation of PGE2 (Choudry et al. 1989). Bradykinin-induced sensitisation of airway nerves has in particular been implicated in the pathogenesis of Angiotensin-Converting Enzyme Inhibitor (ACE-i) cough (Fox et al. 1996). Evidence from animal studies also suggests that these inflammatory mediators can sensitise airway mechanoreceptors (Riccio et al. 1996; Ho et al. 2000).

Neurotrophins

As outlined in the section on pulmonary innervation, neurotrophins are growth factors, expressed and released from cells within the respiratory epithelium. Neurotrophins are the key mediators of neuroplasticity, which is the adjustment of the nervous system to environmental changes, and can be physiological or pathological (Trojan and Pokorny 1999). In particular, nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) have a role in maintaining neurons and can stimulate growth (Trojan and Pokorny 1999). They perform this role by binding to high-affinity tyrosine kinase (TK) receptors on vagal sensory nerve endings and this neurotrophin-TK complex is then transported to the cell body (Helke 2005). Levels of NGF are known to be increased in inflammation (Woolf et al. 1996), and have been shown to be raised in conditions such as asthma and IPF (Bonini et al. 1996; Hope-Gill et al. 2003)

Neurotrophins have been implicated in the sensitisation of afferent fibres and therefore increased cough sensitivity via a number of mechanisms. Firstly, neurotrophins have been shown to result in an increased synthesis and release of neuropeptides (Lindsay and Harmar 1989), which was believed to be a result of gene induction in neurones (Kollarik and Undem 2003). However guinea pig studies have suggested that during the presence of inflammation and or infection, NGF can result in the phenotypic switch of non-neuropeptide containing Aδ fibres to neuropeptide containing fibres, which are known to converge with C- fibres in the central nervous system (CNS) (Carr et al. 2002; Myers et al. 2002). ‘Central sensitisation’ is thought to be a consequence of this functional change as increased neuropeptide release can augment synaptic transmission in the central nervous system; this possibly results in a tachykinin mediated response from a mechanical stimulus (Kollarik and Undem 2003). This phenomenon is widely appreciated in the peripheral sensory system and can result in chronic pain. The process of ‘central sensitisation’ has also been implicated in the association between cough and gastro-oesophageal (GOR) reflux; it is postulated that afferent oesophageal and airway fibres may converge in the central nervous system, and an altered sensitivity in one pathway could alter the CNS response to the other i.e. physiological distal reflux in the distal oesophagus resulting in cough (Woodcock et al. 2010).

Secondly, airway re-modelling with increased smooth muscle in the epithelium of patients with idiopathic chronic cough has been identified, which may be associated with increased neurotrophin levels (Niimi et al. 2003; Niimi and Chung 2004). Also as described previously, neurotrophins have also been shown to have influence on cells involved in the fibrotic process (Micera et al. 2001). Changes in the properties of the lung tissue may also have an influence on nerve sensitivity. For example, changes in the parenchyma may result in activation of stimulatory pulmonary RARs or inactivation of inhibitory RARs; if the mechanical forces within the lung change (Kollarik and Undem 2003).

Finally, neurotrophins are known to induce nerve growth and sprouting in response to tissue damage (Trojan and Pokorny 1999), and although it could be speculated that structural up-regulation of afferent fibres results from tissue injury in lung disease resulting in cough hypersensitivity this is yet to be proven. Using immunohistochemical techniques O’Connell et al. compared bronchial biopsies of patients with chronic cough with control subjects. Although the density of CRGP- immunoreactive nerves present in the epithelium were significantly higher in chronic cough patients there was not a significant difference in total nerve density visualised using a general nerve marker, protein gene product- 9.5 (PGP-9.5) (O' Connell et al. 1995). Evidence for the functional and structural up-regulation of TRP receptors is more conclusive however, and is detailed in the next section.

1.5 THE COUGH CHANNELS

There has been a recent increase in the understanding of the molecular nature of chemical receptors in airway sensory neurons. It is hoped that this will facilitate a better understanding of the neural pathways regulating cough in health and disease.

For the mechanical or chemical stimuli to result in sensory nerve activation and evoke cough it must interact with membrane ion channels in a manner that results in membrane depolarisation in the peripheral terminals of the afferent fibres (Kollarik and Undem 2006). There are a number of identified ion channels, however the key ones involved in this process include: Transient Receptor Potential channels, sodium channels of the Degenerin/Epithelial (ENaC-Deg) family, 5-Hydroxytryptamine$_3$ receptors, Purinergic 2X receptors, Bradykinin B_2 receptors, Adenosine receptors and Nicotinic acetylcholine receptors (Kollarik and Undem 2006). This thesis will only focus on the three receptors below, however a full review can be found in Kollarik and Undem 2006.

1.5.1 Transient Receptor Potential Vanilloid 1

Transient receptor Potential Vanilloid 1 (TRPV-1) is a member of the transient receptor potential (TRP) family of ion channels that modulate sensory and inflammatory information. This proteinacious ion channel, which is expressed on the neuronal plasma membrane, is the molecular target of capsaicin action on sensory neurons, which is why it is otherwise known as the capsaicin receptor. TRPV-1 was first cloned and characterised in 1997 (Caterina et al. 1997), and its role in cough was confirmed in guinea pigs using capsazepine, a capsaicin antagonist (Lalloo et al. 1995).

Immunohistochemical studies have confirmed a heterogeneous expression of TRPV-1 channels in the airways (Groneberg et al. 2004a; Watanabe et al. 2005). In particular TRPV-1 was found to co-localise with SP and CGRP- containing C-fibre afferents in the extra and intra-pulmonary airways in guinea pigs; although TRPV-1 immunoreactive non-neuropeptide containing fibres were also visualised. The TRPV-1 containing axons were present in and around sub-epithelial regions of the airways, including smooth muscle and blood vessels and

within the lower airways down to the alveolar level (Watanabe et al. 2005). Interestingly TRPV-1 was not identified in pulmonary epithelial cells of guinea pigs, whereas it has been identified in human epithelial cells in vivo (Groneberg et al. 2004a; McGarvey et al. 2014) and in vitro (Agopyan et al. 2003) although the significance of this remains unclear at present.

TRPV-1 contains six transmembrane domains, with both the N- and C- termini on the cytosolic side of the cell membrane (Figure 15). These domains assemble as tetramers to form cation-permeable pores. On adequate stimulation the non-selective cation channel, (which is highly permeable to calcium), opens and allows ions to flow through initiating a nerve impulse and the subsequent response from the neuronal pathway (Caterina et al. 1997; Clapham 2003). Being a polymodal receptor, as well as being activated by capsaicin it is also activated by numerous exogenous and endogenous agonists, heat (>42 ºc) and extracellular protons (pH < 6.0) (Surowy et al. 2010). This activation either occurs directly or indirectly via G protein-coupled receptors on the cell membrane which initiate intracellular signalling cascades (Maher et al. 2011). Interestingly, activation of TRPV-1 by singular specific stimuli often produces a sub-maximal activation, whereas the activation threshold is lowered if two or more stimuli are present and act synergistically; for example, at low pH, TRPV-1 can be activated at body temperature (Surowy et al. 2010).

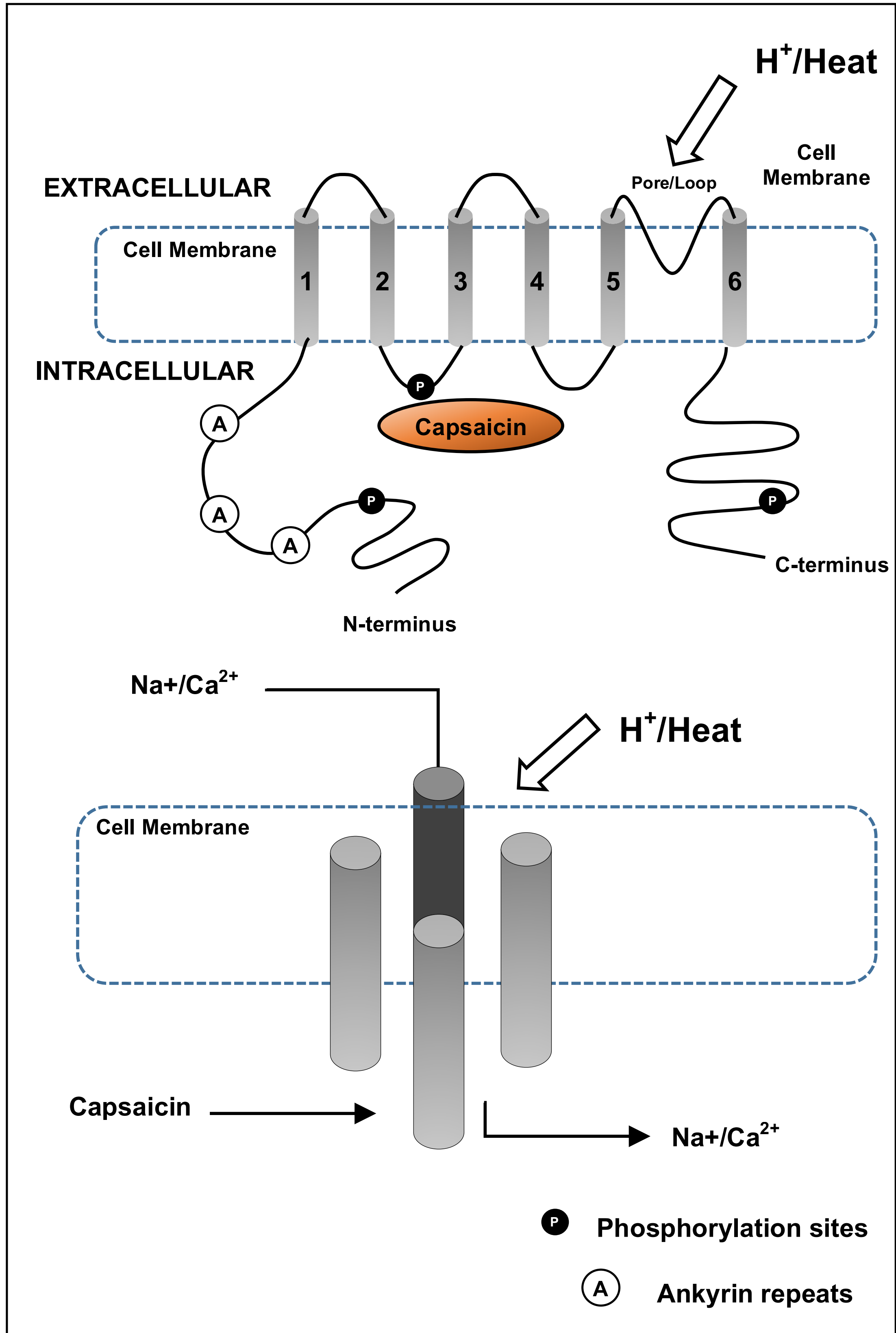


Figure 15 Schematic structure of TRPV-1
Adapted from Toxicology of the lung, 4th edition (Gardner 2006)

As well as desensitisation occurring in TRPV-1 channels, as shown by the inhibition of cough with capsaicin pre-treatment in experiments in guinea pigs, sensitisation can also occur (Surowy et al. 2010). TRPV-1 sensitisation of peripheral nociceptors is believed to be responsible for hyperalgesia in chronic pain, and there is growing evidence for its role in cough (Nilius et al. 2007; Adcock 2009). Endogenous pro-inflammatory mediators including bradykinin, prostaglandins (PGE2) and NGF (which are released during inflammatory processes) can sensitise TRPV-1 which reduces the activation threshold and heightens responsiveness to stimuli, resulting in increased neuronal firing and possibly enhanced cough reflex (Chuang et al. 2001; Gu et al. 2003; Mazzone et al. 2005). Exogenous stimuli such as ethanol have also been found to sensitise this channel (Trevisani et al. 2002). TRPV-1 sensitisation occurs through multiple phospholipase C (PLC) coupled receptor pathways, and phosphorylation through protein kinase C (PKC) and other kinases also play a role; however the exact mechanisms underlying the sensitisation of TRPV-1 are not yet fully understood. Figure 16 illustrates the multiple signal transduction pathways that are known to be involved (Adcock 2009). A key finding that supports the sensitisation of TRPV-1 by inflammatory mediators and its role in cough is the reduced threshold for capsaicin-induced cough in a number of 'inflammatory' airway conditions such as IPF (Doherty et al. 2000a; Hope-Gill et al. 2003), cough variant asthma (Millqvist et al. 2000), COPD (Doherty et al. 2000b) and chronic cough (Cho et al. 2003). The increased cough sensitivity to PGE-2, histamine and bradykinin also supports this mechanism (Choudry et al. 1989).

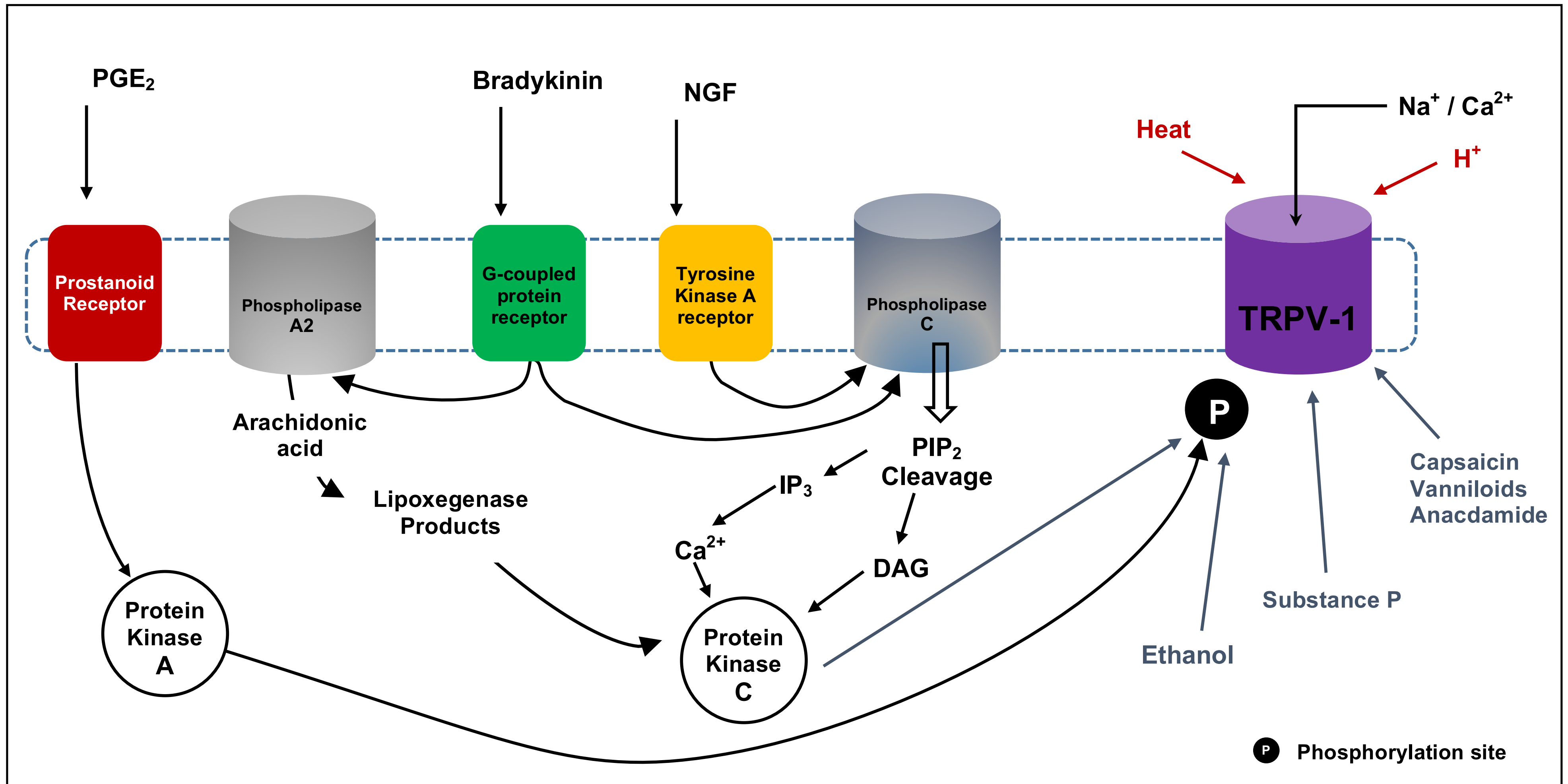


Figure 16 **Proposed mechanisms for sensitising TRPV-1 receptors.**
Adapted from (Adcock 2009)

TRPV-1 is also believed to have a role in ‘neurogenic inflammation’ as SP has also been found to cause sensitisation. As described earlier SP is released from neuropeptide containing C–fibre afferents upon activation, which is mediated by TRPV-1 channels. After release, SP mediates its pro-inflammatory effects via neurokinin-1 receptors (NK-1 receptors). It has been demonstrated that TRPV-1 and NK-1 receptors are co-localized in the dorsal root ganglion neurons of rats, and that capsaicin-induced currents in TRPV-1 were increased on the binding of SP to NK-1 receptors (Zhang et al. 2007). As discussed in a previous section, the role of neurogenic inflammation in human airways is debated due to the apparent lack of neuropeptide containing fibres in humans compared to guinea pigs; however this observation is important as TRPV-1 has also been identified on non-neuropeptide containing C-fibres.

In addition to the physiological up-regulation of TRPV-1, there is also evidence of functional up-regulation of TRPV-1 playing a role in the pathophysiology of cough. Immunohistochemical studies comparing bronchial biopsies of patients with and without chronic cough identified a significant increase in TRPV-1 staining nerve profiles, which was positively correlated with capsaicin tussive response (Groneberg et al. 2004a). A second study failed to show a difference in neuronal TRPV-1 expression, however did find that patients with chronic cough had a significantly higher expression of TRPV-1 in smooth muscle cells, the significance of which remains unclear (Mitchell et al. 2005). More recently, a study of chronic airway inflammation in rats demonstrated that inflammatory mediators can initiate the expression of TRPV-1 ion channels in airway afferent neurons that do not normally express them (Zhang et al. 2008). The neurotrophin, nerve growth factor (NGF) has been shown to up-regulate the expression and function of this channel, and has a key role in neuro-modulation (Chuang et al. 2001; Ji et al. 2002; Zhang et al. 2005).

As outlined above, there is increasing evidence supporting the hypothesis that the expression, activation and modulation of TRPV-1 in sensory neurones is integral to the regulation of cough pathways, although the precise contribution of TRPV-1 in normal and pathological cough has yet to be confirmed.

1.5.2 Transient Receptor Potential Ankyrin 1 (TRPA-1)

TRPA-1 is also a six trans-membrane, calcium preferring, non-selective cation channel which belongs to the TRP family. First isolated in 1999, its distinguishing feature is an extended 14-ankyrin repeat domain in its amino terminus (Jaquemar et al. 1999). TRPA-1 is insensitive to capsaicin, but instead binds allyl isothiocyanate and allicin found in mustard oil and garlic respectively, and is also activated by cold air and a number of environmental irritants such as acrolein, and other electrophilic compounds that are present in air pollution, vehicle exhaust fumes, and cigarette smoke (Taylor-Clark et al. 2009).

Animal studies have shown that TRPA-1 is co-expressed with TRPV-1 on neuropeptide C-fibre afferents, and stimulation of the TRPA-1 channel results in C-fibre activation (Nassenstein et al. 2008; Taylor-Clark et al. 2008; Taylor-Clark et al. 2009). Cultured human pulmonary cells and preliminary immunohistochemical studies on human bronchial biopsy samples have also confirmed the expression of TRPA-1 in the epithelium, smooth muscle bundles and nerve tissue (Anand et al. 2008; Faruqi et al. 2011a). The role of TRPA-1 in cough was established following the stimulation of cough in guinea pigs and humans after exposure to TRPA-1 agonists (Andre et al. 2009; Birrell et al. 2009). This finding was supported by the inhibition of cough in guinea pigs following the administration of TRPA-1 antagonists, which did not occur on administration of TRPV-1 antagonists (Andre et al. 2009).

Similar to TRPV-1, TRPA-1 mediates inflammatory pain; however compared with TRPV-1 the evidence for its role in pathological cough is less conclusive. TRPA-1 is also activated by endogenous inflammatory mediators, however in addition to substances such as bradykinin and PGE_2 released during inflammation, they are also activated by mediators associated with oxidative injury, such as reactive oxygen species (ROS). Animal studies have observed that sensitisation of TRPA-1 may occur via similar signal transduction pathways as seen in TRPV-1 (Dai et al. 2007; Wang et al. 2008a; Grace et al. 2012).

Given the co-localisation of the two TRP channels it is likely that cooperation occurs, however this is currently unproven. TRPA-1 has been found to be

dependent on calcium for activation and it is possible that TRPA-1 could be activated by intra-cellular calcium, from ‘over-spill’ of other activated channels. It is also possible that TRPA-1 could amplify other calcium-mobilising pathways, causing activation and sensitisation of TRPV-1 and neuronal firing (Zurborg et al. 2007; Cavanaugh et al. 2008; Grace and Belvisi 2011).

Despite accumulating evidence which has led to an increased understanding of the molecular role and interactions of the TRPV-1 and TRPA-1 channels, uncertainty remains with regards the regulation of TRPV-1 and TRPA-1 transcription in humans. Transcription is a process in which the enzyme RNA polymerase copies the information contained in a DNA strand into messenger RNA. This process is highly regulated and depends upon the chromatin structure, the presence of activating and repressing protein transcription factors and post-transcriptional modification also occurs through the influence of microRNAs and DNA methylation (Hoopes 2008).

Transcription factors are proteins which regulate the process of transcription and along with RNA polymerase bind to specific promoter sequences on the DNA strand to initiate transcription (Lodish 2000). Analysis of genomic data with algorithms such as DECODE: DECipherment Of DNA Elements (Qiagen, UK) which explore genome databases have predicted a number of regions within TRPV-1 and TRPA-1 that are anticipated to bind a wide range of transcription factors and therefore act as promoter regions. Biological validation of several TRPV-1 transcription factors including specificity protein 1 (Sp1) (Chu et al. 2011), Runt related transcription factor (RUNX1) and CCAAT/enhancer-binding protein beta (C/EBP β) (Ugarte et al. 2013) has been achieved in the murine model, however the promoter sequences are not well understood in humans (Park et al. 2007). Human promoter sequences for TRPA-1 have been identified however, and epigenome-wide association studies have demonstrated differential methylation of the promoter region which is relative to pain sensitivity (Bell et al. 2014), although these mechanisms remain poorly understood (Hatano et al. 2012).

1.5.3 Acid Sensing Ion Channels (ASICs)

Acid sensing ion channels are amiloride sensitive sodium channels that belong to the epithelial Na^+ /degenerin (ENaC-Deg) family of proton-gated ion channels (de la Rosa et al. 2000). This is a relatively newly recognised group of ion channels; the first ASIC was cloned in 1997 (Waldmann et al. 1997). Six different proteins arising from four genes have been cloned to date, ASIC1a, ASIC1b, ASIC2a and ASIC2b, ASIC3 and ASIC4 (Wemmie et al. 2006). Four of these subunits form functional homomultimeric channels, with homomeric and heteromeric ASICs having distinct pH sensitivity, ion selectivity, and channel kinetics (Hesselager et al. 2004). ASICs are expressed widely throughout the central and peripheral nervous system in mammals, on sensory neurons in particular. They are simple ligand gated channels that are transiently activated by rapid extracellular acidification (Waldmann et al. 1997).

In their studies on guinea pigs using immunohistochemistry, Canning et al. identified the presence of sodium channels on the Aδ afferent fibres (Canning et al. 2004). Subsequent patch-clamp experiments on rat vagal pulmonary sensory neurons confirmed that ASICs as well as TRPV-1 were involved in acid signalling. It was noted that an extracellular pH of 7.0 resulted in only a transient inward current which the current-voltage curve indicated was predominantly as a result of sodium influx, whereas a pH of less than 6.5 resulted in a slow, sustained inward current. The transient inward current was subsequently inhibited by amiloride in a dose dependent manner, confirming the role of the ASIC, while the sustained component was inhibited by the TRPV-1 antagonist capsazepine, indicating it was mediated by TRPV-1 (Gu and Lee 2006).

ASICs were initially localised to the rat dorsal root ganglion (DRG) neurons, but more recently ASIC subunits 1a, 1b, 2a, and 3 were found to be expressed in rat pulmonary sensory neurons (de la Rosa et al. 2002; Gu and Lee 2010). Cultured human epithelial cells have been shown to express ASIC1a and ASIC3, however at present no in vivo human lung localisation studies have been completed (Agopyan et al. 2003).

Other than the known outcomes of c-fibre and Aδ fibre stimulation in humans as outlined above, the evidence for the role of ASICs in cough is limited to animal studies, which suggest that like TRPV-1, altered sensitivity and/or expression of ASICs in the airway can alter the excitability of bronchopulmonary sensory nerves (Gu and Lee 2011). Animal studies have identified mechanisms by which this increased sensitivity could occur, as outlined below.

Firstly, Protease-activated 2 receptors (PAR2) have been shown to increase sensitivity. These are G-protein coupled receptors that are found throughout the airways. Endogenous inflammatory mediators and exogenous proteases modulate their activity and PAR2 has been shown to be elevated in a variety of inflammatory lung diseases (Sokolova and Reiser 2007). Patch-clamp experiments with rat vagal pulmonary neurons, which had been pre-incubated with PAR2 agonists resulted in potentiation of the acid-invoked ASIC and TRPV-1 inward currents (Gu and Lee 2010). It is suggested that this mechanism may play a role in inflammatory lung disease, as a result of simultaneous PAR2 activation and tissue acidification (Gu and Lee 2011).

The response of acid-sensing channels in response to temperature was also recently studied and showed the sensitivity of ASIC channels to acid was inhibited at temperatures within physiological range, whereas the sensitivity of TRPV-1 was enhanced by any increase in temperature (Ni and Lee 2008). This difference may have clinical relevance as airway temperature is known to be significantly elevated during exacerbations of asthma and patients with chronic cough often report change in environmental temperature as a trigger factor (Piacentini et al. 2007; Gu and Lee 2011).

Given the recent identification of ASICs on pulmonary neurons it is not surprising that knowledge of their location and mode of activation remains limited. Better knowledge of the cough mechanisms at the molecular level gives us insight into the pathophysiology of cough and may allow the development of targeted therapies, however the pathophysiology of cough has been investigated in some diseases more than others and many aspects of the pathophysiology of cough in IPF remain unclear as discussed in the next section.

1.6 COUGH IN IDIOPATHIC PULMONARY FIBROSIS

Chronic cough (CC) is defined as a cough lasting 8 or more weeks, with most patients complaining of a dry or minimally productive cough (Morice et al. 2006). CC is very common within the general population and is a significant cause of morbidity (Morice 2008). Global prevalence has been estimated at 9.6%, however there is significant heterogeneity in the CC definitions used, which may affect the validity of this estimate (Song et al. 2015). There are many potential causes of CC with the most common thought to be as a result of asthma/eosinophilic bronchitis, rhino-sinusitis and GORD (Morice et al. 2004; Morice et al. 2007). There are various guidelines to aid the assessment and management of chronic cough (Morice et al. 2004; Irwin et al. 2006a; Morice et al. 2007), however despite a systematic approach, no clear cause for cough is found in significant proportion of patients (Haque et al. 2005). Evidence suggests, that in this sub-set of patients with CC of unknown cause (idiopathic chronic cough), that CC is a distinct clinical syndrome characterised by neuronal hypersensitivity, which has been termed cough hypersensitivity syndrome (Morice 2010; Chung 2011; Escamilla and Roche 2014). It has also been demonstrated that patients with chronic cough are less able to suppress cough during a capsaicin cough challenge than normal volunteers (Young et al. 2009). The epidemiological and resultant economic burden of CC has resulted in increased research in an attempt to identify therapeutic targets (Irwin et al. 2006a; Morice et al. 2007; Song et al. 2015), and as a consequence has also aided our understanding of IPF-associated cough, which may be as a result of similar pathogenic mechanisms.

1.6.1 Cough in IPF

Chronic cough is a common symptom described in patients with Idiopathic Pulmonary Fibrosis and impairs quality of life (French et al. 1998; Ryerson et al. 2011). Recent studies using 24 hour cough monitoring were able to quantify the extent of this debilitating symptom, and illustrated the need for a better understanding of the pathogenesis to allow therapeutic developments (Key et al. 2010). It has always seemed inconsistent that cough should be a prominent symptom of a disease that affects the peripheral lung parenchyma, whereas the afferent fibres believed to be associated with cough are concentrated in the

central airways, however as detailed above there are a number of potential mechanisms which could play a role in the increased cough sensitivity seen in IPF patients. The understanding and evidence for the mechanisms involved in IPF and cough remain limited; current understanding is outlined below.

Gastro-oesophageal reflux

Gastro-oesophageal reflux (GOR) and the micro-aspiration of gastric contents into the airways has been implicated in the pathogenesis of cough in IPF as well as the disease itself as already discussed in chapter 1. Patients with IPF have been found to have a high prevalence of GOR on 24 hour oesophageal pH monitoring and manometry, and are often asymptomatic (Tobin et al. 1998; Patti et al. 2005; Raghu et al. 2006a; Noth et al. 2012). There are two theories regarding how GOR can result in cough. One involves the micro-aspiration of gastric fluid into the lungs resulting in sensory afferent activation and inflammation. The second as discussed previously involves afferent oesophageal and airway fibres converging in the central nervous system, with altered sensitivity in one pathway altering the CNS response to the other i.e. physiological distal reflux in the distal oesophagus resulting in cough, which is supported by studies in which acid instilled into the distal oesophagus triggered cough in patients with asthma and chronic cough (Wu et al. 2002; Javorkova et al. 2008), and possibly by the temporal relationship between cough and GOR (Smith et al. 2010). A model of acid GOR has also recently been shown to result in a release of inflammatory mediators and up-regulation of TRPV-1 receptors in oesophageal cells (Ma et al. 2012). In this context TRPV-1 is of interest as it is believed to be involved in the ‘heart burn’ pain often associated with GOR (Guarino et al. 2010). Increased TRPV-1 gene expression was also reported in oesophageal biopsies taken from patients with erosive and non-erosive GOR in keeping with the in-vitro findings, however surprisingly there was no association between acid exposure times measured using oesophageal pH monitoring and TRPV-1 expression (Guarino et al. 2010).

Interestingly, recent studies using a combination of oesophageal pH and impedance monitoring; which is able to characterise all reflux episodes irrespective of pH; showed that non-acid as well as acid reflux occurs in IPF

patients (Savarino et al. 2013; Kilduff et al. 2014). In the study by Kilduff et al the patients underwent this testing with simultaneous 24 hour cough recording pre and post a period of acid suppression which demonstrated that proton pump inhibitors (PPI) had no effect on cough symptoms despite the abolition of acid reflux and yet resulted in an increase in non-acid reflux (Kilduff et al. 2014). Proton pump inhibitors have not found to be completely effective in the treatment of chronic cough and interestingly in these patients both acid and non-acidic reflux result in a heightened cough reflex sensitivity (Chang et al. 2006; Qiu et al. 2011). An in vitro study also provides supporting evidence, with GOR from patients on PPI treatment provoking a greater inflammatory reaction human primary bronchial epithelial cells than GOR from patients not on PPI treatment (Mertens et al. 2010). Together, the evidence suggests that the acid component of gastric refluxate may not be the sole cause of cough in IPF and interest has increased into the other components of gastric refluxate such as pepsin, pancreatic enzymes and bile salts.

Evidence for micro-aspiration of gastric fluid into the lungs is clear in lung transplant patients, with elevated levels of pepsin and bile acids, (key constituents of gastric refluxate), present in BAL fluid (D'Ovidio et al. 2005; Ward et al. 2005). In other respiratory diseases the evidence is not as conclusive, with there being no significant increase in pepsin or bile salts in the induced sputum of patients with chronic cough (Grabowski et al. 2011). It is recognised however that the methods for detecting these substances are limited (Emilsson et al. 2013). Recent studies of BAL pepsin in patients with IPF however have shown that BAL pepsin levels are significantly elevated in patients with an acute exacerbation of IPF, and interestingly most stable IPF patients also have measurable levels of pepsin in BAL fluid (Lee et al. 2012). More recent evidence of proximal reflux events and the presence of pepsin and bile acids in BAL and saliva of IPF patients provides further support (Savarino et al. 2013). This along with the finding that pepsin induces EMT in epithelial cells and bile salts can induce fibrogenesis in airway cells suggests that a link between GOR, IPF and cough may exist (Perng et al. 2008; Ahmad et al. 2009).

It is evident that patients with IPF may have cough that is caused or exacerbated by other co-morbidities, and these often confound the study of cough in IPF

(Harrison 2013). Common co-morbidities include rhino-sinusitis, obstructive airways disease, hypertension treated with angiotensin converting enzyme inhibitor therapy and GOR as already mentioned. A previous small study of 26 separate episodes of chronic cough in 21 patients with interstitial lung disease (8 patients with IPF) reported that chronic cough was found to have a cause other than the disease itself in 54% of cases (Madison and Irwin 2005). More recently obstructive sleep apnoea (OSA) has been increasingly recognised as a co-morbidity associated with IPF (Schiza et al. 2015). One single centre study of 50 patients with IPF and an average BMI of 32.2, reported a diagnosis of OSA in 88% of patients, with 34 (68%) patients having moderate-to-severe OSA (Lancaster et al. 2009). Interestingly, OSA has been associated with chronic cough, with a reported incidence of 33% in one study of 108 patients referred for OSA assessment (Chan et al. 2010). As there is clinical and epidemiological evidence supporting an association between OSA and GOR (Demeter et al. 2005; Jung et al. 2010), it was proposed that IPF, GOR and OSA were linked and resulted in a 'vicious triad' that provoked cough (Harrison 2013). A study investigating oesophageal reflux in 54 patients with fibrotic interstitial lung disease (22 patients with IPF) did not find that GOR measured with oesophageal impedance was higher in patients with OSA than those without OSA, therefore does not provide supporting evidence for this hypothesis (Pillai et al. 2012).

The current evidence suggests that although co-morbidities are an important consideration when performing an assessment of a patient with IPF and cough they alone are not sufficient to explain cough in all patients and thus other mechanisms are proposed.

Increased cough sensitivity

There is evidence for increased expression of neurotrophins in the lungs of patients with IPF. Compared with controls, elevated levels of NGF and BDNF have been demonstrated in induced sputum and BAL fluid in IPF patients (Hope-Gill et al. 2003; Harrison 2013), and neurotrophins have also been demonstrated on interstitial cells of patients with IPF using immuno-histochemical analysis (Ricci et al. 2007) .As detailed in chapter 1, neurotrophins are involved in neuronal differentiation and proliferation and can increase cough sensitivity via a

number of mechanisms. In addition, patients with IPF have a direct cough response to capsaicin and increased levels of the neuropeptide substance P in BAL fluid, which supports the possibility of a functional and or structural up-regulation of sensory afferents in IPF (Takeyama et al. 1996; Hope-Gill et al. 2003). Neurotrophins also influence the expression and sensitivity of the TRP channels in the airways (Chuang et al. 2001; Ji et al. 2002; Zhang et al. 2005), with evidence suggesting TRPV-1 channels are increased in patients with chronic cough (Groneberg et al. 2004a; Mitchell et al. 2005). It is not known whether this is the case in IPF.

Biomechanical factors

Another possible mechanism for the cause of cough in IPF is that mechanical distortion of small airways due to peribronchiolar fibrosis may result in enhanced stimulation of the peripheral RARs or destruction of the peripheral inhibitory nerve fibres. A study which provides evidence for this theory compared the cough response of mechanical stimulation to the posterior chest wall in patients with IPF and controls. Enhanced cough reflex sensitivity to chest wall vibration was observed in IPF patients but was not seen in control subjects (Jones et al. 2011). However, the lack of clear correlation between capsaicin cough sensitivity with measures of lung function (Doherty et al. 2000a; Hope-Gill et al. 2003) and objective measures of cough severity with lung function testing (Key et al. 2010) and disease severity (Kilduff 2013) reduces the likelihood that the mechanical effects of IPF alone are the cause of the enhanced cough reflex.

Genetic factors

A more recent development has been the potential genetic contribution to the IPF patient with cough. A study investigating the MUC5B polymorphism in in patients with IPF has demonstrated that the presence of the MUC5B minor T allele polymorphism was significantly correlated with cough severity as measured by LCQ (Scholand et al. 2014). The MUC5B gene has a role in the production of airway mucus and as described in chapter 1 has also been implicated in the development of IPF (Seibold et al. 2011). Scholand et al. propose that the MUC5B polymorphism may result in the distal airway production of mucin which

could accumulate and trigger a non-productive or productive cough. They suggest that their findings are corroborated by the demonstration of increased MUC5B mucin expressing cells on Immunofluorescence in the distal airway epithelium of IPF patients compared with controls (Seibold et al. 2011). The main limitation of the study by Scholand et al was that they did not control for co-morbid diseases in IPF that can also cause cough and therefore the results need confirmation with further studies. Provisional data proposing that MUC5B genotype does not influence cough severity was presented recently, however full publication is awaited (Saunders and Maher 2015).

1.6.2 Cough and IPF prognosis

There has been very little research into the prognostic value of cough in IPF. An early study found that the presence of cough in patients with CFA had no influence over survival (Turner-Warwick et al. 1980), however a study of 242 patients with IPF has shown cough to be an independent predictor of disease progression on multivariate analysis (OR 4.97, 95% CI 1.25-19.80, p =0.02) (Ryerson et al. 2011). A limitation of this study was that it did not include any subjective or objective measures of cough. A more recent longitudinal study of cough in 67 IPF patients that did include these measures found that although cough may worsen over time, there was no relationship between cough and baseline disease severity or progression of disease (Kilduff 2013).

1.6.3 Therapeutic options for cough in IPF

There have been no large randomised, placebo-controlled studies investigating treatments for cough in IPF to date, however there have been a number of small studies, which have influenced current therapeutic strategies with further studies currently underway. A summary of the drugs used and evidence behind their use is summarised in a table 1.

