THE UNIVERSITY OF HULL

THE PATHOGENESIS OF IDIOPATHIC PULMONARY FIBROSIS

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CONTENTS PAGE

Index of Figures

Index of Tables

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Personal Statement

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Abbreviations

AECA	Anti-endothelial cell antibodies
ANOVA	Analysis of variance
BALF	Bronchoalveolar lavage fluid
BSA	Bovine serum albumin
CEA	Carcinoembryonic antigen
CD40L	CD40 Ligand or CD154
EBC	Exhaled breath condensate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbant assay
EMT	Epithelial mesenchymal transition
FACS	Fluorescence activated cell sorting
FITC	Fluorescein isothiocyanate
FVC	Forced vital capacity
GERD	Gastro-esophageal reflux disease
HARQ	Hull airway reflux questionnaire
HUVEC	Human umbilical vein endothelial cells
IIP	Idiopathic interstitial pneumonia
ILD	Interstitial lung disease
IPF	Idiopathic pulmonary fibrosis
Kco	Gas transfer corrected for alveolar volume
PAR	Protease activated receptor
PBS	Phosphate buffer saline
PECAM	Platelet endothelial cell adhesion molecule
PMC	Platelet-monocyte complexes
PMN	Polymorphonuclear leukocytes
PSGL-1	P-selectin glycoprotein ligand-1
R-PE	R-Phycoerythrin
TLco	Total lung carbon monoxide diffusion capacity

Abstract

Idiopathic pulmonary fibrosis (IPF) is a restrictive pulmonary disorder of unknown aetiology with a relentless disease course and a median survival of 3 years after the diagnosis. It is the most common idiopathic interstitial lung disease (ILD) with a basal and peripheral predominance associated with temporal and geographical heterogeneity. As the pathogenesis of this disease is poorly understood, the aim of this work was to investigate the pathobiology of IPF in a prospective manner. There is evidence of a strong association of gastro-esophageal reflux and vascular disease with IPF. Moreover, a proportion of patients have evidence of immunological antibodies without any evidence of connective tissue or autoimmune disease. The data presented in this thesis suggest that platelet-monocyte complexes may be involved in the pathogenesis of IPF at molecular level as suggested by the flow cytometric data utilizing monoclonal antibodies to platelets (CD42a) and monocytes (CD14). Moreover, expression of CD40L, P-selectin and PSGL-1 on platelets and subpopulation of leukocytes suggested that platelet expression of these molecules is not significantly different in IPF as compared to ILD other than IPF or non-ILD controls. Furthermore, platelet mediated injury hypothesis is supported by significant elevation of platelet endothelial cell adhesion molecule in plasma of IPF patients.

Reflux of gastric secretions into the tracheo-bronchial tree is another attractive hypothesis in light of remarkably high prevalence of gastro-esophageal reflux disease (GERD) in IPF. The data suggest that patients with IPF have significantly higher gastro and extra-esophageal reflux symptoms when assessed by

Hull airway reflux questionnaire (HARQ). However, there was a lack of objective evidence of extra-esophageal reflux measured by exhaled breath pepsin concentration or significantly higher prevalence of *Helicobacter Pylori*. Furthermore, there was evidence of immune mediated injury in IPF by indirect immunofluorescence study of alveolar epithelial (A549) cells as significant membranous enhancement of A549 cells by anti-IgG antibodies was demonstrated in IPF patients' sera. However, Human umbilical vein endothelial cells (HUVEC) did not show any differential staining pattern with either anti-IgG or IgM. Hence, there is a suggestion of alveolar epithelial disruption mediated by immune mechanisms with a predominant involvement of IgG antibodies. Furthermore, epithelial derangement may extend into the respiratory epithelium with release of carcinoembryonic antigen (CEA) in peripheral circulation as evidenced by a significant correlation of raised CEA level and lung function impairment in IPF.

These findings provide clinical and molecular evidence of novel mechanisms of pathogenesis of IPF with increased platelet-monocyte aggregation. Moreover, immune mediated alveolar epithelial dysfunction involving IgG antibodies may provide further insight into the understanding of the pathogenesis and natural history of this fibrotic disease.

CHAPTER 1: INTRODUCTION

1.1 PATHOGENESIS OF IDIOPATHIC PULMONARY FIBROSIS

Idiopathic pulmonary fibrosis (IPF) is an irreversible restrictive fibrotic disorder of the pulmonary interstitium with a dismal prognosis. Despite a number of studies evaluating the pathogenesis of this devastating disease (Chilosi et al. 2002;Ebina et al. 2004;Erlinger et al. 1991;Gabbiani 1998;Pantelidis et al. 2001;Selman, King, & Pardo 2001), the aetiology of IPF remains elusive. The triggering factor for this parenchymal restrictive lung disease is believed to be alveolar epithelial injury with subsequent lung fibrosis. The exact nature of this epithelial cell injury is yet to be elucidated. This uncertainty of exact aetiology of this progressively fatal disease is reflected by a lack of effective disease modifying therapeutic agent in a series of randomised clinical trials (King, Jr. et al. 2005;King, Jr. et al. 2008;Raghu et al. 1991;Raghu et al. 2004;Raghu et al. 2008;Taniguchi et al. 2010;Zisman et al. 2010).

Historically, IPF was believed to be an inflammatory disorder but this hypothesis has been challenged for a number of reasons. First, inflammation is not a prominent histological finding. Second, IPF does not respond well (or at all) to anti-inflammatory therapy in the form of corticosteroids or other immunosuppressive agents. Third, epithelial injury can lead to pulmonary fibrosis even in the absence of significant ongoing inflammation (Selman, King, & Pardo 2001). This epithelial cell injury may by immunological (either antibodies or cell mediated), chemical (eg, reflux of gastric contents), microbial, or particulate (organic or inorganic) [Figure 1.1].

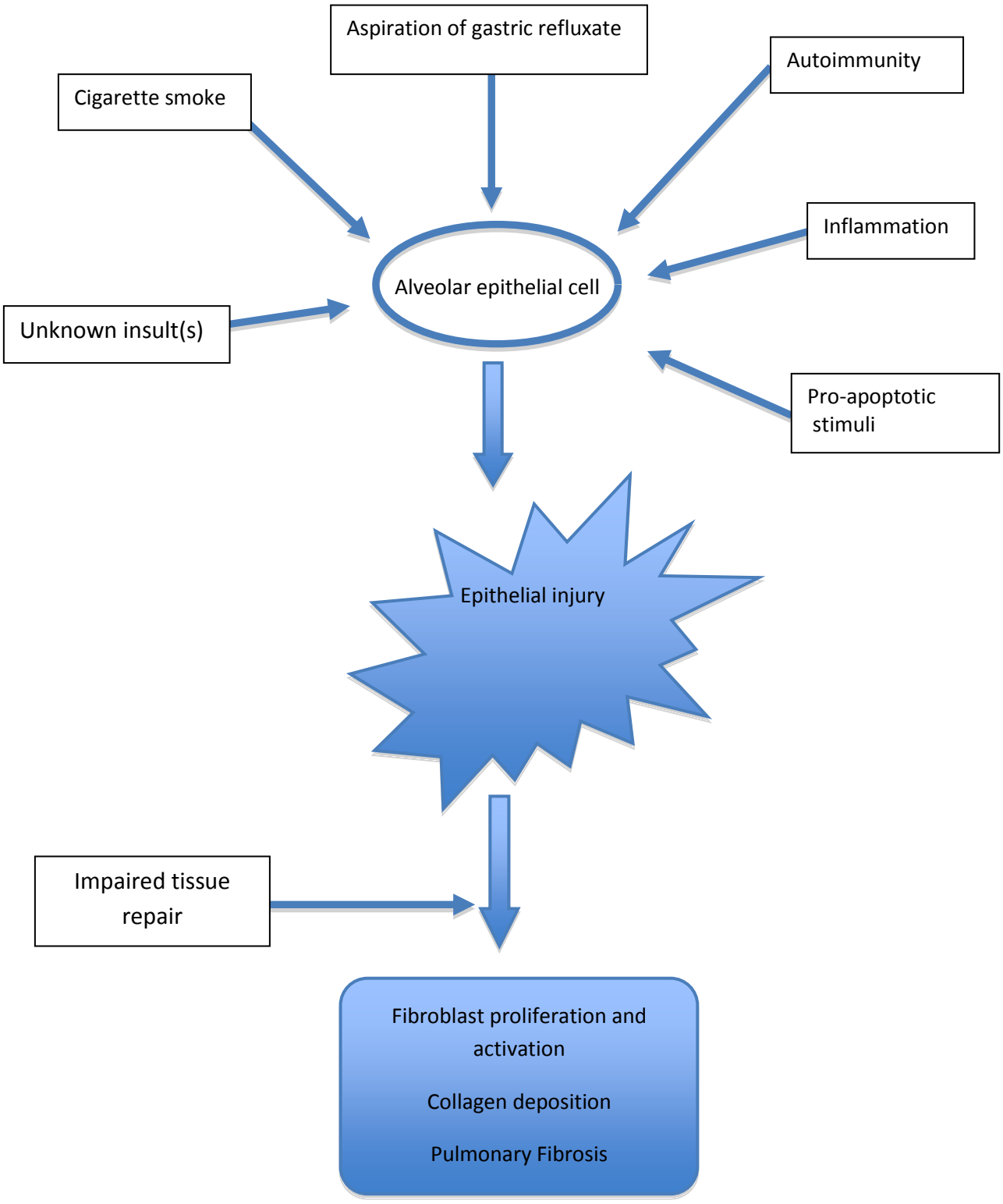

Figure 1.1 *"Epithelial hypothesis" proposed mechanisms of alveolar epithelial injury and fibrosis in IPF.*

Epithelial mesenchymal transition (EMT) is an attractive mechanism for the development of IPF as there is evidence to suggest that abnormal alveolar repair secondary to prolonged epithelial mesenchymal cell interaction (through TGF-β) results in pulmonary fibrosis in IPF (Sheppard 2001). There is transformation of alveolar epithelial cells with loss of polarity and epithelial markers (E-cadherin and zonula occludens-1) to acquire spindle shape morphology and start behaving like mesenchymal cells with acquisition of mesenchymal markers such as alpha smooth muscle actin (α-SMA) (Zavadil & Bottinger 2005), vimentin, desmin and type I collagen. The role of EMT in IPF is further discussed in the following section.

Myofibroblasts: The key effector cells in pulmonary fibrosis

Myofibroblasts are the key effector cells responsible for the production of extracellular matrix and expression of α-SMA (Gabbiani 1998) that contributes to interstitial pulmonary fibrosis. Myofibroblasts display features of both fibroblasts and smooth muscle cells with contractile properties and are an essential component of 'fibroblastic foci' which are recognized as the characteristic histological feature of IPF. Myofibroblasts are derived from a number of sources and have a close interaction with pro-coagulant signalling via thrombin [Figure 1.2]. There are three predominant sources of myofibroblasts. Firstly, resident fibroblasts (Center et al. 1993;Martin 1997) that interact with cytokines and growth factors (Sower et al. 1995) resulting in accumulation and activation of fibroblasts. Secondly, epithelial to mesenchymal transition (EMT) is a process of phenotypic change of alveolar epithelial cell into spindle shape morphology along with functional consequences. These spindle shaped cells contribute to fibroblast expansion in IPF lungs by virtue

of collagen deposition and extracellular matrix accumulation. Moreover, the role of EMT in IPF is strengthened by microarray analysis demonstrating that many EMT related genes including TGF-β3 (Nawshad & Hay 2003;Romano & Runyan 2000) and lymphoid enhancing factor-1 (LaGamba, Nawshad, & Hay 2005) are up-regulated in IPF lungs. Finally, bone marrow derived cells have recently been discovered (Bucala et al. 1994) and are thought to be a potential source of myofibroblasts. These CD45+, Collagen 1+ and CXCR4+ cells show plasticity and traffic to the lung in response to a chemokine CXCL12 (Phillips et al. 2004). However, their role in the pathogenesis of IPF is as yet debatable and there is paucity of robust data on the contribution of these subtypes of leukocytes in perpetuation and progression of pulmonary fibrosis in IPF. It would be of significant importance to prospectively evaluate fibroblasts and myofibroblasts during the course of development of pulmonary fibrosis and attempt to intervene (with anti-fibrotic drugs) at a stage much earlier than the established honeycombing and end stage fibrosis. Figure 1.2 summarizes the role of myofibroblasts in the pathogenesis of IPF and its relationship with coagulation cascade via protease activated receptors (PAR) also known as thrombin receptors. The interaction of pro-fibrotic growth factors with angiogenic and thrombotic mediators may be a novel mechanism in the development of irreversible scarring of pulmonary parenchyma in IPF.

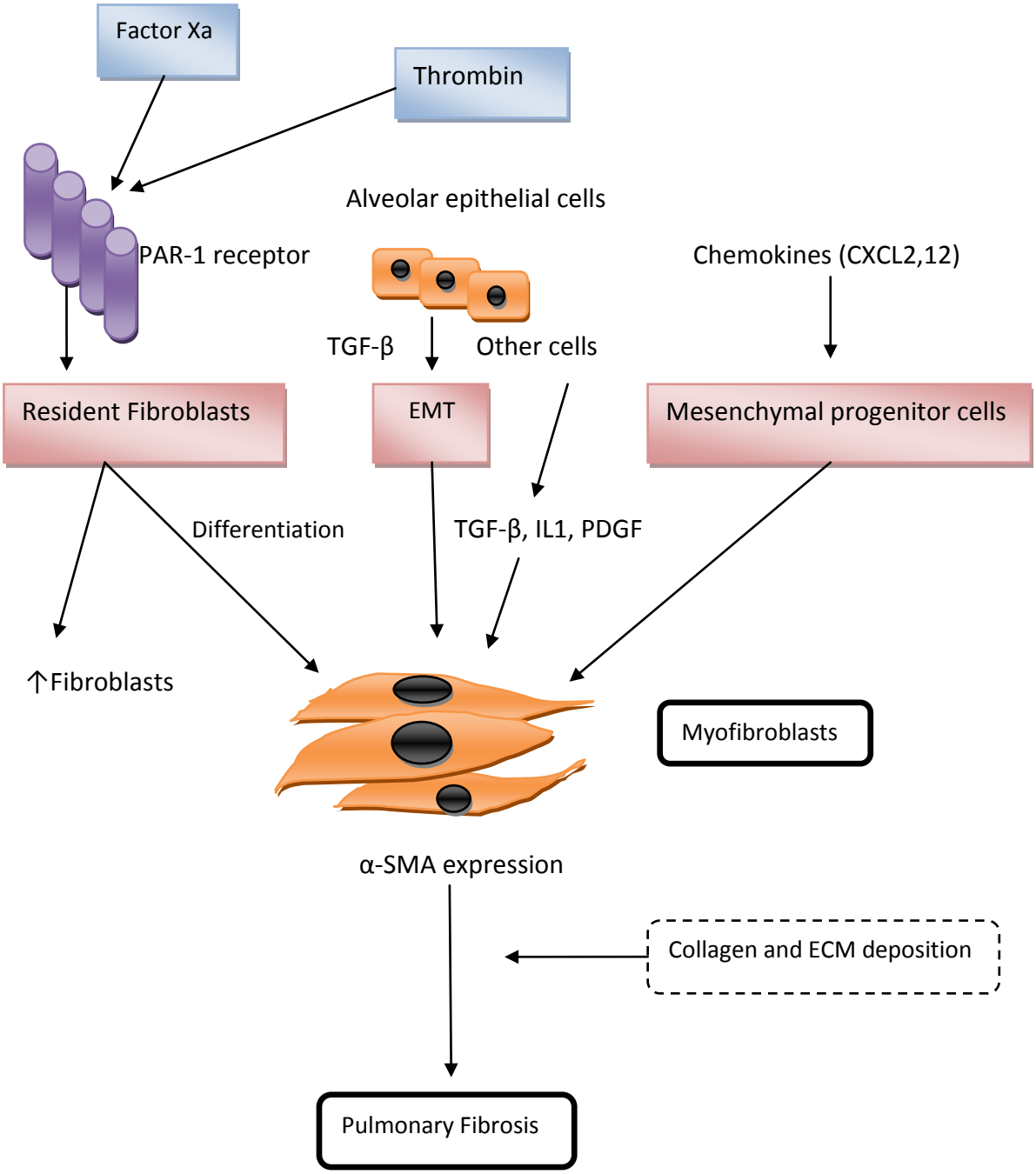

Figure 1.2 ***Myofibroblasts, protease activated receptors and pulmonary fibrosis***

Myofibroblasts are the key component of fibrotic cascade in IPF. There is a close interaction between coagulation and fibrotic cascades in the pathogenesis of lung fibrosis. Thrombin and factor Xa are key agonists of PAR-1 receptor that leads to the activation of fibroblasts and differentiation into myofibroblasts and subsequent biological effects of profibrotic molecules resulting in lung fibrosis. These pro-fibrotic mediators are released by a number of cells including resident epithelial cells, fibroblasts and endothelial cells. PAR Protease activated receptors; EMT Epithelial-mesenchymal transition; SMA Smooth muscle actin; TGF Transforming growth factor; PDGF Platelet derived growth factor; ECM Extracellular matrix.

The gastro-esophageal reflux hypothesis

The gastro-esophageal reflux hypothesis is attractive and intriguing as the histopathological analysis of IPF lung biopsies show geographical heterogeneity with areas of honeycomb lung interspersed with normal lung parenchyma. It is plausible that recurrent episodes of gastro-esophageal reflux (including both acid and non-acid) lead to epithelial injury in bronchocentric and bronchiolocentric distribution as well as part of refluxate may reach alveoli and trigger the fibrotic response. Indeed there is objective evidence of a very high prevalence of classic gastro-esophageal or acid reflux in IPF (Raghu et al. 2006;Tobin et al. 1998). These prospective randomized studies have shown a prevalence of up to 87% of patients having distal reflux and 63% of patients with reflux reaching proximal oesophagus. Furthermore, there is recent evidence of a convincing association of gastro-esophageal reflux with asymmetrical IPF and acute exacerbations (Tcherakian et al. 2011). Significant gastro-esophageal reflux has been documented in patients with scleroderma associated interstitial fibrosis, a disease with radiological and pathological similarities with idiopathic pulmonary fibrosis. Moreover, Interstitial lung fibrosis is a major cause of death in scleroderma (Bryan et al. 1999;Ioannidis et al. 2005;Simeon et al. 2003). Indeed, a contributing factor to gastro-esophageal reflux in scleroderma is esophageal dysmotility. The validity of "reflux" hypothesis as a significant cause of interstitial pulmonary fibrosis is hampered by lack of an ideal investigation with a high sensitivity and specificity. The best available diagnostic modality for detection of GERD is ambulatory pH monitoring coupled with high resolution impedance manometry to detect non-acid reflux. Gastric enzyme pepsin,

if detected in respiratory secretions (either BAL or exhaled breath condensate) may be a useful adjunct to the objective assessment of gastro and extra-esophageal reflux.

As previously shown (Strugala, Dettmar, & Morice 2009), pepsin in exhaled breath condensate is potentially a marker of extra-esophageal reflux and it may have a significant contribution to epithelial injury by means of laryngopharyngeal reflux. Indeed, a relationship between pulmonary aspiration and increased pepsin concentration in BAL has been demonstrated in animal and human studies (Farhath et al. 2006;Farrell et al. 2006;Metheny et al. 2004;Metheny et al. 2006;Starosta et al. 2007;Tasker et al. 2002b;Tasker et al. 2002a;Ufberg et al. 2004;Ward et al. 2005) and is associated with pulmonary inflammation (McNally et al. 2011).

It is unclear why a small proportion of population with reflux develop IPF when the prevalence of gastro-esophageal reflux is fairly high in general population. It will be interesting to explore and identify the susceptibility factors for the development of pulmonary fibrosis in patients with reflux. In this regard, evaluation of non acid reflux may provide further insight into the pathogenesis of pulmonary fibrosis and its relationship with GERD.

There has been much interest in how certain genetic differences might predispose to IPF. For example, NA1/NA2 polymorphism of the IgG receptor CD16B is associated with IPF, supporting a role for an underlying immunological insult (Bournazos et al. 2010a). Moreover, FcgammaRIIa R131H polymorphism has been found to be associated with the severity and progression of IPF (Bournazos et al. 2010b). Gene expression profiling has shown that there are clearly different gene

signatures in IPF patients compared to normal controls. The genes encoding extracellular matrix formation and degradation are significantly increased in fibrotic lungs, the most distinctive being matrix metalloproteinase-7 (Zuo et al. 2002). The only clearly defined genetic mutations in association with IPF are in the gene encoding surfactant protein C *(SFTPC)* and telomerase genes *hTERT* and *TERC* (Nogee et al. 2001;Tsakiri et al. 2007). There is evidence of up-regulation of adenosine 2B receptor and prominin-1/CD133 genes in the rapidly progressive variant of idiopathic pulmonary fibrosis (Selman et al. 2007). Moreover, it is possible to distinguish between familial and idiopathic forms of idiopathic interstitial pneumonias (IIP) on the basis of gene expression (Yang et al. 2007). There is also evidence that shorter telomere length of circulating leukocytes increases the predisposition towards the development of sporadic as well as familial pulmonary fibrosis (Cronkhite et al. 2008). This genetic association of IPF is interesting and provides a basis for further evaluation of certain genetic markers in the pathogenesis of this fibrotic and relentless disease of dismal prognosis.

As there is an association of IPF with vascular disease (acute coronary syndrome, angina and deep vein thrombosis) even after controlling for the confounding factors such as age and smoking history, it is conceivable that platelets may be involved in the pathophysiology and are likely to have a central role in the alveolar epithelial/endothelial injury and fibrosis. Indeed platelet derived serotonin has been linked with vascular disease and tissue fibrosis in systemic sclerosis (Dees et al. 2011). The vascular injury mediated by platelets does need further investigation.

1.2 PLATELET LEUKOCYTE COMPLEXES AND MARKERS OF PLATELET ACTIVATION IN IDIOPATHIC PULMONARY FIBROSIS

Platelet leukocyte interaction plays a key role in inflammatory recruitment of these cells to combat the injurious insult [Figure 1.3]. One of the initial steps in inflammation and atherogenesis is the recruitment of peripheral monocytes into the site of vascular damage (Consigny 1995). These platelet monocyte interactions with activated endothelium are mediated by a number of cell surface molecules called integrins and selectins. These molecules mediate the adhesive interactions of peripheral monocytes with the vascular wall to withstand the local flow at the vascular endothelium. Platelets do bind to peripheral blood leukocytes in diseases such as coronary artery disease (Furman et al. 1998), unstable atherosclerosis (van Zanten et al. 1994) and hypercholesterolemia (Huo et al. 2003). In addition, increased platelet-monocyte binding has been observed following lung transplantation with a significant rise in platelet activation markers as compared to control patients undergoing thoracotomies (Sternberg et al. 2008). P-selectin or CD62p is a 140 kDa transmembrane protein stored in alpha granules of platelets and Weibel-Palade bodies of endothelial cells respectively and is involved in platelet-neutrophil and platelet-monocyte interaction via P-selectin glycoprotein ligand-1 (PSGL-1) on polymorphonuclear leukocytes (PMN) cell surface. Moreover, blockage of PSGL-1 results in inhibition and reversal of platelet-monocyte complexes following platelet stimulation, suggesting a central role of PSGL-1 in platelet-monocyte adhesion (Fernandes et al. 2003). Activated platelets interact with PSGL-1 on monocytes via P-selectin and monocytic MAC-1 via GPIIb/IIIa or GPIbα (Gawaz, Langer, & May 2005)[Figure 1.4].

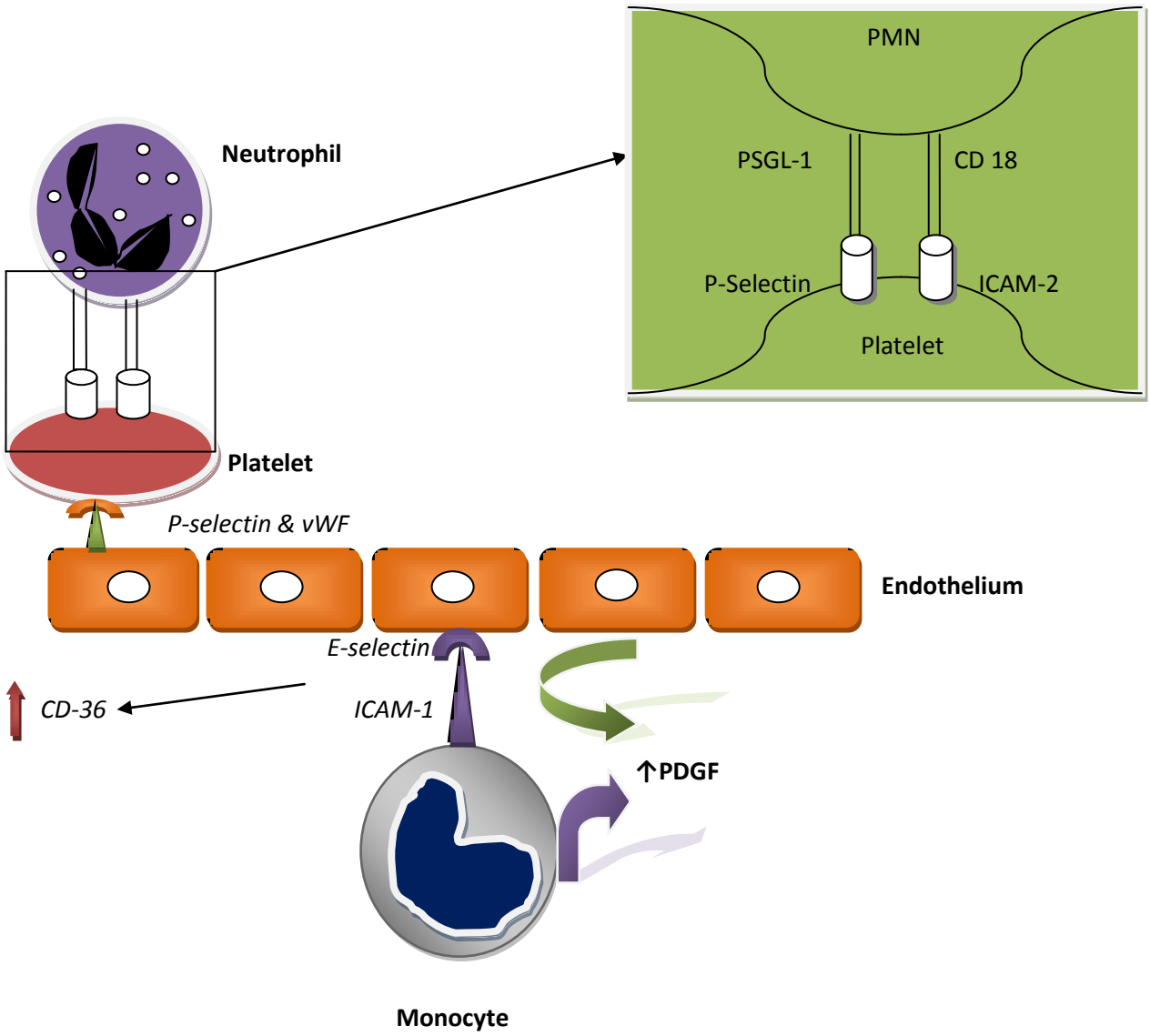

Figure 1.3 ***Platelet-Neutrophil-Monocyte interaction with endothelium.*** *Platelets play a key role in PMN recruitment through PSGL-1 (CD162) and CD18 interaction with P-selectin (CD62p) and ICAM-2 respectively. Platelet monocyte adhesion is of significance in vascular disease and is mediated partially by PSGL-1. The monocyte adhesion with endothelium is mediated by ICAM-1 and other adhesion molecules including E-selectin and is one of the initial steps in atherosclerosis. There is increased expression of CD36 following interaction of E-selectin with monocytes. Moreover, PDGF is released by endothelium as well as monocytes upon activation. PMN Polymorphonuclear leukocytes; PSGL P-selectin glycoprotein ligand; ICAM Intercellular adhesion molecule; PDGF Platelet derived growth factor; vWF von Willibrand factor.*

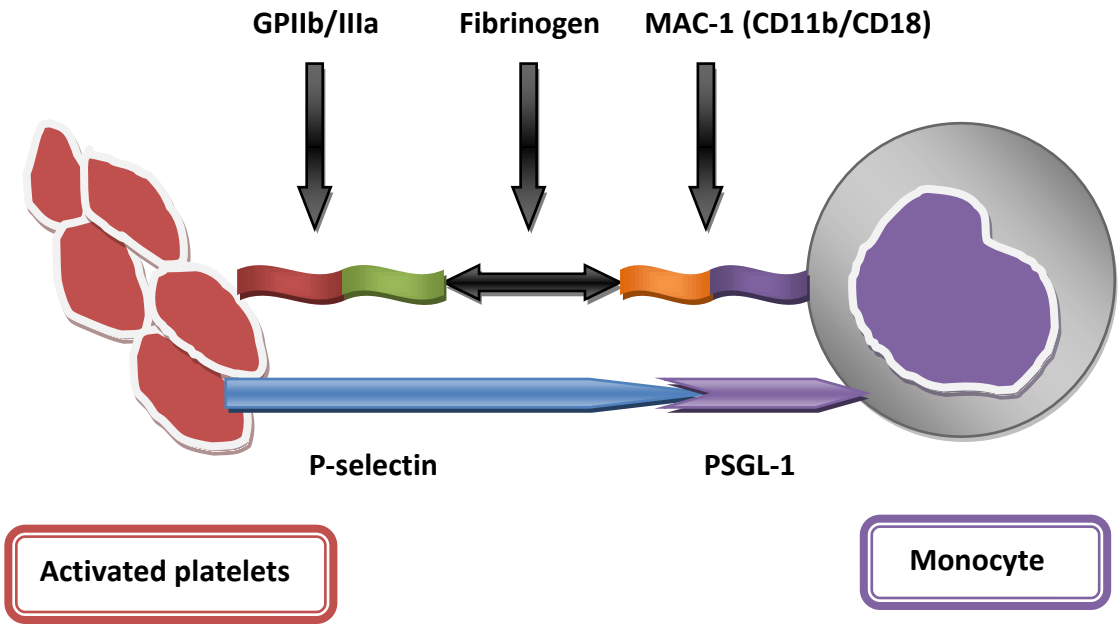

Figure 1.4 ***Platelet Monocyte interaction after activation.***
The platelets in the activated state adhere with monocytes through a number of molecules including GPIIb/IIIa and MAC-1. Fibrinogen acts as a bridge for this interaction. P-selectin on platelet surface interacts with PSGL-1 on monocyte cell surface to form platelet monocyte complexes. MAC-1 Macrophage-1 antigen.

There is evidence of increased risk of vascular disease in idiopathic pulmonary fibrosis in a number of studies. In a large cohort of 920 patients with IPF, an increased risk of acute coronary syndrome (odds ratio [OR], 1.53; 95% confidence interval CI, 1.15-2.03), angina (OR, 1.84; 95% CI, 1.48-2.29) and deep-vein thrombosis (OR, 1.98; 95% CI, 1.13-3.48) has been found in the period before the diagnosis of IPF (Hubbard et al. 2008). Moreover, there was a significantly increased risk of acute coronary syndrome (RR 3.14) and deep venous thrombosis (RR 3.39) in the follow up period. In another study of 630 patients for the evaluation of the prevalence of coronary artery disease in pulmonary fibrosis, there was a significant association between IPF and multi-vessel disease (OR, 4.16; 95% CI, 1.46-11.9). Adjustments were made for traditional risk factors and the association of coronary

artery disease was significant for non-granulomatous fibrotic lung disorders and its subset, idiopathic pulmonary fibrosis (Kizer et al. 2004).

The above findings do support the notion that pulmonary fibrosis in IPF may be a consequence of pro-coagulant state in the lung microenvironment. Indeed there is evidence of reversal of alveolar haemostatic balance in acute lung injury as well as pulmonary fibrosis (Ware et al. 2006;Ware et al. 2007). In normal circumstances, the lung microenvironment is antithrombotic and pro-fibrinolytic. In response to injury, this equilibrium is shifted towards pro-coagulant and anti-fibrinolytic state (Chambers 2008). There is evidence of coagulation cascade activation in a number of interstitial lung diseases (ILD) (Gunther et al. 2000;Hernandez-Rodriguez et al. 1995) including idiopathic pulmonary fibrosis (Imokawa et al. 1997). Moreover, inhibition of thrombin is associated with attenuation of collagen deposition in bleomycin induced lung fibrosis in animal models (Howell et al. 2001). This pro-coagulant environment may be accentuated as a result of platelet-monocyte aggregation or interaction of endothelial surface with activated platelets and subtypes of leukocytes. It will be of interest to evaluate the possible triggers for this pro-coagulant micro-vasculature and target this aspect of lung pathology in the quest for a therapeutic agent in this devastating disease.

In light of the association of idiopathic pulmonary fibrosis with vascular disease, we hypothesized that patients with IPF have increased tendency for platelet-monocyte and platelet-neutrophil interaction and form complexes, in particular platelet-monocyte complexes as evidenced by an investigation in patients with acute coronary syndrome and myocardial infarction (Sarma et al. 2002). There

was evidence of significant P-selectin independent conjugation mechanism to platelet-monocyte adhesion seen in peripheral blood. As platelets are key cells at the site of injury at microvascular compartment with other subsets of leukocytes, we propose that platelets are involved in the pathogenesis of this devastating lung disease of unknown aetiology. The platelet-monocyte complexes may become clumped in the peripheral and basal lung architecture within the microvascular compartment and act as an initiating stimulus for alveolar epithelial injury, the hallmark and one of the earliest events in the pathogenesis of IPF. It will be important to demonstrate a significant difference in the proportion of platelet-monocyte complexes in a cohort of IPF patients as compared to control population. As there is increased thrombotic tendency not only at macroscopic level, but also at microvascular lung architecture level, a significantly increased platelet-monocyte adhesion may have a therapeutic implication, with anti-platelet agents.

1.2.1 Platelet activation markers in pulmonary fibrosis

CD154 or CD40 ligand (CD40L) is a type 2 membrane glycoprotein of 33 kDa in size, that belongs to TNF family of cytokines and is expressed by CD4+ T cells (Armitage et al. 1992;Noelle et al. 1992), B cells (Grammer et al. 1995), dendritic cells (Pinchuk et al. 1996) and platelets (Henn et al. 1998). CD40 is the receptor for CD40L and is expressed by antigen presenting cells (APC) as well as fibroblasts (Fries et al. 1995;Sempowski et al. 1998;Sempowski, Chess, & Phipps 1997) keratinocytes and endothelial cells (Denfeld et al. 1996;Mach et al. 1997). In response to CD40 ligation, human fibroblasts release pro-inflammatory cytokines IL-6 and IL-8 (Sempowski et al. 1998;Sempowski, Chess, & Phipps 1997) with increased expression of adhesion molecules ICAM-1 (intercellular adhesion molecule) and

VCAM-1 (Vascular cell adhesion molecule) (Yellin et al. 1995). Fibroblast expression of these molecules is associated with amplification of inflammatory and repair processes. There is evidence that CD40-CD40L interaction activates human lung fibroblasts in vitro (Cao et al. 1998;Sempowski, Chess, & Phipps 1997) and certainly mouse models support that this pathway plays a crucial role in lung injury in vivo. Furthermore, treatment with anti-CD40L antibody results in blockage of ionizing radiation and oxygen induced lung damage in mice (Adawi et al. 1998). There is evidence of significantly increased expression of CD40L in IPF lung fibroblasts in comparison to non-fibrotic controls. Moreover, blood and BAL fluid CD40L levels in fibrotic patients are significantly higher than controls (Kaufman, Sime, & Phipps 2004). These findings support the role of CD40L in the pathogenesis of lung fibrosis in IPF.

CD40-CD40L interaction may be an important mechanism in human lung fibrosis and could be a potential target for further study in human IPF. In view of the above molecular findings of CD40L in pulmonary fibrosis, the expression of this molecule on human platelets in patients with IPF and other interstitial lung diseases might provide a further insight into the molecular pathogenesis of IPF. We hypothesize that this molecule is differentially expressed on platelets in peripheral blood of IPF patients as compared to non-IPF ILD or patients without interstitial lung disease.

CD162 or P-selectin glycoprotein ligand (PSGL)-1 is a counter receptor present on polymorphonuclear leukocytes and endothelial cells. It is involved in neutrophil rolling on platelets by binding to P-selectin on platelets (Kuwano et al.

2010). PMN rolling on platelets can be completely blocked by monoclonal antibodies (mAb) to one of these molecules. There is increased monocyte binding to inflamed endothelium by activated platelets that is important in atherosclerosis and this increased adhesion of platelets with monocytes is found in patients presenting with acute coronary syndromes (unstable angina and acute myocardial infarction) as compared to patients with non-cardiac chest pain (Sarma et al. 2002). P-selectin-PSGL-1 interactions account for most platelet-monocyte binding and it will be interesting to evaluate the markers of platelet activation and platelet monocyte complexes in IPF as it is a disease with very close association with cardiovascular disease and thrombo-embolic episodes.

1.3 ANTI-EPITHELIAL/ ENDOTHELIAL ANTIBODIES IN IPF

As mentioned above, the triggering or inciting insult in the pathogenesis of IPF is believed to be alveolar epithelial injury. The structural remodelling in IPF is manifested by a range of phenotypic changes in epithelial cells from cuboidal to squamous cells lining the alveolar walls (Carrington et al. 1978) and type II pneumocyte hyperplasia and metaplasia of bronchiolar epithelial cells (Katzenstein & Askin 1982). There is limited data on the role of anti-epithelial antibodies in the aetiology and pathogenesis of this disease. A subset of anticytokeratin antibodies (CK-19), that are expressed in regenerated broncho-epithelial cells, have been demonstrated in IPF and pulmonary fibrosis associated with collagen vascular disease (Fujita et al. 1999a). Furthermore, an antibody against small airway epithelial cell has been demonstrated in pulmonary fibrosis associated with dermatomyositis (Fujita et al. 1999b). In addition, a number of other autoantibodies

have been investigated in patients with pulmonary fibrosis of obscure aetiology (Erlinger et al. 1991;Meliconi et al. 1989;Meliconi et al. 1993;Wallace et al. 1994). Together, these data underscore the significance of epithelial derangement in the pathogenesis of pulmonary fibrosis in IPF and in association with connective tissue diseases where the underlying mechanisms may be immune mediated.

