A Study of Platelets and the Endothelium in Idiopathic Pulmonary Fibrosis

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December 2012

Abstract

Idiopathic pulmonary fibrosis (IPF) is a devastating lung disease associated with significant morbidity and early mortality. Despite this, the pathogenesis remains poorly understood and there is no effective disease modifying treatment. Epidemiological studies demonstrate an association between IPF and vascular disease. Platelets and the endothelium play an important role in maintaining vascular integrity, patency and function. In addition to their role in haemostasis, platelets have significant inflammatory and pro-fibrotic potential. Platelets and the lung have a close relationship in physiology and in disease however the role of platelets in IPF has not previously been investigated.

In this thesis we investigate the link between IPF and vascular disease and consider a potential pathogenic role of platelets in IPF. We do this through the following series of experiments: (1) investigation of the platelet endothelial cell adhesion molecule-1 single nucleotide polymorphisms in IPF and controls; (2) assessment of markers of platelet activation in IPF and controls; (3) investigation of the effect of IPF plasma on control platelets; (4) assessment of platelet function by measurement of platelet-endothelial cell adhesion; and (5) evaluation of plasma markers of endothelial activation and fibrinolysis in IPF.

We demonstrate that IPF patients exhibit increased platelet reactivity and that this can be reproduced in control platelets following incubation in IPF plasma suggesting that a plasma factor is responsible for this phenomenon. In addition, we show that the increased platelet reactivity in IPF is associated with an increased propensity to adhere to vascular endothelium confirming abnormal platelet function in IPF patients and suggesting a potential pathogenic mechanism. We do not demonstrate any difference in plasma levels of endothelial activation markers or fibrinolysis between IPF patients and controls. Similarly, we find no clinically significant difference in the prevalence of the PECAM-1 polymorphisms in IPF and controls.

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Acknowledgements

I would like to thank Dr Simon Hart for his continuing support throughout my MD and my clinical work as my supervisor and mentor. Prof Alyn Morice and Prof Khalid Naseem have provided their invaluable advice and expertise guiding me towards my completed thesis. Dr Laura Sadofsky and Chris Crow have been a great help to me throughout my MD teaching me essential laboratory skills.

I would particularly like to thank my wife Natalie for her unconditional support during my research.

Authors Declaration

I confirm that this work is original and that if any passage(s) or diagram(s) have been copied from academic papers, books, the internet or any other sources these are clearly identified by the use of quotation marks and the reference(s) is fully cited. I certify that, other than where indicated, this is my own work and does not breach the regulations of HYMS, the University of Hull or the University of York regarding plagiarism or academic conduct in examinations. I have read the HYMS Code of Practice on Academic Misconduct, and state that this piece of work is my own and does not contain any unacknowledged work from any other sources. I confirm that any patient information obtained to produce this piece of work has been appropriately anonymised.

Related Publications

Fahim A., Chong MC., Crooks MG., Hart SP. Idiopathic Pulmonary Fibrosis is Associated with Circulating Antiepithelial Antibodies. Lung. 2012 [Epub ahead of print].

Crooks MG., Hart SP. Increased Platelet Reactivity in Idiopathic Pulmonary Fibrosis. Am J Respir Crit Care Med 185;2012:A5162

Crooks MG., Aslam I., Hart SP. Chapter 5: Inflammation and Pulmonary Fibrosis. In: Khatami M., Ed. Inflammatory Diseases – Immunopathology, Clinical and Pharmacological Bases. In-Tech. 2012

Fahim A., Crooks M., Hart SP. Gastroesophageal Reflux and Idiopathic Pulmonary Fibrosis. Pulmonary Medicine. 2011;2011:634613

Fahim A., Crooks M., Hart S. Increased Platelet Binding to Circulating Monocytes in Idiopathic Pulmonary Fibrosis. Am J Respir Crit Care Med. 2011;183:A3550

1. Background

Idiopathic pulmonary fibrosis (IPF) is the most common idiopathic interstitial pneumonia (IIP) and carries a prognosis worse than many cancers with newly diagnosed patients having a median life expectancy of 2.8 years (1). Current classification defines IPF as the histological subtype of IIP, usual interstitial pneumonia (UIP). Patients typically experience symptoms of progressive shortness of breath with or without a dry cough. The progressive nature of this disease results in profound morbidity and early mortality. Despite the significant burden that this disease places on affected individuals it remains poorly understood and without effective disease modifying treatment.

There have been significant changes in IPF research over the years with reclassification of the disease taking account of non-specific interstitial pneumonia (NSIP) as a distinct clinical entity with a different clinical course and better prognosis than UIP. As such, historical studies of IPF (also known as cryptogenic fibrosing alveolitis) grouped a number of the IIP under a single umbrella term and therefore must be interpreted carefully when drawing conclusions applicable to current practice. It is also important to note that the pathological appearance of UIP is not unique to IPF and is also observed in interstitial lung disease associated with asbestos exposure, hypersensitivity pneumonitis and the connective tissue diseases(2).

There has been a shift in hypothesis regarding the pathophysiology of IPF away from a model of chronic inflammation precipitating fibrosis to a new model where initial epithelial and or endothelial injury results in aberrant wound healing/tissue repair. There has been a resulting change in the focus of scientific research with recent advances resulting in appreciation of the process of epithelial mesenchymal transformation in IPF and discovery of the circulating fibroblast precursor of bone marrow origin termed the fibrocyte.

An interesting discovery in recent years is the association between vascular disease and IPF. An increased rate of ischaemic heart disease and thromboembolic disease has been observed in patients prior to and following the diagnosis of IPF (3;4). This leads to important questions about what links these conditions and poses challenges regarding chronology and causality. The endothelium and haemostatic mechanisms are

important in both cardiovascular disease and venous thromboembolic disease and may also be of importance in IPF. It is well established that platelets and the lung are closely linked in physiology and indeed in pathology. Markers of endothelial activation and endothelial autoantibodies have been described in IPF and polymorphisms and serum levels of platelet endothelial cell adhesion molecule-1 (PECAM-1) have been implicated in cardiovascular disease in humans and pulmonary fibrosis in a mouse model. For this reason it is important to further investigate the role of the endothelium, platelets and haemostatic mechanisms in IPF.

The series of experiments described in this thesis aim to investigate platelet, endothelial and haemostatic responses in IPF patients compared with control patients without fibrotic lung disease. The results of these experiments will be discussed in their respective chapters following a review of the literature regarding IPF and its links to the vasculature.

1.1 Disease Classification

Hamman and Rich published a case series in 1935 describing a number of patients presenting to John Hopkins Hospital with diffuse parenchymal lung disease with variable stages of fibrosis evident on autopsy (5). This case series represents an early description of interstitial lung disease, in particular IIP. Our understanding of this group of lung diseases has progressed greatly since this time and continues to evolve; however, until recently there has not been a consensus on the classification of this heterogenous group of disorders.

IPF is the most common interstitial lung disease. Formerly also known as cryptogenic fibrosing alveolitis (CFA), the definition of this interstitial lung disease has changed in recent years with the publication of the 'International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias by the European Respiratory Society (ERS) and American Thoracic Society (ATS) in 2002(6). The evolution of the classification of the IIP's is presented in table 1.

The IIP's were initially classified by Liebow in 1968 describing 5 histological subtypes: usual interstitial pneumonia (UIP), desquamative interstitial pneumonia (DIP), bronchiolitis with interstitial pneumonia, lymphoid interstitial pneumonia (LIP), and

giant cell interstitial pneumonia (GIP). This classification was updated in 1997 by Muller and Colby with the exclusion of LIP because it was felt to represent a lymphoproliferative disorder and GIP which was discovered to be pneumoconiosis (6;7). Despite the classification of the IIP's by histological sub-type there remained widespread grouping of UIP, NSIP and DIP under the umbrella terms IPF or CFA including in the 1999 British Thoracic Society Guidelines (7). However, in 2002 the ATS/ERS published the consensus that defines the current classification of this group of interstitial lung diseases. In this classification the terms IPF or CFA refer only to UIP with NSIP and DIP becoming independent clinical entities (2;6). The British Thoracic Society (BTS) adopted this approach in their 2008 guidelines, discarding the term CFA and adopting IPF as described in the ATS/ERS consensus (2).

The international acceptance of the ATS/ERS consensus classification has reduced the ambiguity in terminology that plagues the historic literature making it easier to interpret and apply current research findings to clinical practice. The diagnostic criteria for IPF were further revised in 2011 in a joint statement from the ATS, ERS, Japanese Respiratory Society (JRS) and the Latin American Thoracic Association (ALAT) (8) but the main principles and classification remain unchanged from the 2002 consensus (9).

1.2 Clinical Course of IPF

The clinical course of IPF can vary widely ranging from rapidly progressive shortness of breath and dry cough with early mortality to asymptomatic fibrotic change noted incidentally on plain chest x-ray. Examination findings vary and can include finger clubbing and 'velcro-type' inspiratory crackles at both lung bases. The diagnosis is multidisciplinary based on clinical presentation, lung function testing and high resolution computed tomography (HRCT) appearance with lung biopsy reserved for cases in which there is diagnostic uncertainty (2).

Liebow 1968*	Müller 1997*	ATS/ERS 2002**
Usual interstitial pneumonia Desquamative interstitial pneumonia Bronchiolitis with interstitial pneumonia Lymphoid interstitial pneumonia Giant cell interstitial pneumonia	Usual interstitial pneumonia Desquamative interstitial pneumonia Bronchiolitis obliterans with organizing pneumonia (BOOP/COP) Acute interstitial pneumonia Non-specific interstitial pneumonia (Lymphoid interstitial pneumonia and giant cell interstitial pneumonia no longer included as felt to represent a lymphoproliferative disorder and pneumoconiosis respectively.)	Idiopathic pulmonary fibrosis (UIP) Non-specific interstitial pneumonia Cryptogenic Organizing pneumonia Acute interstitial pneumonia Respiratory bronchiolitis interstitial lung disease Desquamative interstitial pneumonia Lymphoid interstitial pneumonia

Table 1. Evolution of Classification of IIP

*See reference (7)

** see reference (6)

1.3 Epidemiology of IPF

Our understanding of the epidemiology of IPF has improved in recent years with a growth in the number of published epidemiological studies. Limitations relating to the use of large population data sets and historical changes in disease classification have to be considered when interpreting the data. A study of the incidence and mortality of IPF in the United Kingdom (UK) utilised a computerised General Practice database (the Health Information Network) containing information from 255 centres throughout the country (10). It reported an incidence rate of 4.6 per 100,000 person-years with a mean age of 71 years at diagnosis and male predominance (62%). There was significant geographical variation in incidence rates being higher in Scotland and the North of England and there was an overall rise in incidence by 11% per year throughout the study period. The median survival in this cohort was 3.9 years (10). A more recent UK study utilising the Health Information Network in addition to mortality data published by the Office of National Statistics reported rising incidence of IPF from 5.77 per 100,000 person-years in 2000 to 8.04 per 100,000 person-years in 2008 (11). Mortality attributed to IPF was also found to have increased over time from 0.92 per 100,000 person-years in 1968-1972 to 5.10 per 100,000 person-years in 2005-2008. The incidence and mortality rates were highest in men and with increasing age (11). Similar trends have been observed in hospital admission rates due to IPF in the UK with a 5% annual increase per year between 1998 and 2012 (12). Another European study assessed all pulmonary clinics in Finland and estimated a nationwide prevalence of 16-18 cases per 100,000 with familial cases accounting for 3-4% (13).

A retrospective cohort study carried out in the United States (US) estimated an incidence of 16.3 per 100,000 people and a prevalence of 42.7 per 100,000 people by extrapolating data from a cohort of 2.2million people in 20 states who had made claims through a large US health plan provider(14). These figures are based on evaluation of the number of patients with one or more encounters coded as IPF in the absence of any other encounters coded as a different interstitial lung disease. The authors referred to this as the 'broad definition'. Narrowing the diagnostic criteria to include only those with procedure codes for surgical lung biopsy, transbronchial biopsy or CT thorax ('narrow definition') reduced the estimated incidence and prevalence of IPF to 6.8 and 14 per 100,000 people respectively. Based on the 'broad definition', the

prevalence increased with age from 4 per 100,000 people under 34 years to 227 per 100,000 people over 75 years (14). In light of this association with increasing age the ATS/ERS criteria for diagnosis of IPF in the absence of surgical lung biopsy includes age >50 years as a minor criteria (2). A male predominance was also noted in the American cohort in keeping with the findings in the UK study however this was not confirmed in the Finnish population.

There is an association with smoking with an odds ratio of 1.6 for people who had ever smoked, increasing for current smokers and those with higher cigarette consumption(15). A survey of 1,442 patients with IPF in the US reported a history of cigarette smoking in 68.9% with 3.2% continuing to smoke (16). A history of smoking has a negative impact on prognosis with reduced severity-adjusted survival (17). IPF has been associated with a number of other conditions including gastroesophageal reflux (14;16;18); cardiovascular disease (3); and deep vein thrombosis (DVT) (4).

Gastroesophageal reflux (GER) is prevalent in the general population with a survey of 2,200 individuals aged 25-74 years in the US reporting approximately 20% of individuals experience symptoms of heartburn or acid regurgitation at least once weekly (19). An association between GER and fibrotic lung disease has been described for many years (20;21) however it is only recently that with the development of technology allowing assessment of oesophageal physiology that the relationship has been further investigated. Raghu et al. (22) performed a prospective study of 65 IPF patients and 133 controls with intractable asthma in order to assess the prevalence of GER in this group. They found a significantly greater prevalence of GER in the IPF group with 87% of patients having GER on oesophageal physiology studies but only 47% reporting typical reflux symptoms. The percentage of proximal and distal reflux time did not however correlate with IPF severity (22). Imaging studies have demonstrated that hiatus hernia, a condition associated with GER, is more prevalent in IPF patients compared with patients with other respiratory diseases (23). GER has also been found to be associated with acute exacerbations in IPF patients (24). Examination of bronchoalveolar lavage (BAL) specimens in a case-control study of IPF patients with acute exacerbations compared with controls with stable disease found that pepsin, indicating aspiration of gastric contents, was an indicator of acute exacerbation status in these patients (24). Interestingly, IPF patients with asymmetrical disease on HRCT

have been shown to have a significantly increased rate of GER and acute exacerbations compared to IPF patients with symmetrical disease (25). In this study, the method of diagnosing GER was the presence of symptoms with or without evidence of reflux on endoscopy or PH studies (25). Asymptomatic GER is common in IPF patients (22) and therefore the rate of GER may have been underestimated. There is some suggestion that treatment of GER in IPF patients may improve outcome. The use of GER treatments consisting of acid suppression at the time of IPF diagnosis was associated with less fibrosis on HRCT and increased long-term survival in a retrospective study (26). Although the retrospective nature of this study limits the strength of its conclusions it does provide support to the hypothesis that GER contributes to lung injury in IPF and emphasises the need for a prospective, randomised-controlled trial of GER treatment in IPF. Although GER with microaspiration and resulting lung injury represents an attractive hypothesis for inducing pulmonary fibrosis, the temporal relationship remains unclear with questions remaining whether GER is the primary event leading to IPF or simply results from the increased negative intrathoracic pressure that results from reduced compliance of fibrotic lungs.

A population-based study in the UK used the Health Information Network to assess the relationship between IPF and cardiovascular disease (3). This study included 920 IPF patients and 3,593 age, sex and community matched controls. An increased rate of vascular events including acute coronary syndrome, angina, and DVT were observed in the years preceding the diagnosis of IPF compared with the control group. This increased rate continued following diagnosis with an incidence of new acute coronary events of 19.3 per 1,000 person-years in the IPF group compared with 8.5 per 1,000 person-years in control subjects. Similarly, the rate of DVT was significantly greater following IPF diagnosis with 5.9 per 1,000 person-years compared to 2.1 per 1,000 person-years in controls. The findings of this study were not explained by differences in demographic or smoking status. Therefore, there is evidence that IPF patients have a higher rate of vascular events than age and sex matched controls and that this association precedes the diagnosis of IPF.

The association between vascular events and IPF has been further assessed in a nationwide study of the Danish population over a 27-year period comprising almost 7.5million people (4). This study utilised the Danish Patient Registry and Danish Causes

of Death Registry in order to identify patients with IIP and patients with venous thromboembolic disease including DVT and pulmonary embolism (PE). The risk of developing IIP was significantly higher in individuals with a previous history of venous thromboembolism (VTE) with an incidence of 18 per 100,000 person-years compared with 8 per 100,000 person-years in individuals with no history of VTE. The incidence of IIP was highest in those with a previous history of PE (28 per 100,000 person-years)(4). This association has also been demonstrated in a cohort of over 45 million individuals of whom 218,991 satisfied the criteria for a diagnosis of IPF (27). IPF was associated with a 34% higher risk of VTE compared with the general population with death occurring at a younger age in IPF patients with previous VTE versus those with no history of VTE (27).

These studies confirm an association between vascular events and IPF however the nature of the relationship remains unclear. It is clear that further research is required to improve our understanding of the pathophysiology of IPF in order to better define the relationship between IPF and vascular disease.

1.4 Pathophysiology of IPF

The initial hypothesis that chronic inflammation was central to the pathogenesis of IPF led to traditional treatment strategies targeting this process. Despite this disease having a prognosis worse than many cancers, until recently there has been a paucity of evidence regarding the proposed therapies with a meta-analysis by the Cochrane Collaboration in 2003 studying the use of corticosteroids in IPF failing to find any randomised clinical trials or controlled clinical trials for inclusion (28). Based on current evidence, the treatment regimens based on corticosteroids and other immunosuppressant medications have largely proved ineffective in altering the natural disease progression and resulting mortality and indeed may be harmful(29;30). Evidence from an early study reported that the addition of acetylcysteine to the regimen of corticosteroids and azathioprine slowed the deterioration of lung function. However, this study was underpowered to demonstrate any mortality benefit and only carried a weak recommendation in guidelines (2;31). Subsequently, a large randomised, placebo-controlled trial of prednisolone plus azathioprine plus

acetylcysteine was stopped early due to increased mortality, largely due to deterioration of lung disease, in the treatment arm (30).

New therapies are under investigation with some promising initial results from Pirfenidone; a novel agent that has anti-inflammatory, antioxidative and antifibrotic effects. Early trials have demonstrated a reduced decline in lung function and decreased incidence of acute exacerbations in the treatment group versus controls (32-34). The tyrosine kinase inhibitors are also undergoing investigation in IPF. An early study of Imatinib in IPF was disappointing, failing to demonstrate any benefit in lung function or survival in the intervention arm (35). However, a recent phase 2 trial of the new tyrosine kinase inhibitor BIBF1120, although failing to meet its primary end-point of reduced rate of FVC decline, did demonstrate a reduced decline of FVC from baseline, a reduction in acute exacerbations and a modest improvement in the St George Respiratory Questionnaire with the highest dose of the study drug (36). Despite these recent advances, there remains no widely accepted disease modifying treatment for patients with IPF. In exploring new avenues of treatment it is necessary to improve our understanding of the underlying disease process including its aetiology and pathogenesis.

Over recent years there have been significant changes in our understanding of the pathogenesis of this condition. The focus has moved from the classical hypothesis suggesting a central role of chronic inflammation towards a new hypothesis of epithelial and/or endothelial injury with abnormal tissue repair/wound healing (37). Recent advances have led to the belief that pulmonary fibrosis develops following initial lung injury with loss of the integrity of the alveolar-capillary basement membrane. Subsequent failure of re-epithelialisation and re-endothelialisation with cytokine mediated fibroblast and myofibroblast proliferation leads to collagen deposition (37). Several cytokines have been implicated in this process including platelet-derived growth factor (PDGF) (38-40), transforming growth factor-β (TGF-β) (41;42) insulin-like growth factor (39) and interleukin-1β (Il-1β) (43).

However, the role of inflammation in IPF has not been exhaustively investigated and there remains debate over its involvement. Indeed, NSIP has been demonstrated to coexist with UIP in 26% of individuals with IIP undergoing lung biopsy as part of a

prospective investigation of the histological variability in surgical lung biopsies from multiple lobes (44). Ground-glass opacification on HRCT (thought to represent inflammation on HRCT) is a feature of NSIP, which is also believed to be more steroid responsive than UIP. These findings pose the question whether NSIP, in which inflammation is a feature, represents an earlier stage in the disease process that when untreated progresses to the pathological appearance of UIP. Also, caution must be exercised when dismissing a role for inflammation based on the lack of efficacy of immunosuppressant regimes containing corticosteroids. This approach disregards the possible negative effects of corticosteroids. Corticosteroids reduce alveolar cell proliferation and increase apoptosis with potentially deleterious effects (45). In addition, corticosteroids have negative effects on vascular function by reducing prostaglandin E (PGE) production by vascular smooth muscle cells, enhancing vasoconstriction in response to angiotensin II and reducing vasodilation in response to arachidonic acid (46). The mineralocorticoid aldosterone has been shown to decrease endothelial cell nitric oxide (NO) production through inactivation of endothelin-B receptor (47) with resulting adverse effect on vascular tone. Cotricosteroids have a degree of mineralocorticoid activity in addition to their glucocorticoid effects and therefore may also have this effect. All of the mechanisms described above have the potential to reduce any benefit brought about by the anti-inflammatory properties of corticosteroids. Indeed, hyperoxia induced lung injury has been shown to be worsened by administration of corticosteroids (48). It is therefore important not to dismiss inflammation when considering the pathophysiology of IPF but to acknowledge that its role in the emerging picture of epithelial/endothelial injury with aberrant wound healing remains poorly understood.

The final common pathway in IPF is believed to be the accumulation and activation of fibroblasts and myofibroblasts with resulting extracellular matrix deposition and fibrosis. The origin of fibroblasts in IPF has been the subject of recent research with the discovery of epithelial-mesenchymal transformation (EMT) and the fibrocyte (cells of bone marrow origin that migrate to the lung prior to differentiating into fibroblasts and myofibroblasts) (37).

Chilosi et al. first suggested the process of EMT in IPF in 2003 (49). EMT is the process by which epithelial cells transform into mesenchymal cells through a series of events

including loss of epithelial polarity, detachment from the basement membrane and alteration of cytoskeletal architecture in response to a series of intra and extra cellular events (50). Initiation of EMT is dependent on the interaction between extracellular signals including collagen and hyaluronic acid in the extracellular matrix and soluble growth factors including TGF-β (50). The result is a loss of expression of the key epithelial markers and the adoption of mesenchymal cell markers (table 2).

Epithelial Markers	Mesenchymal Markers
E-cadherin	Fibronectin
Claudins	Vitronectin
Occludins	FSP-1
Desmoplakin	Vimentin
Cytokeratin-8,-9 and -18	Smooth-muscle actin
Mucin-1	FGFR2 IIIb and IIIc splice variants

Table 2. Cell markers characteristic of epithelial and mesenchymal cells(50)

Several *in vitro* and *in vivo* studies have confirmed that type-II pneumocytes undergo EMT in response to TGF-β1 as evidenced by reduced expression of epithelial phenotype markers, increased expression of mesenchymal phenotype markers and increased collagen production (51-54). The epithelial integrin α3β1 has been implicated in this process through its role in the phosphorylation of β-catenin to form pY654-β-catenin/pSmad2 complex resulting in initiation of EMT in response to TGF-β1 (55). In bleomycin induced pulmonary fibrosis, α3 integrin knockout mice failed to produce the pY654-β-catenin/pSmad2 complex and despite exhibiting a normal inflammatory response did not go on to develop fibrosis (55). This process appears to be relevant to IPF with lung tissue from IPF patients demonstrating pY654-β-catenin/pSmad2 complexes not present in control samples (55).

The role of EMT in experimental models of lung fibrosis is well established and the underlying mechanism and its role in IPF remains the subject of research.

Fibrocytes were first described in 1994 as a circulating cell that was morphologically distinct from leukocytes and had characteristics similar to fibroblasts (56). They have a unique phenotype expressing collagen, vimentin and CD34 (56). These cells express a number of chemotaxin receptors including CCR7 and CXCR4, undergoing chemotaxis in response to secondary lymphoid chemokine and CXCL12 respectively and have been demonstrated to localise to the lungs with associated increased collagen deposition in a mouse model of bleomycin induced pulmonary fibrosis (57;58). Elevated CXCL12 has been demonstrated in patients with IPF compared to controls with a negative correlation between CXCL12 levels and carbon monoxide diffusion capacity of the lung (59). There is evidence to suggest that in the mouse model, inhibition of the CXCR4 receptor reduced circulating fibrocyte numbers with a reduction in lung collagen deposition (60). These bone marrow-derived mesenchymal progenitor cells therefore represent an interesting new development in IPF research and indeed there is a suggestion that the presence of increased number of circulating fibrocytes in IPF patients is a marker of poor prognosis with raised mortality (61). However, the fibrocyte is not without controversy and there remains valid concern regarding the robustness of the methodology used in their identification and it has been suggested that rather than representing a unique population of cells (fibrocytes) they represent a range of different leukocytes(62). Further research to clarify the existence of the fibrocyte is required with subsequent investigation into the mechanisms underlying their recruitment and transmigration if their existence is confirmed.

Il-1β is a proinflammatory cytokine implicated in many diseases. Kolb et al (43) investigated its role in tissue fibrosis using adenoviral gene transfer resulting in transient over expression in rat lungs. A vigorous acute inflammatory response was demonstrated with elevation of the profibrotic cytokines PDGF and TGF-β in BAL fluid. Although the acute inflammatory response resolved over 2 weeks, TGF-β remained elevated at 60 days. Associated progressive tissue fibrosis with histological confirmation of myofibroblasts and fibroblastic foci was seen (43). The role of Il-1β in pulmonary fibrosis is supported by evidence that patients with a polymorphism in the Il-1 receptor antagonist gene are at increased risk of developing the disease (63). Il-1β

activates a series of cell adhesion molecules resulting in leukocyte transmigration in response to endothelial activation (64).

Despite ongoing investigation of the origin of fibroblasts in IPF their role is not debated. The same is true for the role of TGF-β despite ongoing research clarifying the specific mechanisms behind its involvement. As previously mentioned, a current hypothesis for IPF is based upon initial injury with altered healing mechanisms leading to the pathological features of the disease. The source of this initial injury is not yet known and may indeed not be consistent. However, it is generally suggested that alveolar epithelial injury is the initial event. This is supported by the development of pulmonary fibrosis following type II alveolar cell injury in a mouse model (65). Alternatively, it is important to consider the possibility that the primary injury or pathology may be endothelial. Indeed the pathological picture of UIP is recognised in response to both inhaled environmental factors (asbestosis) and circulating factors (connective tissue disease). A study of 40 patients with IPF revealed significantly elevated factor VIII levels (a marker of endothelial cell injury) in IPF patients versus controls. These patients were also noted to have positive antiphospholipid antibodies in 37/40 compared to 6/26 in the control group (66). Immunofluorescence and light microscopy of lung biopsies in these patients revealed evidence of immunoglobulin and complement deposition in the interalveolar septae and appearances consistent with immune-mediated microvascular injury (66). A similar study by Matsui et al. (67) assessed the presence of anti-endothelial cell antibodies by enzyme-linked immunosorbent assay (ELISA) using human umbilical vein endothelial cells (HUVEC) in the sera of patients with IIP. This study failed to demonstrate anti-endothelial cell antibodies in patients with IPF however did identify them in patients with NSIP and UIP related to connective tissue diseases (67). These findings do not appear to have been investigated elsewhere in the literature but these studies do highlight the need to consider the importance of the endothelium in the pathogenesis of IPF and its interplay with other circulating cells and molecules.

The existing hypotheses regarding the pathogenesis of IPF are broad and have so far failed to identify a novel pathogenic process or prompt the development of a disease modifying therapy. Furthermore, much of the evidence underlying current concepts in IPF stem from the bleomycin mouse model of pulmonary fibrosis and therefore have

questionable applicability to IPF. It is therefore essential to consider new hypotheses and explore existing hypotheses with greater focus.

The endothelium provides a constant barrier between the circulation and the lung interstitium trafficking circulating cells through the lung. Endothelial cell activation can lead to leukocyte transmigration and resulting inflammatory response. Another circulating cell with significant effects on the lung is platelets. The relationship between platelets and the lung is complex with important physiological functions and association with pathology (68). Platelets have been implicated in lung diseases including cystic fibrosis (69), asthma, pulmonary hypertension and adult respiratory distress syndrome / acute lung injury. (68) The role of platelets in IPF is not however clearly defined.

1.5 Platelets and the Lung

Platelets have a diameter of approximately 2-4 μm and survive in the circulation for approximately 7-9 days. They are formed from the cytoplasm of megakaryocytes that circulate in the blood originally forming long processes termed proplatelets that branch during maturation forming platelets at the tip with subsequent release (70). The lung appears to play an important role in this process with megakaryocytes and proplatelets entering and fragmenting in the pulmonary circulation to produce 2 or more platelets resulting in higher platelet numbers being recorded in the pulmonary venous circulation versus the pulmonary arterial system (71). Using electron microscopy, megakaryocytes have been demonstrated in the pulmonary capillaries of mice at various stages of platelet release following stimulation of thrombopoesis through venesection or administration of thrombopoetin (72).