TABLE 1 A summary of the treatments for IPF-associated cough

Corticosteroids	An uncontrolled, open label study of a four week trial of high dose prednisolone in 6 patients with IPF showed an improvement in cough symptom severity and a reduction in cough reflex sensitivity to inhaled capsaicin (Hope-Gill et al. 2003).
Thalidomide	Thalidomide is an immune-modulating anti-fibrotic agent which has been investigated as a treatment for cough in IPF. 23 patients with IPF were randomly assigned to thalidomide or placebo in a 24 week, double blind crossover trial. The primary end point was an improvement in the Cough Quality of life Questionnaire, which was significantly higher in the thalidomide group, and there was also an improvement seen on VAS score. However adverse events (constipation, dizziness, malaise) were reported in 74% of the thalidomide group compared to 22% in the placebo group (Horton et al. 2012).
Gabapentin	Gabapentin, is an anti-convulsant but is also used to treat chronic pain. In a small randomised controlled study carried out in 62 patients with chronic cough over a 10 week period, gabapentin was found to result in a reduced cough severity and an improved quality of life related to their cough. There was however a relatively high rate of withdrawal from the study (10) (Ryan et al. 2012). The basis for the therapeutic action of this agent is based on the theory of cough in IPF being as a result of central sensitisation.
Interferon- α	In an open-label study investigating the use of interferon-α in slowing the progression of fibrosis in IPF, it was demonstrated that 5 out of 6 patients who had chronic cough had a significant improvement in their cough as assessed by LCQ (Lutherer et al. 2011).
Pirfenidone	In an exploratory analysis following a phase III clinical trial investigating the benefits of pirfenidone in IPF it was found that pirfenidone was associated with a reduction in cough using subjective measures of assessment (Azuma et al. 2011). A subsequent

	retrospective study from the Netherlands has also shown a reduction in cough score in a group of 11/19 patients with IPF-associated cough (Wijsenbeek et al. 2015). A prospective, multi-centre cohort study assessing the effect of pirfenidone on cough in patients with IPF (Cough-IPF) is currently recruiting.
Nintedanib	Little is known about the effect of nintedanib on cough in IPF. One small study reported subjective improvement in cough in 12 out of 36 patients (Bonella et al. 2013).
Azithromycin	Azithromycin is a macrolide antibiotic with immunomodulatory effects. It is believed that it might improve cough related quality of life in patients with IPF. A randomised, placebo-controlled trial is currently recruiting in Berne, Switzerland.
Morphine	In a study of 27 patients with chronic cough in a randomised double-blind placebo-controlled study, there was a significant improvement in cough severity measured using LCQ in the patients taking 5- 10 mg morphine twice daily. However there was no significant change in cough reflex sensitivity to citric acid (Allen et al. 2005). There have been no similar studies in IPF but in a small study of 11 elderly opioid-naïve patients with IPF, morphine was found to be effective and safe.
AF-219	AF-219 is an oral antagonist of the P2X3 cough receptor which is believed to be involved in mediating cough reflex sensitivity. A recent phase 2, randomised, double-blind, placebo-controlled study investigating the use of AF-219 in 24 patients with chronic cough demonstrated a significant reduction in cough frequency measured using 24 hour cough counting compared to placebo (Abdulqawi et al. 2015). The results of a similar study investigating the role of AF-219 in IPF patients are currently awaited.

The majority of these trials are not placebo controlled and therefore need to be interpreted with caution, as cough is a symptom that can be considerably influenced by the placebo effect (Van den Bergh et al. 2012). A number of these medications also have significant side effects and cannot to be tolerated. At present, unless there are associated co-morbidities such as GOR or rhino-sinusitis, which can be treated, the therapeutic options for cough in IPF are limited and palliation with opiates is frequently necessary.

This review highlights the progress made over recent years regarding the neurophysiology of cough and the pathogenesis of cough in IPF, however gaps in our knowledge clearly exist and at present treatments for this troublesome symptom remain limited. Further investigation into the pathogenesis of cough in IPF may provide insight into new therapeutic targets and the disease pathogenesis in general.

1.6.4 Hypotheses

Patients with IPF have been shown to have an enhanced cough reflex and there is accumulating evidence to suggest that this is as a consequence of altered cough neurophysiology. Given the evidence in patients with chronic cough (who also have an augmented cough reflex) supporting a link between cough hypersensitivity and an increase in TRPV-1 receptors, I hypothesise that TRP receptor expression is increased in the central airways of IPF patients.

There is growing evidence to support the link between acid and non-acid GOR, cough and IPF, and a proposed mechanism for chronic cough in patients with IPF is increased cough reflex sensitivity as a result of gastroesophageal reflux. As cough reflex sensitivity is increased to capsaicin, I hypothesise that the constituents of gastric refluxate increase the expression of TRP receptors in airway epithelial cells.

1.6.5 Aims of this Dissertation

The aims of this research project are to investigate the presence TRP receptor expression in the central airways of IPF patients and to assess the consequence of airway epithelium exposure to the constituents of gastric refluxate.

In order to achieve these aims the following specific questions will be addressed:

1. Does airway TRPV-1 and TRPA-1 receptor protein expression differ between subjects with IPF and those with idiopathic chronic cough and controls?
2. Does airway TRPV-1 and TRPA-1 gene expression differ between subjects with IPF and those with idiopathic chronic cough and controls?
3. Does airway epithelium exposure to constituents of gastric refluxate result in an increased TRPV-1 and TRPA-1 gene expression?

Two separate studies have been devised to address these questions; a clinical study of airways innervation in IPF patients and an in vitro study of the effects of lung epithelial cell exposure to gastric refluxate constituents. The methods used

will be described separately in the chapters that follow. Method development and optimisation will also be outlined.

CHAPTER 2: MATERIALS AND METHODS

2.1 A CLINICAL STUDY OF AIRWAYS INNERVATION IN IPF PATIENTS

In order to assess TRPV-1 and TRPA-1 expression in IPF patients, bronchial biopsies from IPF patients were compared with patients with idiopathic chronic cough and controls. Quantitative Reverse Transcription Polymerase Chain Reaction (qPCR) was used to assess TRPV-1 and TRPA-1 at a gene expression level while Immunohistochemistry allowed localisation and analysis of receptor protein expression.

2.1.1 Patient Selection

The patients studied were recruited from the Respiratory Department at University Hospital Llandough, Cardiff, UK between January 2011 and August 2014. Three groups of patients were identified.

1. Patients with Idiopathic Pulmonary Fibrosis (IPF)
2. Patients with Idiopathic Chronic Cough
3. ‘Normal’ Controls

All IPF patients fulfilled the American Thoracic Society criteria for the diagnosis of IPF (Raghu et al. 2011). All patients were discussed at a multi-disciplinary team (MDT) meeting to ensure they had clinical, physiological and radiological features of IPF without evidence of an associated connective tissue disease. All apart from one IPF patient had a HRCT scan showing a typical UIP pattern as described in figure 6. The patient without a typical UIP pattern on HRCT had a UIP pattern confirmed following surgical lung biopsy. Assessment of the HRCT scans by a radiologist with a specialist interest in pulmonary disease at the MDT meeting also allowed co-existing emphysema to be excluded in this group of IPF patients. Bronchoalveolar lavage was performed on all IPF patients as part of their routine clinical investigation in order to exclude alternative diagnoses such as hypersensitivity pneumonitis as shown in appendix 1.

Idiopathic chronic cough patients were identified as those patients with a > 3 month history of cough and a normal chest X-ray after the exclusion of other

causes of chronic cough as per usual clinical practice. All patients with chronic cough had at least a 1-month trial of treatment with a proton pump inhibitor in order to eliminate GOR reflux as a cause of cough. Co-existent bronchial hyper-reactivity was excluded following thorough clinical assessment, in addition reversibility testing or methacholine challenge testing were performed where there was clinical uncertainty.
'Normal' control patients were identified as patients who did not complain of cough but were required to undergo bronchoscopy for evaluation of a non-airways centred disorder e.g. pulmonary nodule/ single episode of haemoptysis.

Potential confounding causes of cough were identified and all three groups were subject to the same exclusion criteria, which meant that all other causes of cough were carefully excluded. The Exclusion criteria are outlined in table 2. Several patients were also recruited into a pilot group to allow for experimental optimisation; these patients were not subject to the exclusion criteria.

A target sample size of 40 patients (20 IPF, 10 chronic cough and 10 control subjects) was selected on the basis that comparison between two or three groups, each of size ten, is powered to detect a difference of 1.1 to 1.25 standard deviations (Professor R. Newcombe – personal communication), which was thought reasonable based on the findings of other similar studies (O' Connell et al. 1995; Groneberg et al. 2004a).

2.1.2 Research and Development and Ethical Approval

The study was approved by the Cardiff and Vale University Local Health Board Research and Development Committee and the Local Research Ethics Committee (10/WSE02/62 and 09/CMC/4620). Study participants provided informed written consent.

Study Exclusion Criteria
Smoking history within 1 year.
Respiratory tract infection within three months.
Current symptoms of gastroesophageal reflux.
Significant rhinosinusitis symptoms.
Other respiratory diagnosis, including COPD, Asthma
Other severe systemic illness.
Angiotensin converting enzyme inhibitor therapy

TABLE 2 Study Exclusion Criteria

2.1.3 Pulmonary Function Tests

All patients with IPF and idiopathic chronic cough underwent spirometric/pulmonary function testing as part of routine clinical care. Pulmonary function was performed by experienced respiratory physiologists in the University Hospital of Llandough, Pulmonary Function Department (MS PFT and MS PFT Box, Jaeger, Germany) and were performed according to current guidelines (BTS and ARTP 1994; Pellegrino et al. 2005). 'Normal' control patients underwent spirometric testing at the discretion of the consulting healthcare professional. All 'Normal' ex-smokers underwent spirometric testing as a minimum.

2.1.4 Assessment of Cough Severity

All patients with IPF and idiopathic chronic cough were asked to complete a Leicester cough questionnaire (LCQ) and Visual analogue score (VAS) prior to bronchoscopy (Appendix 2a and 2b). The LCQ comprises 21 questions which are divided into social, physical and psychological domains. A lower score indicates poorer quality of life. The VAS is a 100 mm linear scale on which patients are asked to mark the severity of their cough. The extremes of the scale were marked 0 (no cough), and 100 (worse cough) (Birring et al. 2003; Morice et al. 2007). All 'Normal' controls were asked about cough symptoms at the time of enrolment, patients were only enrolled if they had no history of cough.

2.1.5 Bronchoscopy

Flexible fibre-optic bronchoscopy was performed in all recruited patients for diagnostic purposes using an Olympus Lucera CV-260 scope, according to established guidelines (Honeybourne et al. 2001). Intravenous midazolam was given pre-procedure and topical anaesthesia of the upper airways and larynx was obtained using 1-2% lignocaine. Oxygen at a flow rate of 2-4 l/minute was administered via nasal prongs. Bronchial biopsies (max. 6) using 2.8mm forceps were taken from the segmental carinae of the right lung in each patient to allow for direct comparison between patients (Jeffery et al. 2003). Biopsies were placed immediately into RNA*later*™ (Ambion, Paisley, UK) or buffered formalin. Biopsies stored in RNA*later*™ were stored at room temperature overnight and then transferred to -80 ºc until all samples had been collected, those stored in buffered

formalin were transferred to the Department of Pathology, University Hospital of Wales where they were subsequently paraffin embedded.

2.1.6 RNA Extraction

RNA extraction was performed on patient biopsies in batches upon completion of recruitment.

RNA Handling

To reduce the risk of RNA enzymatic degradation all surfaces and equipment were thoroughly treated with RNaseZAP™ (Sigma-Aldrich Company Ltd, Dorset, UK). The cabinet and equipment used were designated to RNA work at the time of extraction. Micro centrifuge tubes, pipette tips and the water used was RNAse/ DNAse free.

Method Development

There are several available methods for isolating nucleic acid. In order to ensure the chosen method yielded the best possible quantity and quality of RNA from the patient biopsies (which typically weighed less than 100 mg), RNA extraction was optimised using sacrificed mouse pulmonary tissue, which was gifted from the Haematology research group in The University Hospital of Wales. The mouse pulmonary tissue was dissected and stored in the same manner as the patient biopsies and processed using acid guanidinium thiocyanate-phenol-chloroform (TRIzol) and column-based techniques. The former method produced a greater yield of comparable quality RNA which was confirmed with pilot biopsies, therefore this technique was used for the patient samples as outlined below (Data shown in Appendix 3).

TRIzol RNA Extraction

Biopsies were removed from the RNA*later*™ after a sufficient period of thawing at room temperature and placed into 1 ml of TRIzol (Invitrogen, Paisley, UK). Biopsy material was then homogenised using a TissueRuptor (Qiagen, Hilden, Germany) with disposable probe tips, at full speed for 20-second intervals until

complete homogenisation had occurred. Samples were added to pre-spun Phase Lock Gel (PLG)-Heavy containing tubes (VWR International, UK) and incubated at room temperature for 5 minutes. 200 µl of chloroform was then added to the samples, which were mixed vigorously for 15 seconds and then incubated for a further 5 minutes at room temperature. Sample mixes were centrifuged for 10 minutes (12,000 g, 4 ºC), after which the clear, aqueous phase was removed. 600 µl of 2-propanol was added to the aqueous phase, which was mixed and incubated for 10 minutes at room temperature prior to 10 minutes of centrifugation (12,000 g, 4 ºC). If no pellet was visible at this stage the supernatant was removed and 100 µl of 2-propanol added, mixed and the centrifugation repeated. When a pellet was clearly visible the supernatant was removed and 900 µl of 80% ethanol was added, vortexed and centrifuged for 10 minutes (7,500 g, 4 ºC). The supernatant was removed and this step repeated. After two ethanol washes the supernatant was removed again and the pellet was air-dried and re-suspended in 30 µl of RNAse free H_2O. Samples were incubated for 5 minutes at 65 ºC to facilitate dissolution and then placed on ice for at least 30 minutes prior to quantification. RNA samples were stored at – 80 ºC prior to first strand synthesis.

Nucleic Acid Concentration

RNA concentration was quantified using a ND-1000 spectrophotometer (NanoDrop products, Wilmington, DE, USA.) 1.5 µl RNA was tested against a blank of RNAse/DNAse free water. For patient samples, which yielded less than 1 µg total RNA, samples were vacuum concentrated (Speedvac, Thermo Scientific, UK) to ensure appropriate volumes and concentrations were available for downstream applications.

Nucleic Acid Quality Assessment

The 260/280 and 260/230 absorbance ratios were measured using the ND-1000 spectrophotometer (NanoDrop products, Wilmington, DE, USA.) A 260/280 ratio of ≥ 1.8 was achieved in all samples accept 4 of the samples with reduced concentration of RNA; this is likely to be a consequence of reduced RNA concentration resulting in a larger ratio of impurities compared to RNA. Given the

precious nature of the samples they were used with caution; there did not seem to be any untoward effect on downstream applications.

2.1.7 First strand synthesis (cDNA synthesis)

First strand synthesis was performed using the SuperScript® VILO^{TM} cDNA Synthesis Kit (Invitrogen, Paisley, UK), which uses random primers for first strand cDNA synthesis. 0.5 µg of sample RNA was made up to 17.5 µl with RNAse free water and incubated at 65 ºC for 5 minutes, and then kept on ice for at least 1 minute. The following components of the SuperScript® VILO^{TM} cDNA Synthesis Kit (Invitrogen, Paisley, UK) were then added: 5 µl 5X VILO™ reaction mix and 2.5 µl 10X SuperScript® enzyme mix. This was mixed gently, incubated at 25°C for 10 minutes, 42°C for 60 minutes and then the reaction was terminated at 85°C for 5 minutes. The RT product (cDNA) was stored at -20 °C.

2.1.8 qPCR

A SYBR green based protocol, utilising the Lightcycler® 2.0 Carousel-based system (Roche Diagnostics Ltd, West Sussex, UK) was used to perform qPCR. The Lightcycler® FastStart DNA Master SYBR Green I kit (Roche Diagnostics Ltd, West Sussex, UK) was used for all reactions. This ready-to-use PCR reaction mix uses hot start technology which improves the sensitivity and specificity of qPCR(Diagnostics 2011). The level of fluorescence of SYBR Green I is directly proportional to the amount of double-stranded DNA generated in the reaction as demonstrated in Figure 17.

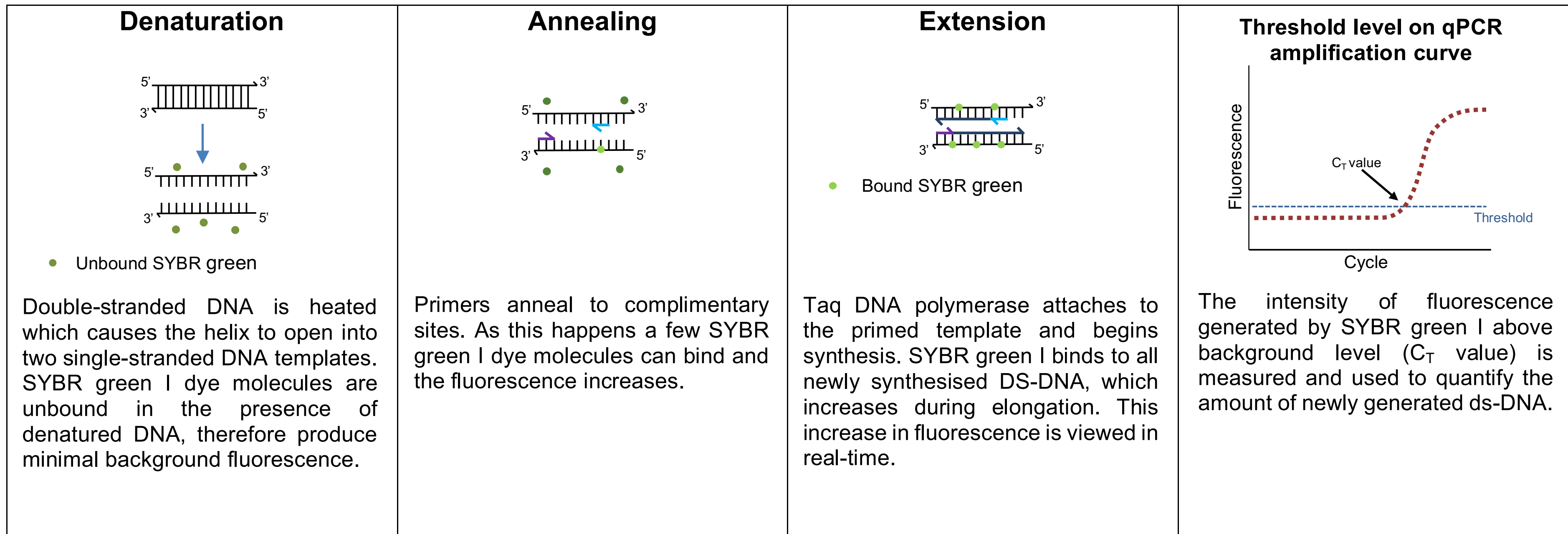


FIGURE 17 SYBR Green based qPCR assay

Protocol

The qPCR master mix was prepared in a cooling block as per the manufacturer's instructions as shown in Table 3.

18 µl of the qPCR master mix was added to each glass Lightcycler® reaction capillary within the cooling block and 2 µl of the appropriately diluted cDNA was added to each capillary prior to capping. The capillaries, within the cooling block adaptors were subjected to a 30 second centrifugation (800g, 4 °C), prior to being loaded onto the Lightcycler® carousel. qPCR was performed according to the conditions shown in Table 4.

TRP Receptor Primer Design

A literature search was conducted to find studies that had previously investigated the gene expression of TRPV-1 and TRPA-1. A study was identified in which TRPA-1 mRNA level was evaluated in A549 cells (Buech et al. 2013). Primers for TRPV-1 were originally chosen from a study that had examined gene expression in clinical material (Guarino et al. 2010) however these primers were found to be non-intron spanning, therefore alternative primers were designed using Primer3Plus Version: 2.3.3 software package (Untergasser et al. 2012). Intron spanning primers, are primers which anneal to separate exons, this type of primer was chosen to avoid amplification of potential contaminating genomic DNA. Primers were assessed for homology using a BLAST search, those unique to the genes of interest were chosen. The TRPV-1 primers were also complimentary to regions included in the documented four transcript variants for the gene. Each primer set was trialled briefly and deemed satisfactory therefore further optimisation was carried out. Primer sequences are shown in Table 5.

	Primer Set		
	HPRT	GAPDH/ TRPA-1	TBP/ TRPV-1
Reagent	Volume (µl) per reaction		
MgCl2 (25 µM)	1.6	2	2.4
Forward primer (5 µM)	2	2	2
Reverse primer (5 µM)	2	2	2
FS Mix*	2	2	2
H_20	10.4	10	9.6
cDNA template	2	2	2
Total	20	20	20

*FastStart reaction Mix (FS Mix): 10 µl of Lightcycler® reagent 1a added to vial 1b. Combined reagent kept at 4°C and not re-frozen. This contains FastStart Taq DNA Polymerase, reaction buffer, dNTP (nucleotide) mix, SYBR green I dye and 10 µM $MgCl_2$.

TABLE 3 qPCR Master Mix Preparation

	Initial Denaturation	Denaturation	Annealing	Extension		**Melting Curve**
Temperature (°C)	95	95	55/58/60*	72	**Temperature (°C)**	65-95
Time (s)	600	10	5	5	**Rate change (°C/s)**	0.1
Cycles	1		60		**Cycles**	1

*Annealing temperatures are specific to each primer set. GAPDH: 55°C, TRPV-1/TRPA-1: 58°C, TBP/HPRT: 60°C.

TABLE 4 qPCR Conditions for each Primer Set

Reference Genes

The use of reference genes is essential to improve reliability of qPCR data. Reference genes are used to normalise the data; the ratio of gene of interest mRNA concentration is reported in relation to mRNA concentration of the reference genes (Bustin et al. 2009). Normalisation allows comparison of mRNA concentration across different samples as it controls for factors including quantity and quality of starting material (cDNA), efficiency of cDNA synthesis and PCR amplification (Bustin et al. 2009). Reference genes ideally are stably expressed, however the expression of reference genes can vary considerably in different tissue or cell types and within the same cell or tissue type under different experimental conditions (Dheda et al. 2004; de Jonge et al. 2007).

Identification of a panel of reference genes that are stably expressed in epithelial cell lines and bronchial tissue under different experimental conditions was outside the scope of this project. Instead, a literature search was completed to identify published geNorm studies that rank the stability of reference genes in lung epithelial cells and/or tissue. This literature review failed to identify a geNorm study for IPF or idiopathic chronic cough patients, so the reference genes were chosen based on their stability in the A549 pulmonary epithelial cell line. This cell line was chosen to act as the positive control in all experiments as it is a commonly studied lung cell line. Also, since patient samples were precious, conditions were optimised using material derived from human cells lines of pulmonary origin. BEAS-2B bronchial epithelial cells as well as the A549 alveolar epithelial cells were used for this purpose. Further details on the cell lines are outlined in section 2.2.1.

In an attempt to improve the reliability of the reference genes in the patient samples, expression levels for three reference genes that cover a range of expression levels were chosen. Using three reference genes allows a geNorm calculation to be performed to assess the suitability of chosen reference genes, and allows exclusion of one or more reference genes if it is found to be less stable in the tissue or cell line studied. Hellemans et al have shown that mean coefficients of variation (CV) and gene stability values (M) of <25% and 0.5, respectively are typical of stably expressed reference genes in a homogenous

sample, with values of <50% and 1.0 in heterogeneous samples, therefore these limits were set in this study (Hellemans et al. 2007).

Two genes, HPRT (Hypoxanthine guanine phosphoribosyl transferase) and TBP (TATA box binding protein) were chosen based both on their stability in A549 cells (Pinhu et al. 2008) and the experience of a neighbouring research group in using them. The primer sets for these assays were chosen from previously published works and optimised by Dr Dean Bryant from the HPV Research group, Institute of Cancer and Genetics, Cardiff University (Allen et al. 2008; Minner and Poumay 2009). GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) was also chosen for its stability in the A549 cell line (Mane et al. 2008; Pinhu et al. 2008). The GAPDH primer set was taken from previously published work (Pinhu et al. 2008). All reference gene assays were intron spanning, which as previously stated was to avoid amplification of potential contaminating genomic DNA. The qPCR primer sequences are detailed in Table 5.

Name	Accession number	Primer sequence	RNA product size (bp)	DNA product size (bp)	Reference
TRPV-1	NM_080704.3	Fwd: TGGTATTCTCCCTGGCCTTG Rv: CTTCCCGTCTTCAATCAGCG	188	3763	Untergasser et al., 2012
TRPA-1	NM_007332.2	Fwd: TCACCATGAGCTAGCAGACTATTT Rv: GAGAGCGTCCTTCAGAATCG	74	1647	Buech et al., 2013
HPRT	NM_000194.2	Fwd: TGACACTGGCAAAACAATGCA Rv: GGTCCTTTTCACCAGCAAGCT	94	4893	Allen et al., 2008
TBP	NM_003194.4	Fwd: TCAAACCCAGAATTGTTCTCCTTAT Rv: CCTGAATCCCTTTAGAATAGGGTAGA	122	803	Minner and Poumay, 2009
GAPDH	NM_002046	Fwd: ACAGTCAGCCGCATCTCTTCTT Rv: TTGACTCCGACCTTCACCTT	81	321	Pinhu et al., 2008

The gene accession numbers were obtained from the NCBI (National Centre for Biotechnology Information) database. All primers were synthesised by Life Technologies (Paisley, UK).

Table 5 Genes and their primer sequences

Optimisation and Specificity

Following selection and design of primer sets the conditions (magnesium ion concentration and annealing temperature) were optimised. Input cDNA amount for cell lines and patient samples were also optimised. cDNA from the RT reaction were diluted 1 / 2 for clinical samples and 1 / 4 for cell lines, which equates to 0.25µg and 0.125µg cDNA respectively.

A limitation of using SYBR Green I is that it is less specific than probe-based technologies, such that any double-stranded DNA (dsDNA) may be detected, including primer dimers and non-specific amplification (Ririe et al. 1997). A post-amplification melting curve plots the change in fluorescence observed when dsDNA dissociates, into single-stranded DNA (ssDNA) and is achieved by increasing the temperature over the melting temperature of the amplicon. When the melting temperature is reached there is a sudden decrease in fluorescence, which is indicative of the T_m of the amplicon. Melting curve analysis was performed following every run to ensure reaction specificity. Umelt^{SM} is a tool that predicts the oligonucleotide's T_m; this was used to ensure the melting curve from the reaction matched what was expected (Dwight et al. 2011). Melting curves that were not consistent with amplicon curves were excluded from further analysis (Figure 18).

Products obtained by a trial of qPCR using primers targeting TRPV-1 and TRPA-1 genes were confirmed by sequencing analysis. PCR conditions were as previously described, using cell line A549 as control template. Amplicons were analysed on a 1% agarose gel-TBE buffer stained with ethidium bromide. Gels were visualised and photographs recorded under UV light with the GelDoc-It^{TM} Imaging System (UVP, UK) as shown in Figure 19.

Bands corresponding to each PCR product were extracted from the gel and purified using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions. Purified DNA concentration was measured using a NanoPhotometer (IMPLEN, Germany) and products with concentrations above 5 ng/µL were sent to Eurofins MWG Operon (Eurofins, Germany) for sequencing.

Sequencing of amplicons resulted in 157 bp and 32 bp nucleotide sequences, respectively. The sequences obtained were compared to GenBank nucleotide data library using the BLAST software provided by the National Centre for Biotechnology Information (NCBI). The nucleotide sequence obtained using TRPV-1 primers was most closely related to the transcripts 1-4 of *Homo sapiens* transient receptor potential cation channel, subfamily V, member 1 (TRPV-1) (accession numbers: NM_080704.3, NM_018727.5, NM_080706.3, NM_080705.3, 98% identity). The PCR-amplified fragment using the TRPA-1 primers was most closely related to *Homo sapiens* transient receptor potential cation channel, subfamily A, member 1 (TRPA-1), (accession number: NM_007332.2, 100% identity).

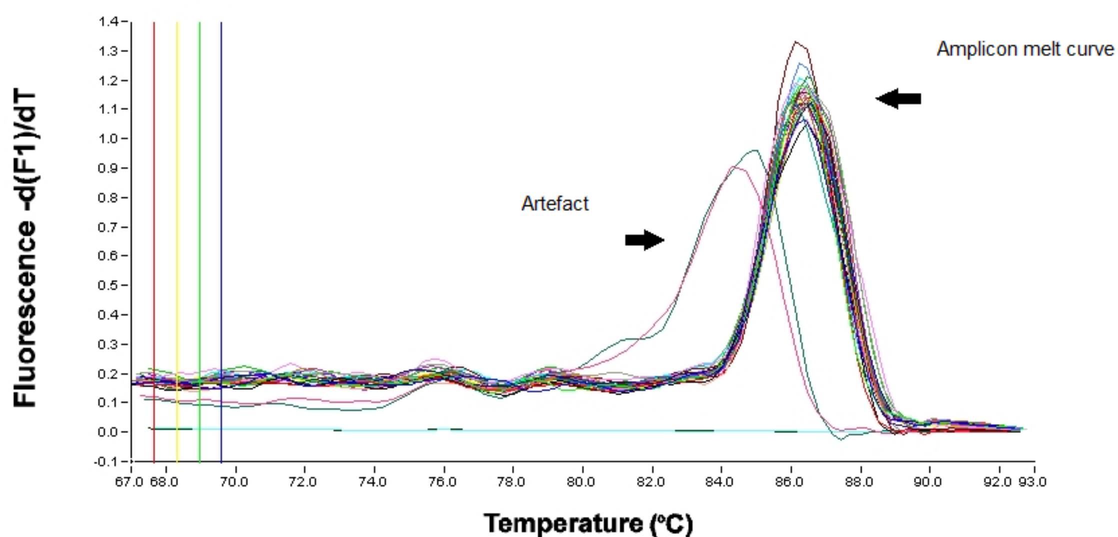


Figure 18 Melting Curve Example

An example of how melting curve analysis was undertaken. Any samples that generated secondary artefact were excluded. Note that non-template controls do not omit fluorescence and appear as flat lines.

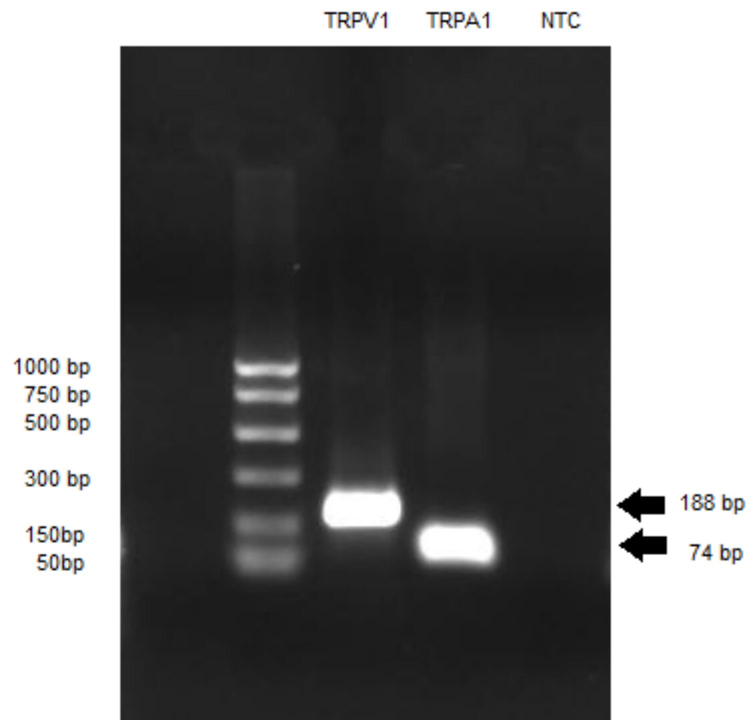


Figure 19 TRPV-1 and TRPA-1 qPCR product gel electrophoresis
An example of TRPV-1 and TRPA-1 in A549 cell line qPCR product run on a 1% agarose gel with TBE. NTC = No-template control.

PCR Efficiency

A relative quantification model was used to measure gene expression levels in this study. To limit potential errors in the calculated expression ratios that can result from variation in the amplification efficiencies of individual assays, PCR efficiency was calculated for each primer set using the calibration curve method. This uses linear regression to produce a formula for the relationship between the resultant Ct (cycle threshold) values of serially diluted input cDNA, where the cycle threshold is defined as the number of PCR cycles required for the fluorescent signal to cross the threshold (background level) (Hellemans et al. 2007).

Calibration curves were constructed in triplicate for each primer set using at least 6 points of a 3 fold dilution (1/3) of A549 cDNA. The calibration curve data was entered into the qBasePlus software, which calculates the amplification efficiency by using linear regression and then takes this and the error (uncertainty) of the calculated efficiency into account for all further calculations (Hellemans et al. 2007; Bustin et al. 2009).

A limitation of this method is that it makes the assumption that efficiency is the same for all dilutions of template cDNA, however when template cDNA is diluted, inhibitors within the sample are also diluted which may lead to more efficient amplification. This limitation is unlikely to have had a significant impact in this experiment as samples were processed in a way to minimise variation between the samples through processing and dilution and all samples were compared with like samples.

The calibration curves are shown in Figure 20 and a summary of the PCR efficiency for each assay with the linear regression equation data is shown in Table 6.

Primer	PCR efficiency	PCR efficiency (%)	Slope (m)	Intercept (c)
TRPV-1	1.9063	90.63	-3.5691	22.3078
TRPA-1	1.9447	94.47	-3.4619	24.5287
HPRT	1.8731	87.31	-3.6688	19.9564
TBP	1.8127	81.27	-3.8712	23.8255
GAPDH	1.9255	92.55	-3.5145	13.7375

Table 6 qPCR Assay Efficiency for each Primer Set

This table shows the average values for 3 independent dilution series for each primer pair.

Linear regression is performed according to the equation: (Pfaffl 2001)

$$Ct = m. (\text{Log}_{10} \text{ DNA concentration}) + c$$

In this way the calibration curve is used to calculate the slope (m), and therefore the efficiency (E) using the equation:

$$E = 10^{(-1/m)}$$

Quality control, Reproducibility and Sensitivity

A number of measures were in place to ensure adequate quality control within and between qPCR runs. All clinical samples were run in triplicate to ensure a mean could be obtained and any outlying results easily identified. Water (PCR grade) was used as a negative control and A549 cDNA, 0.5 µg diluted 1 / 4 was also included in each run as a standard positive control. An A549 RT negative control was also included in each run with the aim of identifying undigested genomic DNA. A final control using the A549 cDNA that was used to produce the standard curves was included in each run at the dilution found to be at the limit of detection (LOD) of each assay i.e. cDNA diluted 1 /177147 was the LOD for the GAPDH assay therefore this control was included with all samples being run with this primer set. This control acted as assay quality assurance.

The calibration curves produced to determine PCR efficiency demonstrate the reproducibility of the assays (Figures 20a-e). The standard curves illustrate the relationship between Ct value and concentration in each of the pairs of qPCR primers. Each colour and point shape represents an experimental repeat. Regression lines for each of the repeats are included. Figure 21 supports this observation, showing little variation between the Ct values of 1 / 4 diluted A549 cDNA in independent qPCR runs for each assay. The sensitivity of the assays was also determined by producing the standard curves and finding the LOD.