The levels of circulating immune complexes are increased with deposition in tissues leading to complement activation in a number of chronic inflammatory diseases (Bodi et al. 1998;Digeon et al. 1979). It is plausible that chronic inflammation of lung parenchyma precedes the interstitial pulmonary fibrosis in an autoimmune mechanism. However, an inflammatory model in the pathogenesis of IPF has not held the promise in view of lack of effectiveness of anti-inflammatory therapy.

In addition to the epithelial disintegration and associated inflammation, abnormal angiogenesis has been linked to the development of pulmonary fibrosis (Cosgrove et al. 2004). Matrix metalloproteinases (MMPs) are involved in extracellular matrix remodelling, angiogenesis and wound healing. These molecules are capable of degrading collagen and have been found in increased levels in BALF of IPF patients (McKeown et al. 2009) and this increased production is associated with abnormal capillary permeability. On the basis of these observations of close interplay between neo-angiogenesis and increased vascular permeability, it is apparent that molecular abnormalities in IPF are not confined to epithelial derangement and endothelial-capillary basement membrane dysfunction is closely associated with the pathogenesis.

Anti-endothelial antibodies have been implicated in the pathogenesis of idiopathic interstitial pneumonias (IIPs) as there is evidence of increased levels of these antibodies in idiopathic non specific interstitial pneumonia (NSIP), the second commonest of the idiopathic interstitial pneumonias. In a study of 24 patients with collagen vascular disease associated ILD and 20 patients with idiopathic interstitial pneumonias (including IPF and idiopathic NSIP), there was increased prevalence of anti-endothelial cell antibodies (AECA) in idiopathic NSIP and CVD-ILD (Matsui et al. 2008). However, there was no evidence of increased levels of these antibodies in IPF. Moreover, there was no correlation between AECA and serum markers of interstitial lung disease namely KL-6 and SP-D. ILD is associated with pulmonary endothelial cell injury as suggested by a study of ^{123}I-MIGB kinetics (Takabatake et al. 2005). There was a significantly reduced washout rate of ^{123}I-MIGB in ILD patients when compared to controls without interstitial lung disease suggesting endothelial cell injury. Moreover, the washout rate correlated with disease severity. A number of studies (Ihn et al. 2000;Orfanos et al. 2001;Wusirika et al. 2003) have suggested a link between anti-endothelial cell antibodies and pulmonary fibrosis in the context of scleroderma; a disease with radiological and pathological similarities with IPF. There is evidence of immune dysregulation in IPF as antiphospholipid antibodies are present in patients with IPF (Magro et al. 2003;Magro et al. 2006;Magro et al. 2007). Expression of anti-endothelial antibodies is increased in sera and lung biopsies in patients with IPF as compared to controls. In addition, there is morphologic evidence of microvascular injury, and ultrastructural studies have demonstrated the changes of endothelial injury and necrosis with basement membrane collagen

deposition and lamellation. These findings are suggestive of a close association of these antibodies with idiopathic pulmonary fibrosis and substantiate the evidence of their role in alveolar epithelial/endothelial injury and progression to lung fibrosis.

1.4 CARCINOEMBRYONIC ANTIGEN AND IPF

Carcinoembryonic antigen (CEA) is a glycoprotein involved in cell adhesion and is produced during foetal development. It was first discovered by Gold and Freedman in 1965 (Gold & Freedman 1965). It is a glycosyl phosphatidyl inositol (GPI)-cell surface anchored glycoprotein that is crucial in metastatic dissemination of colonic carcinoma by acting as colon carcinoma L-selectin and E-selectin ligands. The production of this antigen ceases soon after birth and a very small amount is detectable in normal healthy individuals. However, higher levels of CEA are present in smokers. Its predominant use is as a tumour marker in serum and the most common tumours associated with raised CEA levels are colorectal, gastric, pancreatic, lung and breast. In view of low sensitivity and specificity of this protein, it is not a useful screening tool in colorectal cancer. However, it is of prognostic value in cases of established colorectal malignancy and the serum levels should return to baseline value after complete surgical resection (Goldstein & Mitchell 2005). Raised CEA levels have been demonstrated in IPF patients' sera as well as BAL fluid, suggesting an abnormality of epithelial cell integrity associated with release of this tumour antigen in respiratory secretions and the vascular compartment.

In this thesis, I aim to investigate the pathogenesis of idiopathic pulmonary fibrosis in a variety of studies ranging from gastric reflux and anti-

epithelial/endothelial antibodies to platelet-monocyte aggregates and molecular markers of platelet activation. Moreover, the association of CEA with lung function impairment in IPF will be explored. It will be interesting to evaluate a number of hypotheses in a disease of extremely dismal prognosis without any effective therapeutic option and I believe that this investigation will give me an excellent opportunity to not only improve my understanding of IPF, but it will serve as a stimulus to continue further research in this field.

CHAPTER 2: METHODS

This chapter presents the methods for all the studies in a systematic manner. The studies conducted for the investigation into the pathogenesis of IPF were prospective and were carried out after ethical approval was obtained by Hull and East Riding Research and Ethics Committee (LREC No: 08/H1304/54). All the data has been kept anonymous and password protected.

2.1 Platelet Leukocyte study

2.1.1 Flow-cytometry

Inclusion criteria and blood sampling

The diagnosis of IPF was based on the ATS/ERS guidelines (Flaherty et al. 2004). The control subjects included patients with other ILD, including asbestosis, rheumatoid arthritis associated ILD and idiopathic interstitial pneumonias other than IPF. The control subjects without ILD comprised healthy individuals and patients with COPD and other common medical conditions. Blood from patients with IPF and control subjects was drawn by venepuncture via 19-gauge needle, collected into 6 ml tubes containing 102 IU lithium heparin (BD Ltd, Plymouth, UK) and was gently inverted.

Antibodies, Immunolabelling and Flow Cytometry

Blood (50 µL) was labelled within 30 minutes of collection by incubation with specific antibodies for 20 minutes at room temperature. The antibodies used for labelling the blood samples are shown in Table 2.1.

Monoclonal Antibody	*Fluorescent staining*	*Molecular Marker*
1- Unlabelled Blood	None	None
2- CD42a	FITC conjugated	Platelets
3- CD14	R-PE conjugated	Monocytes
4- CD62p	R-PE conjugated	P-selectin
5- CD162	R-PE conjugated	P selectin glycoprotein ligand-1 (PSGL-1)
6- CD154	R-PE conjugated	(CD40 ligand)
7- Control	FITC control	Negative Control
8- Control	R-PE control	Negative Control

Table 2.1 *The reagents used for labelling the peripheral blood prior to flow cytometry in platelet-monocyte adhesion study*

Monoclonal antibodies (mAbs) directly conjugated to fluorochromes were purchased from the following sources: FITC-conjugated CD42a and R-PE conjugated CD14; Serotec Ltd (Oxford UK). R-PE conjugated CD62p mAb AK-4, CD162 mAb KPL-1, CD40L (CD154) mAb MR-1 and control IgG1 were obtained from BioLegend (San Diego, CA, USA).

Following volumes of mAbs were used for flow cytometry: CD42a 1µL; CD14 1µL; CD62p 5µL; CD162 5µL; CD40L 5µL; FITC control 5 µL and R-PE control 1 µL. 370µL of FACSLyse solution (Becton Dickinson, San Jose, CA, USA) (in 1:10 dilution with distilled water) was added to each sample to lyse the red blood cells. Samples were run on a Becton Dickinson FACSCalibur flow cytometer, and data analysis was

performed using Cell Quest Pro (Becton Dickinson) software. Samples were analyzed with the flow cytometer triggered on forward scatter and again by triggering on FL-1 to select CD42a positive platelets. The subtypes of leukocytes were identified by distinct forward and side scatter properties and are shown in Figures 2.1 and 2.2.

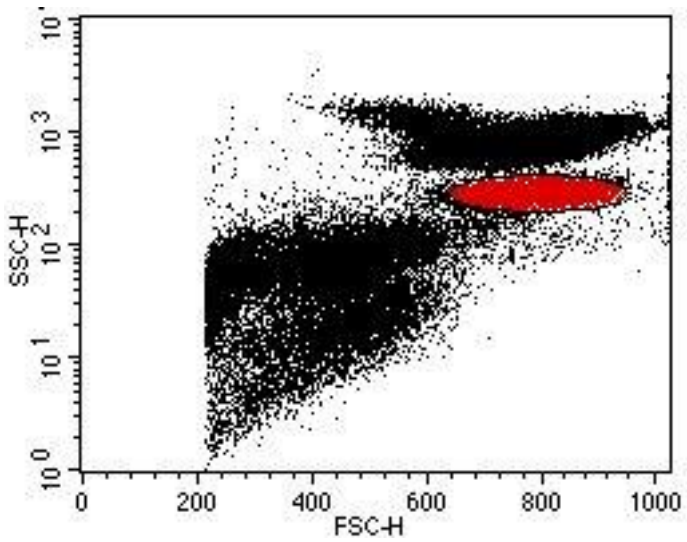

Figure 2.1 *Flow cytometry data focusing on monocytes in red. These cells have similar forward scatter (FSC-H) as neutrophils but lower side scatter (SSC-H).*

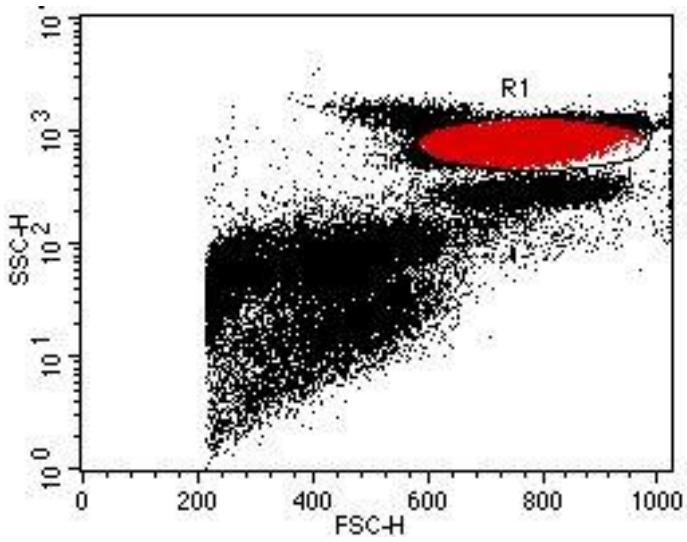

Figure 2.2 *Flow cytometry showing a distinct population of cells with high forward and side scatter identified as neutrophils (gate R1)*

The platelet-monocyte adhesion was determined using directly conjugated CD14-PE and CD42a-FITC mAb. Figures 2.3 and 2.4 shows how neutrophil and monocyte cut-off was used to measure platelet-monocyte complexes. The percentage of platelet-monocyte binding was detected by the proportion of $CD14^{High}/CD42a^{High}$ events (Upper Right quadrant) from $CD42a^{High}$ and $CD42a^{Low}$ events (Upper right and Upper Left quadrants) (Figure 2.5).

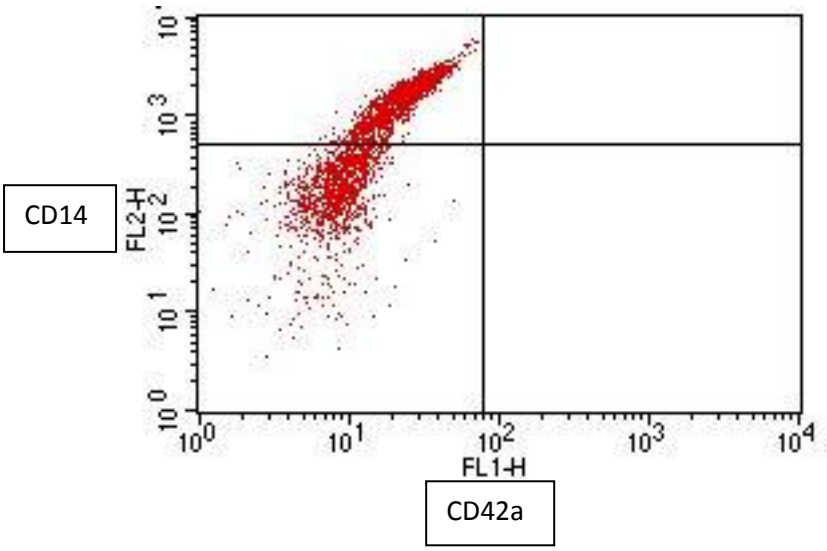

Figure 2.3 *The cut-off used to separate monocytes from platelet monocyte complexes (verticle line)*

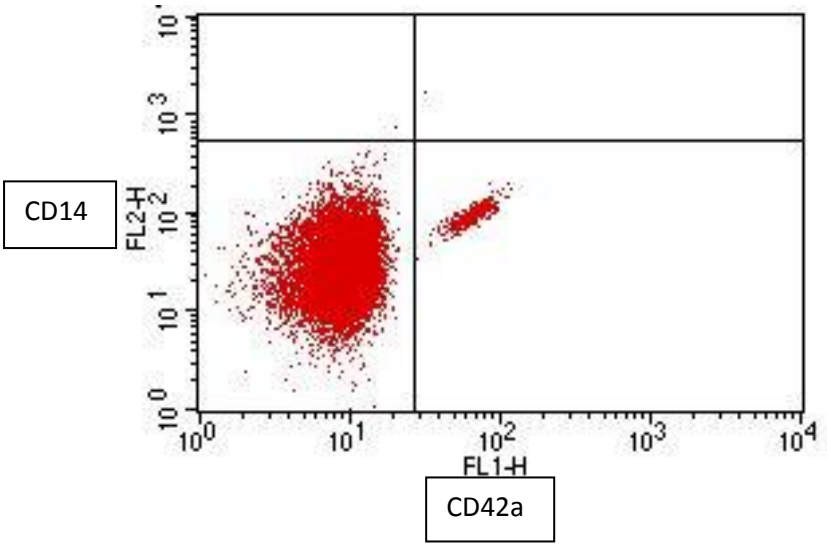

Figure 2.4 *The cut-off used to separate neutrophils (Left lower quadrant) by a horizontal line during flow cytometry where FL2 represents CD14 expression.*

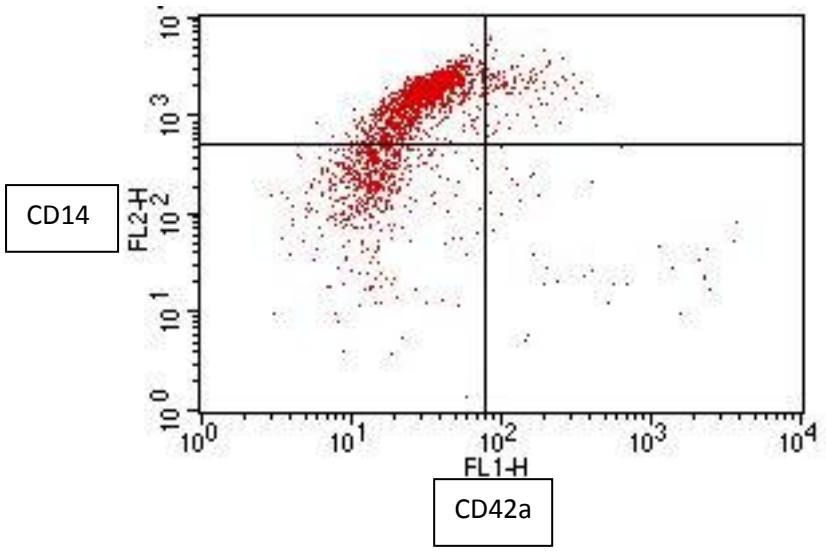

Figure 2.5 *The platelet-monocyte complexes were measured by calculating the proportion of the events in upper right quadrant from the total events in both upper left and upper right quadrants. FL1 denotes CD42a (to mark platelets) and FL2 is consistent with CD14 (to mark monocytes).*

P-selectin, PSGL-1 and CD40L expression was measured by labelling the samples with CD62p, CD162 and CD154 respectively. The samples were run at two settings (high and low forward scatter) to compensate for cell debris. Moreover, both FITC conjugated as well as R-PE conjugated controls were used during the flow cytometry analysis.

2.1.2 ELISA for P-selectin, CD40L and PECAM-1

Plasma levels of P-selectin, CD40L and Platelet endothelial adhesion molecule (PECAM-1) were measured by sandwich ELISA. All reagents were obtained by Bender MedSystems (Vienna, Austria). The plasma samples were obtained by standard phlebotomy and kept at -80^{o}C and were thawed at room temperature prior to centrifuge. A standard 96 wells ELISA plate was used for the measurement of the concentration of all of the above molecules. A good quality absorbance curve

was obtained prior to the measurements. The plasma levels were measured in duplicate and the recommended manufacturer's protocols were followed.

2.2 Gastro-esophageal Reflux and IPF Study

This study evaluated the role of reflux in IPF in a number of subjective and objective parameters. The objective parameters included *Helicobacter Pylori* serology and exhaled breath condensate pepsin. The subjective assessment was done by completing two questionnaires ie Hull airway reflux questionnaire (HARQ) and over-the counter-medication questionnaire.

2.2.1 *Helicobacter Pylori* study

6 ml of peripheral blood was obtained according to the standard protocol. The serum was stored at -80^{o}C immediately after centrifugation (1500xg at 20^{o}C for 10 minutes). The presence of *Helicobacter Pylori* was detected by rapid antibody detection by ELISA (immunoassay for IgG) in serum.

2.2.2 Exhaled Breath Pepsin Study

Exhaled breath condensate (EBC) was collected using a dreschel glass flask (Figure 2.6). The participants were asked to breathe into the device while a nose clip was applied. The condensate (1-2 ml) was collected after 10 minutes of tidal breathing. EBC sample was divided into 250 µL aliquots. The presence of pepsin was detected by lateral flow technique (by using 2 monoclonal antibodies specific to human pepsin). Pepsin positivity in EBC is suggestive of extra-esophageal reflux as it is a gastric enzyme and respiratory secretions do not normally contain this enzyme.

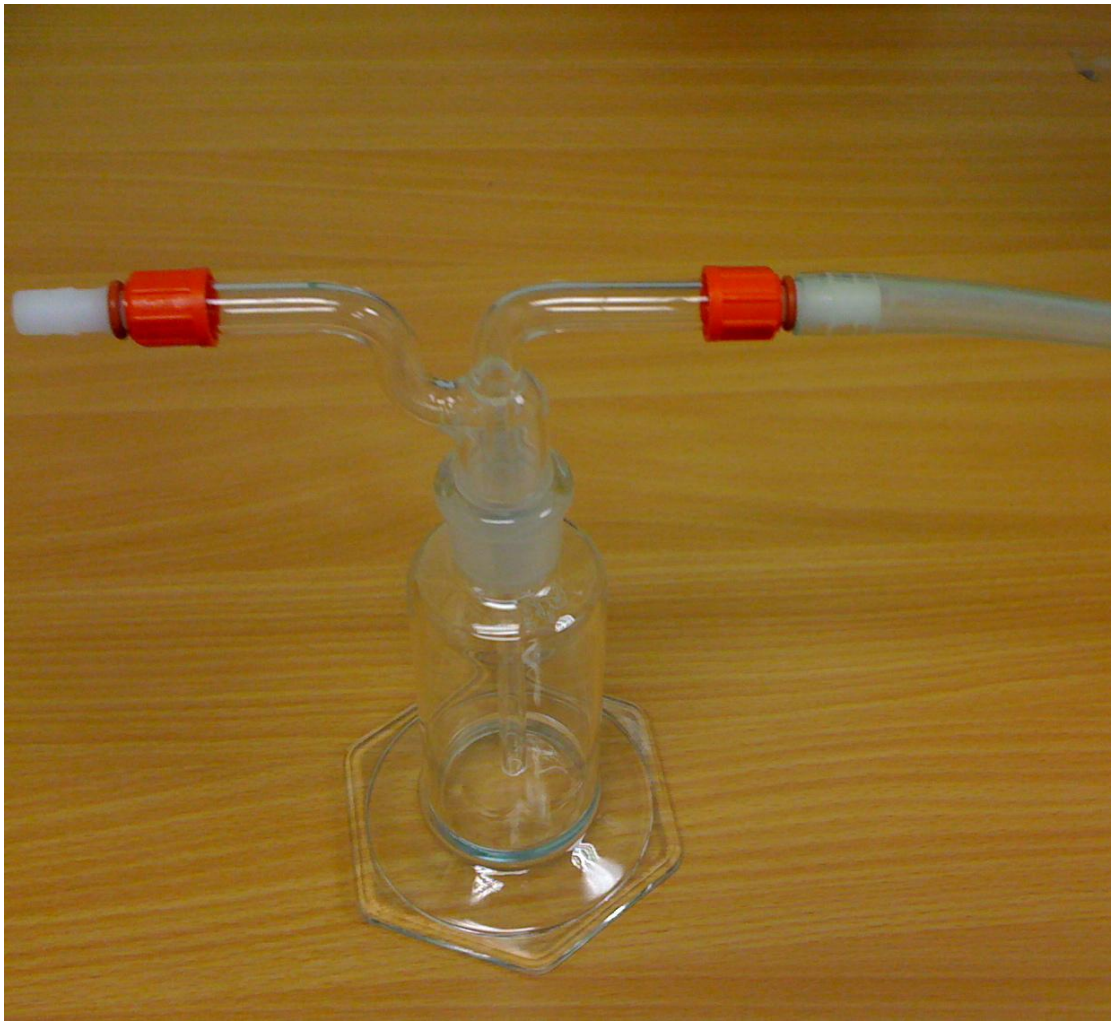

Figure 2.6 *Exhaled breath condensate device used for the collection of sample. A nose clip was used and 1-2 ml of sample was obtained after 10 minutes of normal tidal breathing. The device was kept in an ice box during the collection.*

2.2.3 Over-the-counter medication/Hull airway reflux questionnaire study

As part of investigation of gastro-esophageal reflux in idiopathic pulmonary fibrosis, a questionnaire of over-the-counter medications was developed and was completed by a cohort of IPF patients and controls. The medications included in the questionnaire are shown in Table 2.2. The parameters recorded in the questionnaire attempted to include any previous over the counter remedies and the dosage being taken at present and in the past.

	Have you ever taken it?	If YES, are you still taking it now?	How many/ how much per day?	For how long have you been taking it? (If you have stopped, how long did you take it for?). Please give approximate dates.
Cod Liver Oil	YES / NO	YES / NO		
Omega 3, EPA, or other fish oils	YES / NO	YES / NO		
Echinacea	YES / NO	YES / NO		
Garlic capsules	YES / NO	YES / NO		
Ginko tablets	YES / NO	YES / NO		
Glucosamine (with or without chondroitin)	YES / NO	YES / NO		
Guarana tablets	YES / NO	YES / NO		
Oil of peppermint	YES / NO	YES / NO		
Olbas Oil	YES / NO	YES / NO		
Coenzyme Q10	YES / NO	YES / NO		
St John's Wort	YES / NO	YES / NO		
Turmeric capsules	YES / NO	YES / NO		
Any others?	YES / NO	YES / NO		

Table 2.2 ***Over-the-counter medication questionnaire***

For the assessment of airway reflux symptoms, Hull airway reflux questionnaire was completed. It is a 14 item questionnaire with scores of 0-5 for each item (Table 2.3).

Within the last MONTH, how did the following problems affect you? **0 = no problem and 5 = severe/frequent problem**						
Hoarseness or a problem with your voice	0	1	2	3	4	5
Clearing your throat	0	1	2	3	4	5
The feeling of something dripping down the back of your nose or throat	0	1	2	3	4	5
Retching or vomiting when you cough	0	1	2	3	4	5
Cough on first lying down or bending over	0	1	2	3	4	5
Chest tightness or wheeze when coughing	0	1	2	3	4	5
Heartburn, indigestion, stomach acid coming up (or do you take medications for this, if yes score 5)	0	1	2	3	4	5
A tickle in your throat, or a lump in your throat	0	1	2	3	4	5
Cough with eating (during or soon after meals)	0	1	2	3	4	5
Cough with certain foods	0	1	2	3	4	5
Cough when you get out of bed in the morning	0	1	2	3	4	5
Cough brought on by singing or speaking (for example, on the telephone)	0	1	2	3	4	5
Coughing more when awake rather than asleep	0	1	2	3	4	5
A strange taste in your mouth	0	1	2	3	4	5

Table 2.3 ***Hull airway reflux questionnaire*** *(Courtesy of Prof Morice). This symptom based assessment of airway reflux has been developed to address oesophageal and extra-esophageal reflux symptoms (Everett & Morice 2007;Morice et al. 2011)*

2.3 Anti-epithelial and endothelial antibodies and IPF

Anti-epithelial antibodies

To evaluate the role of anti-epithelial antibodies, we evaluated 56 patients with indirect immunofluorescence. A549 alveolar epithelial cells were used for this purpose. The cells were obtained from ECACC (animal cell line database, Wiltshire, UK) and were cultured in 10% serum with Dulbeco's modified eagle medium (DMEM, Invitrogen, UK). A549 cells were allowed to adhere to monospot glass slides, fixed with paraformaldehyde, and permeablized with 0.1% Triton X-100 for 15 minutes. 1% PBS/BSA was used as a blocking agent and incubated for 30 minutes. This was followed by 30 minutes' incubation with 1:40, 1:160 and 1:640 serum dilutions or with 0.1% PBS/BSA as a control. The slides were washed by distilled water and air dried between each incubation. The secondary antibodies were used in 1:100 dilutions and were incubated for 30 minutes. As there was no change in immunofluorescence pattern with different serum dilutions, the final analyses were carried out with 1:40 dilution. The secondary antibodies used in this investigation were bought from the following sources:

Goat F (ab')2 Anti-Human IgG (gamma) and Goat F (ab')2 Anti-Human IgM (μ) were from Invitrogen CALTAG laboratories, Carlsbad, CA USA. Anti-IgG and Anti-IgD were bought from the same source. We commenced our study looking at IgD, IgA, IgM and IgG antibodies. As the initial pilot study demonstrated no significant difference in immunofluorescence with IgD and IgA antibodies, we evaluated IgM and IgG in the detailed investigation. Vecta Shield (Vector laboratories, CA USA) was used as a mounting medium. Fluorescent antibody complexes were visualized using an Olympus microscope and images were recorded with a digital camera.

Anti-endothelial antibodies

For the evaluation of anti-endothelial antibodies, HUVEC (Human umbilical vein endothelial cells) were cultured in endothelial culture medium (Invitrogen, UK). As they were fixed with methanol, Triton X was not used for permeabilization. The serum dilution used for HUVEC was 1:40 and anti-IgG and IgM antibodies were incubated at 1:100 dilution. The blocking agent used was 1%PBS/BSA and 0.1% PBS in BSA was used as control. The protocol was same as for epithelial cells and anti-IgG and IgM antibodies were from the same source as A549 cells.

Scoring method for immunofluorescence

Quantitative scoring for the intensity of fluorescence was performed by single observer (the author). The fluorescence was scored on a scale of 0-3 for both IgG and IgM. The intensity of staining was scored by taking into account the pattern on the whole slide rather than focusing on one particular area of abnormality. Following were the scores according to fluorescence on each slide:

0= No difference in fluorescence as compared to control

1= Mildly increased fluorescence as compared to control

2= Moderately increased fluorescence as compared to control

3= Severely increased fluorescence as compared to control

2.4 Carcinoembryonic antigen and idiopathic pulmonary fibrosis

To evaluate the association of CEA rise with lung function impairment in IPF, we evaluated serum levels of CEA in a group of 43 patients with IPF. As there is a well defined reference range for CEA in normal subjects (0-5 ng/ml), no healthy controls were evaluated in this study. The samples were collected by standard phlebotomy technique using 20-gauge needle and 6 ml of whole blood was centrifuged at 1500xg for 10 minutes immediately after collection. Following are the methodological details for each component of the study.

(1) Measurement of serum CEA levels

The serum level of carcinoembryonic antigen was measured by 2-site sequential chemiluminescent immunometric assay.

(2) HRCT Evaluation

Thoracic CT scans were evaluated by a single observer (the author). The scans were assessed for pattern of fibrosis including predominant reticulation, predominant honeycombing or mixed pattern. The extent of fibrosis was determined for the entire lung, using four categories (0 = no involvement, 1 = <25% involvement, 2 = 26-50% involvement, 3 = 51-75% involvement, and 4 = 76-100% involvement).

(3) Surgical biopsies and tissue preparation

A lung biopsy specimen from a patient with IPF and raised CEA level (8.21 ng/ml) was stained for CEA. Furthermore, normal lung tissue from a lobectomy specimen was used as a control in this study. The specimens were stained using a standard histopathology laboratory protocol using immunohistochemistry.

CHAPTER 3: PLATELET MONOCYTE COMPLEXES AND IDIOPATHIC PULMONARY FIBROSIS

3.1 Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic interstitial lung disease of unknown aetiology, characterized by irreversible pulmonary fibrosis and a restrictive ventilatory defect. It is the commonest of the idiopathic interstitial pneumonias (IIP) with a median survival of 2.5-3 years after diagnosis (Kim, Collard, & King, Jr. 2006).

IPF has been linked closely with cardiovascular disease (Hubbard et al. 2008) in observational studies and there are similarities in molecular pathogenesis of atherosclerosis and pathological lesions in idiopathic pulmonary fibrosis. For example, monocyte recruitment to the site of vascular damage is one of the initial steps in atherosclerosis (Consigny 1995). Moreover, adhesion of platelet-monocyte complexes to the vessel wall enhances monocyte clustering, which is mediated by P-selectin and PSGL-1 on platelets and monocytes respectively (da Costa et al. 2004). In atherosclerosis, there is an exuberant endothelial fibro-proliferative response with recruitment of leukocytes, extracellular matrix deposition (Katsuda & Kaji 2003) and smooth muscle cell proliferation (Schwartz, Virmani, & Rosenfeld 2000). IPF is characterized by myofibroblast stimulation and release of pro-fibrotic mediators culminating in an exuberant fibro-proliferative response and smooth muscle hypertrophy in the pulmonary vasculature in response to as yet unidentified injury.

There is increased binding of platelets to circulating monocytes in the peripheral blood in patients with myocardial infarction and unstable angina (Sarma et al. 2002). Moreover, there is evidence of peripheral blood monocytosis in

idiopathic interstitial lung diseases possibly reflecting oxidative stress associated with ILD (Varney et al. 2008). This oxidative stress may be exacerbated by cigarette smoke. Indeed smoking is linked with increased platelet-monocyte aggregation and is associated with up regulation of CD40L and its receptor CD40 (Harding et al. 2004).

Circulating platelet-monocyte aggregates are a sensitive marker of in vivo platelet activation and are superior to platelet surface P-selectin (Michelson et al. 2001). Platelet adhesion to monocytes and PMN leukocytes is primarily mediated through PSGL-1-P-selectin interaction. This platelet interaction with leukocytes may alter their activation and recruitment patterns (Merhi et al. 1997;Neumann et al. 1997;Theilmeier et al. 1999). There is evidence of differential platelet binding to monocytes in comparison to PMN as the proportion of monocytes bound to platelets remains the same in whole blood and mononuclear cell fractions (Bournazos et al. 2008b). However, PMN binding to platelets is dramatically reduced in isolated PMN cell preparations. We hypothesized that there may be increased platelet activation in IPF leading to formation of platelet-monocyte complexes, which may initiate the alveolar injury at the vascular endothelial /alveolar epithelial interface.

We hypothesized that there is increased platelet-monocyte adhesion in peripheral blood in IPF. In this study, we used flow cytometry to examine platelet-monocyte binding and markers of platelet activation in peripheral blood. Moreover, plasma levels of P-selectin, CD40L and PECAM-1 were measured to evaluate correlation between these molecules and platelet-monocyte complexes.

3.2 Statistical analysis

All statistical analyses were performed using SPSS (Version 17, Chicago IL, USA). The Kolmogorov and Smirnov method was used to assess data distribution. Comparisons between patient groups were made by one-way ANOVA and independent sample t-tests for normally distributed data. The data with skewed distributions were analysed by non-parametric tests (Mann-Whitney U test and Kruskal-Wallis test). Pearson correlation coefficient was used to evaluate the correlation between lung function and platelet-monocyte adhesion. A multivariate regression model was utilized for the comparison between markers of platelet activation and surface expression of these molecules on leukocytes and platelets. The level of statistical significance was set at 0.05.

The power calculation analysis showed that a total of 45 subjects would provide 80% power to detect a difference of ≥15% in PMC at a significance level of 5%. This calculation was conducted using estimates of effect size based on previously published data (Dotsenko et al. 2007). The threshold of 15% was chosen on the basis of previously published data demonstrating a difference of ≥15% or more in patients with acute coronary syndrome and control subjects (Michelson et al 2001). As there is no previous study evaluating platelet-monocyte aggregation in IPF, a threshold of 15% was considered biologically significant for aforementioned reason.

3.3 Results

The basic demographics of study populations [Table 3.1] show that patients were older in the IPF group. The only other significant difference in the study groups was in blood monocyte counts with higher counts in control patients without interstitial lung disease. The rest of the parameters were similar and the groups were adequately matched. The complete data with respect to diagnoses and platelet-monocyte binding is demonstrated in Table 3.2.

Platelet-monocyte adhesion in Peripheral Blood

The platelet-monocyte interaction was assessed by 2-color flow cytometry to distinguish monocytes and neutrophils by their distinct laser scatter properties. CD14 was used to identify the monocytes to eliminate the possibility of large lymphocytes with similar laser scatter properties to be included in the analysis. Platelets were identified using CD42a (FITC conjugated) and monocytes were identified using CD14 (R-PE conjugated). The platelet-monocyte complexes were assessed by labelling the lysed blood with both CD42a and CD14.

Table 3.1 Selected demographics and clinical characteristics of study participants

	IPF	Non IPF ILD	Non ILD Controls	p Value
Subjects (n)	19	9	14	
Age (years, mean±SD)	69±7	58±9	56±21	0.005*
Gender				0.064
Male (n)	15	6	6	
Female (n)	4	3	8	
Platelet count	246±67	248±72	265±91	0.882
Monocyte count	0.73±0.14	0.59±0.2	0.95±0.17	0.002*
White cell count	7.85±2.2	7.43±2.48	9.36±0.7	0.290
Smoking (n)				0.052
Current	1	1	0	
Ex/ Never	18	8	14	
Hypertension %	42	11	22	0.285
Diabetes %	5	11	29	0.142
Ischemic heart disease %	5	0	11	0.642
Thrombo-embolism %	5	11	7	0.194
Anticoagulation %	5	22	7	0.215
Clopidogrel %	0	0	0	
FVC % predicted	89±18	86±11	NA	0.482
TLco % predicted	57±19	56±15	NA	0.356

Data are presented as mean±SD or percentage % unless stated otherwise. FVC: forced vital capacity; TLco: Total gas transfer coefficient; NA: Not applicable.

* Statistical significance at α-level of 0.05.

Table 3.2 Complete data on basic characteristics, diagnoses and platelet-monocyte complexes (PMC) with and without EDTA. *DIP: Desquamative interstitial pneumonia; FB: Follicular bronchiolitis; HP: Hypersensitivity pneumonitis; NSIP: Non specific interstitial pneumonia; RA-ILD: Rheumatoid arthritis associated interstitial lung disease.*

S.No	Age	Gender	Diagnosis	PMC (No EDTA) %	PMC (EDTA) %
1	78	M	IPF	19.10	4.70
2	62	M	IPF	12.30	3.00
3	72	M	IPF	33.00	8.50
4	68	M	IPF	48.00	3.60
5	78	F	IPF	51.00	9.00
6	83	M	IPF	16.70	1.70
7	77	M	IPF	23.00	2.00
8	73	F	IPF	14.00	2.70
9	72	M	IPF	48.22	7.00
10	75	M	IPF	8.00	2.30
11	58	M	IPF	31.00	7.00
12	61	M	IPF	22.00	4.20
13	57	M	DIP	10.50	3.00
14	51	M	FB	35.30	1.00
15	62	F	RA-ILD	24.00	2.60
16	66	F	RA-ILD	18.00	3.00
17	41	M	HP	42.00	14.00
18	56	M	Occupational ILD	NA	NA
19	31	M	No ILD	32.00	2.10
20	47	F	No ILD	8.80	1.90
21	33	F	No ILD	13.00	4.70

S.No	Age	Gender	Diagnosis	PMC (No EDTA) %	PMC (EDTA) %
22	43	F	No ILD	18.00	2.70
23	37	F	No ILD	NA	NA
24	51	F	No ILD	7.90	0.70
25	82	F	No ILD	17.90	3.20
26	62	F	NSIP	20.00	6.20
27	77	M	COPD	23.00	2.60
28	51	M	Crohns ILD	15.4	1.70
29	72	F	IPF	47.00	3.70
30	69	M	IPF	39.20	6.00
31	45	M	No ILD	12.60	1.20
32	80	F	IPF	65.00	12.28
33	74	F	COPD	4.01	2.80
34	96	F	No ILD	16.20	6.80
35	65	M	IPF	43.90	13.00
36	71	M	IPF	46.00	8.00
37	79	M	IPF	82.40	9.50
38	57	M	IPF	3.00	6.00
39	79	M	IHD	23.00	5.20
40	30	M	No ILD	22.00	4.00
41	72	M	Asbestosis	23.00	2.94
42	50	M	No ILD	NA	NA

The data show that there is significantly increased platelet-monocyte adhesion in IPF as compared to control subjects (IPF 35.6±18%, non IPF-ILD 23.5±10.4%, non-ILD controls 16.5±7.8%; *P*<0.01: one-way ANOVA; Figure 3.1). A post-hoc analysis (Bonferroni) showed that the difference between platelet-monocyte binding between IPF and non-ILD controls was significant (*P*=0.004). However, the aggregation between IPF and non-IPF ILD failed to reach statistical significance (*P*=0.184).