Platelets are anucleate but maintain other cellular cytoplasmic organelles (73). Within the platelet cytoplasm there are several different granules, namely, alpha granules, dense granules and lysosomes that contain a wide range of proinflammatory, profibrotic and prothrombotic factors (70;74). The alpha granules contain numerous adhesion molecules necessary for platelet, leukocyte and endothelial interactions in addition to containing mitogenic factors including PDGF (75) and TGF-β (76); coagulation factors; and protease inhibitors(77). The activation of platelets results in the release of the contents of alpha granules and dense granules through exocytosis

(77;78). In addition, platelets synthesise IL-1β via a regulated post-transcriptional pathway that can be released in microvesicles or into solution with resulting endothelial cell activation, cell adhesion molecule expression and chemokine production culminating in neutrophil accumulation and activation (79). Following activation, platelets continue to circulate and have been shown to have the same lifespan as non-activated platelets (80). Although the surface P-selectin is cleaved within a matter of hours with release of a soluble form into the plasma (80;81), the platelet remains functional for a prolonged period with potential to contribute to inflammatory and thrombotic states (79).

A process similar to leukocyte adhesion and transmigration takes place allowing platelets to adhere to the intact endothelium. The first step in this process is mediated by P-selectin, a member of the selectin family of cell adhesion molecules that is found in the alpha granules of platelets and the Weibel-Palade bodies of endothelial cells (82;83). Through Ca^{2+} dependent translocation P-selectin becomes rapidly expressed on the surface of endothelial cells where it facilitates the rolling of platelets on the endothelial surface (84). Subsequent firm adhesion is mediated by the β_3 integrins, glycoprotein IIb/IIIa (GPIIb/IIIa) and $\alpha_v\beta_3$. Intercellular adhesion molecule-1 (ICAM-1) has also been implicated in this process (85). Through complex intracellular signalling processes the activated, endothelial bound platelets can release potent inflammatory and pro-fibrotic mediators (73).

In addition to the lungs' role in platelet production from proplatelets, the pulmonary microvasculature appears to act as a sieve removing platelets and other circulating cells from the circulation. A study using radiolabeled platelets demonstrated that one third of platelets are extracted on a single pass through healthy rabbit lungs although they remain in the lung for less than ten minutes (86). This leads to the question whether platelet sequestration in the lungs through cell adhesion molecule interactions and resultant release of inflammatory and pro-fibrotic mediators plays a role in chronic fibrotic lung diseases, namely IPF. Platelet trapping in the lung has been demonstrated using indium-111 labelled platelets in response to intravenous bleomycin with an association between platelet trapping and collagen deposition (87). Interestingly, in this study, treatment with antiCD11a monoclonal antibody significantly reduced the number of platelets in contact with the alveolar endothelium

suggesting involvement of the β_2-Integrins in this process. This is further supported by evidence of platelet sequestration in the lung following hyperoxia induced lung injury. On this occasion, treatment with anti-TNF-α resulted in reduced platelet surface CD11a expression and resulting reduced pulmonary sequestration (88). It is therefore reasonable to hypothesise that platelet sequestration in the lungs as a result of cell adhesion molecule interactions and associated platelet activation would result in a local release of known pro-fibrotic mediators.

As previously discussed, there is an increased incidence of cardiovascular and venous thromboembolic events in patients with IPF (3;4). Atherosclerosis underlies the development of cardiovascular disease with development of an atherosclerotic plaque leading to coronary artery luminal narrowing with flow limitation on exertion resulting in clinical symptoms of angina. Disruption of an atherosclerotic plaque exposes the subendothelial matrix leading to platelet thrombus formation with profound luminal narrowing or obstruction resulting in an acute coronary syndrome. Atherosclerosis involves both inflammation (89) and thrombosis (90). These two processes appear to play a role in IPF, although this has not yet been fully defined. In light of the important relationship between platelets and the lungs it is important to consider the relationship between platelets and IPF and the association between IPF and vascular events. Platelet activation and interaction with inflammatory cells is increased in patients with stable coronary artery disease as demonstrated by increased percentage of circulating platelet-monocyte complexes and increased platelet expression of P-selectin (91).

The alpha granules of platelets contain platelet endothelial cell adhesion molecule-1 (PECAM-1)/CD31), a complex molecule found on platelets, leukocytes and endothelial cells with many important functions (92). Therefore, in addition to the direct effect of proinflammatory and fibrotic mediators on the lung, the release of PECAM-1 may have additional consequences. PECAM-1 plays an important role in leukocyte transmigration in coordination with other cell adhesion molecules.

1.6 PECAM-1

PECAM-1 is a 130 kDa glycoprotein that belongs to the Immunoglobulin superfamily. It is coded for by a single copy gene consisting of 65kb divided into 16 exons on chromosome 17 (93). It consists of 6 extracellular domains each coded for by a separate exon (exons 3-8), a transmembrane portion coded on exon 9 and a complex intracytoplasmic tail with immunoreceptor tyrosine-based inhibitory motif (ITIM) domains coded for by 7 different exons (exons 10-16) (93). PECAM-1 is expressed on platelets, leukocytes and endothelial cells (92) and its mRNA is highly expressed in the lung, heart, kidneys, brain and liver (94;95).

A soluble form of PECAM-1 exists as a 100 kDa molecule that is missing the transmembrane and intracytoplasmic components. This soluble form is thought to result from membrane shedding and through alternative mRNA splicing (96). Numerous variants have been described with alternative splicing including a soluble PECAM-1 molecule with deletion of exon 9 (94). The process of shedding from endothelial cells is dependent on the matrix metalloproteinases and is thought to occur in the early stages of cell apoptosis. The complete PECAM-1 molecule appears to protect against apoptosis while the truncated form appears to have a role in the completion of apoptosis (97;98). An alternative source of soluble PECAM-1 production is via cleavage from platelets when exposed to high shear stress. This process has been demonstrated by observing reduced PECAM-1 expression on the surface of platelets exposed to high shear stress and was found to be dependent on the activation of calpain and the glycoprotein Ib (GPIb) – von Willebrand Factor (vWF) interaction (99).

PECAM-1 is a complex molecule with diverse functions, possibly dependent on its form and a complex process of cell signalling and phosphorylation of the intracytoplasmic domains. Indeed, where full length PECAM-1 is known to play an important role in leukocyte transmigration, addition of soluble PECAM-1 blocks this process (100). The role of PECAM-1 in leukocyte transmigration is attributed to its 1st extracellular domain (coded on exon 3) that is felt to mediate homophilic interactions necessary for this process (93). Using a monoclonal antibody to PECAM-1 or recombinant soluble PECAM-1, monocyte and neutrophil transmigration can be reduced by 70-90% (100). A number of heterophilic PECAM-1 ligands have also been identified although the

function of their interaction with PECAM-1 is not understood. Examples of proposed heterophilic PECAM-1 ligands include $\alpha_v\beta_3$ integrin (101), CD38 (102) and CD177 (103) and are felt to interact predominantly with the 2nd extracellular domain coded on exon 4 of the gene.

In addition to leukocyte transmigration numerous other functions have been attributed to PECAM-1 (see Figure 1) including: angiogenesis; mechanosensing and response in vascular stretch (104); regulation of platelet collagen interactions and platelet signalling pathways limiting growth of platelet thrombi (105); regulation of macrophage phagocytosis preventing the engulfing of healthy cells; and clearance of apoptotic cells(94;106).

The role of PECAM-1 has been investigated in a number of different diseases. A number of single nucleotide polymorphisms (SNP's) including Leu125Val on exon 3, Ser563Asn on exon 8 and Gly670Arg on exon 12 of the PECAM-1 gene have been associated with coronary artery disease with variable results (107-110). The Gly670Arg SNP on exon 12 has been shown to have a functional impact on molecular function in an ex vivo study demonstrating increased adhesion and transmigration of monocytes with a Gly/Gly genotype compared with an Arg/Arg genotype (111). The three described SNP's demonstrated strong linkage disequilibrium (107) and therefore the phenotypic difference resulting from the exon 12 SNP is also likely to be observed in association with SNP's in exon 3 and 8.

Elevated soluble PECAM-1 levels have been observed in stable coronary artery disease and during acute myocardial infarction reinforcing the association of this molecule with vascular events (109;112). A similar association has been observed in ischaemic stroke (113).

Expression of PECAM-1 has also been observed in many endothelial and haemotopoetic malignancies with over expression being related to resistance to chemotherapy induced apoptosis (114). As a result PECAM-1 is being investigated as a possible target for anti-cancer drugs. The role of PECAM-1 has not been investigated in fibrotic pulmonary disease.

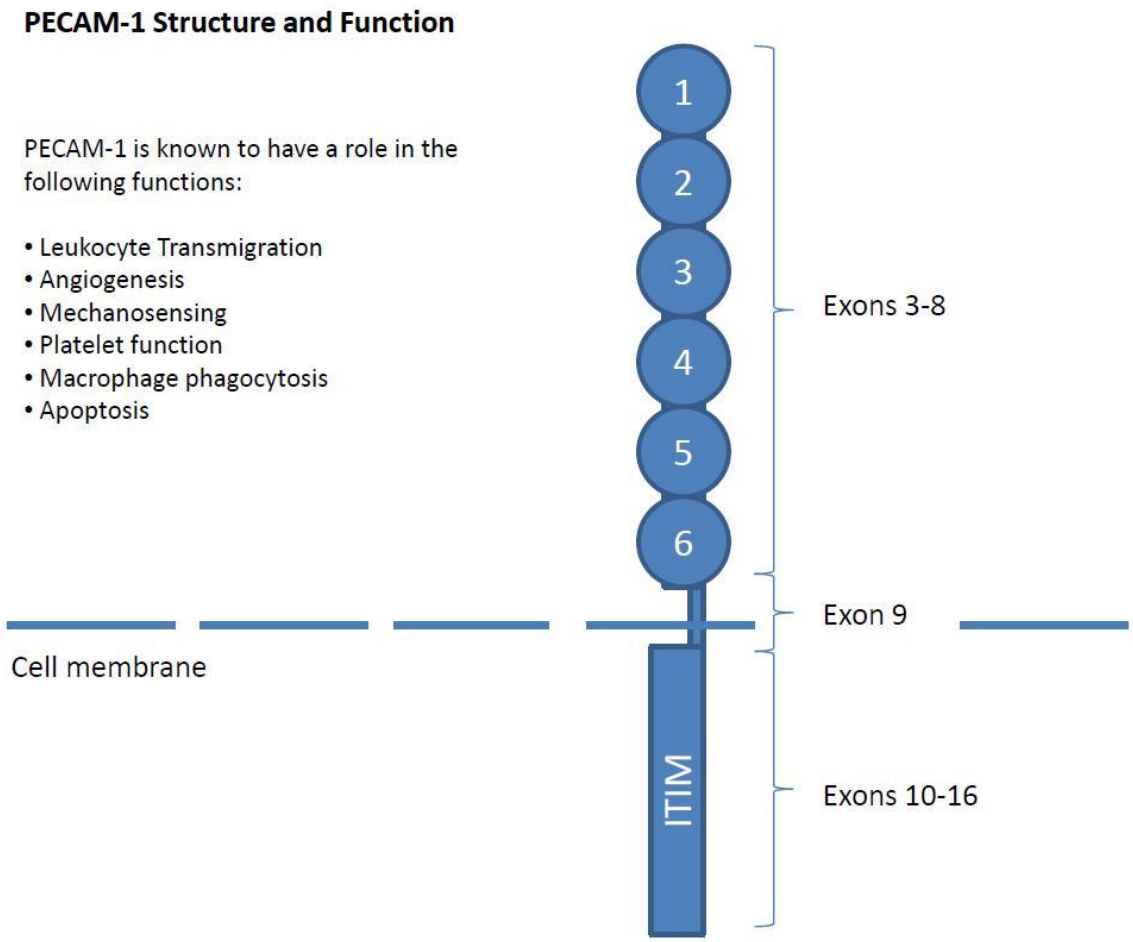

Figure 1. PECAM-1 consists of 6 extracellular domains coded on exons 3-8, a transmembrane portion coded on exon 9 and a complex intracytoplasmic tail with immunoreceptor tyrosine-based inhibitory motif (ITIM) domains coded on exons 10-16.

Interestingly, in PECAM-1 deficient mice, an idiopathic fibrotic pulmonary pathology has been identified. In this study of two strains of PECAM-1 deficient mice (FVB -/- and C57BL/6 -/- strains) the FVB -/- group were noted to develop respiratory distress with subsequent mortality (106). The most common abnormality identified in the lungs of these mice was diffuse alveolar damage with interstitial thickening and in many cases type II pneumocyte hyperplasia, increased numbers of alveolar macrophages and fibrosis with collagen deposition. This disease process was seen in the FVB -/- mice but not in the C57BL/6 -/- mice strain. This suggests that the latter strain may have PECAM-1 independent pathways that protect against the deleterious effects of PECAM-1 deficiency observed in the FVB -/- strain. Indeed, this mouse strain also has a

lesser reduction in acute inflammatory response associated with PECAM-1 deficiency than the other mouse strain (106).

When considering the possible role of PECAM-1 in IPF it is noteworthy that PECAM-1 has been demonstrated to be involved in the transmigration of CD34 positive haematopoietic progenitor cells through the endothelium into bone marrow following haematopoietic stem cell transplantation (115). In this study, immortalized primary endothelial cells pre-treated and activated with Il-1β were used to assess the transmigration of CD34 positive haematopoietic stem cells in response to the chemokine stem cell derived factor-1 (SDF-1). Reduction of transendothelial migration was observed with the addition of PECAM-1 blocking antibody with additional reduction with blockade of $Beta_1$ and $Beta_2$ integrin function demonstrating their involvement in this process (115). This raises the question whether bone marrow derived CD34 positive fibrocytes also undergo PECAM-1 dependent transmigration in the lung in patients with IPF. There has been little investigation of PECAM-1 expression on circulating fibrocytes. In a model of ischaemia/reperfusion cardiomyopathy cardiac fibroblasts were studied after 5 days of ischaemia/reperfusion injury demonstrating a significant proportion expressing CD34 and CD45 with a smaller spindle shaped morphology (116). These cells did not express PECAM-1 (CD31) at this stage expressing alpha-smooth muscle actin, collagen I and vimentin (116). The question remains however whether circulating fibrocytes may express PECAM-1 with loss of expression associated with development into active fibroblasts and myofibroblasts.

1.7 Haemostasis and IPF

Haemostatic mechanisms in health maintain vascular integrity preventing haemorrhage while maintaining vascular patency by preventing inappropriate thrombosis. There is a complex interplay between haemostatic processes, inflammation and cellular/tissue injury resulting in disordered haemostatic processes being implicated in the pathogenesis of many diseases. There are three main mechanisms by which haemostasis is maintained: primary haemostasis involves the formation of a platelet plug; secondary haemostasis involves formation of a fibrin clot through activation of the coagulation cascade; and fibrinolysis promotes the destruction of fibrin clot in order to maintain vascular patency and maintain haemostatic homeostasis. Platelets are central to primary haemostasis and have been

discussed in a previous section and therefore this section will focus on the role of secondary haemostasis and fibrinolysis in IPF.

Secondary Haemostasis: Formation of the Fibrin Clot

Secondary haemostasis can be activated through intrinsic or extrinsic pathways, the latter requiring the interaction of tissue factor (TF) with factors VII and VIIa. TF is a 43kD transmembrane cell surface glycoprotein that interacts with factor VII and VIIa to form a complex that in turn activates factor X with resulting production of thrombin from prothrombin that in turn converts fibrinogen to fibrin. This process is inhibited by tissue factor pathway inhibitor (TFPI) that forms a complex with factor Xa prior to inhibiting the action of TF/factor VIIa complex (117).

Local activation of the coagulation cascade has been demonstrated as a key process in IPF. TF and TFPI have been demonstrated to be increased in BAL samples from patients with IPF with higher levels seen in those with advanced disease (117;118). Despite elevated levels of TFPI corresponding with increased TF expression there was a positive correlation with a procoagulant state suggesting the procoagulant effects of TF outweighed the anticoagulant effects of TFPI (117). Hyperplastic type II pneumocytes, epithelial cells lining fibroblastic foci and macrophages have been demonstrated to express TF with associated fibrin deposition in the lungs of patients with IPF and not in healthy lungs (117;119). A similar pattern was seen in patients with scleroderma related interstitial lung disease and BOOP (119). The occurrence of this process across different diseases suggests that this is a response to lung injury rather than a primary event.

Fibrinolysis

Fibrinolysis is an essential process in maintaining haemostatic homeostasis preventing excess accumulation of fibrin clot and resulting tissue and organ dysfunction. The interplay between factors promoting and inhibiting this process has been demonstrated to be important in lung injury and lung fibrosis. Plasmin is central to the process of fibrinolysis but is a broad-spectrum protease enzyme capable of breaking down many other extracellular proteins in addition to fibrin. It is therefore believed to play an important role in repair following alveolar injury (120). Plasmin is produced from plasminogen in response to plasminogen activator. The alveolar space in health

favours fibrinolysis due to local abundance of urokinase-type plasminogen activator (u-PA) however plasminogen activator inhibitors (PAI) have been demonstrated to be increased in cases of acute lung injury including adult respiratory distress syndrome leading to persistence of intra alveolar extracellular matrix proteins (121). There are two identified PAI but the most important implicated in this process is PAI-type 1 (PAI-1) (121).

PAI-1 is a member of the serpine superfamily and is the main inhibitor of u-PA and tissue-type plasminogen activator (t-PA). It acts by forming a stable 1:1 serine-proteinase/serpine complex. PAI-1 has been investigated in a mouse model of pulmonary fibrosis induced by intratracheal administration of bleomycin using genetically modified mice with up regulated PAI-1 expression (PAI +/+) and down regulated PAI expression (PAI -/-). PAI-1 deficient mice (PAI-/-) were found to be relatively protected from the fibrogenic effects of intratracheal bleomycin with markedly lower collagen deposition measured by lung tissue hydroxyprolene content than PAI+/+ mice. There appeared to be a correlation between gene expression and collagen deposition with heterozygous mice (PAI +/-) having an intermediate fibrotic response to intratracheal bleomycin (120). This finding demonstrates the importance of impaired plasmin production through excessive plasminogen inhibition via PAI-1 in the fibrotic response to lung injury in a mouse model. Intra-nasal administration of small interfering RNA has been utilised to reduce PAI-1 levels in the lungs of mice with resulting attenuation of the fibrotic process following intratracheal bleomycin representing a promising therapeutic option (122). PAI-1 has also been implicated in human disease. A study of bronchoalveolar lavage (BAL) specimens from patients with IPF found significantly elevated levels of PAI-1 and PAI-type 2 (PAI-2) in IPF patients compared with controls. There was no difference in u-PA or t-PA levels in BAL in these patients suggesting a shift in balance from pro-fibrinolytic to anti-fibrinolytic in patients with IPF (118). In IPF, PAI-1 has a similar pattern of expression to TF being localised to hyperplastic type-2 pneumocytes, macrophages and the columnar epithelium lining areas of honeycomb fibrosis (122). Investigation of the prevalence of polymorphisms in the promoter region of the PAI-1 gene in IIP's failed to demonstrate a significant difference however subgroup analysis revealed an association with NSIP but not UIP (123).

PAI-1 has been associated with cardiovascular disease with increased plasma activity observed following acute myocardial infarction (124). Increased PAI-1 production has been demonstrated in response to platelet associated growth factors including epidermal growth factor (EGF) and TGF-β and directly in response to platelet lysates *ex vivo* in cultured human hepatocytes (125) and *in vivo* in a rabbit model (126). This demonstrates that platelet factors known to be released with platelet activation can promote PAI-1 production with resulting inhibition of plasmin production leading to accumulation of fibrin and extracellular matrix proteins as observed in IPF. Despite high TGF-β concentrations in the lung following intravenous administration there was no associated increase in lung PAI-1 mRNA expression with increases limited to the aorta, heart and liver leading the authors to suggest that endothelial cells and hepatocytes are predominantly responsible for the increased plasma levels (126). Given the increased PAI-1 production by hyperplastic type II pneumocytes in patients with IPF it is conceivable that platelet factors could play a role in this process and therefore warrants further investigation.

In addition to promoting PAI-1 production by other cells, platelets retain PAI-1 mRNA and continue to produce the active molecule (127). This represents an alternative source of PAI-1 that warrants further investigation in patients with IPF.

IPF and Anticoagulation

Kubo et al. (128) performed a randomised controlled trial of anticoagulation in addition to steroid therapy in patients with IPF in order to identify the presence of a coagulation disorder in IPF and assess the effect of anticoagulation with warfarin on survival. Sixty-four patients were recruited to the study with 56 patients completing the study (33 in the non-anticoagulant group and 23 in the anticoagulant group). It is noteworthy that all patients dropping out of the study were in the anticoagulant group with 6 fearing side effects of treatment or frequent venepuncture, 1 experiencing purpura and 1 moving away from the locality. Plasma D-Dimer levels on recruitment to the study were elevated in both groups increasing further during acute exacerbations of the disease.

D-Dimer is a fibrin degradation product and is released into the plasma following endogenous fibrinolysis. It therefore represents increased activation of haemostatic

mechanisms and is used clinically in risk stratification in the diagnosis of venous thromboembolic disease but is elevated in a wide range of conditions including but not limited to sepsis, trauma, vascular disease and malignancy. The elevated D-Dimer level at baseline in IPF patients suggests background activation of haemostatic mechanisms.

Patients were randomised to receive prednisolone alone or prednisolone and anticoagulation with outpatient warfarin (target international normalised ratio (INR) of 2.0-3.0) and intravenous low molecular weight heparin during admission with acute exacerbations. The mortality rate was significantly lower in the anticoagulant group with 5/23 deaths during the study period compared with 20/33 deaths in the non-anticoagulant group (p=0.006) with 1-year survival rates of 87% and 58% respectively. The reduced mortality was associated with a significant reduction in d-dimer in the anticoagulant group. Despite the small number of patients included in the study, the lack of blinding and results reported only for those actually receiving the intervention and not on an intention to treat basis these results appeared promising and supported an important role for coagulation/haemostatic mechanisms in disease progression.

Noth et al. did not support these findings in a later study (23) that randomised patients with progressive IPF to warfarin (target INR 2.0-3.0) or placebo for a 48-week treatment period. This study was stopped early after a mean follow-up period of 28 weeks due to increased mortality in the warfarin group (14/72 in the warfarin group and 3/73 in the placebo group). The trial evidence regarding warfarin is therefore inconsistent but the robust methodology in the later study by Noth et al. compared with the original smaller, open label study by Kubo et al. strongly suggests an absence of benefit with warfarin therapy and indeed probable harm. Despite this, it is clear that there is an imbalance within the lungs of IPF patients that favours fibrin and extracellular matrix deposition and inhibition of fibrinolysis.

The finding of Kubo et al. (128) that D-Dimer levels were elevated at baseline and increased during an acute exacerbation supports the importance of haemostatic mechanisms in IPF. D-Dimer directly reflects fibrinolysis and we know from studies of PAI-1 in BAL specimens that fibrinolysis is suppressed in the alveoli of patients with IPF. As previously discussed, studies have identified localised release of prothrombotic mediators including TF in areas of pulmonary fibrosis with increased inhibition of fibrinolysis mediated by PAI-1. However, studies have demonstrated that IPF is

associated with venous thromboembolic disease and cardiovascular disease supporting a systemic prothrombotic tendency. The origin of elevated fibrin degradation products in the circulation of patients with IPF is therefore unclear but has two potential sources: firstly it may originate from the lungs where there is an imbalance between fibrin deposition and fibrinolysis resulting in net fibrin deposition and accumulation of extracellular matrix proteins in the alveolar spaces; or secondly, it may represent a systemic activation of haemostatic mechanisms with increased fibrin deposition and fibrinolysis with localised lung disease due to local imbalance favouring fibrin and extracellular matrix deposition due to PAI-1 release from hyperplastic type II pneumocytes and macrophages.

2. PECAM-1 and Idiopathic Pulmonary Fibrosis

2.1 Introduction

PECAM-1 is a complex molecule with a diverse range of functions including leukocyte transmigration, angiogenesis, vascular mechanosensing, platelet adhesion and apoptosis. A strain of PECAM-1 knockout mice spontaneously developed pulmonary fibrosis similar to IPF raising the possibility of a role for this molecule in IPF pathogenesis (106). The role of PECAM-1 in IPF has not previously been investigated. Interestingly, soluble PECAM-1 has been demonstrated to be elevated in cardiovascular disease (112) and a number of studies have demonstrated an association between the recognised PECAM-1 SNP's and cardiovascular disease. In light of the increased incidence of cardiovascular disease in IPF (3) it is important to consider whether a common PECAM-1 genotype may provide an explanation for this link.

There are 3 recognised SNP's within the PECAM-1 gene: Leu125Val in exon 3, Ser563Asn in exon 8 and Gly670Arg in exon 12. Importantly, an ex vivo study of the Gly670Arg polymorphism in exon 12 demonstrated a phenotypic difference between Gly homozygous and Arg homozygous HUVEC. HUVEC that were Gly homozygous demonstrated significantly increased PECAM-1 tyrosine phosphorylation and monocyte adhesion and transmigration compared with Arg homozygous cells (111). The three PECAM-1 SNP's have been demonstrated to exhibit strong linkage disequilibrium and therefore Gly homozygosity in exon 12 implies association with complementary polymorphisms in exon 3 and 8. These findings support a functional impact of PECAM-1 SNP's leading to the question whether variation in polymorphism frequency predisposes to the development of IPF.

Soluble PECAM-1 (sPECAM-1) can arise from membrane shedding or alternate mRNA splicing with deletion of exon 9. It has been observed to be elevated in cardiovascular and cerebrovascular disease (112;113); however, its role and origin in vascular disease is unknown. The role of sPECAM-1 in IPF has not previously been investigated.

In order to investigate the role of PECAM-1 in IPF we assessed the frequency of the recognised SNP's in exon 3 (Leu125Val), exon 8 (Ser563Asn) and exon 12 (Gly670Arg)

of the PECAM-1 gene. We also measured the plasma concentration sPECAM-1 and correlated this with lung function measurements.

2.2 Method

Patient Selection

IPF patients diagnosed according to ATS/ERS criteria were recruited from the local interstitial lung disease clinic in a large teaching hospital. All patients entering the study were stable at the time of sampling being clinically free from infection or acute exacerbation. Control patients with non-fibrotic respiratory conditions were recruited from both outpatient and in-patient clinical settings. Control patients were clinically well at the time of sampling and free of clinical signs of infection. Those recruited from in-patient settings were awaiting discharge following resolution of the medical complaint leading to admission and were assessed to ensure that there was no evidence of ongoing infection or inflammation. A total of 37 IPF patients and 41 controls entered the study. A random selection of these patients went on to have the sPECAM-1 concentration measured (IPF n=20, Controls n=17). Lung function measurements were available for 15 of the IPF patients undergoing sPECAM-1 measurement. The regional ethics committee approved the study and written informed consent was obtained from all participants.

PECAM-1 Single Nucleotide Polymorphisms

From each of the 37 IPF patients and 41 controls entering the study, samples of whole blood were collected and anticoagulated with Ethylenediaminetetraacetic acid (EDTA). The whole blood was stored at -80^{o}C prior to thawing for DNA extraction using QIAmp DNA Blood Midi Kit (Qiagen, Valencia, CA). The extracted DNA was stored at -20^{o}C prior to being thawed and PCR performed using primers specific for exon 3, exon 8 and exon 12 of the PECAM-1 gene (table 3). The following PCR protocol was used: 95^{o}C for 2 minutes followed by 30 cycles of 95^{o}C for 30 seconds, 55^{o}C for 30 seconds and 72^{o}C for 45 seconds prior to a final step of 72^{o}C for 10 minutes on completion of cycling. The PCR product was then purified using Nucleospin Extract II PCR Clean-up and gel extraction kit (Machary-Nagel, Düren, Germany) and a selection analysed using gel electrophoresis to confirm the presence of a product of correct size. Sequencing was undertaken by Eurofins MWG Operon.

Soluble PECAM-1

A random selection of the original cohort (IPF n=20, Controls n=17) had additional blood collected and anticoagulated with lithium heparin. The samples were centrifuged at 1,500G for 10 minutes at 20^{o}C and the plasma collected. sPECAM-1 concentration was measured using eBioscience Human sPECAM-1 instant ELISA (eBioscience, San Diego, USA). Lung function measurements were available for 15 of the IPF patients. For the purposes of assessing the effect of sPECAM-1 on lung function IPF patients were divided into two groups using an arbitrary TLCO cut off (TLCO >50% predicted and TLCO≤50% predicted) and analysis performed using an unpaired t-test.

Data Analysis

This study had 80% power to detect a difference in allele frequency of 30% with a p-value of <0.05 considered significant. Allele frequencies were analysed using 2x2 contingency tables and Fishers Exact test. The mean sPECAM-1 concentrations were analysed using an unpaired t-test.

Exon 3							
	Sequence (5'->3')	**Strand on template**	**Length**	**Start**	**Stop**	**Tm**	**GC%**
Forward primer	TCACCGGGATGATGTTGTTTCTGAG	Plus	25	1	25	57.06	48.0%
Reverse primer	TGCTCCATCTGCTTGCCGTGC	Minus	22	397	376	59.9	59.1%
Product length	397						
Exon 8							
	Sequence (5'->3')	**Strand on template**	**Length**	**Start**	**Stop**	**Tm**	**GC%**
Forward primer	CTGGTTTTCAGCCCCGGTGGA	Plus	21	511	531	58.96	61.9%
Reverse primer	ACACTGGAGGCGTGGTTGGC	Minus	20	900	881	59.55	65.0%
Product length	390						
Exon 12							
	Sequence (5'->3')	**Strand on template**	**Length**	**Start**	**Stop**	**Tm**	**GC%**
Forward primer	GGACCCAGGACCCAGAGACAGTG	Plus	23	206	228	59.93	65.2%
Reverse primer	GCATCTCTGGTGAAAGCAGCTAGC	Minus	24	575	552	58.15	54.2%
Product length	370						

Table 3. PCR primers for exon 3, exon 8 and exon 12 of the human PECAM-1 gene.