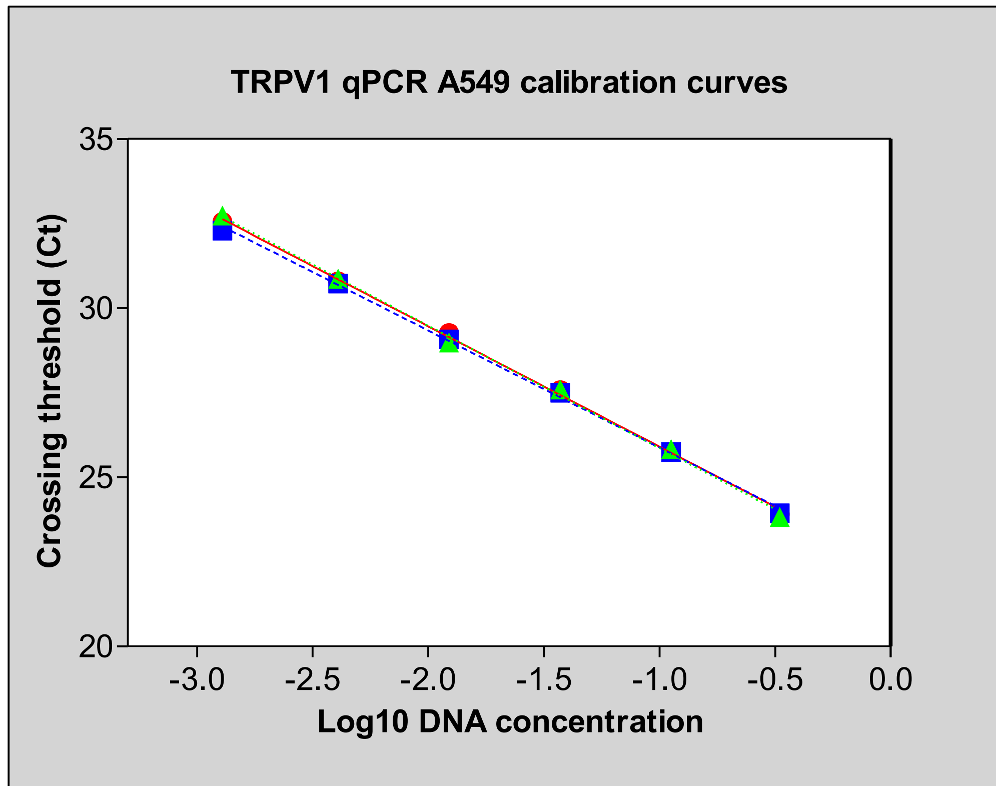


Figure 20a TRPV-1 qPCR primer pair

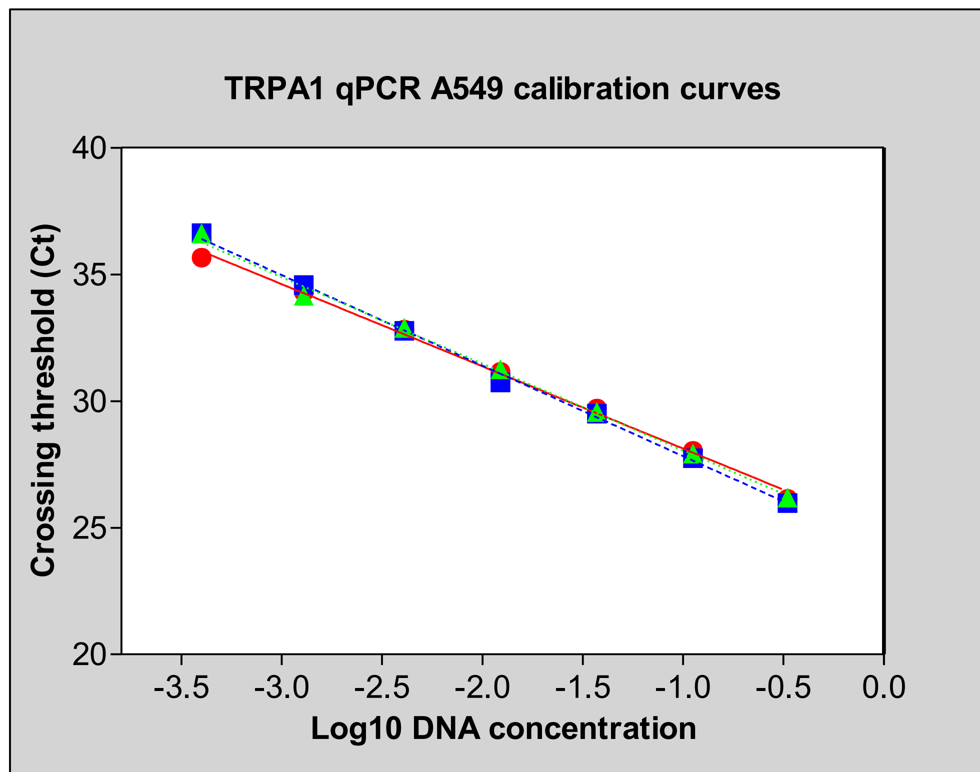


Figure 20b TRPA-1 qPCR primer pair

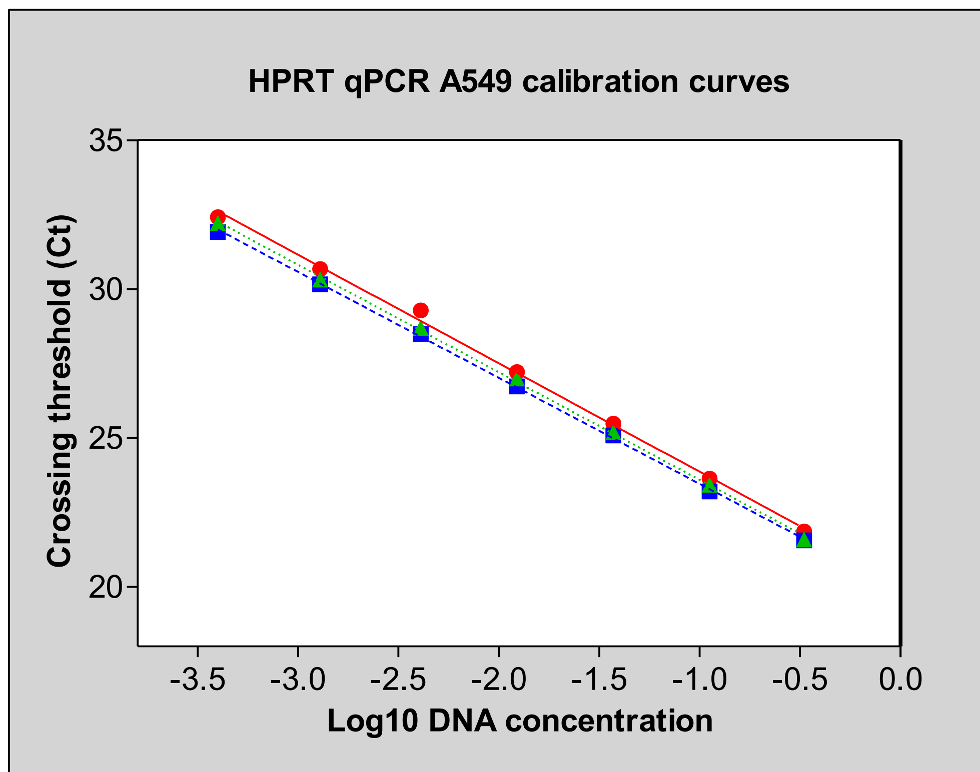


Figure 20c HPRT qPCR primer pair

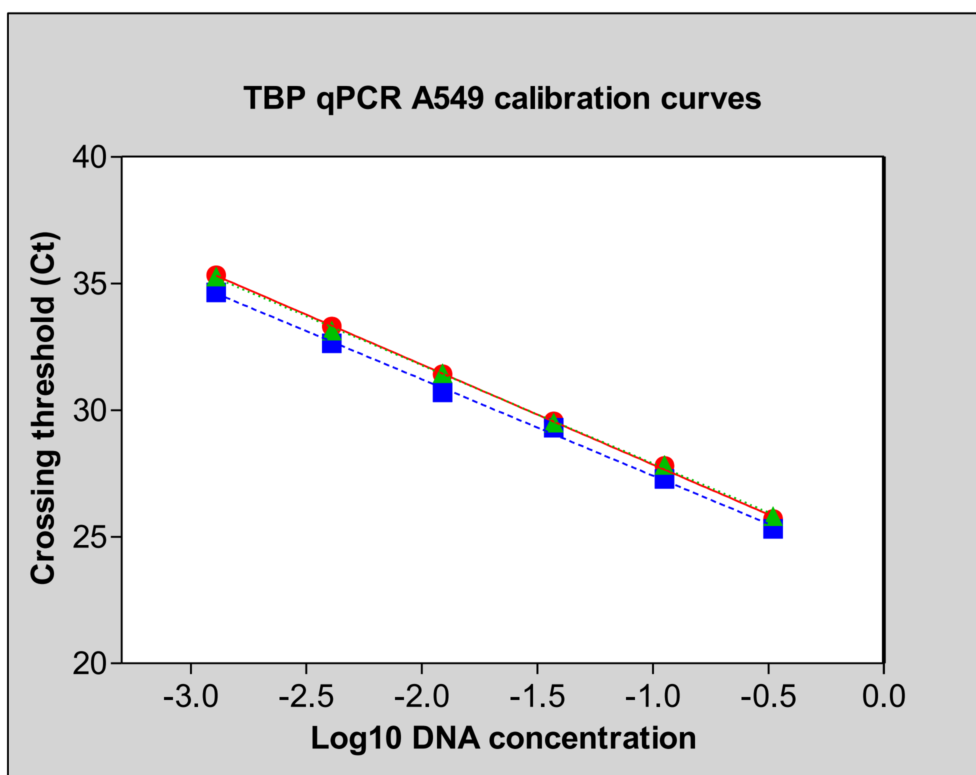


Figure 20d TBP qPCR primer pair

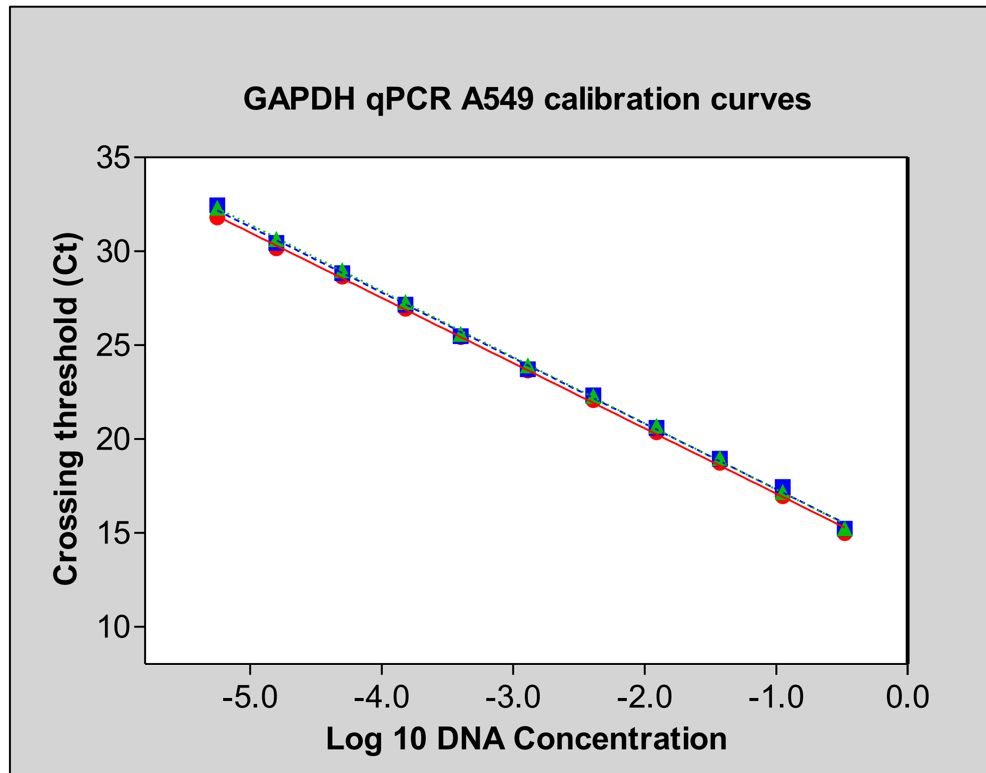


Figure 20e GAPDH qPCR primer pair

Data Analysis / Relative Quantification

The dilution series and experimental data obtained from the Lightcycler® 2.0 (Roche Diagnostics Ltd, West Sussex, UK) was uploaded onto qBasePlus software which determines relative expression of target genes using mathematical models and algorithms (Hellemans et al. 2007).

This software presents the data in a colour-coded list which allows for raw data quality control. Quality control parameters were: sample Ct >38 should be excluded, Ct difference between replicates should be <0.5. 100% of replicates had a Ct difference of <0.5, however 3 patients had CT values of > 38 for TRPA-1 and were therefore excluded.

Default analysis parameters were: amplification efficiency was calculated using ‘target specific amplification efficiency’ and normalisation strategy was set to ‘reference targets’ as previously described. Normalisation was evaluated within qBasePlus by two quality control measures; ‘the coefficient of variation of the normalised reference gene expression levels and the geNorm stability M-value (Hellemans et al. 2007).’ Removing GAPDH from the analysis improved the stability of the reference genes therefore only HPRT and TBP were used for normalisation of patient study samples (Genorm, M value: 0.415, CV: 0.148).

Expression levels of the target genes are shown relative to the cycle quantity (Cq) average (otherwise known as cycle threshold or Ct average). Final expression data is expressed as normalized relative quantity (NRQ) and standard error of the mean (SEM) or 95% confidence interval. The qBasePlus calculation algorithm is shown in appendix 4.

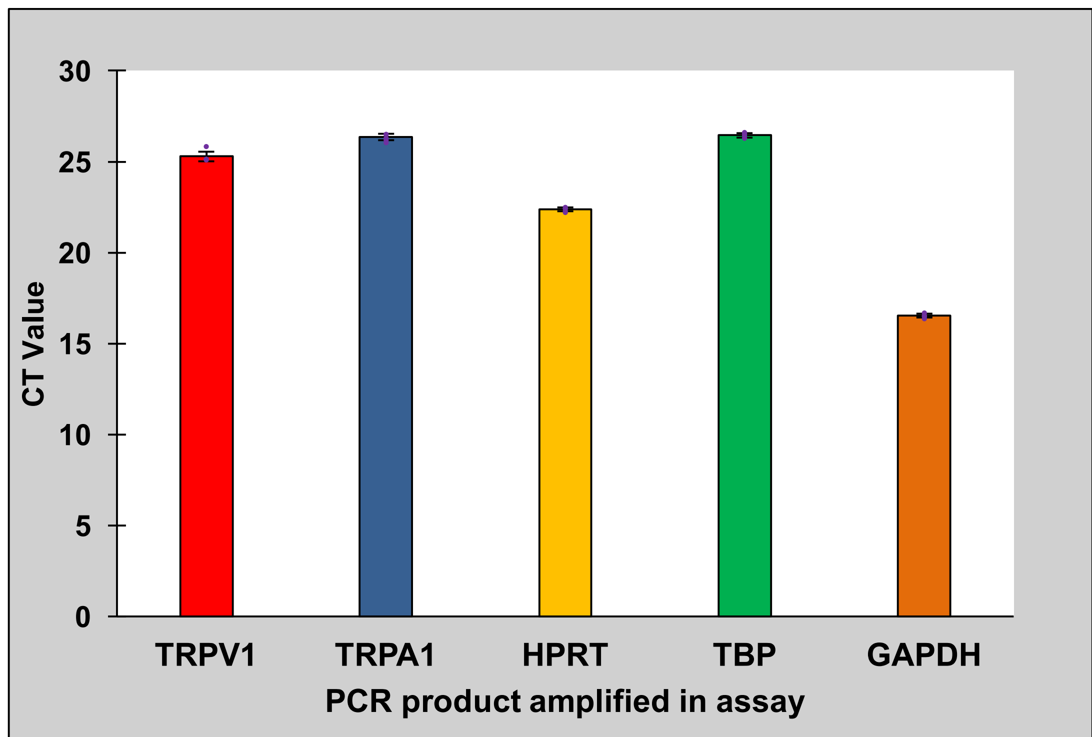


Figure 21 **Ct stability in A549 across independent repeats**
Five full independent qPCR runs are included (repeated in duplicate (i.e. n=10). Coloured columns = overall average, black bars = 95% confidence intervals and purple circles represent individual values

2.1.9 Histopathological Analysis

Following bronchoscopy samples stored in buffered formalin were transferred to the Department of Pathology, University Hospital of Wales, where all laboratory procedures outlined below were undertaken by Sue Wozniac, Chief Biomedical Scientist. Following at least an overnight incubation the formalin-fixed specimens were paraffin embedded.

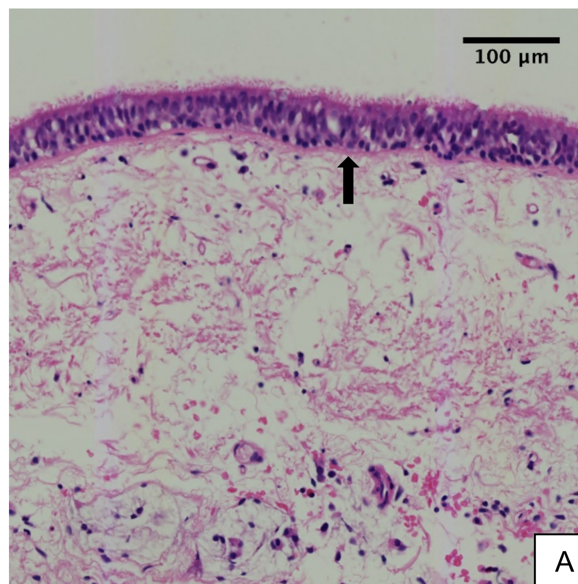
Biopsy morphological examination

4-micrometre sections were routinely stained with haematoxylin and eosin. The specimens were evaluated by myself and Dr Allen Gibbs, an experienced histopathologist with a special interest in pulmonary pathology. All reviewers were unaware of the grouping of each subject. A multi-headed Olympus BX51 microscope was used. Pilot samples were used to decide upon the criteria used for assessment. Examined parameters included the presence and degree of basement membrane thickening, chronic inflammation and mucous cell hyperplasia as shown in table 7. Basement membranes were considered thickened if they were ≥ 8μm (Figure 22). The degree of chronic inflammation (lymphocytic infiltration) was graded 1-3 (1 = mild, 2 = moderate, 3= severe) as shown in Figure 23. A semi-quantitative scale for the number of mucous cells was used to determine the degree of mucous cell hyperplasia (normal = < 20%, mild = 20-40%, moderate = 40 -60 %, severe = >60%) as shown in Figure 24. Slides were examined in batches of ten to prevent observer fatigue.

Chronic inflammation	Basement membrane thickening (≥ 8 µm)	Mucous cell Hyperplasia
No	No	No
Yes: 1- Mild	Yes	Yes: Mild
2- Moderate		Moderate
3- Severe		Severe

Each specimen was evaluated by myself and an experienced histopathologist.

Table 7 Histopathologial Examination



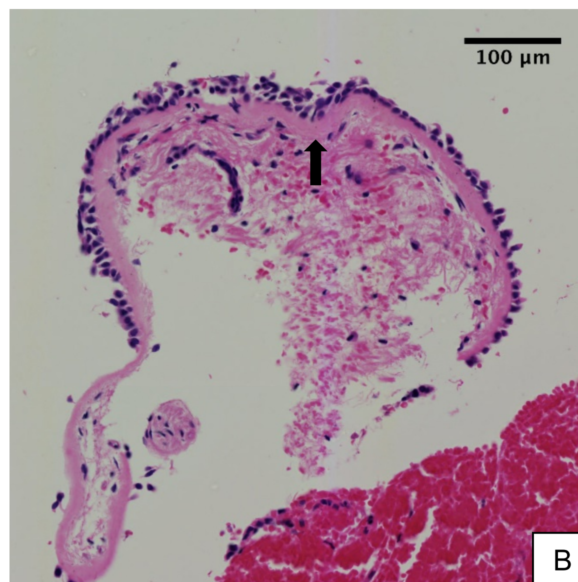


Figure 22 Representative H & E images demonstrating basement membrane thickening
The black arrows indicate the basement membrane. Panel A shows a normal basement membrane (6.9 µm), and panel B shows a thickened basement membrane (19.6 µm).

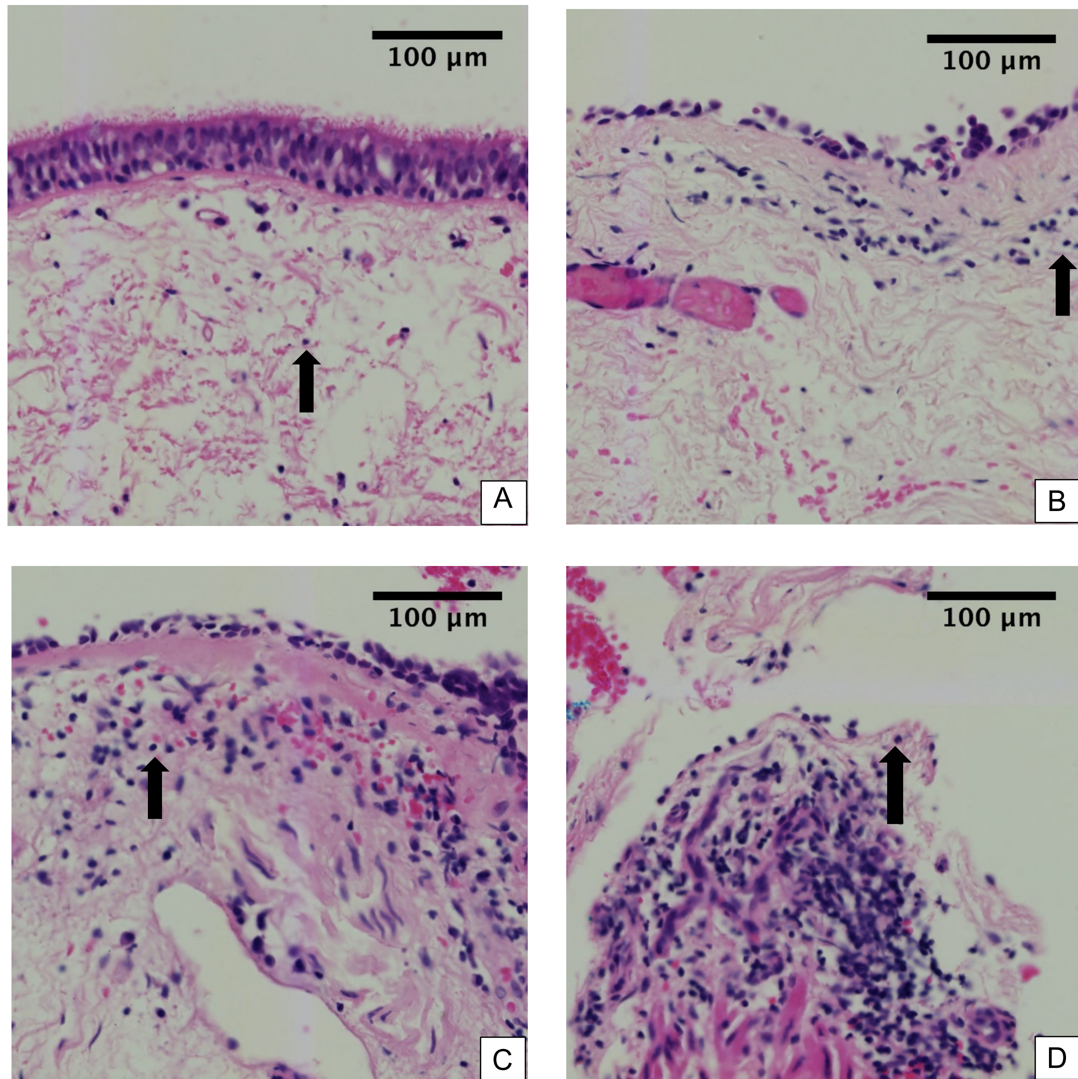


Figure 23 Representative images demonstrating chronic inflammation

Lymphocytes are shown by the black arrows. Panel A does not show chronic inflammation. Panels B, C and D show mild, moderate and severe chronic inflammation respectively.

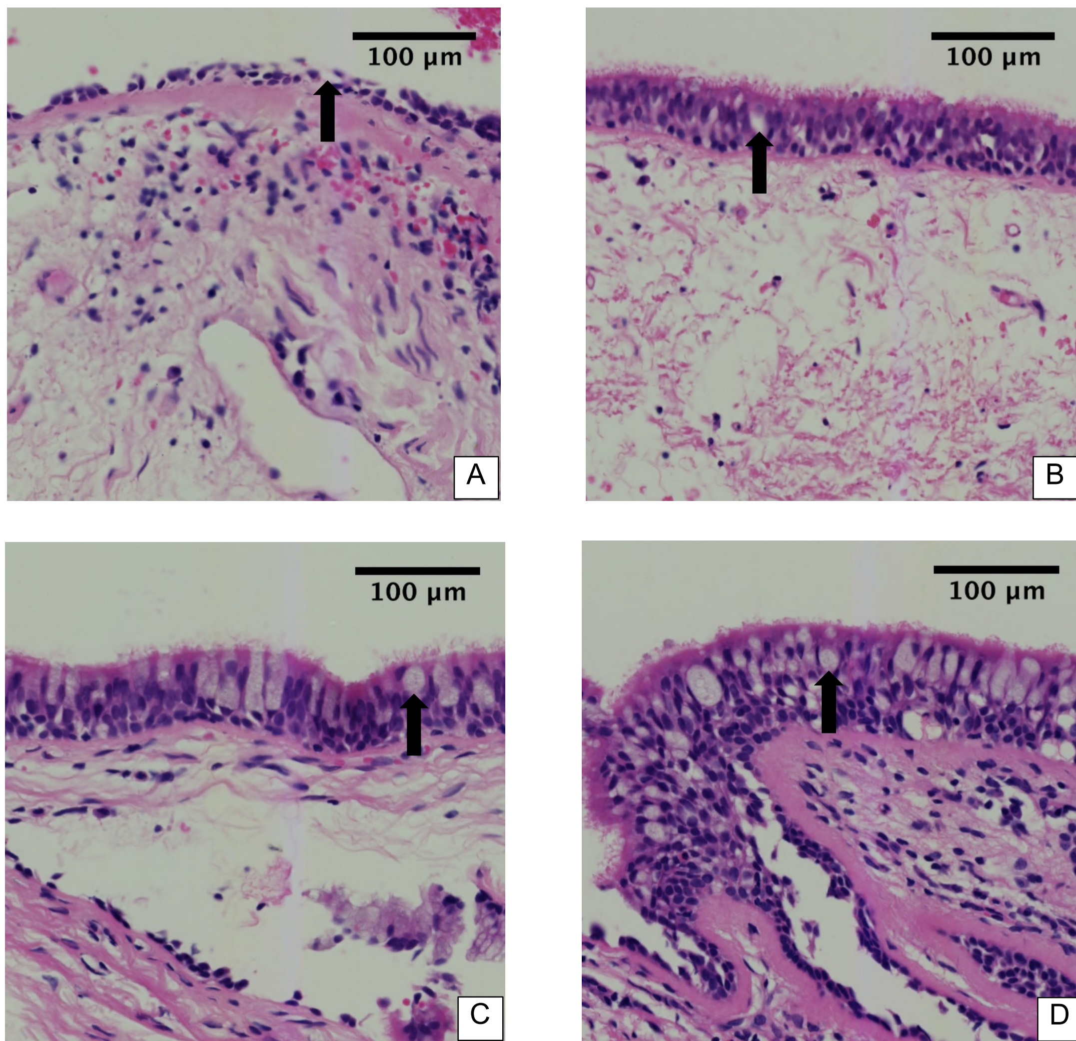


Figure 24 Representative H & E Images demonstrating mucous cell hyperplasia

The black arrows in figures A-D indicate mucous cells. There is no mucous cell hyperplasia in panel A. Panels B, C and D show mild, moderate and severe mucous cell hyperplasia respectively.

2.1.10 Immunohistochemistry

Protocol

Formalin-fixed paraffin-embedded sections were investigated by immunohistochemistry for TRPV-1 and TRPA-1 expression. The sections were also investigated for PGP-9.5 (protein gene product 9.5) positivity, an amino acid protein present in tissues of human neuronal and neuroendocrine origin used as a general nerve marker (Day and Thompson 1987).

Immunohistochemistry was performed using the Dako Flex™ Envision + kit, product code K8002 (Dako, Denmark). The sections were first floated onto 'Flex'™ slides and dried for 1 hour at 60 °c. The slides were then placed into a Dako pre-treatment module chamber containing target retrieval solution (pH dependent on antibody, see Table 8). The slides were heated within the module to 97°c for 20 minutes and then cooled to 65°c before being placed immediately into Dako wash buffer for 5 minutes. This step incorporates de-waxing, rehydration and heat induced epitope retrieval. The slides were loaded onto the Dako Autostainer Link 48 ™ staining machine. A Dako horseradish peroxidase (HRP) block was added for 5 minutes to block any endogenous peroxidase present and then washed twice in buffer solution. The primary antibody was then added (see table 8 for concentrations) for 20 minutes followed by a buffer wash. A second 20 minute incubation with Dako EnVision™ FLEX /HRP polymer allowed detection of the primary antibody. Two 5 minute incubations in Dako substrate working solution followed by a wash allowed visualisation of the antigen antibody complex by the enzymatic reduction of 3, 3-diaminobenzine tetrahydrochloride (DAB). A final 5-minute incubation with Dako Flex Haematoxylin was performed for counterstaining. Slides were then removed from the staining machine and dehydrated (through a series of graded alcohol baths) then 'cleared' (through a series of Xylene baths) and mounted using a DPX mountant (Sigma-Aldrich Company Ltd, Dorset, UK). A negative and positive (appendix tissue) control was included in each run. These control slides ensured background antibody staining could be identified and specificity of the IHC results (Images are shown in section 3.1.5). Appendix tissue was used due to availability within the laboratory, experience of staff in using the tissue type and previous

TRPV-1 and TRPA-1 localisation within this tissue type in The Human Protein Atlas (Uhlen et al. 2015).

Antibody	Concentration	Retrieval solution pH	Product code	Reference
Rabbit anti-PGP9.5	1/1600	High	Z5116 (Dako, Denmark)	(Wick 2000)
Rabbit anti-TRPV-1	1/1000	High	AB5370P (Chemicon, UK)	(Mitchell et al. 2005)
Rabbit anti-TRPA-1	1/600	Low	LS-B177 (Lifespan, Seattle, WA, USA)	(Buech et al. 2013)

Antibodies were diluted to the optimised concentration with Dako antibody diluent (Dako, Denmark). All primary antibodies were originally sourced from Dako however they failed to stain appropriately therefore antibodies were sourced from previously published works as detailed above.

Table 8 Immunohistochemistry primary antibodies

Interpretation of Immunohistochemical results

The specimens were evaluated simultaneously by myself and Dr Allen Gibbs. Any discrepancies in the results were resolved by consensus following reassessment of the slide by both investigators. Images were viewed an Olympus BX51 microscope. PGP-9.5, TRPV-1 and TRPA-1 staining was recorded as either positive or negative and its distribution noted.

Computer-aided quantitative Image analysis

To quantify any difference in immuno-histochemical staining between the three groups a digital method of quantification was used. Bright-field whole slide images were captured and digitalised using an Axio Scan.Z1 slide scanner with a 20X objective lens and a Hitachi HV-F2025CL camera (Zeiss, Cambridge, UK). The images were exported as TIFF (Tag Image File Format) files and analysed using the open source scientific image analysis package ImageJ version 1.50j (Schindelin et al. 2015). The immunohistochemistry (IHC) image analysis toolbox is a semi-automated colour detection system which is available as a plugin on ImageJ (Shu 2013). This system was used to detect the positively stained colour pixels within the area of interest on each slide allowing the area of specific immunostaining and the total area to be measured and therefore the positive neuronal PGP-9.5, TRPV-1 and TRPA-1 staining to be expressed as a percentage of the total area. The areas of specific staining were distinguished from any background staining. Dr Jie Shu (North China, University of Technology) kindly assisted in optimising the IHC toolbox to meet the needs of this project. The group identity of the samples were not available prior to the analysis. Examples of how the programme was 'trained' to pick up the appropriate colour pixels and how the image analysis was performed is shown in Figures 25 and 26 respectively.

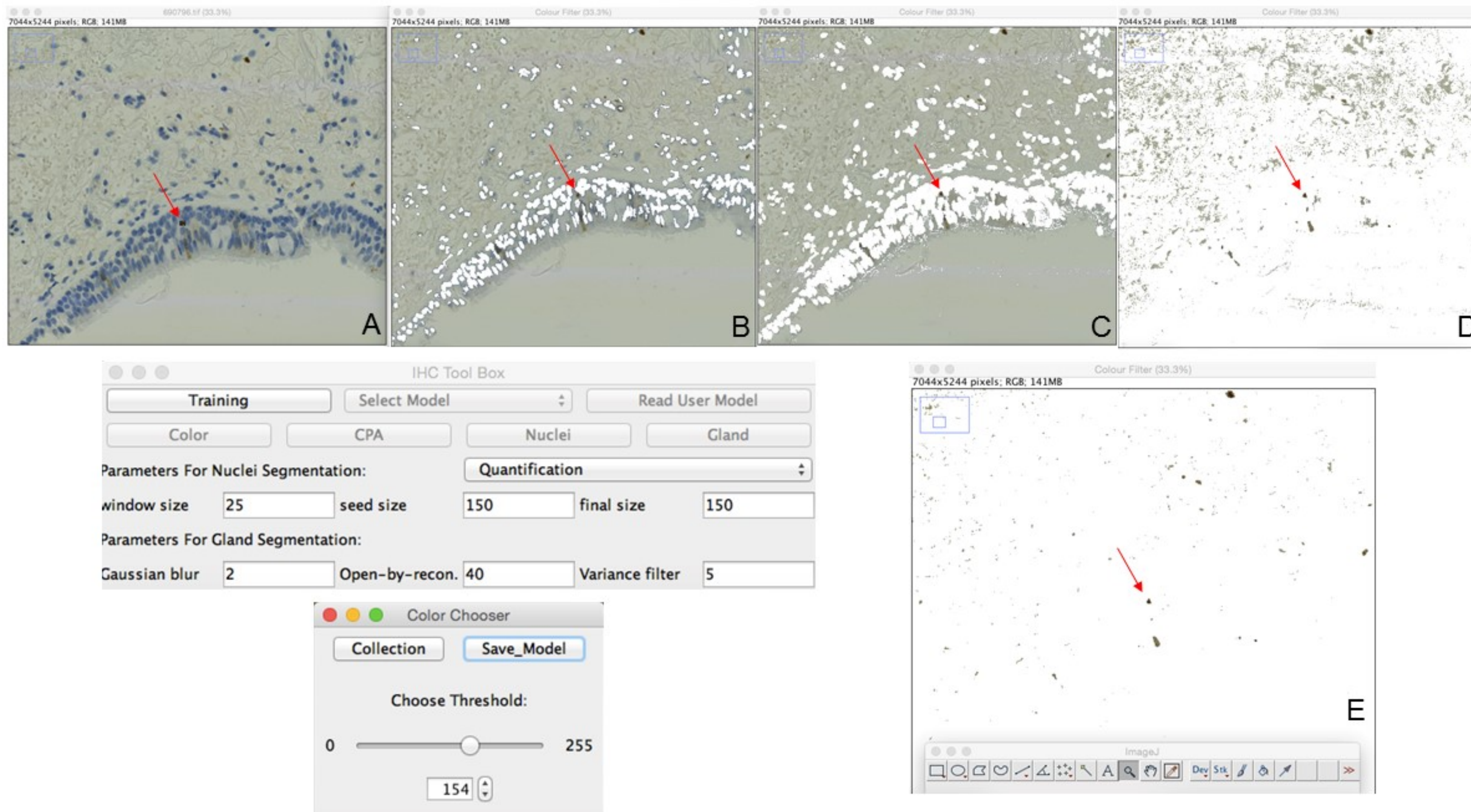


Figure 25 The process of 'training' on ImageJ using the IHC toolbox.
The colour pixels in an area of specific DAB staining (red arrows) were selected to train the statistical colour detection model (panel A). The colour threshold was gradually changed to remove unwanted background pixels (panels B-D). The result image (panel E) contains the reserved colour pixels and minimal background pixels. The data obtained is then collected and the exercise repeated. The combined data is saved as a model and validated on a number of the study images prior to image analysis. Images shown are PGP-9.5 staining on a study subject.

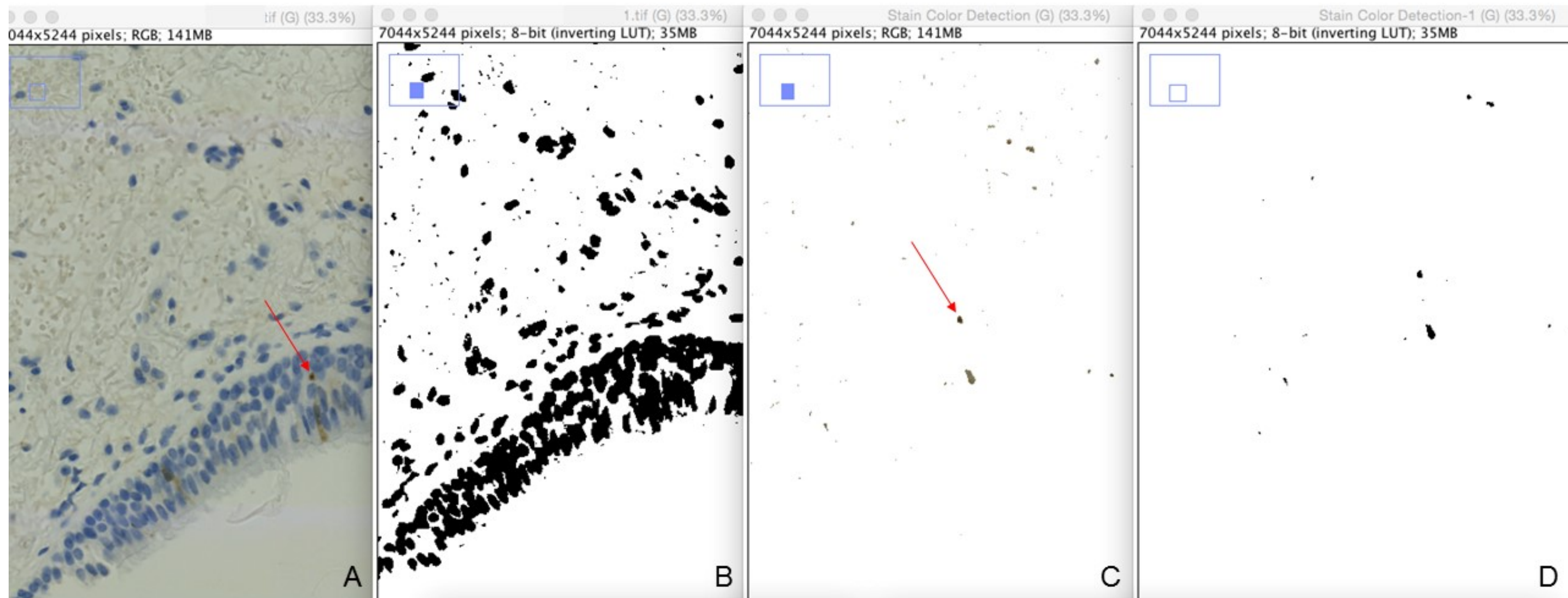


Figure 26: Quantitative IHC image analysis in ImageJ.

2.1.11 Statistical Analysis

Independent statistical advice was sought and obtained from Dr Mark Kelson from the Research Design and Conduct Service (RDCS) run by Cardiff University. SPSS version 20 (SPSS Inc. Chicago. Illinois) was used for data analysis. Statistical significance was considered to be <0.05.

Patient demographics

The Student t test and Mann-Whitney U test were used to compare parametric and non-parametric continuous data between two groups respectively. One-way Analysis of Variance (ANOVA) was used when more than two groups were being compared. The Shapiro-Wilk and Levene tests were used to determine normality and equality of variance respectively. Chi-squared test was used to compare the categorical patient demographic data, whilst Spearman's rank correlation was used to assess association between continuous variables.

qPCR data

All qPCR data was Log_{10} transformed to ensure it fitted a normal distribution. Average gene expression between the groups was assessed using ANOVA, however linear logistic regression was also used to account for the other variables within the study. Spearman's rank correlation was used to assess association between TRPV-1 and TRPA1 gene expression with measures of cough severity.

Histopathological Data

Binary logistic regression was originally chosen to analyse whether PGP-9.5, TRPV-1 and TRPA-1 protein expression could be predicted from the co-variables, however the model was an inadequate fit so Fisher's exact test was used to investigate the association between the categorical histopathological and immunohistochemical data. Group data obtained from quantitative IHC analysis was assessed using Kruskall-Walis and linear logistic regression. The Student t test and Mann-Whitney U test were used to compare parametric and non-parametric continuous data between two groups respectively and Spearman's rank correlation was used to assess association between TRPV-1 and TRPA-1 protein expression with measures of cough severity.

2.2 STUDIES OF TRP RECEPTORS IN RESPIRATORY CELL LINES EXPOSED TO GASTRIC FLUID CONSTITUENTS

In order to assess whether the constituents of gastric refluxate affect the expression of TRP gene expression in airway epithelial cells, an experimental model of gastric reflux was devised using pulmonary epithelial cell lines and the individual components of gastric refluxate.

2.2.1 Cell Culture

Cell lines

To assess the affect gastric refluxate would have on TRP gene expression, cell lines that had previously been found to express the genes of interest were chosen (Agopyan et al. 2003; Reilly et al. 2003; Thomas et al. 2007; Mukhopadhyay et al. 2011; Park et al. 2012; Buech et al. 2013; Shapiro et al. 2013). Epithelial cells from both bronchial and alveolar origin were used to ensure that any changes identified could be linked to an anatomical area within the airways.

Human adenocarcinoma, alveolar epithelial (A549) cells and normal bronchial epithelial (BEAS-2B) cells were obtained from the European Collection of Cell Cultures (ECACC). The A549 cell line was originally derived from a 58 year Caucasian male with adenocarcinoma of the lung, whereas the BEAS-2B cell line are SV-40 adenovirus transformed, immortalised normal human bronchial epithelial cells.

Culture technique

A549 cells were grown in Dulbecco's Modified Eagle's medium (DMEM) (Life technologies, Paisly, UK) and BEAS-2B in F12K medium (Life technologies, Paisly, UK) containing 10% foetal bovine serum. Both media were supplemented with 100 units/ml of penicillin and 100 mg/ml of streptomycin. Cells were maintained at 37°C in a humidified incubator with an atmosphere of 5% CO_2.

Cell passage

Cell lines were sub-cultured when sub-confluent (70-80%) under aseptic conditions using 0.25% trypsin (Life technologies, Paisly, UK). The passage number was kept to a minimum during each experiment; A549 cells were used

between passage numbers 10-20 and BEAS-2B between passage numbers 50-61.

2.2.2 Method Development

RT-PCR

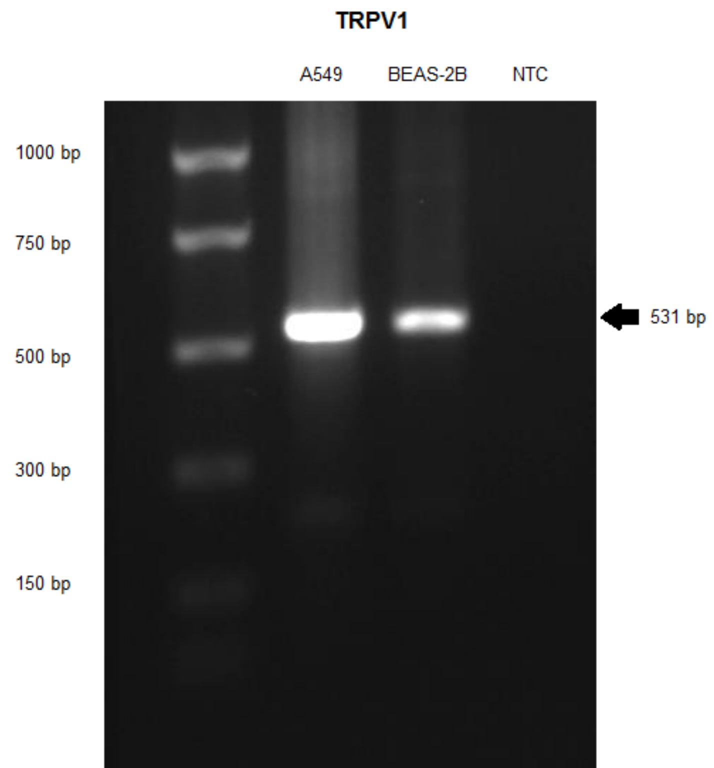
RT-PCR (Reverse transcription-PCR) was performed to ensure expression of the genes of interest in the chosen cell lines.