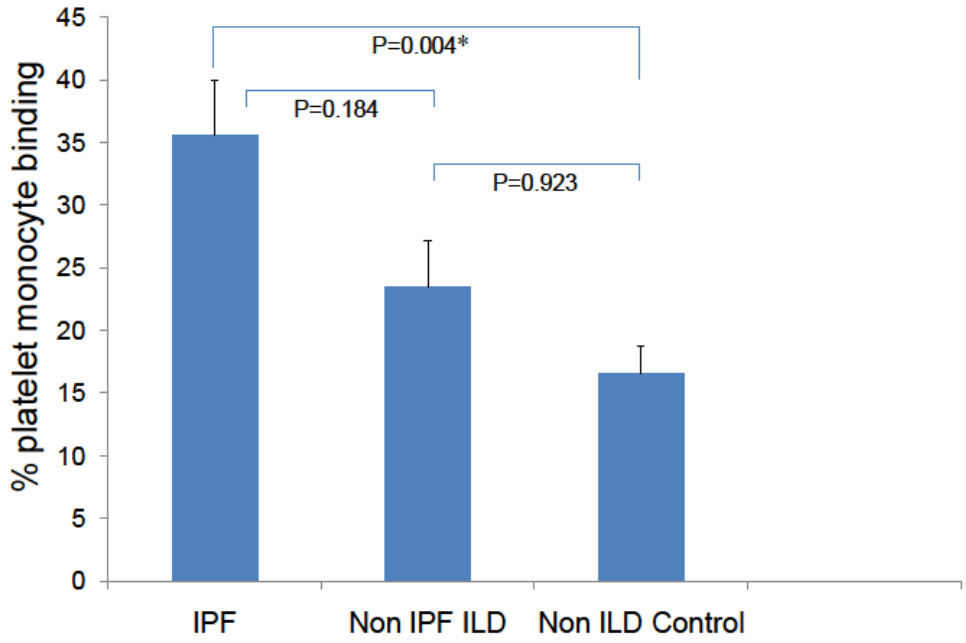

Figure 3.1 *The proportions of mean platelet-monocyte complexes (PMC) observed in flow cytometry analysis showing a significantly higher percentage of platelet binding to monocytes in IPF group (35%) in comparison to non-ILD control subjects (16%).*

During this study, we evaluated the platelet-monocyte adhesion with and without the addition of Ethylenediaminetetraacetic acid (EDTA) to demonstrate the divalent cation-dependent nature of platelet-monocyte binding [Figures 3.2 and 3.3]. An

example of a high percentage of platelet-monocyte aggregates is depicted in Figure 3.4.

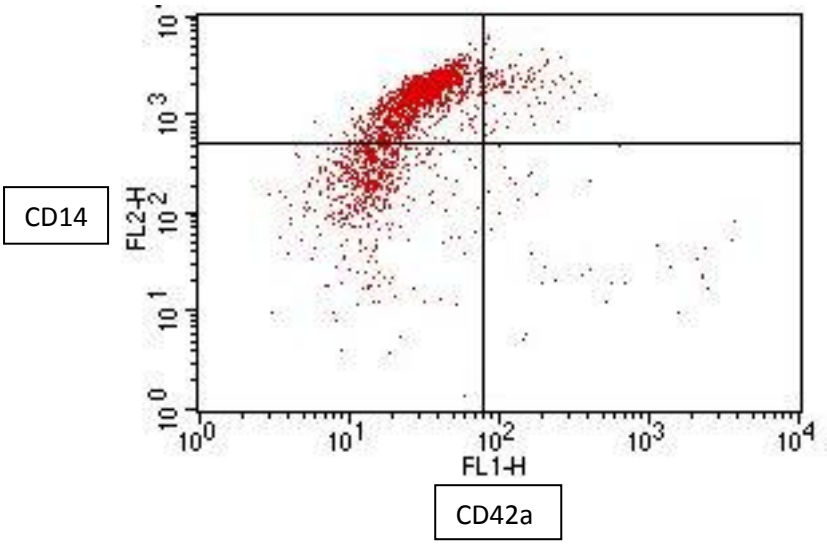

Figure 3.2 *Platelet-monocyte complexes without EDTA show a proportion of 12% in a patient with IPF.*

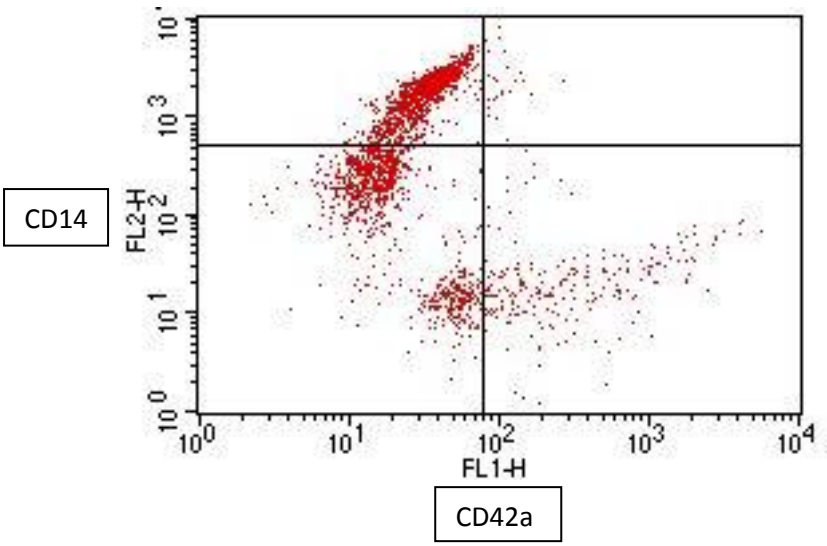

Figure 3.3 *Platelet-monocyte complexes with the addition of EDTA show a reduction in the proportion of aggregates to 3% demonstrating the cation dependent nature of this adhesion.*

Our findings show that there is a distinct population of CD14/CD42a-positive events, clearly evident in the absence of EDTA with only a small proportion of

complexes seen with the addition of EDTA (IPF 6.01% ± 3.3; non-IPF ILD 4.3% ± 4.1; non-ILD controls 3.1% ± 1.7) [Figure 3.5]. However, the proportion of platelet-monocyte adhesion in the presence of EDTA did not reach statistical significance in the three groups evaluated in this study (*P*=0.058; one way ANOVA).

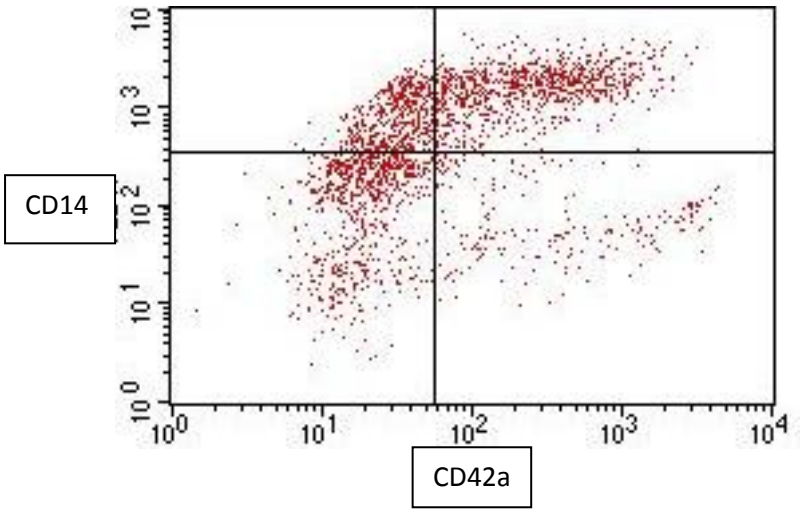

Figure 3.4 *Platelet-monocyte binding of 65% in a patient with IPF.*

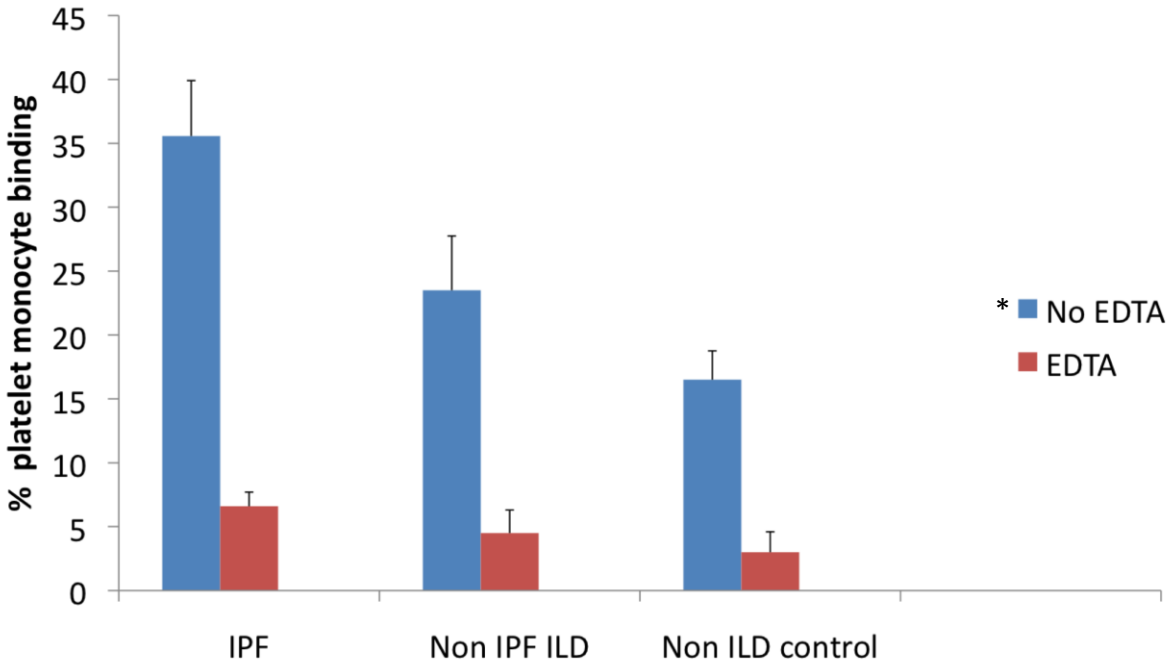

Figure 3.5 *Platelet-monocyte complexes in three study groups with (shown in Red) and without EDTA (shown in Blue). There is marked reduction in platelet-monocyte binding following the addition of EDTA. * Statistically significant*.

Expression of platelet activation markers on leukocytes

Figures 3.6 and 3.7 demonstrate the expression of PSGL-1 and CD40L on monocytes respectively.

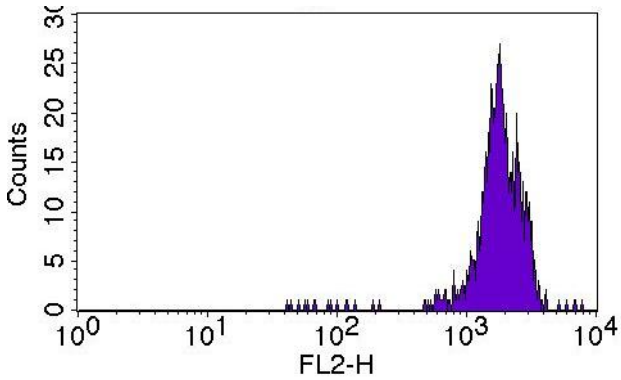

Figure 3.6 *Monocyte expression of PSGL-1. The expression was calculated by the fluorescent peak on the extreme right of the graph. FL2 denotes the R-PE conjugated fluorescent antibody (CD162).*

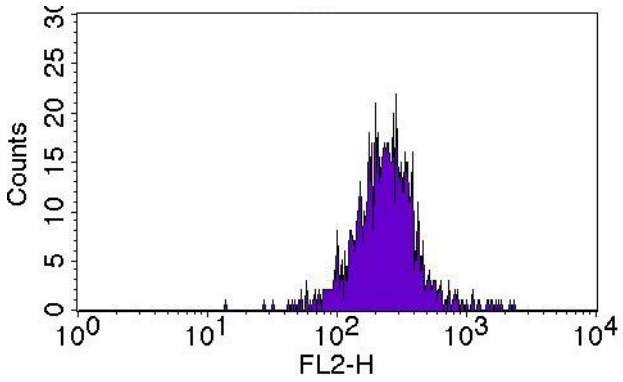

Figure 3.7 *Histogram showing an example of CD40L expression on monocytes in a patient with IPF.*

The platelet expression of activation markers was analysed at a different forward scatter threshold to accurately assess the surface expression of CD62p, CD162 and CD40L. Figures 3.8 and 3.9 demonstrate the flow cytometric settings to evaluate the expression of these activation markers on platelets at a different forward scatter threshold.

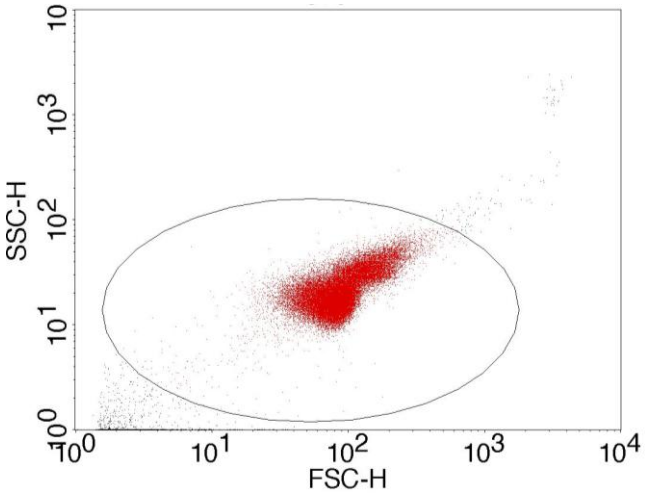

Figure 3.8 *Platelet setting showing the events selected (circle) to evaluate the expression of platelet activation markers on platelets.*

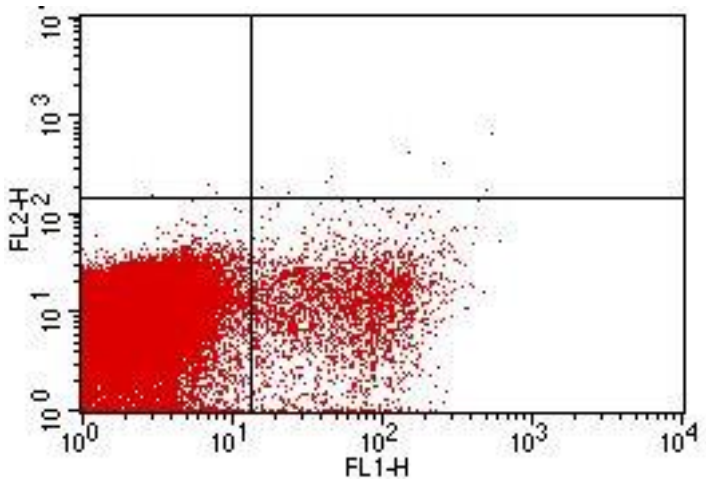

Figure 3.9 *The FL-1 threshold used to select CD42a positive and negative events (vertical line). FL-2 denotes expression of CD62p, CD162 or CD40L.*

PSGL-1 and CD40L expression in different population of leukocytes

As PSGL-1 has a key role in platelet-leukocyte-endothelial interactions and is constitutively expressed by all leukocytes, the expression of this molecule on different subsets of leukocytes (Figure 3.10) and platelets was studied during this investigation. There was no differential expression of PSGL-1 on any of the leukocyte sub-populations (ANOVA).

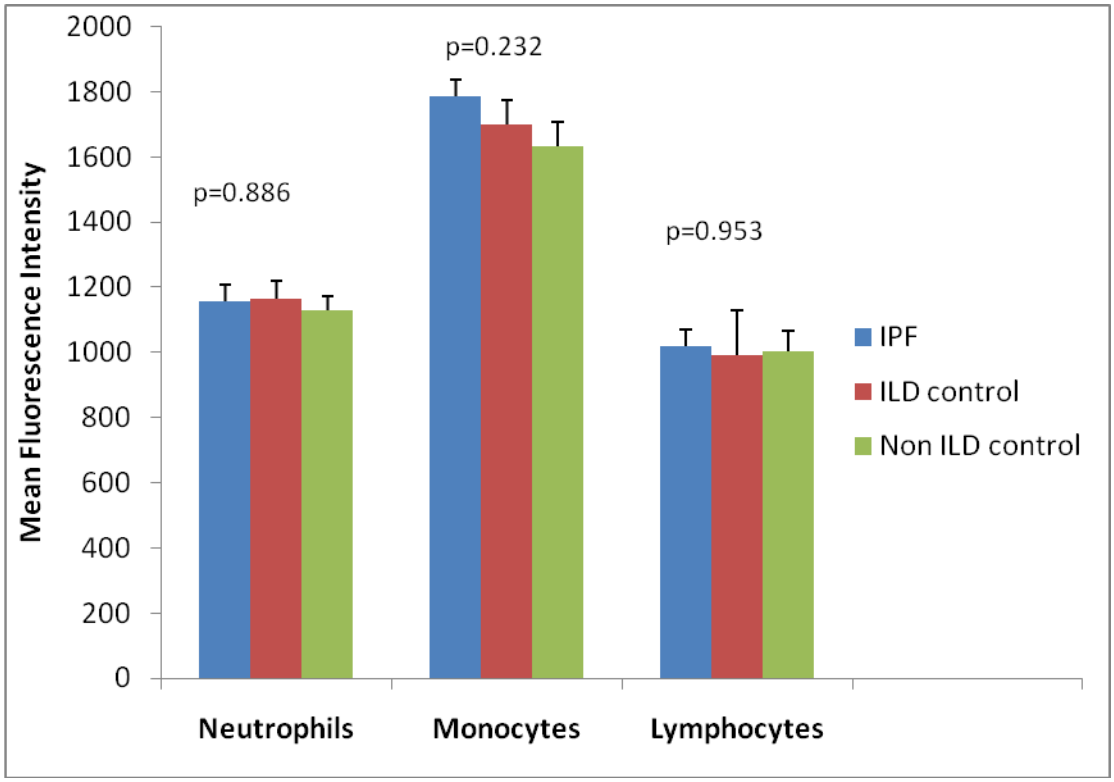

Figure 3.10 *PSGL-1 expression on different subsets of leukocytes in the study groups. There is no significant difference in the surface expression of this molecule between groups.*

The expression of CD40L on different subset of leukocytes is shown in Figure 3.11. Although there was no differential expression of this glycoprotein on monocytes, neutrophils and lymphocytes, there was a trend towards higher CD40L expression on neutrophils and monocytes in non-IPF ILD as compared to IPF or non-ILD control subjects (Kruskal-Wallis test).

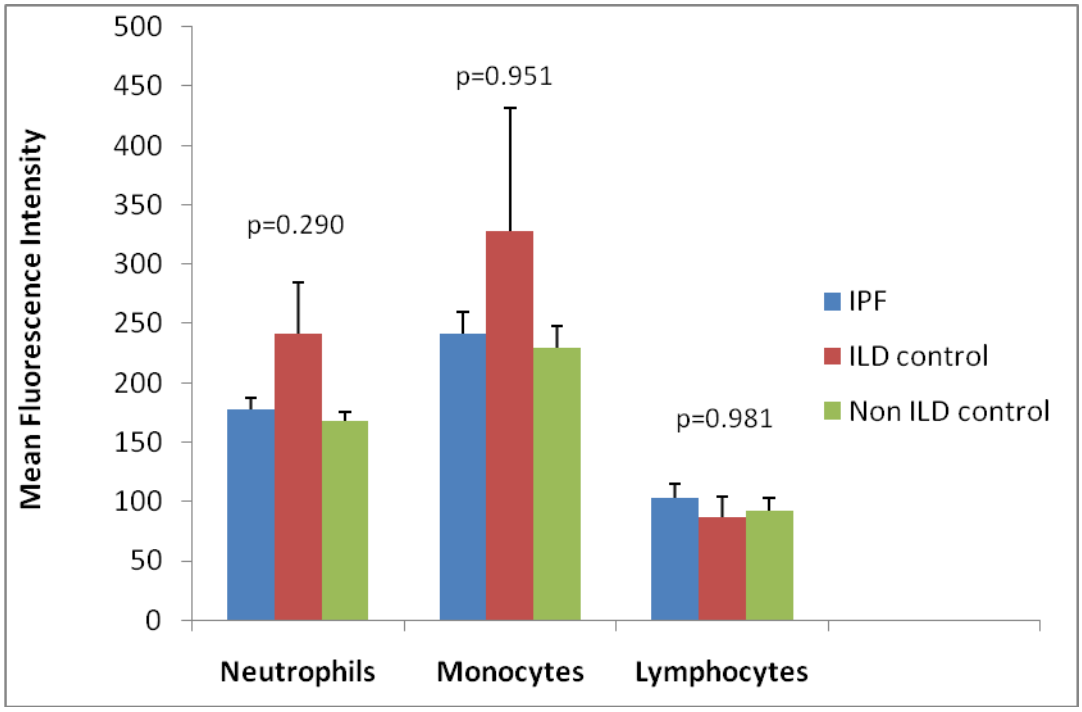

Figure 3.11 *CD40L data reflect a non-significant trend towards higher mean fluorescence on neutrophils and monocytes in non-IPF ILD.*

Platelet expression of P-Selectin, PSGL-1 and CD40L

Similar to the data of platelet activation markers on leukocytes, the platelet expression of CD62p (P-selectin), CD162 (PSGL-1) and CD40L was not significantly different in IPF patients as compared to control subjects (Figure 3.12).

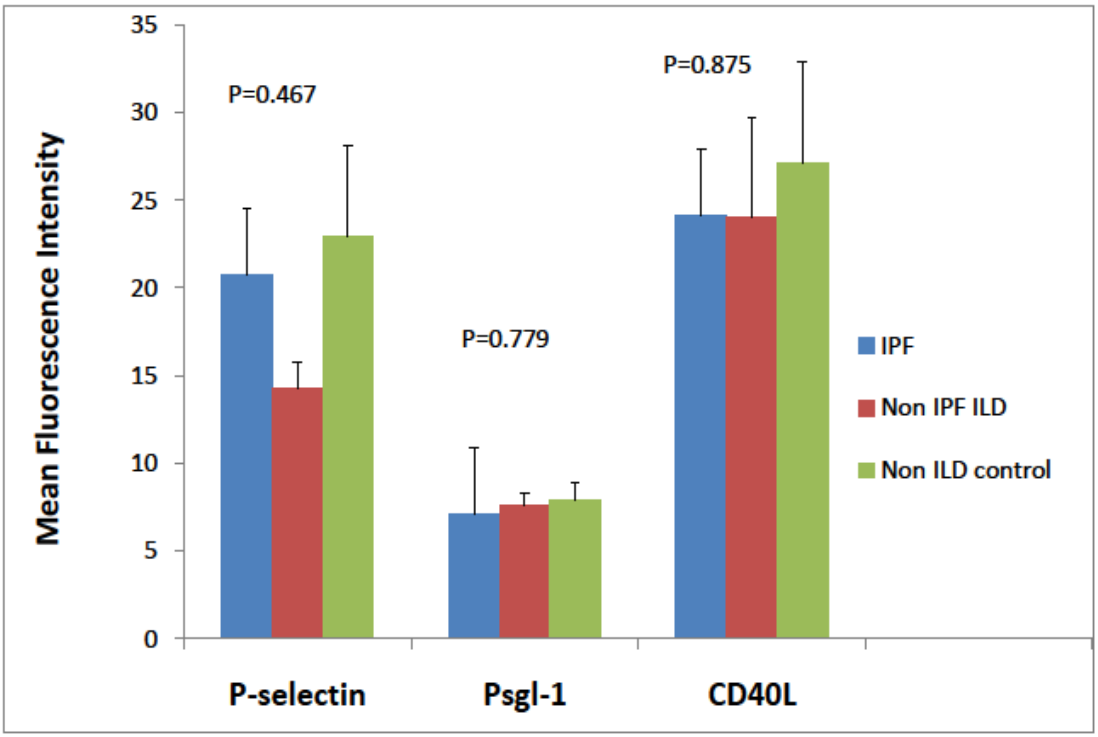

Figure 3.12 *The platelet expression of activation markers demonstrating no differential expression in three study groups.*

Platelet-monocyte complexes and lung function parameters

As part of the investigation of platelet-monocyte aggregates in IPF, the correlation between platelet-monocyte binding and lung function parameters was evaluated. These data show that there was a lack of significant correlation between percentage platelet-monocyte binding and percent predicted FVC (P=0.150, R^2=0.12) or percent predicted TLco (P=0.876, R^2=0.01). These correlations are demonstrated in Figures 3.13 and 3.14.

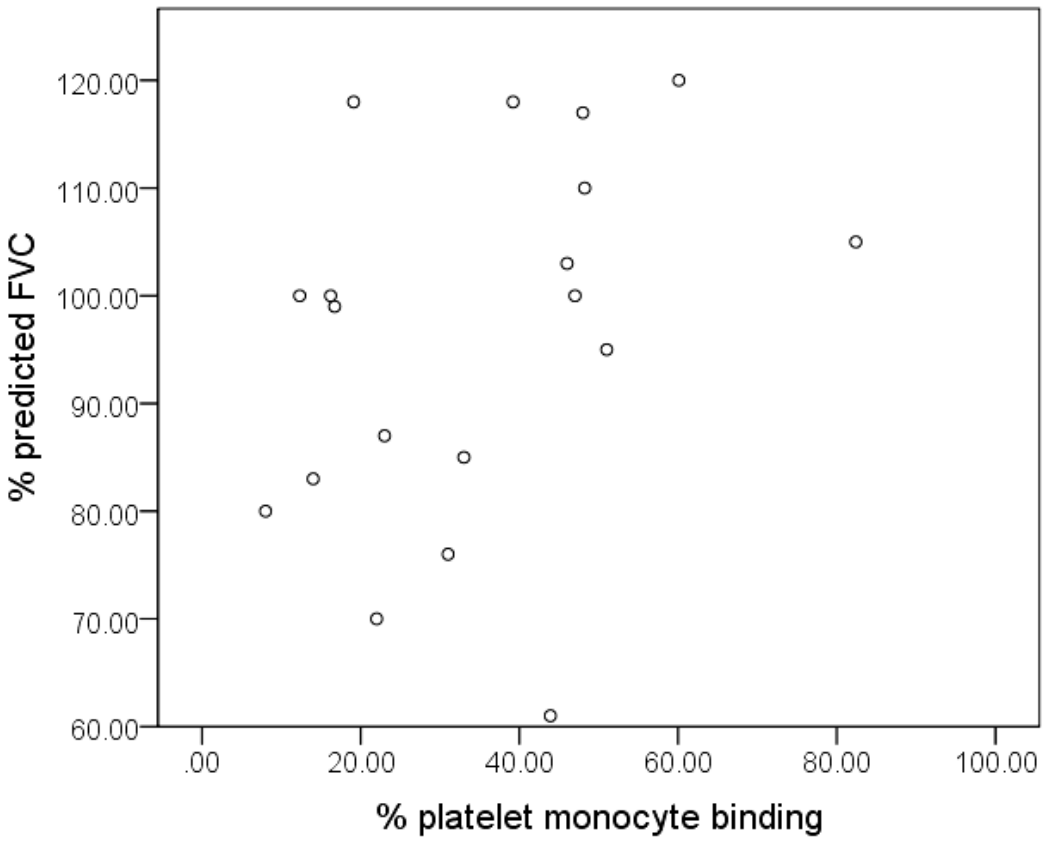

Figure 3.13 *The correlation between platelet monocyte binding and percent predicted FVC in IPF. There was no significant correlation between these parameters (R^2=0.12).*

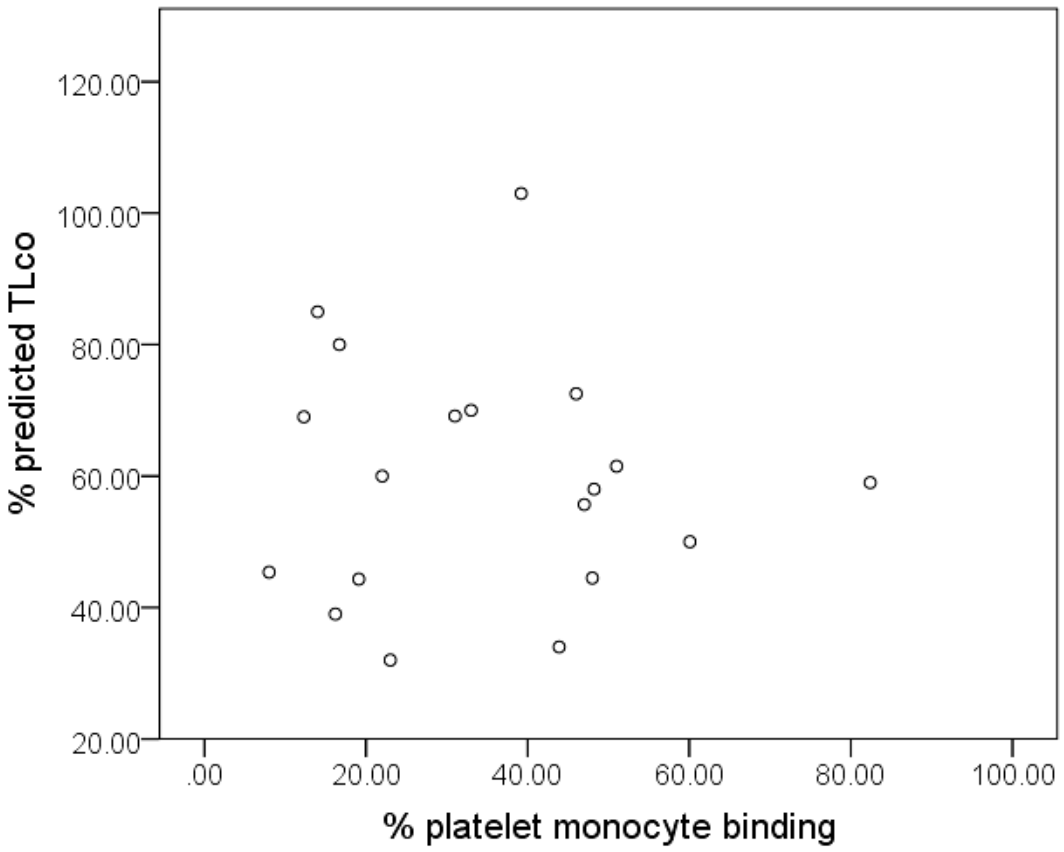

Figure 3.14 *There was no significant correlation between platelet monocyte binding and total gas transfer for carbon monoxide (R^2=0.01).*

A subgroup analysis of platelet-monocyte complexes after stratification of lung function (TLco<40% and ≥40%) showed no differential adhesion of platelets with monocytes in relation to gas transfer (P=0.88, data not shown). These data suggest that platelet monocyte adhesion in independent of lung function impairment in IPF.

Plasma levels of CD40L (CD154), P-selectin (CD62p) and PECAM-1 (CD31)

As part of the investigation of platelet activation markers expression in IPF, concentrations of CD40L and soluble P-selectin were measured in plasma. The distribution of plasma levels of P-selectin and CD40L are shown in Figures 3.15 and 3.16.

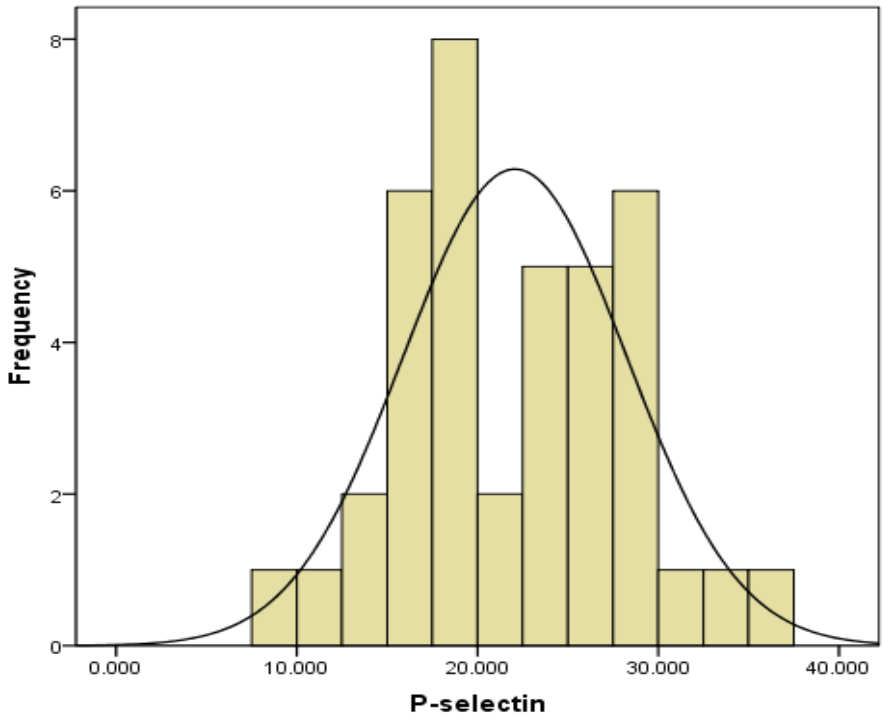

Figure 3.15 *The histogram of plasma CD62p (P-selectin) showing a normal distribution. Hence ANOVA was used for statistical analysis.*

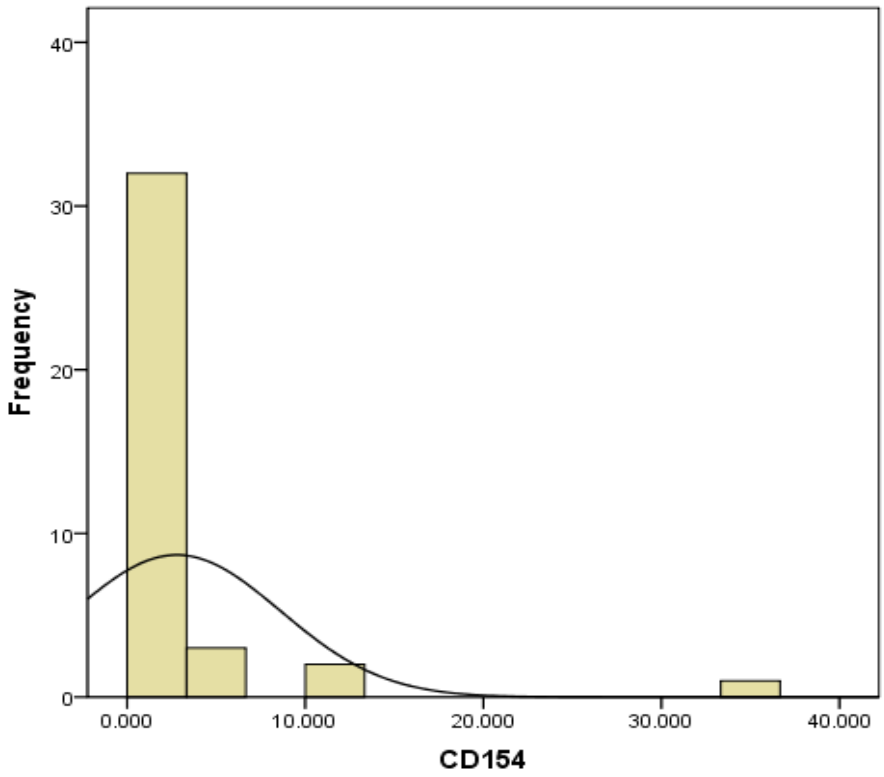

Figure 3.16 *The histogram of plasma CD40L (CD154) showing a skewed distribution. Hence the data were analyzed by non-parametric tests.*

The plasma levels of P-selectin and CD40L in IPF and control subjects are shown in Figures 3.17 and 3.18 respectively.

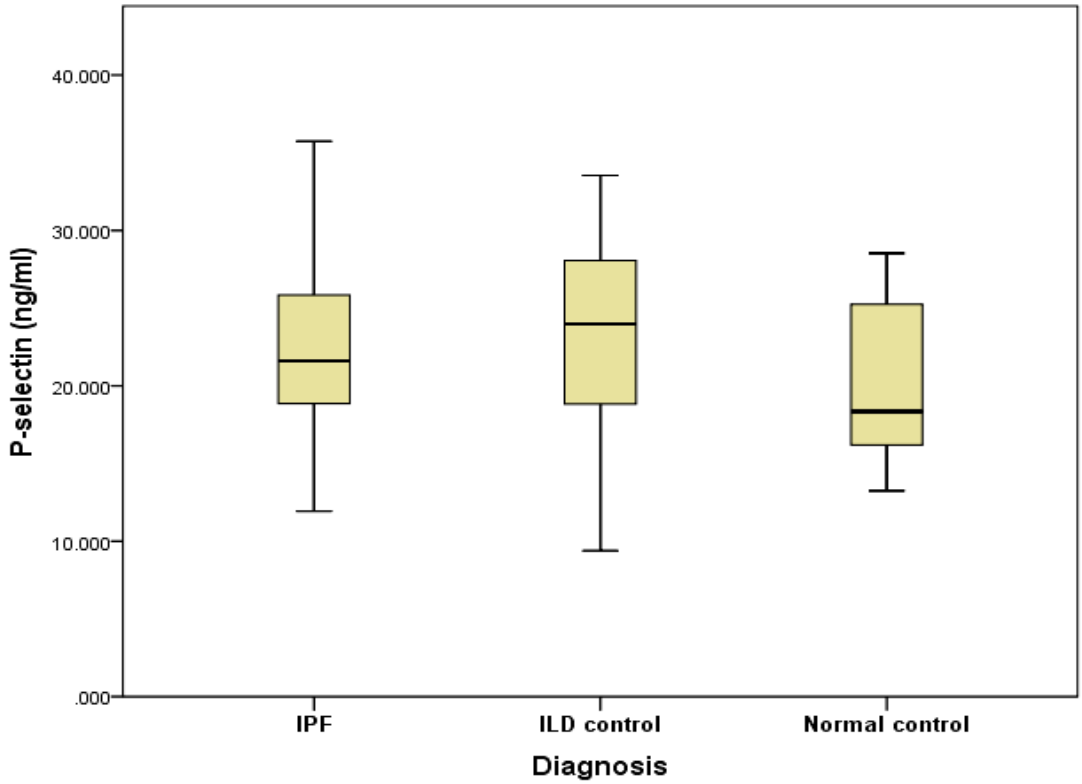

Figure 3.17 *Plasma P-selectin levels in three study groups. There was no statistically significant difference in plasma level in IPF or control subjects. Horizontal bars represent median value.*

The mean plasma P-selectin levels in IPF, non-IPF ILD and non-ILD control subjects were 22.5±6.24, 23.3±7.18 and 20.3±5.38 (ng/ml) respectively (p=0.532; one way ANOVA). There was no significant difference in plasma levels of CD40L across three groups (p=0.621; Kruskal Wallis test). Furthermore, there was no significant correlation between plasma P-selectin, CD40L and platelet monocyte adhesion or lung function parameters.