2.3 Results

IPF patients were older than controls (p=0.01) and there was an increased prevalence of COPD in the control group (p=0.001). Otherwise, the groups were well matched for all variables and all participants were Caucasian (table 4). There was no significant difference in allele frequency for the SNP's on exon 3 (p=0.33), exon 8 (p=0.33) or exon 12 (p=0.30) of the PECAM-1 gene (table 5).

	IPF	**Controls**	**P-value**
Number (n)	37	41	
Age in years – Mean (SD)	74.1 (7.67)	66.1 (15.45)	0.01
Gender (%)			
- Male	26 (70.3)	22 (53.7)	0.16
- Female	11 (29.7)	19 (46.3)	0.16
Comorbidities (%)			
- IHD	11 (29.7)	8 (19.5)	0.42
- TIA/CVA	4 (10.8)	3 (7.3)	0.69
- PVD	3 (8.1)	1 (2.4)	0.34
- Hypertension	10 (27.0)	5 (12.2)	0.15
- Diabetes mellitus	3 (8.1)	8 (19.5)	0.20
- Hypercholesterolaemia	2 (5.4)	3 (7.3)	1.00
- COPD	3 (8.1)	24 (58.6)	0.001
- Asthma	0 (0)	2 (4.9)	0.49
- Sleep apnoea	0 (0)	1 (2.4)	1.00
- DVT/PE	2 (5.4)	3 (7.3)	1.00
Smoking Status (%)			
- Current	4 (10.8)	8 (19.5)	0.35
- Ex-smoker	18 (48.7)	16 (39.0)	0.49
- Non-smoker	3 (8.1)	5 (12.2)	0.71
- Unknown	12 (32.4)	12 (29.3)	0.81
FVC – Mean % predicted (SD)*	100.1 (17.5)	-	
TLCO – Mean % predicted (SD)*	53.8 (12.0)	-	

Table 4. Baseline characteristics of patients with IPF and controls.

***Lung function data for the IPF patients undergoing sPECAM-1 measurement for whom lung function data was available (n=15)**

SNP	Exon	Genotype	IPF n=37	Controls n=41	p Value
Leu125Val	3	C / No C	28 / 9	26 / 15	0.33
		G / No G	29 / 8	32 / 9	1.00
Ser563Asn	8	G / No G	28 / 9	26 / 15	0.33
		A / No A	26 / 11	33 / 8	0.43
Gly670Arg	12	G / No G	27 / 10	31 / 10	0.80
		A / No A	30 / 7	28 / 13	0.30

Table 5. The prevalence of single nucleotide polymorphisms in exon 3, exon 8 and exon 12 of the PECAM-1 Gene

IPF and control patients within the subgroup of participants undergoing analysis of soluble PECAM-1 concentration were well matched with no significant age difference (p=0.06). Soluble PECAM-1 was significantly higher in the IPF group versus controls (106.18ng/ml versus 84.33ng/ml respectively) p=0.04 (figure 2).

The mean sPECAM-1 concentration was 130.38ng/ml in the TLCO>50% predicted group versus 86.78ng/ml in the TLCO≤50% predicted group (p=0.04) (figures 3 and 4)

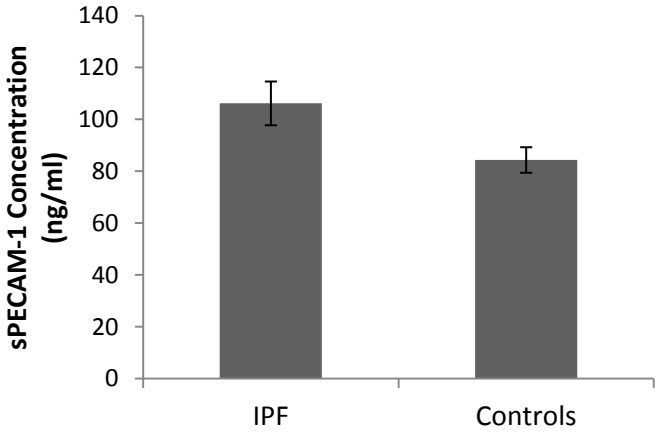

Figure 2. Soluble PECAM-1 concentration in IPF (n=20) versus Controls (n=17) (p=0.04)

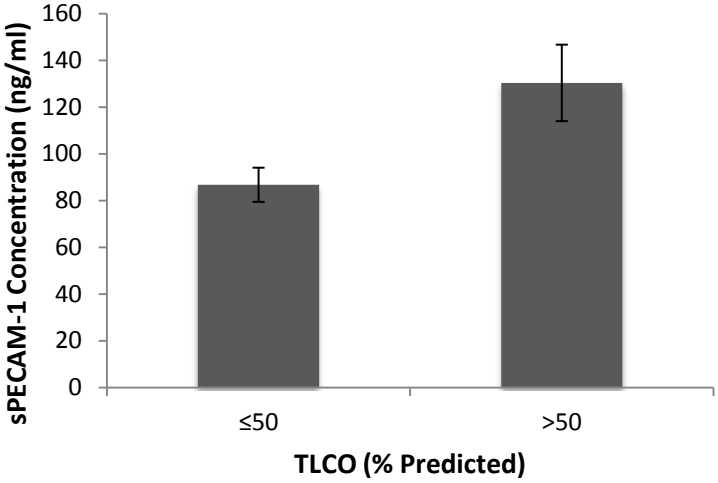

Figure 3. Soluble PECAM-1 concentration in IPF patients with TLCO >50% predicted versus IPF patients with TLCO ≤50% predicted (p=0.04) (n=15)

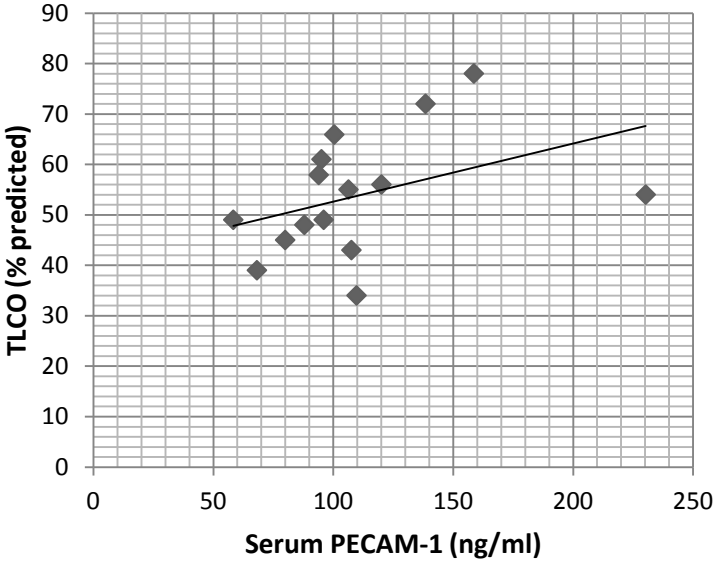

Figure 4. Correlation between soluble PECAM-1 concentration and TLCO (R^2=0.16, p=0.14)

2.4 Discussion

This study does not demonstrate any significant difference in SNP frequency between IPF patients and controls. Although this study was underpowered to detect small differences in genotype we suggest that if a PECAM-1 polymorphism was clinically significant it would be evident in the vast majority of IPF patients. We therefore conclude that there is no clinically significant difference in SNP frequency between the groups.

The mean age in the IPF group was older than the mean age in the control group. It could be argued that participants in the control group may later go on to develop IPF, however, the low incidence of IPF in the general population makes it very unlikely that this would have a significant impact on the results. It is important to note that there was no significant age difference between IPF patients and controls in the subgroup undergoing analysis of sPECAM-1. The increased prevalence of COPD in the control group reflects the method of recruitment of control subjects from respiratory outpatient clinics and inpatients awaiting discharge.

It is noteworthy that there appears to be geographical variation in the prevalence of PECAM-1 polymorphisms with genotypes varying significantly between Slovenian and Indian populations in two separate studies of the Leu125Val SNP on exon 3 in cardiovascular disease (109;129). The genotype frequencies (CC/CG/GG) in the control groups of the Slovenian and Indian studies were 17.1%/53.5%/29.4% and 42.7%/47.3%/10% respectively. In these studies, cardiovascular disease was found to be associated with the C allele in a Slovenian population and the G allele in an Indian population (109;129). Conclusions must therefore be drawn with care with the appreciation that a lack of association in this white British population cannot be generalised to other patient populations.

sPECAM-1 can result from membrane shedding of the extracellular component or through alternate mRNA splicing with loss of the transmembrane portion coded on exon 9 and therefore consisting of the extracellular component and intracytoplasmic tail. The individual roles of these distinct forms of sPECAM-1 have not been established. We demonstrate an increased plasma concentration of sPECAM-1 in IPF compared with controls with higher levels associated with preserved lung function.

However, the nature of the increased sPECAM-1 and its functional implications have not been established. A potential mechanism for preserved lung function associated with higher sPECAM-1 is through blockade of homophilic interaction of membrane bound forms of the molecule by occupying the binding sites. Goldberger et al. (96) demonstrated that sPECAM-1 at a concentration of 10µg/ml resulted in almost complete blockade of membrane bound PECAM-1 interactions with lower concentrations resulting in partial blockade. It is however important to note that the concentrations studied in this experiment are significantly greater than the plasma concentrations observed in this study.

2.5 Conclusion

Despite the spontaneous development of pulmonary fibrosis in PECAM-1 knockout mice there was no clinically significant difference in PECAM-1 polymorphism frequency in IPF patients and controls in the studied population. However, the plasma concentration of sPECAM-1 was significantly higher in the IPF group compared with controls with higher concentrations associated with preserved lung function. The origin and functional impact of the observed elevation of sPECAM-1 in IPF requires further investigation.

3. Platelet Reactivity in Idiopathic Pulmonary Fibrosis

3.1 Assessment of Markers of Platelet Activation in Idiopathic Pulmonary Fibrosis and Controls at Basal Levels and Following Agonist Induced Platelet Activation

3.1.1 Introduction

Platelets are anucleate cells that survive in the circulation for 7-9 days and have important roles in haemostasis, wound healing and inflammation. Platelets are recognised to be closely linked with the lungs in health and in disease. In physiology, platelets are produced from megakaryocytes and proplatelets within the pulmonary circulation (71). However, a number of respiratory diseases have been associated with platelets in animal and human models.

Platelet trapping in the lung has been demonstrated following intravenous bleomycin and is strongly correlated with collagen deposition (87). Seven days following bleomycin administration, increased platelet adherence to the pulmonary endothelium was observed using electron microscopy. These changes persisted for approximately three weeks. Platelet adhesion to the endothelium in this model is believed to be mediated by CD11a as addition of a blocking monoclonal antibody largely reversed this process (87). Platelets have also been implicated in human lung diseases including adult respiratory distress syndrome (68) and cystic fibrosis (69;130). Patients with cystic fibrosis have been demonstrated to have increased platelet activation in basal conditions and increased platelet reactivity in the presence of a platelet agonist. This was found to be secondary to plasma factors and intrinsic platelet mechanisms and was demonstrated by increased levels of circulating platelet-leukocyte complexes and increased platelet surface P-selectin expression (130). Similarly, studies of patients with ARDS have revealed markers of increased platelet activation, platelet sequestration within the lung and microthrombi within alveolar capillaries (68;131). This recognised association with respiratory disease including animal models of pulmonary fibrosis led us to question the role of platelets in IPF.

There is a recognised association between vascular disease and IPF with an increased incidence of cardiovascular disease and venous thromboembolism (3;4). Increased platelet activation and reactivity is recognised in both stable coronary artery disease

and acute coronary syndromes (91). We hypothesise that platelet activation and reactivity will be increased in patients with IPF with implications for its pathogenesis and providing a link with cardiovascular disease.

3.1.2 Method

Patient Selection

Patients with IPF diagnosed in accordance with ATS/ERS criteria (9) were recruited from the interstitial lung disease clinic at a large University Hospital. All patients were stable at the time of sampling being free from infection and/or disease exacerbation. Age and sex matched controls without interstitial lung disease were selected from out-patient clinics within the same hospital and stable inpatients awaiting discharge. A total of 13 IPF patients and 12 controls were recruited into the study. Basic demographic information was collected in addition to details of comorbidities, antiplatelet drug use, smoking status and lung function data when available (table 6). For the purposes of this study current smokers were classified as those who smoked in the preceding six months prior to sampling, ex-smokers as those who had stopped smoking prior to six months before the study and non-smokers as those who had never smoked. In light of the well-recognized increased platelet-monocyte binding and platelet activation in ischaemic heart disease (91), patients with a known history of ischaemic heart disease were not eligible for inclusion in this study. The local ethics committee approved the study and written informed consent was obtained from all participants.

Demographic	IPF (%)	Controls (%)	p-value
Number	**n=13**	**n=12**	
Age (mean)	**70.3**	**66.2**	0.22
<50	0 (0)	0 (0)	-
51-60	0 (0)	4 (33.3)	-
61-70	8 (61.5)	3 (25)	-
71-80	5 (38.5)	4 (33.3)	-
>80	0 (0)	1 (8.3)	-
Gender			
Male	9 (69.2)	8 (66.7)	1.00
Female	4 (30.8)	4 (33.3)	1.00
Comorbidities			
COPD	2 (15.4)	6 (50)	0.09
Prev. Malignancy	2 (15.4)	1 (8.3)	1.00
Hypertension	4 (30.8)	1 (8.3)	0.30
Diabetes mellitus	0 (0)	2 (16.7)	0.22
TIA	3 (23.1)	0 (0)	0.22
CVA	0 (0)	0 (0)	1.00
Atrial Fibrillation	1 (7.7)	2 (16.7)	0.58
Anti-platelet drug use			
Aspirin	4 (30.8)	0 (0)	0.09
Clopidogrel	0 (0)	0 (0)	1.00
Dipyridamole	1 (7.7)	0 (0)	1.00
Other	0 (0)	0 (0)	1.00
Smoking Status			
Non-smoker	1 (7.7)	6 (50)	0.03
Ex-smoker	10 (77.9)	4 (33.3)	0.04
Current Smoker	2 (15.4)	2 (16.7)	1.00

Table 6. Baseline Characteristics of IPF patients and controls following exclusions.

Blood Collection

Blood was collected using a 21-gauge butterfly needle (Becton Dickinson Vacutainer Systems, UK) into two 4.5ml vacutainer tubes anticoagulated with 0.105 Molar Sodium Citrate (Becton Dickinson Vacutainer Systems, UK). The first tube was discarded in order to prevent inadvertent platelet activation at the time of venepuncture influencing the results. All samples were used within 20 minutes of venesection and flow cytometry performed within 2 hours. Platelet monocyte complexes and platelet activation were assessed using the following protocols.

Platelet-Monocyte Complexes

50µl of whole blood was incubated for 20 minutes in basal conditions and in the presence of varying concentrations of adenosine diphosphate (ADP) or the protease activated receptor - 1 (PAR-1) agonist TFLLR (0.1, 1 and 10µM ADP and 1, 5 and 10µM TFLLR) with 10µl FITC-conjugated anti CD42b (BD Biosciences, UK) and 5µl RPE-conjugated anti CD14 (AbD Serotec, UK). Each sample was prepared in duplicate with the addition of 5µl of 100mM EDTA to alternate tubes in order to measure the presence of cation dependent platelet-monocyte binding. Red blood cells were then lysed using 500µl FACS Lyse solution (BD Biosciences, UK). Samples were further diluted at a ratio of 1:2 with phosphate buffered saline (PBS) prior to performing two-colour flow cytometry. Monocytes were identified by their specific forward and side scatter properties and confirmed by positive CD14 binding (figure 5). The percentage of monocytes (CD14+) forming platelet-monocyte complexes (CD14+, CD42b+) was calculated. Statistical significance was assessed using an unpaired t-test with a p value of <0.05 considered significant.

Markers of Platelet Activation

5µl of whole blood was incubated for 20 minutes in basal conditions and in the presence of ADP or TFLLR of varying concentrations (0.1, 1 and 10µM ADP and 1, 5 and 10µM TFLLR) with PE-conjugated anti-human CD62P (Biolegend, San Diego, California) or FITC-conjugated anti-fibrinogen antibodies (Dako, UK). A separate sample was incubated with FITC-conjugated CD42b to confirm that the gated population was platelets. The samples were made up to 50µl with PBS. Following incubation samples were fixed with 500µl of 1% paraformaldehyde prior to flow cytometry. Data was

expressed as the percentage of platelets expressing CD62p and fibrinogen (figure 6). Statistical significance was assessed using an unpaired t-test with a p value of <0.05 considered significant.

Flow Cytometry

Flow cytometry was performed using a Becton Dickinson FACS Calibur flow cytometer (Becton Dickinson, UK). Data were analyses using CellQuest Pro software (Becton Dickinson, UK).

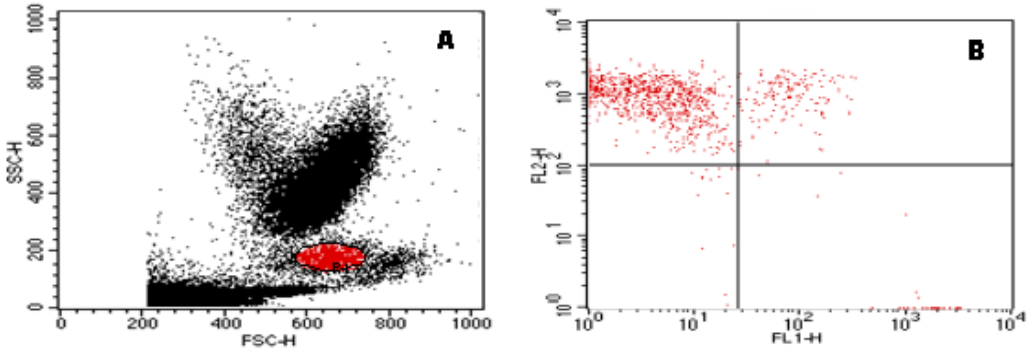

Figure 5. A. Scatter plot demonstrating whole blood flow cytometry gated on monocytes. B. Quadrant plot demonstrating CD14 positive cells (monocytes) in the upper two quadrants (FL2 high) and CD42b positive cells (platelets) in the right two quadrants (FL1 high) with platelet-monocyte complexes (FL2 high and FL1 high) in the right upper quadrant.

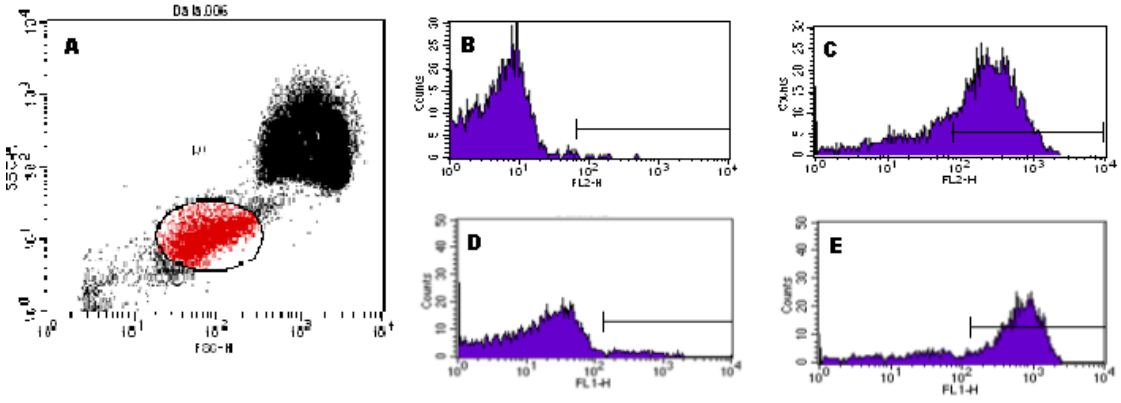

Figure 6. A. Scatter plot demonstrating whole blood flow cytometry using platelet settings gated on platelets. B-E Histograms demonstrating resting platelets with low P-selectin and fibrinogen expression (B and D respectively) and activated platelets with significantly increased P-selectin expression and fibrinogen binding (C and E respectively).

3.1.3 Results

Platelet-Monocyte Complexes

There were a greater percentage of platelet-monocyte complexes in the IPF group compared with controls reaching statistical significance at higher levels of stimulation. The percentage of monocytes forming platelet complexes in IPF and controls were 18.2% and 13.7% at basal levels (p=0.302), 24.1% and 15.3% with 0.1µM ADP (p=0.097), 29.4% and 16.1% with 1µM ADP (p=0.007), and 44.8% and 32.1% with 10µM ADP (p=0.014). A similar trend was observed with platelet stimulation by TFLLR with platelet-monocyte complexes in IPF and controls of 28.6% and 18.6% with 1µM TFLLR (p=0.06), 41.7% and 27% with 5µM TFLLR (p=0.002), and 61% and 41.1% with 10µM TFLLR (p=0.001) respectively. The percentage of platelet-monocyte complexes is demonstrated in table 7 and figure 7. The addition of EDTA resulted in a significant decrease in percentage of platelet-monocyte complexes. In the presence of EDTA there was no difference in proportion of platelet monocyte complexes between groups or with addition of platelet agonist (Figure 8).

	Percentage of Platelet-Monocyte Complexes (SD)		
Platelet Agonist	**IPF (n=13)**	**Controls (n=12)**	**p-value**
Basal	**18.19 (13.99)**	**13.71 (4.68)**	**0.30**
0.1µM ADP	**24.08 (16.87)**	**15.28 (5.16)**	**0.09**
1µM ADP	**29.40 (14.85)**	**16.10 (5.25)**	**<0.01**
10µM ADP	**44.83 (11.81)**	**32.11 (12.09)**	**0.01**
1µM TFLLR	**28.59 (16.38)**	**18.65 (6.19)**	**0.06**
5µM TFLLR	**41.73 (8.92)**	**27.03 (11.93)**	**<0.01**
10µM TFLLR	**61.05 (14.16)**	**41.09 (12.14)**	**<0.01**

Table 7. Platelet-monocyte complex formation in basal and stimulated conditions.

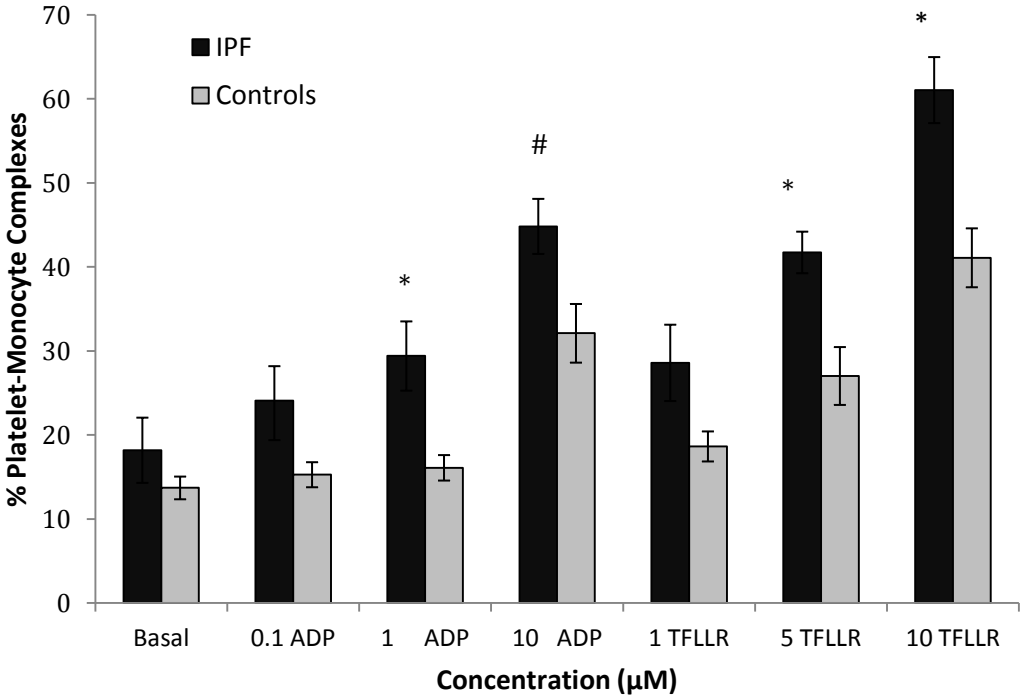

Figure 7. Percentage of monocytes forming complexes with platelets in basal conditions and when stimulated with 0.1µM ADP, 1µM ADP, 10µM ADP, 1µM TFLLR, 5µM TFLLR and 10µM TFLLR in IPF and controls. # p=0.01, * p=<0.01

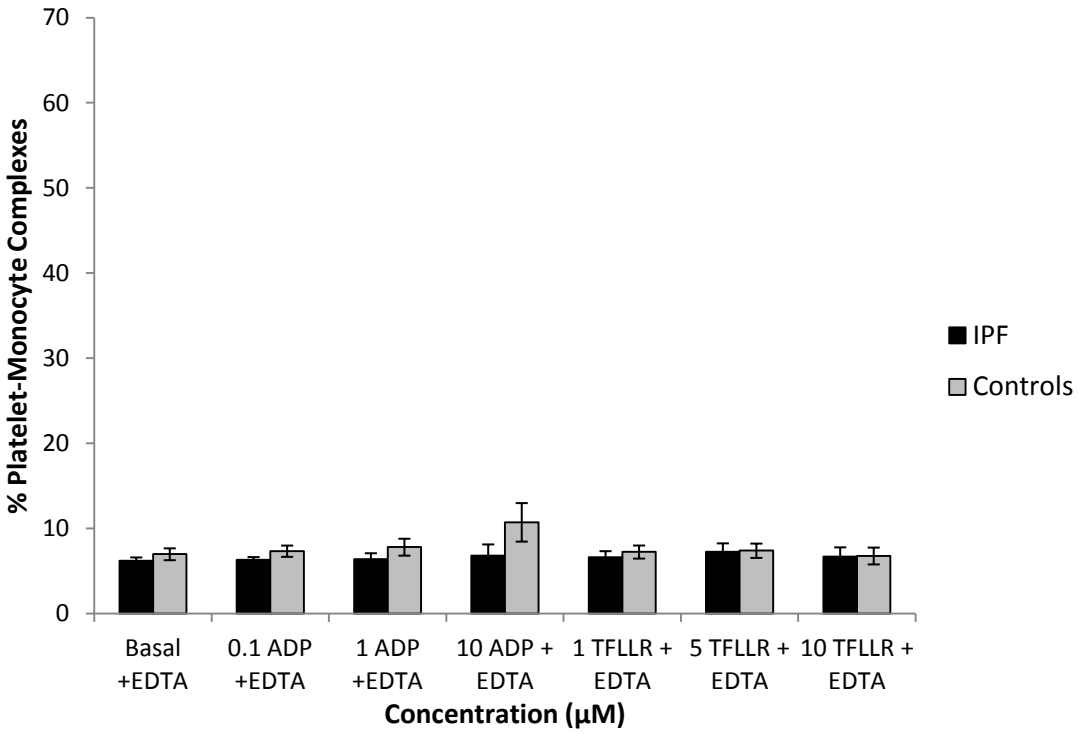

Figure 8. Percentage of monocytes forming complexes with platelets in the presence of 10mM EDTA in basal conditions (p=0.33) and when stimulated with 0.1µM ADP (p=0.17), 1µM ADP (p=0.25), 10µM ADP (p=0.14), 1µM TFLLR (p=0.57), 5µM TFLLR (p=0.93) and 10µM TFLLR (p=0.95) in IPF and controls.

Markers of Platelet Activation

In basal conditions there was no significant increase in platelet expression of P-selectin or fibrinogen in IPF patients compared to controls (0.99% versus 0.70%, p=0.25 and 19.92% versus 8.67%, p=0.13 respectively). However, with increasing stimulation the difference in P-selectin expression becomes significant as demonstrated in table 8 and figure 9. The difference in fibrinogen expression when stimulated with ADP reaches statistical significance at moderate levels losing significance as both groups reach maximal stimulation. A similar trend was observed with TFLLR however it failed to reach statistical significance. Fibrinogen expression in the two groups is demonstrated in table 9 and figure 10.

	Percentage of platelets positive for P-selectin (SD)		
Platelet Agonist	**IPF (n=13)**	**Controls (n=12)**	**p-value**
Basal	**0.99 (0.52)**	**0.70 (0.64)**	**0.25**
0.1µM ADP	**1.86 (1.65)**	**0.72 (0.44)**	**0.03**
1µM ADP	**9.78 (4.52)**	**3.25 (2.66)**	**<0.01**
10µM ADP	**41.27 (15.16)**	**22.52 (9.15)**	**<0.01**
1µM TFLLR	**3.05 (5.99)**	**0.99 (0.87)**	**0.25**
5µM TFLLR	**16.81 (19.06)**	**5.28 (7.13)**	**0.06**
10µM TFLLR	**51.53 (33.32)**	**23.10 (18.98)**	**0.02**

Table 9. Platelet expression of P-selectin in basal and stimulated conditions.