Following the manufacturer's protocol and as detailed previously, total RNA was isolated from cultured cells (up to 6 x 10^6) using TRIzol® (Life technologies, Paisley, UK). Quantification was performed using a ND-1000 spectrophotometer (NanoDrop products, Wilmington, DE, USA). Using 2.5 μg of RNA, first-strand synthesis was carried out using SuperScript® VILO™ cDNA Synthesis Kit as detailed previously (Invitrogen, Paisley, UK). PCR was set up with the cDNA as template with the Platinum® Taq DNA Polymerase kit (Life technologies, Paisley, UK). The primers used are described in table 9. Negative controls were treated in the same way as positive controls except water was added in place of cDNA. The PCR reaction was performed using the following cycling program: 95°c for 2 min, 30 cycles at 95°c for 1 min, 55°c for 1 min, and 72°c for 1 min followed by a 10-min incubation at 72°c. Amplicons were analysed on a 1% agarose gel-TBE buffer stained with ethidium bromide. Gels were visualised and photographs recorded under UV light with the GelDoc-It™ Imaging System (UVP, UK) as shown in Figure 27.

Name	Accession number	Primer sequence	Product size (bp)	Reference
TRPV-1	NM_080704.3	Fwd: GCAAGAACATCTGCAAGCTGC Rv: GCTGACAGAGCACTGGTGTTC	531	(Yu et al. 2012)
TRPA-1	NM_007332.2	Fwd: CCCCTCTGCATTGTGCTGTAG Rv: CCATTGTCCAGGCACATTTTG	478	(Wang et al. 2008b)

All RT-PCR primers were synthesised by Life Technologies (Paisley, UK).

Table 9 RT-PCR Primers



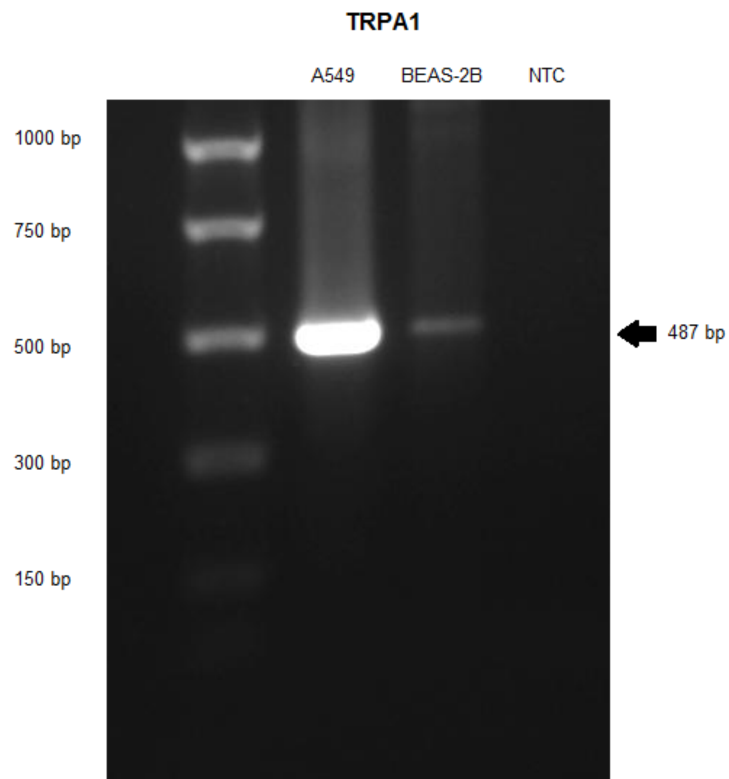


FIGURE 27 TRPV-1 and TRPA-1 RT-PCR product gel electrophoresis

Gastric constituents

Up to 1.5 litres of gastric juice is secreted per day and this is essential for the initiation of digestion of food-stuff that enters the stomach. Gastric juice contains acid, pepsin, bacteria and occasionally duodenal factors such as bile acids and pancreatic enzymes. Reflux of gastric juice through the lower oesophageal sphincter into the oesophagus is a normal physiological event. The complex mixture of these components results in a liquid or gas, which may be an alkaline, acidic or neutral pH. The pH of the refluxate is an important factor as it influences the cellular toxicity, enzymatic activity and constitution of the separate components.

The individual components of gastric refluxate used in this experiment to determine which if any increase the expression of TRP gene expression in airway epithelial cells are outlined below:

- **pH**
  An acidic environment was created by altering the pH of media with the addition of sterile, filtered 2M-filtered Hydrochloric acid (HCl). As oesophageal impedance reflex events are defined according to pH with acid reflux, pH <4; weakly acid reflux, pH 4 – 6.9; non-acid reflux, pH≥7, preliminary experiments were carried out with media at pH2, pH4, pH6 and pH7-8 (Hirano et al. 2007; Cho 2010).

- **Pepsin**
  Pepsin is a proteolytic enzyme secreted as inactive plasminogen by gastric chief and mucous cells, and has been implicated as a cause of oesophageal damage during GOR. Various concentrations of pepsin (Sigma, UK) were made by dissolution in sterile growth media (0.5mg/ml-10mg/ml). Preliminary experiments were carried out using pepsin concentrations based on those previously studied (Bathoorn et al. 2011; Johnston et al. 2012). The activity and stability of pepsin is affected by the pH of its solution (Piper and Fenton 1965; Bardhan et al. 2012), with increased peptic activity at lower pH and inactivation of the enzyme at pH > 8. The pepsin concentration (1mg/ml), chosen based on the findings

from preliminary experiments and the clinical relevance of the concentration (Bathoorn et al. 2011) was therefore dissolved in media at pH2, pH4, pH6 and pH7-8 to assess the possible difference in levels of pepsin activity.

- **Bile salts**

  Bile acids can be found in gastric juice as a result of duodenal reflux into the stomach through the pylorus. Patients with GOR disease have higher levels of bile acids present in oesophageal aspirates compared to patients without GOR disease (Kauer et al. 1997) In humans bile acids exist in several forms: as free (unconjugated) bile acids, bile acids bound to sodium or potassium salts, or bile salts that are conjugated with glycine, taurine, sodium or potassium (Pearson and Parikh 2011). Human bile consists of mainly conjugated bile salts with only a trace of unconjugated bile salts being present, however in patients on acid suppression (proton-pump inhibitors for example) bile salts can become de-conjugated through a process of bacterial overgrowth, and then have an increased role (Hofmann and Small 1967; Pearson and Parikh 2011). In this study unconjugated chenodeoxycholic acid (CD) and conjugated glycochenodeoxycholic acid (GD) (Sigma, UK) were used. Animal studies have demonstrated that conjugated bile salts are injurious to the oesophageal mucosa at a lower pH, whereas unconjugated bile acids cause injury at a neutral pH (Katz 2000). However bile salts are poorly soluble in acid therefore could not be studied at acidic pH and were instead dissolved in growth media and used at pH 7-8. CD was first dissolved in 80 % ethanol due to poor solubility. The ethanol carrier was originally at 5% however preliminary experiments showed that at this concentration the ethanol carrier had an adverse effect on cell viability therefore the carrier was reduced to 0.5% which was not found to have any adverse effect. Preliminary experiments were carried out using concentrations based on clinically applicable concentrations that were studied previously (50-500 μM) (Nehra et al. 1999; Jaiswal et al. 2006; Perng et al. 2008).

Final experimental concentrations and time of cell exposure to the various gastric constituents was decided following a series of preliminary experiments as

outlined below. Clinically applicable concentrations were chosen for each constituent following confirmation that the concentration and time of exposure did not biologically or statistically affect the viability of the cells in a significant way (Figure 28 shows this process). It was essential to ensure that any subsequent changes in receptor gene expression levels were as a consequence of the effect of the gastric constituent exposure rather than as a result of cell death or proliferation.

1. **Cell viability assay** - Cell titre 96

Test Doses	Time Intervals 24 hour continuous	1 hour/day 3 days	1 hour/day 1 day	5 & 15 minutes/day 3 days	5 minutes/day 1 day
Pepsin: 0.5mg/ml-10mg/ml	✓	✓			
CD/GD: 50μM-500μM	✓	✓			
Ethanol carrier 0.5-5%	✓	✓			
pH: 2, 4, 6, 8	✓	✓	✓	✓	✓
Pepsin 1mg/ml pH 2-8	✓	✓	✓	✓	✓

2. **Cellular viability and apoptosis assay** - Flow cytometric assay

Test Doses	Time Intervals 24 hour continuous	1 hour/day 3 days	1 hour/day 1 day	15 minutes/day 1 day	5 minutes/day 1 day
Pepsin: 1mg/ml		✓	✓		
CD/GD: 50μM		✓	✓		
Ethanol carrier 0.5%		✓	✓		
pH: 6, 8		✓	✓		
pH: 4, 6, 8					✓
Pepsin 1mg/ml pH6-8		✓	✓		
Pepsin 1mg/ml pH 6					✓

3. **qPCR**

	Time Intervals 24 hour continuous	1 hour/day 3 days	1 hour/day 1 day	15 minutes/day 1 day	5 minutes/day 1 day
Pepsin: 1mg/ml		✓			
CD/GD: 50μM		✓			
Ethanol carrier 0.5%		✓			
pH: 6, 8		✓			
pH: 4, 6, 8					✓
Pepsin 1mg/ml pH6-8		✓			
Pepsin 1mg/ml pH 6					✓

FIGURE 28 Preliminary experiments for gastric constituent exposures

2.2.3 Cell Viability Assay

The CellTiter96® AQ_{ueous} One Solution cell proliferation assay (Promega, Southampton, UK) was used to assess cell viability following gastric constituent exposure. This assay uses the MTS tetrazolium compound, which is bio-reduced by metabolically active cells and results in a tissue culture medium colour change which can be detected and quantified using a 96-well plate reader (Figure 29).

Briefly, cells were counted using a haemocytometer and 100 µl of cell suspension (A549 and BEAS-2B) were then seeded in 96-well culture plates (1500 cells/well) and allowed to adhere overnight. The cells were treated with various concentrations of gastric constituents as outlined above and for various time intervals to establish an in-vitro model of gastric reflux that would be suitable for the cell types used. All treatments of cells were carried out aseptically and the time spent out of the incubator was kept to a minimum. Each treatment was timed precisely with a stopwatch and following treatment each well was rinsed with warm fresh media before the addition of new media. There were at least 3 biological replicates for each concentration used. On the final day of cell exposure following 1 hour of incubation in fresh media, 20µl of CellTiter 96 AQueous One Solution Reagent® (Promega) was added and the cells were further incubated at 37°C for 1–2 h. Cell viability was measured by reading the absorbance at a wavelength of 490 nm on a Titretec plate reader (ThermoScientific, Leicester, UK). Viability measured by absorbance was calculated as a percentage of the control sample and each experiment was repeated at least in triplicate.

It was noted that some concentrations/ exposure times had a significant effect on the cells biologically when examined microscopically but the difference in absorbance failed to reach statistical significance, therefore the cells were clearly damaged or distressed but remained metabolically active (Figure 42 in section 3.2.1). The concentrations and time of exposure that showed no significant difference microscopically or statistically in terms of cell viability were then further assessed for cell viability, necrotic and apoptotic death, since the CellTiter96® assay is indicative of cell viability but not definitive.

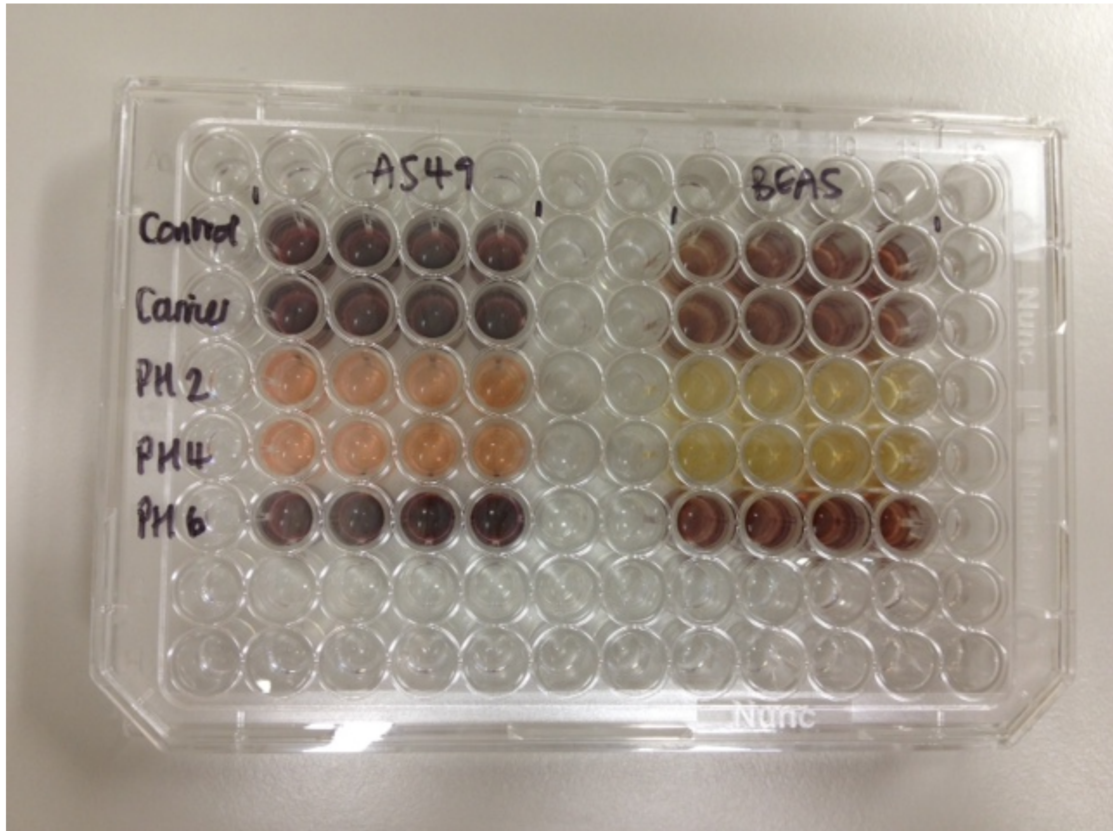


Figure 29 A 96 well plate showing CellTiter96® AQueous One Solution cell proliferation assay

This 96 well plate shows the colour change seen in this cell proliferation assay. The darker wells indicate more metabolically active cells. In this example A549 and BEAS-2B cells have been exposed to acidic media as indicated for 1 hour.

2.2.4 Cellular Viability and Apoptosis Assay

A flow cytometric assay was used to investigate cells for evidence of apoptosis, following exposure to each gastric constituent. Due to the increased number of cells needed for this experiment, cells were seeded in 6 well plates at the observed optimum density of 100,000 cells per well and allowed to adhere overnight. The cells were exposed to the gastric constituents for 1 hour per day for 3 days (bile salts and pepsin) or for 5 minutes for one day (acidified media and pepsin in an acidified media) based on the results of the cell viability experiment as detailed above and shown in Figure 28. Following exposure cells were rinsed once with fresh media and then incubated with further fresh media for one hour. The 3-day experiments were designed to allow for analysis after each daily exposure and therefore allowed for analysis following daily as well as repeated exposures. Cells were analysed for apoptosis using Annexin V-Cy5 and Propidium iodide staining.

The Annexin V stain detects cells undergoing apoptosis by binding to membrane phosphatidyl- serine (PS), which is translocated by the cell from the inner surface of the plasma membrane to the cell surface shortly after the initiation of apoptosis. Propidium iodide (PI) is often used in conjunction with Annexin V to ascertain whether cells are viable, apoptotic or necrotic (Vermes et al. 2000). PI cannot enter into cells with an intact cell membrane and therefore it cannot stain alive or early apoptotic cells however in late apoptosis or necrosis where the permeability of the membrane is compromised, PI can freely enter and intercalate with double-stranded DNA or RNA and result in positive staining (Vermes et al. 2000).

Briefly following the 1 hour incubation in fresh media the media was removed and the cells rinsed with Phosphate-buffered saline (PBS) prior to the addition of Trypsin EDTA 0.25% (Life technologies, Paisley, UK). Cells were pelleted, washed in cold PBS twice, and re-suspended in binding buffers containing the Annexin-V Cy5 (Biovision, Cambridge, UK) and Propidium iodide (Sigma-Aldrich Company Ltd, Dorset, UK) stains according to the manufacturer’s instructions. The samples were analysed for apoptosis immediately using an Accuri™ C6

cytometer (BDbiosciences). 20,000 events (cells) were counted for each sample (10,000 for positive control).

Each run contained a negative (untreated) control and positive control. Initial experiments using rotenone (Sigma-Aldrich Company Ltd, Dorset, UK) and serum-free media as a positive control failed to provide adequate results therefore cisplatin 100 µM (Accord healthcare limited, UK) for 48 hours was used (Liu et al. 2013). All samples were run in duplicate and experiments repeated three times. Data was analysed using FCS express version 4 (De Novo software). The quadrants and gates were set based on unstained cells and debris (determined by cell size) was excluded from analysis. Figure 30 shows examples of how the data was analysed.

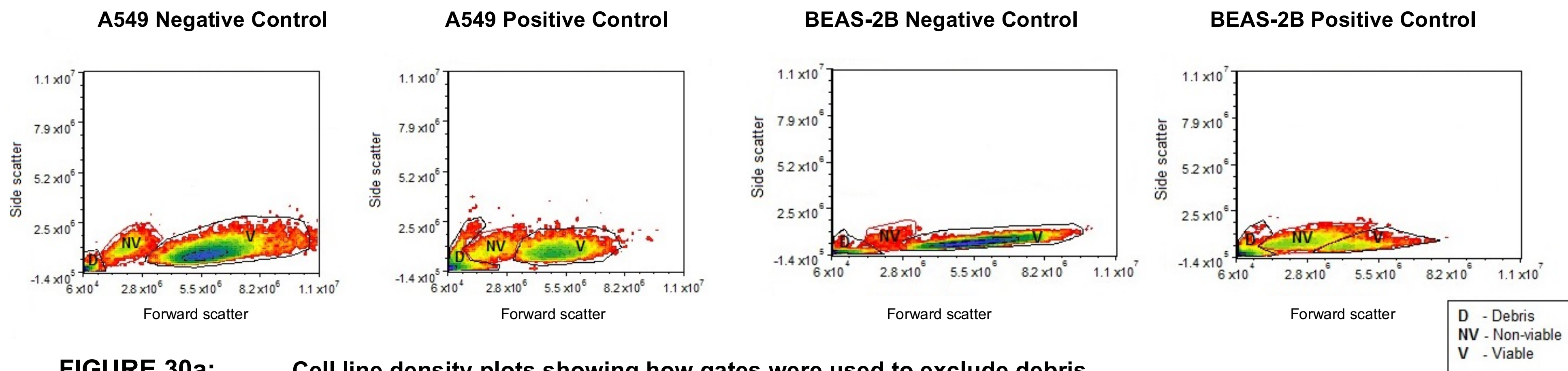


FIGURE 30a: Cell line density plots showing how gates were used to exclude debris

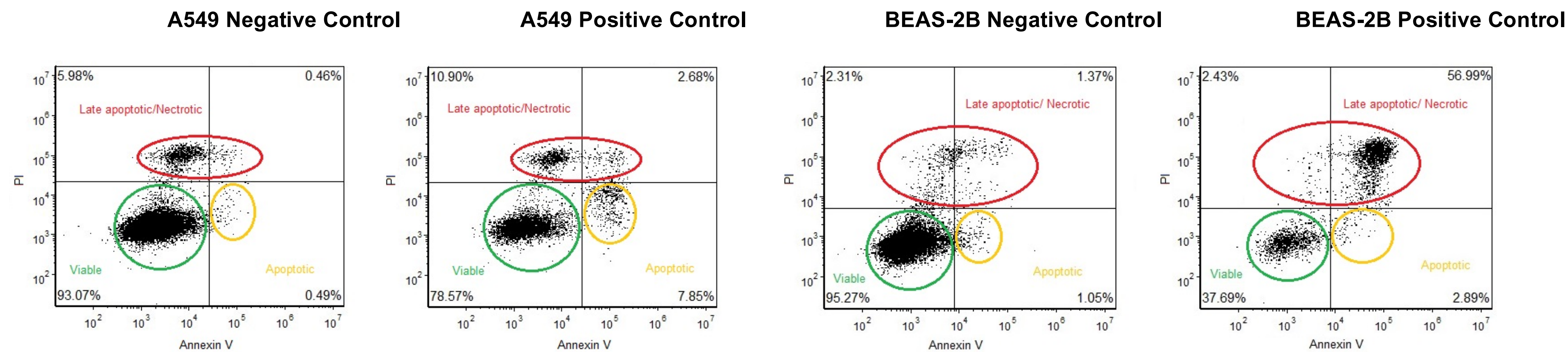


FIGURE 30b Cell line scatter plots showing Annexin-V Cy5 and Propidium Iodide (PI) staining (debris excluded)

2.2.5 Experimental Procedure

The preliminary experiments allowed for the optimisation of the concentrations and exposure times of each component of gastric refluxate but also allowed experience to be gained in cell culture technique and the critical timings of the experiment.

Ideally our model of gastric reflux would include repetitive exposures as occurs in vivo however this was not possible for all test conditions despite trialling several different regimes as it was not tolerated by the A549 and BEAS-2B cell lines. This in particular was the case for the exposures to pH <6 with and without pepsin. The tolerated concentrations and times of exposure were taken forward in order to gain as much information as possible regarding the expression of TRP receptors in response to the acid and non-acidic components of gastric refluxate.

Following these preliminary experiments the final test concentrations were as follows:

- pH
    - pH7-8: 1 hour per day on 3 consecutive days (control)
    - pH6: 1 hour per day on 3 consecutive days.
    - pH6: 5 minutes on 1 day.
    - pH4: 5 minutes on 1 day.
- Pepsin
    - 1mg/ml pH 7-8: 1 hour per day on 3 consecutive days
    - 1mg/ml pH 6: 1 hour per day on 3 consecutive days and 5 minutes on 1 day.
- Bile salts
    - CD: 50μM, 1 hour per day on 3 consecutive days.
    - GD: 50μM, 1 hour per day on 3 consecutive days.

As per the cell apoptosis study, cells were seeded in 6 well plates at the observed optimum density of 100,000 cells per well and allowed to adhere overnight. The cells were exposed to the gastric constituents for 1 hour per day for 3 days or for 5 minutes for one day as detailed above. Following exposure cells were rinsed once with fresh media and then incubated with further fresh media for one hour

prior to RNA extraction. A negative control and if applicable a carrier control was included in each run. The carrier control was included to ensure any potential osmotic effect of the diluted media by the addition of the constituent was controlled for. Each experiment was completed in triplicate.

The following methodologies are a summary only as the full details are outlined in the methods section of the study of airways innervation in IPF patients. Any differences in methodology have been described.

2.2.6 RNA extraction / first strand synthesis/ qPCR

Following completion of the cell exposures as detailed above, 1ml of TRIzol (Invitrogen, Paisley, UK) was added to each well and pipetted to lyse cells. Samples were then snap frozen in TRIzol using liquid nitrogen. RNA extraction as detailed in section 2.1.6 was then completed in batches.

Quantification was performed using a ND-1000 spectrophotometer (NanoDrop products, Wilmington, DE, USA.). Only samples with > 1 µg total RNA were used. The BEAS-2B cell line following 1 day exposure failed to yield adequate RNA for downstream experiments therefore were not used.

A 260/280 absorbance ratio of ≥ 1.8 was achieved. Another way of assessing total RNA quality is by assigning a RNA integrity number (RIN). The RIN is a numbering system from 1 to 10 which reflects the degree of RNA degradation; 1 being degraded and 10 being most intact (Biomedical Genomics 2007). 1 µl of seven random samples were assessed by Mrs Amanda Gilkes (Research Assistant, Institute of Cancer and Genetics, Cardiff University) using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). The average RIN analysed was 9.5, which suggests good quality, intact RNA.

RNA samples were stored at – 80 ºC prior to first strand synthesis which was carried out on 0.5µg of RNA using SuperScript® VILO™ cDNA Synthesis Kit as detailed previously (Invitrogen, Paisley, UK). The RT product (cDNA) was stored at -20 °C.

qPCR was performed using the Lightcycler® 2.0 Crousel-based system (Roche Diagnostics Ltd, West Sussex, UK) using the same primer templates and conditions as previously described. cDNA from the RT reaction was diluted 1 / 4 (0.125 µg cDNA) for cell line samples. All samples were run in duplicate with a H_20 negative control, RT negative control, A549 cDNA 0.5 µg diluted 1 / 4 standard positive control, and a serially dilute A549 sample which acted as assay quality assurance.

Data Analysis / Relative Quantification

The data was analysed using the qBasePlus software package (Hellemans et al. 2007). Each experiment (N=3) was analysed using this software separately in order to enable the normalisation strategy to be set to the control (untreated sample). The amplification efficiency was calculated using 'target specific amplification efficiency'. Normalisation was performed using TBP, HPRT and GAPDH as reference genes evaluated within qBasePlus produced a genorm value of <0.5 and CV value of <0.2 for all cell line experiments.

2.2.7 IL-6 and IL-8 Enzyme-linked Immunosorbent Assay (ELISA)

Supernatants were taken from the A549 and BEAS-2B cells at 24 and 48 hour intervals following their exposure to the gastric constituents. The supernatants were stored at a temperature of -80°C before use in the assay.

IL-8 was measured in the A549 cells only. Nunc MaxiSorp® microliter 96-well plates were coated with 4 µg/ml (100 µl total) of IL-8 monoclonal mouse anti-human antibody (MAB208 – capture antibody, R & D Systems, Abingdon, UK) for 24 hours. Following 3 washes with wash buffer (Phosphate buffered saline and Tween 20 at pH 7.4), a block buffer (1% sucrose and 1% bovine serum albumin (BSA)) was added and incubated for 1 hour at room temperature. A serial dilution of a recombinant human IL-8 standard (R & D Systems, Abingdon, UK) was prepared using TBS-T (Tris-buffered saline 0.1%, BSA 0.05% and Tween 20 at pH 7.3) with a final standard curve range of 31.25- 2000 pg/ml. 100 µl of the samples and standards were then added to the wells in duplicate and incubated at room temperature for 2 hours. 100 µl of biotinylated goat anti-human IL-8 (BAF208 – detection antibody, R & D Systems, Abingdon, UK) at a concentration

of 20ng/ml was then added to each well for a further 2 hour incubation. This was followed by 3 washes and the addition of 100 µl of a 1/ 20,000 dilution of HRP-conjugated streptavidin (Life technologies, paisley, UK) and a 20 minute room temperature incubation. After a further three washes in wash buffer, 100 µl of the chromogenic substrate Tetramethylbenzidine (TMB) was added and incubated for 30 minutes. The reaction was stopped by the addition of 100 µl of 0.5 M sulphuric acid (stop solution) to each well.

The Thermo Scientific™ Human IL-6 ELISA kit (Thermo Fisher Scientific, USA) was used to measure IL-6 in both cell types as described in the manufacturer's instructions. Briefly, 50 µl of the biotinylated antibody reagent was added to each well of an anti-human IL-6 96-well strip plate. Standards were reconstituted and 50 µl was added to the plate in order to construct a standard curve as instructed (range: 10.24-400pg/ml). 50 µl of each supernatant was added. The samples were incubated for 2 hours at room temperature. Following the incubation period, the plates were washed three times with wash buffer. Streptavidin-HRP solution was then added to each well followed by a further 30 minute incubation at room temperature, followed by the addition of TMB and the subsequently the stop solution as described above.

The ELISA plates were then evaluated within 30 minutes using a Titretec plate reader set at 450nm (ThermoScientific, Leicester, UK). In both experiments the interleukin standards were used to plot a standard curve. The quantity of human IL-6 and IL-8 in each sample was determined by interpolating from the absorbance value (Y axis) to the IL concentration (X-axis) using the standard curve ($y = mx + c$). The mean absorbance of the duplicate samples was used and unless indicated otherwise all experiments were performed at least twice.

2.2.8 Statistical Methods

SPSS version 20 (SPSS Inc. Chicago. Illinois) was used for data analysis. For the preliminary experiments, ANOVA was used to compare the means of the samples. ANOVA does not perform a comparison of means between pairs of groups, therefore if a statistically significant difference was found a comparison using the Tukey- Kramer test was performed. For the preliminary studies, not all

data was normally distributed, which violates an assumption of the ANOVA test, however hypothesis generating studies often lower the requirements of the ANOVA, accepting the violations but interpreting the results with caution (Bowker 2007). As the aim of these experiments was to exclude concentrations where there was a biologically or statistically significant change in absorbance from control, parametric testing was deemed more sensitive despite violating the assumption of normality. All qPCR data was Log_{10} transformed to ensure it fitted a normal distribution. Shapiro-Wilk and Levene's test were performed to confirm normality and equality of variance respectively. Average gene expression between the control and test samples were assessed using ANOVA, whereas Interleukin analysis was assessed using Kruskall-Wallis. Statistical significance was considered to be <0.05.

CHAPTER 3: RESULTS

3.1 A CLINICAL STUDY OF AIRWAYS INNERVATION IN IPF PATIENTS

3.1.1 Baseline characteristics

39 patients were initially recruited from the interstitial Lung Diseases Clinic at University Hospital Llandough, Cardiff, UK according to the criteria described in Chapter 2. Four patients were subsequently moved to the pilot group, leaving a total of 35 patients (two patients had bronchoalveolar (BAL) results suggestive of hypersensitivity pneumonitis and two patients had evidence of obstructive airways disease on subsequent investigations that were not available at the time of recruitment.)

Five patients were recruited into a pilot group to allow for experimental optimisation; these patients were not included in the final analysis.

Demographic and pulmonary function data for study subjects are compared in table 10 and a more detailed demographic profile of the IPF patients is shown in appendix 1. Compared to the 'normal' control and chronic cough patients, the IPF patients were older. Patients with chronic cough were predominantly female and IPF patients predominantly male. There was no evidence of airflow obstruction in the patients studied and as would be expected patients with IPF had a significantly lower FVC % ($p = 0.026$) and TLCO % ($p = 0.002$) compared with chronic cough patients.

Baseline cough assessments are shown in table 11. Cough was a predominant symptom in all IPF patients studied. Cough assessments were not carried out on 'normal' controls as all patients denied cough at the time of recruitment. Patients with chronic cough had significantly greater cough symptom severity assessed by VAS and LCQ compared to IPF patients ($p < 0.05$). There was good correlation between the two methods of measuring cough severity (Figure 31), however cough severity measures did not correlate with baseline lung function in either group (Table 12).

	CONTROL	IPF	CHRONIC COUGH
Number	8	16	11
Mean age: years (Min, Max)	46 (24, 69)	70 (56, 82)*	60 (48, 73)¥
Gender (M:F)	5:3	14:2	2:9 * ¥
Mean FEV1: % predicted (Min, Max)	94.85 (84.9, 110.0)ǂ	87.21 (59.7,126.4)	107.29 (72.0,145.0)
Mean FVC: % predicted (Min, Max)	100.8 (80.0,133.0)ǂ	82.29 (51.9,113.9)	108.65 (78.2,142.3)¥
Median TLCO: % predicted		41.15	81¥

* $p < 0.05$ compared with control group

¥ $p < 0.05$ compared with IPF group

ǂ Number=4

Definition of abbreviations: F = female; M = male; 95% CI = 95 % confidence interval; FEV1 = forced expiratory volume in 1 second; FVC = forced vital capacity; TLCO = gas transfer.

Table 10 Study Subject Characteristics

	IPF	CHRONIC COUGH
Median VAS: mm (25th, 75th quartile)	40 (24.8, 62)	77 (49, 89) *
Mean Total LCQ score (95% CI)	14.88 (12.6, 17.1)	10.91 (9.5, 12.3) *
Mean LCQ -physical domain (95% CI)	4.9 (4.3,5.6)	3.8 (3.3, 4.3) *
Mean LCQ -psychological domain (95% CI)	4.9 (4.0, 5.8)	3.2 (2.5,3.8) *
Mean LCQ -social domain (95% CI)	5 (4.2, 5.9)	3.5 (2.8,4.2) *

* $p < 0.05$ compared with IPF group

Definition of abbreviations: VAS = Visual analogue score; LCQ = Leicester cough questionnaire; 95% CI = 95 % confidence interval.

Table 11 Baseline Cough Assessments

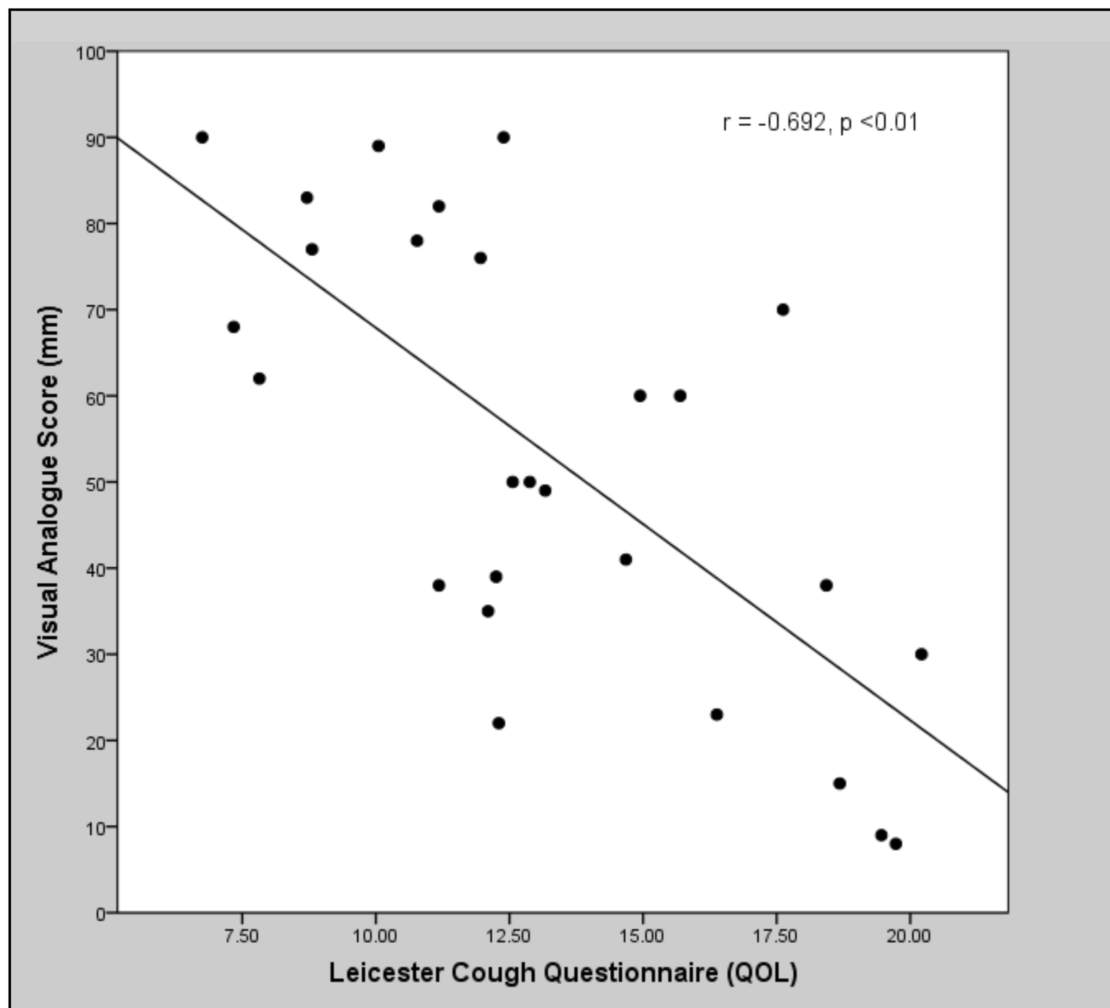


Figure 31 There was a significant correlation between the cough VAS and the LCQ total score. This graph combines the score of the IPF and chronic cough patients. IPF patients alone r = -0.703, p <0.01, CC patients alone r = -0.556, p 0.07.
NB a lower LCQ score indicates worse cough related quality of life resulting in a negative correlation co-efficient.

	VAS		LCQ	
	IPF	CC	IPF	CC
FEV1%	r = 0.149 p = 0.58	r = 0.523 p = 0.12	r = 0.023 p = 0.91	r = 0.030 p = 0.93
FVC%	r = -0.166 p = 0.54	r = 0.523 p = 0.12	r = -0.096 p = 0.64	r = -0.030 p = 0.93
TLCO%	r = -0.105 p = 0.77	r = 0.314 p = 0.54	r = -0.066 p = 0.77	r = -0.029 p = 0.96

Table 12 Correlations between measures of cough severity and lung function

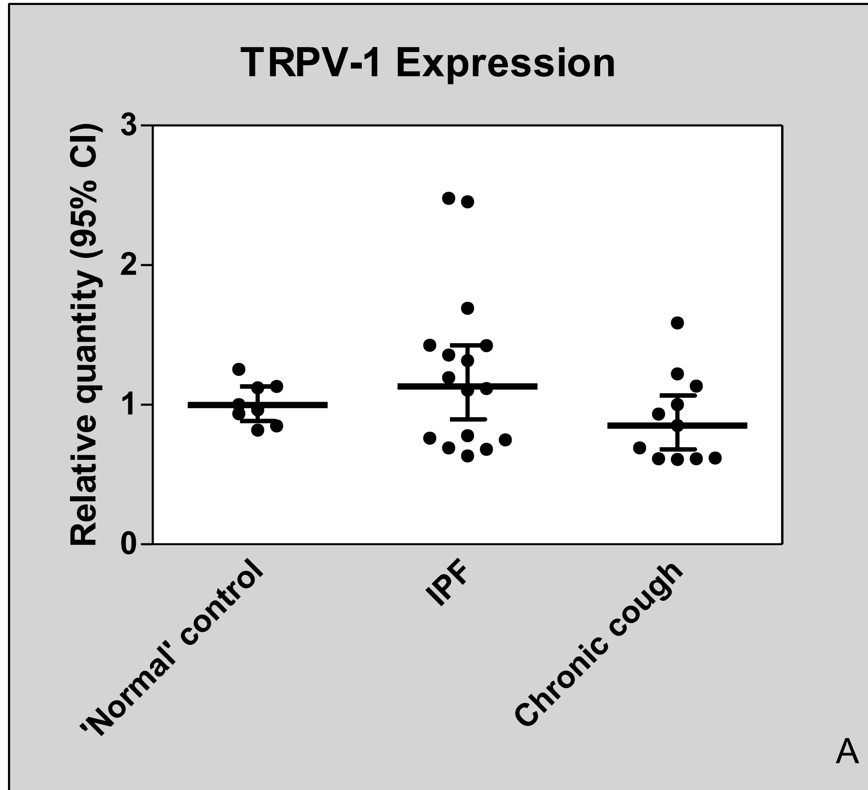
There was no significant correlation between cough symptom severity assessed by VAS score and LCQ score with % predicted FEV_1, % predicted FVC or % predicted TLCO in IPF or chronic cough patients.