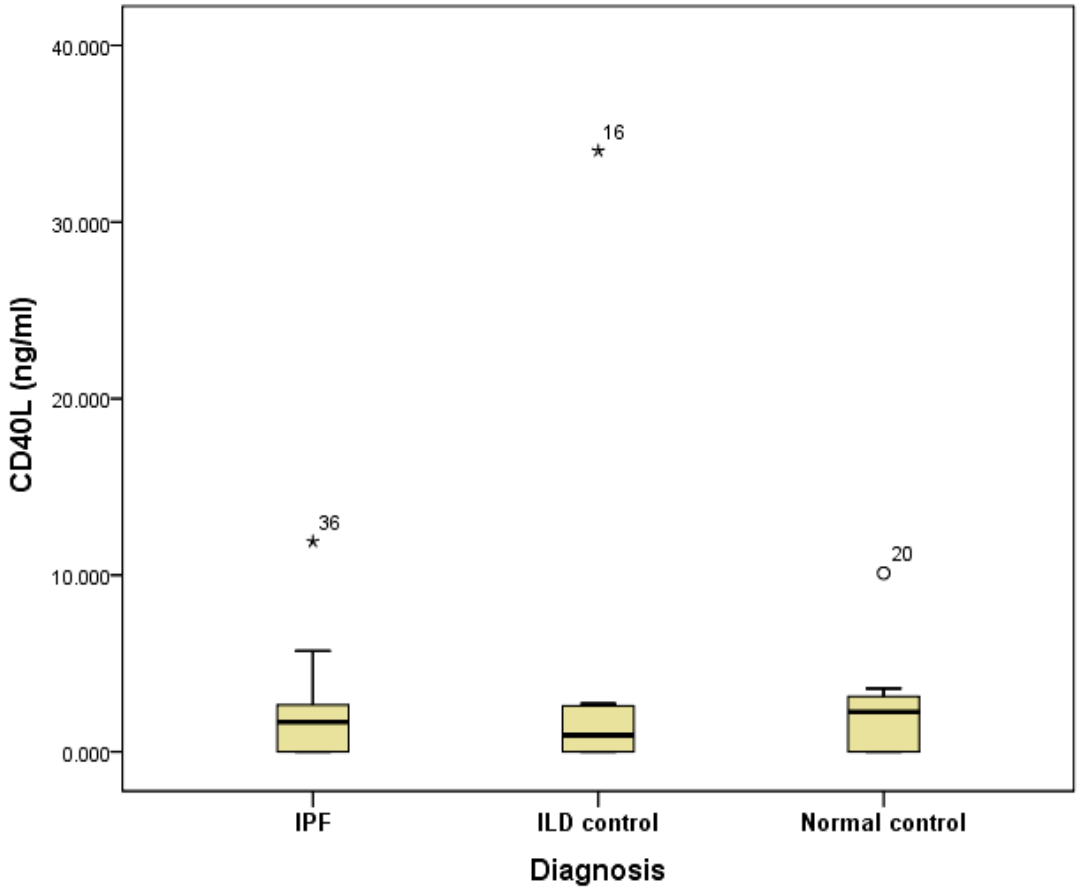

Figure 3.18 *Plasma levels of CD40L (CD154) in three study groups. There was no statistically significant difference in the plasma level of this molecule between groups.*

As part of platelet involvement in the pathogenesis of IPF, we evaluated plasma levels of platelet endothelial adhesion molecule (PECAM-1). This study population was slightly different than CD40L and P-selectin participants as data for PECAM-1 was collected from two groups (IPF and non-IPF group) rather than three groups. As shown in Figure 3.19, plasma levels of PECAM-1 were significantly higher in IPF (106±37 ng/ml) as compared to control population (84±20 ng/ml)[$P<0.05$, unpaired t-test].

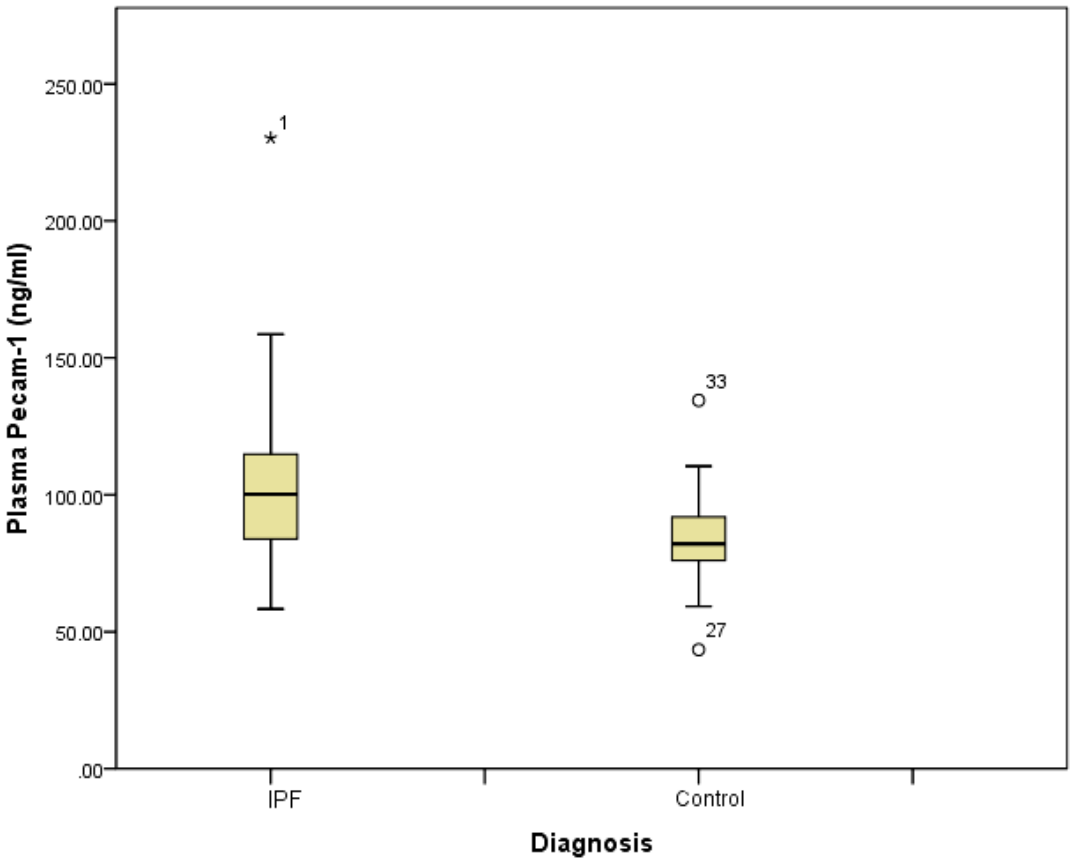

Figure 3.19 *Plasma level of Pecam-1 (ng/ml) in the study groups. There was a statistically significant difference in plasma level of this molecule with higher levels in IPF group.*

3.4 Discussion

To our knowledge, this study is the first investigation of platelet-monocyte interaction in idiopathic pulmonary fibrosis. Monocytes and macrophages are involved in the production of cytokines and tissue remodelling in many inflammatory disorders and indeed in atherosclerosis (Fischer-Betz, Halle, & Schneider 2010). Monocytes are unique in having functional plasticity and their differentiation into different tissue macrophage phenotypes makes them a key cellular component in a number of diseases. There is increased platelet-monocyte

adhesion in end stage renal disease associated with cardiovascular complications (Ashman et al. 2003) as well as in COPD (Maclay et al. 2011). Moreover, there is evidence of progressive accumulation of monocytes in mouse atheroma, which is proportional to the extent of atherosclerosis (Swirski et al. 2006). In view of the fact that there is increased risk of acute coronary syndrome and deep vein thrombosis before and after the diagnosis of IPF, it is possible that these patients are in a pro-thrombotic state that predisposes them to have micro-emboli and subsequent pulmonary vascular injury. Indeed, individuals homozygous for the pro-coagulant factor V R506Q Leiden have increased tendency to have severe dyspnoea as well as decreased lung function with a restrictive ventilatory defect (Juul et al. 2005). Furthermore, there is evidence of activation of the clotting cascade in pulmonary fibrosis (Chambers & Laurent 2002). In light of the above evidence of pro-coagulant environment in pulmonary fibrosis, our findings of increased platelet-monocyte adhesion support the findings of pro-thrombotic state in IPF. Furthermore, these complexes may be the inciting source of alveolar injury culminating in fibrosis and irreversible scarring in IPF.

Our findings are in accordance with a flow cytometric study in cardiovascular patients evaluating platelet-monocyte aggregates in patients with chest pain with and without myocardial infarction (Michelson et al. 2001). Interestingly, the proportions of PMC in acute myocardial infarction and control patients were parallel to the proportions in IPF and controls respectively. These findings further support the link and close association of IPF with vascular dysfunction.

The choice of anticoagulant can have a significant effect on the extent of platelet-monocyte adhesion (Bournazos et al. 2008a) as there is evidence of significantly reduced platelet-monocyte binding with sodium citrate compared with heparin or hirudin. Hence, heparin was used as anticoagulant for this particular investigation.

CD40L is a glycoprotein expressed by CD4+ T cells (Armitage et al. 1992;Noelle et al. 1992), B cells (Grammer et al. 1995), dendritic cells (Pinchuk et al. 1996) and platelets (Henn et al. 1998). We did not find any significant difference in plasma levels of CD40L in IPF or control subjects. It is possible that CD40L is differentially expressed and released in respiratory epithelium as compared to plasma and simultaneous measurement of this molecule in blood and BAL fluid may provide further insight into the interplay between this glycoprotein and platelet-monocyte adhesion in the pathogenesis of IPF.

As smoking may be associated with increased platelet-monocyte binding (Harding et al. 2004), it could be speculated that the differential platelet-monocyte adhesion may be confounded by smokers in IPF group. However, there were no significantly different proportions of current smokers in either group to account for smoking associated increase in platelet-monocyte adhesion. Hence the observed increase in platelet-monocyte complex formation is likely to be independent to the effect of nicotine in our study population.

As platelet-monocyte adhesion has an association with cardiovascular disease, the finding of increased platelet-monocyte binding in IPF could be a mere reflection of the associated cardiovascular complications. Hence, we evaluated the

prevalence of cardiovascular and thromboembolic events in the study population and found no significant difference in these events in either IPF or non-IPF population included in our study. It suggests that the significant increase in platelet-monocyte complexes in idiopathic fibrosis group is independent of their cardiovascular risk and thrombo-embolic event profile in this cohort of participants.

It is interesting to note that platelet-monocyte adhesion had no significant correlation with percent predicted FVC or TLco. This finding of a lack of correlation supports the hypothesis that platelet activation is a possible cause rather than a consequence of pulmonary fibrosis in IPF. It will be of interest to evaluate physiologic parameters of lung function impairment (including FVC and TLco) with platelet activation during the course of IPF to further characterize the association.

Limitations of the study

Although we have shown that platelet-monocyte binding is significantly increased in IPF and provide a possible pathologic mechanism in this relentless fibrotic disorder, we cannot confirm that increased monocyte adhesion to platelets is not a secondary event as a result of lung fibrosis and injury. However, the finding of relatively lower percentages of platelet complexes with monocytes in patients with ILD other than IPF with a similar degree of impairment of gas exchange (data not shown) refutes this potential speculation. The patients in IPF group were older than control subjects that may suggest that the increased platelet-monocyte adhesion observed in this study may be related to older age. However a subgroup analysis of control subjects after stratification into younger (age <70) and older age groups (age >70) suggested that the platelet-monocyte binding did not differ in

either age group (Older group 19.8±10.6%, younger group 18.5±7%; *P*=0.79 unpaired t-test). Hence, it is unlikely that age per se would have had an effect on platelet-monocyte adhesion.

Conclusion

This study suggests that there is differential platelet-monocyte adhesion in idiopathic pulmonary fibrosis in comparison to control subjects without interstitial lung fibrosis. Moreover, PECAM-1 is significantly elevated in plasma of IPF patients. The findings of this study highlight a potentially important mechanism of increased pro-coagulant state that may be related to increased platelet-monocyte aggregation in peripheral blood. It would be crucial to evaluate the pathogenesis of this relentless fibrotic disorder at microvascular level for better understanding of the aetiology and potential therapy.

CHAPTER 4: GASTRO AND EXTRA-ESOPHAGEAL REFLUX AND IPF

4.1 Introduction

In view of a significantly high prevalence of gastro-esophageal reflux in IPF, we evaluated the hypothesis that patients with IPF have higher symptom prevalence of acid as well as non acid reflux (assessed by the Hull airway reflux questionnaire). Moreover, a questionnaire was designed to address over-the-counter remedies on the basis of our clinical observation that patients with ILD and particularly with IPF take cod liver oil on a regular basis. We enquired the frequency and dosage of the most common over-the-counter remedies taken by this cohort of patients and compared it with a cohort of COPD patients as they have similar profile of co-morbidities.

The objective measurement of *H. Pylori* prevalence was undertaken to test the hypothesis that there is increased prevalence of *H. Pylori* in IPF with subsequent reflux into respiratory tract resulting in alveolar epithelial injury as this bacterium is associated with epithelial injury of the columnar epithelium of gastric mucosa (Hatakeyama 2008) that may be direct injury or an immune mediated phenomenon (Ivie et al. 2008).

Pepsin is an enzyme released by chief cells of the epithelial lining of gastric mucosa (Figure 4.1) and was discovered by Theodor Schwann in 1836. It is released in pro-enzyme form pepsinogen. In the acidic environment of stomach created by hydrochloric acid (HCL), pepsinogen is cleaved into its active form pepsin. The main function of this enzyme is degradation of food proteins into peptides and amino acids.

Gastric enzyme pepsin and respiratory tract "Reflux hypothesis"

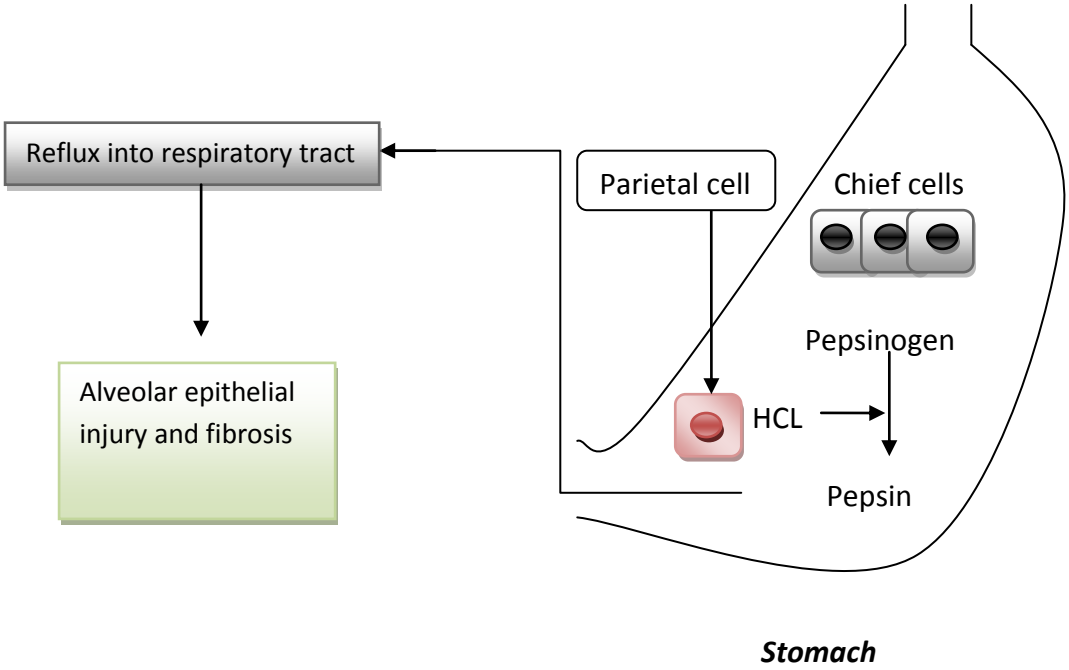

Figure 4.1 *Pathophysiological mechanism proposed by "reflux hypothesis" as a potential cause of interstitial pulmonary fibrosis. Exhaled breath condensate pepsin may provide an additional support for objective assessment of reflux. HCL: Hydrochloric acid.*

We hypothesized that patients with IPF have episodes of gastro-esophageal reflux leading to alveolar epithelial injury that is repeated and might be associated with micro-aspiration. There is evidence of a significant proportion of IPF patients having gastro-esophageal reflux disease on 24-hour oesophageal pH testing. The rationale for measurement of pepsin in exhaled breath condensate is the evidence that this enzyme as a reliable marker of extra-esophageal reflux (Strugala, Dettmar, & Morice 2009).

4.2 Statistical Analysis

The statistical analyses were performed using SPSS (version 17, Chicago IL). The data with normal distribution were analysed with unpaired t-tests and skewed

data were analysed with the Mann-Whitney U test. Categorical data were analysed by Pearson Chi-square and α-value of 5% was considered statistically significant.

4.3 Results

4.3.1 Hull Airway Reflux Questionnaire and over-the-counter medication survey

The main demographics of study participants of the airway reflux questionnaire study are shown in Table 4.1. The mean scores on reflux cough questionnaire were markedly higher in the IPF group as compared to the control subjects (IPF 19.6 ± 12.4; controls 3 ± 2.9; P<0.001) [Figure 4.2]. The upper limit of normal score on HARQ is 13 (for total score) with a mean of 4.

Table 4.1 Baseline characteristics of HARQ study participants

	IPF (n=40)	CONTROL (n=50)	p Value
Age	70±12	68±15	0.210
Gender (M/F)	31/9	31/19	0.221
HARQ score	19.6±12.4	3.0±2.9	<0.01*
FVC %	88±16	NA	
TLco %	56±19	NA	
Medications (n, %)			
Prednisolone	11 (27)	6 (12)	0.055
N-acetylcysteine	3 (6)	0 (0)	0.084
Azathioprine	6 (15)	2 (4)	0.074
PPI	14 (35)	5 (10)	0.004*
Diagnoses in control group (n)		Healthy controls (40)	
		COPD (6)	
		Rheumatoid lung (4)	

Data are presented as mean±SD unless stated otherwise. PPI: Proton pump inhibitors; COPD: Chronic obstructive pulmonary disease; FVC: Forced vital capacity; TLco: Total gas transfer for carbon monoxide; NA: Not applicable. * Statistically significant.

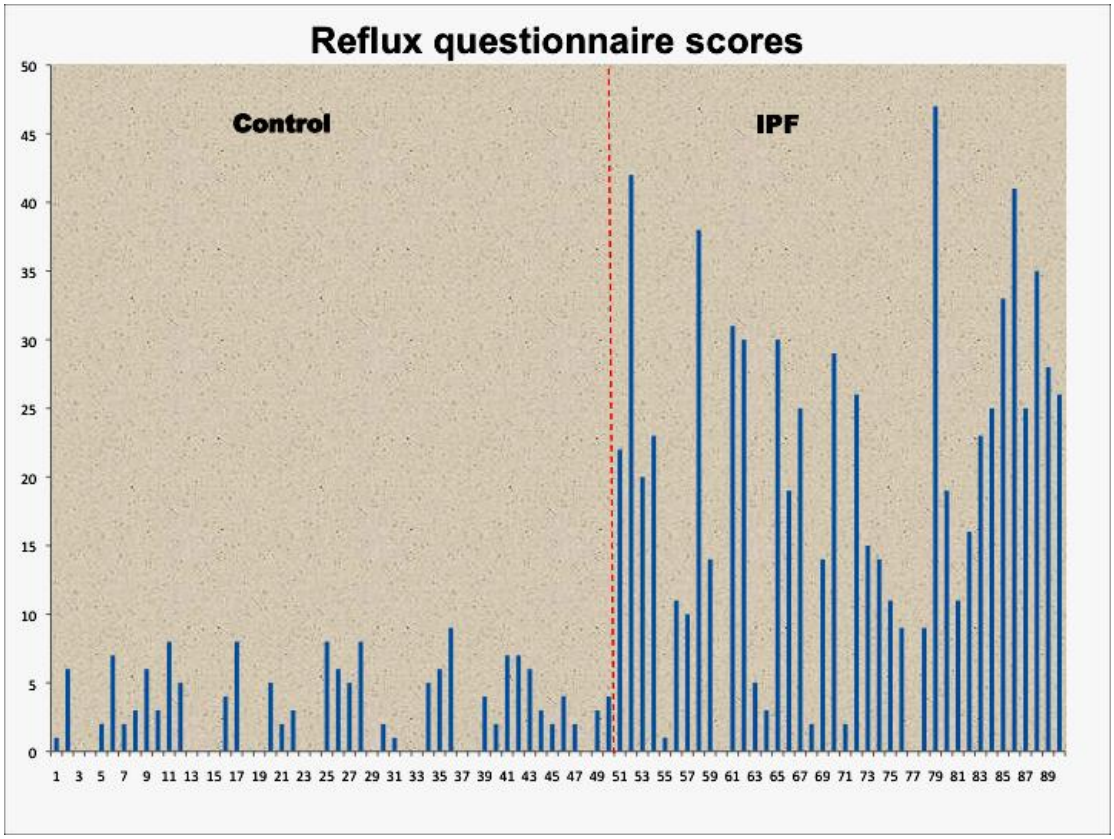

Figure 4.2 *The reflux scores in IPF and control subjects. The bars show individual patients with height of each bar demonstrating the total score on the questionnaire for that participant. The data suggest a marked difference in airway reflux symptoms in the idiopathic fibrosis group with mean score of 19.6 as compared to 3 in control population.*

The findings of significantly worse symptom scores on the questionnaire do support the hypothesis that GERD might play a significant role in the pathogenesis of idiopathic pulmonary fibrosis. The over the counter survey was undertaken in 152 adults with a larger cohort of patients with COPD (n=100) and other interstitial lung diseases to compare the intake of over the counter medications with IPF population. The data show that there was no significant difference in the intake of these remedies over time in either group studied during this survey (Table 4.2; Pearson Chi-square).

Table 4.2 Over-the-counter medications survey

Diagnosis	Cod Liver Oil/Omega 3	No OTC Mediciation
IPF (n=34)	19 (56%)	11 (32%)
Other ILD (n=18)	11 (61%)	6 (33%)
COPD (n=100)	49 (49%)	35 (35%)
		P=0.569

The data are presented as number and percentages. The data were divided into two groups after evaluation of a whole range of remedies as there were a very small number of participants taking remedies other than Cod liver oil/Omega 3 capsules. OTC: over-the-counter.

4.3.2 Helicobacter Pylori study

A total of 57 patients were included in the study. The main demographics of the study population are shown in Table 4.3. The main findings of the serology are shown in Table 4.4. There was no significant difference in the proportion of patients with IPF and controls in terms of *H. Pylori* infection (*P*=0.419 Pearson Chi-square).

Table 4.3 Baseline characteristics of *Helicobacter Pylori* Study participants*

	IPF (n=34)	CONTROL (n=23)	p Value
Age	71±10	67±13	0.032*
Gender (M/F)	29/5	9/14	0.001*
FVC %	81±21	81±23	0.988
TLco %	44±20	64±28	0.212
Medications (n, %)			
Prednisolone	11 (32)	6 (26)	0.419
N-acetylcysteine	1 (3)	0 (0)	0.596
Azathioprine	5 (17)	3 (13)	0.590
PPI	15 (44)	6 (26)	0.047*
Diagnoses in control group (n)		COPD (8)	
		Rheumatoid lung (5)	
		Sarcoidosis (2)	
		Respiratory infection (4)	
		Miscellaneous (4)	

Data are presented as mean±SD unless stated otherwise. * Statistically significant.

Table 4.4 Cross-tabulation of *H. Pylori* status against the diagnosis

	***H. Pylori* status**		
Diagnosis	Positive	Negative	Total
IPF	17	17	34
Control	14	9	23
Total	31	26	57

H. Pylori positivity in relation to proton pump inhibitor intake is shown in Table 4.5.

Table 4.5 Subgroup analysis of *H. Pylori* positivity and PPI usage.

			PPI usage		
Diagnosis			Yes	No	Total
	H Pylori	Positive	7	10	17
IPF		Negative	8	9	17
	Total		15	19	34
Controls	*H Pylori*	Positive	2	12	14
		Negative	4	5	9
	Total		6	17	23

P=0.212

4.3.3 Exhaled Breath Condensate Study

The main demographics of the study population are shown in Table 4.6. The analysis of EBC pepsin showed that there was no significant difference in pepsin positivity between IPF and control patients (IPF 2/17, controls 0/6; *P*=0.379; Chi square).

Table 4.6 Baseline characteristics of Exhaled Breath Condensate study participants

	IPF (n=17)	CONTROL (n=6)	p Value
Age	72±7	52±15	0.004*
Gender, n (M/F)	10/7	4/2	0.717
FVC %	86.2±18.8	97.6±20	0.932
TLco %	63.2±19.5	62±17	0.520
Medications (n, %)			
Prednisolone	3 (17)	1 (17)	0.730
N-acetylcysteine	1 (6)	0 (0)	0.739
Azathioprine	2 (12)	0 (0)	0.538
PPI	6 (35)	1 (17)	0.382
Pepsin positivity (n, %)	2 (12)	0 (0)	0.379

Data are presented as mean±SD unless stated otherwise. ***** Statistically significant.

4.4 Discussion

The present study of investigation of gastro-esophageal and extra-esophageal reflux in idiopathic pulmonary fibrosis has shown significantly increased airway reflux symptoms in IPF population. However, there is no objective evidence of significant difference in *H. Pylori* infection or extra-esophageal reflux detected by exhaled breath pepsin analysis. The association of GERD and interstitial pulmonary fibrosis of obscure aetiology has been documented long before the term IPF was even described (Belcher 1949). More recently, Tobin and colleagues (Tobin et al. 1998) investigated 17 patients with biopsy proven IPF and 8 control patients with other interstitial lung diseases in a prospective study. There were significantly higher proportions of IPF patients (16 out of 17) who had abnormal distal and/or proximal oesophageal acid exposure as compared to controls (4 out of 8). Raghu and colleagues (Raghu et al. 2006) evaluated 46 IPF patients and 133 intractable

asthmatics with 24-hr pH testing. There was a significantly increased prevalence of abnormal acid exposure in IPF group (87% Vs 68%, *P*=0.014). Moreover, the odds of having IPF in patients with abnormal acid exposure were 3.19 (OR). These studies suggest that gastro-esophageal reflux is very common on objective measurements in IPF. However, classic GERD symptoms are not observed in the majority and were only seen in 25 - 47% of IPF patients.

HARQ data show a marked difference in reflux scores between IPF and control subjects in our study. Hull airway reflux questionnaire is designed to pick up non-acid airway reflux (laryngo-pharyngeal reflux) that can be brief and occur at repeated intervals and looks at these symptoms in the last four weeks. This symptom based assessment provides a comprehensive evaluation of both acid and non acid reflux episodes. The rationale of investigating these symptoms in the evaluation of reflux is based on a clinical study of gastro-esophageal reflux (Everett & Morice 2007) and is part of standard evaluation in every patient presenting with chronic cough at our institution.

The exhaled breath pepsin measurement was taken in outpatient clinic (single measurement for each patient) whereas the episodes of reflux over a period of 24 hours indicated by Hull airway reflux questionnaire may have been occurring infrequently and we possibly missed them. Moreover, the addition of 0.01M citric acid prior to collection of EBC may improve the diagnostic yield as it stabilizes the pH of EBC sample.

The prevalence of *H. Pylori* infection varies with geographical location and the laboratory method to identify the infection. In an Australian study (Moujaber et

al. 2008), a prevalence of 15% has been reported, while a study of Italian villagers showed the prevalence to be as high as 58% (Zagari et al. 2008).

In our study, there were significantly higher proportions of patients taking proton pump inhibitors (PPI) in IPF group as compared to controls (44% Vs 26% respectively). However, a subgroup analysis showed that being on PPI did not have a significant effect on *H. Pylori* positivity. Hence, it is unlikely that patients' exclusion on the basis of PPI usage would have had an effect on *Helicobacter* status. Moreover, *H. Pylori* infection can itself be associated with achlorhydria with a reduction in acid reflux and could potentially be an explanation for failure to find a difference in *H. Pylori* status in IPF and control subjects. Secondly, the relatively lower sensitivity of *H. Pylori* serology to detect present infection in comparison to faecal stool antigen test may be another factor. The finding of a significantly increased prevalence of both acid and non-acid reflux symptoms in the study population is interesting and is in contrast to the studies of gastro-esophageal reflux and IPF. It may be the result of inclusion of a comprehensive evaluation of reflux symptoms by HARQ in contrast to only addressing the typical heartburn/acid reflux symptoms in previous studies (Bandeira et al. 2009;Raghu et al. 2006;Tobin et al. 1998).

We lack a gold standard test to objectively assess the gastro and extra-esophageal reflux and it will be important to utilize novel techniques of airway reflux assessment, such as pharyngeal pH testing by a minimally invasive probe. This newer technique, which detects liquid as well as aerosolized acid, is inserted transnasally and rests just behind the soft palate for between 24 and 48 hours and

the data is transmitted by wireless telemetry to a recorder (Restech Dx-pH measurement system, Respiratory Technology Corporation, San Diego, California, USA). It has detected upper airway reflux in a series of patients in whom 24-hr oesophageal pH testing failed to detect any significant gastro-esophageal acid reflux (Molyneux, Morice, & Jackson 2010).

Conclusion

This study of investigation of gastro and extra-esophageal reflux in IPF demonstrates that IPF patients have a high prevalence of airway reflux symptoms. However, *H. Pylori* infection or single exhaled breath pepsin analysis is not helpful in the diagnosis of gastro-esophageal reflux. As the measurement of exhaled breath pepsin is dependent on the timing of reflux symptoms, repeated sampling with a portable device immediately after the symptoms of reflux may increase the diagnostic yield. A larger prospective study to evaluate the contribution of reflux, by both detailed subjective and objective measurements including airway pH analysis might be able to address the association in greater detail.

CHAPTER 5: ANTI-EPITHELIAL AND ENDOTHELIAL ANTIBODIES AND PULMONARY FIBROSIS

5.1 Introduction

This indirect immunofluorescence study evaluated the role of anti-epithelial and endothelial antibodies in the pathogenesis of IPF. A549 (alveolar epithelial cells of an adenocarcinoma lung cancer line) and HUVEC (Human umbilical vein endothelial cells) were used to evaluate anti-epithelial and anti-endothelial antibodies respectively. The aim of this investigation was to evaluate if there is any evidence of a significant difference in membranous, cytoplasmic, nuclear or mixed fluorescent staining in IPF, non-IPF ILD and non-ILD control subjects.

IPF or control patients' sera in 1:40 dilution were followed by incubation with FITC conjugated anti-IgG or anti-IgM to detect antibody binding to either alveolar epithelial or endothelial cells. An example of membranous staining with A549 cells is shown in Figure 5.1. Cytoplasmic staining pattern is depicted in Figure 5.2. The intensity of magnification is shown in bottom right corner of the slides.

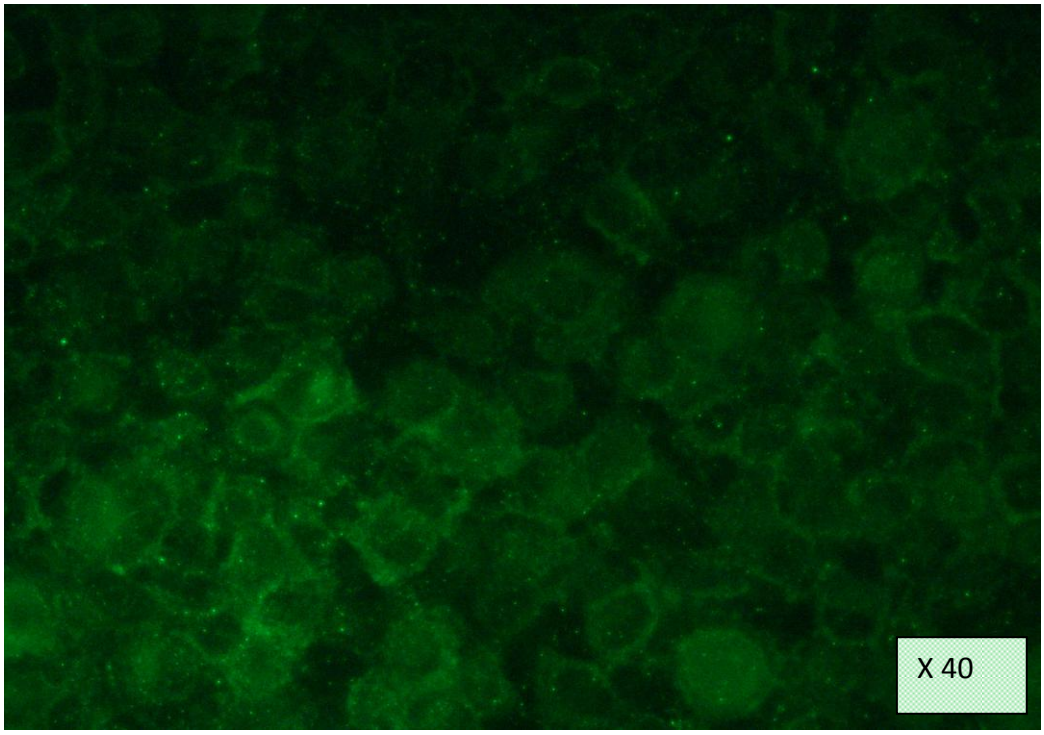

Figure 5.1 *Membranous staining of A549 (alveolar epithelial) cells with FITC conjugated anti-IgG antibody.*

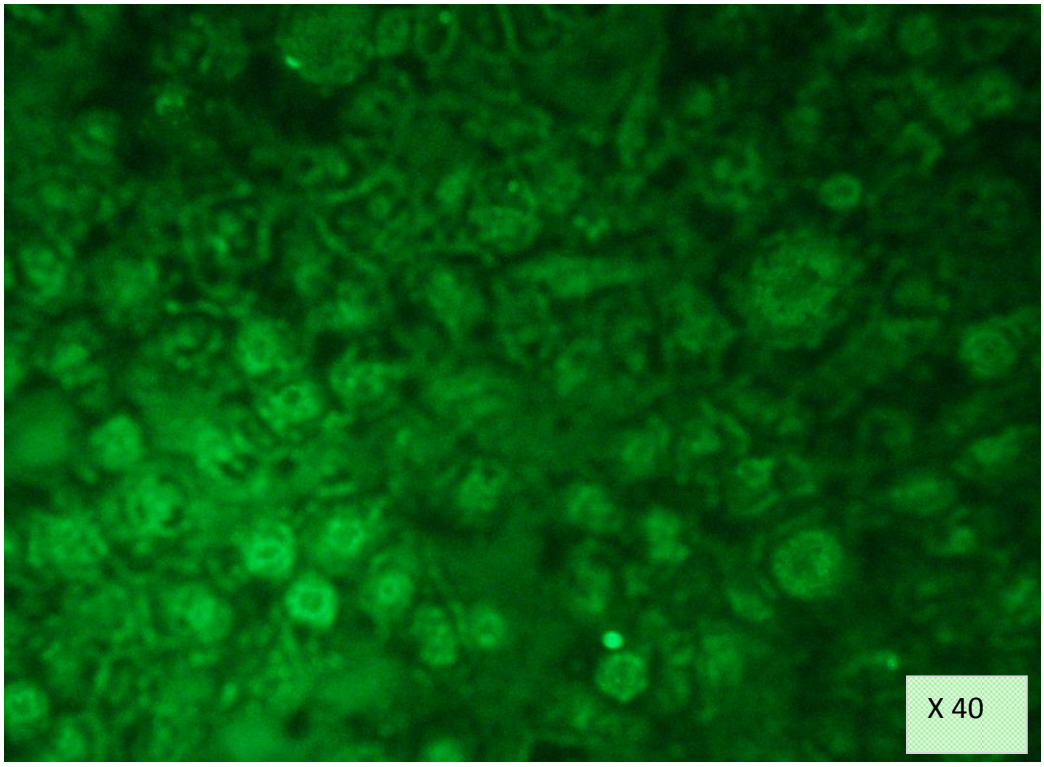

Figure 5.2 *An example of cytoplasmic and membranous staining with anti-IgG antibody. There is a relative lack of nuclear staining in majority of cells.*

5.2 Statistical analysis

As the data in this study was categorical according to the grading of immunofluorescence staining, the Chi-square test was used to compare the intensity of fluorescence staining in the study groups. A General Linear Model (univariate) was used to evaluate the relationship of the severity of immunofluorescence and lung function impairment measured by percent predicted forced vital capacity (FVC) and carbon monoxide gas transfer (TLco).

5.3 Results

We used FITC conjugated anti-IgM and anti-IgG antibodies in this study to detect binding of immunoglobulins in patients' sera to A549 or HUVEC. There was no significant difference in the staining pattern at serum dilutions of 1:40, 1:160 and 1:640 during the pilot study (Figures 5.3-5.5). Hence, the subsequent analyses were carried out using 1:40 dilution.