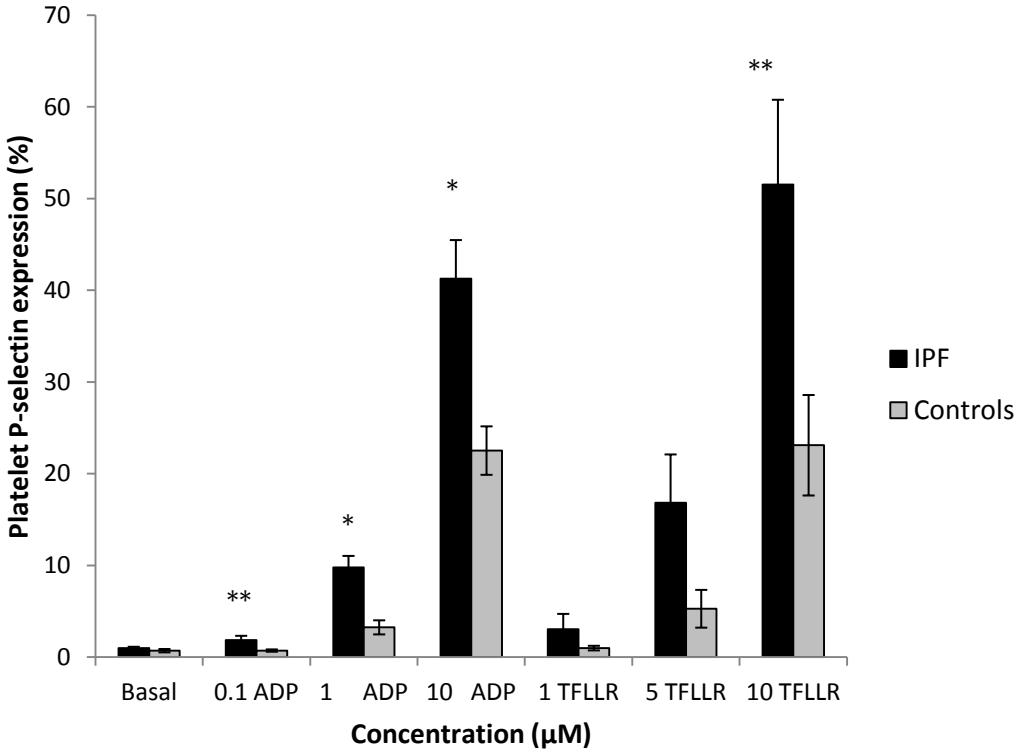

Figure 9. Platelet expression of P-selectin in basal conditions and when stimulated with 0.1µM ADP, 1µM ADP, 10µM ADP, 1µM TFLLR, 5µM TFLLR and 10µM TFLLR in IPF and controls. * p=<0.01 ** p=<0.05

	Percentage of platelets positive for fibrinogen (SD)		
Platelet Agonist	**IPF (n=13)**	**Controls (n=12)**	**p-value**
Basal	**19.92 (21.49)**	**8.67 (11.05)**	**0.13**
0.1µM ADP	**50.28 (31.91)**	**17.48 (21.23)**	**<0.01**
1µM ADP	**77.92 (16.70)**	**56.24 (22.62)**	**<0.01**
10µM ADP	**92.13 (6.09)**	**86.36 (8.81)**	**0.07**
1µM TFLLR	**49.30 (32.23)**	**31.82 (24.23)**	**0.15**
5µM TFLLR	**68.49 (27.81)**	**46.61 (26.34)**	**0.06**
10µM TFLLR	**83.10 (19.09)**	**71.78 (21.65)**	**0.18**

Table 9. Platelet expression of fibrinogen in basal and stimulated conditions.

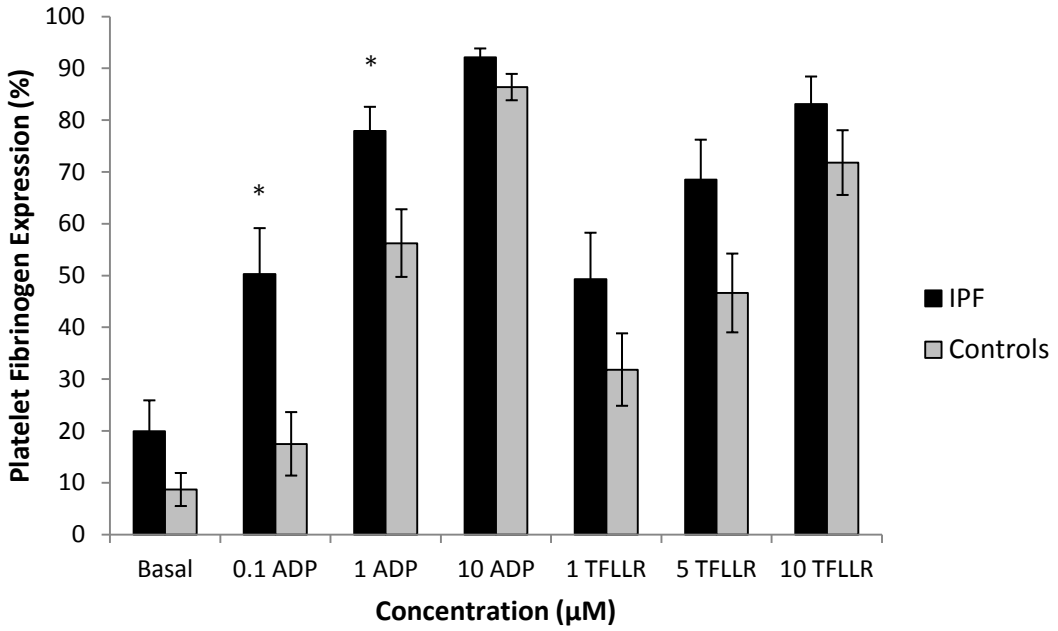

Figure 10. Platelet expression of fibrinogen in basal conditions and when stimulated with 0.1µM ADP, 1µM ADP, 10µM ADP, 1µM TFLLR, 5µM TFLLR and 10µM TFLLR in IPF and controls. * p=<0.01

3.1.4 Discussion

This study demonstrates significantly increased platelet reactivity in IPF patients compared to controls as demonstrated by a concentration dependent increase in platelet-monocyte complex formation, platelet P-selectin expression and platelet fibrinogen binding in the presence of` the platelet agonists ADP and TFLLR. The reproducibility using two different platelet agonists demonstrates that this is not restricted to a single pathway of activation. The addition of EDTA returned platelet-monocyte complex formation to basal levels with no difference between IPF patients and controls confirming that this interaction is divalent cation dependent consistent with P-selectin mediated adhesion.

At basal levels there was a trend to increased platelet activation that was reproducible across the measured activation markers however this did not reach statistical significance. Due to the small sample size and the inter-individual variability this study was underpowered to detect a difference at basal levels however the use of citrate anticoagulation during sample preparation may have contributed.

It is recognised that the choice of anticoagulant used in sample preparation can affect platelet-monocyte binding. Although there is no consensus on the best anticoagulant to use for this purpose it is important to recognise the influence that the anticoagulant can have on the result. Heparin based anticoagulants are known to cause platelet activation by binding to the integrin $\alpha_{IIb}\beta_3$ (GPIIb/IIIa) resulting in an enhanced response to platelet agonists (132). The use of heparin anticoagulation therefore has the potential to overestimate platelet activation by spuriously increasing platelet monocyte complex formation, platelet P-selectin expression and platelet fibrinogen binding. Conversely, anticoagulation with citrate results in a degree of calcium chelation. The interaction between platelet P-selectin and P-selectin glycoprotein ligand-1 (PSGL-1) expressed on monocytes is divalent cation dependent and therefore citrate anticoagulation causes platelet-monocyte complex formation to be spuriously low (133). In order to prevent a potential false positive result due to the use of heparin anticoagulation we chose to use citrate anticoagulation. This may have reduced our capability to identify a difference at basal levels. Despite this, this study clearly demonstrates increased platelet activation in a concentration dependent manner that is reproducible across the three markers of platelet activation and two pathways of

activation. This provides compelling evidence of increased platelet reactivity in IPF and emphasises the importance of interactions between the lung, the vasculature and circulating cells. Additionally, it provides insight into the recognised link between IPF and vascular diseases.

This study does not establish the causal relationship of this phenomenon. It is therefore not possible to conclude whether the increased platelet reactivity precedes the development of pulmonary fibrosis and therefore is important in the pathogenesis of the condition or whether it is a secondary effect of the pulmonary fibrotic process. However, it is recognised that the increased incidence of cardiovascular and venous thromboembolic disease in IPF patients precedes the diagnosis of their lung disease (3;4). It could therefore be postulated that patients with IPF have a prothrombotic tendency that predates their lung disease and that this may be associated with the increased platelet reactivity demonstrated in this study. Although this is by no means confirmed by this observation there is little doubt that activated platelets have the potential to drive fibrosis.

During platelet activation they degranulate releasing numerous profibrotic cytokines including TGF-β (76) and PDGF (75) that are recognised to be important in the pathogenesis of IPF. Abnormal platelet activation has been demonstrated in connective tissue disease related interstitial lung disease adding further support to a possible pathogenic role. Interstitial lung disease secondary to scleroderma has been shown to be associated with increased platelet activation demonstrated by elevated plasma platelet factor 4 (PF4) levels, not observed in scleroderma patients without interstitial lung disease (134). It is therefore plausible that the observed increased platelet reactivity in IPF contributes to the fibrotic process through local activation and degranulation with release of proinflammatory and profibrotic mediators within the pulmonary circulation.

The two groups in this study were well matched with equal sex distribution and similar age. However, although not statistically significant, there was a higher prevalence of cerebrovascular disease in the IPF group with 3 patients having suffered a TIA in the past. There is a recognised association between cerebrovascular disease and platelet activation (135), however repeat analysis of the data with exclusion of patients with cerebrovascular disease did not alter the results and therefore they have been

included in the final analysis. The increased use of antiplatelet drugs in the IPF group reflects the inclusion of patients with cerebrovascular disease. The platelet assays used in this study are not affected by the specific antiplatelet medications being used by the patients in the IPF group.

There is a similar distribution of cardiovascular risk factors between the two groups. However, a greater proportion of the IPF group were ex-smokers as would be expected with the disease being more prevalent in this group. The four current smokers in this study were all still smoking at the time of sampling and were evenly distributed between both groups meaning that the recognised increased platelet activation and surface expression of P-selectin in smokers (136) will not have affected the results in this study.

Following completion of this study a report was published suggesting that prednisolone has the potential to reduce platelet activation when added to whole blood following venepuncture (137). This was only observed in response to ADP and not other platelet agonists and following addition of prednisolone and not other corticosteroids. In order to ensure that this did not influence our findings, a retrospective review of corticosteroid use was performed. Two subjects were taking low-dose maintenance prednisolone in this study (1 in the IPF group and 1 in the control group). A further 2 patients in the control group had been treated for an acute exacerbation of COPD within the preceding 2 weeks and therefore are likely to have received high-dose corticosteroids as part of their treatment. Reanalysis of the data excluding these patients did not alter the results (data not shown) and therefore they were included in the final analysis.

Patients with COPD have recently been demonstrated to have increased levels of circulating platelet-monocyte complexes compared to age and smoking status matched controls with a further increase observed during an acute exacerbation (138). In the exacerbating group, blood was sampled during the acute hospitalisation and therefore these patients are likely to have been receiving oral prednisolone as part of their treatment. The increased platelet-monocyte complex formation in this group does not support any significant effect of prednisolone on platelet activation in patients. Furthermore, the high prevalence of COPD in the control group in this study may result in the difference between the groups being underestimated. This adds

further support to the significance of the observed increased platelet reactivity in patients with IPF.

3.1.5 Conclusion

The results of this study demonstrate increased platelet reactivity in patients with IPF compared to age and sex matched non-interstitial lung disease controls. This demonstration of an abnormal platelet response in IPF patients provides insight into the link between IPF and vascular disease and the need for further investigation of the role of the vasculature in IPF. This study does not establish cause and effect and further investigation is required to better understand the mechanism underlying the observed increased platelet reactivity and the functional consequences.

3.2 The Effect of Plasma on Platelet Activation in Idiopathic Pulmonary Fibrosis

3.2.1 Introduction

Patients with IPF demonstrate increased platelet reactivity compared with age and sex matched non-interstitial lung disease controls as demonstrated in the previous experiment. It is important to establish whether this is due to an intrinsic platelet defect that predisposes patients to develop fibrotic disease or whether the increased reactivity is due to an external plasma factor that primes an otherwise normal platelet for activation. In order to assess this, a plasma swap approach was used.

3.2.2 Method

Blood collection

Venous blood was collected using a 21-gauge butterfly needle (Becton Dickinson Vacutainer Systems, UK) from 7 control patients without evidence of interstitial lung disease, other fibrotic condition or cardiovascular disease. The first 6mls of blood was collected into a vacutainer tube anticoagulated with 102 IU lithium heparin (Becton Dickinson Vacutainer Systems, UK) and was used for isolation of patient plasma. Plasma was stored at -80^{o}C and thawed at room temperature prior to use. Subsequent blood was collected in four 4.5ml vacutainer tubes anticoagulated with 0.105 Molar Sodium Citrate (Becton Dickinson Vacutainer Systems, UK) and used for isolation of platelets.

Blood was collected in the same way from 7 patients with IPF diagnosed in accordance with the ATS/ERS criteria that were clinically stable at the time of sampling and free from any acute intercurrent illness. Blood collected into a vacutainer tube anticoagulated with 102 IU lithium heparin (Becton Dickinson Vacutainer Systems, UK) was used for isolation of patient plasma. Plasma was stored at -80^{o}C and thawed at room temperature prior to use.

Preparation of Washed Platelets

Citrate anticoagulated blood was centrifuged at 900 rpm for 20 minutes at 20^{o}C to produce platelet rich plasma (PRP). 0.3M citric acid was added to the PRP to achieve a PH of 6.4 in order to prevent platelet activation during the washing process. The PRP

was further centrifuged at 1,900 rpm for 12 minutes at 20^{o}C to produce a platelet pellet and the supernatant was discarded. The platelet pellet was suspended in wash buffer (PH 6.5) by gentle pipetting prior to a final centrifugation at 2,200 rpm for 10 minutes at 20^{o}C. The supernatant was discarded and the washed platelet pellet was suspended in 300µl PBS. The washed platelets were used immediately following preparation.

Isolation of Plasma

Lithium heparin anticoagulated blood was centrifuged at 1,500G for 10 minutes at 20^{o}C and the plasma supernatant was collected. The plasma was separated into 100µl aliquots and stored at -80^{o}C until needed. The plasma was thawed and incubated at room temperature prior to further centrifugation at 13,000rpm for 2 minutes to ensure any cellular debris was removed.

Plasma swap Protocol

Washed platelets suspended in PBS were separated into three 100µl aliquots. 100µl of autologous plasma was added to the first aliquot of washed platelets, 100µl of plasma from a control patient was added to the second aliquot and 100µl of plasma from an IPF patient was added to the final aliquot resulting in 3 suspensions of washed platelets constituting an Autologous Control, an Allogeneic Control and an IPF sample (see Figure 11). 5µl of each sample was individually incubated for 20 minutes in basal conditions and in the presence of ADP (0.1, 1 and 10µM) with PE-conjugated anti-human CD62P (Biolegend, San Diego, California) and separately with FITC-conjugated anti-fibrinogen antibodies (Dako, UK). A separate sample was incubated with FITC-conjugated CD42b to confirm that the gated population was platelets. The samples were made up to 50µl with PBS. Following incubation samples were fixed with 500µl of 1% paraformaldehyde prior to flow cytometry. Data were expressed as the percentage of platelets expressing P-selectin or fibrinogen in the three groups and as the difference in expression of P-selectin/fibrinogen binding compared with the autologous control. Statistical significance was assessed using a paired t-test with a p value of <0.05 considered significant.

Flow cytometry was performed using a Becton Dickinson FACS Calibur flow cytometer and data were analysed using CellQuest Pro software (Becton Dickinson, UK).

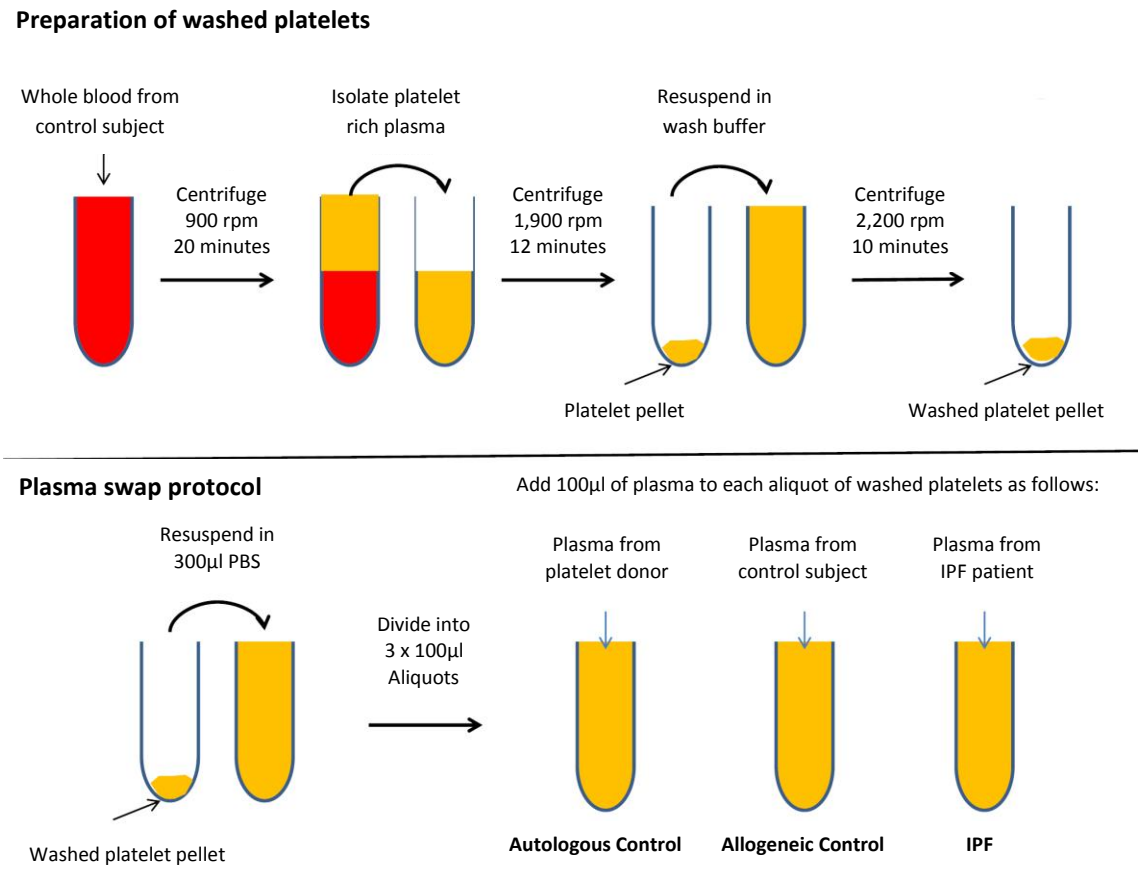

Figure 11. Illustration of procedure for the preparation of washed platelets and the plasma swap protocol.

3.2.3 Results

Platelet P-selectin expression was significantly greater following incubation in IPF plasma compared with platelets incubated in autologous control and allogeneic control plasma at basal levels and following stimulation with ADP at concentrations of 0.1 and 1 µM. Following stimulation with 10µM ADP there was no significant difference between the groups as maximal stimulation was reached (figure 12 – 16). Further analysis of the data was performed to compare the change in platelet P-selectin expression in the Allogeneic Control and IPF groups from baseline taken as the platelet P-selectin expression in the Autologous Control group. The change in platelet P-selectin expression in the IPF group was significantly greater than the Control group at basal level and following stimulation with all concentrations of ADP (figure 17).

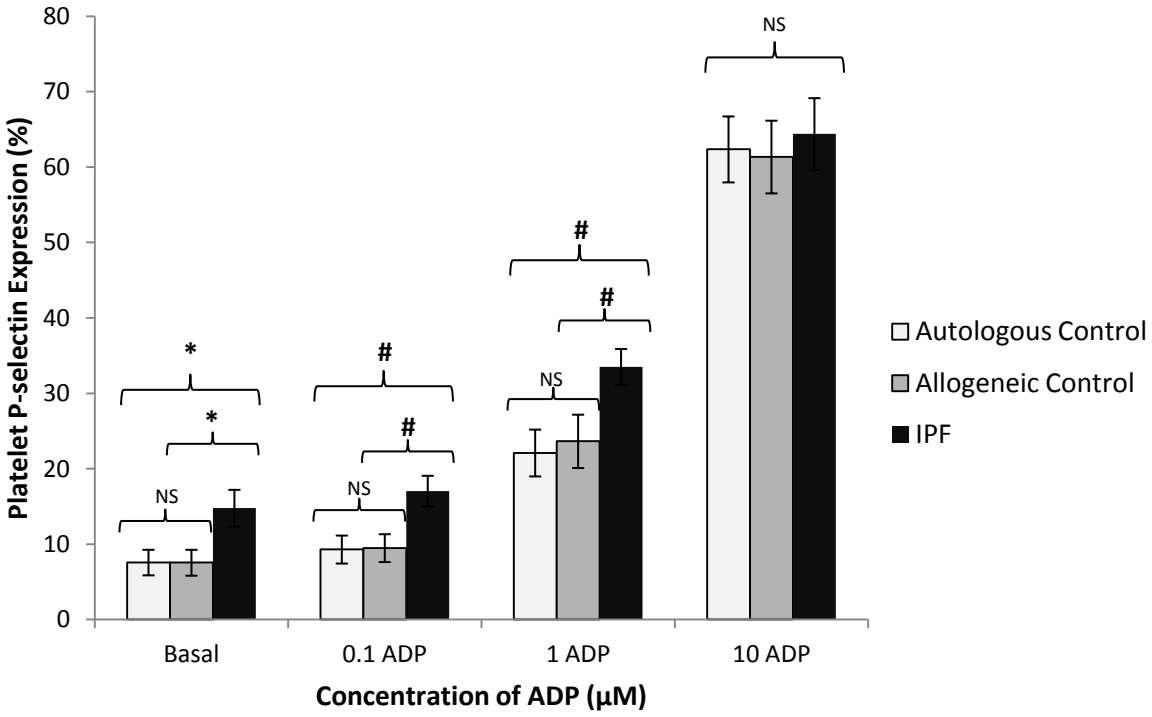

Figure 12. Platelet P-selectin expression following incubation in autologous plasma (Autologous Control), control patient plasma (Allogeneic Control) and IPF patient plasma (IPF) in basal conditions and following stimulation with ADP (0.1, 1 and 10µM). Data expressed as percentage of platelets positive for P-selectin ± SEM. (n=7). * P= <0.05, # P= ≤0.01, NS = no significant difference.

	IPF	Controls	P-value
Number (n)	7	7	
Age in years – Mean (SD)	74 (8)	65 (10)	0.07
Gender (%)			
- Male	6	4	0.56
- Female	1	3	0.56
Comorbidities (%)			
- IHD	1	0	1.00
- TIA/CVA	1	0	1.00
- Hypertension	1	2	1.00
- Diabetes mellitus	1	0	1.00
- GER	2	2	1.00
- COPD	0	2	0.46
- Sleep apnoea	0	2	0.46
Smoking Status (%)			
- Current	0	0	1.00
- Ex-smoker	4	4	1.00
- Non-smoker	2	2	1.00
- Unknown	1	1	1.00

Table 10. Baseline characteristics for IPF and control groups in plasma swap experiment.

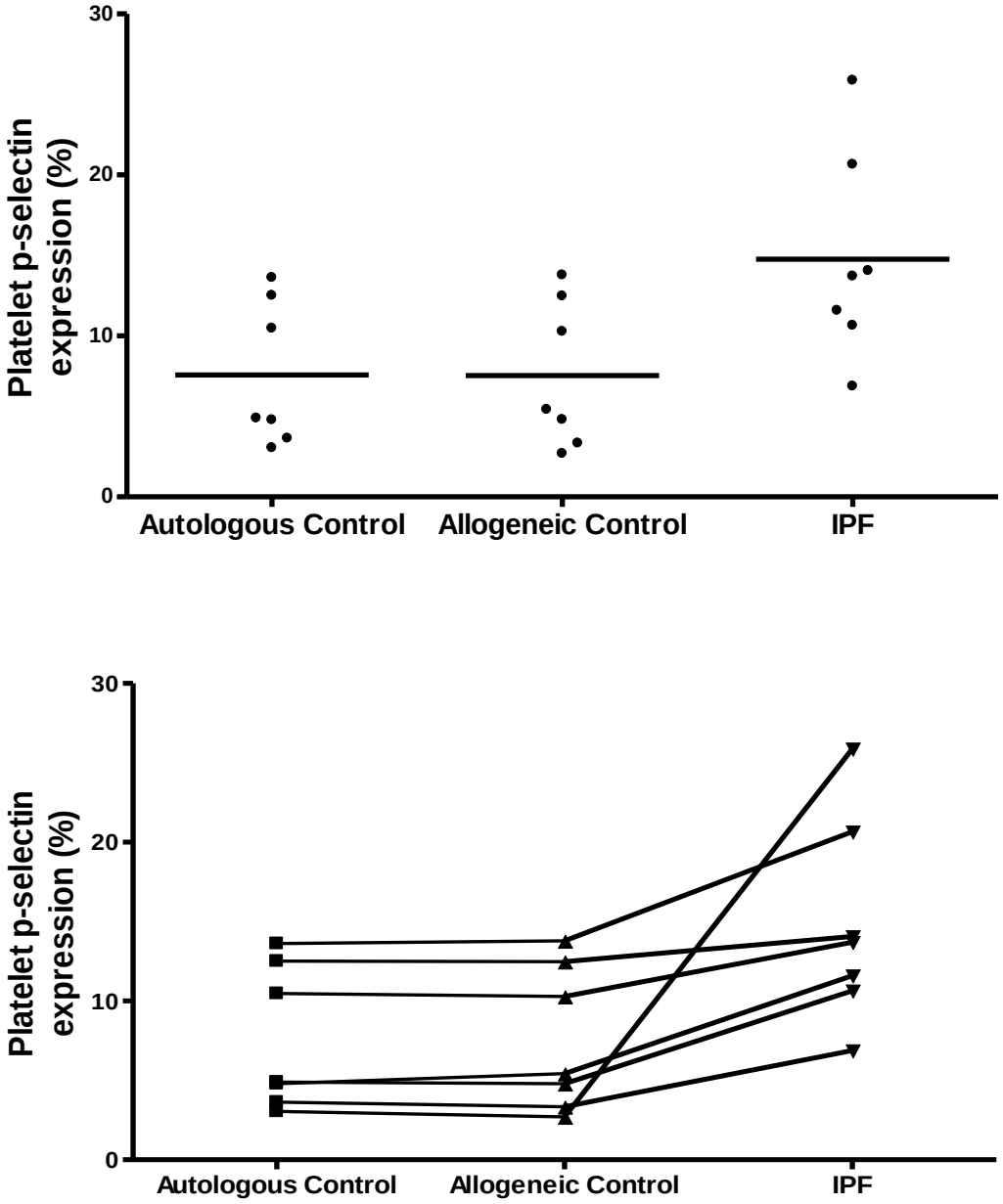

Figure 13. Dot plot demonstrating platelet P-selectin expression following incubation with autologous plasma (Autologous Control), control patient plasma (Allogeneic Control) and IPF patient plasma (IPF) at basal levels. The mean P-selectin expression was 7.56 ± 1.69% in the Autologous Control group, 7.54 ± 1.72% in the Allogeneic Control group and 14.78 ± 2.44% in the IPF group. The platelet P-selectin expression in the IPF group was significantly greater than in the Autologous Control group (p=<0.05) and the Allogeneic Control group (p=<0.05). There was no significant difference between the Autologous Control and Allogeneic Control groups (p=0.86).