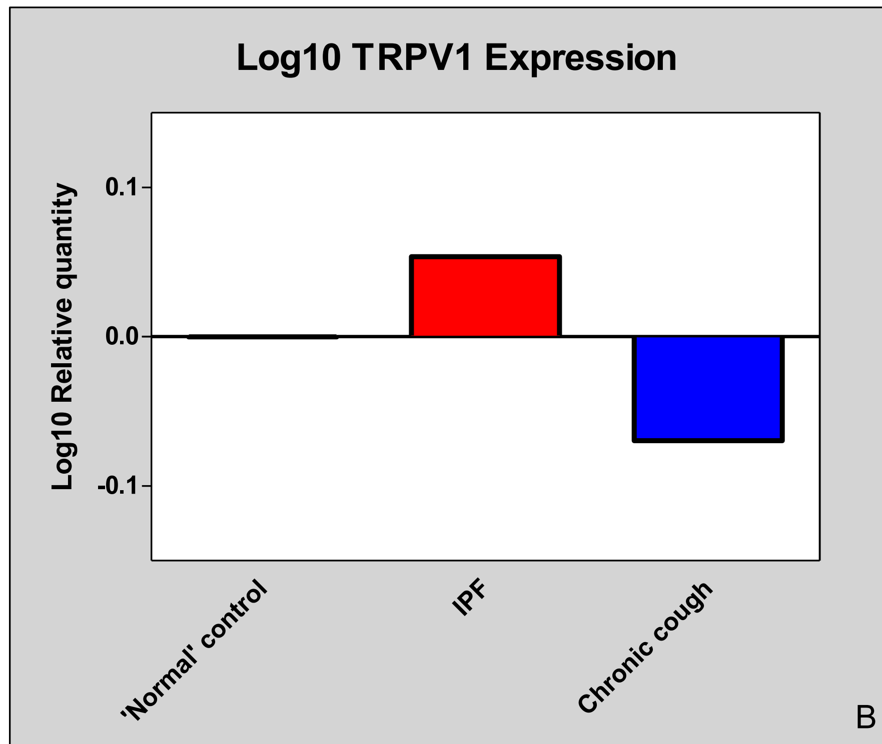
There were fewer ex-smokers in the chronic cough group but no significant difference between the groups studied (IPF: 7/16, CC 2/11, NC: 3/8, $p = 0.379$). All patients had stopped smoking for at least 12 months prior to bronchoscopy.

All chronic cough patients and 62.5% of patients with IPF had at least a 1 month trial of proton pump inhibitor prior to bronchoscopy, no patients had active symptoms of gastro-oesophageal reflux or rhinosinusitis as per the study exclusion criteria.

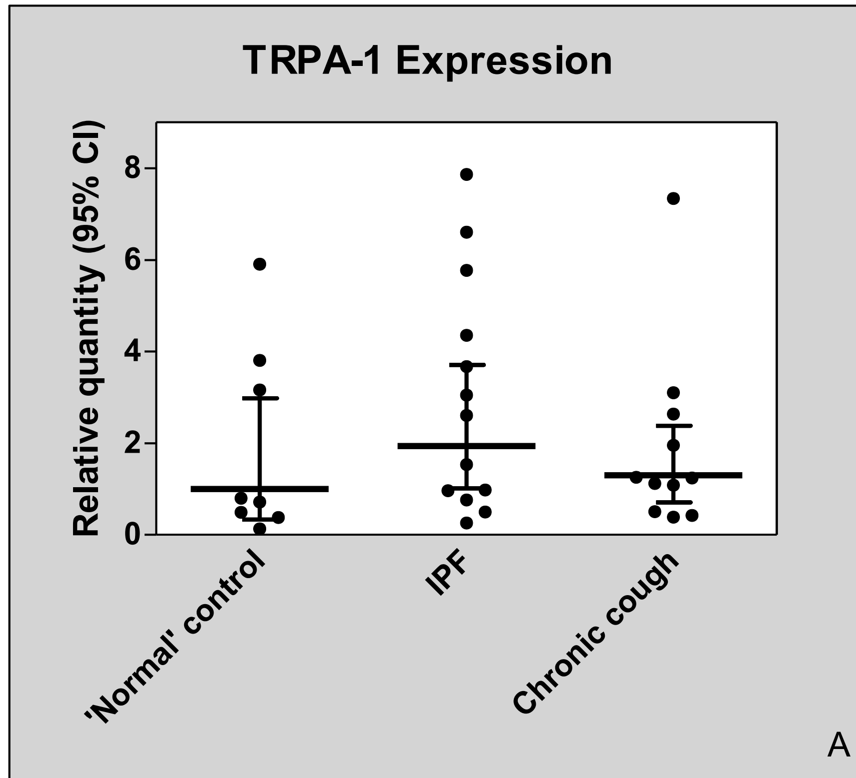
3.1.2 TRPV-1 and TRPA-1 receptor gene expression

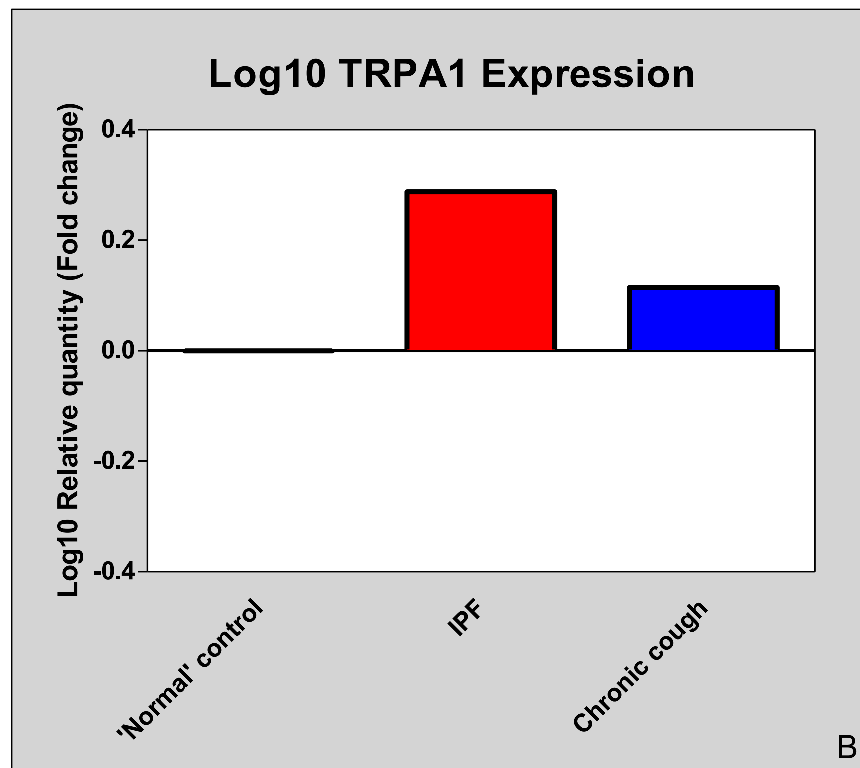
Gene expression is presented as relative quantity and represents a fold difference in gene expression compared to the calibrator sample (ratio), normalised against the chosen reference genes HPRT and TBP as detailed in section 2.1.8. The choice of calibrator sample does not influence the relative quantity, as although the average quantity may be different the fold changes between the groups remain the same (Hellemans et al. 2007). The relative quantities of TRPV-1 and TRPA-1 in individual study subjects (circles) and the mean of each patient group (horizontal line) are shown in figures 32a and 33a respectively, with the 'normal' control group as the calibrator. The same data is presented on a Log_{10} scale in figures 32b and 33b as this allows easier interpretation of the data; such that a relative quantity lower than the 'normal' control appears as a negative deflection.





FIGURES 32A and 32B Airway TRPV-1 Expression





FIGURES 33A and 33B Airway TRPA-1 Expression

Mean airway TRPV-1 gene expression was higher in IPF patients compared with 'normal' controls, with a 1.132 fold increase. Gene expression was lower in the chronic cough group however, with a 0.852 fold reduction in gene expression compared with 'normal' controls.

The difference in relative expression of TRPV-1 between the IPF group and the control group when gender, ex-smoking status and age were taken into account failed to reach statistical significance (β = 0.137, 95% CI = -0.025, 0.299, p = 0.093). Interestingly, age was a statistically significant predictor of TRPV-1 expression; there was a statistically significant increase in relative expression of TRPV-1 in those < 65 years of age (β = 0.127, 95% CI = 0.001, 0.254, p = 0.050), with a 13.5% increase in TRPV-1 relative quantity in those in the <65 age group (R^2 = 0.109). The relevance of this finding is unclear and should be interpreted with caution as the study was not designed to investigate TRPV-1 expression with age a priori, and as the study groups differed in ages the assumption of independence of the co-variate is violated. When co-variants were not accounted for and statistical significance was analysed with analysis of variance (ANOVA) there was not a statistically significant difference in TRPV-1 expression between the study groups (p = 0.146).

Mean TRPA-1 gene expression was higher in the both groups compared with 'normal' controls, with a 1.939 fold increase in the IPF group and 1.300 fold increase in the chronic cough group. There was not a statistically significant difference in relative TRPA-1 expression between the IPF group and the control group (β = 0.150, 95% CI = - 0.399, 0.698, p = 0.579), or the CC group and the control group (β = 0.190, 95% CI = -0.338, 0.718, p = 0.466) when gender, ex-smoking status and age were taken into account (R^2 = 0.065).

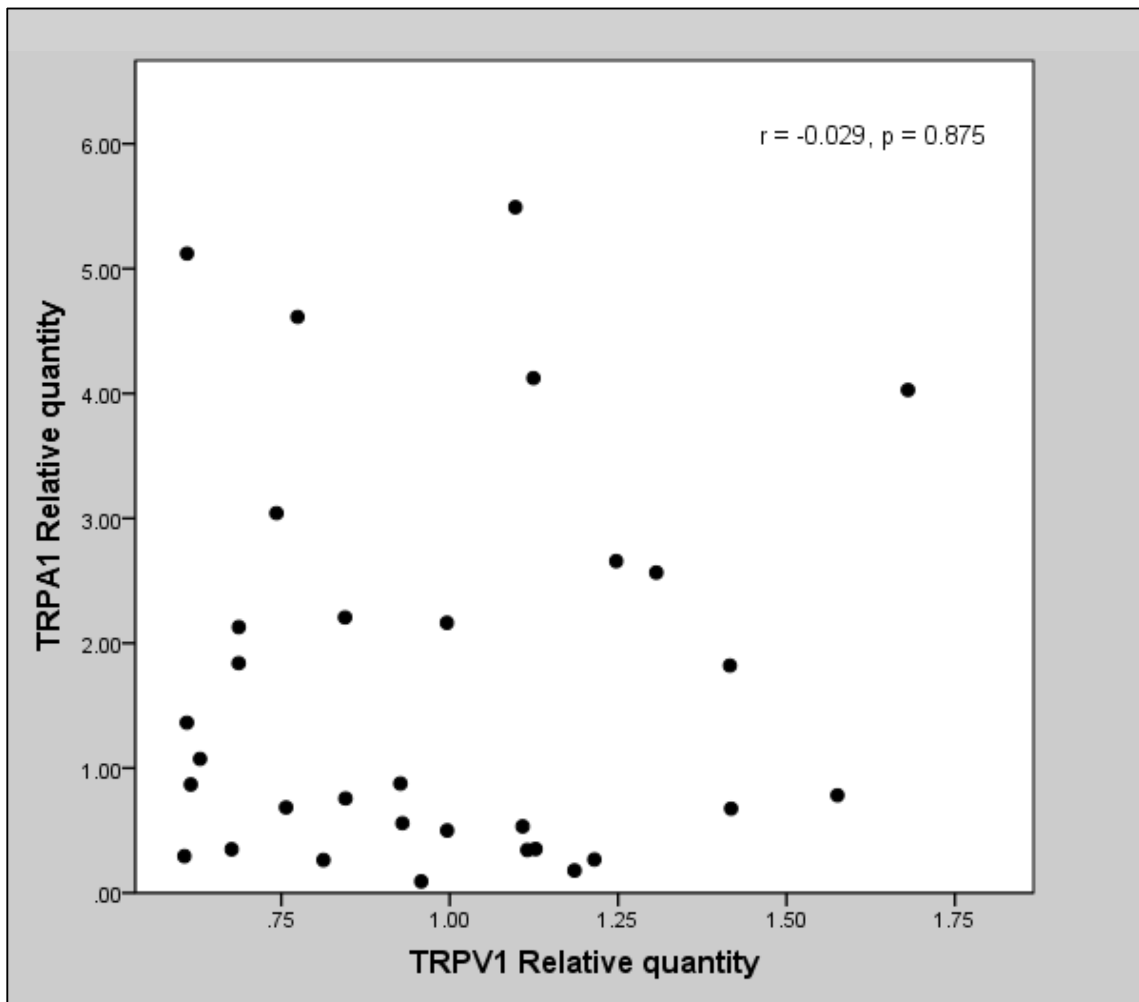


FIGURE 34 TRPV-1 and TRPA-1 airway expression correlation plot

TRPV-1 and TRPA-1 receptors are often co-expressed in sensory ganglia. This study failed to demonstrate a correlation between the relative quantities of airway TRPV-1 and TRPA-1 gene expression. Figure 34 shows all patients combined, however separation of the groups shows the same result.

3.1.3 Correlation of gene expression and measures of cough severity

Assessment for correlation between the methods used to assess cough severity and gene expression was carried out on the chronic cough and IPF groups combined and on each study group individually as presented in Table 13. There was no significant correlation between LCQ or VAS score and TRPA-1 gene expression in either study group or with the both groups combined.

Interestingly there were significant negative correlations between TRPV-1 gene expression and both measures of cough severity when the groups were combined. On separation of the groups the correlation between TRPV-1 gene expression and LCQ failed to reach significance. However, a significant negative correlation is evident between cough severity measured by VAS score and TRPV-1 gene expression in the IPF group; as cough severity increased, relative TRPV-1 gene expression was lower.

	TRPV-1			TRPA-1		
	All	IPF	CC	All	IPF	CC
LCQ	r = 0.427 **p = 0.026**	r = 0.415 p = 0.110	r = -0.036 p = 0.915	r = 0.309 p = 0.142	r = 0.297 p = 0.325	r = 0.327 p = 0.326
VAS	r = -0.454 **p = 0.017**	r = -0.657 **p = 0.006**	r = 0.005 p = 0.989	r = -0.305 p = 0.147	r = -0.342 p = 0.253	r = -0.178 p = 0.601

TABLE 13 Correlation of gene expression and measures of cough severity

3.1.4 Histopathological Analysis

All patients gave consent to have up to ten biopsies taken at the time of bronchoscopy, however the priority during the procedure was to obtain the samples required for clinical care. Samples for research purposes were therefore only obtained once the usual diagnostic procedure had been completed and providing the patient tolerated the procedure well. Samples were placed into RNA*later*^{TM} (Ambion, Paisley, UK) as a priority therefore if the patient failed to tolerate the procedure a matching sample was not obtained for histopathological and immunohistochemical analysis. This occurred in 3 out of 35 patients. A further 5 samples had insufficient material for adequate assessment, therefore biopsy inflammatory status was assessed with haematoxylin and eosin staining in 14 IPF patients, 7 chronic cough patients and 6 ‘normal’ controls in total.

Examined parameters included the presence and degree of chronic inflammation, basement membrane thickening and mucous cell hyperplasia as outlined in Chapter 2.1.9.

No patients had evidence of acute inflammation on H & E staining, however, surprisingly the majority of patients had some degree of chronic inflammation composed of a lymphocytic infiltration. There was also evidence of basement membrane thickening, mucous cell hyperplasia and areas of focal squamous metaplasia in all three groups as shown in Table 14. There were no significant differences in the morphological features of the samples between the three groups.

		NC n=6	IPF n= 14	CC n=7	P value
Chronic inflammation	No	1 (17%)	2 (14%)	1 (14%)	0.528
	Mild	2 (33%)	8 (57%)	3 (43%)	
	Moderate	2 (33%)	4 (29%)	3 (43%)	
	Severe	1 (17%)	0	0	
Basement membrane thickening	No	4 (67%)	9 (64%)	5 (71%)	0.999
	Yes	2 (33%)	5 (36%)	2 (29%)	
Epithelium abnormality	Focal squamous metaplasia	1 (17%)	4 (29%)	1 (14%)	0.845
	Mucous cell hyperplasia	2 (33%)	3 (21%)	3 (43%)	0.555

Table 14 Histopathological Characteristics

3.1.5 Immunohistochemical Analysis

Immuno-histochemical (IHC) staining was undertaken in 14 IPF, 7 chronic cough and 6 'normal' control patients. Initially PGP-9.5, TRPV-1 and TRPA-1 staining was recorded as either positive or negative and the distribution noted.

Staining the biopsies for PGP-9.5 revealed nerve profile specific staining in the epithelium and sub-epithelium (Figure 35). TRPV-1 immunohistochemistry also demonstrated specific staining of nerve profiles in the sub-epithelial and epithelial layers (Figure 36). There was very occasional positive TRPV-1 staining identified in epithelial cells and no staining in smooth muscle myocytes. In contrast staining for TRPA-1 revealed specific staining of epithelial cells, smooth muscle myocytes as well as sub-epithelial nerve profiles (Figure 37). The bronchial epithelium in particular exhibited a pronounced expression of TRPA-1, with all samples having positive staining.

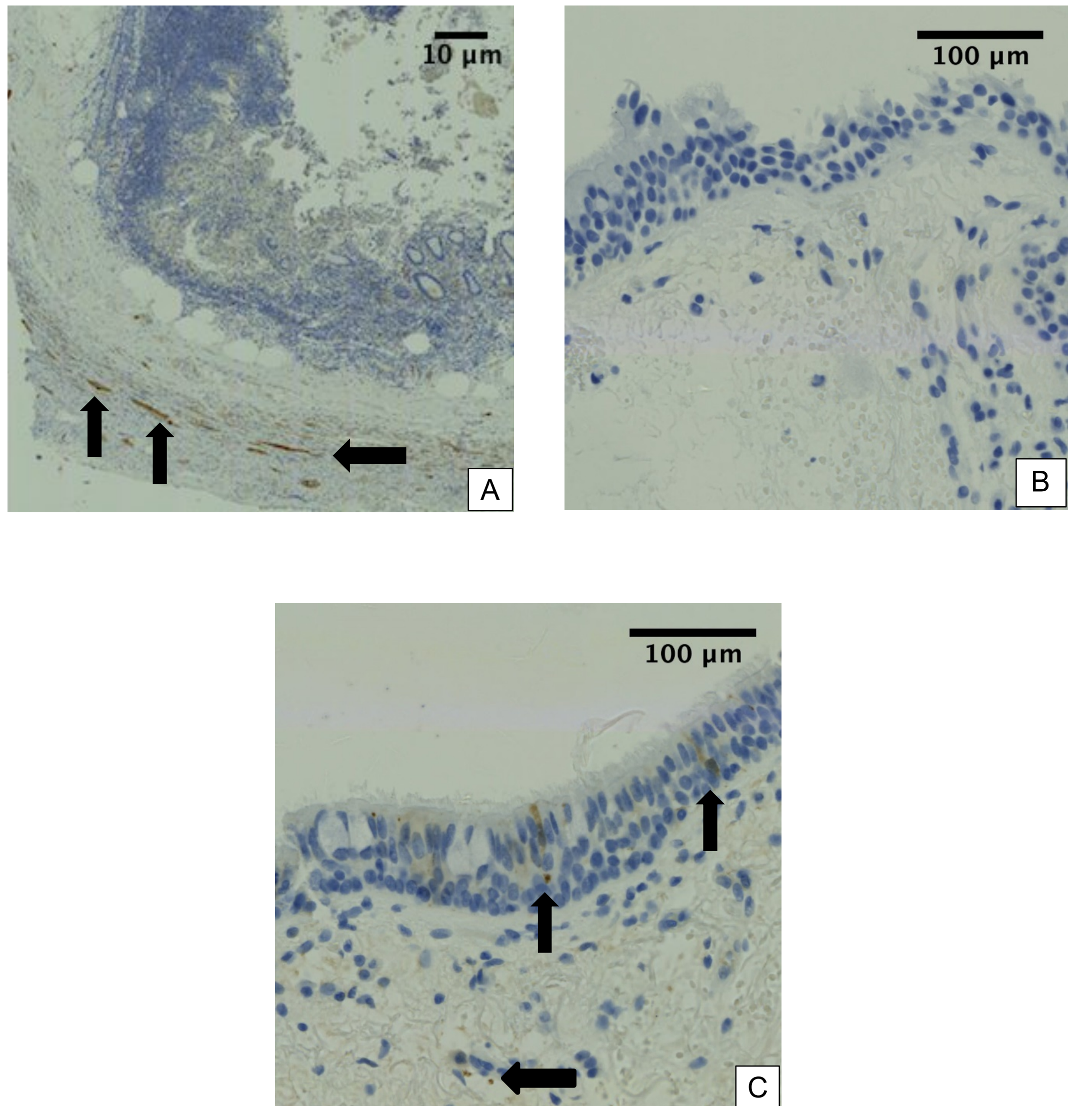


Figure 35 Immunohistochemical staining for PGP-9.5
The positive control (appendix tissue) is shown in panel A, with the black arrows denoting PGP-9.5 specific staining. Bronchial sections incubated in the absence of the primary antibody showed no staining, a representative image is shown in panel B. Panel C shows IHC staining of airway nerves in a bronchial biopsy with an anti-PGP-9.5 antibody from a study patient.

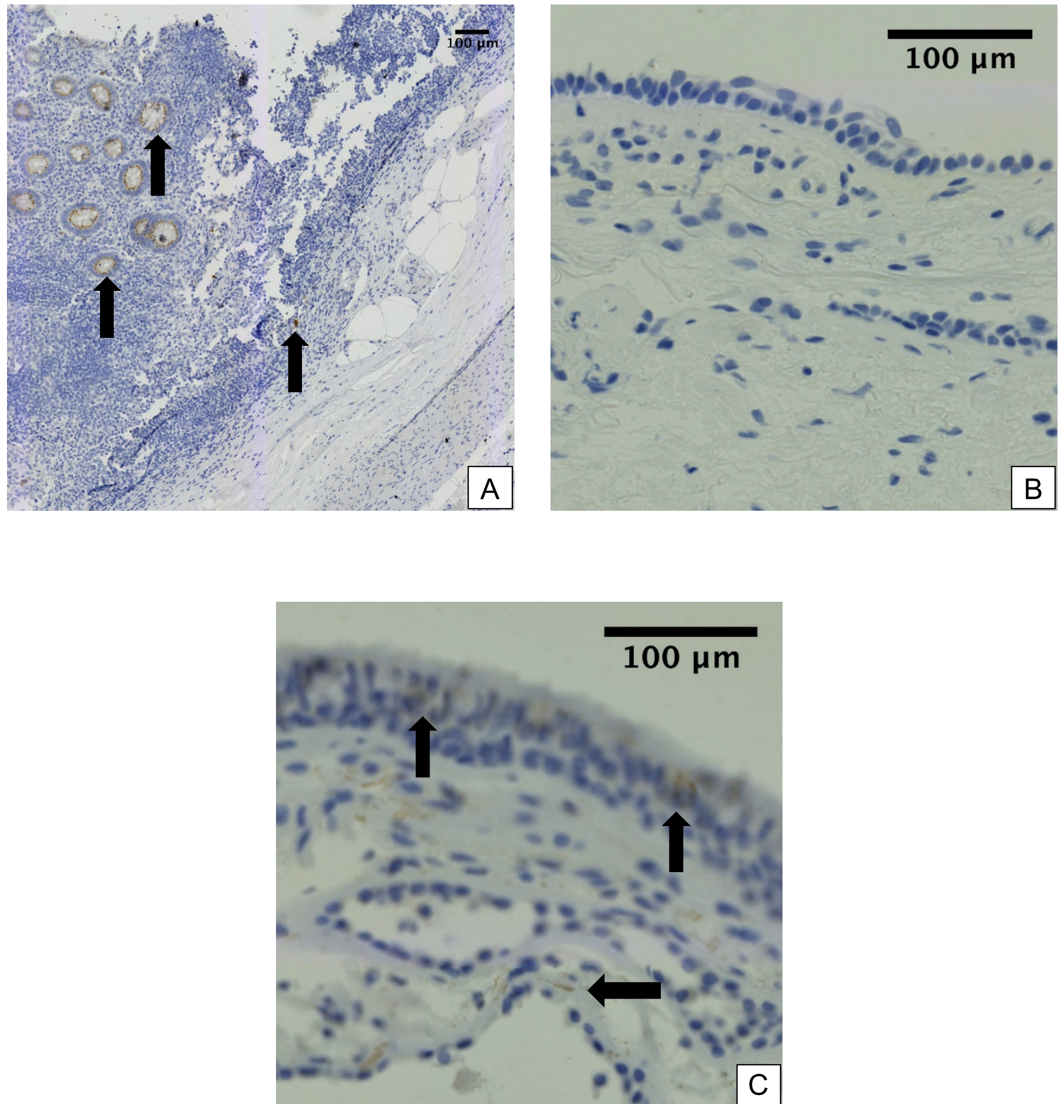


Figure 36 Immunohistochemical staining for TRPV-1
The positive control (appendix tissue) is shown in panel A, with the black arrows denoting TRPV-1 specific staining. Bronchial sections incubated in the absence of the primary antibody showed no staining, a representative image is shown in panel B. Panel C shows IHC staining of airway nerves (vertical arrows) and spindle cells in the sub-mucosa (horizontal arrow) in a bronchial biopsy with an anti-TRPV-1 antibody from a study patient with IPF.

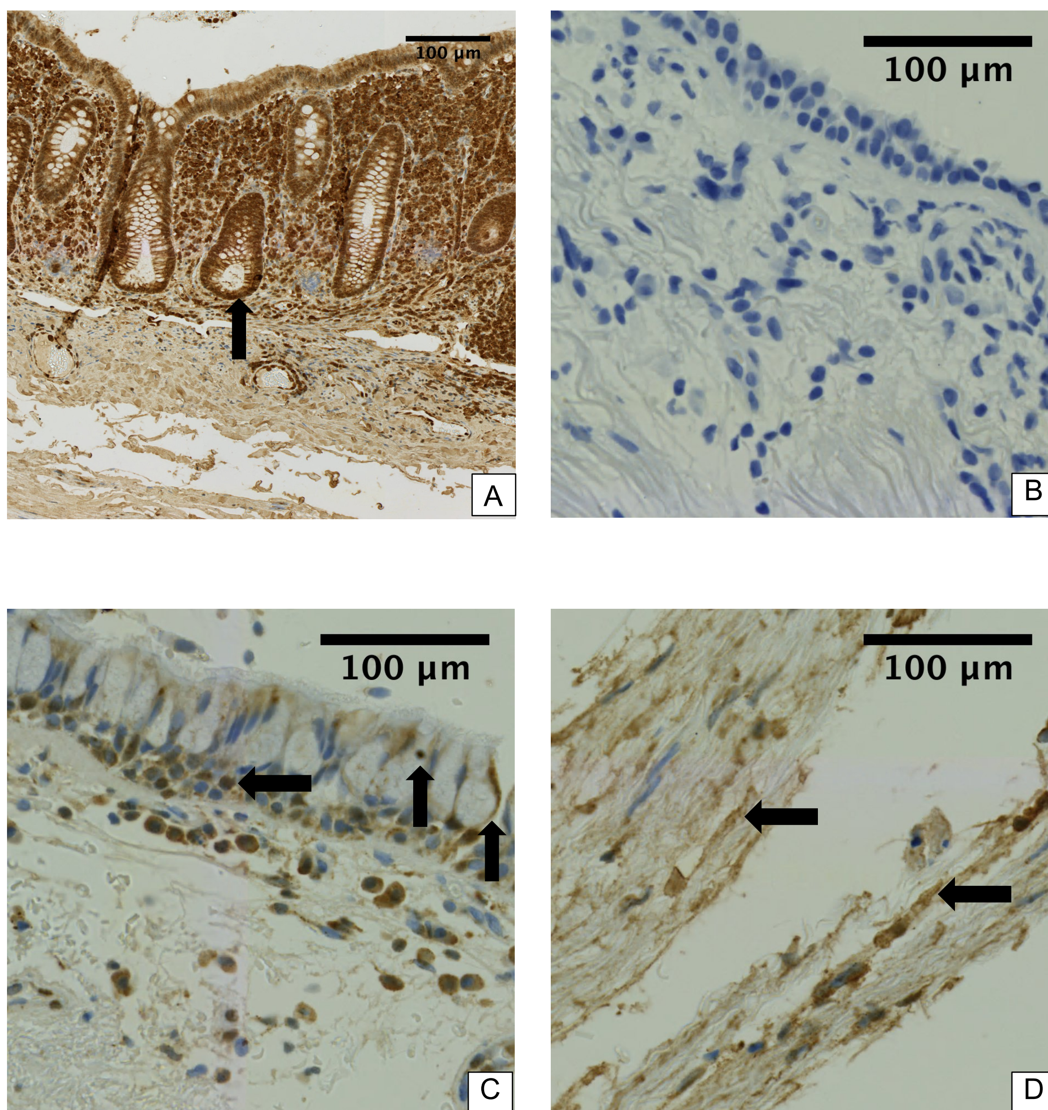


Figure 37 Immunohistochemical staining for TRPA-1
The positive control (appendix tissue) is shown in panel A, with the black arrows denoting TRPA-1 specific staining. Bronchial sections incubated in the absence of the primary antibody showed no staining, a representative image is shown in panel B. Panel C shows IHC staining of airway nerves (vertical arrows) and epithelial cells (horizontal arrow) in a bronchial biopsy with an anti-TRPA-1 antibody from a study patient with IPF. Panel D shows TRPA-1 staining within the smooth muscle.

The initial manual immunohistochemical analysis is summarised in table 15a-c. Airway PGP- 9.5 nerve profile staining is summarised in Table 15a. There was no PGP-9.5 nerve profile staining in 7 samples. All samples were re-analysed and this was thought to be a true result.

TRPV-1 airway nerve profile staining is summarised in table 15b. There was a slight increase in positive staining in the IPF group and reduction in the CC group, as seen with the gene expression however these differences were not statistically significant.

All samples had positive TRPA-1 airway epithelial cell staining; table 15c summarises the TRPA-1 nerve profile and smooth muscle staining. Compared to the NC group, there was increased TRPA-1 expression in nerve and smooth muscle myocytes in the IPF and CC groups, however there was no statistically significant difference.

Binary logistic regression was used to assess whether the positivity of PGP-9.5, TRPV-1 and TRPA-1 could be predicted from the co-variables in the study other than study group (age, gender, ex-smoking status), however the results are not displayed as the model was an inadequate fit.

	PGP-9.5		
	Positive	Negative	
NC	4/6 (67%)	2/6 (33%)	P value
IPF	10/14 (71%)	4/14 (29%)	0.999
CC	5/6* (83%)	1/6 (17%)	0.999

* There was sub-optimal PGP-9.5 staining in one CC sample.

TABLE 15a Airway nerve profile PGP-9.5 staining

	TRPV-1		
	Positive	Negative	
NC	3/6 (50%)	3/6 (50%)	P value
IPF	9/14 (64%)	5/14 (36%)	0.642
CC	3/7 (43%)	4/7 (57%)	0.999

TABLE 15b Airway nerve profile TRPV-1 staining

	TRPA-1					
	Nerve			Smooth muscle		
	Positive	Negative		Positive	Negative	
NC	1/6 (17%)	5/6 (83%)	P value	2/5 (40%)*	3/5 (60%)*	P value
IPF	9/14 (64%)	5/14 (36%)	0.141	8/14 (57%)	6/14 (43%)	0.628
CC	3/7 (43%)	4/7 (57%)	0.559	3/7 (43%)	4/7 (57%)	0.999

* There was inadequate smooth muscle tissue in one NC sample to adequately assess staining.

TABLE 15c Airway nerve profile and smooth muscle TRPA-1 staining

Following the manual immunohistochemical assessment digital quantification was performed using the scientific image analysis package ImageJ, with immunostaining recorded as a percentage of the total area as outlined in Chapter 2.

In agreement with previous studies, neuronal immunostaining for the general nerve marker PGP-9.5 varied among cases and between groups (O' Connell et al. 1995; Groneberg et al. 2004a). The median (range) total nerve density of PGP-9.5 in IPF patients was 0.053% (0.002 to 0.29%) and 0.082% (0.009 to 0.63%) in CC patients. The median in the NC group was 0.056% (0.006 to 0.19%) with no statistically significant difference in PGP-9.5 immunostaining between the controls and patients with IPF or CC (Figure 38).

Median TRPV-1 immunostaining was 0.124 % (0.01-0.29 %) in IPF patients, which was higher than the median percentage seen in CC and NC patients, however this this difference was minimal and did not reach statistical significance ($p = 0.53$) as shown in (Figure 39). The difference in TRPV-1 IHC staining between the IPF group and control group also failed to reach statistical significance when gender, ex-smoking status and age were taken into account ($\beta = -0.056$, 95% CI = -0.155, 0.044, $p = 0.256$). We also quantified the expression of TRPV-1 in the biopsies as a ratio of the PGP-9.5 expression measured in the adjacent section for each subject. There was no statistically significant difference in TRPV-1 to PGP-9.5 ratio between NC and IPF patients ($p = 0.904$) or NC and CC patients ($p = 0.234$).

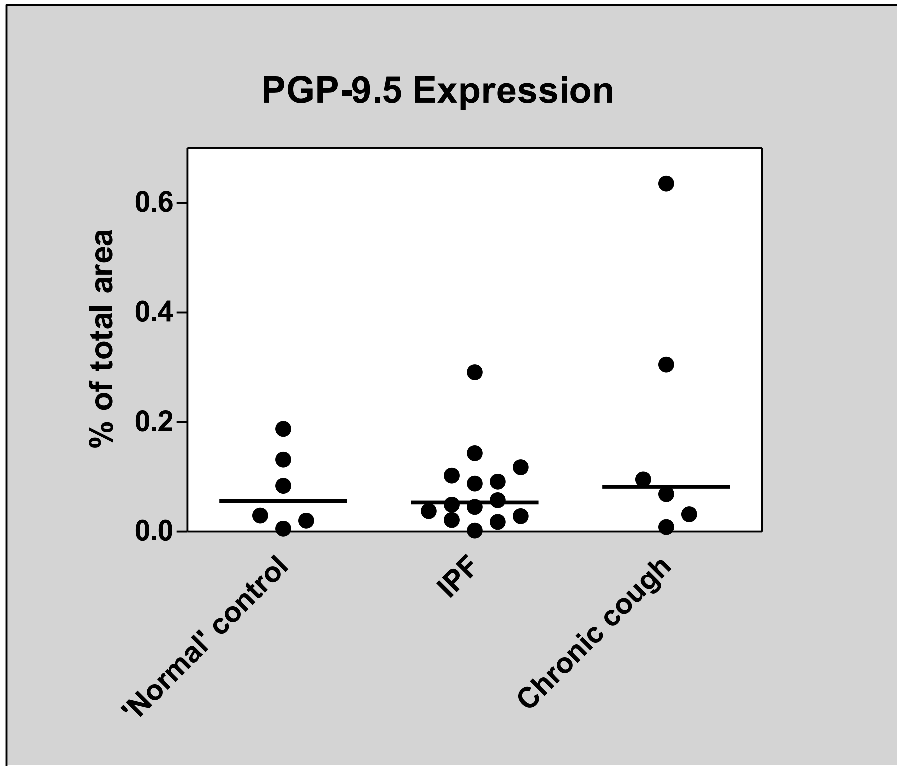


Figure 38 PGP-9.5 Expression

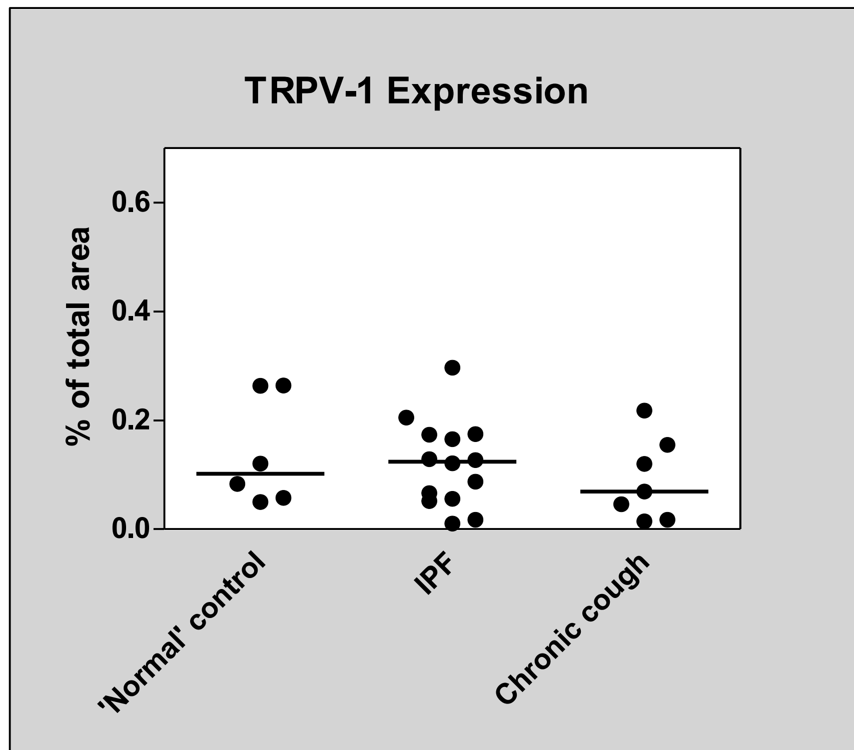


Figure 39 TRPV-1 Expression

Quantification of TRPA-1 immunostaining was more difficult as there was epithelial cell and stromal cell specific staining within the smooth muscle as well as neuronal staining. It was not possible to separate the two for analysis, therefore total immunostaining was measured. There was no statistically significant difference in median TRPA-1 immunostaining as demonstrated in (Figure 40). The difference in TRPA-1 IHC staining between the IPF group and control group also failed to reach statistical significance when gender, ex-smoking status and age were taken into account (β =-8.170, 95% CI -18.49, 2.153, $p = 0.115$).

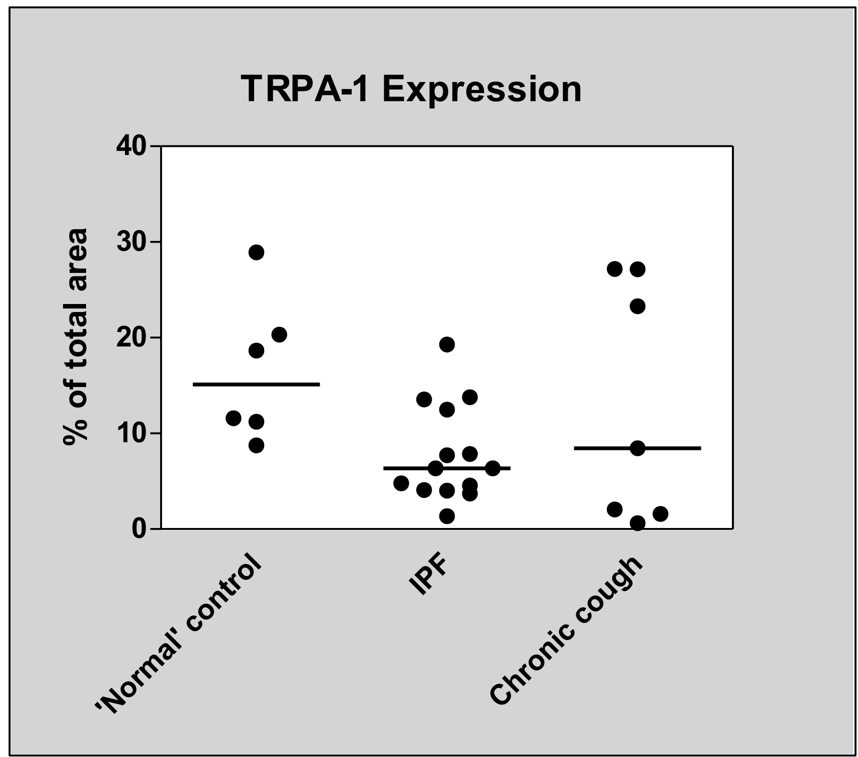


Figure 40 TRPA-1 Expression

These results however are not an accurate reflection of neuronal staining as are influenced by the proportion of epithelial cells in the section studied. Also, it was not possible to quantify the expression of TRPA-1 as a ratio of the PGP-9.5 expression due to the inclusion of the epithelial and stromal cells in the quantification. In an attempt to assess the differences more accurately a manual semi-quantitative analysis of TRPA-1 expression was performed as shown in (Figures 41a and b). Although there was a higher number of patients with grade 2 (<10%) of smooth muscle immunostaining in the IPF group there was no statistically significant difference between the groups for grade of smooth muscle ($p = 0.71$) or epithelial ($p = 0.18$) TRPA-1 staining.