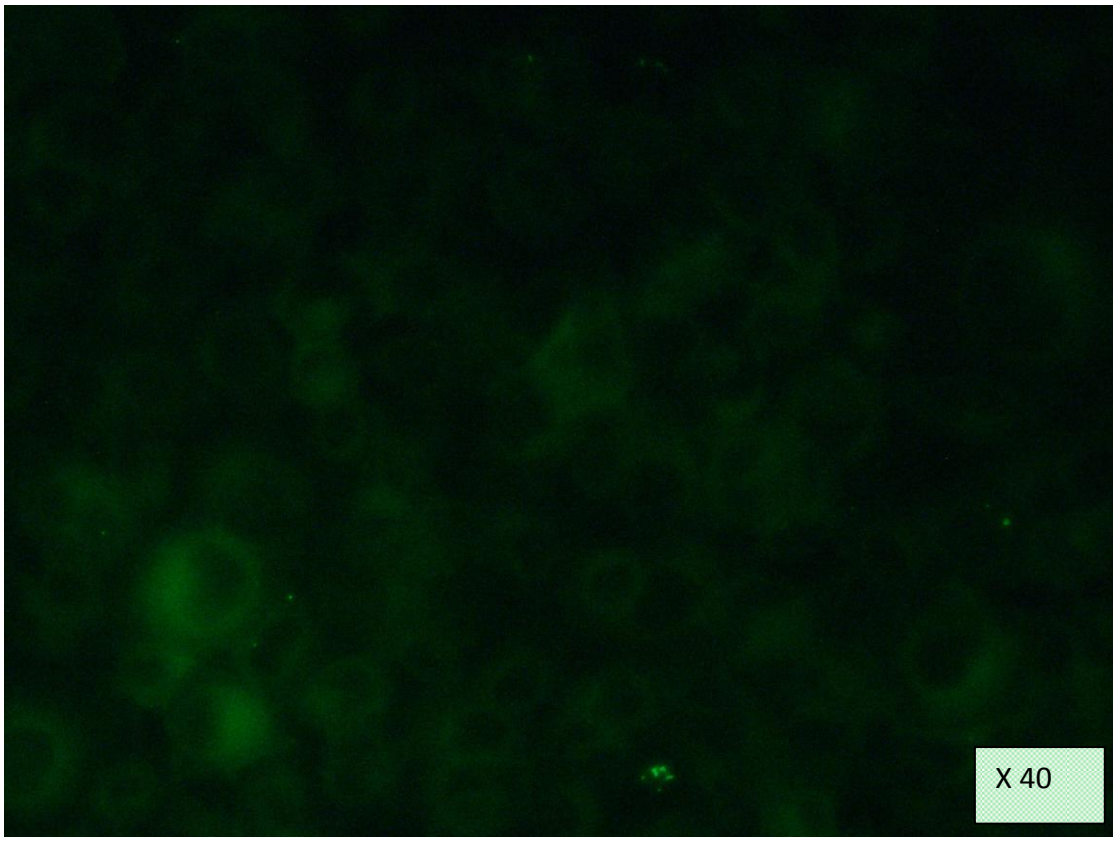

Figure 5.3 *An example of immunofluorescence pattern in a membranous distribution in A549 cells incubated with serum (1:40) from an IPF patient, followed by FITC conjugated anti-IgG antibody.*

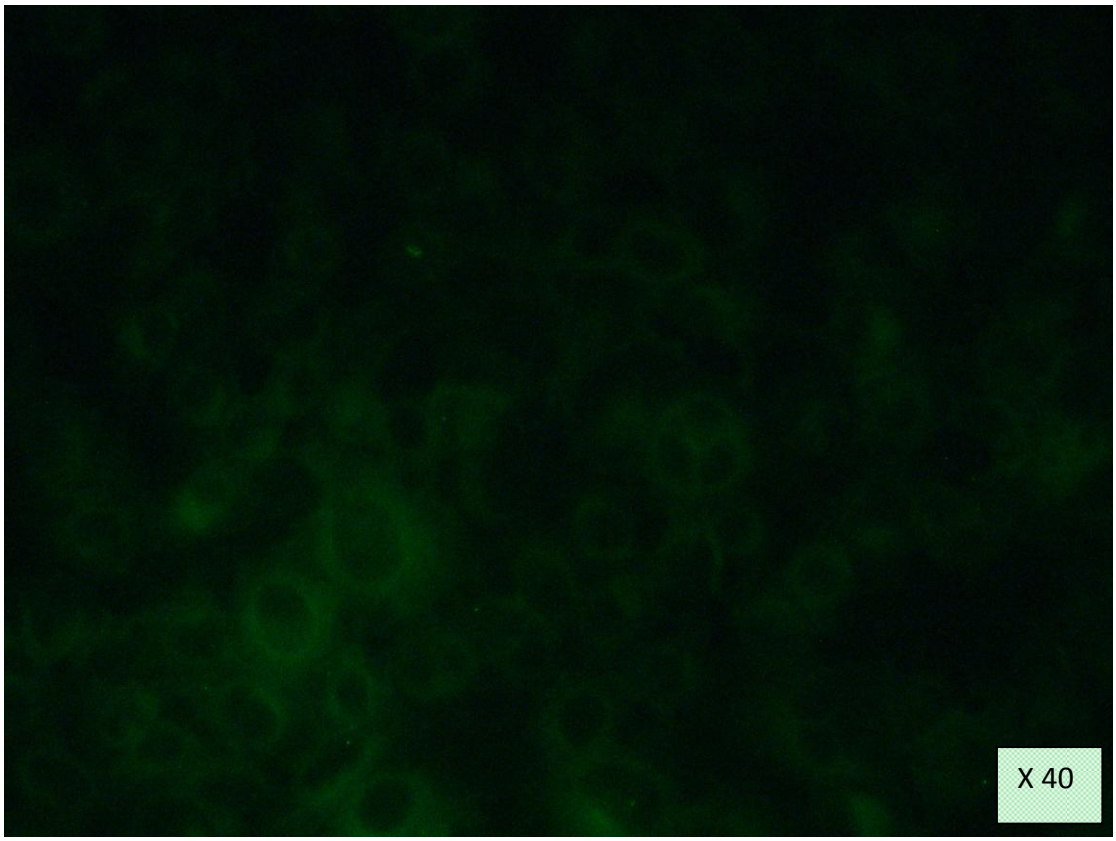

Figure 5.4 *The serum sample diluted at 1:160 without significant change in the fluorescence intensity.*

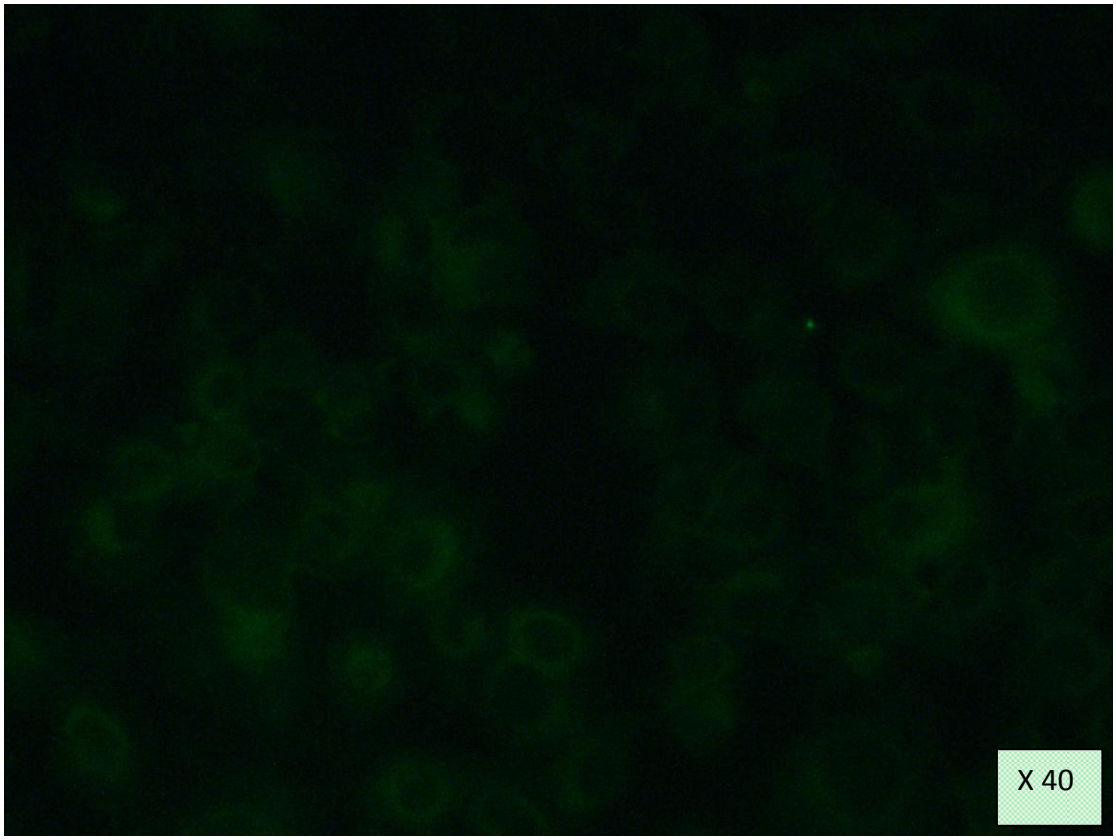

Figure 5.5 *The sample as shown in figures 5.3 and 5.4 at a serum dilution of 1:640. There has been no significant difference at three different levels of serum dilution.*

Immunofluorescence staining on alveolar epithelial (A549) cells

The baseline characteristics and demographics of study population are shown in Table 5.1. The diagnoses in control groups are shown in Table 5.2. The data show that there was significantly increased immunofluorescence with serum IgG antibodies in idiopathic pulmonary fibrosis as compared to interstitial lung diseases other than IPF and control subjects without ILD (Table 5.3, *P*=0.03; Pearson Chi-square). Examples of differential fluorescence of IPF patients' sera in comparison to control serum IgG and IgM on alveolar epithelial cells are shown in Figures 5.6-5.20. The pattern of immunofluorescence was membranous in the majority of samples.

However, one patient had evidence of conspicuous nuclear staining (Figure 5.16). There was no statistically significant difference in immunofluorescence with serum IgM antibodies (*P*=0.349; Chi-square).

Table 5.1 Selected demographics of participants in anti-epithelial antibody study

	IPF	Non IPF ILD	Non ILD Controls	*P* Value
Subjects (n)	28	9	19	
Age (years, mean±SD)	72±8.4	65±10	67±12	0.087
Gender				0.045*
Male (n)	22	4	9	
Female (n)	6	5	10	
IgG (g/L)	14±7	14±2	9±4	0.334
IgM (g/L)	0.7±0.3	1.2±0.3	0.92±.0.2	0.286
IgA (g/L)	3.5±2.1	3.8±2.3	2.9±1.5	0.852
FEV1%	88±14	81±24	74±29	0.386
FVC%	84±16	71±21	91±9.0	0.290
TLco%	58±20	45±10	80±14.7	0.083

Data are presented as mean±SD unless otherwise stated. * Statistically significant

Table 5.2 The diagnoses in control subjects

Non IPF interstitial lung diseases (n=9)
Cryptogenic organizing pneumonia n=2
Non specific interstitial pneumonia n=2
Hypersensitivity pneumonitis n=1
Radiation pneumonitis n=1
Rheumatoid lung n=1
Amiodarone lung n=1
Sjogren's syndrome n=1
Non ILD control subjects (n=19)
Chronic obstructive pulmonary disease n=12
Healthy controls n=4
Lower respiratory tract infection n=1
Asthma n=1
Congestive cardiac failure n=1

Table 5.3 Staining of A549 cells with anti-IgG in IPF and control subjects

Diagnoses	**Grading of immunofluorescence staining**				
	0	**1**	**2**	**3**	**Total**
IPF (n)	1	13	6	8	28
Non IPF ILD (n)	1	4	0	4	9
Non ILD Controls (n)	10	7	1	1	19
Total	12	24	7	13	56

P=0.034 Chi-square

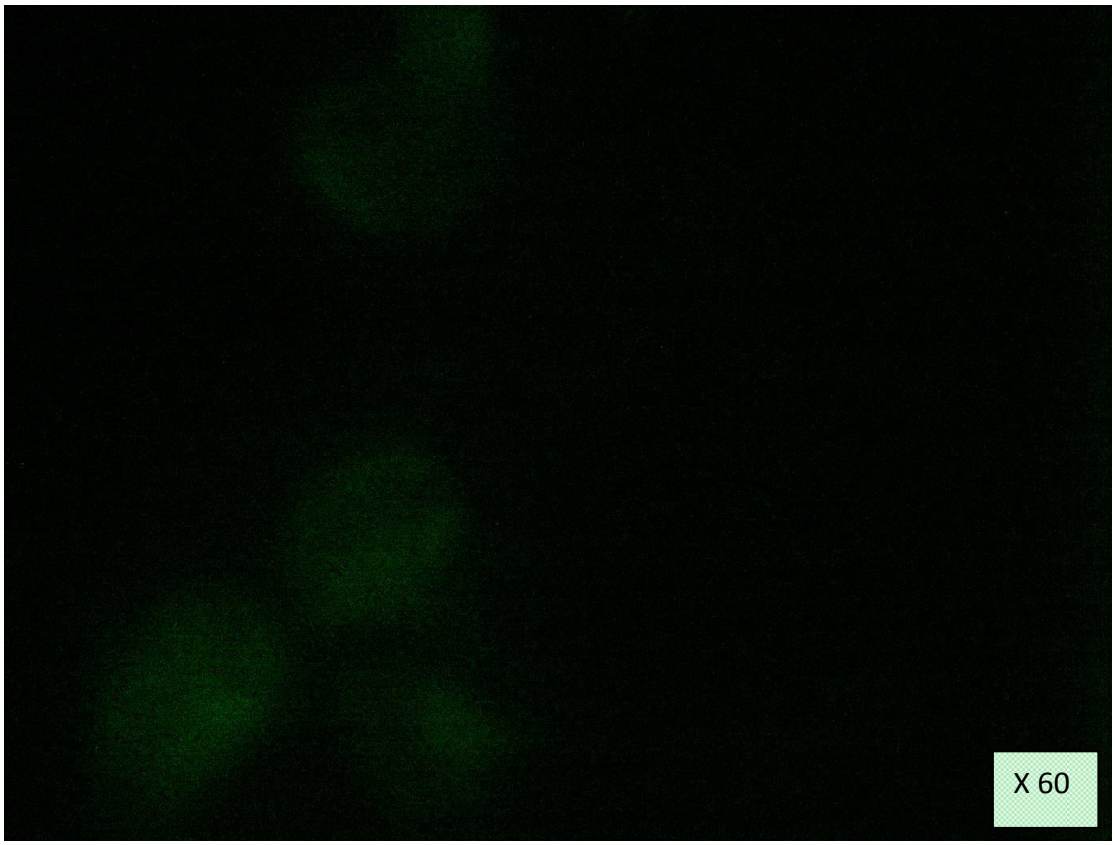

Figure 5.6 *An example of PBS control without serum followed by FITC conjugated anti-IgG demonstrating minimal fluorescence on A549 cells.*

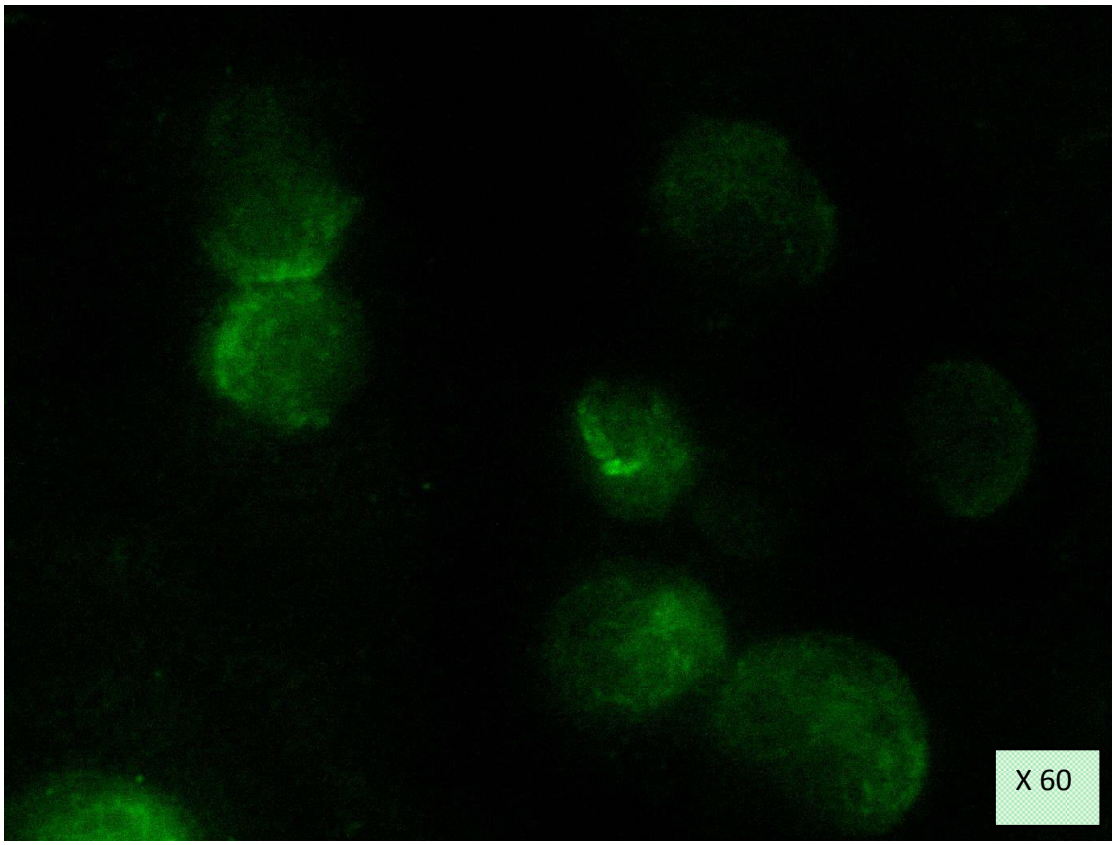

Figure 5.7 *An example of PBS control without serum followed by FITC conjugated anti-IgM demonstrating minimal fluorescence on A549 cells.*

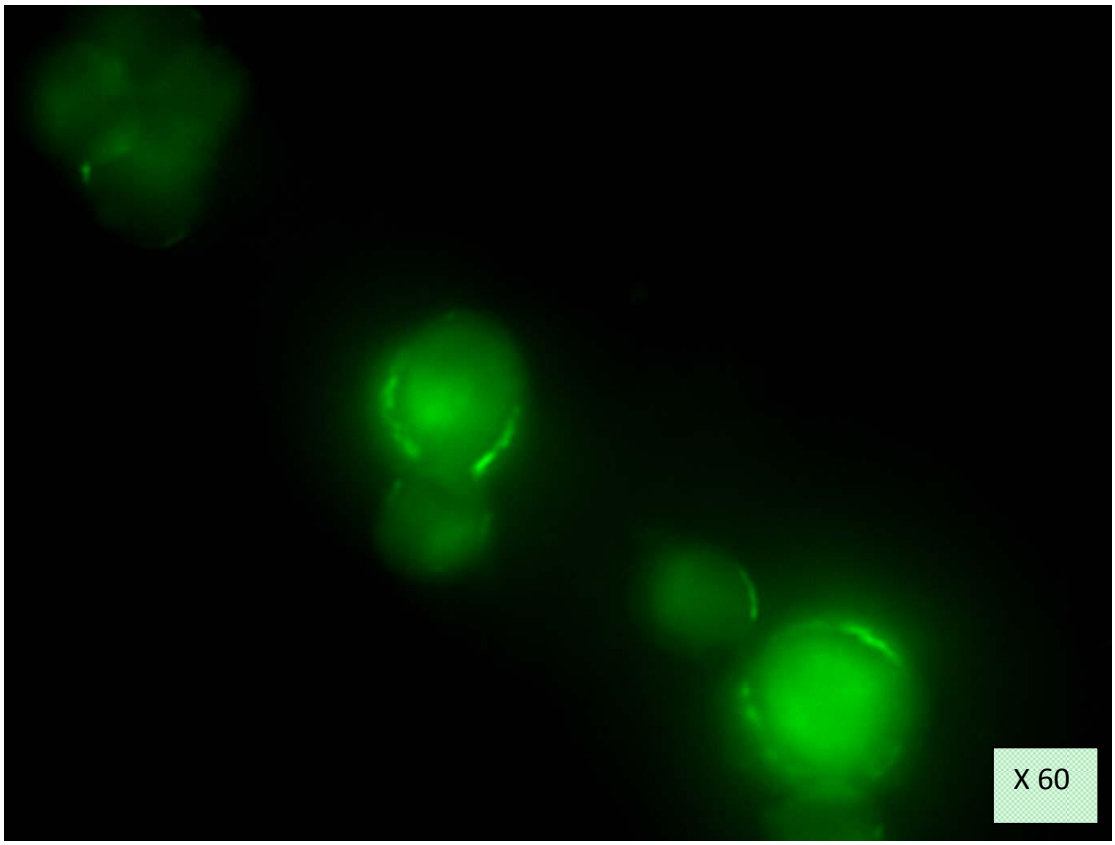

Figure 5.8 *Immunofluorescence in a membranous and cytoplasmic pattern following incubation with FITC conjugated anti-IgG in a patient with IPF. It was scored 2 on fluorescence severity grade.*

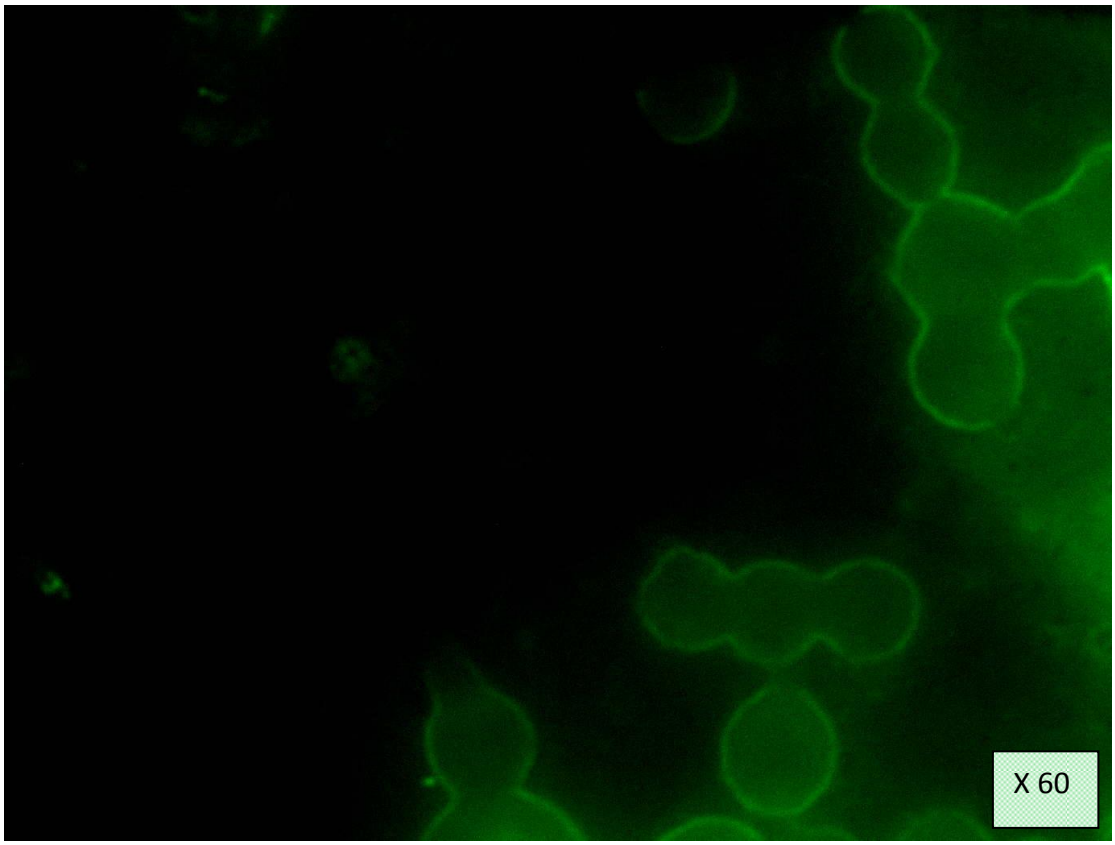

Figure 5.9 *Anti-IgG antibodies in another IPF patient demonstrating a remarkably striking immunofluorescence in a membranous pattern. This was scored 3 on the fluorescence severity grade.*

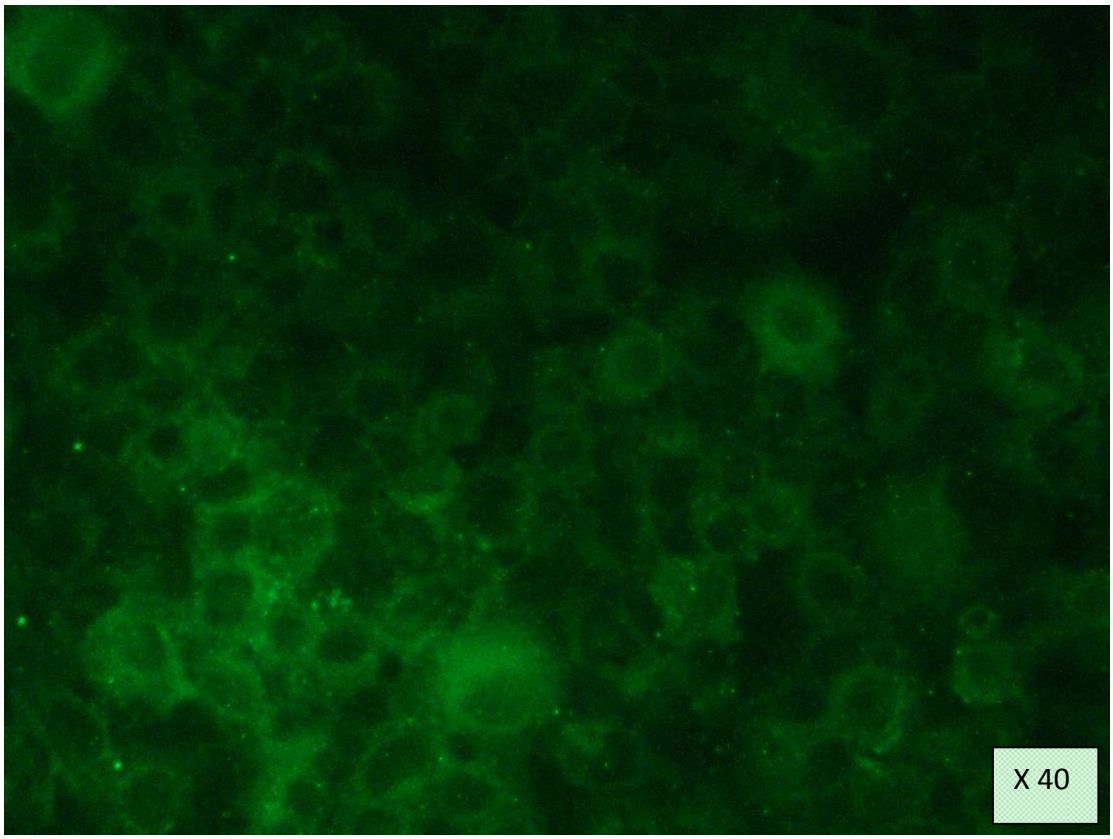

Figure 5.10 *Alveolar epithelial cells incubated with serum from an IPF patient followed by FITC conjugated anti-IgG antibody demonstrating immunofluorescence in a membranous pattern.*

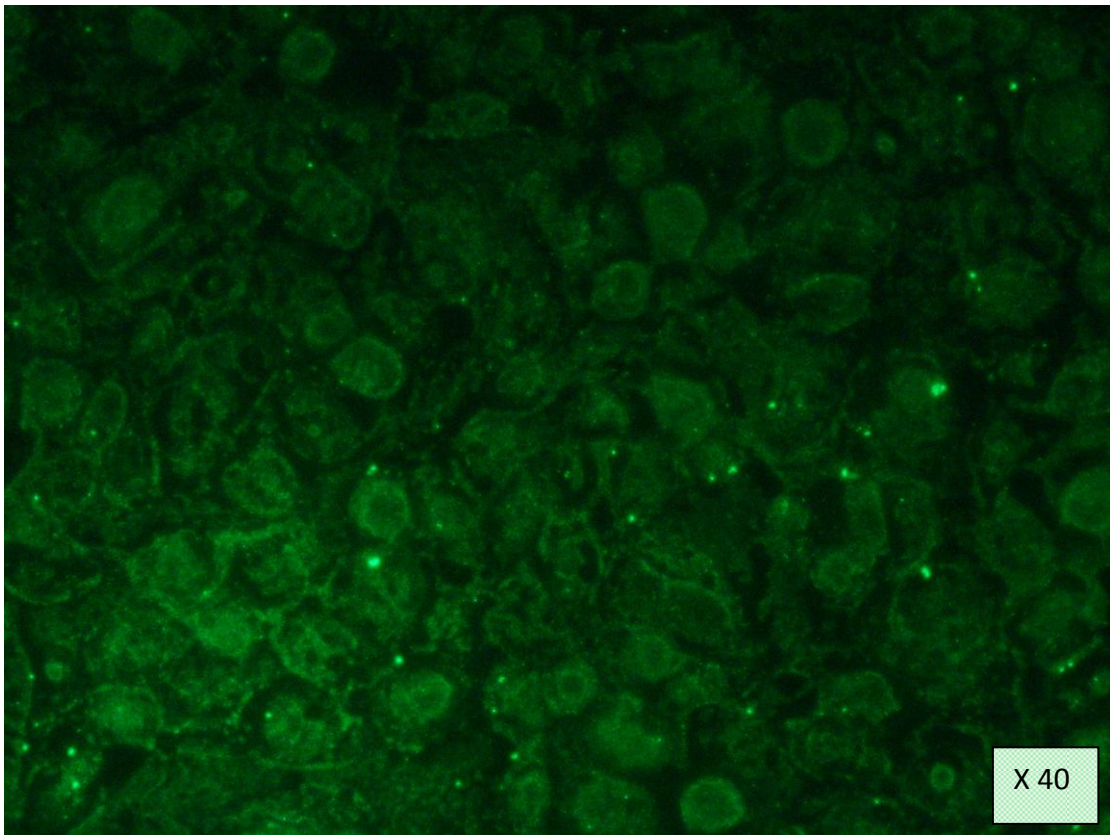

Figure 5.11 *Another example of anti-IgG antibodies in an IPF patient demonstrating immunofluorescence in a membranous pattern.*

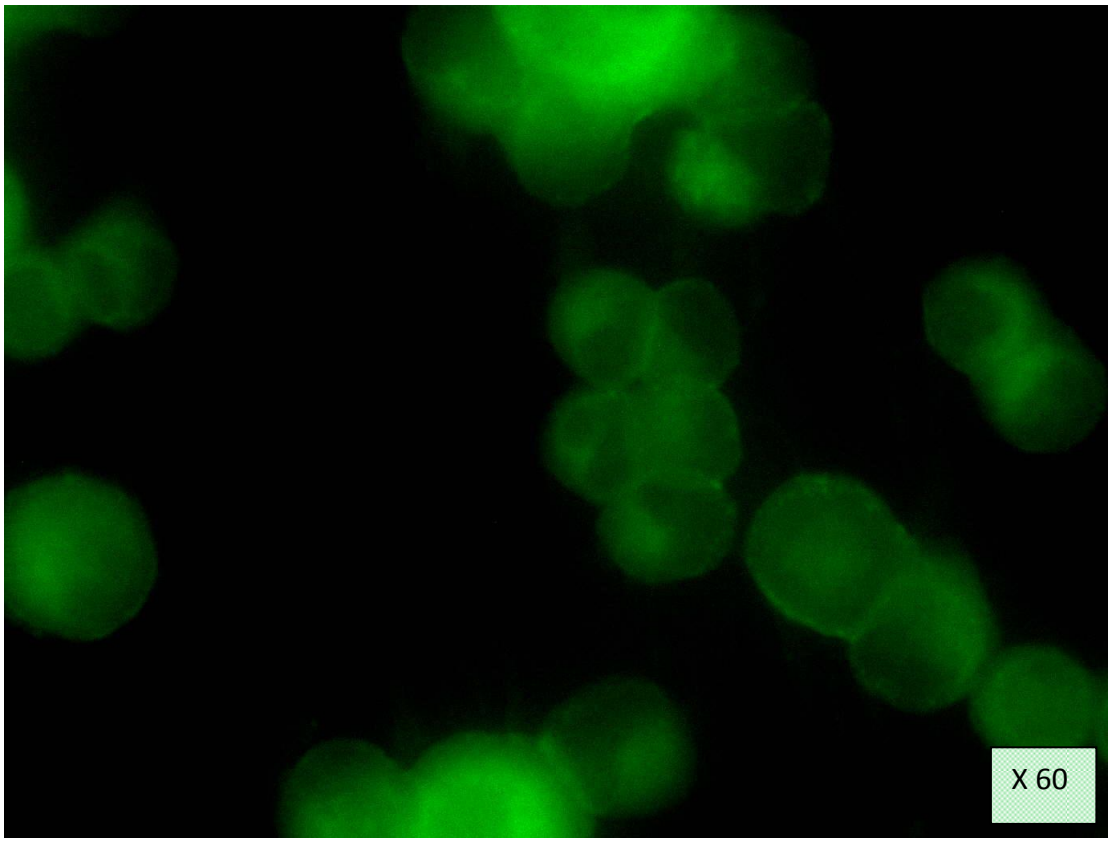

Figure 5.12 *FITC conjugated anti-IgG in a control patient with COPD demonstrating cytoplasmic staining*.