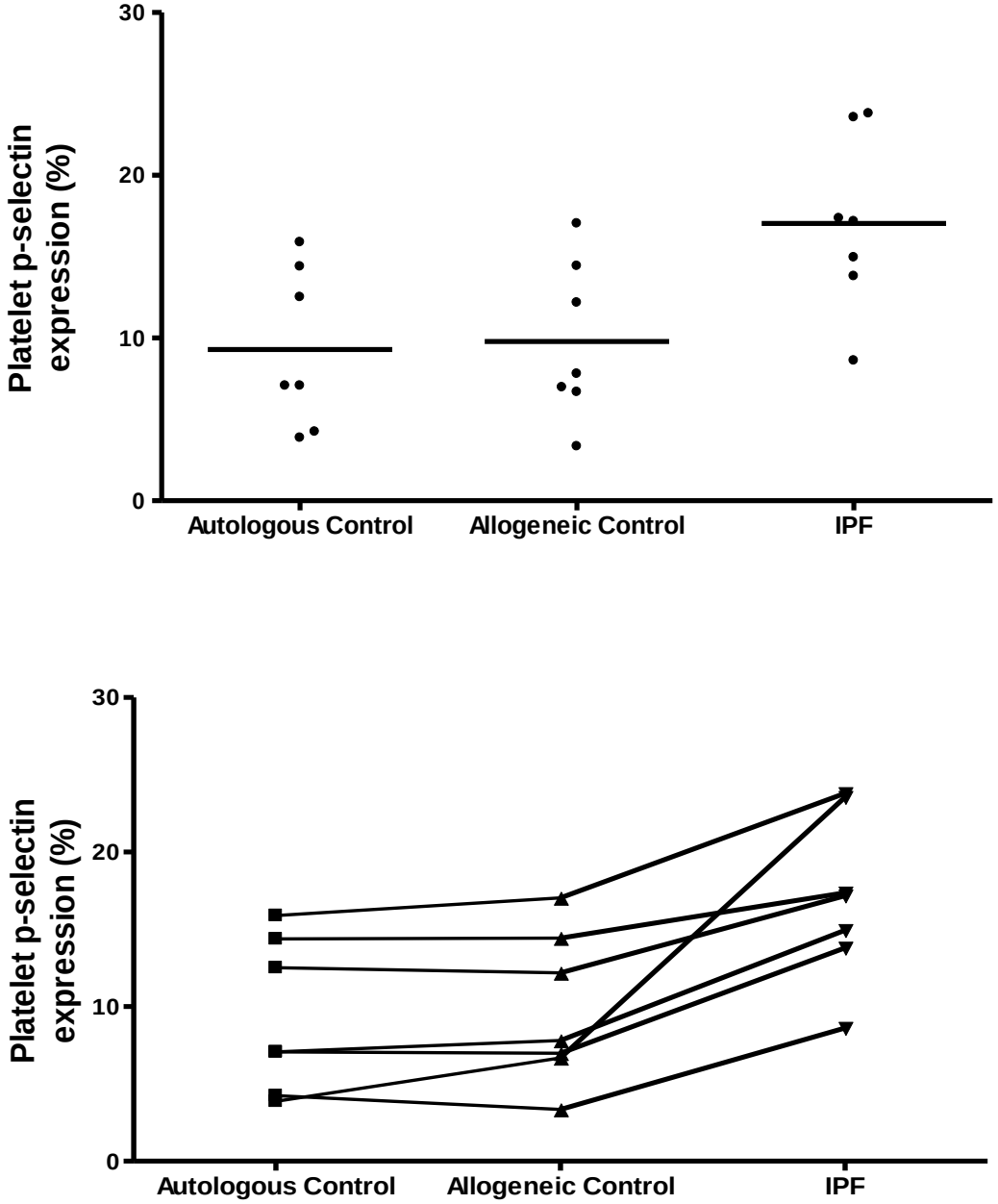

Figure 14. Dot plot demonstrating platelet P-selectin expression following stimulation with 0.1µM ADP and incubation with autologous plasma (Autologous Control), control patient plasma (Allogeneic Control) and IPF patient plasma (IPF). The mean P-selectin expression was 9.29 ± 1.86% in the Autologous Control group, 9.78 ± 1.84% in the Allogeneic Control group and 17.04 ± 2.04% in the IPF group. The platelet P-selectin expression in the IPF group was significantly greater than in the Autologous Control group (p=0.01) and the Allogeneic Control group (p=0.005). There was no significant difference between the Autologous and Allogeneic Control groups (p=0.33).

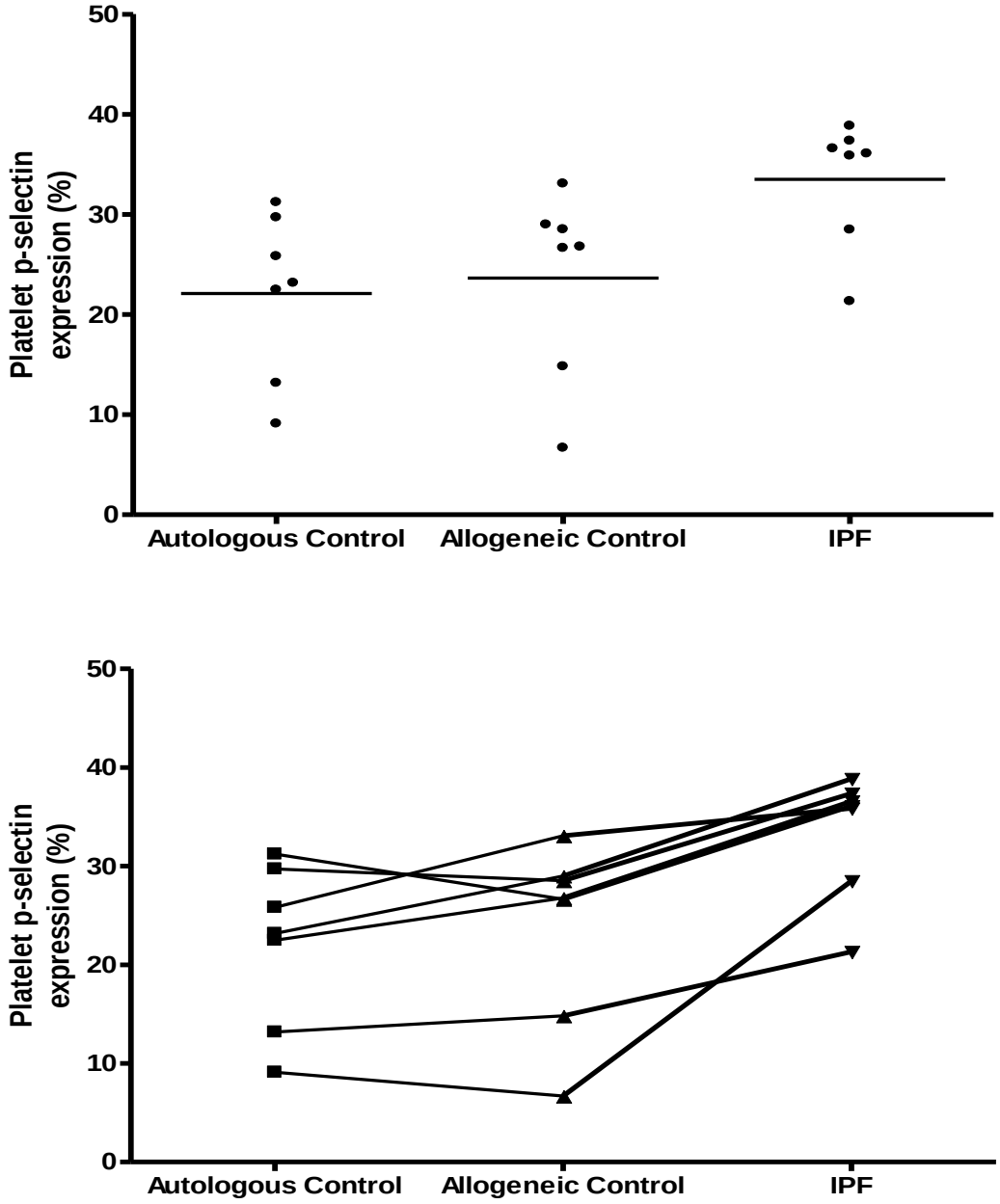

Figure 15. Dot plot demonstrating platelet P-selectin expression following stimulation with 1µM ADP and incubation with autologous plasma (Autologous Control), control patient plasma (Allogeneic Control) and IPF patient plasma (IPF). The mean P-selectin expression was 22.1 ± 3.11 % in the Autologous Control group, 23.64 ± 3.54% in the Allogeneic Control group and 33.51 ± 2.39% in the IPF group. The platelet P-selectin expression in the IPF group was significantly greater than in the Autologous Control group (p=0.001) and the Allogeneic Control group (p=0.004). There was no significant difference between the Autologous and Allogeneic Control groups (p=0.4).

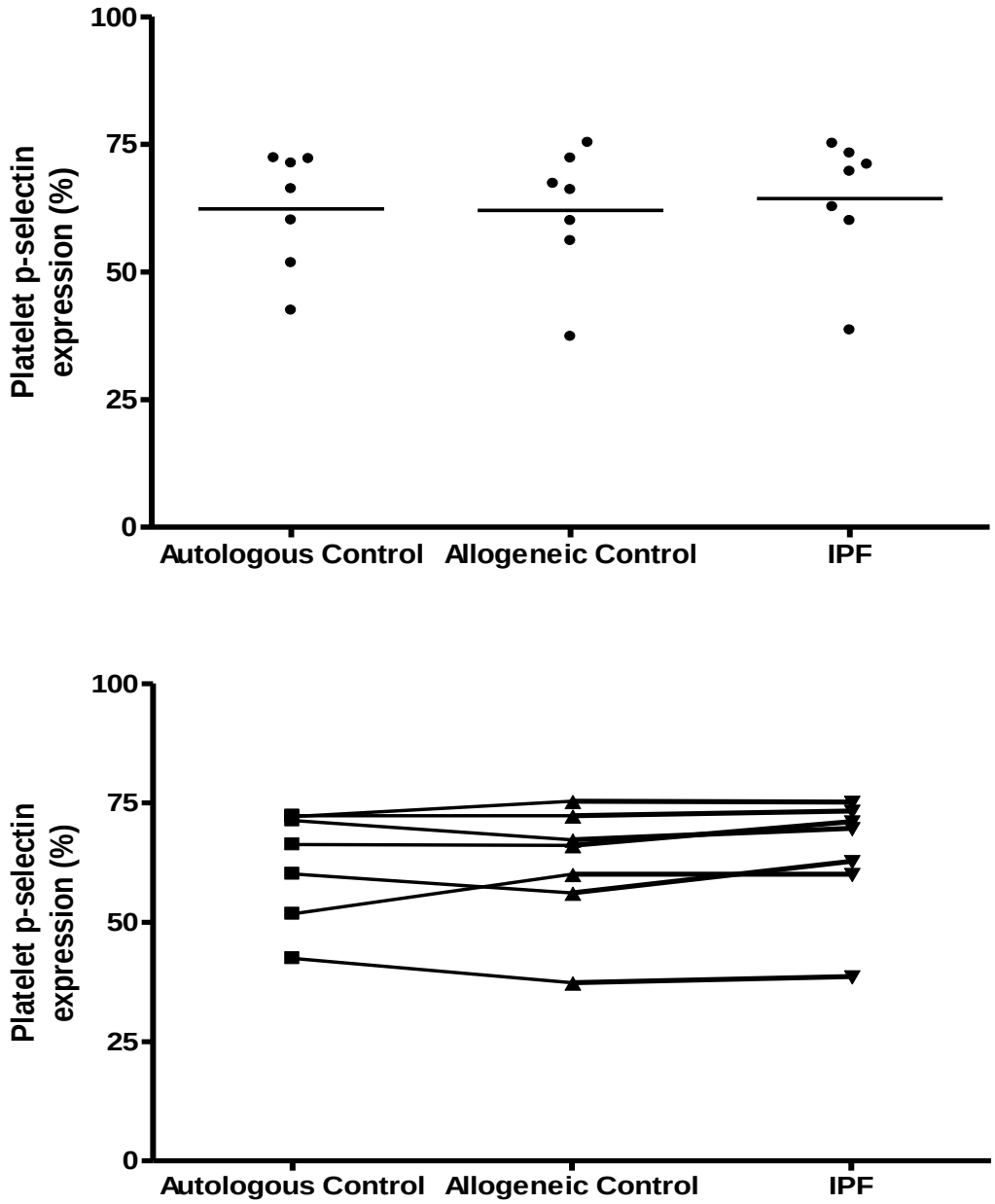

Figure 16. Dot plot demonstrating platelet P-selectin expression following stimulation with 10µM ADP and incubation with autologous plasma (Autologous Control), control patient plasma (Allogeneic Control) and IPF patient plasma (IPF). The mean P-selectin expression was 62.36 ± 4.38% in the Autologous Control group, 62.09 ± 4.82% in the Allogeneic Control group and 64.39 ± 4.76% in the IPF group. There was no significant difference between platelet P-selectin expression in the IPF group compared with the Autologous Control group (p=0.23) and the Allogeneic Control group (p=0.06). There was no significant difference between the Autologous and Allogeneic Control groups (p=0.88).

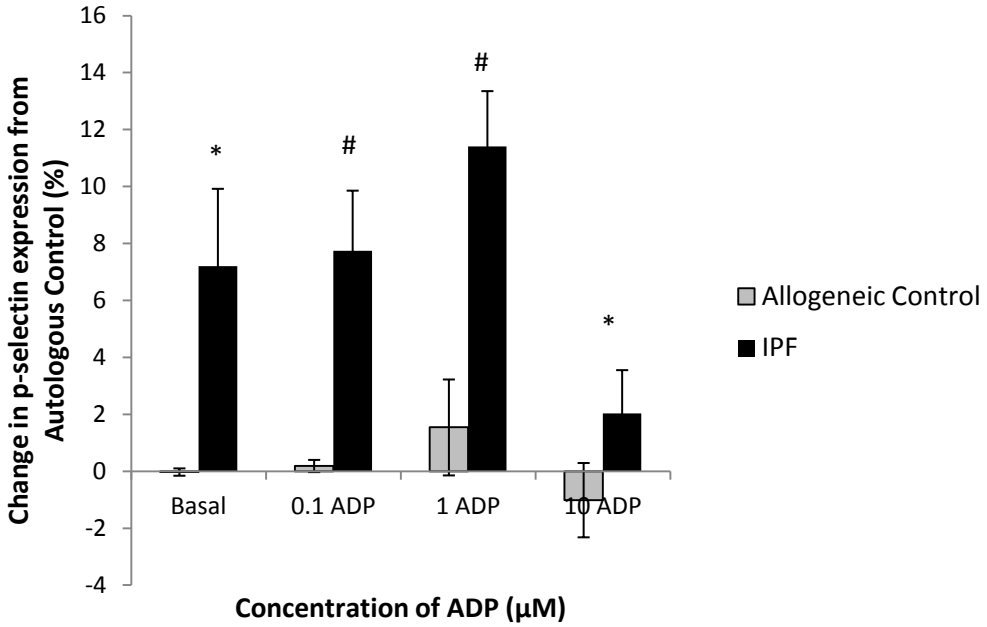

Figure 17. Change in platelet P-selectin expression from the Autologous Control following incubation with Allogeneic Control plasma and IPF plasma. Data expressed as percentage of platelets positive for P-selectin ± SEM. * P=<0.05, # P=≤0.01.

There was no significant difference in fibrinogen binding between the three groups (figure 18-22). The difference in platelet fibrinogen binding reached statistical significance between the Allogeneic Control and IPF groups following stimulation with 1µM ADP with greater binding in the Allogeneic Control group. However, there was no statistically significant difference between either of these groups and the Autologous Control group. The same findings are true when the data is analysed to compare the change in fibrinogen binding in the Allogeneic Control and IPF groups from baseline taken as the fibrinogen binding in the Autologous Control group (figure 23).

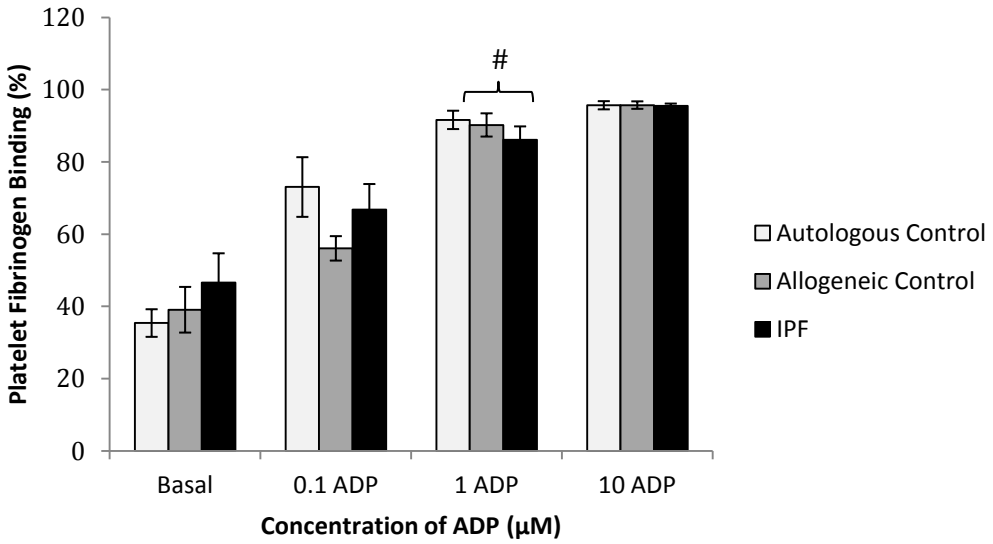

Figure 18. Platelet fibrinogen binding following incubation in autologous plasma (Autologous Control), control patient plasma (Allogeneic Control) and IPF patient plasma (IPF) in basal conditions and following stimulation with ADP (0.1, 1 and 10µM). Data expressed as percentage of platelets positive for fibrinogen ± SEM. # P=≤0.01. Unless stated otherwise there was no statistically significant difference noted between the groups.

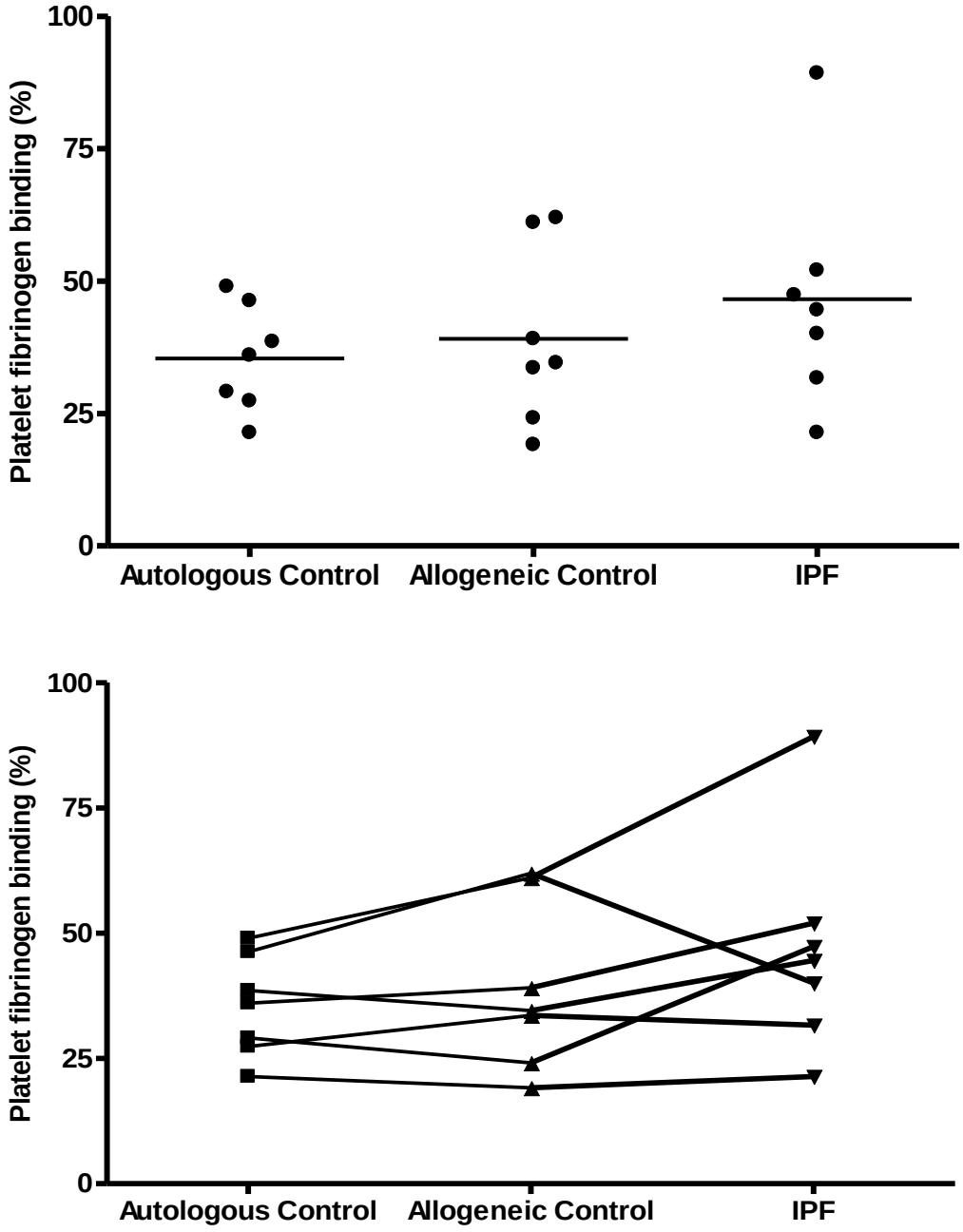

Figure 19. Dot plot demonstrating platelet fibrinogen binding following incubation with autologous plasma (Autologous Control), control patient plasma (Allogeneic Control) and IPF patient plasma (IPF) at basal levels.

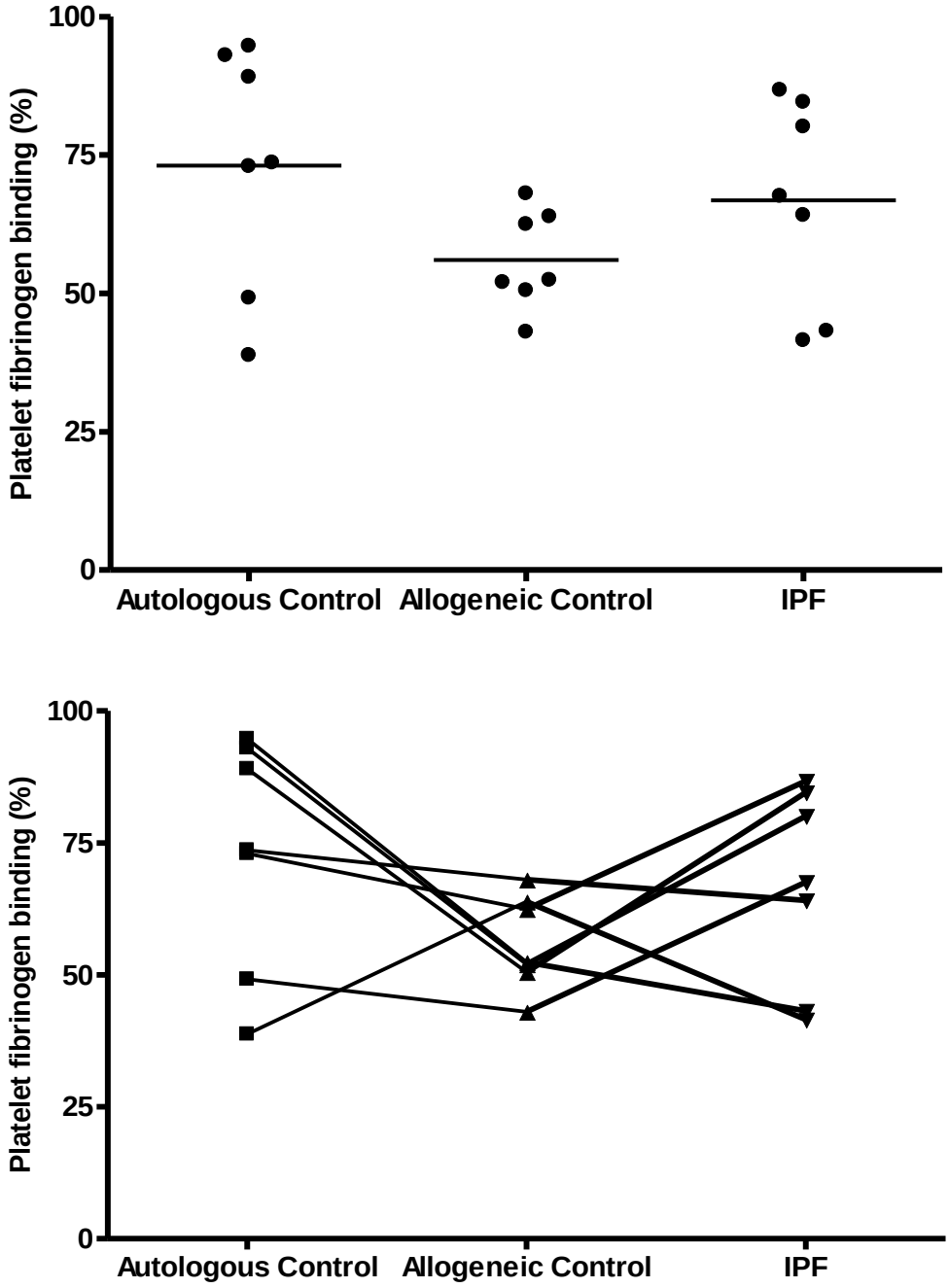

Figure 20. Dot plot demonstrating platelet fibrinogen binding following stimulation with 0.1µM ADP and incubation with autologous plasma (Autologous Control), control patient plasma (Allogeneic Control) and IPF patient plasma (IPF).

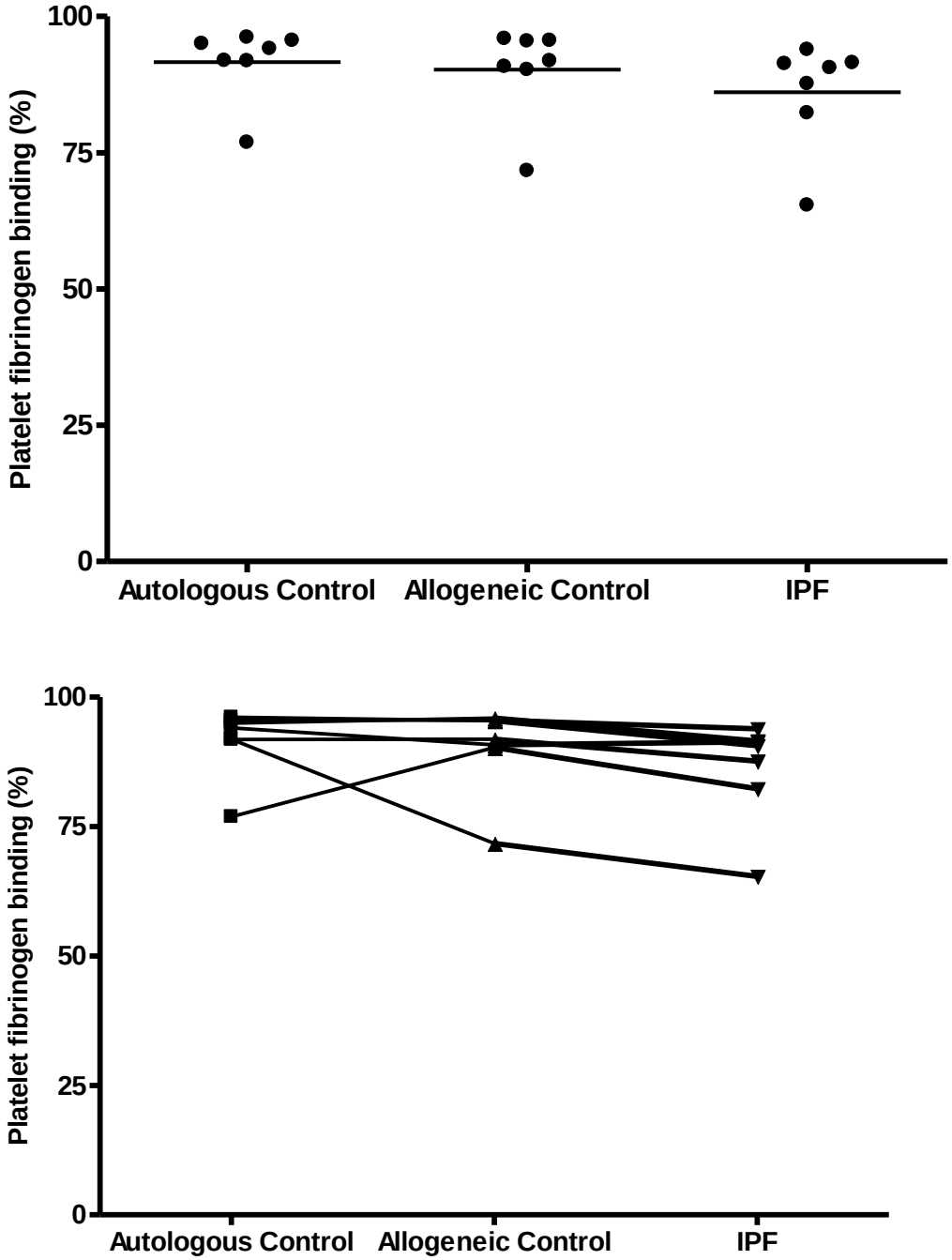

Figure 21. Dot plot demonstrating platelet fibrinogen binding following stimulation with 1µM ADP and incubation with autologous plasma (Autologous Control), control patient plasma (Allogeneic Control) and IPF patient plasma (IPF).