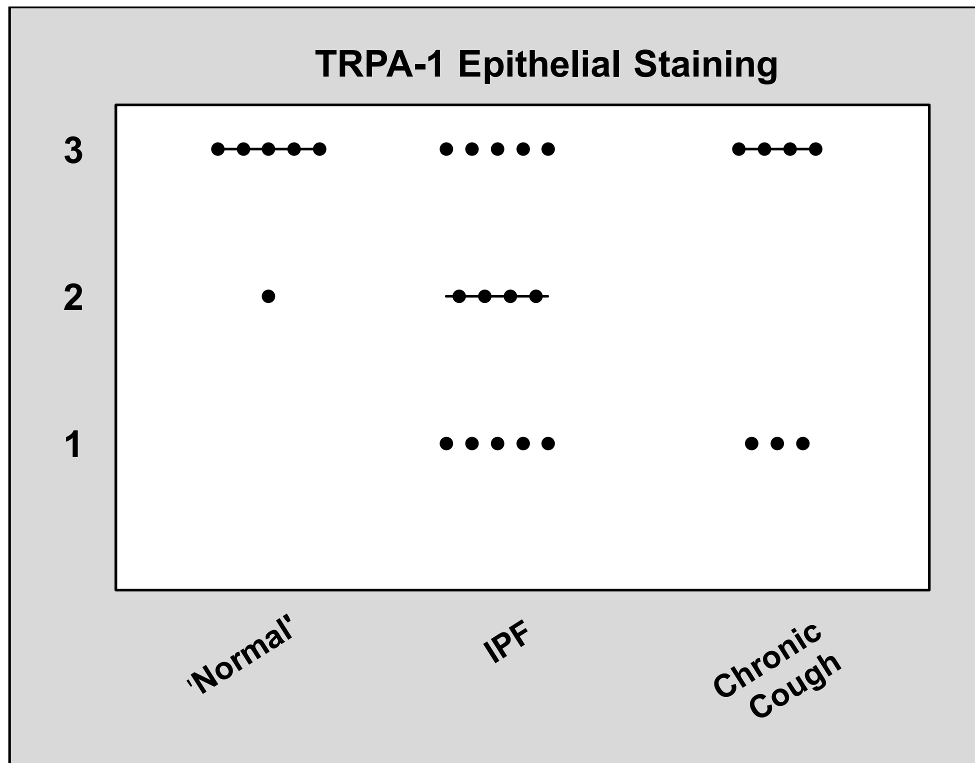


Figure 41a Epithelial TRPA-1 staining was graded on a semi-quantitative scale. Grade 1 = <10% cells, Grade 2 = 10-50% cells, Grade 3 = > 50% cells. Individual subjects and the median are displayed.

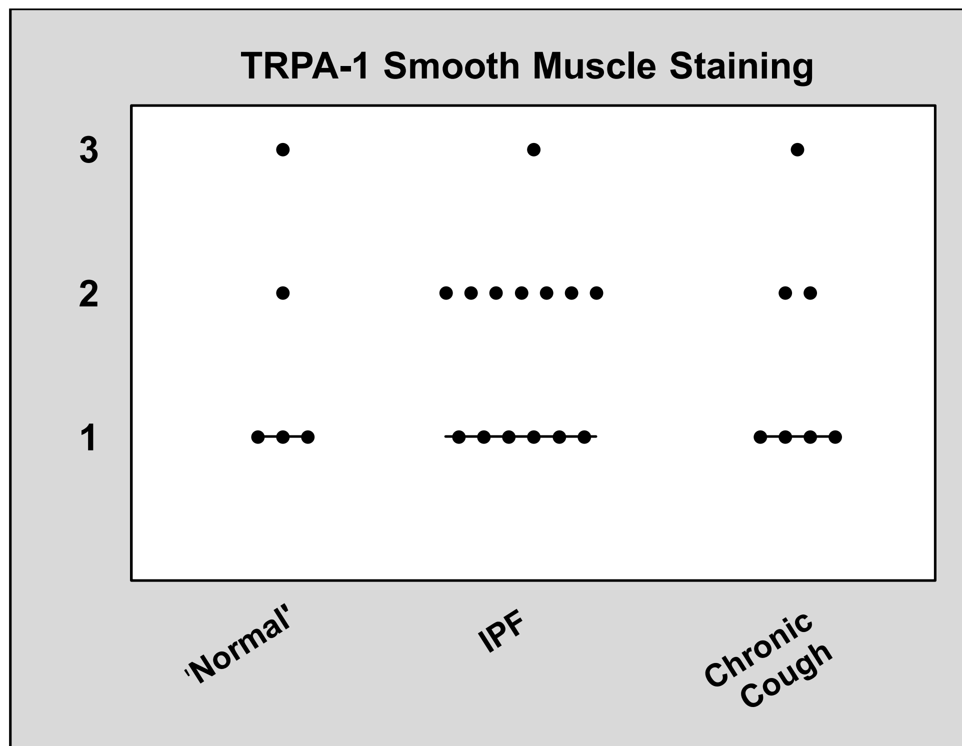


Figure 41b Smooth muscle TRPA-1 staining was graded on a semi-quantitative scale. Grade 1 = Negative, Grade 2 = <10%, Grade 3 = 10-50%, Grade 4 >50%. Individual subjects and the median are displayed.

There was evidence of agreement between the manual assessment of IHC staining and the digital quantification as the sections assessed as positive for PGP-9.5 had a median total nerve density of 0.09 % compared with 0.01% in the sections that were assessed as negative (p = **0.013**). This was also apparent for TRPV-1 with median total nerve density of 0.13% in positive sections compared to 0.07% in negative samples, however this difference failed to reach statistical significance (p = 0.167).

There was no significant correlation between neuronal TRPV-1 and TRPA-1 IHC staining using manual assessment (p = 0.999).

Interestingly, there was a statistically significant increase in TRPV-1 immunostaining observed in IPF patients who did not receive a trial of a PPI. Median TRPV-1 nerve density in subjects who received a PPI was 0.066%, compared to 0.173% in those who did not receive a PPI (p = **0.042**). It is important to note that none of the patients had active symptoms of GOR and that the study was not designed to investigate the relationship between TRPV-1 expression and PPI treatment a priori. There was no significant difference in VAS and LCQ scores between patients who received a PPI trial and those who did not.

3.1.6 Correlation between Protein Expression and Inflammation

As there were only 4 samples in total without any evidence of inflammation, investigating a correlation between TRP expression and the presence of inflammation was unsuccessful. Of note from the four samples that did not have evidence of inflammation, there was TRPV-1 nerve and TRPA-1 smooth muscle staining in two, but no TRPA-1 nerve profile staining.

There was no statistically significant difference in the expression assessed manually or nerve density analysed digitally of TRPV-1 and the degree of inflammation present (p = 0.44 and p = 0.10 respectively).

There was no significant difference in the expression of TRPA-1 in smooth muscle and the degree of inflammation present in the samples (p = 0.66). Interestingly, there was a clear trend between TRPA-1 nerve profile staining and

the degree of inflammation which almost reached the level of statistical significance ($p = 0.05$). There was increased TRPA-1 nerve profile staining in the presence of mild inflammation (positive: 10, negative: 3), but decreased staining in moderate (positive: 3, negative: 6) and severe (positive: 0, negative: 1) inflammation. This trend was only observed when all samples were combined, there was no statistical difference between the groups and there was no statistically significant difference in cough severity ($p = 0.29$) or cough related QOL ($p=0.68$) and degree of inflammation present.

3.1.7 Association between Protein Expression and Measures of Cough Severity

Table 16 shows the staining profile of PGP-9.5, TRPV-1 and TRPA-1 assessed manually and the mean or median, LCQ and VAS scores respectively. There was no significant difference in cough severity measured by VAS and LCQ and the expression of either TRP receptor statistically or clinically in terms of minimal important difference (MID). Correlation between cough severity and quantitative TRP receptor expression was also assessed and no significant correlations were identified (data not shown).

	TRPV-1 (Nerve)		
	Positive	Negative	P value
Median VAS	50	50.5	0.999
Mean LCQ	13.2	13.7	0.759
	TRPA-1 (Nerve)		
Median VAS	45.3	60	0.169
Mean LCQ	13.9	12.8	0.515
	TRPA-1 (Smooth muscle)		
Median VAS	50	51.5	0.809
Mean LCQ	13.7	13.2	0.758
	PGP-9.5 (Nerve)		
Median VAS	50	39	0.933
Mean LCQ	13.5	13.6	0.981

TABLE 16 Association between measures of cough severity and protein expression

This table shows the combined results of the CC and IPF patients. There remains no significant difference in cough severity measured by VAS and LCQ and the expression of either TRP receptor when the groups are analysed independently.

3.1.8 Summary of results

1. In this cohort, patients with chronic cough had greater cough symptom severity as assessed by VAS and LCQ when compared to IPF patients.

2. There was a trend towards increased airway TRPV-1 and TRPA-1 gene expression in IPF patients. However, this result failed to reach statistical significance between patients with IPF, chronic cough and normal controls when gender, ex-smoking status and age were taken into account.

3. IPF patients less than 65 years of age had significantly higher TRPV-1 gene expression compared with controls.

4. No correlation was observed between TRPV-1 and TRPA-1 airway gene expression in this cohort of patients.

5. Increased cough severity as assessed by VAS and LCQ was not associated with increased airway gene expression of TRPV-1 and TRPA-1.

6. No significant difference in airway TRPV-1 or TRPA-1 protein expression was demonstrated between patients with IPF, chronic cough and controls.

7. There was a statistically significant increase in TRPV-1 nerve density observed in a small cohort of IPF patients who did not receive PPI therapy.

8. There was evidence of a trend between the degree of inflammation present and the expression of TRPA-1 protein in airway nerves, with an increased expression in sections with milder chronic inflammation.

9. No correlation was observed between TRP receptor protein expression and cough symptom severity.

3.2 STUDIES OF TRP RECEPTORS IN RESPIRATORY CELL LINES EXPOSED TO GASTRIC FLUID CONSTITUENTS

3.2.1 Preliminary experiment results - Cell viability assay (Cell titre 96)

Preliminary experiments were carried out on each of the pulmonary epithelial cell lines to ensure the concentration of gastric constituents and exposure time was tolerated as described in section 2.2.2. These experiments aimed to exclude doses and exposure times that resulted in a biologically or statistically significant change in cell viability, so that any change seen in receptor gene expression level could not be attributed to cell death or proliferation.

Initial test doses were based on previously used concentrations. Cell lines were at first exposed to the various constituents continuously for a 24-hour period. Both cells lines showed evidence of a reduction in the percentage of metabolically active cells on exposure to higher concentrations of CD, GD and Pepsin compared with the control sample. Both cell lines were unable to tolerate a continuous exposure to media at a pH <6. Continuous exposure cell viability is shown in appendix 5.

The experiments were repeated with a reduction in exposure time to one hour but with repeated exposures over 3 consecutive days in an attempt to model gastric reflux in vivo. Table 17 shows the average (N=3) viability (measured using absorbance) of A549 and BEAS-2B cells calculated as a percentage of the control sample, and the corresponding p-value of each test dose compared with the control sample. Pepsin at various pHs was tested at a number of concentrations, however the 1mg/ml dose is displayed. The main finding was that even at a reduced time exposure there was a biologically and statistically significant reduction in cell viability when exposed to a pH < 6.

	Absorbance/ Viability (%)					
	Control	Ethanol carrier	CD 50 µM	CD 100 µM	CD 250 µM	CD 500 µM
A549	100.00	104.44	109.69	106.40	101.72	63.47
P value		*0.99*	*0.77*	*0.95*	*0.99*	***0.01***
BEAS-2B	100.00	106.13	105.26	112.66	101.99	61.76
P value		*0.99*	*0.61*	*0.77*	*0.99*	***0.04***

	Control	H_20 carrier	GD 50 µM	GD 100 µM	GD 250 µM	GD 500 µM
A549	100.00	95.02	98.23	95.17	94.41	93.93
P value		*0.89*	*0.99*	*0.73*	*0.88*	*0.96*
BEAS-2B	100.00	99.74	105.33	103.17	110.74	99.27
P value		*0.99*	*0.98*	*0.86*	*0.15*	*0.99*

	Control	Pepsin 0.5 mg/ml	Pepsin 1mg/ml	Pepsin 2mg/ml	Pepsin 5mg/ml	Pepsin 10mg/ml
A549	100.00	112.04	108.28	105.17	96.38	93.56
P value		*0.17*	*0.51*	*0.86*	*0.99*	*0.94*
BEAS-2B	100.00	108.42	110.05	113.05	115.40	92.34
P value		*0.88*	*0.79*	*0.58*	*0.7*	*0.97*

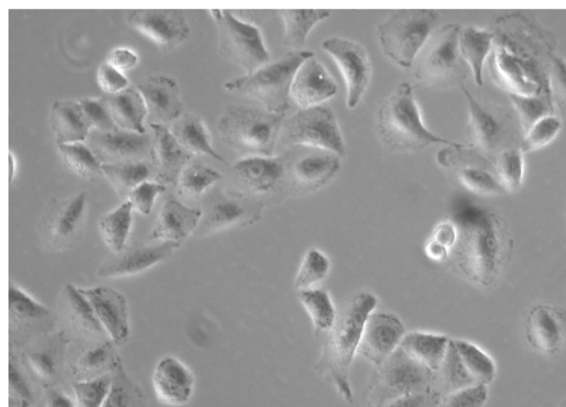
	Control	H_20 carrier	Pepsin pH8	Pepsin pH6	Pepsin pH4	Pepsin pH2
A549	100.00	110.39	110.89	101.23	27.90	27.74
P value		*0.94*	*0.97*	*0.99*	***0.01***	***0.01***
BEAS-2B	100.00	96.70	105.95	131.26	31.56	29.99
P value		*0.99*	*0.99*	*0.53*	***0.005***	***0.004***

	Control	H_20 carrier	pH6	pH4	pH2
A549	100.00	97.36	108.91	40.83	20.38
P value		*0.69*	*0.69*	*0.08*	***0.03***
BEAS-2B	100.00	101.71	94.90	26.53	26.94
P value		*0.71*	*0.71*	***0.03***	***0.04***

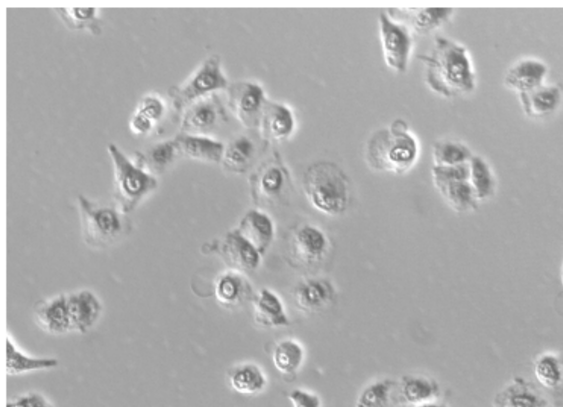
TABLE 17 Cell Viability – Exposure for 1 hour/day for 3 consecutive days

Repeated exposures to acidic pH with and without pepsin for shorter time exposures (15 minutes on 3 consecutive days and 5 mins on 3 consecutive days) still resulted in a significant reduction in viability at a pH <6 (data not shown). A single 1 hour exposure also resulted in reduced viability as shown in in Figure 42.

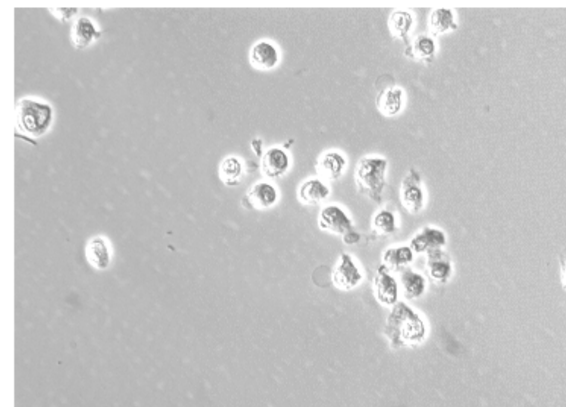
Reducing the time exposure to 5 minutes for 1 day only also resulted in a reduction in viability by almost half in both cell lines on exposure to pepsin at pH4 as shown in table 18, however the cells tolerated this short exposure to pH4 alone. Despite being unable to expose the cell lines to repeated acidic environment, the tolerated exposure times were taken forward in order to gain as much information as possible.



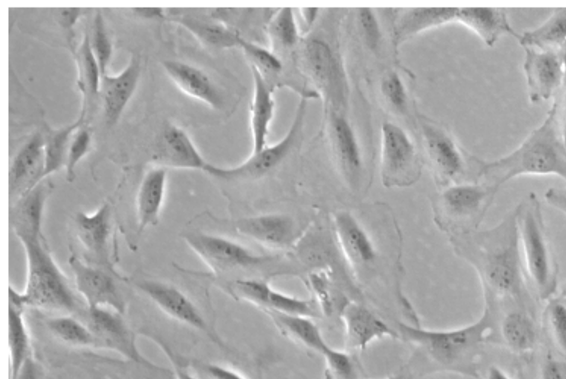
A549 Control



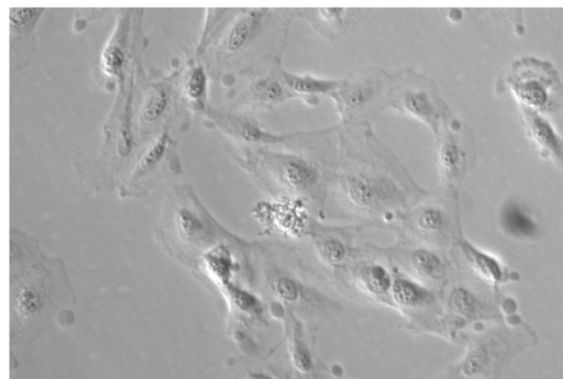
A549 pH4



A549 pepsin 1mg/ml pH4



BEAS-2B Control



BEAS-2B pH4

BEAS-2B pepsin 1mg/ml pH4

Figure 42 A549 and BEAS-2B cells following exposure to media at pH4 and pH4 with 1mg/ml pepsin for 1 hour. The cells exposed to pH4 with and without pepsin show loss of cell-cell interaction and abnormal morphology. The BEAS-2B pepsin 1mg/ml pH4 image is blurred as a result of excess cell debris. Pictures were taken with an Axiovert 35M microscope at X10 magnification.

	Control	H_20 carrier	Pepsin pH8	Pepsin pH6	Pepsin pH4	Pepsin pH2	pH6	pH4	pH2
A549	100.00	104.67	110.13	103.69	52.48	42.17	99.93	91.75	81.55
P value		*0.99*	*0.97*	*0.99*	***0.01***	***0.01***	*0.99*	*0.90*	***0.04***
BEAS-2B	100.00	97.33	101.72	101.47	60.81	52.05	96.54	88.12	68.48
P value		*0.60*	*0.73*	*0.81*	***0.02***	***0.01***	*0.52*	*0.20*	***0.03***

TABLE 18 Cell Viability – Exposure for 5 minutes for 1 day

There was a clear reduction in cell viability when both cell lines were exposed to pH2 and pepsin at pH2 and pH4 therefore these test doses were not used in downstream experiments (Pepsin = 1mg/ml).

3.2.2 Preliminary experiment results – Cell viability and apoptosis assay (Flow cytometric assay)

Following the cell viability assay, clinically relevant concentrations were chosen from those that did not biologically or statistically affect the cells in the viability assay. These concentrations were then further assessed for cell viability, necrosis and apoptosis using a flow cytometric assay as detailed in section 2.2.4.

The average (N=3) percentage cell viability, necrosis and apoptosis for the three consecutive day, 1 hour exposures are shown in tables 19a and b, with the one day, 5 minute exposures shown in table 20.

The positive control had less viable and more apoptotic and necrotic cells as would be expected. There was no statistically or biologically significant difference between the negative control and each test dose. The optimised test conditions were then used for the qPCR experiment.

		Gastric constituent					
		Negative control	Positive control	Ethanol carrier	H_20 carrier	CD 50 µM	GD 50 µM
A549	Viable (%)	94.00	76.85	93.91	94.43	91.86	93.99
	Apoptotic (%)	0.40	12.48	0.32	0.39	0.31	0.34
	Necrotic (%)	5.60	10.70	5.77	5.18	7.83	5.67
BEAS-2B	Viable (%)	95.26	31.93	95.44	95.23	93.20	93.37
	Apoptotic (%)	1.31	12.91	1.03	0.85	0.91	1.02
	Necrotic (%)	3.43	54.99	3.53	3.92	5.89	5.61

		Gastric constituent				
		Negative control	Positive control	Pepsin 1mg/ml	Pepsin pH6	pH6
A549	Viable (%)	95.01	84.64	91.49	90.74	95.32
	Apoptotic (%)	0.57	4.20	0.35	0.86	0.90
	Necrotic (%)	4.42	11.17	8.16	8.41	3.79
BEAS-2B	Viable (%)	95.91	60.40	92.44	95.97	95.98
	Apoptotic (%)	0.94	9.89	1.02	0.81	0.89
	Necrotic (%)	3.15	29.71	6.54	3.23	3.13

TABLES 19a and 19b Cell Viability and Apoptosis – Exposure for 1 hour/day for 3 consecutive days

		Gastric constituent					
		Negative control	Positive control	H_20 carrier	Pepsin pH6	pH6	pH4
A549	Viable (%)	92.81	70.25	94.69	94.27	92.03	88.67
	Apoptotic (%)	0.81	15.74	0.55	0.39	0.62	0.74
	Necrotic (%)	6.41	14.01	4.76	5.34	7.37	10.60
BEAS-2B	Viable (%)	91.52	34.21	94.90	92.59	94.87	93.90
	Apoptotic (%)	1.01	16.49	0.97	1.33	0.61	0.69
	Necrotic (%)	7.47	48.56	4.15	6.09	4.53	5.42

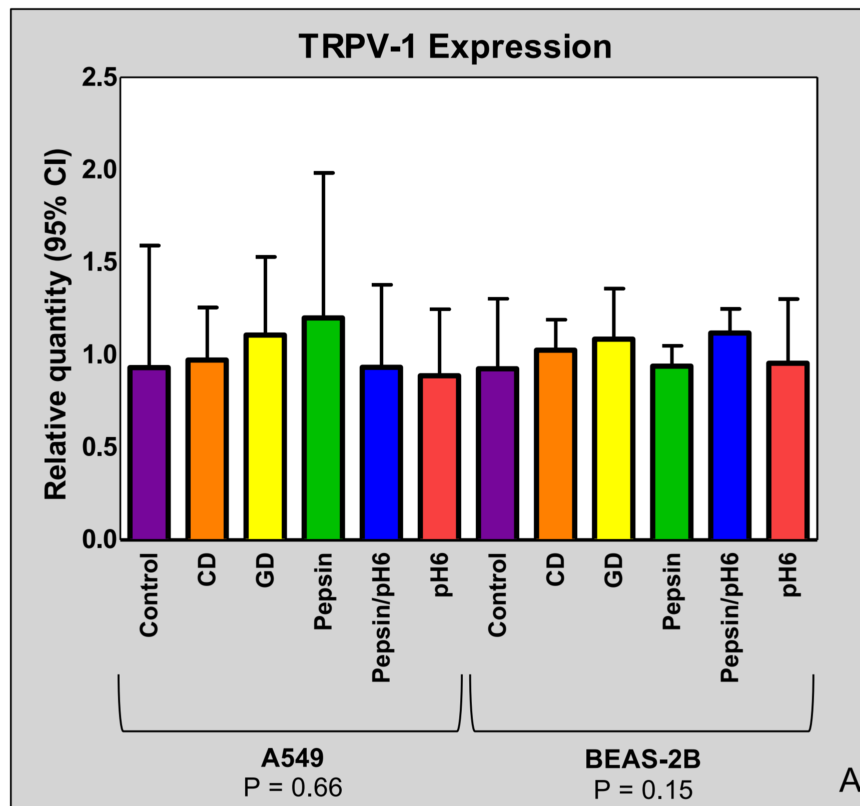
TABLE 20 Cell Viability and Apoptosis – Exposure for 5 minutes for 1 day

3.2.3 TRP gene expression in pulmonary epithelial cell lines

Gene expression is presented as relative quantity and represents a fold difference in gene expression (otherwise known as the ratio) compared to the calibrator sample, normalised against the chosen reference genes HPRT, TBP and GAPDH as detailed in chapter 2. The mean relative quantities of each TRP receptor from three independent experiments in both cell lines are shown in figures 43 and 44. The samples are calibrated to the average to allow visualisation of the biological variability (Figure 43a and 44a). The data is also presented with the control sample as the calibrator on a Log_{10} scale, such that the control sample is set at zero and a relative quantity lower or higher than the control appears as a negative or positive deflection respectively (Figure 43b and 44b). Statistical analysis was carried out on the data calibrated to the average to enable variance to be calculated. As previously discussed the choice of calibrator sample does not influence the relative quantity, as although the average quantity may be different the fold changes (ratio) between the groups remain the same (Hellemans et al. 2007).

TRPV-1 expression in A549 and BEAS-2B pulmonary epithelial cells following one-hour exposures to various gastric refluxate constituents on 3 consecutive days is shown in Figure 43a and 43b. Compared with the control sample TRPV-1 expression was increased in the A549 cells exposed to CD, GD and pepsin but reduced in those exposed to pH6 and pepsin at pH6, whereas TRPV-1 expression was increased in all exposed BEAS-2B cells. Figure 44a and 44b shows TRPA-1 expression in both cell lines. Again the expression pattern was different in each cell line, with TRPA-1 expression reduced compared with the control in the A549 cells but increased in all but the GD exposed in BEAS-2B cells.

The fold changes were small and there was no statistically significant difference in TRPV-1 or TRPA-1 expression in either cell line following the repeated exposure to various constituents of gastric refluxate.



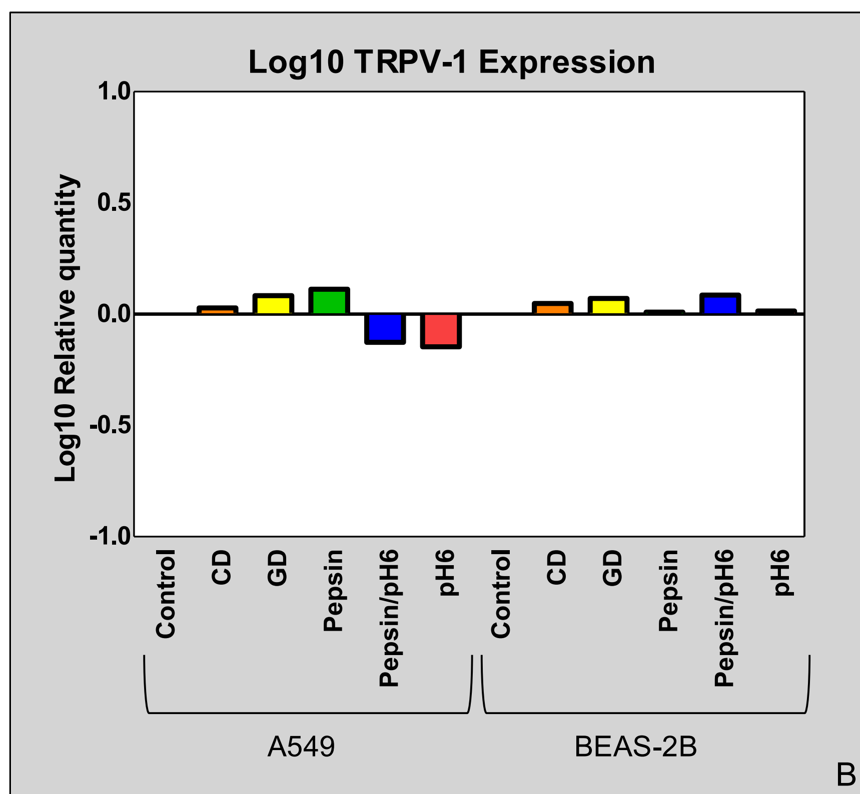
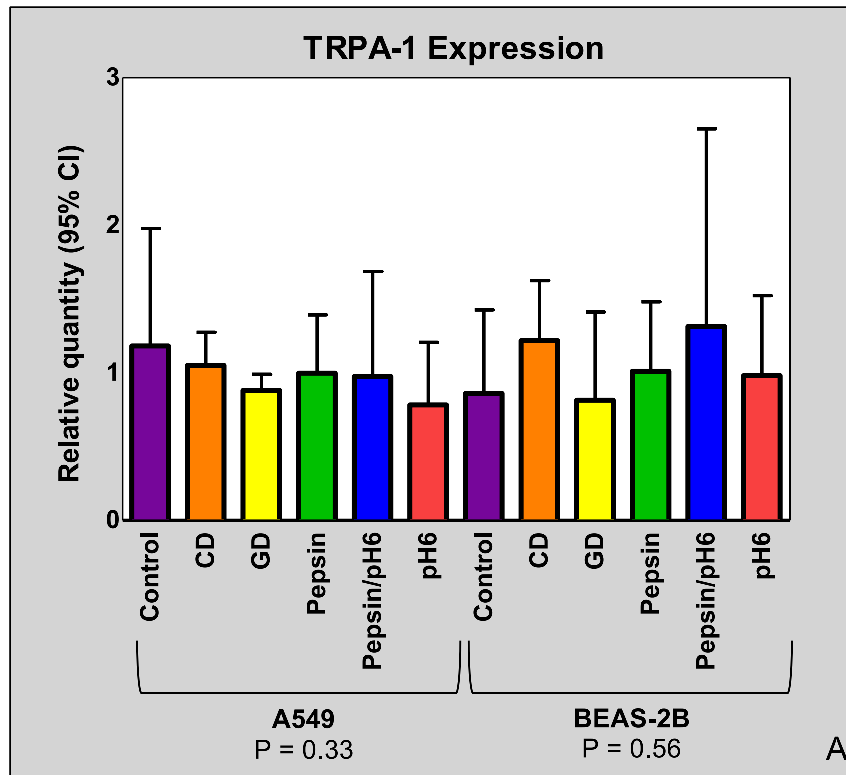


FIGURE 43a and 43b **TRPV-1 expression in A549 and BEAS-2B pulmonary epithelial cell lines following repetitive exposure to gastric reflux constituents**



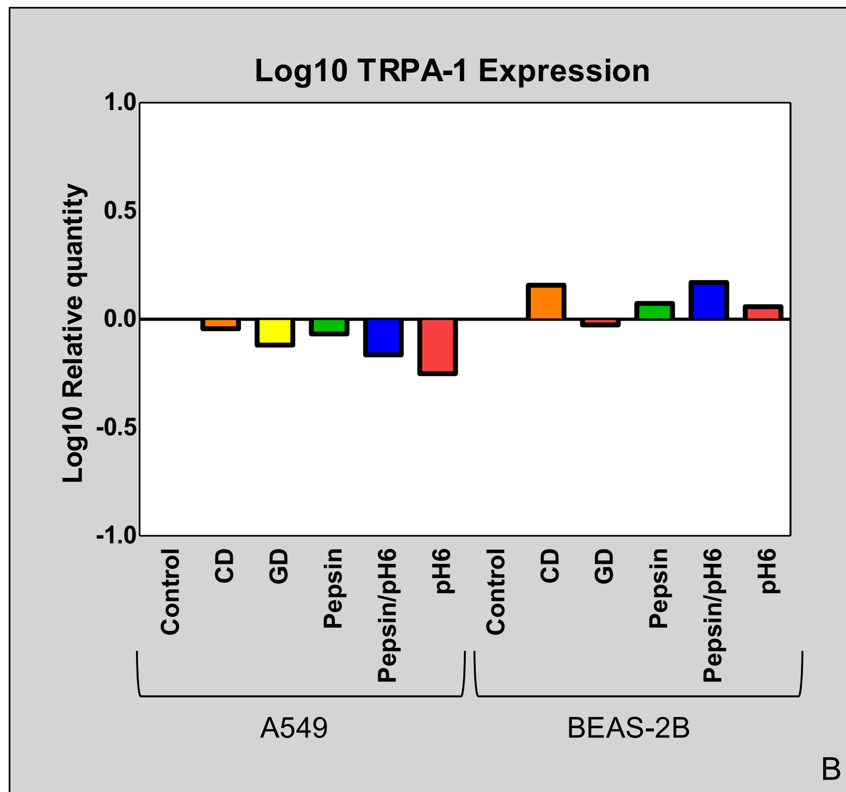
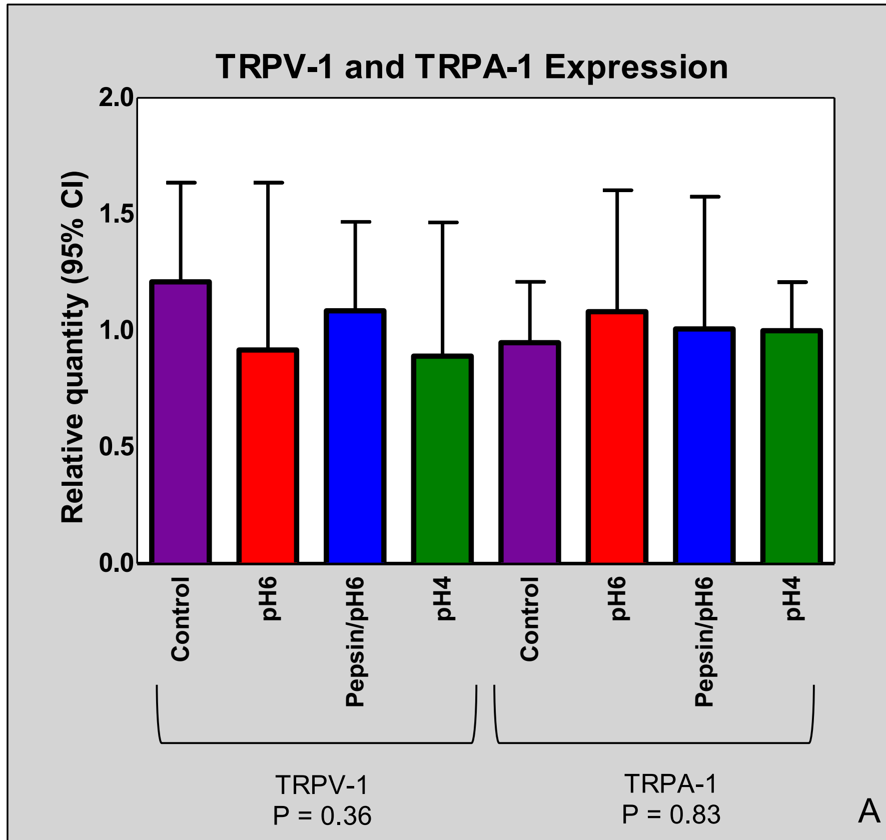


FIGURE 44a and 44b **TRPA-1 expression in A549 and BEAS-2B pulmonary epithelial cell lines following repetitive exposure to gastric reflux constituents.**

TRPV-1 and TRPA-1 expression in A549 cells following exposure to media at pH4, pH6 and pepsin at pH6 for 5 minutes on one occasion is shown in Figure 45a and 45b. As detailed earlier, there was excess cell death with repetitive acidic exposures and with exposure to a pH <6 when pepsin was present therefore receptor expression could not be assessed in these conditions. The BEAS-2B cell line failed to yield adequate RNA for downstream experiments following the 1 day exposure therefore could not be used. Compared to the control sample, TRPV-1 expression was reduced and TRPA-1 expression increased in the cells exposed, however again the fold changes were small and there was no statistically significant difference seen.



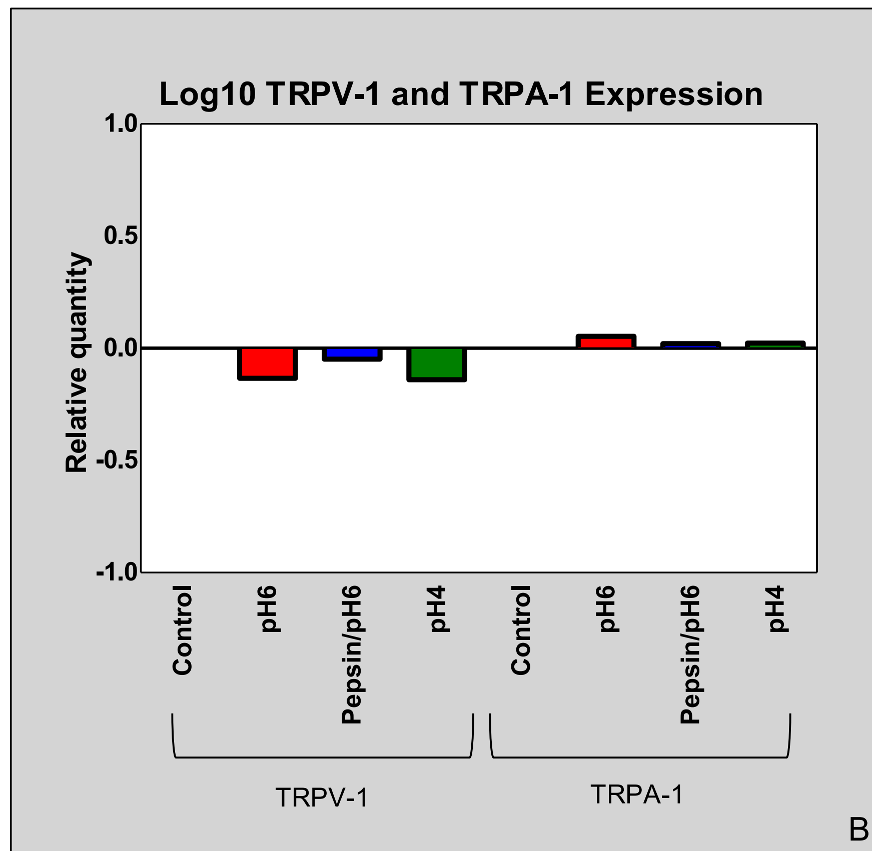


FIGURE 45a and 45b **TRPV-1 and TRPA-1 expression in A549 epithelial cells following a single exposure to gastric fluid constituents**

3.2.4 Il-6 and IL-8 ELISA Assay

The average IL-6 and IL-8 concentrations taken from the supernatants of exposed A549 and BEAS cell lines are presented in tables 21 and 22 respectively (N = 2 unless otherwise stated). The various gastric constituents failed to induce increased secretion of the interleukins in either cell type when compared to the control samples.

	A549		BEAS-2B	
	IL- 6 Concentration (pg/ml)			
	24 hours	48 hours	24 hours*	48 hours
Control	13.7	22.6	5.9	10.7
Ethanol carrier	16.6	38.1	11.3	11.1
H_20 carrier	22.0	44.2	10.4	10.1
CD	16.4	35.5	14.3	9.7
GD	21.8	30.8	11.6	10.7
Pepsin	22.5	33.9	20.8	21.8
Pepsin/pH6	13.7	19.3	41.7	27.4
pH6	9.5	21.6	30.2	28.7
P value	*0.601*	*0.275*	*0.429*	*0.143*

TABLE 21 IL-6 production in supernatant following gastric constituent exposure

* Single experiment only

	A549	
	IL- 8 Concentration (pg/ml)	
	24 hours	48 hours
Control	39.2	58.4
Ethanol carrier	53.4	86.2
H_20 carrier	49.7	80.4
CD	42.8	77.68
GD	47.6	89.1
Pepsin	54.9	88.9
Pepsin/pH6	40.9	50.2
pH6	51.2	47.2
P value	*0.346*	*0.601*

TABLE 22 IL-8 production in supernatant following gastric constituent exposure

3.2.4 Summary of results

1. TRPV-1 and TRPA-1 expression has been demonstrated in A549 and BEAS-2B pulmonary epithelial cell lines.

2. Repeated or prolonged exposure of A549 and BEAS-2B cell lines to an acidic environment results in reduction in cell viability, therefore these cell lines are unsuitable for experiments assessing these conditions.