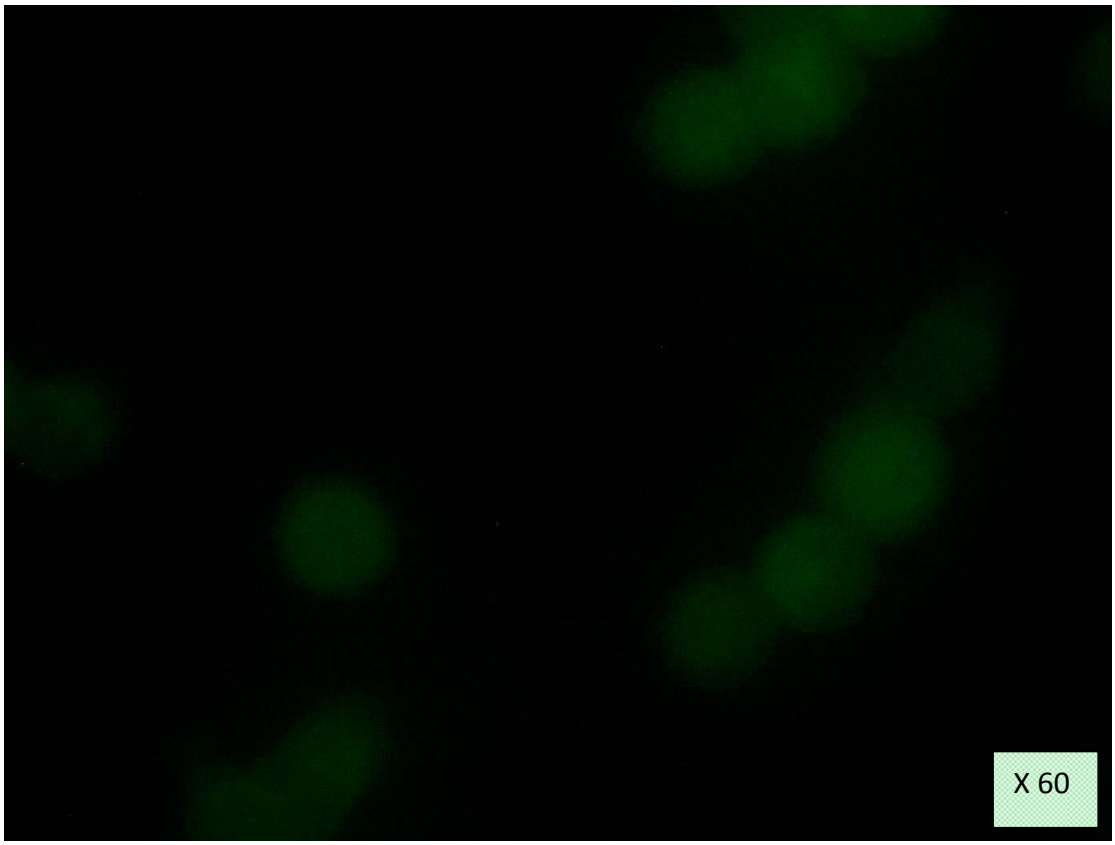

Figure 5.13 *A549 cells incubated with serum from a control subject without interstitial lung disease, followed by FITC conjugated anti-IgM antibody. There is no significant fluorescence staining.*

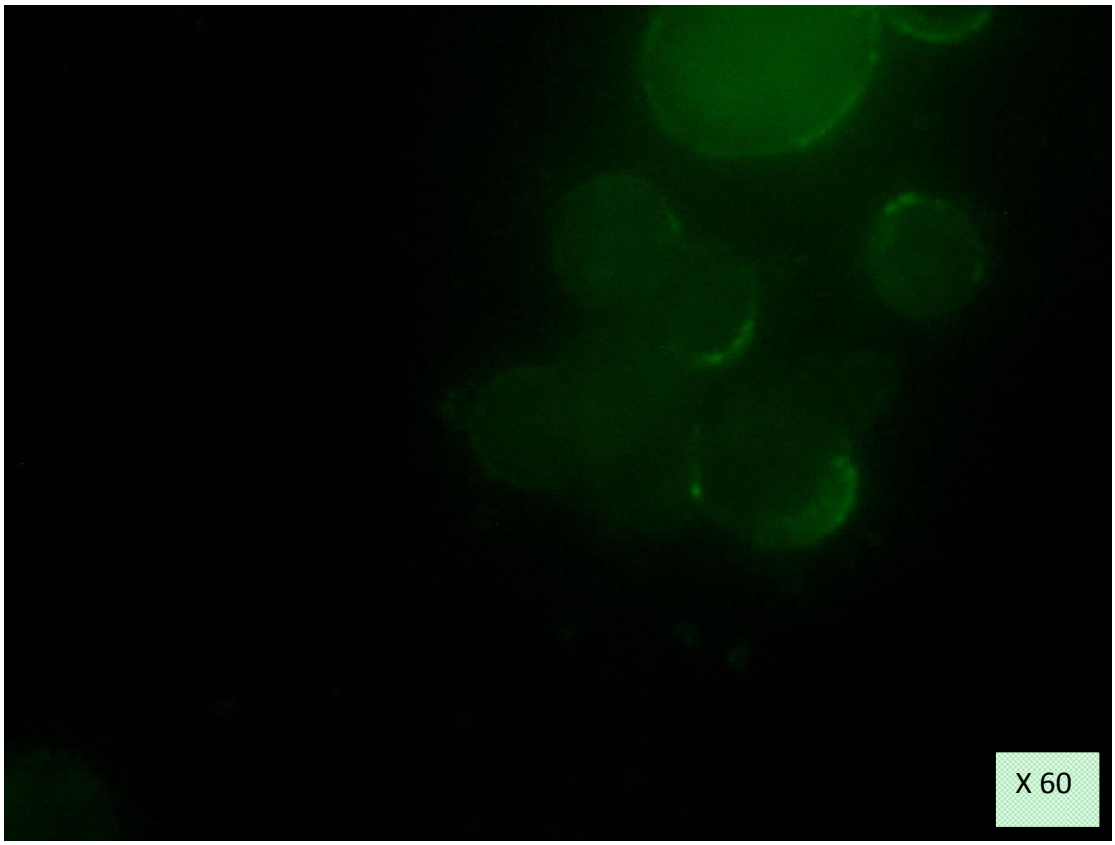

Figure 5.14 *Another example of anti-IgM antibodies on alveolar epithelial cells (A549) demonstrating mild fluorescence in a membranous pattern in a control patient with COPD.*

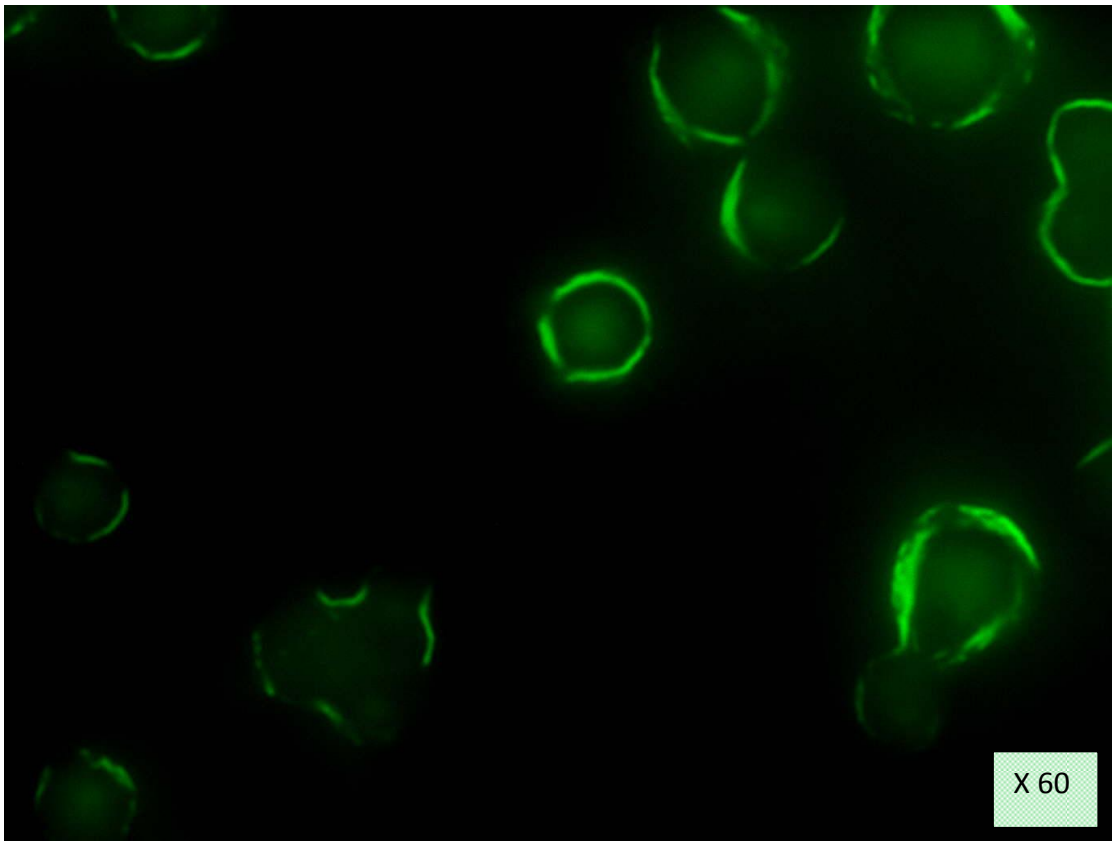

Figure 5.15 *Anti-IgG antibodies with a very strong staining on alveolar epithelial cells (A549) demonstrating a very prominent fluorescence in a membranous pattern in a patient with IPF.*

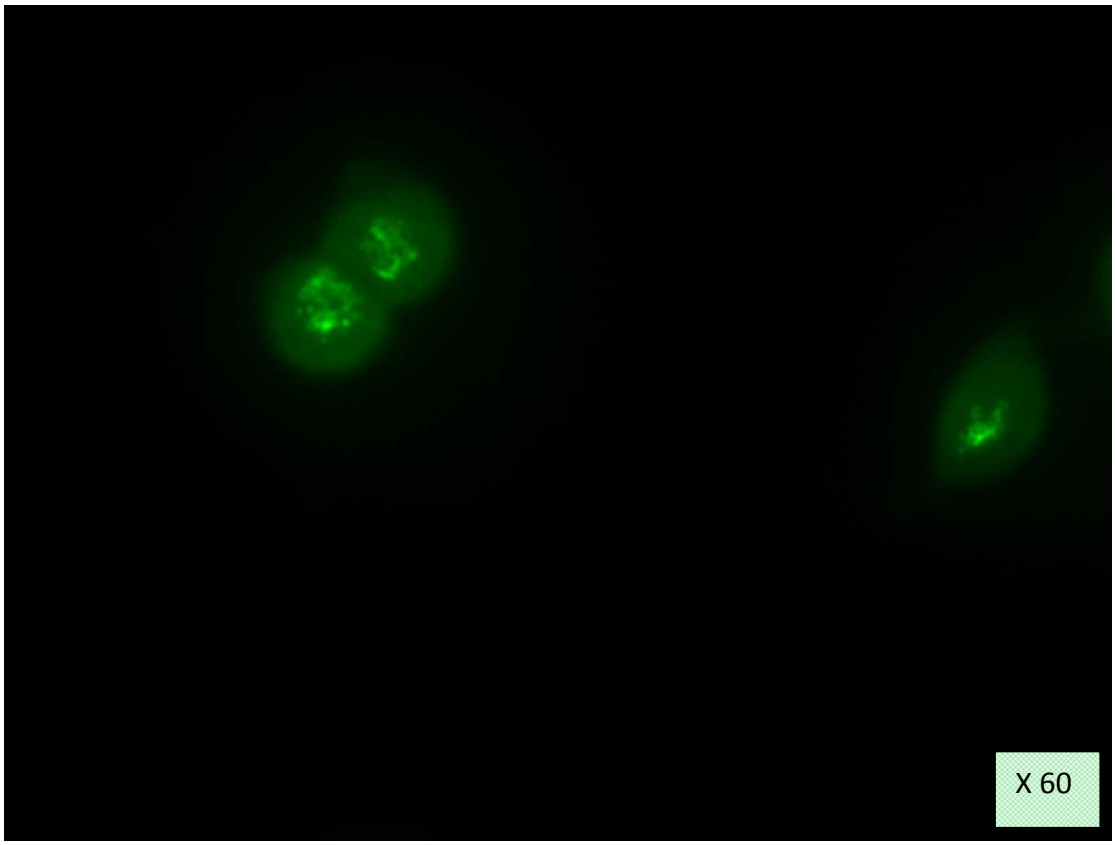

Figure 5.16 *A549 cells incubated with serum from an IPF patient, followed by FITC conjugated anti-IgG antibody demonstrating prominent nuclear immunofluorescence staining pattern.*

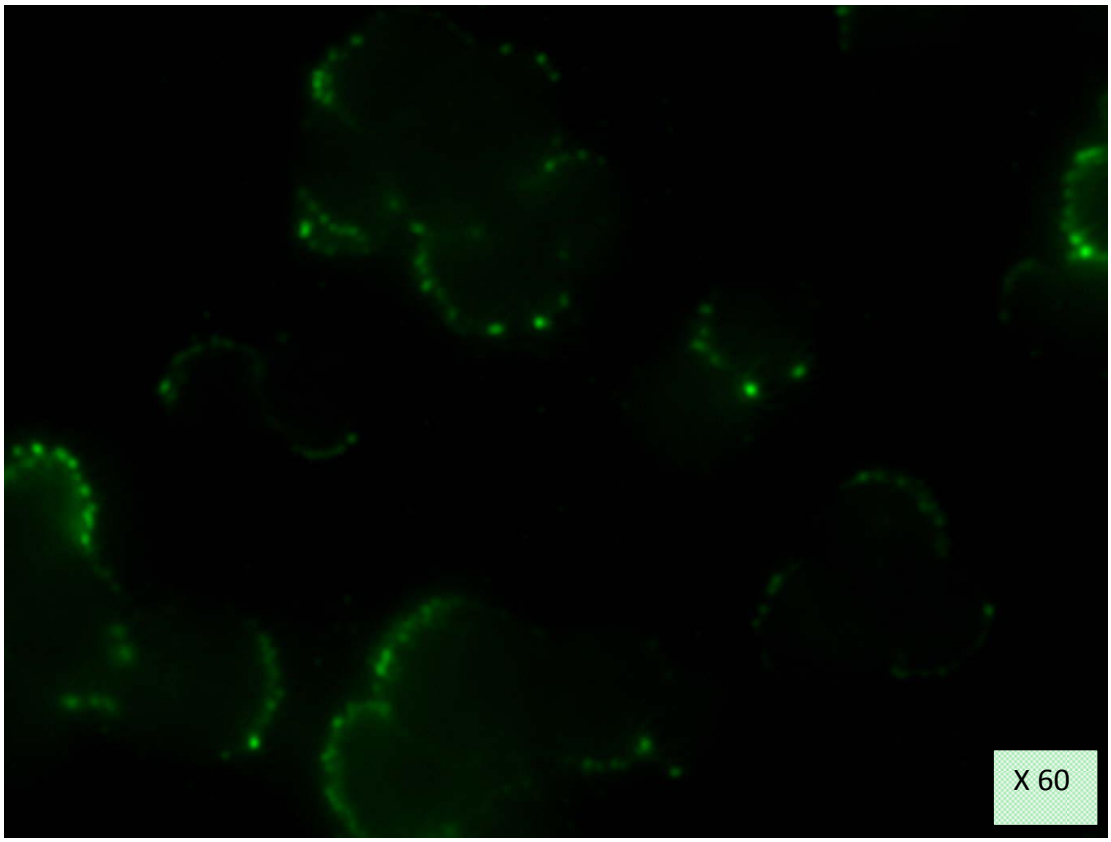

Figure 5.17 *FITC conjugated anti-IgG antibodies in a patient with Sjogren's associated interstitial pulmonary fibrosis demonstrating focal membranous fluorescence.*

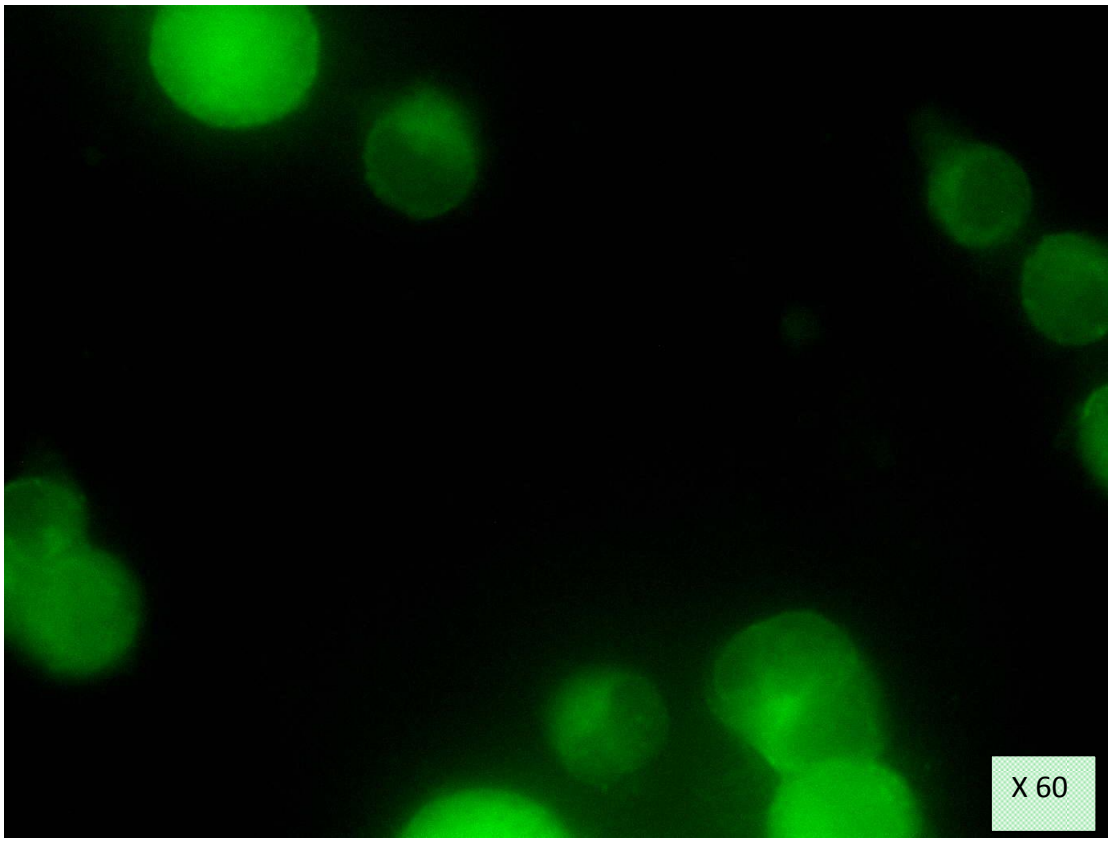

Figure 5.18 *Anti-IgG antibodies in a patient with IPF with minimal fluorescence on alveolar epithelial cells.*

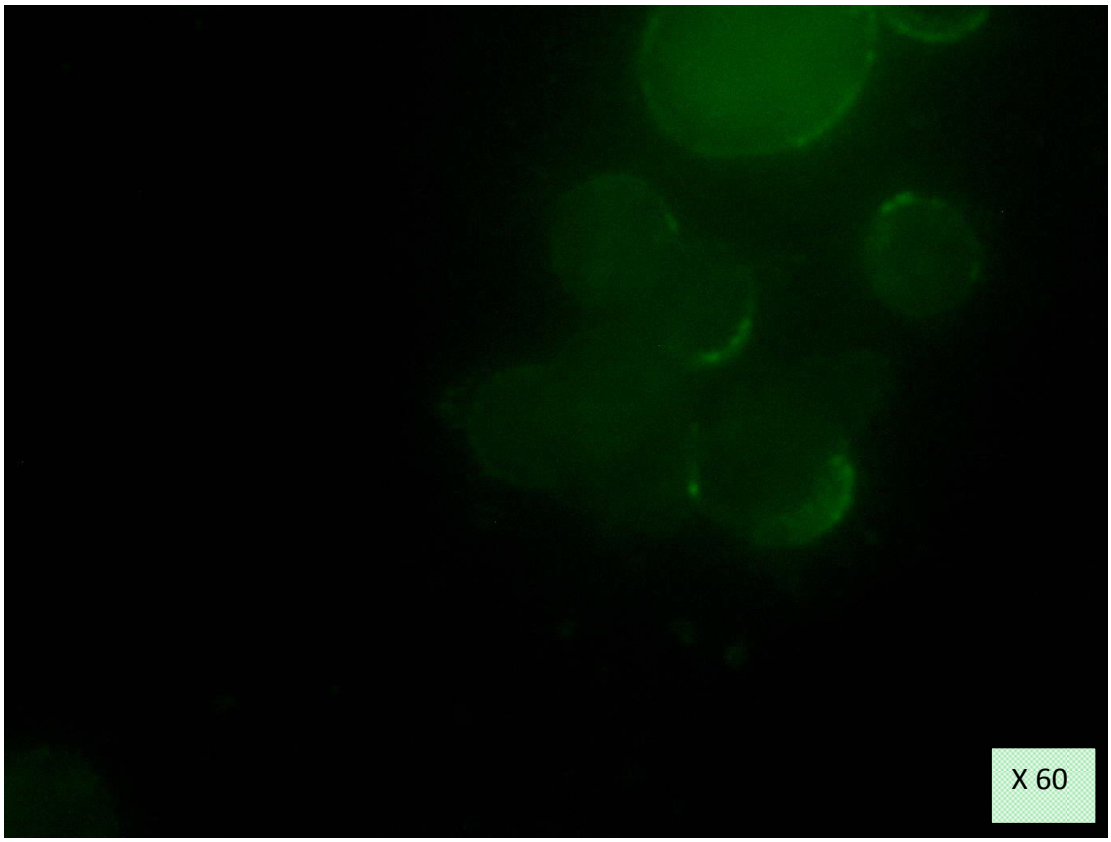

Figure 5.19 *Anti-IgM antibodies on alveolar epithelial cells demonstrating minimal membranous fluorescence in a non-ILD control patient with COPD.*

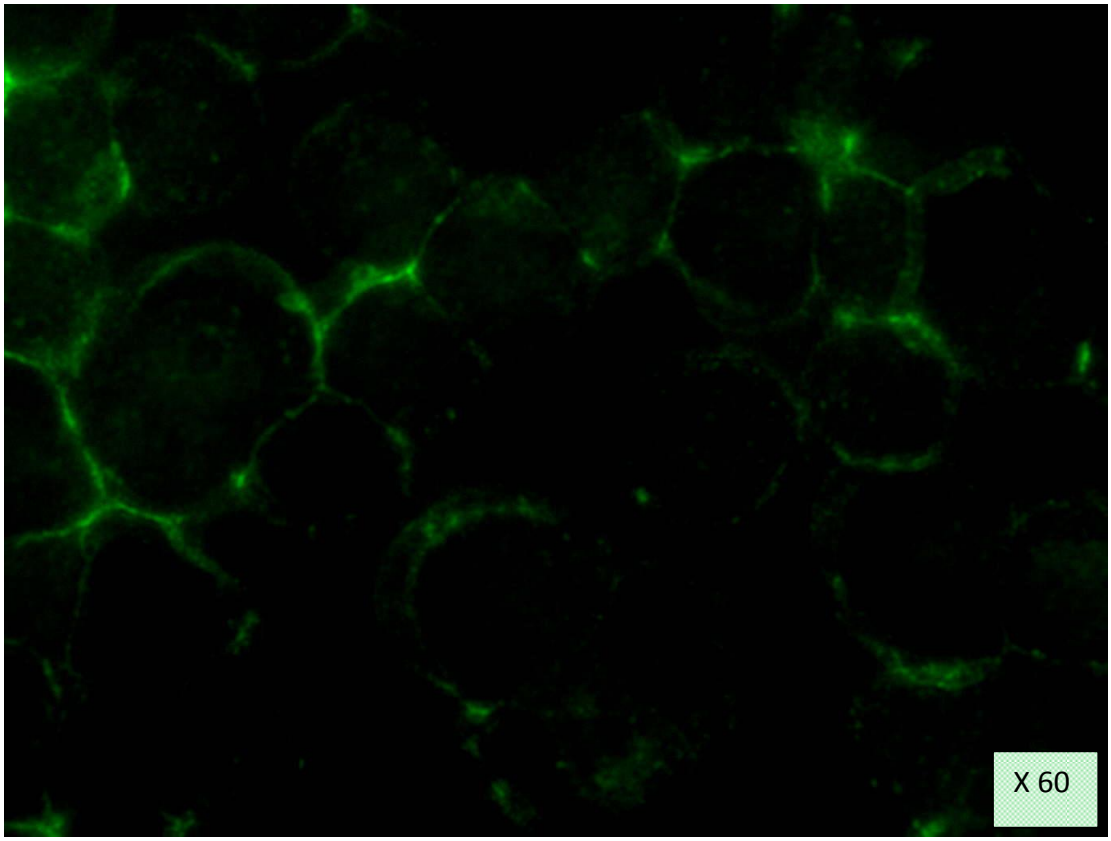

Figure 5.20 *Anti-IgM antibodies on alveolar epithelial cells demonstrating membranous fluorescence in a non-ILD control patient with COPD.*

Immunofluorescence staining on Human umbilical vein endothelial cells

The baseline characteristics and demographics of study population are shown in Table 5.4. In contrast to A549 cells, HUVEC failed to show a differential pattern of fluorescence in IPF and control groups both in terms of IgG (Table 5.5; *P*=0.165 Pearson Chi-square) and IgM antibodies (Table 5.6; *P*=0.297 Pearson Chi-square). Examples of FITC conjugated anti-IgG and IgM on HUVEC in IPF and control patients' sera are shown in Figures 5.21-5.33.

Table 5.4 Selected demographics of participants in anti-endothelial antibody study

	IPF	Non IPF ILD	Non ILD Controls	*P* Value
Subjects (n)	12	3	13	
Age (years, mean±SD)	73±10	62±8	69±11	0.292
Gender				0.542
Male (n)	9	2	7	
Female (n)	3	1	6	
FVC%	83±16 (n=10)	87±7(n=2)	NA	0.569
TLCO%	51±20 (n=10)	75±16 (n=6)	NA	0.340

Data are presented as mean±SD unless stated otherwise.

Table 5.5 Staining of anti-IgG (HUVEC) in IPF and control subjects

Diagnoses	**Immunofluorescence staining**				
	0	**1**	**2**	**3**	**Total**
IPF (n)	3	2	3	4	12
Non IPF ILD (n)	1	1	1	0	3
Non ILD Controls (n)	3	8	2	0	13
Total	7	11	6	4	28

P=0.165 Chi-square

Table 5.6 Staining of anti-IgM (HUVEC) in IPF and control subjects

Diagnoses	Immunofluorescence staining				
	0	**1**	**2**	**3**	**Total**
IPF (n)	5	4	2	1	12
Non IPF ILD (n)	3	0	0	0	3
Non ILD Controls (n)	11	1	1	0	13
Total	19	5	3	1	28

P=0.297 Chi-square

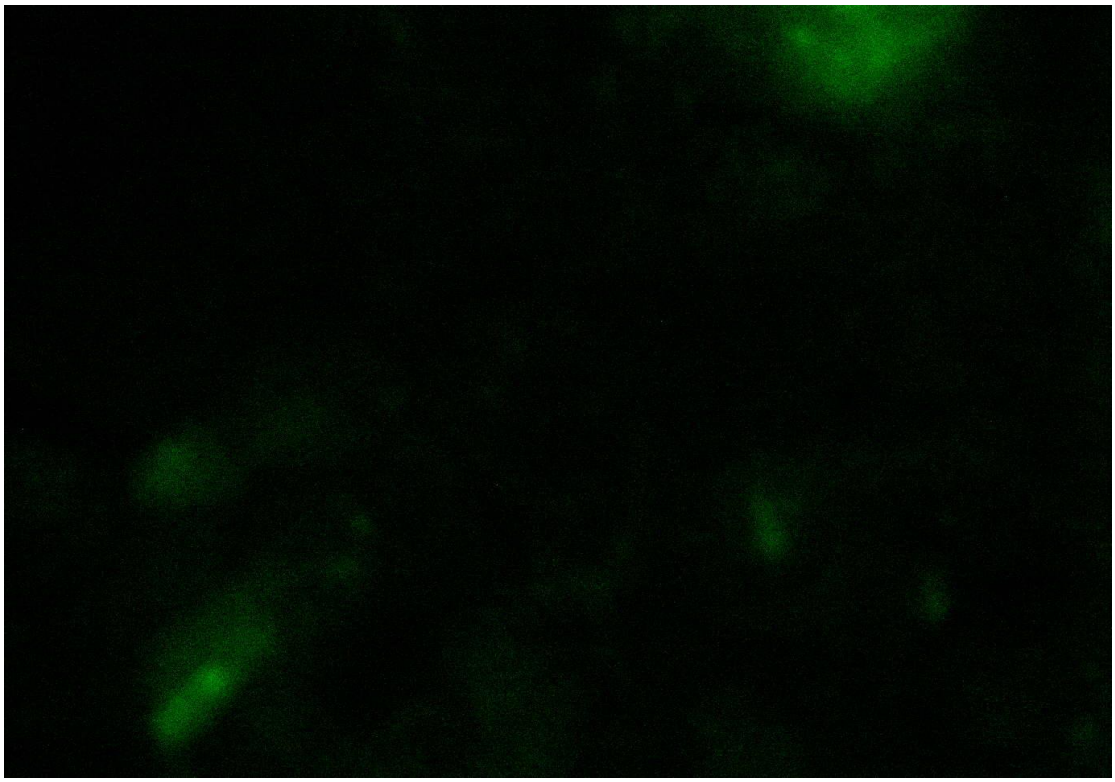

Figure 5.21 *An example of PBS control without serum with minimal fluorescence on endothelial cells following incubation with FITC conjugated anti-IgG antibody.*

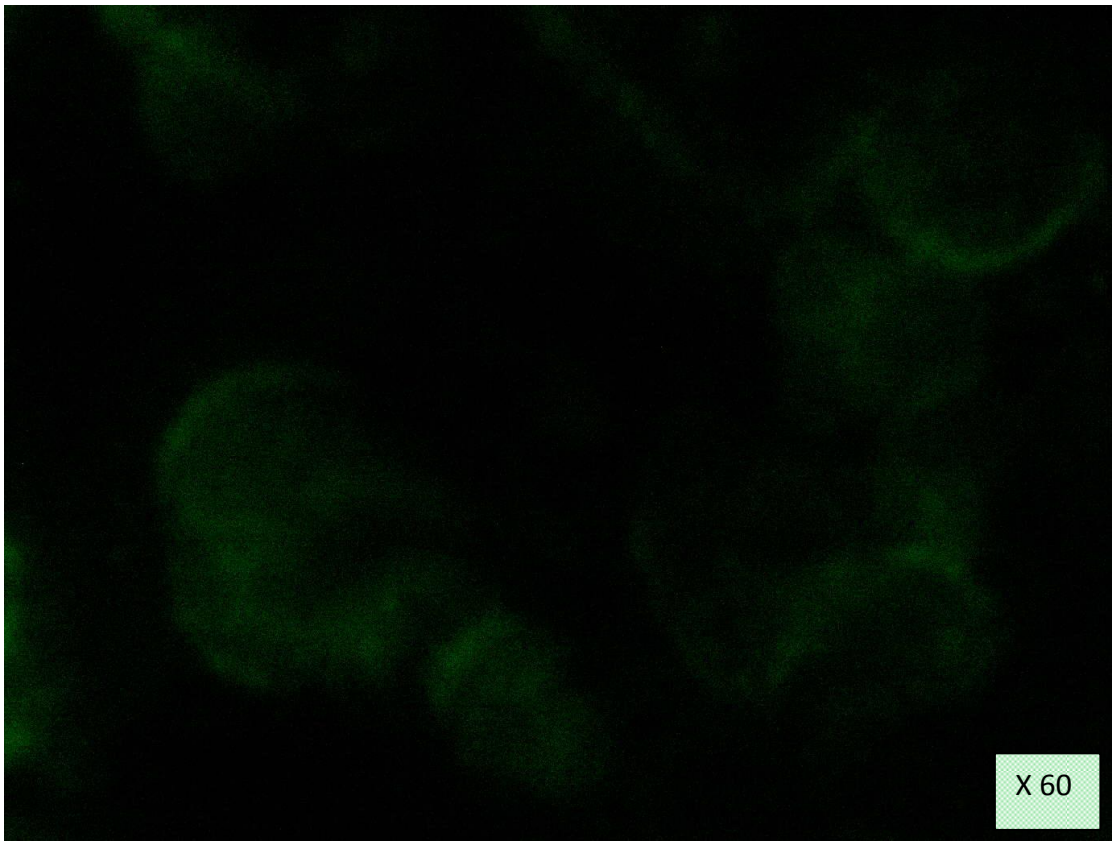

Figure 5.22 *An example of PBS control without serum with minimal fluorescence on endothelial cells following incubation with FITC conjugated anti-IgM antibody.*

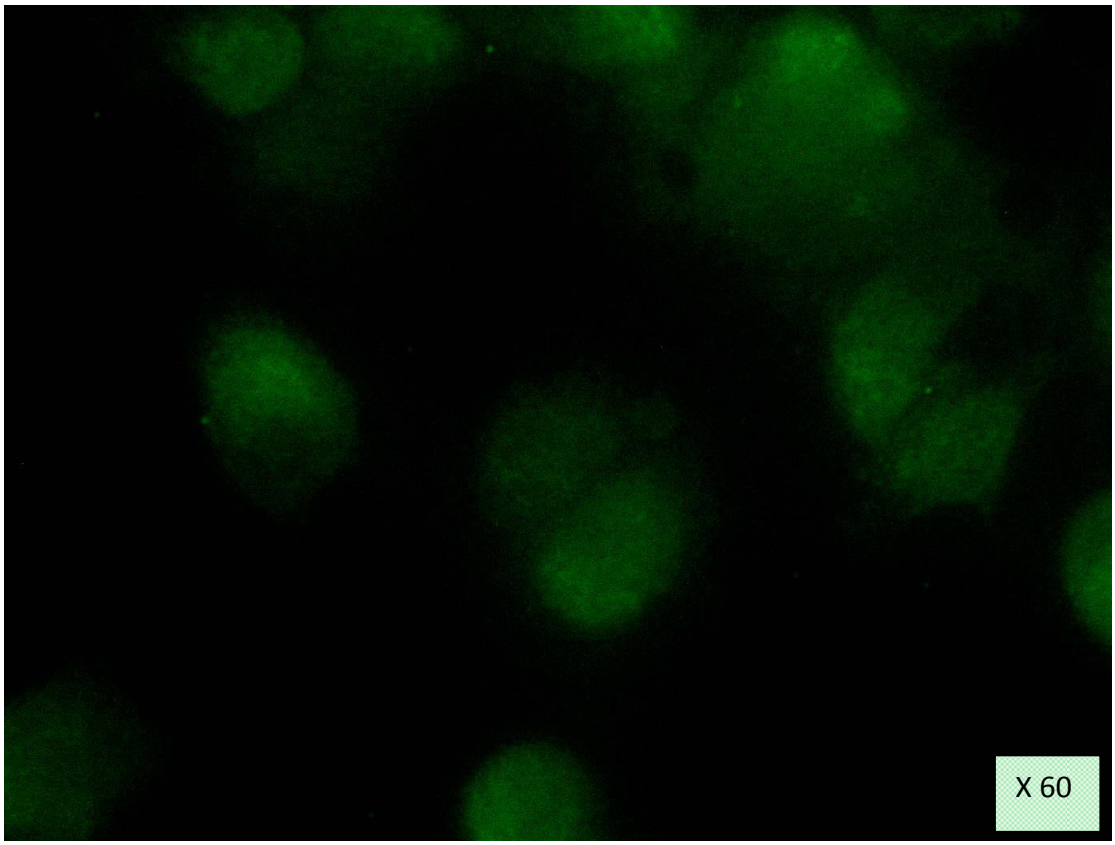

Figure 5.23 *FITC conjugated anti-IgM antibodies in a control patient without ILD demonstrating minimal fluorescence on HUVEC.*

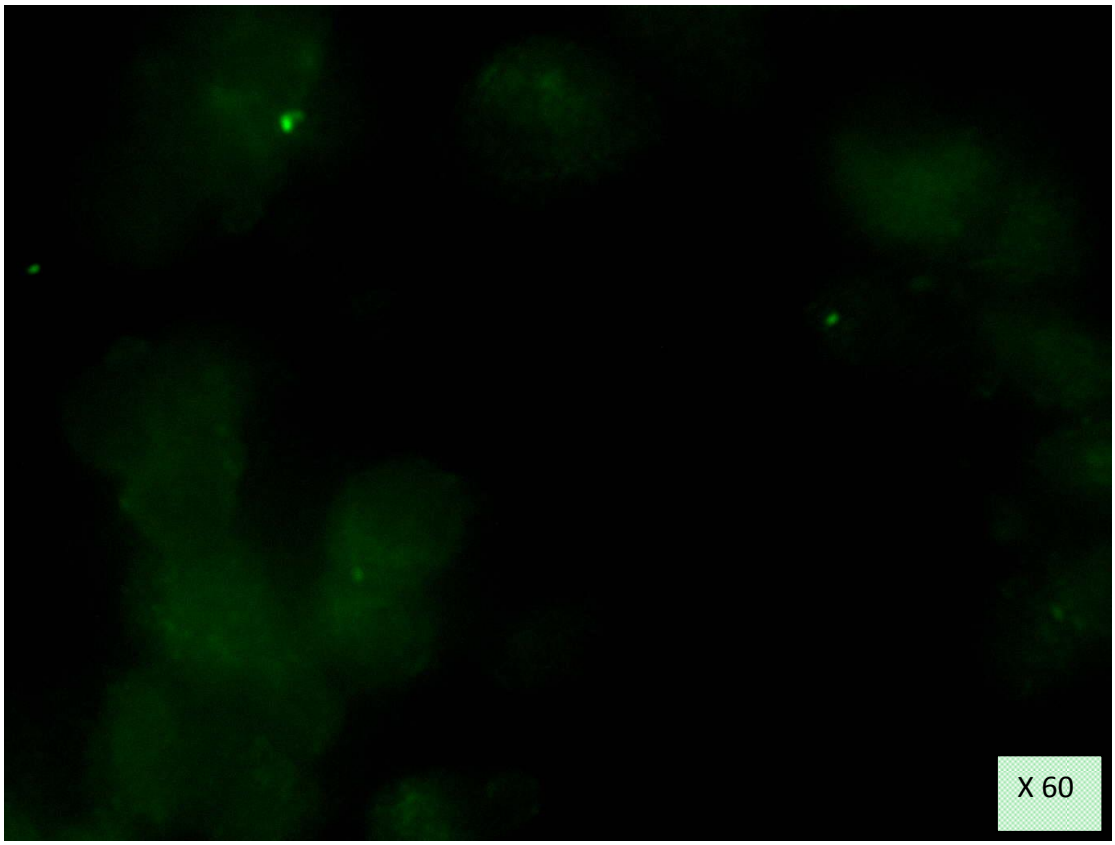

Figure 5.24 *HUVEC incubated with serum from a patient with Rheumatoid associated ILD, followed by FITC conjugated anti-IgG antibody demonstrating no significantly increased immunofluorescence.*

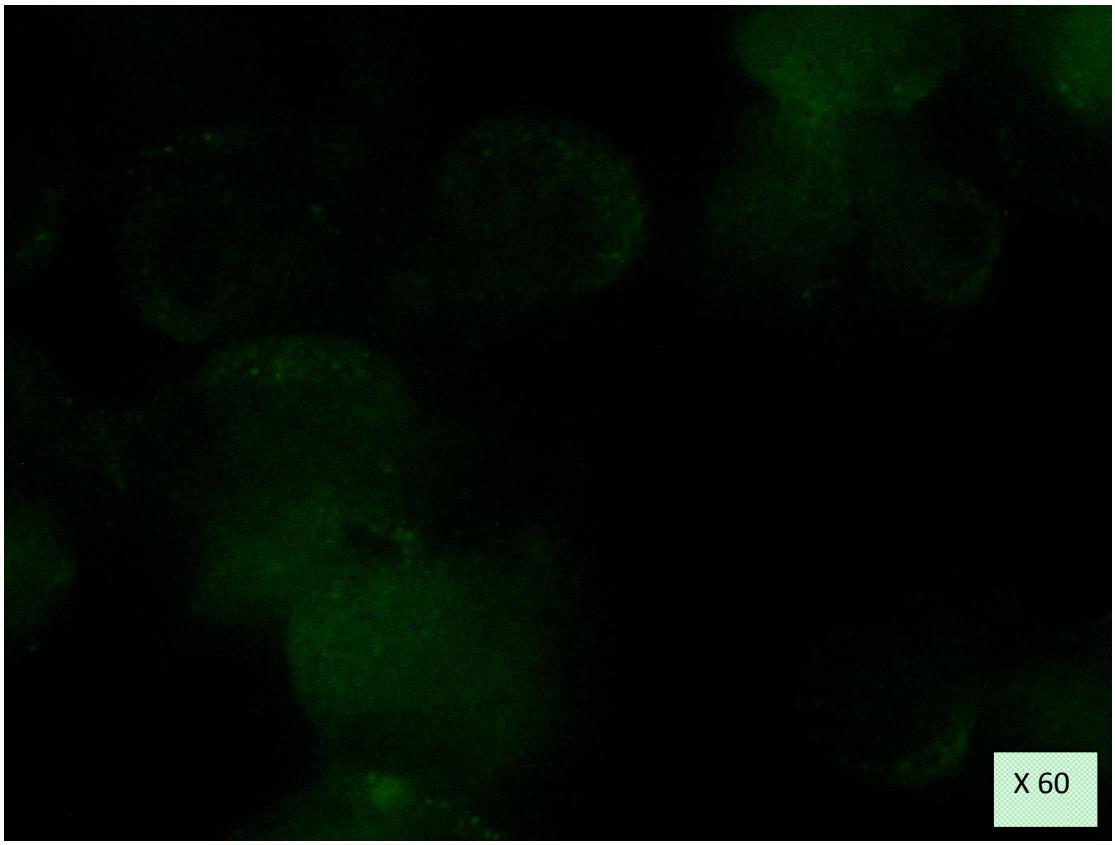

Figure 5.25 *HUVEC incubated with serum from a patient with IPF, followed by FITC conjugated anti-IgM antibodies showing no significant immunofluorescence.*