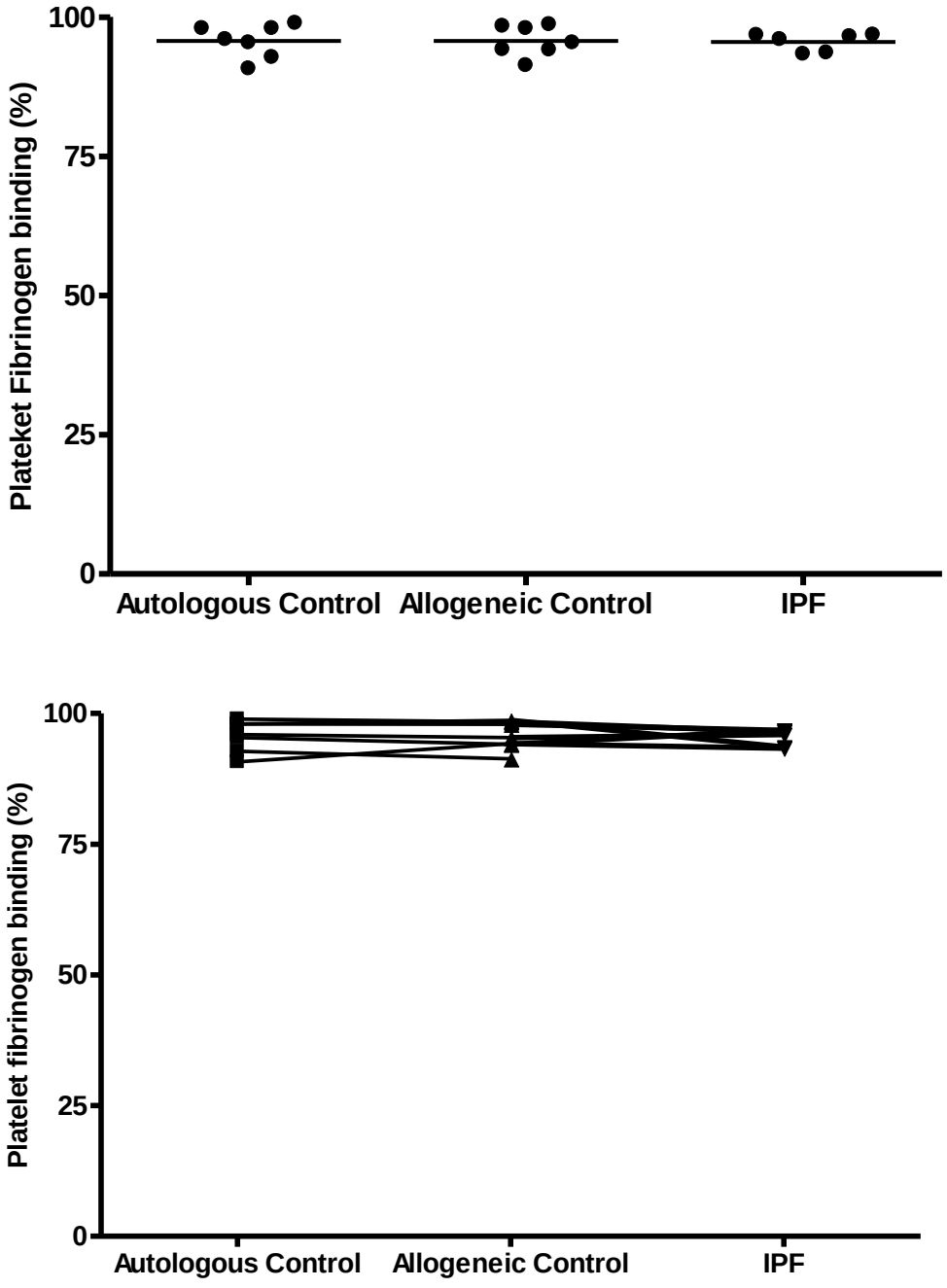

Figure 22. Dot plot demonstrating platelet fibrinogen binding following stimulation with 10µM ADP and incubation with autologous plasma (Autologous Control), control patient plasma (Allogeneic Control) and IPF patient plasma (IPF).

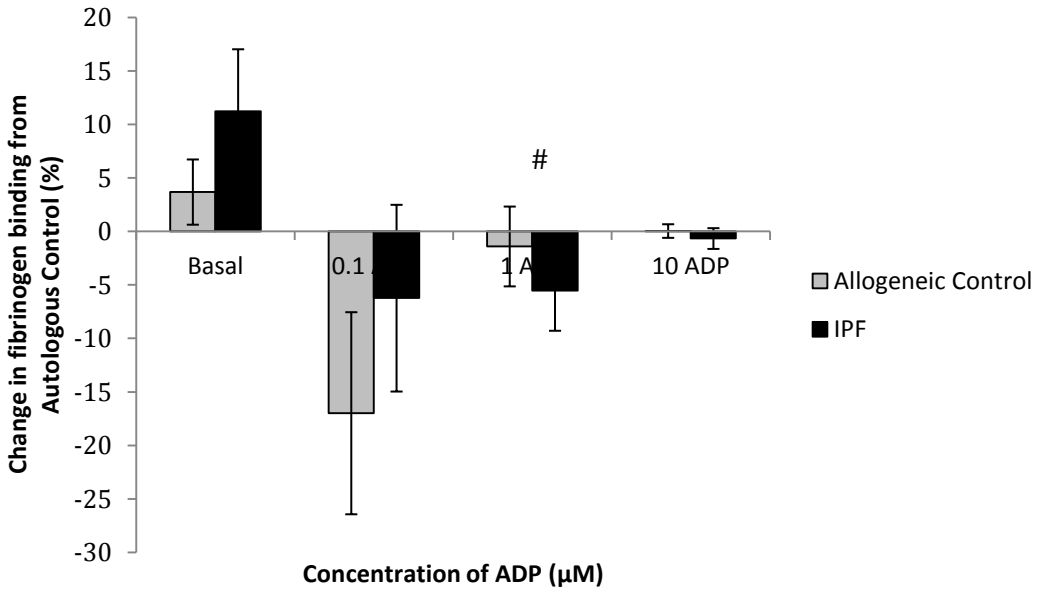

Figure 23. Change in platelet fibrinogen binding from the Autologous Control following incubation with Allogeneic Control plasma and IPF plasma. Data expressed as percentage of platelets positive for fibrinogen ± SEM. # P=≤0.01. Unless stated otherwise there was no statistically significant difference noted between the groups.

3.2.4 Discussion

Platelet activation measured by P-selectin expression was significantly greater following incubation in IPF plasma compared with autologous and allogeneic control plasma at basal levels and following stimulation with 0.1µM and 1µM ADP. Following stimulation with 10µM ADP there was no significant difference in P-selectin expression between the groups however the change in P-selectin expression compared with the autologous control was significantly greater following incubation in IPF plasma compared with allogeneic control plasma. This finding indicates the presence of a factor in IPF patient plasma resulting in increased platelet activation at basal levels and in response to platelet agonists.

Fibrinogen binding was not significantly different between autologous controls, allogeneic controls or IPF patients at basal levels or in response to stimulation with ADP. There was marked variability between control samples in this assay and maximum stimulation was achieved in some samples following stimulation with 0.1µM ADP making it difficult to assess the validity of these results.

The allogeneic control and IPF groups were well matched across all variables. Although the IPF group were slightly older than the allogeneic control group the difference was not statistically significant. Although it could be argued that this may still be clinically significant we did not identify any evidence that older age was associated with increased platelet activation in this patient cohort.

Platelet P-selectin expression was significantly increased at basal levels following incubation in IPF plasma compared with incubation in allogeneic plasma. However, platelet P-selectin expression in all groups was higher than observed in the initial assay using whole blood flow cytometry. It is likely that the process of preparing washed platelets resulted in a degree of platelet activation and resulting increased P-selectin expression. Therefore the observed increase in the IPF group likely represents increased platelet reactivity as previously demonstrated.

Although from these results it is not possible to completely exclude a co-existing primary platelet defect in IPF patients contributing to the increased platelet reactivity demonstrated in the initial whole blood assay, the demonstration that control platelets exhibit this same response following incubation in IPF plasma strongly indicates the presence of a plasma factor (soluble factor or microparticle) increasing platelet reactivity. Although this finding does not indicate whether this is important in the pathogenesis of the disease or a consequence of the fibrotic process, it further emphasises the important relationship between the lung and the vasculature.

Further research is required to identify the plasma factor responsible for increased platelet activation and reactivity in IPF. This should include assessment of the plasma concentration of known platelet agonists and inhibitors. If this fails to identify a culprit, serum fractionation and subsequent mass spectrometry could be used to identify the responsible plasma protein (139).

3.2.5 Conclusion

Incubation of control platelets in IPF patient plasma results in increased activation compared to incubation in autologous and allogeneic control plasma at basal levels and following stimulation with ADP. This provides evidence that the increased reactivity observed in IPF patients is due to a plasma factor. Further research is required to identify the responsible plasma factor and the functional consequences.

4. Assessment of Platelet Function: Platelet-Endothelial Cell Adhesion

4.1 Introduction

Platelets can adhere to the vessel walls either via direct interaction with the endothelial cell or via interaction with endothelial bound leukocytes (140). Platelet-endothelial interactions in the microvasculature have been studied most commonly in ischaemia-reperfusion injury however it has also been described in a wide range of disease states including: hypercholesterolaemia, sickle cell disease, malaria, endotoxemia, and experimental colitis (140). In most models the platelet adhesion to the vessel wall is confined to the post capillary venules although there are reports of adhesion to arteriole (141) and capillary (142) endothelium (140). Endothelial cell activation appears to play an important role in platelet endothelial interactions in the microcirculation (140).

Ischaemia-reperfusion injury is the most studied model with the extent of platelet adhesion being dependent on the duration of ischaemia. ICAM-1 mediated endothelial fibrinogen deposition has been described as a prominent feature during the reperfusion phase of ischaemia-reperfusion injury and provides a backbone for platelet GPIIb/IIIa mediated firm adhesion (143;144). GPIIb/IIIa has been shown to mediate platelet-endothelial cell adhesion in venules in a model of ischaemia-reperfusion injury (141;144). This mechanism has been demonstrated by attenuation of platelet-endothelial cell interaction following treatment with anti-fibrinogen antibodies, in ICAM-1 deficient mice and using platelets isolated from patients with Glanzmann disease (platelets deficient in GPIIb/IIIa) (141).

P-selectin and its ligands have also been implicated in platelet rolling and firm adhesion to the endothelium (145-147). P-selectin is a lectin-like adhesion glycoprotein that in resting conditions is stored in the alpha granules of platelets and the weibel-palade bodies of endothelial cells. On platelet activation P-selectin moves to and is expressed on the surface of the platelet. Likewise, during endothelial cell activation, P-selectin becomes mobilised from the weibel-palade bodies becoming exposed on the cell surface. Addition of a blocking antibody against P-selectin in a mouse model significantly attenuated platelet rolling and adhesion (145;146). However, it remains unclear to what extent this phenomenon is due to platelet P-selectin or endothelial P-

selectin. Massberg et al. reported that platelet-endothelial adhesion was not affected when P-selectin deficient platelets were studied with wild-type endothelium. However, platelet-endothelial adhesion was almost completely absent when wild-type platelets were assessed with P-selectin deficient endothelium (145). Cooper et al. undertook a similar study of platelet-endothelial adhesion in a mouse model of ischaemia-reperfusion injury and concluded that both platelet and endothelial P-selectin play a role in platelet-endothelial adhesion. However, Cooper et al. described platelet P-selectin as having a more important role (146). A study of platelet-endothelial interaction in rabbit lung using an ischaemia-reperfusion model confirmed increased platelet rolling and firm adhesion that was inhibited by blocking P-selectin interactions (148). Endothelial P-selectin has also been implicated in a rat model of retinal ischaemia with attenuation of platelet-endothelial interactions following blockade of endothelial P-selectin (147). Platelet PSGL-1 and GPIbα (CD42b) are important ligands expressed by platelets that are felt to mediate the interaction with endothelial derived P-selectin in venules (140).

Different studies have demonstrated different times to peak platelet–endothelial interaction following reperfusion. Ischaemia-reperfusion models with rapid onset of platelet interaction with the vessel walls are associated with platelet-endothelial adhesion in arterioles and venules however models with slower onset result in platelet–endothelial adhesion confined to the venules (146).

Shear forces generated by blood flow significantly affect the behaviour of circulating platelets and leukocytes. Laminar flow in microvessels leads red blood cells to push platelets and leukocytes toward the vessel wall. Low shear rates promote blood cell-endothelial cell interactions with a reduction in shear rates resulting in an increase in platelet adhesion to the vessel wall (149). This effect was attenuated by blocking P-selectin interactions but also by inducing neutropenia or blocking CD11/CD18 demonstrating that leukocytes can form a platform for platelet-endothelial cell interactions (149).

GP1bα, in addition to functioning as a ligand for endothelial derived P-selectin, has the ability to adhere to endothelial cells in a P-selectin independent manner at low shear stress via interactions with vWF expressed on the surface of activated endothelial cells (150;151).

The degree of shear force mediates the type of adhesion molecules involved in the adhesive process(152). At low shear stress GPIIb/IIIa has been shown to mediate firm adhesion via bound fibrinogen to endothelial ICAM-1(141;153). In the setting of high shear stress as seen in arteries, platelet-endothelial adhesion is mediated by GPIbα receptors binding with matrix vWF (153;154). Platelet binding to exposed subendothelial matrix is mediated by GPIbα and GPIIb/IIIa binding to vWF, collagen and fibronectin. This process is vital in order to maintain haemostasis through thrombus formation; however inappropriate thrombus formation within the vasculature can lead to thromboemboli.

Endothelium	Intermediary	Platelet
ICAM-1	Fibrinogen	GPIIb/IIIa
P-selectin	-	PSGL-1
P-selectin	-	GPIbα
vWF	-	GPIbα
P-selectin	Leukocyte PSGL-1	P-selectin
ICAM-1	Leukocyte CD18 Leukocyte PSGL-1	P-selectin
ICAM-1	Leukocyte CD11b/CD18	GPIbα
a_vb_3	-	GPIIb/IIIa
CD11b/CD18	-	GPIIb/IIIa
CD11b/CD18	-	P-selectin

Table 11. Summary of described mechanisms of platelet-endothelial cell adhesion including intermediaries (152).

Leukocytes appear to have the potential to potentiate platelet-endothelial cell interactions through a variety of mechanisms. Neutrophils have been demonstrated to contribute to the platelet-endothelial cell adhesion induced by LPS through superoxide production. Attenuation of platelet-endothelial interactions can be achieved by induction of neutropenia and blockade of neutrophil NADPH oxidase (155;156). In a model of experimental colitis, it was demonstrated that 97.6% of adherent platelets

were associated with adherent leukocytes in the inflamed colonic venules (157). Similarly, a model of ischaemia-reperfusion injury in small intestine demonstrated approximately 75% of adherent platelets being associated with adherent leukocytes and 25% of platelet adhering independently (156).

Platelet adhesion to endothelial cells is influenced by chemical mediators with nitric oxide inhibiting the response and superoxide promoting adhesion (155;158). Both platelets and endothelial cells produce nitric oxide by endothelial Nitric Oxide Synthase (eNOS) (159;160). Nitric oxide inhibits platelet activation by increasing intracellular cGMP concentration ultimately leading to a reduction in intracellular calcium (159). Superoxide production occurs in platelets, endothelial cells and leukocytes through NADPH oxidase (155). Therefore, the balance between nitric oxide and superoxide in the microvasculature may be important in modulating platelet adhesion.

A study of LPS induced platelet-endothelial cell adhesion in intestinal venules of mice demonstrated a marked increase in platelet adhesion following activation with LPS (161). Inhibition of endogenous nitric oxide (NO) production by L-NG-Nitroarginine Methyl Ester (L-NAME) resulted in a significant increase in platelet-endothelial cell adhesion. Conversely, the addition of the NO donor diethylenetriamine nitric oxide (DETA-NO) attenuated LPS induced platelet-endothelial cell adhesion through activation of soluble guanylyl cyclase (sGC) (161). In order to assess the source of NO responsible for attenuating platelet-endothelial cell interaction, eNOS deficient and wild-type mice were injected with eNOS deficient and wild-type platelets. Significantly increased platelet-endothelial adhesion was observed in eNOS deficient mice following treatment with LPS compared with wild-type mice however no difference was observed between administration of eNOS deficient platelets or wild-type platelets. This supports the theory that endothelial cell derived NO is responsible for attenuating platelet adhesion. The inhibitory effect of NO has been further investigated and supported by demonstrating the importance of downstream signalling molecules including platelet cyclic guanosine monophosphate kinase-1 (cGK-1) (143) and vasodilator-stimulated phosphoprotein (VASP) (162) in inhibiting platelet activation and platelet-endothelial cell interactions.

As previously described, inhibition of GPIIb/IIIa is associated with reduced platelet adhesion to the microvasculature in a mouse model of ischaemia/reperfusion injury

(141). In addition, GPIIb/IIIa inhibition attenuates leukocyte recruitment to venules following ischaemia-reperfusion injury (163). Therefore, where some studies have demonstrated leukocytes to play an important role in promoting platelet-endothelial cell adhesion it also appears that platelets play an important role in facilitating leukocyte-endothelial cell interactions. Studies of ischaemia-reperfusion injury in mice mesentery and myocardium have shown that leukocyte-endothelial interactions were significantly attenuated by P-selectin and GPIIb/IIIa depletion (163;164). This effect could be reversed by infusion of wild-type platelets but not P-selectin deficient platelets suggesting that platelet P-selectin expression is central to this effect (164). In mouse mesentery, P-selectin expression is markedly increased following ischaemia-reperfusion injury (163). This is attenuated by induction of thrombocytopenia or treatment with anti GPIIb/IIIa or fibrinogen monoclonal antibodies (163).

Neutrophil accumulation following ischaemia-reperfusion injury measured by tissue myeloperoxidase activity was also attenuated by inducing thrombocytopenia or treatment with GPIIb/IIIa or fibrinogen monoclonal antibodies and importantly also by treatment with P-selectin blocking antibodies (163). This suggests that leukocyte adhesion to the endothelial surface in this model is facilitated by initial platelet-endothelial cell adhesion through endothelial ICAM-1 binding fibrinogen which is subsequently bound by platelet GPIIb/IIIa with P-selectin dependent platelet-leukocyte binding. This mechanism is further supported in a mouse model of acute postischaemic renal failure where neutrophil accumulation was attenuated in mice lacking platelet P-selectin expression but not in mice lacking endothelial P-selectin expression (165).

Platelet-endothelial interactions are important in considering platelets potential pathogenic role in IPF. Direct interaction with the pulmonary microvascular endothelium resulting in platelet trapping in the lung would facilitate local release of profibrotic mediators during platelet activation. In addition, platelet endothelial interaction may facilitate the recruitment of circulating inflammatory cells with further increase in release of pro-inflammatory and pro-fibrotic mediators with resulting tissue injury and fibrosis. A simple platelet-endothelial adhesion assay was designed and utilised in order to identify if the observed increased platelet reactivity in IPF patients alters the platelet function in terms of endothelial cell interactions. This study

investigates platelet-endothelial cell adhesion in solution following the incubation of control platelets in autologous control, allogeneic control and IPF plasma.

4.2 Method

Platelet-endothelial cell adhesion in IPF and controls was assessed ex-vivo in suspension. In light of earlier findings that the increased platelet activation and reactivity observed in IPF patients is secondary to the effects of IPF plasma, a plasma swap technique was used. Control platelets suspended in plasma were added to HUVEC in suspension and adhesion was assessed using flow cytometry.

Blood collection

Venous blood was collected from an antecubital vein using a 21 gauge butterfly needle (Becton Dickinson Vacutainer Systems, UK). Blood samples were collected from 5 control patients without evidence of interstitial lung disease, other fibrotic conditions or known vascular disease for preparation of washed platelets. Blood was collected from 10 clinically stable IPF patients and a further 10 control patients for preparation of platelet poor plasma. All IPF patients were diagnosed in accordance with ATS/ERS criteria and were free from any acute intercurrent illness at the time of sampling.

Six mls of blood was taken into a vacutainer tube anticoagulated with 102 IU lithium heparin (Becton Dickinson Vacutainer Systems, UK) and used for preparation of platelet poor plasma.

Washed platelets were prepared from blood collected in four 4.5ml vacutainer tubes anticoagulated with 0.105 Molar Sodium Citrate (Becton Dickinson Vacutainer Systems, UK).

Preparation of Platelet Poor Plasma

Lithium heparin anticoagulated blood was used for the preparation of platelet poor plasma. Blood was centrifuged at 1,500G for 10 minutes at 20^{o}C and the plasma supernatant collected. Plasma was stored in 300µl aliquots at -80^{o}C until required. Plasma samples were then thawed at room temperature prior to further centrifugation at maximum speed for 2 minutes to ensure all cellular debris was removed prior to use.

Preparation of Washed Platelets

The first 4.5 mls of blood collected was discarded to avoid inadvertent platelet activation during venepuncture affecting the results. The remaining citrate anticoagulated blood was centrifuged at 900 rpm for 20 minutes at 20^{o}C with no brake to produce PRP. 0.3M citric acid was added to the PRP to achieve a PH of 6.4 in order to prevent unwanted platelet activation during the washing process. The PRP was further centrifuged at 1,900 rpm for 12 minutes at 20^{o}C to produce a platelet pellet and the supernatant was discarded. The platelet pellet was suspended in wash buffer (PH 6.5) by gentle pipetting prior to a final centrifugation at 2,200 rpm for 10 minutes at 20^{o}C. The supernatant was discarded and the washed platelet pellet was suspended in PBS to achieve the desired platelet concentration. The washed platelets were used immediately following preparation.

Plasma swap Protocol

Washed platelets suspended in PBS were separated into three 100µl aliquots. The first aliquot was added to 100µl of autologous plasma, the second aliquot was added to 100µl of allogeneic control plasma and the third aliquot was added to 100µl of plasma from an IPF patient. The end result was 3 platelet suspensions representing autologous control, allogeneic control and IPF samples.

Preparation of HUVEC

HUVEC were cultured in Endothelial Cell culture medium containing supplied supplements (Promocell GmbH, Heidelberg, Germany) (table 12). HUVEC derived from passages four to five were grown to confluence in T75 cell culture flasks at 37^{o}C in an atmosphere containing 5% CO_2 before being harvested using trypsin. Serum free media was added to abort the action of trypsin and the HUVEC in suspension were centrifuged at 1,500G for 5 minutes to form a pellet. The supernatant was discarded and the HUVEC were suspended in serum free media to obtain the desired concentration. HUVEC in suspension were agitated continuously to prevent adhesion to the walls of the container prior to use in the platelet-endothelial cell adhesion assay.

Foetal Calf Serum	0.02ml/ml
Endothelial cell growth supplement	0.004ml/ml
Recombinant human epidermal growth factor	0.1ng/ml
Recombinant human fibroblast growth factor	1ng/ml
Hydrocortisone	1µg/ml
Penicillin	100µg/ml
Streptomycin	100U/ml

Table 12. Endothelial cell culture medium supplements

Platelet-Endothelial Adhesion Assay

Platelet-endothelial adhesion was assessed in suspension. Twenty microliters of platelets suspended in autologous control, allogeneic control and IPF plasma were added to separate eppindorfs containing 20µl of HUVEC suspended in serum free media. The ratio of platelets to HUVEC in each suspension was 10 to 1. The suspensions containing platelets and HUVEC were incubated with 5µl FITC-conjugated anti-CD42b (BD Biosciences, UK) and varying concentrations of the platelet agonist ADP (0.1, 1 and 10µM). Samples were made up to 50µl using PBS. Following 20 minutes incubation samples were fixed by adding 250µl 1% paraformaldehyde. HUVEC were identified on flow-cytometry by their forward and side scatter characteristics and confirmed by labelling a separate sample with PE conjugated anti-CD31 (BD Biosciences, UK). A separate experiment confirmed that no unbound platelets appeared within the HUVEC gate in resting state or following activation with 0.1, 1 and 10µM ADP. Additionally, it was confirmed that the FL-1 characteristics of HUVEC in the presence of FITC-labelled anti CD42b were unchanged following incubation with ADP. Therefore, HUVEC can be reliably identified using their forward and side scatter characteristics with CD42b positive events representing adherent platelets (figure 24). Platelet-endothelial cell adhesion was expressed as the percentage of endothelial cells with one or more bound platelets.

Flow Cytometry and Data Analysis

Flow cytometry was performed using a Becton Dickinson FACS Calibur flow cytometer (Becton Dickinson, UK). Data were analysed using CellQuest Pro software (Becton Dickinson, UK). Statistical analysis was performed using a paired t-test with a p-value of <0.05 considered significant.

4.3 Results

The 10 IPF patients and 10 allogeneic controls were well matched across all variables (table 13).

The percentage of endothelial cells (±SEM) with one or more adherent platelet was significantly increased following incubation of control platelets in IPF plasma compared with allogeneic control plasma at basal levels (0.86 ± 0.03% compared to 0.15 ± 0.03% respectively, p=0.04) and following platelet stimulation with 0.1µM ADP (0.88 ± 0.3% compared to 0.12 ± 0.04% respectively, p=0.04). There was a trend to increased platelet endothelial cell adhesion following incubation in IPF patient plasma compared with allogeneic control plasma in the presence of 1µM ADP (1.73 ± 0.7% compared to 0.21 ± 0.04% respectively, p=0.07) and 10µM ADP (0.71 ± 0.21% compared to 0.34 ± 0.1% respectively, p=0.06) however this failed to reach statistical significance.

Similarly, platelet endothelial cell adhesion was significantly greater following incubation of control platelets in IPF plasma compared to autologous plasma at basal levels (0.86 ± 0.03% compared to 0.09 ± 0.03% respectively, p=0.03) and following platelet stimulation with 0.1µM ADP (0.88 ± 0.3% compared to 0.16 ± 0.04%, p=0.03) and 10µM ADP (0.71 ± 0.21% compared to 0.26 ± 0.08%, p=0.03).

There was no significant difference in platelet-endothelial adhesion between control platelet incubated in autologous and allogeneic control plasma at basal levels (0.09 ± 0.03% compared to 0.15 ± 0.03% respectively, p=0.05) and following stimulation with 0.1µM ADP (0.16 ± 0.04% compared to 0.12 ± 0.04% respectively, p=0.4), 1µM ADP (0.41 ± 0.14% compared to 0.21 ± 0.04% respectively, p=0.11), and 10µM ADP (0.26 ± 0.08% compared to 0.34 ± 0.1 respectively, p=0.13). Platelet-endothelial cell adhesion at basal levels and following platelet stimulation with ADP is displayed in figures 25-29).

	IPF	Controls	P-value
Number (n)	10	10	-
Age in years – Mean (SD)	74(8)	72 (11)	0.60
Gender (%)			
- Male	6(60)	7(70)	0.86
- Female	4(40)	3(30)	0.86
Comorbidities			
- Malignancy	0	1	1.00
- TIA/CVA	1	0	1.00
- Hypertension	1	0	1.00
- Diabetes mellitus	1	0	1.00
- GER	0	1	1.00
- COPD	2	3	1.00
- Atrial Fibrillation	1	0	1.00
Smoking Status			
- Current	2	0	0.47
- Ex-smoker	1	4	0.30
- Non-smoker	4	4	1.35
- Unknown	3	2	1.00

Table 13. Baseline characteristics of IPF patients and allogeneic controls.

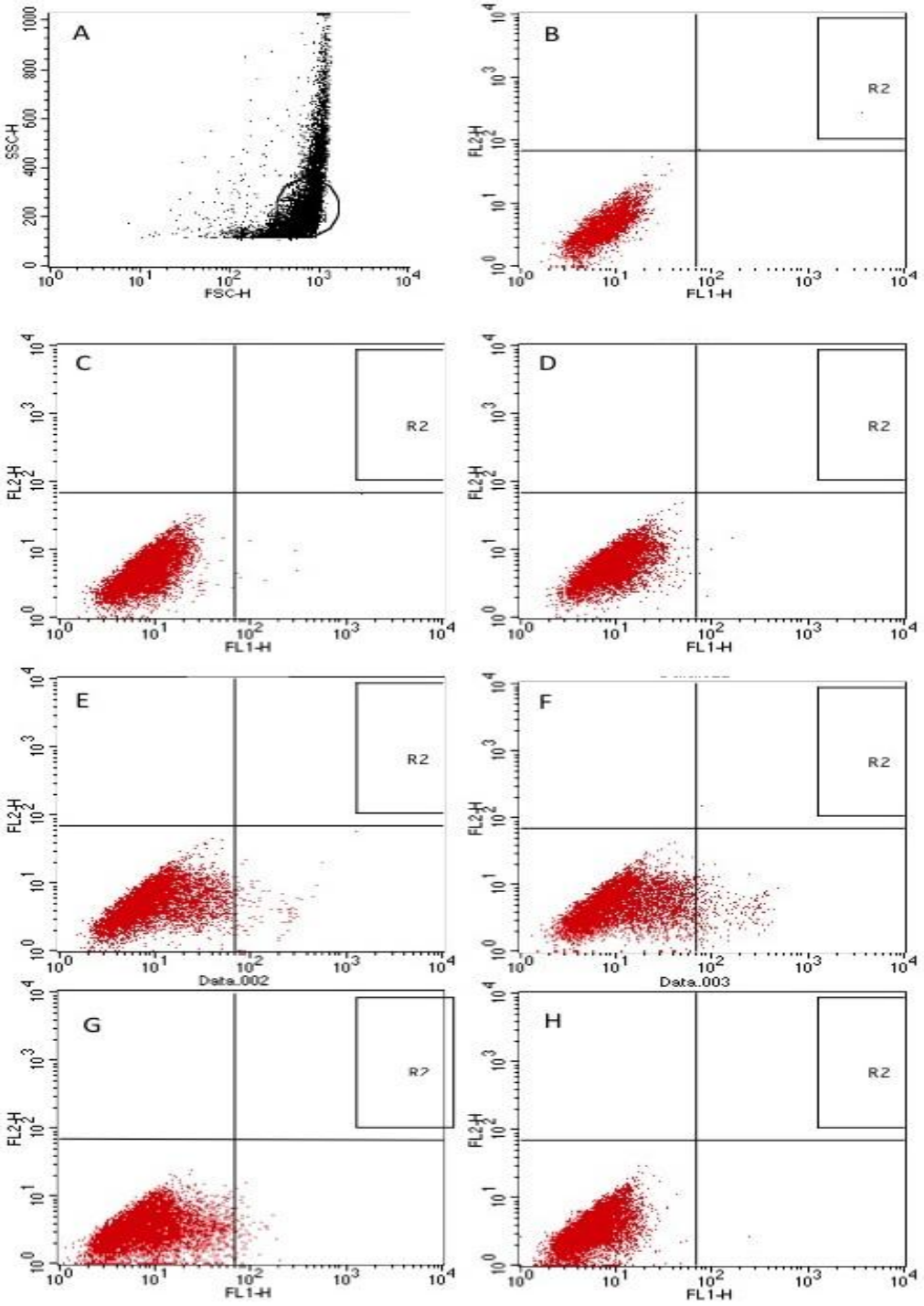

Figure 24. Scatter plot demonstrating forward and side scatter characteristics of HUVEC (A) and quadrant plot demonstarting FL-1 and FL-2 characterisitcs of HUVEC labelled with FITC-conjugated IgG isotype control (B). (C-H) Quadrant plots demonstrating FL-1 and FL-2 characterisitcs of HUVEC and control platelets labelled with FITC-conjugated CD42b suspended in allogeneic control plasma in basal conditions (C) and the same sample with the addition of 1µM ADP (D), IPF plasma in basal conditions (E) and the same sample with the addition of 1µM ADP (F), IPF plasma in basal conditions (G) and the same sample with the addition of EDTA (H).