3. There are small fold increases in TRPV-1 expression on exposure to bile salts and pepsin in both A549 and BEAS-2B pulmonary epithelial cell lines.

4. However, there is no statistically significant difference in TRPV-1 or TRPA-1 expression in A549 or BEAS-2B pulmonary epithelial cell lines when exposed to the major constituents of gastric refluxate used in this study.

5. There was no statistically significant change in IL-6 or IL-8 secretion in either pulmonary epithelial cell line following exposure to the gastric constituents used at concentrations that do not significantly reduce cell viability in this study.

CHAPTER 4: DISCUSSION

4.1 AIRWAYS INNERVATION IN IPF PATIENTS

This study has not demonstrated a significant increase in TRPV-1 and TRPA-1 receptor expression in the large central airways of patients with IPF. In addition there was no significant difference in nerve profile staining for the pan-neuronal marker PGP-9.5 in central airway biopsies of patients with IPF compared to normal controls.

4.1.1 TRP receptor expression

The rationale for investigating the TRP receptors in IPF is clear as there is evidence for the physiological up-regulation of this pathway; IPF patients have a greater cough reflex sensitivity to inhaled capsaicin (Doherty et al. 2000a; Hope-Gill et al. 2003), and elevated levels of the neurotrophins NGF and BDNF in induced sputum and BAL fluid (Hope-Gill et al. 2003; Harrison 2013), which are involved in neuronal differentiation and proliferation.

This is the first time these receptors have been studied in IPF. Two previous studies have investigated the expression of TRPV-1 in bronchial biopsies of patients with chronic cough, which is one reason why this group was used as a 'positive' control. These studies both quantified TRPV-1 expression at protein level; one used IHC to identify positive or negative staining (Mitchell et al. 2005) whilst the other used immunofluorescence (IF) and a digital imaging system to quantify the density of TRPV-1 and PGP-9.5 positive staining nerves as a percentage of the epithelial area (Groneberg et al. 2004a). The first study failed to demonstrate a difference in the neuronal expression of TRPV-1, however found a significant difference in the staining of smooth muscle myocytes between patients with chronic cough and normal controls (Mitchell et al. 2005). The second study by Groneberg et al (Groneberg et al. 2004a) showed an increase in TRPV-1 positive nerve profiles, with a 4.4-fold increase in the ratio of TRPV-1 to PGP-9.5 staining in chronic cough patients when compared to controls, however they did not find a difference between PGP-9.5 staining in the two groups and did not demonstrate smooth muscle TRPV-1 staining. The current study did not show a

significant difference in airway TRPV-1 expression in chronic cough patients compared to controls, therefore the studies report contrasting findings in patients with chronic cough.

There are a number of differences between the methodologies of the current study and previous studies however that should be taken into consideration:

Patient selection

The studies by Groneberg et al and Mitchell et al investigated patients with chronic cough of any aetiology and included patients with asthma, GOR, rhinosinusitis, bronchiectasis, interstitial lung disease and post-infective cough as well as chronic idiopathic cough (unexplained cough) (Groneberg et al. 2004a; Mitchell et al. 2005). Groneberg et al. comment that there was no significant difference in TRPV-1 or PGP-9.5 expression between the patients with idiopathic chronic cough (N = 14) and patients with chronic cough of known cause (N =15), however they do not state whether the difference between the chronic cough group and normal controls would remain significant if only the patients with idiopathic chronic cough were included in the analysis (Groneberg et al. 2004a). Mitchell et al. also fail to comment on this (Mitchell et al. 2005). This difference in the characteristics of the patients included in the studies is an important consideration when undertaking any comparison.

As the focus of our study is on cough in IPF and whether aberrant innervation within the central airways is responsible for the enhanced cough reflex despite the absence of disease centrally, care was taken to exclude subjects with symptoms of cough of other aetiologies in both chronic cough and IPF patient groups. In the current study a thorough history was taken, with specific questions asked regarding exclusion criteria and the rhinosinusitis task force questionnaire (appendix 6) was completed in all patients considered for inclusion in the current study as detailed in chapter 2.

Gastroesophageal reflux (GOR) is a common cause of chronic cough and is common in IPF even in the absence of specific reflux symptoms, with studies demonstrating that non-acid as well as acid reflux occurs (Savarino et al. 2013;

Kilduff et al. 2014). Like IPF and chronic cough patients; patients without cough, but who have acid reflux have an enhanced cough reflex sensitivity to inhaled capsaicin (Ferrari et al. 1995). The relationship between GOR and cough remains poorly understood as although GOR is common, chronic cough is not and the presence of symptoms, abnormalities on endoscopy, pH and or impedance testing do not prove causality or predict response to treatment with PPIs (Ferrari et al. 1995; Madanick 2013). Current guidelines suggest GOR cannot be excluded by a trial of treatment alone (Irwin 2006), and although performing 24-hour oesophageal pH- impedance monitoring to quantify the presence and nature of any GOR in all patients in this study would have been ideal, this was not feasible and the evidence suggests that the pragmatic approach we took, whereby all patients with chronic cough were given at least a 1-month trial of PPI therapy to treat acid reflux was sufficient.

It was originally planned that all IPF patients would also be treated in this way independent of symptomatology, however, it was noted that this was resulting in delays in performing bronchoscopy which could potentially impact on the patients clinical care, so this precondition was removed for IPF patients without symptoms of GOR. This decision was also taken in the light of new research from within our research team which demonstrated that cough frequency in IPF patients did not change despite PPI therapy resulting in a reduction in the number of acid reflux events (Kilduff et al. 2014). Although this could be considered a confounding factor we believe it has ensured the IPF cohort are representative of the IPF population seen in clinical practice.

The finding in this study that neural TRPV-1 staining was significantly increased in IPF patients who did not receive a trial of a PPI is interesting, despite the small sample size. Unfortunately the investigators of TRPV-1 in chronic cough did not report on corresponding data in their publications (Groneberg et al. 2004a; Mitchell et al. 2005). The clinical significance of this result is unclear in the light of the previous study demonstrating that treatment of cough in IPF patients with PPI did not reduce cough severity (Kilduff et al. 2014), but it does correspond with the lack of difference in cough severity in the untreated group compared with the treated group shown here. This finding supports the complex link between GOR, IPF and cough and warrants further investigation.

Co-existent bronchial hyper-reactivity was excluded following thorough clinical assessment (history, clinical examination and spirometric testing or full pulmonary function testing) and reversibility testing or methacholine challenge testing was performed where there was clinical uncertainty (5/16 IPF patients, and 4/11 CC patients). There was also no evidence of significant eosinophilia seen on BAL or biopsy samples therefore, this is unlikely to be a significant confounding factor in the current study. The exclusion of co-existing emphysema by assessment of the HRCT scans in the IPF group is also a key strength in this study. Similarly all patients were assessed for a previous respiratory tract infection by taking a detailed history, and were excluded if an infection had been diagnosed or treated within the last 3 months.

Control group selection

The two aforementioned studies differed in their selection of their ‘normal’ control group. Groneberg et al investigated healthy volunteers with no history of cough (Groneberg et al. 2004a), whereas Mitchell et al investigated patients who were not symptomatic of cough but who were undergoing bronchoscopy as a potential screening procedure for bronchogenic carcinoma (Mitchell et al. 2005).

The ‘normal’ control patients in the current study were identified as patients who did not complain of cough but were required to undergo bronchoscopy for evaluation of a non-airways centred disorder e.g. pulmonary nodule/ haemoptysis. This was advantageous from an ethical and research and development funding perspective, however had a number of disadvantages.

One disadvantage is that the control group in this study made recruitment challenging. As already discussed the ‘normal’ controls were also subject to the exclusion criteria outlined in table 1. These strict criteria, in particular the smoking status resulted in difficulties with recruitment into this group as patients under investigation for a pulmonary nodule or haemoptysis were commonly smokers or recent (< 12 months) ex-smokers. Mitchell et al. did not control for smoking status and reported a significant difference in smoking status between the chronic cough and control group (Mitchell et al. 2005). Cigarette smoking in the absence of chronic lung disease has been shown to reduce cough reflex sensitivity to

capsaicin and this gradually increases following smoking cessation (Dicpinigaitis et al. 2006; Kanezaki et al. 2012). The underlying mechanisms for the down-regulation of cough reflex sensitivity to capsaicin in current smokers are not fully understood therefore we felt that current smoking was a potential confounding factor, and ensured that this was excluded.

A second disadvantage of using a control group that are undergoing bronchoscopy for another medical reason is that they are not ‘normal’ controls, in that they are not healthy and therefore may introduce confounders by their very nature. Every effort was made to ensure any confounding factors were kept to a minimum by using the exclusion criteria, however exclusion of airways disease in particular was in some patients only carried out by taking a detailed history and performing a clinical examination as not all patients underwent spirometric or pulmonary function testing. These patients were often referred for bronchoscopy and for consideration of study participation via the rapid access lung cancer pathway by physicians not directly involved in the study. The presenting complaint and history given by the patient meant that in 50% of cases spirometry and pulmonary function testing was not deemed necessary. In retrospect ethical approval should have been obtained for tests that were not part of the routine clinical care in this patient group, to ensure a complete data set. Importantly there was no significant difference in TRPV-1 or TRPA-1 expression in the patients who did not have spirometry compared to those who did in the current study, therefore this is unlikely to have had a meaningful impact on the results.

Mitchell et al do not provide any evidence that they attempted to exclude airways disease in their control group, and as they had a significantly different smoking status and were being investigated for possible bronchogenic carcinoma it is likely that a proportion of the patients had chronic obstructive airways disease. This is important to consider as a recent study has demonstrated increased TRPV-1 mRNA expression in lung tissue samples from patients with COPD (Baxter et al. 2014). It is possible that the different method used to recruit the control group in Groneberg study resulted in a significant difference in the level of TRPV-1 expression, as their control group was truly ‘normal’ (Groneberg et al. 2004a; Mitchell et al. 2005).

A third disadvantage of not using healthy volunteers is that the control group could not be age and gender matched to the IPF or chronic cough groups. This would have been very difficult to achieve even if healthy volunteers had been used however, given the different demographics of the IPF and chronic cough groups. IPF is a condition that predominantly affects males, who have an average age of 74.3 years at the time of diagnosis (Bradley et al. 2008; Navaratnam et al. 2011), whereas chronic cough predominantly affects mid-life women (Morice et al. 2007). The patients in our study therefore had the expected demographic profiles. The effect of age and gender on the expression of airway TRPV-1 and TRPA-1 expression has not been investigated.

Mitchell et al and Groneberg et al also failed to ensure an equal balance for age and gender between the control group and chronic cough group when looking at airway TRPV-1 expression. In the second study the authors acknowledged this but felt that this did not influence the results they found (Groneberg et al. 2004a); there was no significant difference in TRPV-1 or PGP-9.5 expression between men and women within the normal control or chronic cough group and no significant difference in correlation between age and TRPV-1 expression in normal volunteers. Importantly they did not look at the difference in expression of TRPV-1 between the groups taking age into account and made no comment on the correlation between age and TRPV-1 expression in the chronic cough group.

In the current study, it was surprising to find that age was a significant predictor of TRPV-1 gene expression in the IPF group; there was a statistically significant increase in relative expression of airway TRPV-1 in those below the age of 65 years of age, with a 13.5% increase in the relative quantity of TRPV-1 in patients from this group. The relevance of this finding is uncertain for a number of reasons. Firstly, the study was not designed to investigate the association between TRPV-1 expression and age a priori. Secondly, the study groups differed in ages therefore the assumption of independence of the co-variate was violated, however following analysis and discussion, an independent statistician advised that it would be better practice to include age in the model and violate this assumption than to exclude it for the analysis and not account for the confounding effect. Thirdly the number of patients with IPF in the less than 65 age group was small (4) so the clinical relevance of this increase is uncertain. Finally, age was not a significant predictor of TRPV-1 protein expression, which implies the difference seen at a genetic level may not transfer to what is observed at a

functional level. However, this remains an interesting observation and in any future studies investigating TRP receptors age should be taken into consideration.

Sample size

The sample size is the main limiting factor in this study. This study is smaller than the two studies investigating TRPV-1 in chronic cough as Groneberg et al recruited 45 patients in total; 29 chronic cough (14 idiopathic) and 16 controls, and Mitchell et al recruited 40 in total; 19 chronic cough (5 idiopathic) and 21 controls.

The original calculation for sample size was based on a comparison between 2 or 3 groups, each containing 10 patients, being powered to detect a difference of 1.1 to 1.25 standard deviations, which was felt to be plausible based on previous studies (O' Connell et al. 1995; Hope-Gill et al. 2003; Groneberg et al. 2004a). We aimed to recruit 20 patients in the IPF group and 10 in the control and chronic cough groups. 44 patients were recruited in total, however 5 of these patients were all-comers whose samples were used to pilot the experimental work and 4 patients were moved to the pilot group following enrolment as the result of investigations that were not available at the time of recruitment.

35 patients were therefore recruited (16 IPF, 11 CC and 8 controls), however total numbers were affected by a failure to collect samples in formalin for 3 patients and insufficient material for adequate H & E and IHC assessment in 5 samples. In hindsight, an estimated biopsy failure rate should have been included in the sample size calculation.

Recruitment of NC patients in particular proved to be most difficult and the reliance on incident rather than prevalent cases of IPF and chronic cough was also a limiting factor. This was necessary as it was only incident cases who were undergoing bronchoscopy as part of their routine clinical assessment. It is unlikely that increasing the control group size to 10 would have influenced the study outcome, however a total larger sample size may have allowed the smaller differences seen between the groups of interest to have reached statistical

significance. Despite the fact that this study included only a small number of patients, due to the strict exclusion criteria, the patients in the IPF and chronic cough groups all have well characterised disease which is a strength of this study which many other studies fail to achieve.

Experimental procedure

a. Bronchoscopy

Endobronchial biopsy is considered by some to be the standard method of investigating airway inflammation (Jeffery et al. 2003), and has been used by a number of investigators to describe the innervation of the airways (O' Connell et al. 1995; Groneberg et al. 2004a; Mitchell et al. 2005; McGarvey et al. 2014). It allows the investigation of the structural and functional components of the airway, however does have restrictions. The key limitation is the size of the biopsy attainable, typically 1-2 mm, which can introduce difficulties with downstream applications and results in sampling bias as the sample will not be representative of the whole airway (Jeffery et al. 2003). In this study several steps were taken in an attempt to reduce the impact of these potential limitations; bronchial biopsies were taken with the largest available biopsy forceps, the segmental carinae of the right lung was used in each patient as the carinae are a convenient place to biopsy and this method of sampling also allowed for direct comparison between patients within this study and with other studies that used similar methods (O' Connell et al. 1995; Groneberg et al. 2004a).

b. IHC

This is the first study to investigate the airway expression of both TRPV-1 and TRPA-1 in patients with IPF and chronic cough compared with controls and is also the first study to demonstrate mRNA expression of TRPV-1 and TRPA-1 from airway bronchial biopsies as well as protein expression.

Previous studies have utilised Immunohistochemistry (Mitchell et al. 2005; Buech et al. 2013; McGarvey et al. 2014) or immunofluorescence (IF) (Groneberg et al. 2004b) to determine TRPV-1 and TRPA-1 protein expression in bronchial tissue.

Both methods involve the binding of an antibody to a cellular or tissue antigen of interest. The difference being that IF uses fluorescent-labelled antibodies which can be detected by using a fluorescent microscope. The availability of equipment and expertise were the main reasons IHC was used over IF in this study. However, IHC also has the benefits of allowing tissue morphology to be assessed at the same time as antigen localisation and provides a permanent record of staining rather than the temporary fluorescence achieved with IF.

In this study the IHC was initially analysed as it traditionally has been, manually, with a decision made regarding positive or negative staining and antigen localisation (Taylor and Levenson 2006; Walker 2006; Rizzardi et al. 2012). There are a number of limitations associated with the manual analysis of IHC samples, in particular; increased cost, inter and intra-observer variability, subjectivity and observer fatigue (Taylor and Levenson 2006; Walker 2006). Also the output data is binary or ordinal rather than continuous which has implications to the statistical analysis and sample size (Noordzij et al. 2010). Every effort was made to reduce the limitations associated with the manual interpretation of IHC; the criteria for assessment were set beforehand using the pilot samples, two observers reviewed the slides with any discrepancies in the results resolved by consensus following reassessment of the slide by both investigators and slides were assessed in batches of ten to limit observer fatigue.

Computerised image analysis was used in addition to the manual assessment to limit these factors and allowed the percentage of TRPV-1, TRPA-1 and PGP-9.5 positive staining tissue/ cells to be expressed as a percentage of the total section area. Thus quantitative, less subjective results were achieved, however there are also limitations to this methodology. In particular the effect of regional heterogeneity can still be missed (Jensen 2013), and the heterogeneity of samples following micro-sectioning is likely to be the cause of a number of samples having TRPV-1 and or TRPA-1 neuronal positive staining but no staining for the pan-neuronal marker PGP-9.5. Also an intrinsic limitation of an image-detection program is the inability to distinguish between similar colours which can result in difficulty interpreting results as seen with the TRPA-1 immunostaining in this study (Taylor and Levenson 2006). This can also result in an under or over-estimation of staining depending on how the training thresholds were set. The

analysis of each section in the same way will have reduced the risk of introducing this potential bias and the micro-sectioning and staining of the sections in batches by the same individual will have also increased the accuracy of the quantitative image analysis in the current study.

In order to be able to compare our results with the previous studies in this field attempts were made to locate and acquire the antibodies used in the aforementioned studies, however the TRPV-1 antibody used by Groneberg et al (GlaxoSmithKline, Harlow, UK) had been discontinued and was no longer available, therefore the antibody used by Mitchell et al was used (AB5370P, Chemicon, UK). Compared with control patients we found a slight increase in TRPV-1 staining in the neuronal tissue of IPF patients, but a reduction in patients with chronic cough. Like Mitchell et al we failed to demonstrate a statistically significant difference in TRPV-1 neuronal staining, however in contrast, like Groneberg et al we did not identify any smooth muscle myocyte TRPV-1 staining, and found no difference in PGP-9.5 staining between the chronic cough and control group. Our findings for airway expression of TRPV-1 in chronic cough patients therefore differed from the previous studies.

The differences in the distribution of antibody staining (smooth muscle myocytes) between the three studies brings into question the reproducibility of the findings in the study by Mitchell et al. The antibody concentrations for our study were optimised by a very experienced biomedical scientist, and no smooth muscle staining was seen when the background levels of staining were at an appropriately low level. Failure of our technique is unlikely as TRPV-1 immunoreactivity was detected in the positive control and within neuronal tissue. There have been no other reports of TRPV-1 staining in bronchial smooth muscle.

A separate study of bronchial TRPV-1 expression in patients with asthma demonstrated expression in the bronchial epithelium of patients with asthma and controls (McGarvey et al. 2014) , which is in contrast to this and other studies which have shown only occasional (<1%) or no epithelial cell staining (Groneberg et al. 2004a; Mitchell et al. 2005) . However, non-neuronal TRPV-1 expression has been confirmed at mRNA and protein level in a number of pulmonary epithelial cell lines in vitro (Agopyan et al. 2003; Reilly et al. 2003; Thomas et al.

2007) .The differences may be as a result of the antibodies used, which are known to be difficult to identify and optimise (personal communication Dr L, Sadofsky, co-author with Mitchell et al (Mitchell et al. 2005)), the method of tissue processing and the clinical phenotype of the patient under investigation, however again this brings into question the reliability and reproducibility of studies investigating protein expression of TRP receptors. Of note the IHC in the studies by Mitchell et al and McGarvey et al. were manually assessed, whereas computer-aided image analysis was used by Groneberg et al.

In contrast to TRPV-1, this study confirmed the non-neuronal expression of TRPA-1 in bronchial epithelial cells and smooth muscle myocyte observed in other studies (Faruqi et al. 2011a; Buech et al. 2013). Compared to the NC group, there was increased TRPA-1 expression in nerve and smooth muscle myocytes in the IPF and CC groups, however there was no statistically significant difference with manual or computer-aided assessment of immune-staining. The positive staining in the bronchial epithelium for TRPA-1 in all samples was a particularly surprising finding. The role of TRPA-1 in non-neuronal tissue is unclear, however there is evidence to suggest that non-neuronal TRPA-1 could be involved in triggering airway inflammation (Fernandes et al. 2012; Grace et al. 2014). The evidence for this is largely taken from in vitro work, in which non-neuronal cell lines have been exposed to smoke particulates and have resulted in cell activation or interleukin release in a TRPA-1 dependent manner (Mukhopadhyay et al. 2011; Nassini et al. 2012; Buech et al. 2013; Shapiro et al. 2013). The non-neuronal expression of TRPA-1 was independent of the degree of chronic inflammation, however there was an association between the degree of chronic inflammation present and the neuronal expression of TRPA-1 in the airway. There were only 4 samples in total without any chronic inflammation, all of which had no TRPA-1 nerve profile staining, and surprisingly more patients with mild inflammation had positive staining but there was less positive staining in patients with moderate inflammation. Given the evidence for the role of TRPA-1 in a number of inflammatory conditions it is very likely that it plays a role but what remains unclear is whether TRPA-1 has a role in initiating a pro-inflammatory process as seen in in-vitro and rodent studies or whether the inflammatory environment sensitizes and or up-regulates receptor expression (Bessac and Jordt 2008; Geppetti et al. 2010). The degree of inflammation and TRPA-1 airway

receptor expression has not been investigated. The clinical relevance of TRPA-1 and mild inflammation in this study is unclear as the result approaching statistical significance was only observed when all samples were combined, with no statistical difference between the groups.

c. QPCR

Quantitative PCR was used in addition to IHC in this study in an attempt to improve the sensitivity and specificity of the experiment given the limitation of subjectivity and difficulties with identifying and optimising the TRP receptor antibodies with IHC. The benefits of qPCR are that the technique can detect and allow quantification of very small amounts of nucleic acids in a variety of tissues, quickly with good accuracy, if the assay is appropriately optimised and the results correctly analysed (Bustin et al. 2009). The two methods therefore complement each other well, as information can be obtained on quantitative gene expression, protein expression and protein localisation.

Every effort was made to follow the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines and the extensive steps taken to optimise the qPCR assays and ensure appropriate analysis as described in section 2.1 is a strength of this study (Bustin et al. 2009).

One weakness, highlighted by these guidelines was in the assessment of the RNA following extraction. Obtaining RNA which is of optimal quality and integrity is known to be particularly difficult with bronchial tissue (Yahaya et al. 2011). The quality of RNA as assessed using spectrophotometric absorbance (260/280 ratio) was sub-optimal in 4 patients, despite attempts to optimise the process of RNA extraction using sacrificed mouse tissue and pilot patient samples. RNA of poor quality or that is partially degraded may reduce the assays sensitivity to detect low-level transcripts (Bustin et al. 2009). 260/280 ratio is frequently used to assess RNA quality however the MIQE guidelines recommend the use of RNA gel electrophoresis and the quantification of RNA integrity using a RNA integrity number (RIN); although there is not a universally agreed, applicable or cost-effective way of assessing RNA quality and integrity (Bustin et al. 2009).

Bronchial biopsies have been previously described to have low RINs, which is typically a result of small biopsy size and is thought to be a consequence of the composition of bronchial tissue (Yahaya et al. 2011). Yahaya and colleagues found that increasing the amount of starting tissue resulted in higher RINs, (Yahaya et al. 2011) which was not possible with this study as recruitment was complete. As previous studies suggested that a high RIN was not a necessity for qPCR (Biomedical Genomics 2007), and performing RINs on all patient samples was not practical (due to low RNA yield) or financially viable, the samples were used with caution. Using a reference gene normalisation strategy increased the reliability of the qPCR assay as it controls for variations in the amplification efficiency (which can be affected by differential RNA quality), and extraction yield (Bustin et al. 2009). No untoward effect was apparent on downstream applications.

It was also noted that the TRPA-1 crossing times (Ct) of the patients were near to the limit of detection of the assay, and three IPF patients were removed from the analysis by the software as the Ct was greater that the pre-set value of 38. This suggests that TRPA-1 is expressed at low levels in these tissues, and although the efficiency, technical replicates and reproducibility of the assay was acceptable it is likely that the sensitivity of the assay was lower which is likely to have resulted in the larger confidence intervals.

As previously mentioned no other studies have investigated the gene expression of TRPV-1 and TRPA-1 in the airways of patients with IPF. Although this study found a higher mean airway TRPV-1 and TRPA-1 gene expression in IPF patients compared with ‘normal’ controls, this failed to reach statistical significance and therefore could have occurred by chance. The IHC staining supported this finding as the difference in neuronal expression between the control and study groups seen in both TRPV-1 and TRPA-1 in this study corresponds with the differences seen in mRNA expression; this concordance provides validation.

Interestingly gene expression does not always correlate with protein expression, which is likely to be the result of complex gene regulatory mechanisms (Gry et al. 2009). The study which investigated airway TRPV-1 expression in patients with asthma did not show a correlation between gene expression and protein

expression, however the authors compared the protein expression from bronchial tissue biopsies with the gene expression (using qPCR) from bronchial brush samples (McGarvey et al. 2014). The bronchial brush samples would have only sampled bronchial epithelial cells therefore mRNA expression would have been derived from these cells alone, which may explain the difference. Also, only one control gene was used in the qPCR experiments, which reduces the reliability of the study (Bustin et al. 2009). Studies of TRPV-1 and TRPA-1 in other tissue types have shown a good correlation between gene and protein expression (Diogenes et al. 2007; Guarino et al. 2010), therefore the consistency seen overall in this study is encouraging.

d. Biopsy Inflammatory status

Like Mitchell et al, it was thought that given the potential role of TRP receptors in the inflammatory cascade, it would be interesting to investigate whether the expression of TRP receptors in the airway correlated with the presence or absence of chronic inflammation in bronchial epithelium. (Mitchell et al. 2005). There was no significant difference in the airway morphology or significant correlation between level of TRP expression and degree of inflammation between the groups. The presence and extent of chronic inflammation and morphological abnormality in the 'normal' controls however was an unexpected finding.

Only 24% of the bronchial biopsies from the control patients in the study by Mitchell et al had evidence of chronic inflammation compared to 83% of the control patients in the current study (Mitchell et al. 2005). Two of the three NC patients who had moderate or severe chronic inflammation and evidence of mucous cell hyperplasia were ex-smokers. Studies that have investigated bronchial biopsy inflammation in smokers and ex-smokers with and without COPD have found evidence of chronic inflammation (Willemse et al. 2004; Lapperre et al. 2006), therefore this is likely to be a contributing factor. Also one NC with evidence of basement membrane thickening and focal metaplasia had neutrophils seen on a bronchial washing sent as part of his clinical investigation although there was no bacterial growth and no evidence of acute neutrophilic inflammation on H and E evaluation. As previously mentioned all patients were questioned and excluded if they had recently had a respiratory tract infection,

however, we cannot exclude the possibility of a subclinical infection at the time of the biopsy or in the recent preceding period, which could have also accounted for some of the histopathological changes observed. Another concept to consider is that endobronchial biopsies are more difficult to obtain from normal tissues and as a consequence are more likely to suffer tissue damage; In repeated procedures the procedure can result in scar and a cellular inflammatory response may be initiated prior to the sample being fixated (Jeffery et al. 2003). Although it would be expected that local trauma would result in evidence of acute inflammation rather than chronic inflammation.

Other studies comparing chronic cough patients and controls have shown mild chronic inflammation in all samples but deemed this to be within normal limits (O' Connell et al. 1995). Inflammation is typically graded as mild, moderate and severe and as with all of the morphological features assessment is not quantitative and is limited by subjectivity. To quantify the degree of inflammation we could have used IHC to localise and count the number of lymphocytes, however the priority in the study was to investigate airway innervation with IHC therefore the samples were prioritised. The classification of normal and mild inflammation is therefore likely to vary between different observers and between studies, however the finding of moderate and severe chronic inflammation in our NC patients is likely to be a valid result. A study investigating the variability of inflammatory cell counts on bronchial biopsies in normal subjects (non-atopic, non- smoking individuals), however found large variability in the number of inflammatory cells and concluded that the presence of inflammatory cells could be in part normal variation and related to environmental exposures. They also confirmed variability in basement membrane thickness as they identified that it could be up to 8.78 μm in normal controls (Turcotte et al. 2003). The small size of the NC group and this potential high variability are likely to have resulted in lack of difference in histological morphology between the groups and again highlights a limitation of the normal control group used in this study.

Evidence of chronic inflammation on bronchial biopsy has been identified in patients with asthma (Niimi and Chung 2004; Labonte et al. 2008), COPD (Sutherland and Martin 2003) and chronic cough of various aetiologies (Lee et al. 2001; Irwin et al. 2006b). The chronic infiltrate seen in the endobronchial biopsies

in this study was lymphocytic, which was expected given the chronicity of the cough in the CC and IPF groups and for chronic cough is in keeping with previous studies (Irwin et al. 2006b). As endobronchial biopsies are not commonly taken for IPF as the disease process affects the peripheral lung parenchyma rather than the central airways, the histopathology of endobronchial biopsies in IPF is not clearly defined. Twelve (86%) IPF patients had evidence of chronic inflammation in the central airways and although 5 patients were ex-smokers, they had all stopped smoking for longer than 12 months, had no clinical evidence of chronic bronchitis and none of the IPF patients had a productive cough, making a confounding diagnosis unlikely. Although chronic inflammation was increased in all groups, the current study still adds to the evidence for proximal airway inflammation in IPF (Hope-Gill et al. 2003). What remains unclear is whether the inflammatory changes seen in the central airways of patients with chronic cough of any aetiology are a cause or a result of the cough, one author has suggested that the inflammatory results are a consequence of the trauma of coughing and should be interpreted with caution (Irwin et al. 2006b).

4.1.2 Measures of Cough

All patients with CC and IPF in this study described cough as their main symptom. Two subjective measures of cough were used, the LCQ which measures cough related quality of life (QOL) and a VAS which is a general measure of cough severity. At the time of study conception there were no validated subjective measures of cough symptom severity or quality of life in IPF. VAS and LCQ were chosen as they have previously been used in IPF (Key et al. 2010) and have been validated in the chronic cough population (Birring et al. 2003; Birring et al. 2008; Faruqi et al. 2011b). In keeping with previous studies in the chronic cough population we observed strong correlations between the different measures of cough severity, which suggests these subjective measures of cough are good surrogates for cough frequency monitoring (Decalmer et al. 2007; Faruqi et al. 2011b).

The IPF patients had comparable, if not slightly worse cough severity as measured using VAS and LCQ compared to IPF patients in other studies (Key et al. 2010; Scholand et al. 2014). In this cohort, patients with chronic cough had

greater cough symptom severity than IPF, with a statistically significant increase in the VAS score and lower LCQ scores in all domains. The median VAS scores in particular were higher than those observed in other studies investigating chronic cough patients (Decalmer et al. 2007; Faruqi et al. 2011b). This is in contrast to a previous study showing no statistical difference in the objective assessment of cough frequency using an ambulatory 24 hour cough monitor between these two groups of patients (Key et al. 2010). This may be as a consequence of an increased ‘urge to cough’ in this group, where there is a heightened consciously perceived compulsion to cough, however this may not translate into a physical cough (Mazzone et al. 2007; Davenport 2008).

The female preponderance in the CC group in this study may also play a role as median VAS in men was 66, compared to 77 in women. CC is known to predominantly affect women (Morice et al. 2007) and studies investigating chronic cough frequently have a higher proportion of women than men, however the proportion of women (78%) in this study was higher than that seen in larger studies (Decalmer et al. 2007; Kelsall et al. 2009; Faruqi et al. 2011b). This is likely to be the result of the strict exclusion criteria in this study, to ensure only idiopathic chronic cough was studied. Women with chronic cough have been found to have increased cough on 24 hour ambulatory monitoring (Kelsall et al. 2009) and a higher cough reflex sensitivity in response to inhaled capsaicin (Fujimura et al. 1996). There are conflicting results with regards to the effect of gender on cough related quality of life (French et al. 2004; Polley et al. 2008; Kelsall et al. 2009) however, and no study has specifically looked at cough VAS and gender in adults, but gender did not significantly affect the VAS cough score in children with chronic cough (Chang et al. 2011). Also, although a study investigating the capsaicin evoked urge to cough in normal patients failed to find an increased urge rating in females (Mazzone et al. 2007), another study investigating chronic cough in which the majority (71%) of patients were women identified a severe ‘urge to cough’ in response to a variety of precipitants (Hilton et al. 2015). The urge to cough has not yet been quantified in IPF patients, therefore its role in this condition is unclear.

The Cough Quality of Life questionnaire has now been validated for the use in patients with IPF and should be used in any future studies investigating cough in IPF (Lechtzin et al. 2013).

4.1.3 Measures of Cough and TRP receptor expression

There was no significant correlation between airway TRPA-1 gene or protein expression and cough severity measured with LCQ or VAS in patients with IPF or CC. In contrast there was a significant negative correlation between airway TRPV-1 gene expression and both measures of cough severity when the groups were combined. On separation of the groups the correlation between TRPV-1 gene expression and LCQ failed to reach significance in IPF and CC. However, a significant negative correlation was observed between TRPV-1 expression and VAS score in the IPF group; as cough severity increased, relative TRPV-1 gene expression was lower. This correlation was not evident in the protein expression data.

No previous studies have attempted to correlate the expression of airway TRP receptors at gene or protein level with the cough VAS or the LCQ. The negative correlation between TRPV-1 gene expression and VAS score was an unexpected finding, however in retrospect it is not really surprising that the genetic expression of airway receptors do not reflect the severity of cough based on subjective measures. One might expect a correlation to be seen at a protein level however as a previous study has identified a correlation between TRPV-1 protein expression and cough reflex sensitivity. The study by Groneberg et al demonstrated a significant correlation between the percentage of TRPV-1 positive staining nerve profiles and the capsaicin tussive response in patients with chronic cough; the concentration of capsaicin required to induce five coughs or more (PC_5 response) was lower in patients with increased TRPV-1 expression (Groneberg et al. 2004a).

A limitation of the current study is that objective measures of cough severity and a measure of cough reflex sensitivity was not used. Ethical approval was obtained to perform ambulatory 24 hour cough monitoring on all patients with CC and IPF, and a capsaicin cough challenge on all patients, however patients were not

prepared to consent to this number of tests as it required multiple hospital visits. This was in particular unacceptable to the patients with IPF, who are frequently elderly and frail. In this context it is not unsurprising that despite recommendations for chronic cough to be investigated with a combination of subjective and objective measures (Chung et al. 2003; Decalmer et al. 2007) only two studies investigating cough and IPF have reported ambulatory cough monitoring (Key et al. 2010; Kilduff et al. 2014). It is also reported that in patients with chronic cough, objective cough counts were significantly lower on repeated 24-hour cough monitoring and as a result a 'run-in period' was recommended (Faruqi et al. 2011b). This observation therefore limits the use of a single 24-hour ambulatory recording. The capsaicin challenge test however, has good short term reproducibility and can be performed on a single visit (Dicpinigaitis 2003). Rather than a measure of the severity or frequency of cough the capsaicin cough challenge is a provocation test which measures the sensitivity of the cough reflex and enables the pathophysiology of cough to be investigated (Chung et al. 2003). Although this information would have added to the strength of this study, as the heightened cough reflex sensitivity is well established in IPF patients (Doherty et al. 2000a; Hope-Gill et al. 2003) and patients with chronic cough (Choudry and Fuller 1992; Groneberg et al. 2004a) it would not have added significantly to our current understanding of the pathophysiology of cough in IPF.

Interestingly despite subjective and objective measures of cough severity having a strong correlation, cough reflex sensitivity and subjective measures of cough in particular the VAS do not correlate (Doherty et al. 2000a; Decalmer et al. 2007; Faruqi et al. 2011b). This adds to the argument that it is not unsurprising to see a poor correlation between TRP receptor gene expression and cough VAS and LCQ. Also a recent double-blind randomised controlled trial of a TRPV-1 receptor antagonist was investigated in patients with chronic cough. Surprisingly despite a reduction in capsaicin cough reflex sensitivity at 2 and 24 hours following drug therapy, there was no improvement in subjective measures of cough severity or 24 hour ambulatory cough monitoring (Khalid et al. 2014). Thus the lack of correlation between cough reflex sensitivity and subjective measures of cough may be in part the result of the patient's perception of symptom severity, however the mechanism is likely to be more complex. The lack of correlation and recent drug trial work questions the clinical relevance of these receptors from a cough

severity perspective. However it is possible that the antagonist used had sub-optimal pharmacokinetics to fully occupy the target (Bonvini et al. 2015), and perhaps more likely that a number of receptors and mechanisms are involved in the clinical symptom of cough, and that targeting a single receptor is likely to be ineffective. It is also evident that objective measures of cough are required as outcome measures in any pharmacological study investigating cough.

4.1.4 TRP receptor expression and cough in IPF

The IPF patients did not have a significant increase in TRPV-1 and TRPA-1 receptor expression at gene or protein level when compared to controls, however the small sample size is a limitation.

There is however good evidence for the role of TRP receptors in the cough reflex and in particular that they play a role in the pathogenesis of cough in IPF (Doherty et al. 2000a; Hope-Gill et al. 2003). The current findings of TRPV-1 and TRPA-1 gene and protein expression in neuronal and non-neuronal cells of airway bronchial biopsies provides further evidence for the role of these receptors in afferent respiratory reflexes.

Despite all patients with IPF having a significant cough, the expected correlation between cough severity and TRP receptor expression was not observed. In spite of the limitations of this study as already mentioned, our findings do not suggest that there is gross up-regulation of TRPV-1 or TRPA-1 in the central airways of patients with IPF, however it is not possible to comment on subtle changes and their importance clinically with certainty. It is also not possible to draw firm conclusions between the relationships between TRP receptor expression and cough severity measured using VAS and LCQ.

It is important to consider that this study only investigated the expression of TRPV-1 and TRPA-1 receptors. These receptors are two members of the TRP receptor family which has 28 members divided into 6 subfamilies (Clapham 2003). There is evidence that other TRP receptors particularly TRPV-4 and transient receptor potential melastatin-8 (TRPM-8) could also play a role (Grace et al. 2014; Bonvini et al. 2015). The interactions between these receptors and

others such as the ASICs are poorly understood and as previously mentioned, it is likely that a number of receptors and interactions are involved in the cough reflex.

The level at which the airways were sampled is also an important consideration as although the central airways are known to have increased innervation and were biopsied for practical reasons, it is possible that there is increased TRP receptor expression at the site of disease activity in the peripheral parenchyma in patients of IPF. Evidence of increased expression of neurotrophins (Hope-Gill et al. 2003; Harrison 2013) peripherally provide support for this hypothesis.

It is also possible that a physiological up-regulation of TRP receptors through sensitisation results in an increased cough reflex sensitivity rather than a significant structural up-regulation. Although in vitro studies have suggested that the relative amount of TRPV-1 expression affects the threshold for the initiation of inflammatory processes (Reilly et al. 2003), inflammatory mediators are known to cause TRP receptor sensitisation therefore small differences in TRPV-1 expression may result in large differences in TRPV-1 sensitivity (Chuang et al. 2001; Gu et al. 2003; Mazzone et al. 2005).