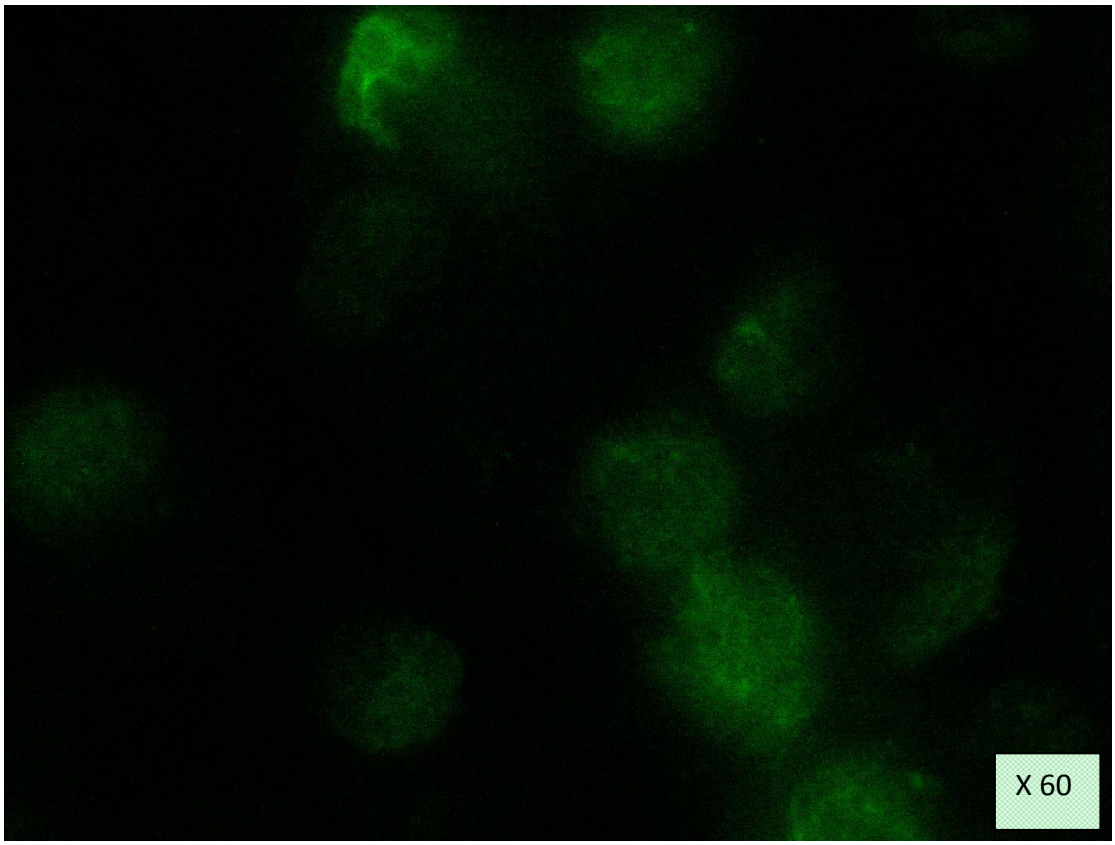

Figure 5.26 *Endothelial cells incubated with serum from an IPF patient, followed by anti-IgG antibody. There is minimal fluorescence.*

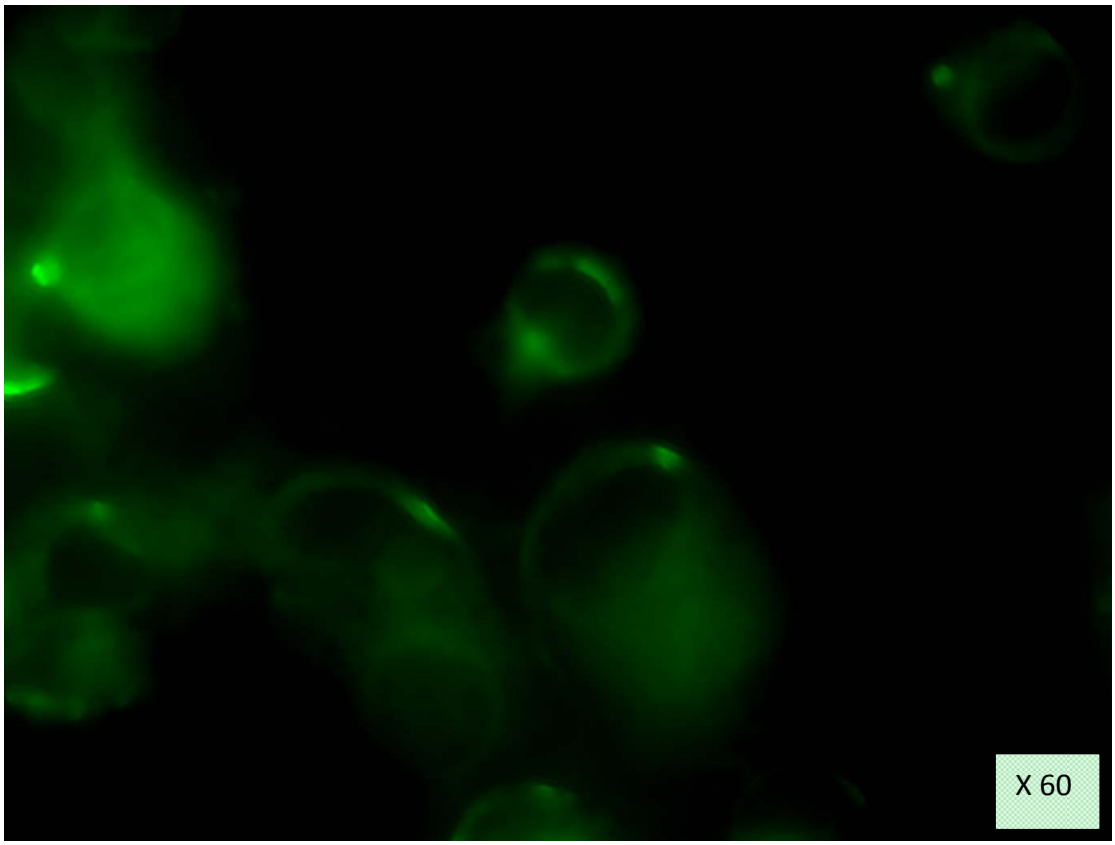

Figure 5.27 *Anti-IgG antibodies in a control patient with COPD demonstrating mild immunofluorescence on HUVEC in a membranous pattern.*

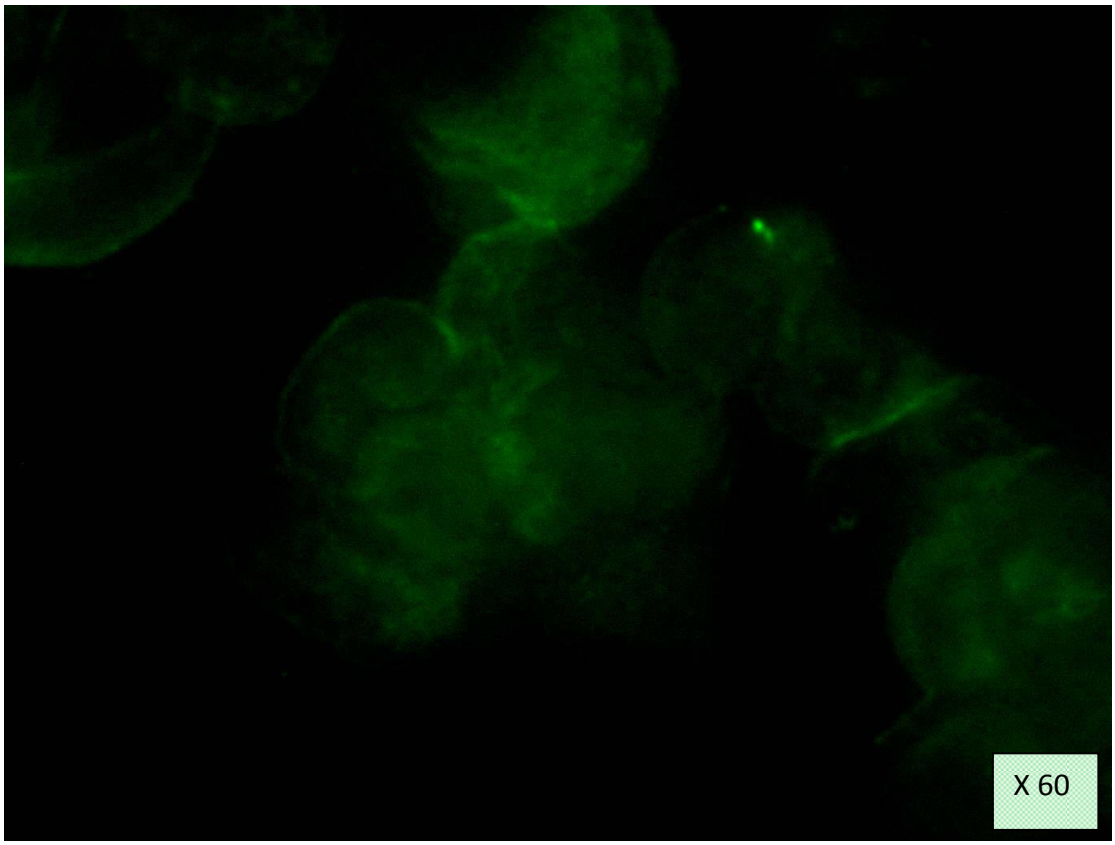

Figure 5.28 *Another example of FITC conjugated anti-IgG antibodies in a patient with COPD demonstrating mildly increased immunofluorescence on HUVEC in predominantly a membranous pattern.*

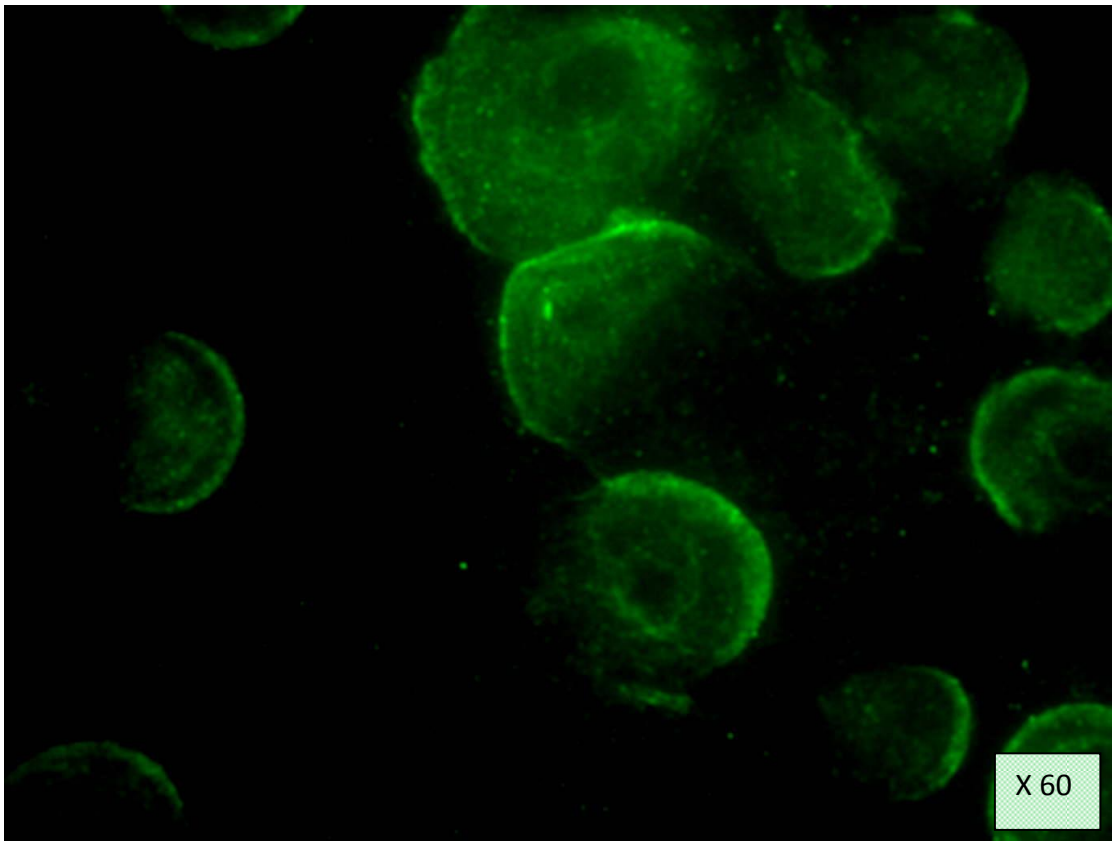

Figure 5.29 *Anti-IgM antibodies in an IPF patient demonstrating moderate immunofluorescence on HUVEC.*

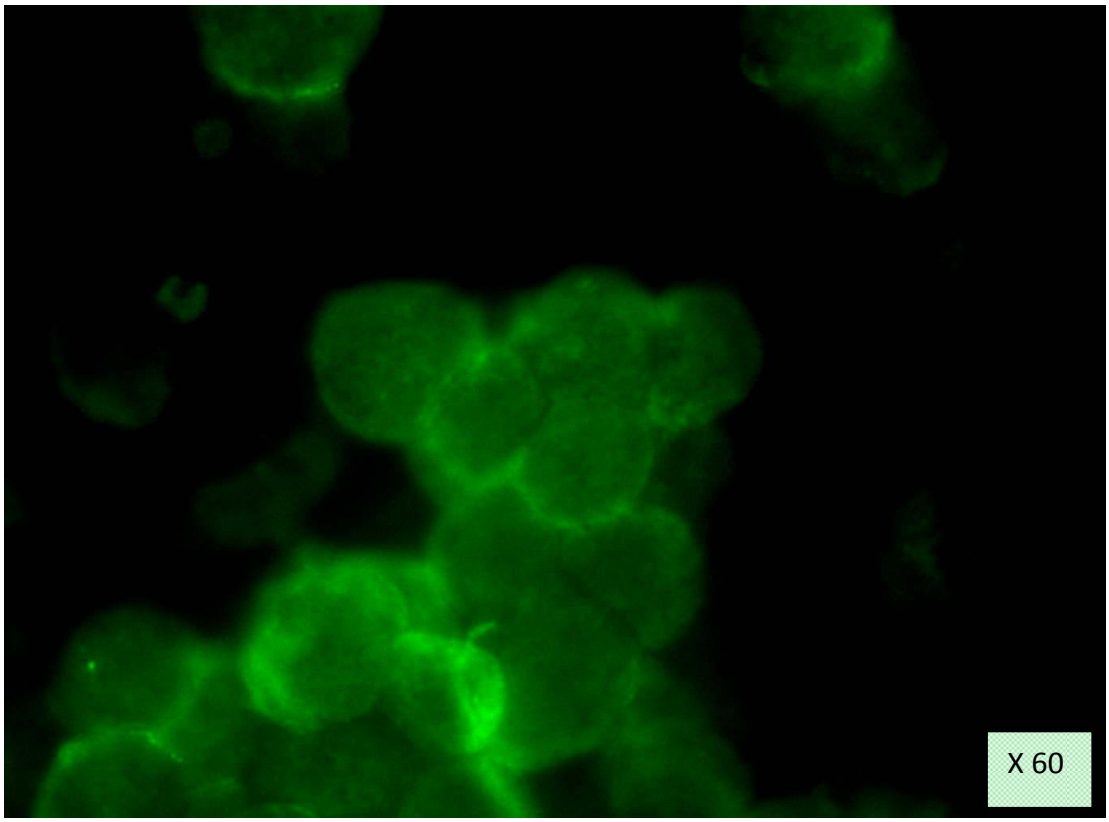

Figure 5.30 *An example of moderate fluorescence in a patient with IPF following incubation with FITC conjugated anti-IgG antibody on endothelial cells.*

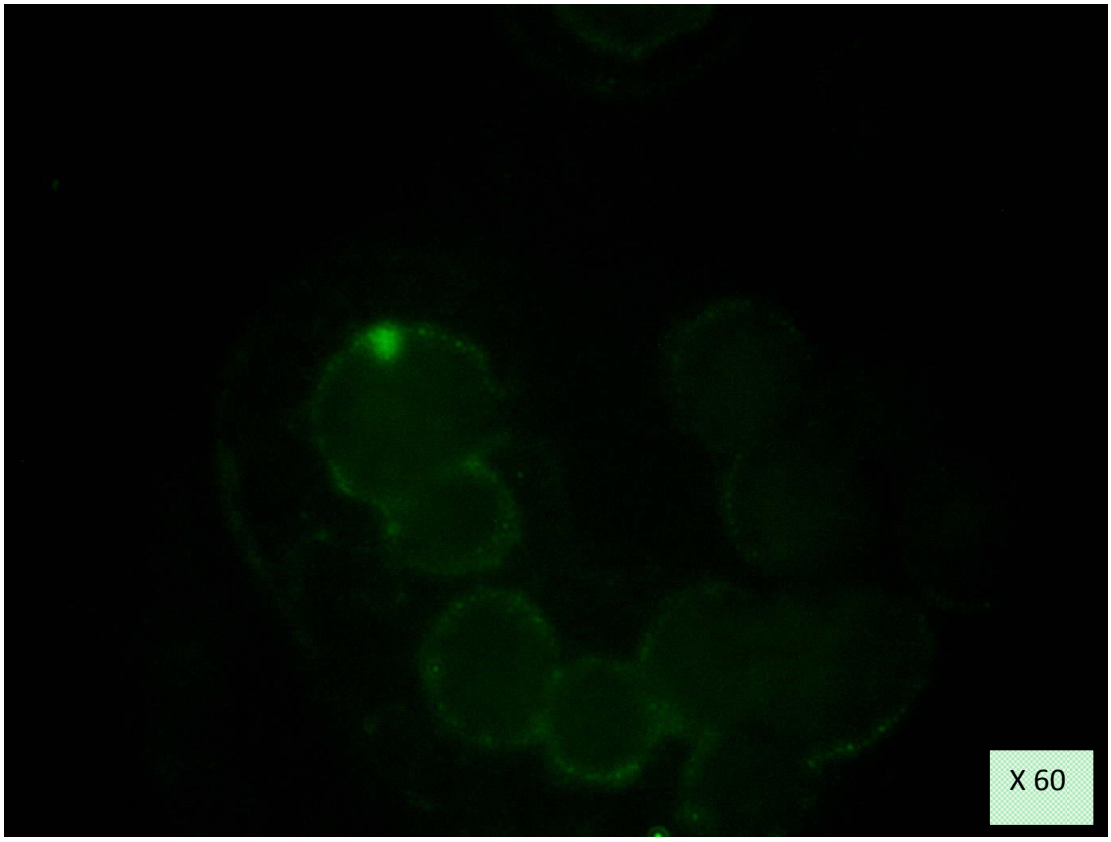

Figure 5.31 *Endothelial cells demonstrating a membranous pattern of staining following incubation with FITC conjugated anti-IgM antibody in a patient with idiopathic pulmonary fibrosis.*

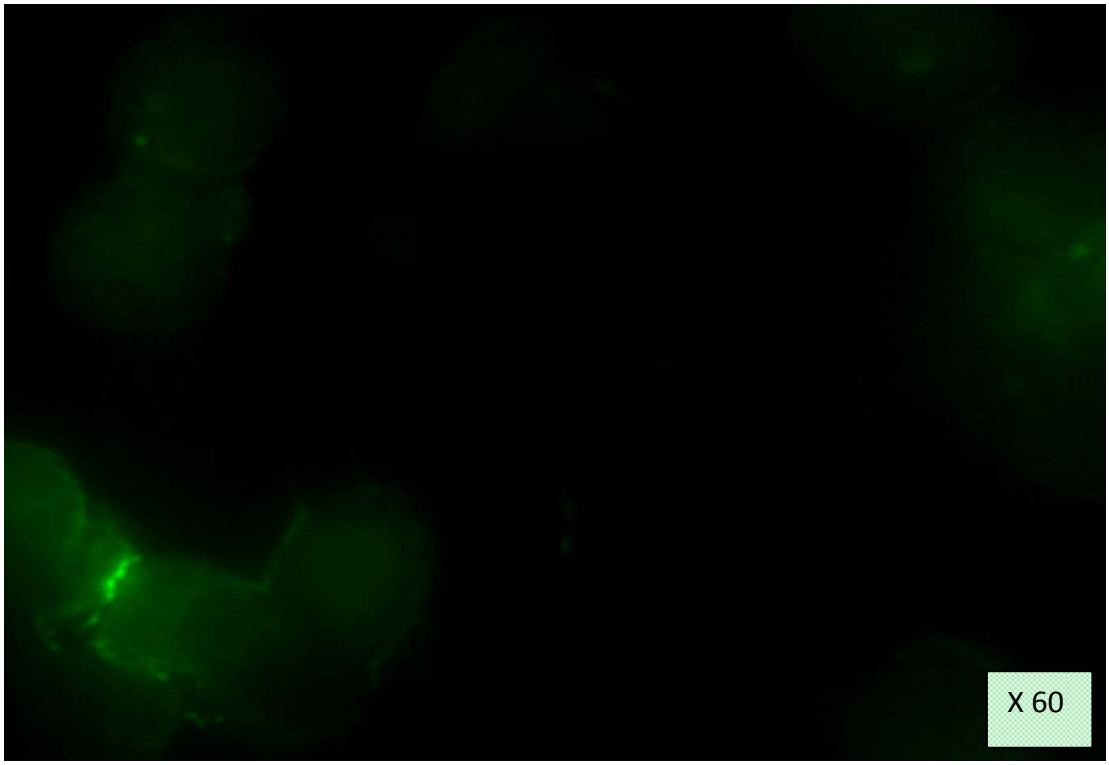

Figure 5.32 *Anti-IgG antibodies in a control patient without ILD demonstrating minimal fluorescence on endothelial cells.*

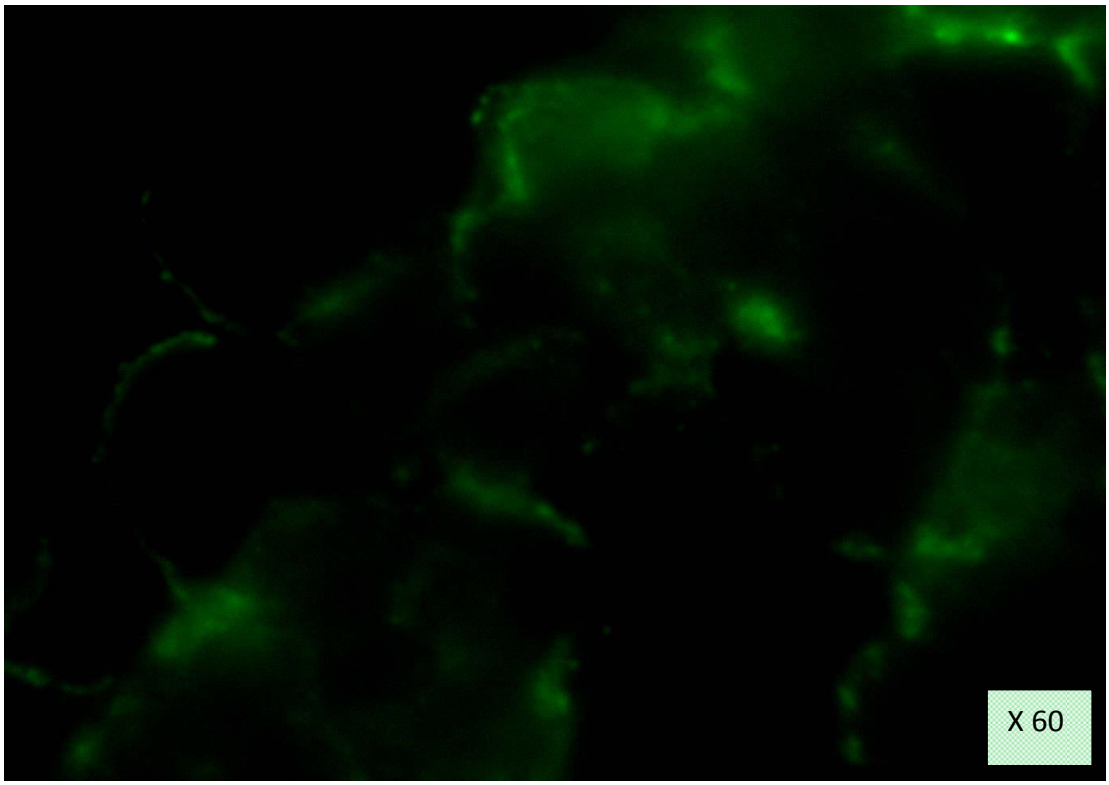

Figure 5.33 *HUVEC demonstrating minimal fluorescence following incubation with FITC conjugated anti-IgG antibodies in a patient with Sjogren's associated ILD.*

Anti-epithelial antibodies and pulmonary function

As part of the evaluation of immune mediated injury, I examined the relationship of anti-epithelial antibodies with lung function impairment measured by percent predicted FVC and TLco. The data showed that there was a significant negative correlation between IgG antibodies and percent predicted FVC (*P*=0.031; General linear model for correlation; Figure 5.34a) but not with carbon monoxide gas transfer TLco (*P*=0.653; Figure 5.34b). Furthermore, there was no significant correlation between IgM antibodies and pulmonary function parameters (data not shown).

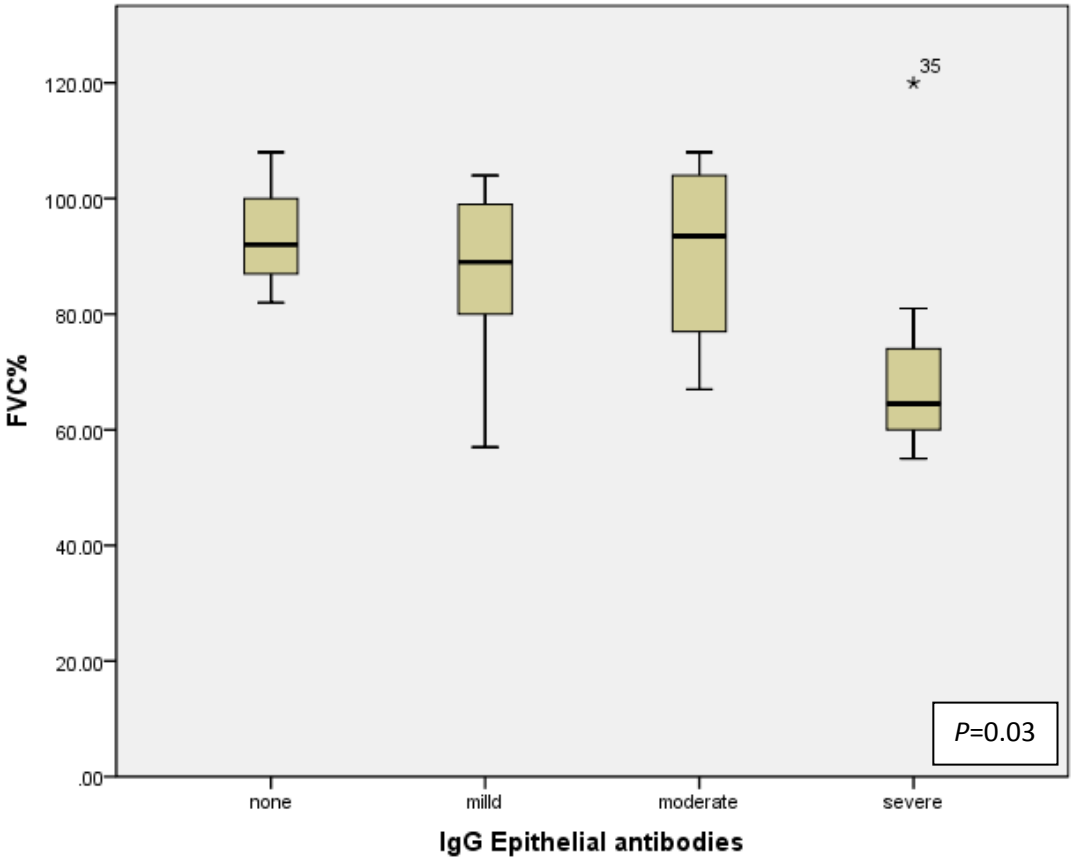

Figure 5.34a *The correlation between IgG antibodies against A549 cells and percent predicted FVC was significant*.

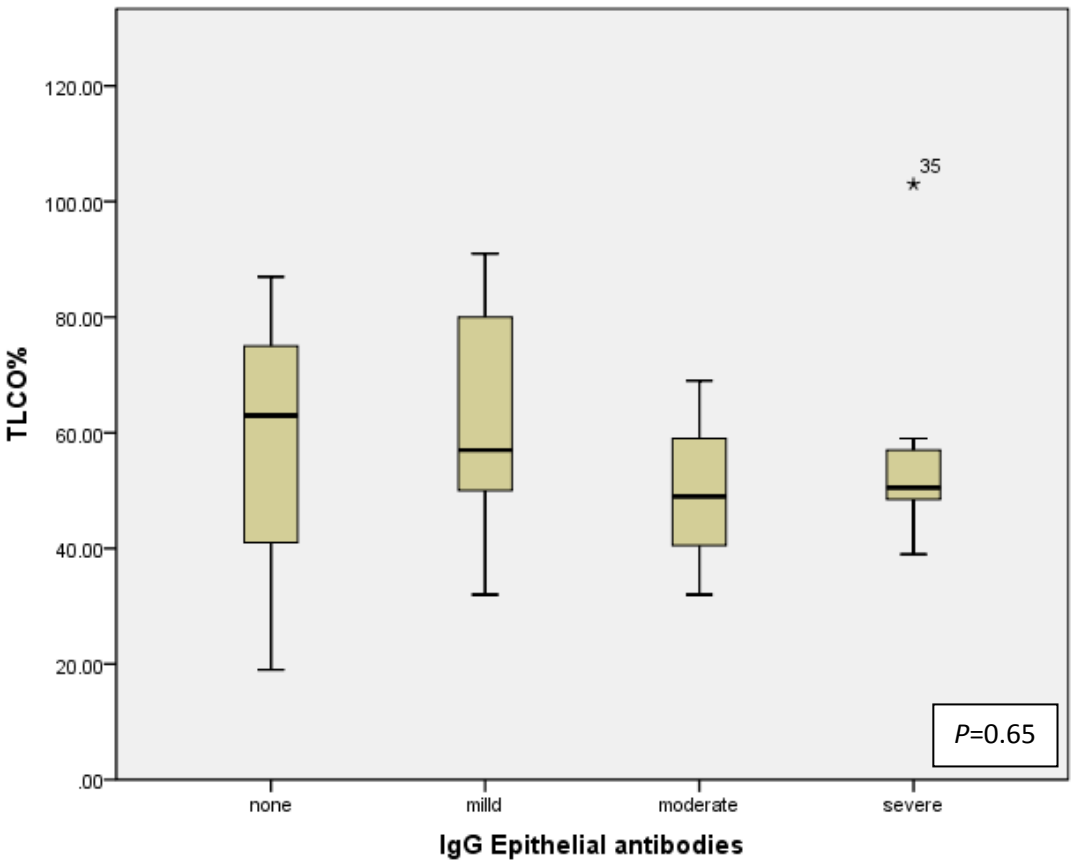

Figure 5.34b *The correlation between IgG antibodies against A549 cells and percent predicted TLco was not significant*.

Anti-endothelial antibodies and pulmonary function

In contrast to anti-epithelial antibodies, anti-endothelial antibodies did not have a significant correlation with parameters of lung function including percent predicted FVC and TLco (Table 5.7).

Table 5.7 Anti-endothelial antibodies and lung function

Antibody		Lung Function parameter	*P* Value
IgG	Vs	FVC	0.43
IgG	Vs	TLco	0.29
IgM	Vs	FVC	0.42
IgM	Vs	TLco	0.88

5.4 Discussion

The conventional theory regarding the initial molecular mechanisms in IPF had focused on a predominant inflammatory model with subsequent fibrosis. However, the lack of steroid responsiveness has argued against this model and the current understanding of IPF pathogenesis focuses upon abnormal wound healing response to an as yet unidentified insult(s). An immunological mechanism of alveolar epithelial injury in response to multiple hits to pulmonary parenchyma followed by imbalance favouring T-helper type II immunity, failure of re-epithelialization and release of pro-fibrotic growth factors is plausible (Strieter 2005).

In the present study, we have demonstrated a possible role of IgG antibodies against alveolar epithelial cells in the pathogenesis of IPF. As alveolar epithelial injury is believed to be one of the earliest events in the fibrotic process in IPF, the finding of significantly increased immunofluorescence with anti-IgG antibodies in a membranous pattern supports this “epithelial hypothesis”. Although the exact nature of this epithelial cell injury is unknown, there is evidence of a role of immune

mediated mechanisms culminating in a fibrotic response after lung injury in IPF (Ward 1979). Furthermore, administration of immune complexes may induce pulmonary fibrosis in animal models (Bellon et al. 1982) and elevated levels of immune complexes have been demonstrated in bronchoalveolar lavage fluid (BALF) in IPF (Dall'Aglio et al. 1988;Dreisin et al. 1978;Haslam et al. 1979).

Anti-epithelial cell antibodies have been described in the pathogenesis of IPF (Dobashi et al. 1999;Dobashi et al. 2000b;Dobashi et al. 2000a;Fujita et al. 1999a) and other interstitial lung diseases, such as radiation pneumonitis and pulmonary fibrosis associated with dermatomyositis (Fujita et al. 1999b). Fujita and colleagues (Fujita et al. 2000;Fujita et al. 2004) evaluated cytokeratin-19 fragment and cytokeratin 8 and 18 in a series of patients with radiation pneumonitis. There were significantly elevated levels of cytokeratin-19 antibody in patients with radiation pneumonitis with a significant correlation with disease progression. Dobashi and colleagues (Dobashi et al. 2000a) evaluated antibodies to cytokeratin-18 in IPF and found significantly elevated levels of this antibody by Western blot. Moreover, there was evidence of a significant increase in CK18: anti-CK 18 immune complexes in sera of IPF demonstrated by ELISA. Together, these findings do support the role of immune mediated mechanisms in the pathogenesis of usual interstitial pneumonia (UIP), the histological counterpart of IPF; as well as in connective tissue disease associated pulmonary parenchymal injury.

The findings of differential fluorescence and a significant relationship of IgG anti-epithelial antibodies with percent predicted forced vital capacity is thought provoking and the pathophysiological basis of this observation could be speculated.

It is possible that there is a linear relationship between epithelial cell injury and lung function impairment which has been demonstrated by this association. However, failure of TLco being significantly correlated with antibodies to alveolar epithelial cells somewhat reduces the credibility of this hypothesis. Indeed TLco may be too insensitive to show a significant association with epithelial damage as FVC is a reliable predictor of poor outcome in IPF (Zappala et al. 2010) and is probably more sensitive than TLco which is dependent on a number of other co-existing factors, such as emphysema.

Antibodies to endothelial cells have been investigated in the pathogenesis of different autoimmune diseases. Magro and co-workers (Magro et al. 2007) investigated 16 patients with connective tissue disease associated pulmonary fibrosis for immune mediated microvascular injury. Interestingly, there were significantly increased IgA anti-endothelial antibodies in patients with Rheumatoid arthritis. Moreover, there was serologic and pathologic evidence of immune based microvascular injury in that cohort of patients. Indeed, the microvascular injury has been demonstrated in the evolution of IPF by a study of 19 patients (Magro et al. 2003) showing septal capillary injury along with immunofluorescent evidence of antibody mediated injury to pulmonary microvasculature. Furthermore, circulating antiphospholipid antibodies were present in 18 patients tested for these antibodies. These data support the role of vascular injury in the pathogenesis of idiopathic pulmonary fibrosis. Although our study failed to show a differential fluorescence with endothelial cells, a subset of patients did have some membranous

enhancement (Figures 5.29-5.31) and a larger study may be able to evaluate endothelial dysfunction at cellular level in greater detail.

In conclusion, the findings of this study provide evidence in support of epithelial cell injury hypothesis in association with circulating IgG antibodies in IPF. As our understanding of IPF pathogenesis unfolds, it is becoming apparent that it is a disease of alveolar epithelial/endothelial interface and microvascular dysfunction secondary to immune dysregulation. This aspect of the pathogenesis must be explored further to expand our knowledge of this devastating fibrotic disorder.

CHAPTER 6: CARCINOEMBRYONIC ANTIGEN AND IDIOPATHIC PULMONARY FIBROSIS

6.1 Introduction

Carcinoembryonic Antigen (CEA) is a glycoprotein involved in cell adhesion and is produced during foetal development. The production of this antigen ceases soon after birth and a very small amount is detectable in normal healthy individuals. However, higher levels of CEA are present in smokers (Stevens & Mackay 1973) and this effect is pronounced in male smokers (Fukuda, Yamakado, & Kiyose 1998). There are several tumours associated with raised CEA level in serum such as colorectal, gastric, pancreatic, lung and breast carcinoma (Figure 6.1). In addition, raised level of this tumour marker has also been found in non-malignant conditions such as ulcerative colitis, pancreatitis, cirrhosis and ascites. The high level of CEA in lung malignancy reflects a marker of histological aberration in the lower respiratory tract and may be a hallmark of epithelial dysplasia and metaplasia. Recently, squamous cell carcinoma antigen has been demonstrated to be expressed by metaplastic epithelium in IPF. The primary aim of this study was to evaluate correlation between CEA concentration in serum and pulmonary function impairment as measured by percent predicted FVC and TLco. Moreover, a lung biopsy specimen with UIP pattern associated with IPF and raised CEA level was stained to explore the probable source of CEA rise in IPF.