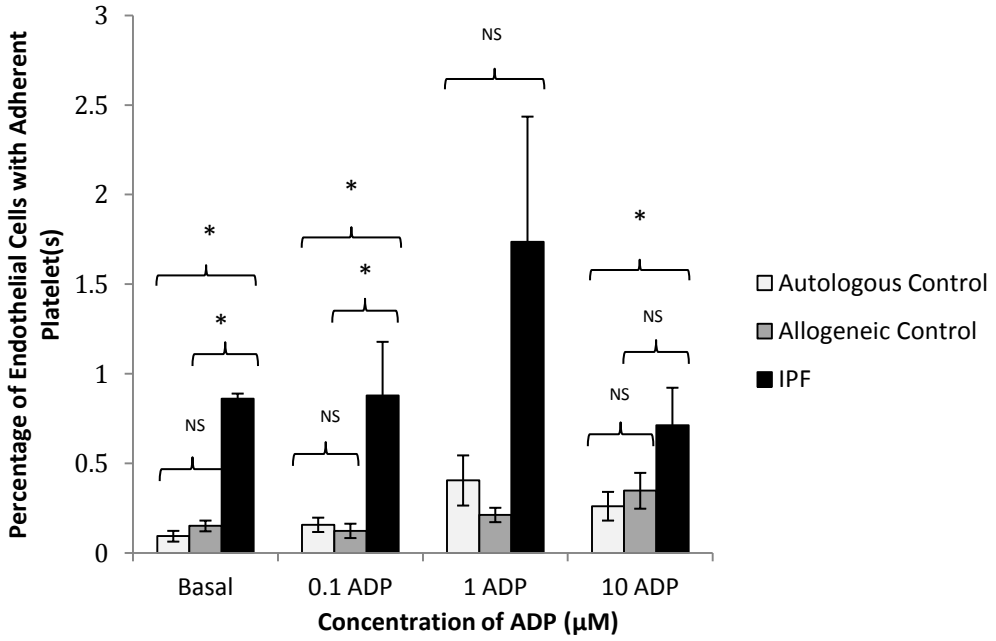

Figure 25. Platelet-endothelial adhesion expressed as the percentage of endothelial cells with one or more adherent platelets. * p = <0.05, NS p = ≥0.05

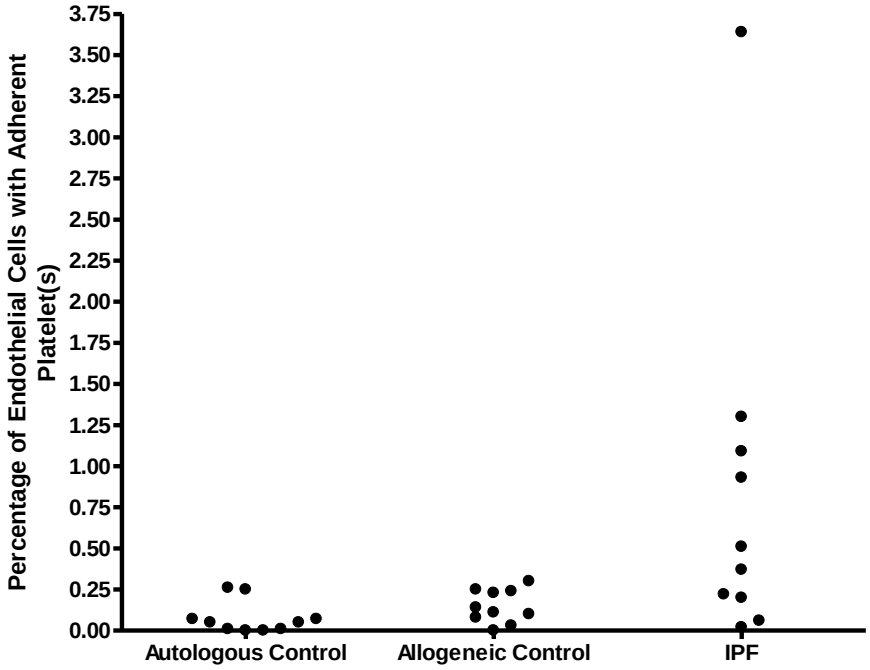

Figure 26. Dot plot demonstrating the percentage of endothelial cells with one or more adherent platelets following incubation of control platelets in autologous, allogeneic and IPF plasma in basal conditions.

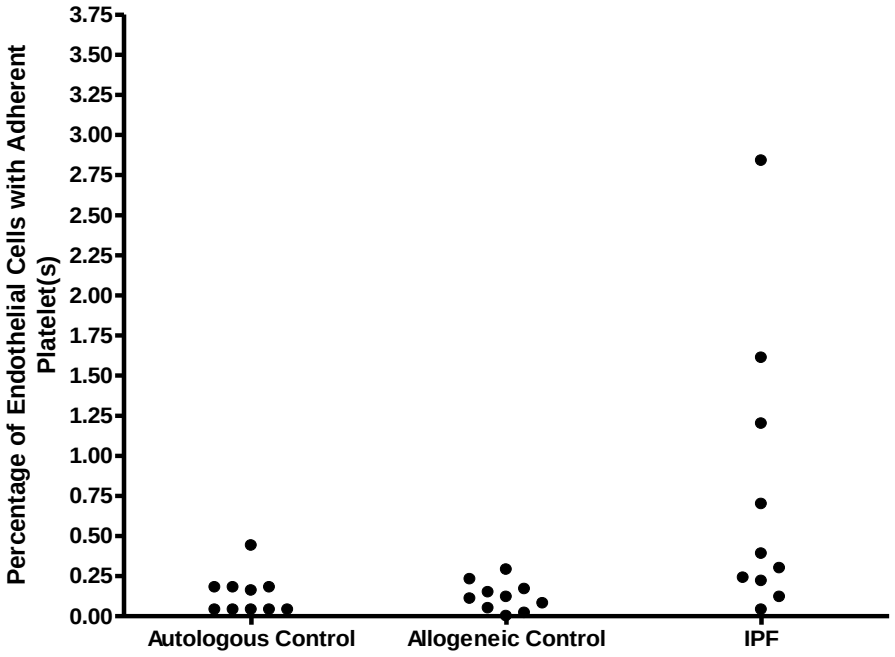

Figure 27. Dot plot demonstrating the percentage of endothelial cells with one or more adherent platelets following incubation of control platelets in autologous, allogeneic and IPF plasma following platelet stimulation with 0.1µM ADP.

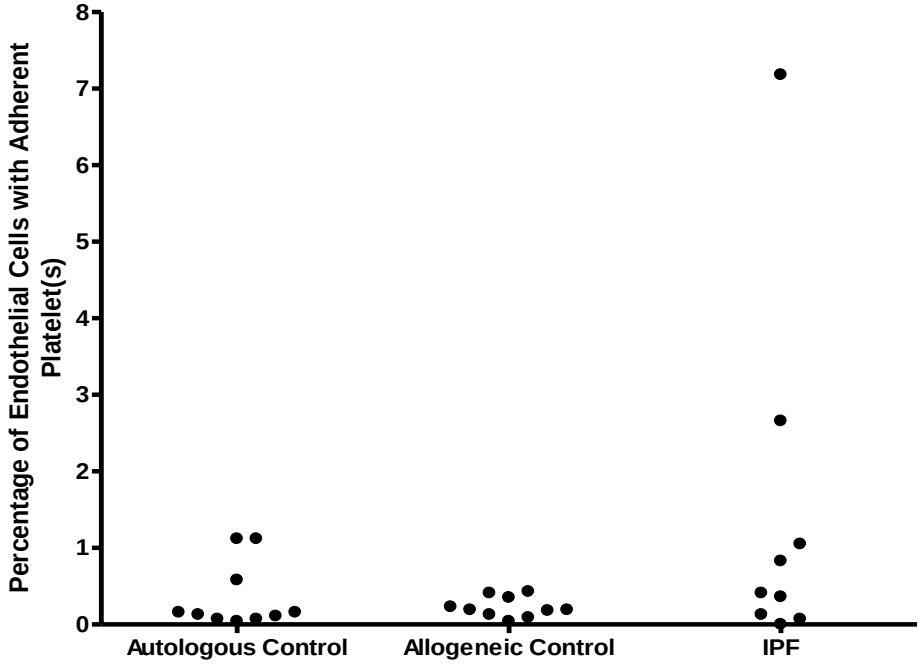

Figure 28. Dot plot demonstrating the percentage of endothelial cells with one or more adherent platelets following incubation of control platelets in autologous, allogeneic and IPF plasma following platelet stimulation with 1µM ADP.

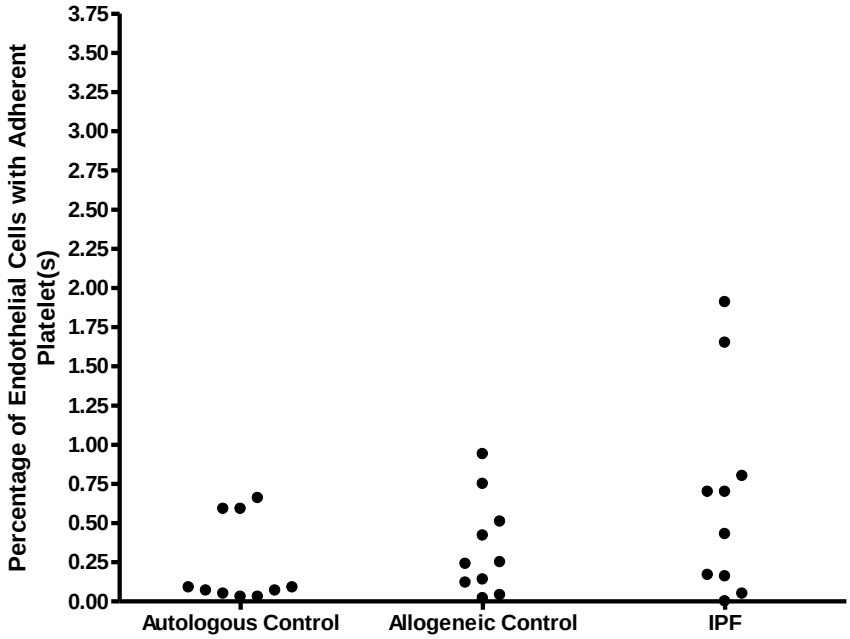

Figure 29. Dot plot demonstrating the percentage of endothelial cells with one or more adherent platelets following incubation of control platelets in autologous, allogeneic and IPF plasma following platelet stimulation with 10µM ADP.

4.4 Discussion

This study demonstrates increased platelet-endothelial cell adhesion following the incubation of control platelets in IPF plasma compared to incubation in autologous and allogeneic control plasma. This confirms that the previously described increased platelet reactivity in IPF patients has a functional effect and provides evidence of a potential pathogenic mechanism.

Patients and controls were well matched in this experiment with no significant difference in age, gender or co morbidities.

In this study, FITC-labelled CD42b monoclonal antibody (BD Biosciences, UK) was used to label platelets. It is important to consider that HUVEC have been reported to express CD42b (GPIbα) (166) and it has been implicated in platelet-endothelial adhesion (85). However, incubation of HUVEC with the FITC-labelled CD42b monoclonal antibody (BD Biosciences, UK) in the absence of platelets did not result in any change in the FL-1 characteristics at basal levels or following stimulation with ADP (data not shown). It is therefore safe to conclude that the change in FL-1

characteristics following incubation with platelets in plasma is due to platelet adhesion and not due to a change in HUVEC CD42b expression.

Platelet-endothelial adhesion assays are often performed under flow conditions using a flow-chamber in an attempt to replicate conditions in physiological and disease states. However, it is recognised that platelets predominantly adhere to matrix proteins and therefore measuring platelet adhesion to endothelial cells that are adherent to wells makes it difficult to differentiate platelet-endothelial binding from platelets adhering to exposed matrix. Some investigators use fixed endothelial cells to maintain confluence and try to overcome this. However, this method can have an effect on receptor function and does not completely eliminate the problem (85). Assessing platelet-endothelial adhesion in suspension removes the potential for platelet-matrix interactions and therefore allows an accurate assessment (85). In this study HUVEC were harvested and suspended in serum free media prior to incubation with control platelets suspended in IPF or control plasma. This is a novel method of measuring platelet-endothelial cell adhesion with previously described assays performing the incubation step in HUVEC coated wells prior to mechanical harvesting, suspension and flow cytometry (85). However, for the purposes of this study, in order to prevent a perceived risk of dissociation of platelets and endothelial cells during harvesting, it was decided to perform the incubation step in suspension and therefore without the need for further manipulation prior to flow cytometry. This does have the potential drawback that matrix proteins that are normally minimally expressed on the luminal surface of endothelial cells but more readily expressed on the abluminal surface may have been exposed (85). However, the purpose of this study was not to identify the mechanism of platelet-endothelial adhesion or to replicate physiological conditions in the pulmonary microvasculature. This study aimed to test the hypothesis that control platelets incubated in IPF plasma would exhibit greater adhesion to endothelial cells than platelets incubated in control plasma. Therefore, despite the methodological limitations, this study has achieved its aim and confirmed the hypothesis.

In keeping with previous studies of resting platelets and HUVEC, the observed platelet-endothelial adhesion was low in all 3 groups (85). It is worth noting that the observed platelet-endothelial adhesion was divalent cation dependent as evidenced by the

absence of adhesion in the presence of EDTA. Therefore, the use of citrate as an anticoagulant following venepuncture may have resulted in lower than expected levels of adhesion as described in chapter 3. Interestingly, platelet-endothelial adhesion did not alter significantly following platelet stimulation with the agonist ADP. A previous study of platelet-endothelial cell adhesion in suspension demonstrated increased adhesion in response to platelet stimulation with thrombin (85). This mechanism of platelet activation was not investigated in this study because platelet stimulation with the thrombin analogue TFLLR in previous assays had resulted in wider variation and less reproducibility than ADP.

Platelet-endothelial cell interactions have a number of potential consequences. It is recognised that activated platelets can alter endothelial cell expression of cell adhesion molecules and the release of chemotactic cytokines. An ex vivo study of HUVEC incubated for 6 hours with ADP stimulated platelets demonstrated increased ICAM-1 expression on the endothelial cell surface and increased release of monocyte chemotactic protein-1 (MCP-1), a cytokine that is important in the recruitment of macrophages (167). This was shown to be due to activation of the transcription factor nuclear factor - κB (NF-κB) that is known to regulate MCP-1 and ICAM-1 gene expression (167). These changes were not observed following incubation of HUVEC with resting platelets or ADP alone confirming that this effect is due to the activated platelets. Resting platelets can also affect endothelial cell expression of surface adhesion molecules (168). In a model of platelet-endothelial adhesion using brain microvascular endothelial cells (MVECs), few resting platelets were noted to adhere to the endothelial surface. When the MVECs were stimulated with TNF, increased platelet adhesion was observed followed by fusion of the platelet and endothelial membranes resulting in the formation of dense granules and incorporation of a portion of the platelet membrane into the luminal membrane of the endothelial cell (168).

Therefore, platelet-endothelial adhesion in the presence of either platelet activation or endothelial activation can result in altered endothelial adhesive properties. Up-regulation of ICAM-1 is recognised to play an important role in leukocyte adhesion and transmigration (169;170) and has been implicated in platelet-endothelial adhesion (144;171). Interestingly, platelet binding and fusion with TNF activated microvascular endothelial cells also increases leukocyte adhesion but also resulted in endothelial

injury that was related to the number of platelets and concentration of TNF-α (168). In studies of platelet-leukocyte adhesion, it has been demonstrated that adherence of activated platelets to neutrophils and monocytes also results in activation of NF-κB and is associated with increased production of inflammatory cytokines including Il-1β, Il-8 and MCP-1 (172). This effect is reduced when platelet-leukocyte adhesion is inhibited by P-selectin blockade (172). As previously described, over expression of Il-1β in the lung has been implicated in local inflammation with tissue injury and subsequent fibrosis associated with transient elevation of PDGF and prolonged elevation of TGF-β in bronchoalveolar lavage fluid of mice (43). In addition to platelets ability to increase leukocyte production of Il-1β, platelets also contain mRNA for the Il-1β precursor pro-Il-1β (79). Following activation, platelets have been demonstrated to produce and release Il-1β with resulting alteration of endothelial cell adhesive properties increasing leukocyte adhesion and transmigration (79).

4.5 Conclusion

The series of experiments in chapter 3 demonstrate increased platelet-monocyte binding following agonist induced platelet activation in IPF patients. The experiment in this chapter demonstrates increased platelet-endothelial binding in platelets incubated in IPF patient's plasma. Adherence of activated platelets to leukocytes is known to contribute to local inflammation (172) and adherence of activated platelets to endothelial cells alters endothelial adhesive and signalling properties (167). Although it is not possible to conclude whether the increased platelet reactivity in IPF is a primary event resulting in fibrosis or a response to the pulmonary fibrotic process, the observed increase in platelet-monocyte and platelet-endothelial adhesion has potential functional consequences. Similar effects have been described in patients with vascular disease (172;173) and therefore this finding in IPF provides important insight into the epidemiological association between these conditions. Indeed, it also provides a potential mechanism by which the pulmonary fibrotic process may be perpetuated: platelet-endothelial adhesion in the pulmonary microcirculation; platelet activation and degranulation with release of pro-inflammatory and pro-fibrotic mediators (e.g. Il-1β, PDGF, and TGF-β); and platelet-leukocyte interactions with resulting potentiation of their inflammatory potential.

5. Plasma Factors in Idiopathic Pulmonary Fibrosis

5.1 Evaluation of plasma D-dimer as a marker of fibrinolysis in idiopathic pulmonary fibrosis

5.1.1 Introduction

D-dimer is the final breakdown product of cross-linked fibrin and represents endogenous fibrinolysis. The D-dimer antigen is produced during the formation of the fibrin clot and can be detected by commercially available assays following fibrinolysis. The first step in fibrin clot formation is thrombin mediated cleavage of fibrinogen to produce fibrin monomers. Each monomer has two D-domains separated by a single E-domain. Fibrin monomers have a high affinity to form non-covalent bonds between the D-domain and the D or E-domain on adjacent monomers forming a fibrin protofibril. Thrombin remains associated with fibrin throughout this process and mediates factor XIII activation to factor XIIIa. Factor XIIIa promotes the formation of covalent bonds between D-domains of adjacent fibrin monomers in the fibrin protofibril to form the D-dimer antigen and create a cross-linked fibrin polymer. The D-dimer antigen remains undetectable until plasmin mediated cleavage of the fibrin polymer occurs releasing fibrin degradation products with exposed D-dimer antigen. This can occur prior to formation of an insoluble fibrin clot (ie plasmin mediated cleavage of a soluble cross-linked fibrin polymer prior to its incorporation into an insoluble fibrin clot) or during degradation of an established insoluble fibrin clot (174). D-dimer assays utilize monoclonal antibodies that recognise an exposed epitope on the cross-linked D-domain of fibrin. D-dimer detection therefore requires the formation of a cross-linked fibrin polymer with D-dimer formation mediated by thrombin and factor XIIIa and subsequent cleavage by plasmin (174).

Measurement of blood D-dimer level is widely used in clinical practice to exclude VTE in patients with a low probability stratified using clinical scoring systems. However, an elevated D-dimer is not specific to thromboembolic disease and will be present in any condition resulting in fibrin formation including but not limited to sepsis, acute cardiovascular events, malignancy and pregnancy. It is therefore useful as an indicator of activation of the coagulation system. Kubo et al. observed that D-dimer levels were elevated in IPF patients and that it increased during an acute exacerbation in a

randomised control trial of anticoagulation in IPF (128). It is recognised that there is an imbalance in haemostatic mechanisms in the lungs of patients with IPF with the environment in the alveolar compartment favouring fibrin deposition (175). However, despite epidemiological studies demonstrating an increased prevalence of thrombotic vascular disease in IPF patients the systemic mechanisms underlying this have not previously been described.

D-Dimer levels have previously been studied in patients with sarcoidosis and have been found to be elevated in 30-39% of patients with elevated levels being associated with lung parenchymal involvement and resulting symptoms of breathlessness and abnormal lung function measurements (176;177). A study of BAL fluid in sarcoidosis demonstrated that D-Dimer was detectable in the BAL fluid of patients but not in controls and that the D-Dimer levels correlated with the degree of lymphocytic alveolitis (178). This supports a possible role for D-dimer measurement in the evaluation of patients with interstitial lung disease and suggests a relationship between activation of the coagulation system and parenchymal lung disease. However, the nature of this relationship is unclear and it is not possible to conclude whether it is cause or effect.

It is possible that D-dimer elevation may have prognostic implications. A study of D-dimer in systemic lupus erythematosus, a connective tissue disease with multisystem involvement, reported an increased rate of thrombotic events in those with an elevated D-dimer level measured during routine follow-up (179). This has important implications for patient management and it is not clear whether these patients would benefit from routine anticoagulation. The role of D-dimer in IPF for predicting future thrombotic events has not previously been investigated.

We have measured the plasma D-dimer concentration of stable IPF patients and prospectively followed their course over 2 years to assess disease progression defined by lung function decline, incidence of acute exacerbations of IPF, incidence of thromboembolic disease, incidence of cardiovascular disease and death.

5.1.2 Method

Patient Selection

Patients were recruited from the interstitial lung disease clinic at a large University Hospital. A total of 29 patients with a diagnosis of IPF in accordance with the ATS/ERS criteria (9) entered the study. 20 control patients without fibrotic lung disease were recruited from other hospital out-patient clinics. All patients were stable at the time of blood sampling being clinically free from an exacerbation or any other acute inflammatory, neoplastic or thrombotic process. Patients were followed up prospectively for a two year period from the time of blood sampling and assessed for the following: acute exacerbation, venous thromboembolic event, acute cardiovascular event, disease progression defined by lung function decline, and death. Baseline lung function was taken as the lung function measurements performed closest to the time of blood sampling. Due to inter individual variation in the length of time between baseline and subsequent lung function measurements the lung function change is expressed as change in FVC and TLCO over 1 year.

Blood Sampling and D-Dimer Assay

Venous blood was collected from an antecubital vein using a 21 gauge butterfly needle (Becton Dickinson Vacutainer Systems, UK) and collected into a vacutainer tube anticoagulated with 102 IU lithium heparin (Becton Dickinson Vacutainer Systems, UK). Plasma was collected by centrifuging the blood at 1,500G for 10 minutes at 20^{o}C and the supernatant was collected. The plasma was stored in 300µl aliquots at -80^{o}C prior to thawing at room temperature prior to testing. HemosIL D-dimer HS automated latex enhanced immunoassay was used to measure plasma D-dimer level (Instrumentation Laboratory Company, Bedford, MA). A cut off of 250 ng/ml was used as the upper limit of normal in keeping with local clinical practice for the exclusion of VTE.

The difference in proportion of patients with an elevated D-dimer in IPF patients and controls was assessed using a 2 x 2 contingency table and Fishers Exact Test. The difference between mean D-dimer levels in IPF patients compared with controls was assessed using an unpaired t-test. A p-value of <0.05 was considered statistically significant.

5.1.3 Results

There was no significant difference in age, gender, prevalence of comorbid conditions or smoking status between IPF patients and controls (table 14).

The D-dimer was elevated above the 250 ng/ml cut off in 12/29 IPF patients. The mean D-dimer level (±SEM) was 253.1±27.2 ng/ml. The D-dimer was elevated above 500ng/ml in 2/29 IPF patients.

The D-dimer was elevated above the 250 ng/ml cut off in 4/20 control patients with 1/20 control patients having a D-dimer above 500 ng/ml. The difference in the number of IPF patients and controls with a D-dimer above 250 ng/ml was not statistically significant (p=0.14). The mean D-dimer in the control group was 198.2±28.1 ng/ml and did not differ significantly from IPF patients (p=0.18) (figure 30). Baseline lung function data was available for 25/29 patients. Serial lung function measurements were available for 18/29 patients. There was no difference in baseline lung function measurements or change in lung function over time between IPF patients with an elevated D-dimer and IPF patients with a D-dimer within normal range (table 15).

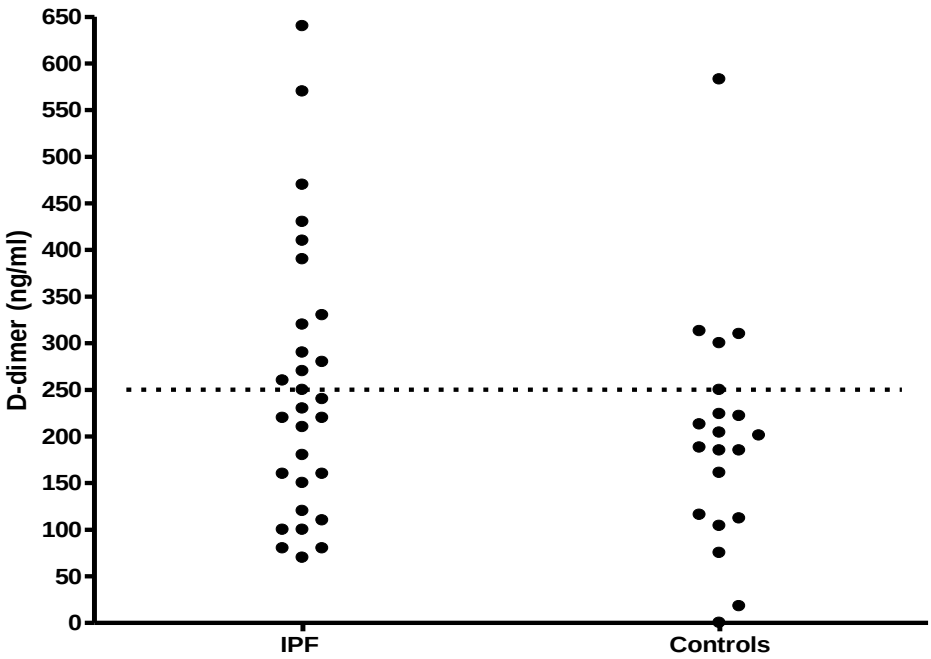

Figure 30. Scatter plot demonstrating the distribution of D-dimer levels (ng/ml) in IPF and controls. Dotted line represents the 250 ng/ml cut off.

	IPF	Controls	P-value
Number (n)	29	20	-
Age in years – Mean (SD)	69.1 (8.3)	66.5 (9.8)	0.33
Gender (%)			
- Male	21(72.4)	11(55)	0.24
- Female	8(27.6)	9(45)	0.24
Comorbidities (%)			
- Malignancy	0(0)	0(0)	-
- TIA/CVA	4(13.8)	0(0)	0.14
- Hypertension	6(20.7)	1(5)	0.22
- Diabetes mellitus	1(3.4)	1(5)	1.00
- GER	3(10.3)	1(5)	0.64
- COPD	5(17.2)	5(25)	0.72
- Sleep apnoea	0(0)	2(10)	0.16
- IHD	7(24.1)	1(5)	0.12
Smoking Status (%)			
- Current	8(27.6)	1(5)	0.06
- Ex-smoker	13(44.8)	10(50)	0.78
- Non-smoker	5(17.2)	7(35)	0.19
- Unknown	3(10.3)	2(10)	1.00

Table 14. Baseline characteristics of IPF patients and controls

Lung Function	Elevated D-dimer	Normal D-dimer	p-value
Baseline			
- FVC (L)	2.98	3.39	0.33
%predicted	88.67	100.86	
- TLCO (ml/mmHg/Mi)	3.81	3.88	0.93
% predicted	48.55	48.29	
Change over 1 year			
- FVC (L)	-0.26	-0.19	0.68
- TLCO (ml/mmHg/Mi)	-0.5	-1.6	0.23

Table 15. Lung function at baseline (litres) and lung function decline in 1 year in IPF patients with elevated D-dimer (>250ng/ml) and normal D-dimer (≤250ng/ml)

The all-cause mortality was 4/12 in the elevated D-dimer group and 3/17 in IPF patients with D-dimer within normal range (p=0.40). There was no difference between the groups with regard to acute exacerbations, venous thromboembolism or acute cardiac events (table 16). One patient in the elevated D-dimer group was admitted with an acute exacerbation of IPF with no acute exacerbations occurring in the normal D-dimer group (p=0.40). One patient in the normal D-dimer group had a deep vein thrombosis during the study period while no patients with an elevated D-dimer at baseline had a venous thromboembolic event (p=1.00). One patient in the normal D-dimer group had an acute non-ST elevation myocardial infarction during the study period with no acute cardiac events occurring in the elevated D-dimer group (p=1.00).