Also in support of sensitisation is the mechanism of desensitisation. Desensitisation is when there is loss of activity at the level of the receptors, which can happen acutely as a result of a conformational change in the channel and closing of its pore following agonist binding, or gradually as a result of repeated administration of an appropriate agonist (tachyphylaxis) (Szallasi and Blumberg 1999; Jara-Oseguera et al. 2008). Desensitisation of the TRPV-1 and TRPA-1 receptors using capsaicin and other agonists has been demonstrated in vitro (Akopian et al. 2007) and has been used widely in chronic pain, particularly neuropathic pain (Szallasi and Blumberg 1999; Sawynok 2005). A recent study has shown the use of desensitisation using oral capsaicin in chronic cough patients, resulted in a statistically significant reduction in cough reflex sensitivity and cough symptom scores although an objective assessment was not made (Ternesten-Hasseus et al. 2015).

The evidence for inflammation in the central airways, heightened cough reflex sensitivity and the presence of neurotrophins in the sputum of IPF patients also support the sensitisation of the TRP receptors in this group. It is possible that the non-neuronal and neuronal functions of TRP receptors play different but synergistic roles in this process. This studys demonstration of the presence of these receptors in the IPF population will now facilitate further investigation into this complex, debilitating symptom within this group of patients as well as those with idiopathic chronic cough.

4.2 TRP RECEPTORS IN RESPIRATORY CELL LINES EXPOSED TO GASTRIC FLUID CONSTITUENTS

This study has not shown a statistically significant increase in TRPV-1 or TRPA-1 mRNA expression following the exposure of two pulmonary epithelial cell lines to acid (pH6), pepsin, CD or GD. However, small fold increases in TRPV-1 in both cell lines were demonstrated. This is the first study to investigate the effect of the individual components of gastric refluxate on cellular viability and expression of TRP receptors in A549 and BEAS-2B cell lines. There are a number of experimental aspects to discuss when considering these results.

4.2.1 Cell line TRP receptor expression

This study confirmed the findings of previous studies by demonstrating the gene expression of TRPV-1 (Agopyan et al. 2003; Reilly et al. 2003; Thomas et al. 2007) and TRPA-1 (Mukhopadhyay et al. 2011; Park et al. 2012; Buech et al. 2013; Shapiro et al. 2013) in pulmonary epithelial cell lines. The presence of these receptors in non-neuronal pulmonary cells has been implicated in modulating airway inflammatory responses in health and disease, however their exact role remains unclear (Gardner 2006). The study also confirms the expression of these receptors throughout the airway as epithelial cells of bronchial (BEAS-2B) and alveolar (A549) origin were used. A number of different pulmonary cell lines have been used in a variety of experiments, and TRPV-1 expression has also been observed in lung primary cells (Thomas et al. 2007; McGarvey et al. 2014). Primary cells are those isolated directly from human tissue and have the benefit of being genetically and phenotypically representative of the cells in vivo however, primary cells compared to cell lines are heterogeneous and are therefore more difficult to compare when used in multiple experiments, and the results are less reproducible. They are also more difficult to culture and have a finite life-expectancy (Pan et al. 2009; Geraghty et al. 2014). As a result of these disadvantages established cell lines were used in the current study to improve reproducibility and reliability, however the potential failure of the cell lines to be representative of cells in vivo was an accepted limitation of the study.

4.2.2 Gastric constituent exposure and TRP receptor expression

The majority of in vitro experiments investigating the constituents of gastric refluxate have been in oesophageal cell lines, and there does not seem to be a universally accepted cell model of gastric reflux, with a variety of concentrations and exposure times used. In the model of reflux used in this study, exposure to the individual constituents of gastric refluxate was not associated with a statistically significant increase in gene expression of TRPV-1 or TRPA-1.

The initial concentrations of the gastric reflux constituents were based on concentrations that were thought to be clinically relevant and were consistent with those used previously, (Nehra et al. 1999; Jaiswal et al. 2006; Perng et al. 2008; Bathoorn et al. 2011; Johnston et al. 2012); however, it is difficult to ascertain the clinical relevance of these concentrations given the variable degree of reflux and composition of gastric refluxate in each individual (Gotley et al. 1991; Woodland and Sifrim 2010). It is also questionable whether the concentrations and timings used in such studies are physiologically relevant in the airway, as although there is evidence of micro-aspiration of gastric fluid into the lungs of patients with a variety of respiratory conditions including IPF (Lee et al. 2012), precise quantification of the exposure is not possible (Emilsson et al. 2013). For example, the final concentration of pepsin used in this experiment was 1mg/ml because the concentration of pepsin in human gastric fluid ranges from 0.5-1 mg/ml (Bathoorn et al. 2011) and it was used in previous studies investigating pepsin in bronchial epithelial cell lines (Bathoorn et al. 2011) and laryngeal cells (Johnston et al. 2012). A study which has used bronchoalveolar lavage to quantify the levels of pepsin in the airways of IPF patients during an acute exacerbation has found the levels to be much lower than this however, with the maximum level in the study reaching only 92 ng/ml^{-1} (Lee et al. 2012). Therefore it is unlikely that airway cells in vivo will be exposed to such high concentrations, however this is a limitation of most in vitro studies.

The effect of pepsin at varying pHs on the airway epithelium and expression of TRP receptor expression was of particular interest in this study as IPF patients who suffer with cough are often given a PPI which has been found to increase non-acid reflux (Kilduff et al. 2014). The activity and stability of pepsin is known

to be affected by the pH of its solution and traditionally pepsin was thought to become inactive at pH ≥ 4 and denatured above pH ≥ 5.5, however it is now appreciated that pepsin does not become completely inactivated until pH >8 (Piper and Fenton 1965; Bardhan et al. 2012). Pepsin has been shown to be toxic to the respiratory epithelium, with the inflammatory effect being pH dependent and a greater effect at lower pH (Bathoorn et al. 2011). It has also been shown to be have toxic cellular effects at neutral pH in cultured hypopharyngeal cells however, which provides support for the role of non-acid reflux in the pathogenesis of various conditions (Johnston et al. 2009). Unfortunately as seen in previous experiments investigating the cytotoxic effects of pepsin, (Bathoorn et al. 2011) repeated and prolonged exposure of the epithelial cells to an acidic environment resulted in reduction in cell viability particularly in the presence of pepsin therefore these cell lines are unsuitable for experiments assessing these conditions. Rather than testing pepsin at a scale of activity levels we were therefore only able to test active and inactive pepsin. However, this strategy still encompasses what would be classed as weakly acidic reflux (pH 4-6.9) and non-acidic reflux (pH >7) in vivo (Hirano et al. 2007; Cho 2010).

For the same reasons it was not possible to assess the TRP receptor expression following repeated exposures to a pH less than 6 or on a single exposure at a pH less than 4 which is unfortunate given that low pH is known to activate the TRPV-1 receptor in particular. The acidic exposures that were used in this study did not result in a statistically significant difference in TRPV-1 expression as expected which is in contrast to an experiment performed using HCl acid in an HET-1a human oesophageal cell line (Ma et al. 2012). In the model of gastroesophageal reflux use by Ma et al, oesophageal cells were exposed to weakly acidic media (pH 5) 7 times over a 48 hour period. The duration of exposure varied from 2 to 16 minutes, and they identified that TRPV-1 mRNA expression was maximal at 12 minutes exposures. They confirmed this at mRNA (qPCR) and protein level (Western blot analysis) and were able to demonstrate that this was an ATP-dependent process. The acidic exposure also resulted in an increased level of inflammatory mediators and cytokines supporting a role for TRPV-1 mediated inflammation. The functional role of the TRPV-1 receptor was confirmed using the TRPV-1 agonist capsaicin and two TRPV-1 antagonists, which resulted in an

upregulation and inhibition of the inflammatory cytokines respectively. A limitation of this study is that only one reference gene was used in qPCR quantification, with no reference to its stability in the experimental conditions. GAPDH is a commonly used reference gene, however there is great variation in its expression between different cell and tissue types, and unless a differential response in expression to acid exposure is excluded the results may be unreliable (Dheda et al. 2005). In support of their results however, a number of studies have identified the gene and protein expression of TRPV-1 in oesophageal biopsies (Matthews et al. 2004; Bhat and Bielefeldt 2006; Guarino et al. 2010), with increased expression in patients with erosive and non-erosive gastroesophageal disease. The correlation between TRPV1 expression and acid exposure time demonstrated in the in vitro study has not been confirmed in vivo however, with conflicting results between the studies.
In the study by Ma et al, cell death measured using the trypan blue exclusion method (trypan blue as added to the cells in culture, only non-viable cells with a defective cell membrane allow the absorption of the trypan and are stained blue) resulted in <2 % cell death over the exposure period (Ma et al. 2012). Although the pH used differed between the studies, it is unlikely that the A549 and BEAS-2B cell lines would have tolerated the protocol used by Ma et al. It is the variable tolerability of various cell lines in acidic solutions that limit such experiments in vitro, whereas in vivo, the cells have protection from continuous blood perfusion which acts as a buffer to the toxic constituents of gastric refluxate.

A key strength of this study was the great effort made to ensure that the concentrations and time of exposure did not affect the viability of the cells exposed. All of the constituents used are known to have cytotoxic effects and it was vital to ensure that any change in the TRP receptor expression levels could be attributed to the effect of the exposure rather than a result of an overall change in cell number as a result of cell death or proliferation. A number of quality control steps were also in place to ensure the data could be considered robust; for example unlike other studies which have relied on one reference gene to quantify their gene of interest, three appropriate reference genes were used as indicated by the genorm CV and M values obtained, carrier controls were used to negate the possible dilutional effect of the added constituent to the media, and all

experiments were performed at least in triplicate with technical repeats in place, to ensure reproducibility.

The results of the qPCR and IL-6/IL-8 ELISA studies may be related to gastric constituent exposure times and doses used as there was no significant increase in interleukin production in either cell line following exposure to the various gastric constituents when compared with the controls. It was expected that the gastric constituents would cause an inflammatory response with inflammatory mediator release. This work was done as an addendum to the cell study work in an attempt to indirectly assess TRPV-1 functionality. With hindsight these experiments should have been done in parallel to the cell study work as this would have resulted in a larger and more complete data set covering a wider range of exposure times and concentrations of gastric constituents.

The TRP receptors are highly permeable to calcium, and stimulation has been shown to result in production of pro-inflammatory cytokines/chemokines therefore this property is frequently exploited as a test of functionality, with the known TRP receptor agonists and antagonists used to stimulate and inhibit the receptors respectively. Previous studies have demonstrated calcium-dependent production of IL-6 in A549 and BEAS-2B cell lines following exposure to the TRPV-1 agonist capsaicin, therefore demonstrating TRPV-1 functional activation (Reilly et al. 2003; Seki et al. 2007). Other studies have also demonstrated that IL-6 and or IL-8 production is upregulated when cells are exposed to HCl acid (Ma et al. 2012), pepsin (Bathoorn et al. 2011) and bile acids (Mertens et al. 2010), indicating that this may be a TRPV-1 mediated response. The lack of a significant increase in IL-6 and IL-8 secretion from the cell lines exposed in this experiment is unlikely to be as a result of assay failure as although a positive control was not used the assay function was confirmed with the standard curves and the experiments were carried out with well-established protocols. Instead it is likely that the lack of significant difference in TRP receptor expression between the samples is reflected in the quantity of IL-6 and IL-8 released, therefore the interleukin studies support the qPCR findings. However, inflammatory stimuli have been shown to up-regulate TRPV-1 (Mitchell et al. 2005), therefore the inflammatory response may be the stimulus for increased TRP receptor expression.

It is therefore possible that although the gastric constituent concentrations and time exposures were chosen to ensure they did not result in a significant change in cell viability, they may have been insufficient to induce an upregulation of the TRP receptors and an inflammatory response. It is however a limitation that a positive control was not used as this would have excluded a failure of the cells to mount an inflammatory response. Interestingly a study that investigated the exposure of gastric juice to primary bronchial epithelial cell lines (PBECs) found that cells exposed to gastric juice from patients who were on a PPI had a significant inflammatory response as measured using IL-8, however there was no significant increase in IL-8 production or correlation with dose when cells were exposed to the individual components: pepsin and bile acids (Mertens et al. 2010). In retrospect to ensure the clinical relevance of the concentrations, the experiment could have been conducted using filtered whole gastric juice from patients with IPF who had undergone 24 hour impedance and pH manometry and controls. The use of PPI medication could be controlled for allowing the investigation of any differences in acidic and non-acidic reflux. However, this method would have also resulted in a number of challenges, namely in obtaining ethical approval, patient recruitment and quantification of gastric fluid constituents.

A complete functional assessment of the TRP receptors in this and the patient study was beyond the scope of this thesis but is a limitation. A number of methods have been used to demonstrate the functionality of the TRP receptors in a variety of cell and tissue types, with most studies taking advantage of the known agonists and antagonists, the calcium permeability of the receptor or its voltage-gated function in patch-clamp experiments. However, a number of studies have already demonstrated the functionality of the TRPV-1 and TRPA-1 receptors in A549 (Reilly et al. 2003; Mukhopadhyay et al. 2011; Buech et al. 2013; Shapiro et al. 2013), BEAS-2B cell lines (Reilly et al. 2003; Park and Jang 2011; Park et al. 2013; Jia et al. 2014) and patients (McGarvey et al. 2014). Therefore although demonstrating that the receptors were functional would have added to the strength of the study it is unlikely that it would have furthered our current understanding significantly.

It is also unfortunate that this study was unable to demonstrate whether there was any change in protein expression at a cellular level. Attempts were made to quantify the protein expression using Western blot analysis however preliminary experiments were unsuccessful despite trials with a number of different TRPV-1 and TRPA-1 antibodies. The lack of selective antibodies for accurate staining is a commonly encountered problem with studies investigating these receptors (Bonvini et al. 2015).

4.2.3 Gastro-oesophageal reflux and Cough in IPF

The link between IPF, GOR and cough is controversial and poorly understood, however there is growing evidence to support this association. As previously described, patients with IPF have been found to have a high prevalence of GOR despite often being asymptomatic (Tobin et al. 1998; Patti et al. 2005; Raghu et al. 2006a; Noth et al. 2012), plus there is evidence of micro-aspiration (Lee et al. 2012; Savarino et al. 2013). GOR is a known cause of chronic cough (Irwin et al. 1981) and one of the proposed mechanisms for chronic cough in patients with IPF is increased cough reflex sensitivity is as a result of gastroesophageal reflux induced neuronal changes within the airways. A study investigating cough in IPF and GOR recently demonstrated abolition of acid GOR but no improvement in cough with high dose PPI treatment (Kilduff et al. 2014). This and a second study both identified a significant increase in non-acid reflux in IPF patients (Savarino et al. 2013; Kilduff et al. 2014), suggesting that acid-reflux alone is not solely present in most IPF patients, with the other constituents of gastric refluxate then potentially implicated.

It is important to note that this in vitro study has only investigated expression in epithelial cells and the role of these non-neuronal cells remains unclear. It is believed that the non-neuronal receptors mediate inflammation through the release of inflammatory cytokines, which are thought to induce up-regulation, neurogenic inflammation and modulate nociceptive signalling. The conflicting results with regards the expression of TRP receptors in non-neuronal cells between experiments in cell culture and human studies is likely to be a consequence of genetic and phenotypic variation in the cells being studied in vitro. It is possible that the inflammatory response mediated by the non-neuronal

cells sensitises or up-regulates the neuronal TRP receptors in vivo, however this remains poorly understood. This study was not designed to test the theory that micro-aspiration results in a heightened cough reflex sensitivity or to investigate the hypothesis that GOR results in cough through a vagally-mediated oesophageal-airway reflex, which may be a promising mechanism given the increased expression of TRPV-1 receptors in oesophageal cells in vitro and in vivo (Ing et al. 1994; Ma et al. 2012).

This study did demonstrate small fold increases in TRPV-1 expression in both A549 and BEAS-2B cell types in response to pepsin and bile salts, however there was no other clear trend in expression pattern seen and importantly no statistically significant change in TRP receptor expression in pulmonary epithelial cells from the central (BEAS-2B) or distal airways (A549). The findings of this study therefore do not support the hypothesis that the individual constituents of gastric refluxate increase the expression of TRP receptors in airway epithelial cells.

The importance of the small fold increases as a result of pepsin and bile acid exposure in both cell types is unclear and although a link could be suggested between the non-acidic components of gastric refluxate, the current study does not provide sufficient evidence for this. The increased expression of TRPV-1 in IPF patients who did not receive a PPI in the clinical study also fails to support this hypothesis, although it is likely that the mechanism is far more complex than we can currently appreciate with simple in vitro models.

Although the study by Mertens et al uses different methodologies and posed different experimental questions the results can be used to help support and understand the findings in this current study. As briefly described previously Mertens et al. investigated the IL-8 production by PBECs following exposure to gastric juice from patients with and without a PPI (Mertens et al. 2010). This study did not show a significant inflammatory response (IL-8 release) when cells were exposed to the individual components of refluxate (bile salts or pepsin) but did show significant correlation between the inflammatory response and gastric juice with PPI (Mertens et al. 2010). Interestingly the gastric juice with PPI had a significantly higher pH and levels of endotoxins which are lipopolysaccharides

produced by bacteria. Patients on PPIs have been shown to have small intestinal bacterial overgrowth (Lombardo et al. 2010) and therefore the raised endotoxin and IL-8 secretion in these patients could be related to this, but as the inflammatory response was higher in un-filtered gastric juice and gastric juice on PPI than gastric juice off PPI it was concluded that it was the combination of factors including the presence of food particles that was likely to be responsible rather than individual elements (Mertens et al. 2010). Therefore, this experiment supports and may explain the current findings that the individual components of gastric refluxate did not provoke the expected inflammatory response, but also supports the link between acid and non-acid GOR and respiratory disease.

The results of this in vitro experiment cannot be directly extrapolated to the in vivo situation or human disease, however the study does deliver robust preliminary results that can be used to develop further studies to investigate this challenging symptom. It is likely that GOR contributes to cough in IPF, and given the complex factors involved in initiating the cough reflex in health and the potential number of influences on cough reflex sensitivity, it is probable that a number of mechanisms are involved. Although the evidence is currently conflicting it is likely that anti-reflux procedures such as Nissen fundoplication which targets GOR as a whole, rather than PPIs which reduce acidity and volume of reflux but do not eradicate reflux, are required to treat this challenging problem (Blondeau et al. 2008).

CHAPTER 5: CONCLUSIONS

Evidence for altered cough neurophysiology in patients with IPF has gradually accumulated, however this is an area of limited data and the exact mechanisms remain unclear. This study has demonstrated the difficulties in investigating the innervation of the airways in patients with IPF, however has provided further evidence for central airways inflammation; and for the first time demonstrated TRPV-1 and TRPA-1 gene and protein expression in neuronal and non-neuronal cells of airway bronchial biopsies in IPF. In relation to the potential role of GOR in IPF-associated cough, the in vitro cell culture work used in this study has provided an insight into the pathogenic mechanisms of GOR induced cough and has demonstrated that the individual components of gastric refluxate do not significantly increase TRP receptor expression in pulmonary epithelial cells. The findings of the current studies brought together with the evidence regarding GOR and cough presented throughout this thesis suggests that targeting the individual components of GOR and empirical PPI for IPF patients with cough needs further investigation, and targeting GOR as a whole with anti-reflux surgical procedures should be considered as an alternative option.

5.1 PROPOSED MECHANISM OF COUGH IN IPF

This study has not shown evidence of a significant increase in TRPV-1 and TRPA-1 gene and protein expression in the airways of patients with IPF. This suggests that a structural up-regulation of airway TRP receptors alone may not be the mechanism for cough in IPF patients. However the evidence for a heightened cough reflex sensitivity to capsaicin in IPF suggests that an alternative mechanism that involves the TRP receptors is at work, and there are a number of possibilities:

1. Physiological up-regulation of TRP receptors through sensitisation may result in a lower threshold for initiation of an action potential and increased cough reflex sensitivity. Although there was no significant increase in TRP receptors, studies suggest that small differences in TRPV-1 expression may result in large differences in TRPV-1 sensitivity through the release of inflammatory mediators and local (peripheral) sensitisation. TRPA-1 is co-expressed and can be sensitised as a result of increased TRPV-1

activity. The presence of chronic inflammation in the central airways of IPF patients in this study supports this, however whether the inflammation is a cause or consequence of the cough remains unclear.

2. It is possible that there is structural up-regulation of TRP receptors in the distal airways that are involved in the fibrotic process. TRPV-1 receptors have been demonstrated at alveolar level (Watanabe et al. 2005), and although the peripheral C-fibres are thought to be inhibitory, there is evidence for increased expression of neurotrophins distally from BAL samples in IPF. Neurotrophins have been implicated in adaptive responses within sensory neurones, including fibre phenotype switching, and increased expression and functionality of TRP receptors.

3. The lack of correlation between TRP receptor expression, cough reflex sensitivity and cough symptomatology raises the possibility that other components of the cough reflex such as central sensitisation may also play a role in cough in IPF patients. Central sensitisation could also be implicated in the relationship between GOR and IPF.

Figure 46 is a schematic diagram illustrating these possible mechanisms. There is however, likely to be a complex interplay of a number of different mechanisms causing cough in this condition. Age, gender, genetic susceptibility and the activation of other receptors such as the ASICs should also be taken into consideration as evidence is emerging that they may also have a role to play.

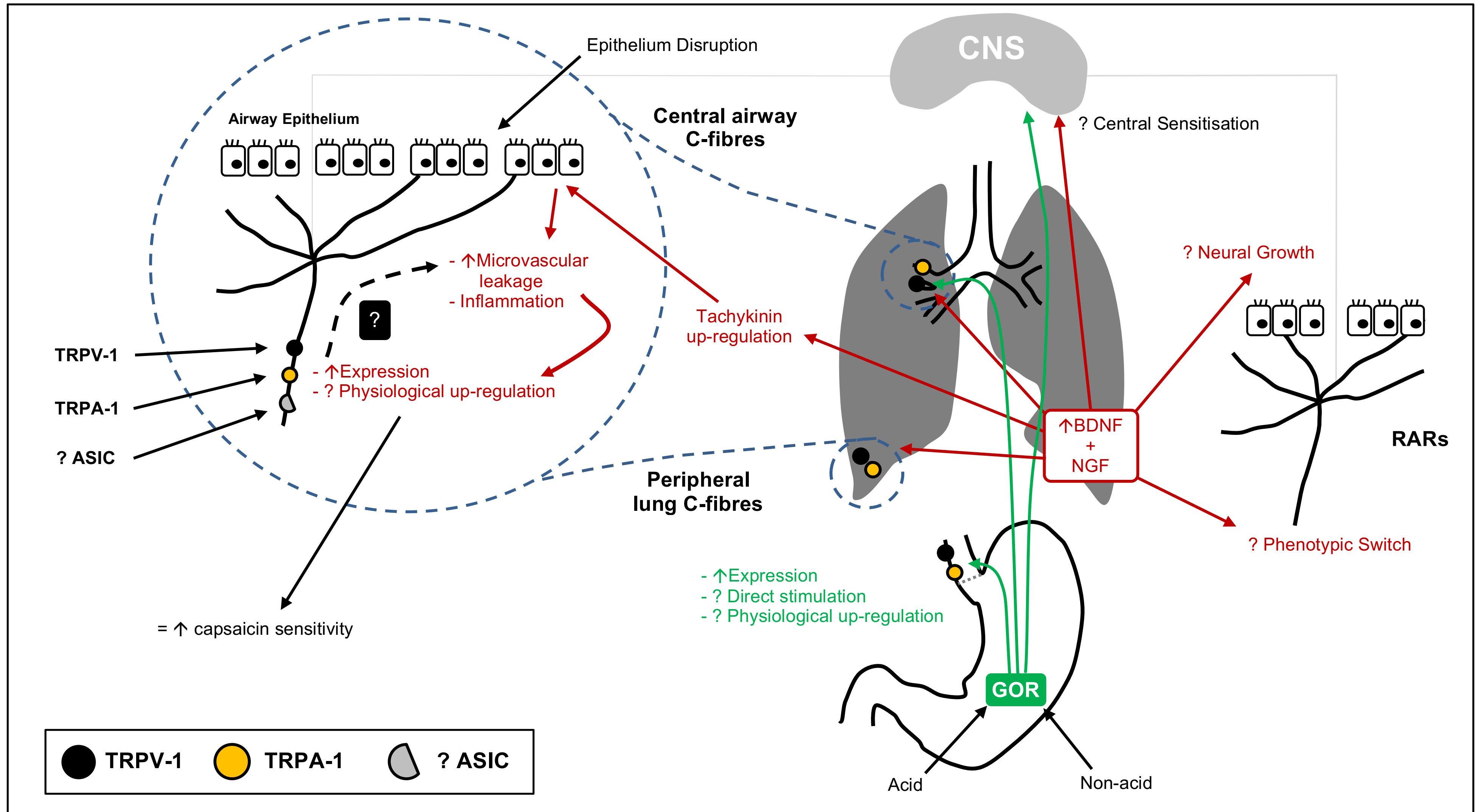


Figure 46 **Proposed model of pathogenesis of cough in idiopathic pulmonary fibrosis.** *Adapted from* (Hope-Gill 2004)

CHAPTER 6: DIRECTIONS FOR FUTURE STUDIES

This thesis highlights the need for further studies investigating the mechanisms of cough in IPF, and points towards potential future experiments as outlined below.

This study highlights the difficulty in obtaining good quality specimens from the airways of patients with IPF and control subjects. Obtaining biopsies from the parenchyma of patients with IPF to determine whether there is distal structural TRP receptor up-regulation will be equally if not more difficult as with the improved resolution of CT scans and the recognised morbidity associated with surgical lung biopsy, a tissue biopsy is obtained less often than previously to diagnose IPF. Trans-bronchial biopsies are occasionally taken, however these often yield small samples. Surgical lung biopsies would allow large amounts of tissue to be analysed which would give a more accurate representation of the part of the airway in question, however this form of biopsy is generally only carried out when there is diagnostic uncertainty and may therefore represent patients with a different spectrum of disease. Ideally larger clinical studies would be carried out which have the power to detect smaller differences between IPF patients and controls, however this also represents a significant challenge given the prevalence of the condition and the frequency of confounding comorbidities. Future studies in this field may benefit from the use of tissue from national tissue bio-banks and the animal model may be an alternative approach which would allow whole lung examination and more directed future human studies.

Although there are limitations to in vitro work as described, such experiments can provide valuable insight into the mechanisms involved in cough without the need for patient recruitment. Further in vitro experiments, using whole gastric refluxate, may provide further understanding of the link between GOR and the expression of TRP receptors in non-neuronal respiratory epithelial cells. Developing specific antibodies to allow protein quantification, and a robust cell model to assess function of these receptors would also be beneficial to aid our understanding of the role of these receptors within the cell. Primary lung cells would allow for more meaningful extrapolation of the results to the in vivo environment, however to investigate the link between GOR, IPF and the theory of central sensitisation a

clinical study would be ideal. This could involve comparing TRP receptor expression in oesophageal biopsies and 24 hour pH monitoring and impedance in patients with IPF against control patients. However an alternative study could involve the oral administration of capsaicin in patients with IPF to determine whether there is an improvement in cough. This has been shown to be effective in chronic cough and the authors postulate that the systemic absorption of capsaicin reduces cough reflex hypersensitivity in a mechanism of central desensitisation (Ternesten-Hasseus et al. 2015), however others argue that the repeated exposure to the lower oesophagus results in local desensitisation (Faruqi and Morice 2015). These theories offer potential therapeutic options for IPF patients.

The demonstration of TRP receptors in the airways of patients in IPF will also allow the exploration of other therapeutic avenues. There are a number of TRPV-1 and TRPA-1 antagonists in development for the treatment for chronic cough. Although their success has been limited in chronic cough, there may be benefits in other conditions such as IPF. However, the demonstration of TRP receptor expression correlating poorly with symptomatic measures of cough, which has also been found with cough reflex sensitivity, suggests that the development of a more practical way to objectively assess cough in clinical practice, research and in pharmaceutical studies is necessary.

As the understanding of normal cough physiology improves, it is likely that further molecular targets will be discovered, such as the ASICs. Further research to develop primers and specific antibodies to detect and locate these receptors should improve our understanding of the mechanisms of cough in health and disease and allow the development of targeted therapy for this debilitating condition in the future.

PUBLICATIONS AND PRESENTATIONS

This research has been presented at the following local, regional and International meetings:

- Lung Research Wales annual meeting. Studies of Pathogenesis and Cough in Idiopathic Pulmonary Fibrosis. (November 2012)
- Ireland, Wales and South-West interstitial lung disease meeting. Cough in Idiopathic Pulmonary Fibrosis (January 2014)
- American Thoracic Society Poster presentation: Airway expression of Transient Receptor Potential channels in Idiopathic Pulmonary Fibrosis (May 2016)
- American Thoracic Society Poster presentation: Expression of Transient Receptor Potential Channels in pulmonary epithelial cells exposed to gastric fluid constituents (May 2016)

APPENDICES

APPENDIX 1

IPF patient demographics

Patient	Age	Gender	Smoking status	FVC	TLCO	HRCT	BAL cell differential (%)			
							M	N	L	E
1	75	Male	Non-smoker	51.9	27.4	UIP pattern	88	2	9	1
2	72	Male	Non-smoker	74.0	33.6	UIP pattern	69	12	18	1
3	72	Male	Non-smoker	79.5	36.0	UIP pattern	Neutrophil predominant- too scant for differential			
4	56	Male	Ex-smoker	76.0	40.0	UIP pattern	91	1	0	0

Patient	Age	Gender	Smoking status	FVC	TLCO	HRCT	BAL cell differential (%)			
							M	N	L	E
5	74	Male	Ex-smoker	85.8	46.5	UIP pattern	66	18	15	1
6	71	Male	Ex-smoker	82.8	41.1	UIP pattern	86	Not reported		
7	67	Female	Ex-smoker	108.3	57.7	UIP pattern	79	9	12	0
8	74	Male	Non-smoker	90.8	60.6	Atypical for UIP*	65		29	
							*UIP pattern confirmed on surgical lung biopsy			
9	74	Male	Ex-smoker	84.2	37.7	UIP pattern	68	26	6	<1
10	70	Male	Non-smoker	61.2	63.4	UIP pattern	92	8	0	0

Patient	Age	Gender	Smoking status	FVC	TLCO	HRCT	BAL cell differential (%)			
							M	N	L	E
11	62	Male	Non-smoker	73.2	37.8	UIP pattern	90-92	7-8	1-2	0
12	65	Male	Non-smoker	77.9	75.7	UIP pattern	94	4	1	1
13	60	Male	Non-smoker	65.8	41.2	UIP pattern	78	12	10	0
14	77	Male	Non-smoker	113.9	134.0	UIP pattern	73	10	11	6
15	69	Female	Ex-smoker	100.7	40.5	UIP pattern	82	9	7	2
16	82	Male	Ex-smoker	90.6	76.3	UIP pattern	77	22		

M = macrophage N = neutrophil L = lymphocyte E = eosinophil

FVC = % predicted forced vital capacity

TLCO = % predicted transfer factor for carbon monoxide

APPENDIX 2

2a Leicester cough-specific health status questionnaire.

Reproduced from (Birring et al. 2008) with permission from BMJ Publishing Group Ltd.

	SCORE						
ACTIVITY:	**1**	**2**	**3**	**4**	**5**	**6**	**7**
1. In last 2 weeks have you had chest or stomach pain as a result of cough?	All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time	Hardly any of the time	None of the time
2. In last 2 weeks have you had sputum production on coughing?	Every time	Most times	Several times	Some-times	Occasion-ally	Rarely	Never
3. In last 2 weeks have you been tired because of coughing?	All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time	Hardly any of the time	None of the time
4. In the last 2 weeks have you felt in control of your cough?	None of the time	Hardly any of the time	A little of the time	Some of the time	A good bit of the time	Most of the time	All of the time
5. How often during the last 2 weeks have you felt embarrassed by your cough?	All of the time.	Most of the time.	A good bit of the time.	Some of the time.	A little of the time	Hardly any of the time	None of the time.
6. In the last 2 weeks my cough has made me feel anxious.	All of the time.	Most of the time.	A good bit of the time.	Some of the time.	A little of the time.	Hardly any of the time	None of the time.
7. In the last 2 weeks I felt my cough has interfered with my job or other tasks.	All of the time.	Most of the time.	A good bit of the time.	Some of the time.	A little of the time.	Hardly any of the time	None of the time.
8. In the last 2 weeks I felt my cough interfered with my overall enjoyment of life.	All of the time.	Most of the time.	A good bit of the time.	Some of the time.	A little of the time.	Hardly any of the time	None of the time.
9. In the last 2 weeks exposure to paints or fumes has made me cough.	All of the time.	Most of the time.	A good bit of the time.	Some of the time.	A little of the time.	Hardly any of the time	None of the time.
10. In the last 2 weeks has your cough disturbed your sleep?	All of the time.	Most of the time.	A good bit of the time.	Some of the time.	A little of the time.	Hardly any of the time	None of the time.

SCORE

ACTIVITY:	1	2	3	4	5	6	7
11. In the last 2 weeks how many times each day have you had coughing bouts?	All of the time	Most times during the day	Several times during the day	Sometimes during the day	Occasional -ly through the day	Rarely	None
12. In the last 2 weeks my cough has made me feel frustrated.	All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time	Hardly any of the time	None of the time
13. In the last 2 weeks my cough has made me feel fed up.	All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time	Hardly any of the time	None of the time
14. In the last 2 weeks have you suffered from a hoarse voice as a result of your cough?	All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time	Hardly any of the time	None of the time
15. In the last 2 weeks have you had a lot of energy?	None of the time	Hardly any of the time	A little of the time	Some of the time	A good bit of the time	Most of the time	All of the time
16. In the last 2 weeks have you worried that your cough may mean serious illness?	All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time	Hardly any of the time	None of the time
17. In the last 2 weeks have you been concerned what others think is wrong with you because of your cough?	All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time	Hardly any of the time	None of the time
18. In the last 2 weeks my cough has interrupted conversation or telephone calls.	Every time	Most times	A good bit of the time	Some of the time	A little of the time	Hardly any of the time	None of the time
19. In the last 2 weeks, I feel that my cough has annoyed my partner, family or friends.	Every time I cough	Most times I cough	Several times when I cough	Some times when I cough	Occasional -ly when I cough	Rarely	Never

Scoring of LCQ :

(1) Domains (questions):

(a) Physical: 1,2,3,9,10,11,14,15

(b) Psychological: 4,5,6,12,13,16,17

(c) Social: 7,8,18,19

(2) Domain scores: total score from items in domain/number of items in domain (range 1–7).

(3) Total scores: addition of domain scores (range 3–21).

2b Visual Analogue Score:

Please mark the severity of your cough, zero being no cough and 100 being the worse cough imaginable.

Cough severity (VAS 0 -100):

0 ____ 100

APPENDIX 3

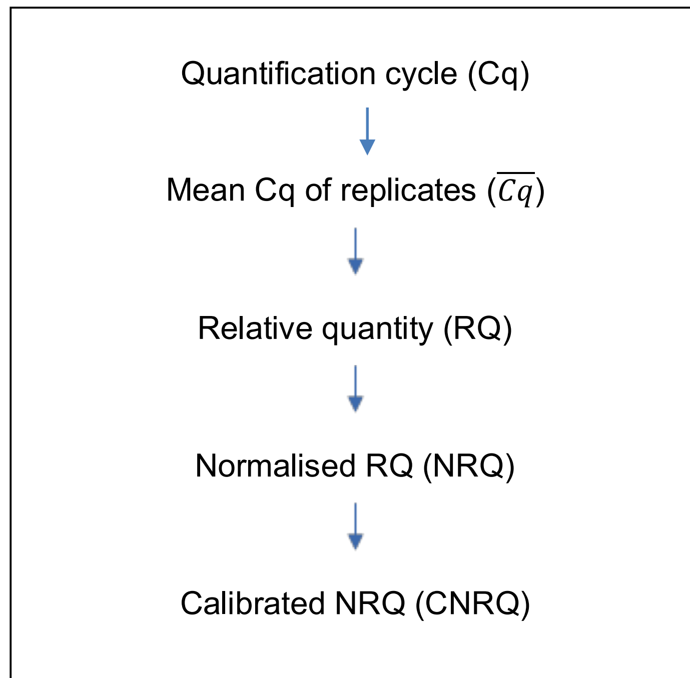
Isolating nucleic acid: method development using mouse pulmonary tissue

Sample	Extraction Technique	RNA Quantity (ng/µl)	260/280 ratio
Mouse tissue (10mg)	TRIzol	48.16	2.04
Mouse tissue (10mg)	Column-based	27.47	1.99
Pilot sample (15mg)	TRIzol	70.9	2.03
Pilot sample (15mg)	Column-based	50.9	1.94

Sacrificed mouse pulmonary tissue was used to assess two methods of RNA extraction. The mouse pulmonary tissue was dissected and stored in the same manner as the patient biopsies and processed using TRIzol (Invitrogen, Paisley, UK) and the RNeasy-mini column-based techniques (Qiagen, Hilden, Germany) as per the manufacturer's instructions. RNA yield was of upmost importance given the small size of patient biopsies.

APPENDIX 4

qBasePlus calculation algorithm



The qBasePlus software uses the algorithm above to automatically calculate the relative quantities of each target gene (Hellemans et al. 2007).

APPENDIX 5

Cell viability following continuous gastric constituent exposure

Absorbance/ Viability (%)

	Control	Ethanol carrier	CD 50 µM	CD 100 µM	CD 250 µM	CD 500 µM
A549	100.00	102.00	105.13	111.28	90.40	34.71
P value		*0.99*	*0.99*	*0.94*	*0.97*	*0.16*
BEAS-2B	100.00	110.95	114.15	119.72	73.97	33.54
P value		*0.96*	*0.90*	*0.74*	*0.52*	***0.03***

	Control	H20 carrier	GD 50 µM	GD 100 µM	GD 250 µM	GD 500 µM
A549	100.00	103.00	109.55	118.49	114.31	90.01
P value		*0.99*	*0.94*	*0.93*	*0.97*	*0.93*
BEAS-2B	100.00	108.42	111.49	107.88	105.90	39.96
P value		*0.98*	*0.96*	*0.99*	*0.97*	*0.09*

	Control	Pepsin 1mg/ml	Pepsin 2mg/ml	Pepsin 10mg/ml
A549	100.00	127.59	112.13	84.76
P value		*0.53*	*0.91*	*0.84*
BEAS-2B	100.00	129.92	128.84	71.39
P value		*0.67*	*0.69*	*0.70*

	Control	Pepsin pH6	Pepsin pH4	Pepsin pH2
A549	100.00	88.84	42.27	35.17
P value		*0.96*	*0.16*	*0.10*
BEAS-2B	100.00	130.11	35.49	34.65
P value		*0.21*	***0.01***	***0.01***

	Control	pH6	pH4	pH2
A549	100.00	77.87	20.24	31.63
P value		*0.90*	***0.04***	***0.08***
BEAS-2B	100.00	108.33	28.25	37.52
P value		*0.98*	***0.01***	***0.01***

APPENDIX 6

Rhinosinusitis Task Force Questionnaire

MAJOR FACTORS	MINOR FACTORS
Facial pain/pressure Nasal obstruction/blockage Nasal discharge/purulence/discoloured postnasal drainage Hyposmia/anosmia Purulence in nasal cavity on examination Fever	Headache Fever Halitosis Fatigue Dental pain Ear pain/pressure/fullness

Adapted from Lanza et al. (Lanza and Kennedy 1997)

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