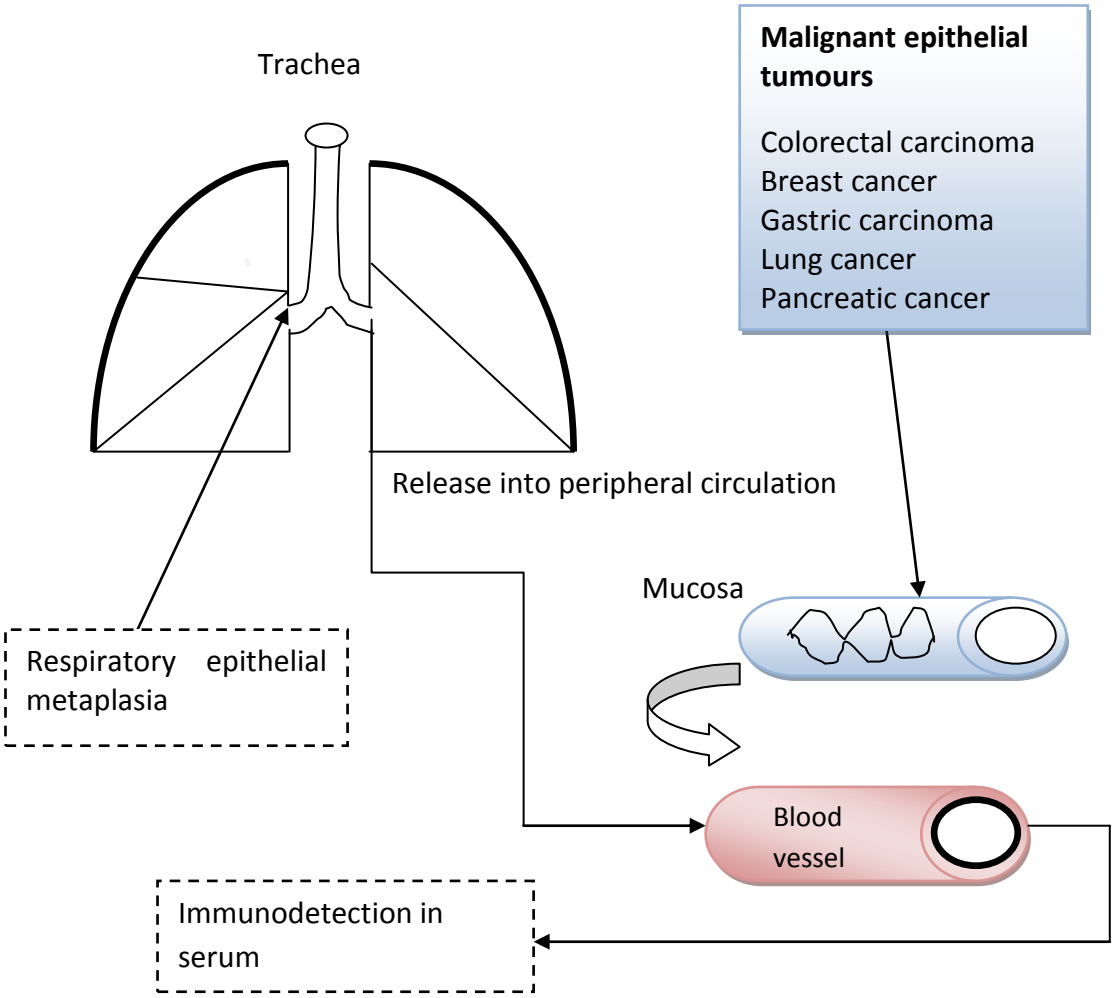

Figure 6.1 *The suspected pathopysiology of carcinoembryonic antigen production and release from malignant tumours and mucosal lining of gastrointestinal and respiratory tract. The speculated mechanism of elevated CEA level in pulmonary fibrosis is the release of this molecule from metaplastic respiratory epithelium.*

Epitheilal tumour markers and Pulmonary Fibrosis

There is increased incidence of lung cancer reported in patients with pulmonary fibrosis of uncertain aetiology (Fraire & Greenberg 1973;Haddad & Massaro 1968;Turner-Warwick et al. 1980). Moreover, there is evidence of raised CEA level in BALF of IPF patients (Takahashi et al. 1985). The exact mechanism for raised serum level of this antigen in IPF is not known. However, atypical epithelial proliferation and squamous metaplasia seen in lung biopsy specimens of patients

with idiopathic interstitial fibrosis (Haddad & Massaro 1968) may be one of the mechanisms responsible for CEA elevation in IPF. The prevalence of raised CEA level in heavy smokers is 13.6% as compared to 1.8% in non smokers (Stevens & Mackay 1973). The rise of CEA in heavy smokers may reflect a marker of dysplasia and or metaplasia of respiratory epithelium secondary to cigarette smoke.

There is evidence of significantly raised levels of carbohydrate antigen CA15-3 in UIP/IPF and advanced sarcoidosis (Ricci et al. 2009). This rise in CA 15-3 does have a significant correlation with decreased diffusion capacity and worsening fibrosis scores on HRCT scan. This finding of a close association of tumour markers with interstitial pulmonary fibrosis was the rationale to investigate this interesting association in further detail.

In this study, we measured the serum level of CEA in a cohort of IPF patients and evaluated any possible correlations between CEA level with lung function impairment, measured by forced vital capacity (FVC) and carbon monoxide diffusion capacity (TLco) and fibrosis scores on HRCT scan. Furthermore, the subjects were sub-categorized into two groups on the basis of total gas transfer impairment threshold of 40% as it is indicative of severe disease in IPF (Bradley et al. 2008). A subgroup analysis was undertaken to evaluate any significant difference in CEA level between these two groups.

6.2 Statistical analysis

The data were analysed by SPSS (Version 17, Chicago, IL). The correlations between serum CEA, lung function parameters and fibrosis extent were evaluated by Pearson correlation. An α-value of 0.05 was considered statistically significant.

6.3 Results

The main demographics and clinical characteristics of the study population are shown in Table 6.1. Two patients were excluded in view of the diagnoses of rectal adenocarcinoma and pancreatic carcinoma.

Table 6.1

Selected demographics of study participants

	IPF (ATS/ERS criteria)
Subjects (n)	43
Excluded (n)	2
Age (years, mean±SD)	73±7.1
Gender	
Male (n)	29
Female (n)	12
Smoking (n)	
Ex	39
Never	2
Current	0
FVC (L)	3.0±0.9
FVC (% predicted)	88±20
TLco (% predicted)	52±19
Fibrosis score on HRCT (n, %)	
Minimal (0-25%)	25 (61%)
Moderate (26-50%)	8 (19%)
Severe (51-75%)	6 (15%)
End stage (76-100%)	2 (5%)
Pattern of fibrosis (n, %)	
Reticular	9 (22%)
Honeycombing	10 (24%)
Mixed	22 (54%)

Data are presented as mean±SD or percentage % unless stated otherwise
FVC: forced vital capacity; TLco: Total gas transfer for carbon monoxide.

The mean CEA level (±SD) in the study cohort was 5.9±4.7 (ng/ml) (Figure 6.2). Normal range for CEA is 0-5 ng/ml.

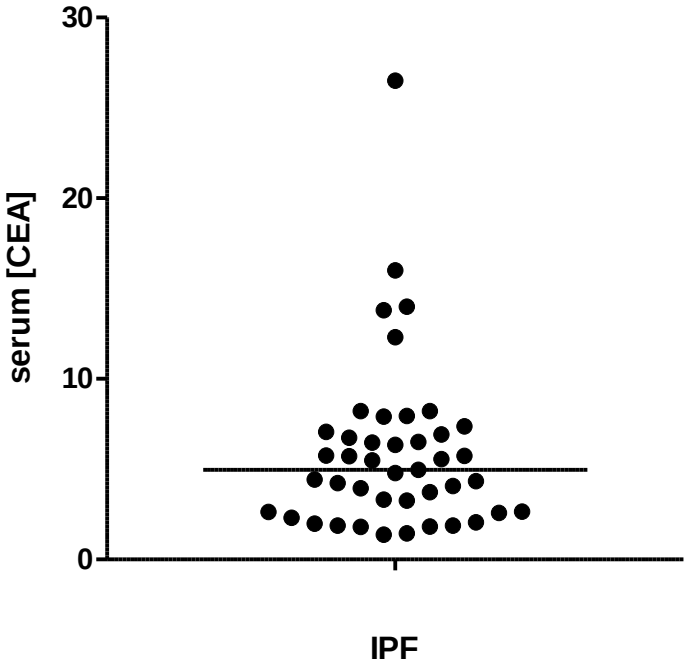

Figure 6.2 *Serum CEA concentrations (ng/ml) in the study cohort of IPF patients. Bar = median.*

Carcinoembryonic antigen in relation to gas exchange

To evaluate CEA level in relation to the degree of pulmonary gas exchange abnormality, the study group was divided into two categories with respect to impairment of gas exchange (TLco) for carbon monoxide at a threshold of <40% of predicted and ≥40%. The data show that CEA level was significantly raised in the group with worse gas exchange abnormality (Figure 6.3).

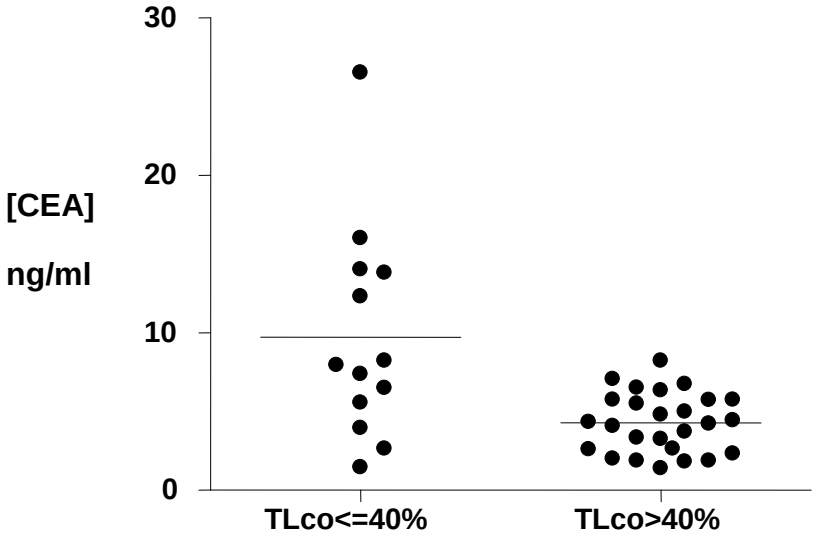

Figure 6.3 *Comparison of serum CEA concentration in IPF patients with TLco ≤ 40% predicted (severe disease) and TLco > 40% predicted. CEA is significantly higher in the severe disease group (p=0.006)*

There was evidence of a significant correlation between CEA and percent predicted FVC (Figure 6.4; R^2=0.19, *P*<0.01) as well as TLco (Figure 6.5; R^2=0.20, *P*<0.01). Furthermore, there was a significant correlation between CEA level and corrected gas transfer coefficient of carbon monoxide, Kco (*P*=0.029; data not shown).

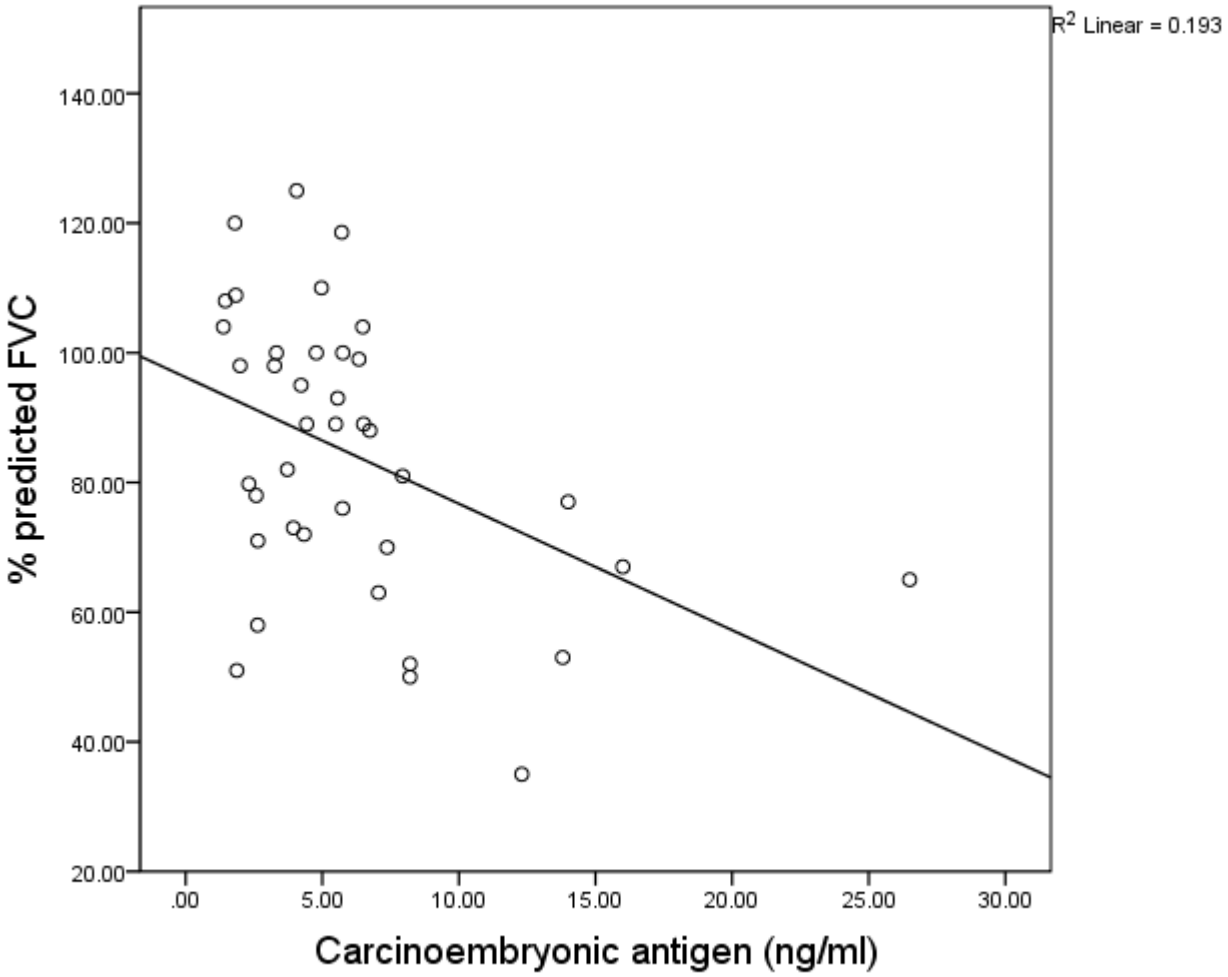

Figure 6.4 *The correlation between serum CEA concentrations and percent predicted FVC (P=0.005)*

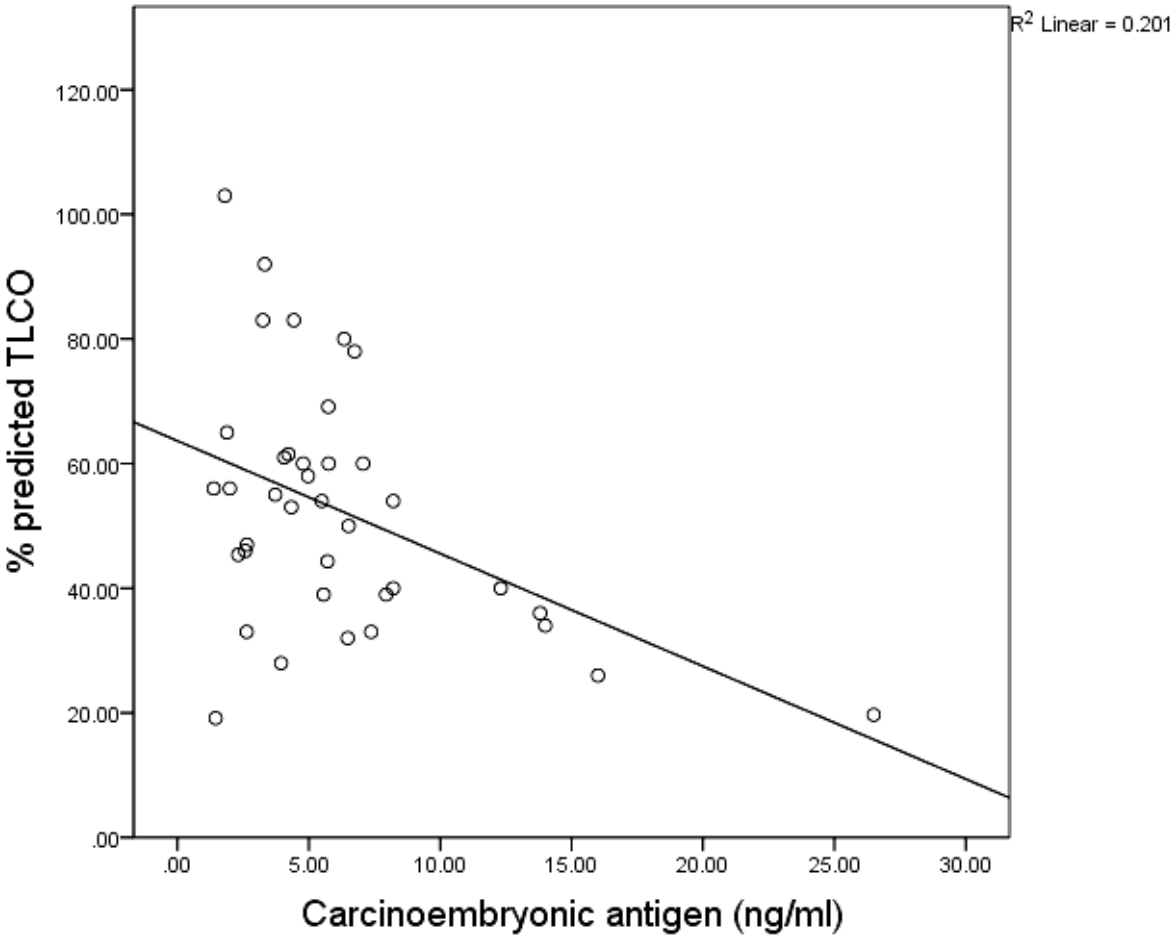

Figure 6.5 *The correlation between serum CEA concentrations and percent predicted TLco (P=0.006)*

HRCT fibrosis scores and CEA level

The extent of fibrosis correlated significantly with carcinoembryonic antigen concentration (*P*=0.027; Pearson correlation; Figure 6.6). However, the pattern of fibrosis did no correlate significantly with CEA level (*P*=0.946; data not shown).

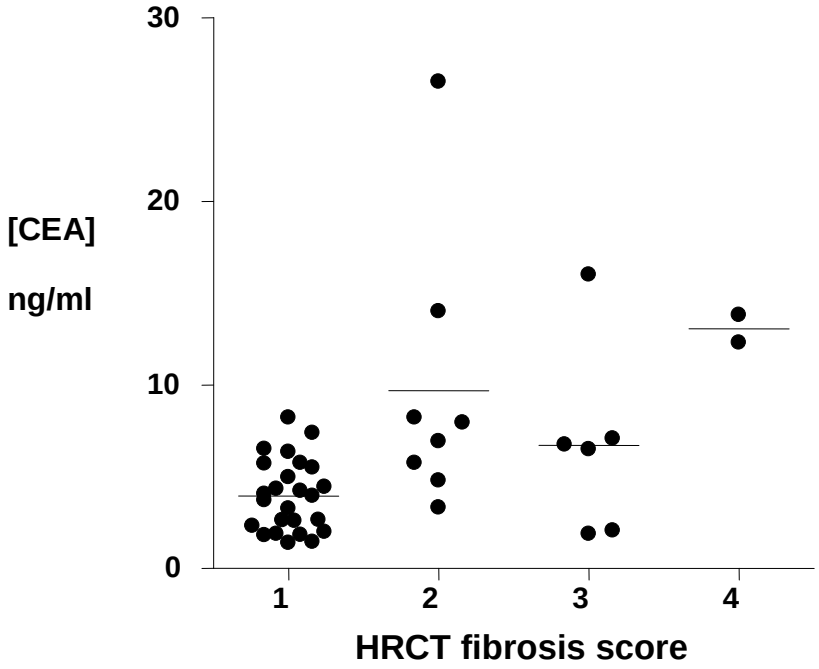

Figure 6.6 *The serum concentrations of CEA in relation to the extent of fibrosis on HRCT scan. The four categories refer to worsening interstitial pulmonary fibrosis where 1=minimal fibrosis and 4=advanced fibrosis affecting 76-100% of lung parenchyma.*

Similar to pulmonary function parameters, the association of CEA with pulmonary fibrosis extent was weak and a larger sample size might be able to clarify the link between rising CEA and lung fibrosis more accurately. The association of lung fibrosis severity with raised CEA level provides a further evidence of abnormal epithelial proliferation at pulmonary micro-alveolar level with the release of carcinoembryonic antigen.

Immunohistochemistry

As shown in Figure 6.7, there is evidence of a strong CEA staining of metaplastic epithelium lining the honeycomb cysts as well as respiratory bronchioles. Furthermore, there is no significant staining observed in normal lung tissue. A lung biopsy specimen from a patient who had undergone lobectomy was used as a control. There was no evidence of significant CEA staining observed in control biopsy specimen (Figure 6.8).

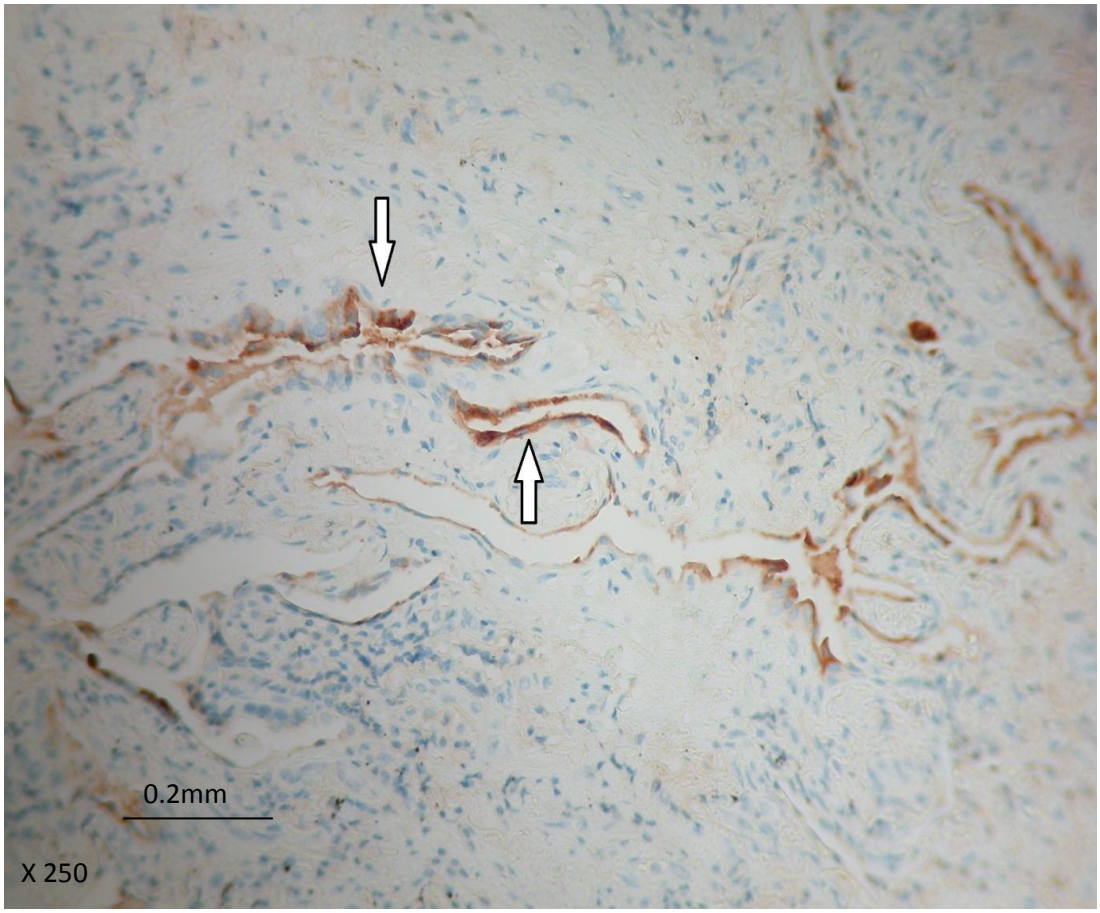

Figure 6.7a *Expression of carcinoembryonic antigen in lung biopsy specimen in epithelial lining of respiratory bronchioles (arrows) in a patient with IPF. (Photomicrograph provided by Dr A. Campbell)*

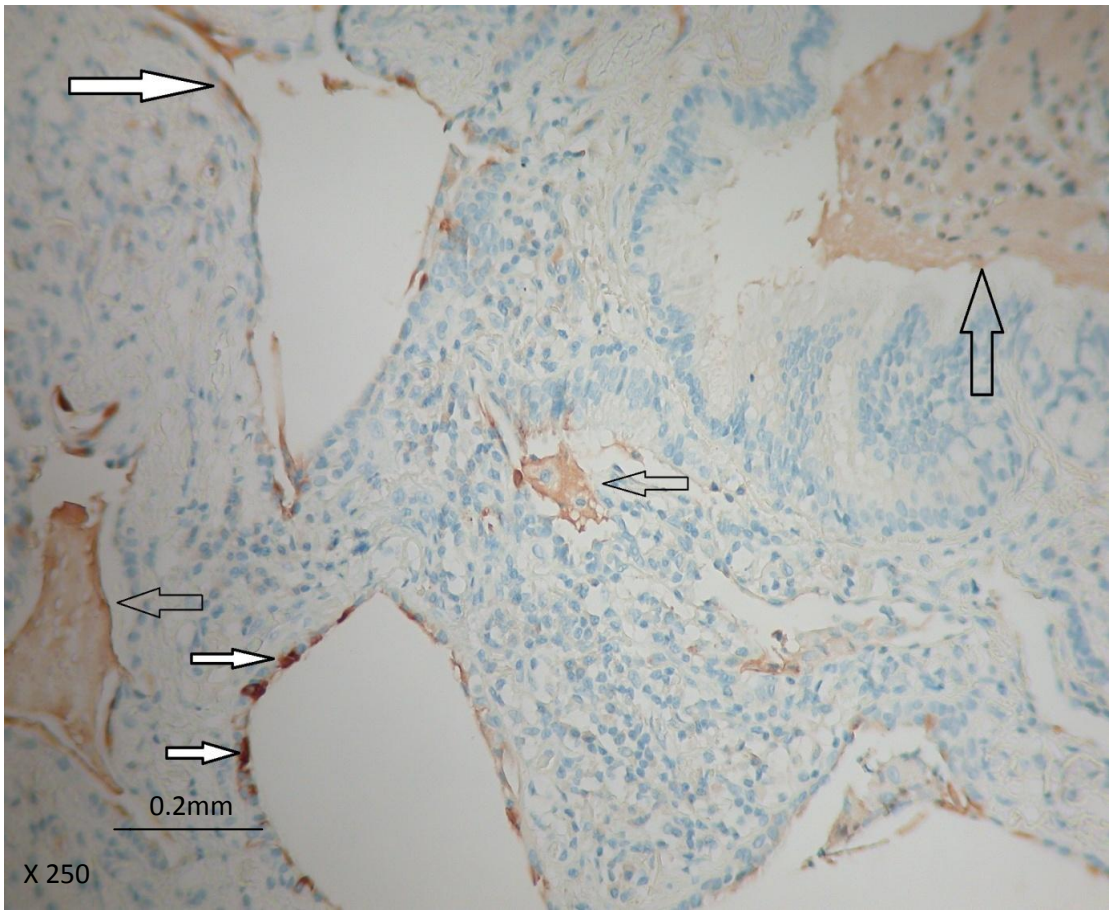

Figure 6.7b *There is strong staining of metaplastic epithelial cells lining the honeycomb cysts (dark brown colour; white arrows). The more diffuse light brown non specific staining is likely to be secondary to mucus (black arrows). (Photomicrograph provided by Dr A. Campbell)*

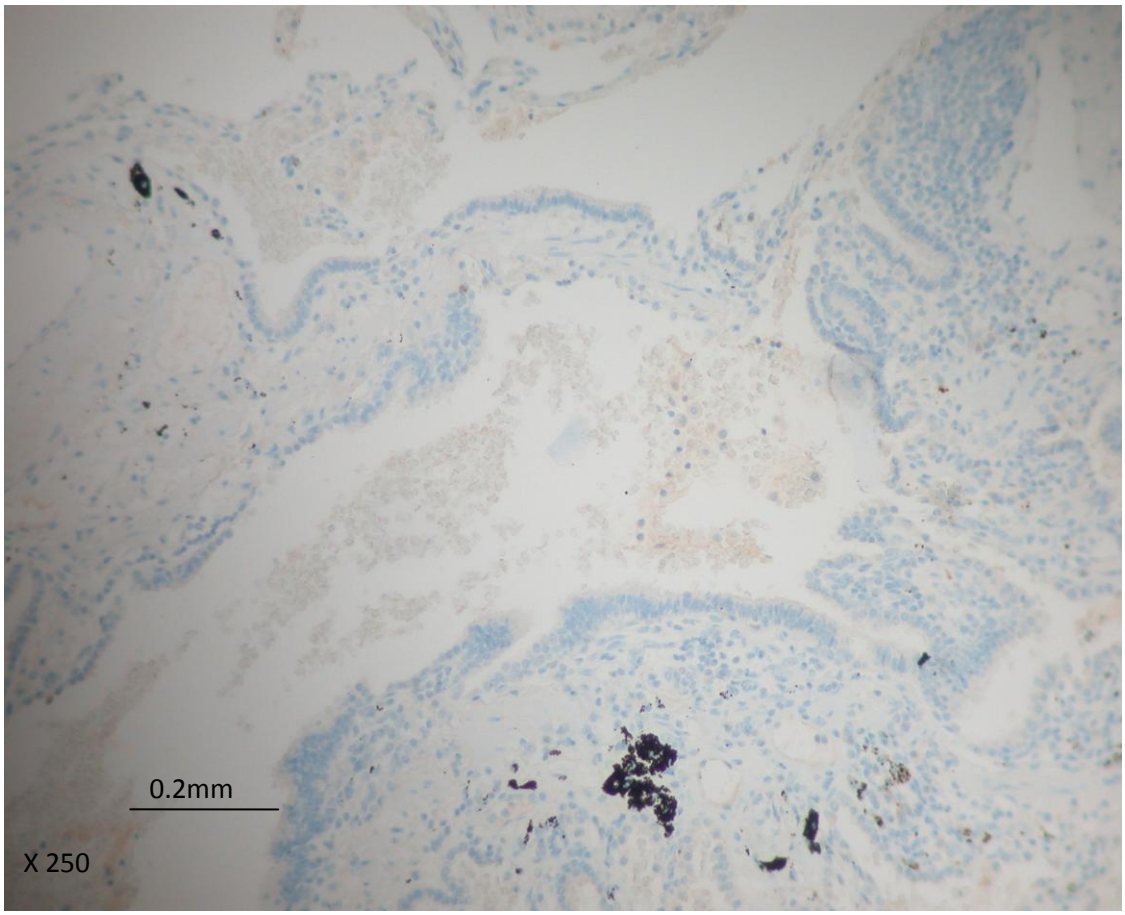

Figure 6.8 *The control lung biopsy specimen from a patient undergone lobectomy. There is no significant CEA staining of bronchiolar epithelium in this tissue section. (Photomicrograph provided by Dr A. Campbell)*

6.4 Discussion

This prospective study of evaluation of carcinoembryonic antigen in idiopathic pulmonary fibrosis suggests that there is evidence of high serum level of this tumour marker in peripheral blood of patients with pulmonary fibrosis. Moreover, the raised CEA level has a significant correlation with lung function impairment, measured by forced vital capacity and carbon monoxide gas transfer.

Our findings are in accordance with a retrospective study of patients undergoing lung transplantation. Hadjiliadis and colleagues (Hadjiliadis et al. 2001) evaluated the prognostic significance of serum CEA in 200 patients who underwent lung or heart-lung transplant at a University Hospital. The IPF group had the highest pre-transplant CEA level among the other indications for lung or heart-lung transplantation. However, rising CEA levels were unable to predict post-transplant survival.

The association of abnormal epithelial proliferation with idiopathic interstitial fibrosis has been recognized for more than 40 years. Haddad and Massaro (Haddad & Massaro 1968) evaluated 8 cases of diffuse idiopathic pulmonary fibrosis and found evidence of squamous metaplasia, epithelial stratification and atypical proliferation in the majority. It is plausible that the cuboidal epithelium lining the honeycomb areas observed in IPF may be the source of this antigen release into the respiratory secretions and subsequent elevation in the serum of these patients with pulmonary fibrosis. Abnormal re-epithelilalization plays a role in the pathogenesis of IPF as suggested by the presence of bronchiolization and the presence of abnormal proliferation of epithelial cells at

bronchiole-alveolar junction (Chilosi et al. 2002). Furthermore, there is evidence of an association between terminal bronchiolar carcinoma and interstitial pulmonary fibrosis (Spain 1957).

The close association of respiratory epithelial damage with the release of CEA in IPF is strengthened by a study of BALF and serum measurement of CEA in 26 patients with histologically confirmed IPF (Takahashi et al. 1985). The ratio of carcinoembryonic antigen to albumin (CEA/Alb, ng/mg) was measured in BALF in patients with IPF, smoking control subjects, sarcoidosis, hypersensitivity pneumonitis and lung cancer. The serum CEA was significantly higher in IPF group with or without lung carcinoma. CEA/Alb ratio was high in smoking controls, IPF and primary lung cancer than other interstitial lung diseases. Moreover, CEA/Alb ratio of BALF was significantly higher than serum, suggesting that majority of CEA in BALF is derived from pulmonary parenchyma. However, there was no correlation between BALF CEA/Alb ratio and serum CEA values.

IPF is associated with interstitial inflammation and structural remodelling of pulmonary interstitium. During the process of fibrosis, epithelial cells undergo a range of morphological changes from large cells to cuboidal, columnar or squamous cells (Carrington et al. 1978), cytoskeletal changes, loss or expression of adhesion molecules and formation of transitional phenotypes between type II and type I cells with limited regenerative capacity (Kasper & Haroske 1996). Moreover, there is diversity of molecular expression of pneumocytes during the proliferation and metaplastic changes in the process of interstitial fibrosis (Fujita et al. 1999a;Lappi-Blanco et al. 2006;Papadopoulos et al. 1993;Ricci et al. 2009). Dobashi and

colleagues (Dobashi et al. 1999) showed that proliferating type II pneumocytes express anti-human cytokeratin 19 antibodies in interstitial fibrosis. Moreover, intense staining of carbohydrate antigen CA 15-3 has been demonstrated in fibroblasts within the fibroblastic foci and cell cultures of lung fibroblasts from IPF lungs (Ricci et al. 2009). However, CEA expression by alveolar epithelium in IPF lungs has not been described in the literature.

This study demonstrates that the metaplastic epithelium lining the honeycomb areas is the likely source of CEA release into the respiratory secretions. Immunohistochemical analysis of lung tissue with UIP pattern and significantly elevated serum CEA has clearly delineated a strong CEA staining with no significant uptake of this antigen by normal lung tissue. As cuboidal pneumocytes are the predominant source of epithelial renewal in severe lung damage and fibrosis (Kawanami, Ferrans, & Crystal 1982), these cells are an attractive source of CEA release in IPF/UIP. To our knowledge, it is the first demonstration of CEA positivity in honeycombed bronchioles in IPF and provides the evidence of a novel mechanism of CEA rise in BALF and subsequently in the peripheral circulation.

Limitations of the study

It is important to acknowledge the fact that although the correlation between CEA level and pulmonary function is statistically significant, it is a weak correlation and a larger sample size might be able to evaluate this association in greater detail. Secondly, the fibrosis scores to correlate with CEA level were done by a single observer. The scoring of lung fibrosis by multiple observers would have increased the credibility of the observed association between carcinoembryonic

antigen and interstitial pulmonary fibrosis. Finally, the measurement of CEA was carried out once for each patient and it will be interesting to evaluate the association of CEA with lung function decline with repeated measurements of this serum tumour antigen parallel to pulmonary function assessment.

In conclusion, this study has demonstrated a negative correlation between serum elevation of carcinoembryonic antigen and pulmonary function in idiopathic pulmonary fibrosis. These findings do provide us with further insight into the pathophysiology of gas exchange abnormality and epithelial aberration in this restrictive lung disease of poor prognosis. On the basis of significant negative correlation between lung function impairment and CEA, it may be a potentially useful biomarker during the course of IPF and may be utilized as an additional guide to commence therapy in patients with progressively worsening pulmonary function.

CHAPTER 7: CONCLUDING REMARKS

The spectrum of alveolar injury in IPF is protean and different aspects of alveolar epithelial and endothelial involvement were evaluated during this work. The association of vascular derangement in form of platelet-monocyte aggregates was investigated in a flow cytometry based analysis. Platelet-monocyte adhesion was found to be significantly increased in IPF and this aggregation was independent of cardiovascular risk factors in the IPF population. Although this thesis did not explore the detailed molecular mechanisms of this binding, it has provided supportive data for a vascular injury hypothesis in line with similar observations in cardiovascular diseases. The findings of this study provide corroborative evidence of the role of platelets in this fibrotic disease with a strong association with vascular disease.

The immune mediated 'epithelial hypothesis' investigation revealed that IgG antibodies are likely to be involved in the pathogenesis of alveolar epithelial injury. These IgG antibodies had a significant correlation with declining lung function measured by FVC, supporting the pathogenic role of the immune mediated mechanisms responsible for epithelial injury and pulmonary fibrosis. Moreover, respiratory epithelial disruption at the level of respiratory bronchioles and honeycomb cysts could be the mechanism responsible for carcinoembryonic antigen rise and an association of the elevated CEA and lung function impairment was demonstrated in IPF. The significant correlation of fibrosis scores with CEA rise substantiates the evidence of close relationship of epithelial aberration and lung fibrosis in IPF.

The role of gastro-esophageal reflux in the pathogenesis of IPF has been debated for a long time. An association of reflux with IPF was confirmed on subjective assessment of symptom based questionnaire addressing both acid and non-acid reflux and it is likely that non-acid reflux might play a significant role in perpetuating the aspiration related alveolar epithelial injury. The novel techniques for the assessment of this type of reflux would provide further insight into the pathophysiology of reflux induced pulmonary parenchymal injury. It will be of crucial importance to evaluate reflux by means of upper airway pH analysis and repeated measurement of exhaled breath markers of gastro and extra-esophageal reflux such as pepsin and bile salts.

This thesis has substantiated the evidence of the role of immune mediated mechanisms in the pathogenesis of IPF. Platelets in conjugation with monocytes are a potential source of endothelial injury and pro-thrombotic state and further studies evaluating clotting factors and Von Willebrand Factor in relation to platelet activation may provide us with further insight into the pathophysiology of IPF at microscopic and cellular level. Furthermore, it will be important to study the effects of platelet agonists such as adenine di-phosphate and thrombin on platelet-monocyte adhesion.

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