Outcome	Elevated D-dimer	Normal D-dimer	p-value
Number of patients (n)	12	17	-
All-Cause Mortality	4	3	0.40
Acute Exacerbation of IPF	1	0	0.40
Cardiovascular Event	0	1	1.00
VTE	0	1	1.00

Table 16. Adverse events in IPF patients with elevated D-dimer (>250ng/ml) and normal D-dimer (≤250ng/ml)

5.1.4 Discussion

The plasma D-dimer level was elevated in 41% of IPF patients indicating increased activation of the coagulation and fibrinolytic systems. Interestingly the plasma D-dimer level was also elevated in 20% of control patients and the difference between the two groups did not reach statistical significance. However, an elevated D-dimer represents increased activation of the coagulation system and the resulting presence of increased concentration of fibrinogen degradation products.

In this study we did not demonstrate any association between an elevated D-dimer and adverse clinical outcomes or with disease severity assessed by lung function at baseline or decline over time. During the follow-up period we observed fewer adverse events than expected with only 1 participant experiencing an acute exacerbation, 1 participant having an acute cardiovascular event and 1 participant having a deep vein thrombosis. Likewise, the mortality rate in this study was lower than expected with a total of 8 deaths during the 2-year follow-up period representing 26% two-year mortality. In light of the low incidence of adverse events it was underpowered to detect a difference between the two groups.

The rate of lung function decline observed in our cohort was lower than reported in a number of large randomised trials of potential new IPF therapies (180;181). A previous study of anticoagulation in 56 IPF patients with 3-year follow up reported a 57% mortality rate with 33 admissions with acute exacerbations of IPF (128). The patients in this study were all treated with prednisolone with half being randomised to additionally receive anticoagulation. In contrast, none of the patients reported in our study were taking corticosteroids or anticoagulants. A recent randomised, placebo-controlled trial was stopped early due to increased mortality and hospitalisation in the group receiving immunosuppression (30). This suggests that immunosuppression including corticosteroids may have a deleterious effect on outcome in IPF patients. Additionally, a recent randomised, placebo controlled trial of the anticoagulant warfarin in IPF was stopped early due to increased mortality in the warfarin group (23). Therefore, the increased adverse events noted by Kubo et al. may be in part due to their drug therapy potentially explaining the comparatively small number of adverse events in our untreated cohort of IPF patients.

5.1.5 Conclusion

There was no significant difference in D-dimer concentration between IPF patients and controls in this study. Despite this, the plasma D-dimer was elevated in 41% of IPF patients indicating increased activation of the coagulation and fibrinolytic systems in the systemic circulation. The interplay between thrombotic and fibrinolytic systems appear important in IPF. However, it is clear that the mechanisms underlying this and potential therapeutic targets remain incompletely understood. In light of the recognised association with thromboembolic disease it would seem reasonable to hypothesise that anticoagulation would confer benefit. However, this has recently been shown to be associated with adverse outcomes using the anticoagulant warfarin (23). It is noteworthy that the reported adverse outcomes were respiratory in origin and not related to bleeding. This poses the question whether warfarin is having a deleterious effect beyond the desired effect of preventing thrombosis. Therefore, the role of anticoagulation warrants further consideration in IPF in order to identify potential mechanisms of harm caused by warfarin and consider the role of alternative means of anticoagulation.

5.2 Evaluation of plasma markers of endothelial cell activation and injury

5.2.1 Introduction

The endothelium forms a barrier between the pulmonary circulation and the lung parenchyma. The endothelium has an important role in regulating the adhesion and trafficking of inflammatory cells, preventing unwanted platelet activation and thrombus formation. Maintenance of normal endothelial function is therefore important in maintaining vascular integrity, patency and facilitating appropriate transfer of substances between the vascular space and the interstitium. Endothelial dysfunction can therefore have a number of adverse affects including inappropriate platelet activation, thrombus formation and adhesion and transmigration of inflammatory cells. In addition to causing local tissue injury there is the potential for endothelial dysfunction to lead to systemic effects through altered function of circulating cells. In order to investigate the potential role of endothelial dysfunction in IPF as a potential link with vascular disease and a potential cause for the abnormal platelet responses demonstrated in the previous experiments this study will measure soluble thrombomodulin, soluble vascular endothelial adhesion molecule - 1 (sVCAM-1) and vWF in the plasma of IPF patients and controls.

Soluble Thrombomodulin

Thrombomodulin is a transmembrane glycoprotein expressed on the surface of endothelial cells. It acts as a receptor for thrombin and has important anticoagulant properties through activation of protein C (182;183) and reducing the ability of thrombin to form fibrin clots and activate platelets (183). In addition to the protective effects thrombomodulin exerts against thrombosis, through activation of protein C it also has numerous cytoprotective and anti-inflammatory affects mediated by protease activated receptor-1 (PAR-1) and endothelial protein C receptor (EPCR) (184). One of the cytoprotective affects of activated protein C is the alteration of endothelial cell gene expression of pro-inflammatory pathways including down regulation of NF-κB subunits resulting in decreased NF-κB binding and down regulation of downstream genes including ICAM-1, VCAM and E-selectin (185). Activated protein C also exerts protective affects through regulating endothelial cell apoptosis and preserving endothelial barrier function (184;185). Thrombomodulin also has anti-inflammatory

effects that are not dependent on activated protein C by directly inhibiting neutrophil binding through ICAM-1 dependent and independent pathways (186). This affect is mediated by thrombomodulins NH(2)-terminal domain that has homology to the C-type lectins (186). Mice deficient in this extracellular domain have reduced survival and increased neutrophil accumulation in the lung following lipopolysaccharide (LPS) inhalation (186). Therefore, thrombomodulin expression on endothelial cells has important roles in protecting against thrombosis and inflammation.

In addition to the membrane bound glycoprotein, a soluble form of thrombomodulin is found in the plasma, serum and urine (187). TNF-α is known to be associated with reduced cell surface expression of thrombomodulin with suggestion that this occurs due to alterations in gene expression and internalisation of the surface molecule (187). However, diseases associated with an inflammatory response, for example systemic lupus erythematosus, have been associated with increased serum thrombomodulin (188). This has subsequently been demonstrated to be due to neutrophil dependent damage of TNF-α stimulated endothelial cells and resulting thrombomodulin release (187). Therefore, in certain conditions associated with endothelial activation and injury, endothelial cell surface expression of thrombomodulin is decreased and this is associated with a release of soluble thrombomodulin into the plasma.

Soluble thrombomodulin levels have been found to be elevated in patients with vascular diseases including stroke (189) and in patients with atrial fibrillation who develop thrombotic events including stroke, myocardial infarction and pulmonary embolism (190). Soluble thrombomodulin has been used as an indicator of endothelial injury and dysfunction in conditions including vascular disease (191), renal disease (192) including following renal transplant (193) and connective tissue disease (194-196). Soluble thrombomodulin has been shown to correlate with the number of circulating endothelial cells, another surrogate marker of vascular injury (197).

Soluble Vascular Endothelial Cell Adhesion Molecule (sVCAM)

VCAM-1 is a member of the immunoglobulin gene superfamily that is expressed on endothelial cells where it is responsible for adhesion and transmigration of monocytes and lymphocytes through interactions with its ligand, very late activation antigen-4 (VLA-4) (198). VCAM-1 expression on endothelial cells occurs in association with ICAM-

1 and E-selectin following stimulation with TNF-α, Il-1β and LPS but VCAM-1 also has specific regulatory mechanisms, for example Il-4 up regulates TNF-α induced VCAM-1 expression while down regulating ICAM-1 and E-selectin expression (198;199). VCAM-1 expression is felt to be particularly sensitive to oxidative stress (198;200). TNF-α induced endothelial adhesion molecule expression is associated with NF-κB activation and can be suppressed by treatment with antioxidants and NO (200;201).

Increased VCAM-1 expression has been described in areas of the vascular endothelium prone to the development of atherosclerotic plaques and in areas bordering existing plaques (202). In an animal model of atherogenesis using apolipoprotein E knockout mice with varying degree of VCAM-1 gene knockout there was a dose dependent reduction in VCAM-1 expression associated with reduced monocyte adherence and atherogenesis demonstrating an important role for VCAM-1 in the development of vascular diseases including cardiovascular disease (203). Additionally, a study of glomeruli in end stage renal failure secondary to age related glomerulosclerosis found that old glomeruli have increased VCAM-1 expression in addition to other adhesion molecules that was dependent on NF-κB up-regulation with a resulting phenotype that was pro-inflammatory, profibrotic and procoagulable (204). This described phenotype appears to have a number of parallels with IPF.

A previous study assessed VCAM-1 expression in lung tissue from 9 IPF patients using immunohistochemistry and did not detect any increased expression (205). However, increased VCAM-1 expression on endothelial cells is known to be associated with pulmonary fibrosis secondary to irradiation (206).

sVCAM-1 is used as a marker of endothelial dysfunction and has been investigated in a number of systemic diseases (207). Soluble adhesion molecules including ICAM-1, E-selectin and VCAM-1 have been shown to be elevated in patients with systemic sclerosis, a connective tissue disease often associated with interstitial lung disease (208). A study of plasma concentrations of adhesion molecules in a large cohort of IPF patients found that elevated levels of sVCAM were associated with worse outcomes including reduced overall survival and progression free survival (209).

Von Willebrand Factor (vWF)

vWF is a multimeric glycoprotein that ranges in molecular weight from 500-10,000kDa depending on the number of subunits in the molecule (210). It is present in the plasma and is contained within platelet granules and endothelial cell Weibel-Palade bodies being released on activation. vWF has two roles that are essential in maintaining normal haemostasis: firstly, vWF binds to exposed subendothelial matrix where it interacts with platelet receptors resulting in the activation of GPIIb/IIIa which mediates platelet binding and growth of the platelet thrombus through accumulation of circulating platelets (210); secondly, vWF forms a complex with factor VIII in the circulation and therefore has an important role in secondary haemostasis.

Multimeric vWF is produced in endothelial cells from pro-pro-vWF which is cleaved to produce pro-vWF and subsequently vWF following a complex series of processing events (211). Endothelial cell produced vWF is released into the subendothelial matrix and the plasma in addition to being stored in Weibel-Palade bodies. Megakaryocytes produce vWF in the same way as endothelial cells however all of it is stored in α-granules for release following platelet activation (210). As a result, plasma vWF originates from direct secretion from endothelial cells following production, release from endothelial cell Weibel-Palade bodies following endothelial activation and release form α-granules of platelets following platelet activation.

This study investigates the levels of the recognised markers of endothelial cell activation: soluble thrombomodulin, sVCAM and vWF in patients with IPF and controls.

5.2.2 Methods

Blood was collected from 26 IPF patients diagnosed according to ATS/ERS consensus criteria (9) and 19 control patients with no evidence of fibrotic disease. All patients were clinically stable at the time of sampling being free from any evidence of active inflammation or other acute inter current illness. Patients were recruited from the interstitial lung disease clinic at a large university hospital. Control patients were recruited from other hospital out-patient clinics. Soluble thrombomodulin was measured in 23 IPF patients and 17 controls. sVCAM and vWF were measured in 21 IPF patients and 17 controls.

Blood was collected using a 21-gauge butterfly needle from a large antecubital vein and collected into a vacutainer tube anticoagulated with 102 IU lithium heparin (Becton Dickinson Vacutainer Systems, UK). Plasma was isolated following centrifugation at 1500G for 10 minutes at 20^{o}C prior to freezing at -80^{o}C until ready for use. Plasma levels of soluble thrombomodulin and sVCAM were measured using ELISA (R&D systems, UK).

The difference in mean concentrations of soluble thrombomodulin and sVCAM were tested for statistical significance using an unpaired t-test. A p-value of <0.05 was considered statistically significant.

5.2.3 Results

Patients were well matched for age and gender. COPD was more prevalent in the control group (p=0.02) and more patients in the IPF group were current smokers (p=0.03) (table 17).

The mean (±SEM) plasma thrombomodulin concentration was 5483.1 ± 407.6 pg/ml in IPF patients compared with 5267.9 ± 266.7 pg/ml in controls (figure 31). The difference was not statistically significant (p=0.69).

The mean (±SEM) plasma sVCAM concentration was 885.84 ± 104.75 ng/ml in IPF patients compared with 739.40 ± 57.852 ng/ml in controls (figure 32). The difference between the two groups was not statistically significant (p=0.13).

Measurement of the plasma concentration of vWF using ELISA was unsuccessful with concentrations in the IPF samples and controls being greater than the highest standard in the assay.

	IPF	Controls	P-value
Number (n)	26	19	-
Age in years – Mean (SD)	70.1(7)	69.2(10.3)	0.73
Gender (%)			
- Male	17(65.4)	11(57.9)	0.76
- Female	9(34.6)	8(42.1)	0.76
Comorbidities			
- Active Malignancy	0(0)	2(10.5)	0.17
- TIA/CVA	2(7.7)	1(5.3)	1.00
- Hypertension	7(26.9)	1(5.3)	0.11
- Diabetes mellitus	2(7.7)	1(5.3)	1.00
- GER	3(11.5)	1(5.3)	0.63
- COPD	2(7.7)	7(5.3)	**0.02**
- Sleep apnoea	0(0)	2(5.3)	0.17
- Atrial Fibrillation	1(3.8)	0(5.3)	1.00
Smoking Status			
- Current	6(23.1)	0(0)	**0.03**
- Ex-smoker	9(34.6)	11(57.9)	0.14
- Non-smoker	7(26.9)	4(21.1)	0.74
- Unknown	4(15.4)	4(21.1)	0.70

Table 17. Baseline characteristics of IPF patients and Controls

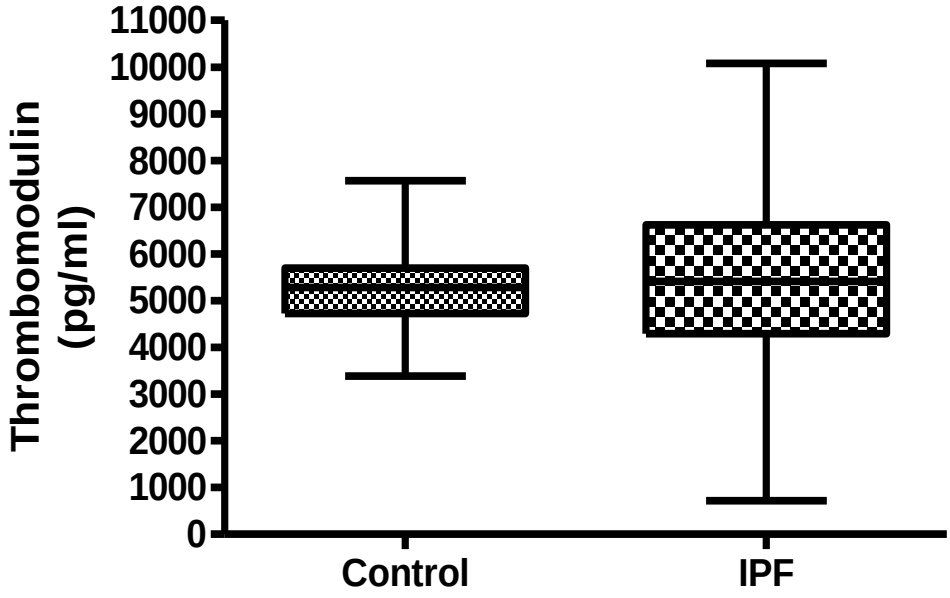

Figure 31. Plasma thrombomodulin concentration (pg/ml) in IPF patients and controls.

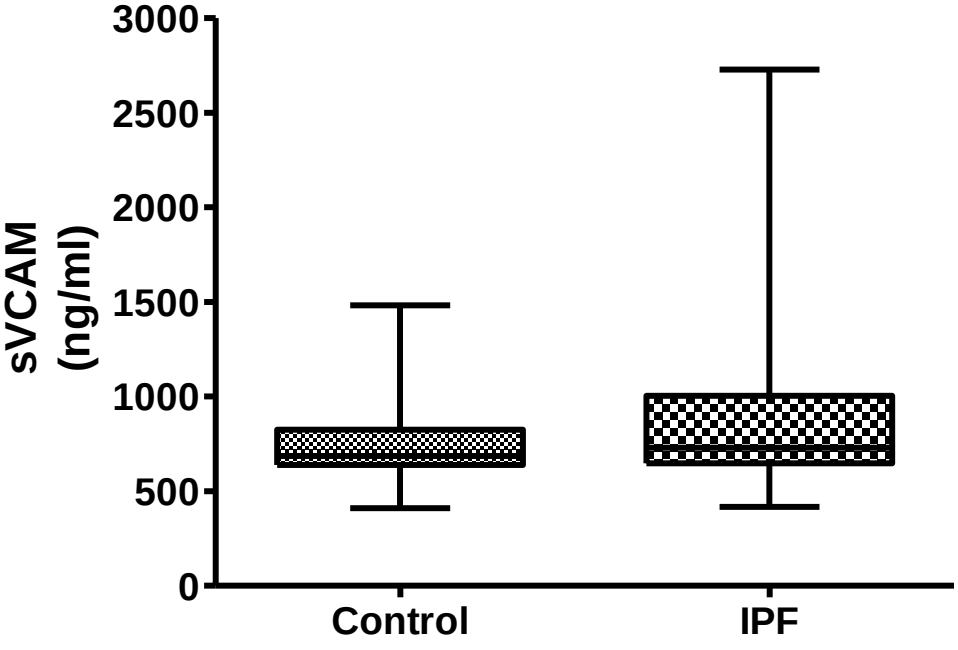

Figure 32. Plasma sVCAM concentration (ng/ml) in IPF patients and controls.

5.2.4 Discussion

This study investigated the plasma concentrations of soluble thrombomodulin, sVCAM and vWF in IPF patients and controls. There was a slightly higher concentration of soluble thrombomodulin and sVCAM-1 in IPF but this was not statistically significant. This suggests that the previously described increased platelet reactivity is not a result of endothelial dysfunction. However, the small sample size in this study makes it difficult to draw firm conclusions regarding the presence or absence of endothelial dysfunction in IPF. Indeed, it is likely that IPF has a prolonged preclinical phase prior to the diagnosis being made. It is conceivable that a small difference in endothelial activation could be present for a prolonged period of time with a cumulative effect resulting in the self-perpetuating fibrotic process. It is also true that there is significant heterogeneity in IPF phenotype between individuals that should be considered when analysing the results of small studies. Therefore, despite this study not demonstrating a significant difference in markers of endothelial activation it is important not to dismiss this possibility completely.

There was a higher proportion of patients with COPD in the control group compared with the IPF group in this study. This reflects the method of recruitment of control subjects from other hospital outpatient clinics. COPD is known to be associated with increased platelet activation (138) and inflammation within the lung so it is essential to consider the possible implications of the increased prevalence of COPD on the measured markers of endothelial activation. There is limited evidence regarding the effects of COPD on VCAM expression with data being extrapolated from studies of smokers and non-smokers (212). However, sVCAM-1 has been demonstrated to be increased during an acute exacerbation of COPD but returned to control levels following treatment (213). The patients in this study were all free from an exacerbation at the time of blood sampling and therefore any potential effect as a result of comorbid COPD will be minimised. It is also important to consider the affect of COPD on soluble thrombomodulin. A study of COPD patients and controls found that there was no difference in soluble thrombomodulin levels at rest however following exercise there was a significant increase in soluble thrombomodulin in COPD patients with a bronchitic phenotype compared to those with predominant emphysema and controls (214). All patients included in this study were rested prior to

blood sampling and therefore the described increase in thrombomodulin on exertion in COPD is not relevant to this patient cohort.

There was a higher proportion of IPF patients that were current smokers at the time of blood sampling compared to controls. Previous studies of the effect of inhaling cigarette smoke acutely and in chronic smokers during cessation demonstrated that cigarette smoke significantly increased plasma levels of vWF that improved during smoking cessation (215;216) but they did not demonstrate any significant difference in soluble thrombomodulin concentrations (216-218). However, the ability of thrombomodulin to bind thrombin does appear to be impaired by cigarette smoke (219). The increased proportion of current smokers in the IPF group may therefore have affected the plasma vWF level however it is not likely to have affected soluble thrombomodulin levels. There is conflicting evidence regarding the effects of cigarette smoking on VCAM-1 expression. Experimental models have demonstrated increased VCAM-1 expression on endothelial cells exposed to cigarette smoke (220;221) and smokers with cardiovascular disease have been shown to have increased plasma levels of sVCAM compared with non-smokers with cardiovascular disease (222). However, a study of pulmonary endothelial VCAM expression in lung biopsy specimens from smokers and non-smokers found that there was no difference between the two groups (223). It is therefore difficult to conclude whether the increased proportion of current smokers in the IPF group will affect the sVCAM-1 concentration measured in this study.

Unfortunately measurement of plasma vWF was unsuccessful due to the measured plasma concentration of all samples being greater than the highest concentration of standard. As a result it was not possible to accurately measure sample concentration of vWF. In order to resolve this it will be necessary to repeat the ELISA with further sample dilution however this was not possible during the timeframe of this study.

A recent study of 241 IPF patients identified sVCAM-1 as a predictor of poor outcome when plasma concentrations measured using Luminex technology exceeded a threshold of 390 to 418 ng/ml (209). It is noteworthy that the mean plasma concentration of sVCAM-1 in both IPF and control groups in this study were almost double the level of this cut off. The reason for the difference in sVCAM-1 in this study is not clear although a different methodology was used to quantify the concentration of sVCAM. Another recent study of sVCAM-1 in a cohort of patients with rheumatoid

arthritis and controls yielded sVCAM-1 concentrations in the control group similar to those observed in this study (224).

In order to definitively assess the role of the endothelium in IPF it will be necessary to perform a study with a large, well matched sample of IPF patients and controls with adequate power to identify an intergroup difference of the magnitude described in this study. It would also be prudent to perform assessment of endothelial dysfunction by measurement of peripheral arterial tone changes following a period of ischemia. This can be measured non-invasively in the peripheral arterial beds using Endo-PAT.

5.2.5 Conclusion

This study did not demonstrate a significant difference in the measured markers of endothelial dysfunction in IPF patients and controls. A study with larger sample size is required to conclusively assess markers of endothelial dysfunction in IPF with greater power to detect small intergroup differences.

6. Concluding Remarks

In this thesis we report the first description of abnormal platelet responses in patients with IPF. We conclusively demonstrate increased platelet reactivity in IPF patients compared with controls that was reproducible across the three measured markers of platelet activation and two activation pathways. This observation is considered even more significant in light of the high proportion of patients with COPD in the control group; a condition also recognised to be associated with increased platelet reactivity. The observation that incubation in IPF plasma can induce the same effect in control platelets suggests that there is a factor in the plasma of IPF patients that is resulting in platelet activation or priming platelets for activation in response to another stimulus. In light of the recognised proinflammatory and profibrotic potential of platelets and the ability of platelets to alter the phenotype of leukocytes and endothelial cells there is little doubt that if inappropriately activated platelets have the potential to drive or perpetuate a fibrotic process. The demonstration that IPF platelets have increased propensity to adhere to endothelial cells confirms a functional effect of the observed increased platelet reactivity but also provides evidence of a potential pathogenic role for platelets in perpetuating fibrosis in IPF.

The endothelium plays an important role in regulating platelet function. We did not demonstrate any evidence of endothelial cell activation in IPF patients compared with controls. However, the limited sample size, inter group differences in terms of comorbid COPD and smoking status, and technical difficulties in assessing vWF make it difficult to draw firm conclusions from this experiment. We also did not demonstrate any difference in plasma D-dimer compared with controls but the D-dimer level was elevated in almost half of IPF patients suggesting that there is increased activation of haemostatic mechanisms. This has been the subject of investigation in large randomised, controlled trials with recent evidence to suggest a harmful effect of warfarin treatment. This requires further exploration.

In addition to confirming an abnormal platelet response in IPF patients, the experiments described in this thesis lead to further questions guiding future research. There are a number of critical factors that require further investigation. It is essential to identify the plasma factor responsible for the increased platelet reactivity observed in IPF patients. This can be achieved by measuring the known endogenous platelet

agonists and inhibitors with a view to serum fractionation and mass spectrometry if initial studies prove inconclusive. Platelet function in IPF patients needs to be further assessed to include platelet aggregation assays and more detailed studies of platelet-endothelial adhesion under flow conditions and in the presence of blocking antibodies. This would provide greater understanding of the functional implication of the observed increased platelet reactivity in IPF and identify the mechanism of increased platelet-endothelial adhesion. A well conducted randomised controlled trial of antiplatelet drugs in IPF would assess the impact of these drugs on clinical outcomes and identify the utility of targeting individual platelet pathways. In addition, further evaluation of endothelial activation markers in a large IPF patient cohort and non-invasive measurement of change in peripheral arterial tone in response to ischaemia would provide more definitive evidence of the role of the endothelium in IPF.

In summary, the series of experiments described in this thesis conclusively demonstrate increased platelet reactivity in IPF patients that can be reproduced in control platelets following incubation in IPF plasma. There is a resulting alteration in platelet function with increased propensity to adhere to endothelial cells. These findings open a new avenue of research in IPF with the potential to explore new treatment strategies.

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Definitions

ADP	Adenosine Diphosphate
ALAT	The Latin American Thoracic Association
ATS	American Thoracic Society
BAL	Bronchoalveolar Lavage
BOOP	Bronchiolitis Obliterans Organising Pneumonia
BTS	British Thoracic Society
cGK-1	Cyclin Guanosine Monoposphate Kinase-1
COP	Cryptogenic Organising Pneumonia
CFA	Cryptogenic Fibrosing Alveolitis
COPD	Chronci Obstructive Pulmonary Disease
CO_2	Carbon Dioxide
CVA	Cerebrovascular Accident
DETA-NO	Diethylenetriamine Nitric Oxide
DIP	Desquamative Interstitial Pneumonia
DVT	Deep Vein Thrombosis
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
ELISA	Enzyme Linked Immunsorbent Assay
EMT	Epithelial Mesenchymal Transformation
eNOS	Endothelial Nitric Oxide Synthase
EPCR	Endothelial Protein C Receptor
ERS	European Respiratory Society
FITC	Fluorescein isothiocyanate
FVC	Forced Vital Capacity
GER	Gastroesophageal Reflux

GIP	Giant Cell Interstitial Pneumonia
GP	Glycoprotein
HRCT	High Resolution Computerised Tomography
HUVEC	Human Umbilical Vein Endothelial Cell(s)
ICAM-1	Intercellular adhesion molecule - 1
IHD	Ischaemic Heart Disease
IIP	Idiopathic Interstitial Pneumonia
Il	Interleukin
INR	International Normalised Ratio
IPF	Idiopathic Pulmonary Fibrosis
ITIM	Immunoreceptor Tyrosine-Based Inhibitory Motif
JRS	Japanese Respiratory Society
LPS	Lipopolysaccharide
LIP	Lymphoid Interstitial Pneumonia
L-NAME	L-NG-Nitroarginine Methyl Ester
MCP-1	Monocyte Chemotactic Protein-1
mRNA	Messenger Ribonucleic Acid
MVECs	Microvascular Endothelial Cells
NF-κB	Nuclear Factor - κB
NSIP	Non Specific Interstitial Pneumonia
PAR-1	Protease Activated Receptor-1
PAI	Plasminogen Activator Inhibitor
PAI-1	Plasminogen Activator Inhibitor type 1
PAI-2	Plasminogen Activator Inhibitor type 2
PBS	Phosphate Buffered Saline
PDGF	Platelet Derived Growth Factor
PE	Pulmonary Embolism

PE-	Phycoerythrin
PECAM-1	Platelet Endothelial Cell Adhesion Molecule -1
PF4	Platelet Factor 4
PRP	Platelet Rich Plasma
PSGL-1	P-Selectin Glycoprotein Ligand - 1
PVD	Peripheral Vascular Disease
RNA	Ribonucleic Acid
SD	Standard Deviation
SDF-1	Stem Cell Derived Factor -1
SEM	Standard Error of the Mean
SNP	Single Nucleotide Polymorphism
sGC	Soluble Guanylyl Cyclase
sPECAM-1	Soluble Platelet Endothelial Cell Adhesion Molecule
sVCAM	Soluble Vascular Cell Adhesion Molecule
TF	Tissue Factor
TFPI	Tissue Factor Pathway Inhibitor
TGF-β	Transforming Growth Factor
TIA	Transient Ischaemic Attack
TLCO	Carbon Monoxide Transfer Factor
TNF-α	Tumour Necrosis Factor - α
tPA	Tissue-Type Plasminogen Activator
UIP	Usual Interstitial Pneumonia
UK	United Kingdom
uPA	urokinase-type Plasminogen Activator
VASP	Vasodilator-Stimulated Phosphoprotein
VCAM	Vascular Cell Adhesion Molecule
sVCAM	Soluble Vascular Cell Adhesion Molecule

VLA-4	Very Late Activation Antigen-4
VTE	Venous Thromboembolism
vWF	von Willebrand Factor