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Exploring the role and association of monocytes with progression of Idiopathic Pulmonary Fibrosis

by

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Thesis for the degree of DM

[Infection, inflammation, and immunity]

University of Southampton

and

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Abstract

Faculty of Medicine

Doctor of Medicine

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Introduction: Idiopathic pulmonary fibrosis (IPF) is a chronic fibroproliferative disease characterised by accumulation of scar tissue within the lung parenchyma. The clinical course of IPF is heterogenous and disease severity at presentation often varies among patients. Mortality and FVC decline may not accurately reflect progression of fibrosis. As such, there is an unmet need for biomarkers to prognosticate IPF. The pathogenic mechanisms underlying the disease remain incompletely understood but involve aberrations in repair pathways. The role of monocytes in IPF has gained interest due to their role in orchestrating repair. I hypothesised that monocytes, measured from standard full blood count analysis, are associated with adverse clinical outcomes in IPF.

Methods: I performed three retrospective clinical studies. In Study 1, I explored association between blood leukocytes (monocytes, neutrophils, and lymphocytes) with (i) progression of the Indeterminate of Usual interstitial pneumonia (iUIP) CT pattern to IPF and (ii) progression of established IPF. In study 2, I explored blood leukocyte association with progression of early fibrotic-interstitial lung abnormalities (EF-ILA) and in Study 3, I explored leukocyte association with radiological progression of fibrosis per se quantified by automated CT (CALIPER). Patients were enrolled from the Oxford Interstitial lung disease (ILD) service or Oxford Radiology service. Analyses were conducted using multivariate Cox proportional hazard regression.

Results: In Study 1 monocyte and neutrophil counts were associated with progression of iUIP to IPF. In cases with established IPF neutrophils, lymphopaenia and neutrophil:lymphocyte ratio were associated with FVC decline; while both monocytes and neutrophil levels (and derived indexes) were associated with all-cause mortality. In Study 2 EF-ILA was significantly associated with all-cause mortality. Monocytes and neutrophils (and derived indexes) were significantly associated with progression of EF-ILA and mortality. In Study 3 neutrophil count demonstrated significant association with progression of total lung fibrosis, as did all leukocyte indexes, but not absolute monocyte and lymphocyte counts.

Conclusion: The association between monocytes, neutrophils and lymphocytes with mortality and disease progression across these cohorts suggests a role for these cells in the pathogenesis of IPF and sub-clinical ILD.

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Research Thesis: Declaration of Authorship

Print name: Andrew Achaiah

Title of thesis: Exploring the role and association of monocytes with progression of idiopathic pulmonary fibrosis

I declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

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Definitions and Abbreviations

AEC I ..... Type I alveolar epithelial cells

AEC II ..... Type II alveolar epithelial cells

AEIPF ..... Acute Exacerbation of Idiopathic Pulmonary Fibrosis

AGES ..... Age/Gene Environment Susceptibility

AM ..... Alveolar macrophage

BAL ..... Broncho-alveolar lavage

BM ..... Bone marrow

CALIPER ..... Computer-Aided Lung Informatics Pathology Evaluation and Ratings

COPDGene ..... Genetic Epidemiology of COPD

CoV ..... Coefficient of variation

DAMP ..... Damage-associated molecular pattern

ECLIPSE ..... Evaluation of COPD to Identify Predictive Surrogate End-points

ECM ..... Extra-cellular Matrix

EF-ILA ..... Early fibrotic-ILA

ERK ..... Extracellular signal-regulated kinase

FEV1 ..... Forced expiratory volume in 1 second

FGF ..... Fibroblast growth factor

FIP ..... Familial Interstitial Pneumonia

FVC ..... Forced vital capacity

GWAS ..... Genome-wide associated studies

ICD ..... International classification of disease

IQR ..... Inter-quartile range

IRF ..... IFN regulatory factor

IFNγ ..... Interferon-γ

ILA ..... Interstitial Lung Abnormality

ILD ..... Interstitial Lung disease

Definitions and Abbreviations

IFN..... Interferon

IFN-I ..... Type 1 Interferon

IQR ..... Inter-quartile range

ISG..... Interferon stimulated gene

IL- ..... Interleukin-

IPF ..... Idiopathic Pulmonary Fibrosis

iUIP..... Indeterminate for UIP

JNK ..... c-Jun N-terminal kinase

LAA..... Low attenuation area

LPS..... Lipopolysaccharide

MESA ..... Multi-Ethnic Study of Atherosclerosis

MFI..... Mean florescent intensity

MHC ..... Major histocompatibility complex

MLR..... Monocyte:Lymphocyte ratio

MMI ..... Mean metal intensity

MRI..... Magnetic resonance imaging

NE..... Neutrophil elastase

NF-kB ..... Nuclear factor kappa-light-chain-enhancer of activated B cells

NLR..... Neutrophil:Lymphocyte ratio

p38..... p38 MAP kinase

PAMP ..... Pathogen-associated molecular pattern

PDGF ..... Platelet-derived growth factor

PFT ..... Pulmonary function tests

PI3-K..... Phosphoinositide 3-kinase

pSTAT ..... Phosphorylated - signal transducer and activator of transcription

PVV..... Pulmonary vessel volume

qCT..... Quantitative CT

RA..... Rheumatoid arthritis

ROC.....Receiver operating characteristic

ROS.....Reactive oxygen species

α-SMA .....Smooth muscle actin

S.D. .....Standard deviation

SIRI .....Systemic inflammation response index

SLE .....Systemic lupus erythematosus

STAT .....Signal transducer and activator of transcription

TBx.....Traction bronchiectasis

TLco .....Transfer factor for carbon monoxide

TLF .....Total lung fibrosis score

TGF-β.....Transforming growth factor β

TNF-α.....Tumour necrosis factor α

UIP.....Usual Interstitial Pneumonia

VEGF.....Vascular endothelial growth factor

Xe .....Xenon

Chapter 1 Introduction

1.1 Idiopathic pulmonary fibrosis

1.1.1 Background

Idiopathic Pulmonary fibrosis (IPF) is a chronic progressive fibrotic lung disease that exclusively affects the lungs. It is characterised by the irreversible deposition of fibrous tissue which gradually replaces normal functioning lung parenchyma. As disease progresses the accumulation of non-functioning scar tissue causes impaired gas exchange, which manifests clinically as respiratory failure and ultimately causes death.[1]

IPF is more common in men and median age of diagnosis is 65 years. It is considered rare in patients below the age of 50. Although once classified as a rare disease it is now considered the most common of the Interstitial pneumonias and accounts for 20-50% of all cases of interstitial lung disease (ILD).[2] Both prevalence and incidence are rising and data from the latest UK ILD registry suggests there to be an estimated 5000 IPF diagnoses per year and a UK prevalence of 30,000 cases. Sadly, disease prevalence is limited by the high mortality rates observed among cases. Although disease course is variable and somewhat unpredictable median survival from time of diagnosis for IPF in untreated disease is 3-5 years.[3]

By definition the aetiology of IPF is unknown. It was first recognised as its own clinical entity at the turn of the 21st century. Historically it was considered a chronic inflammatory condition which over time proceeded to fibrosis. However, at the turn of the century, and following recognition that anti-inflammatory therapy did not improve outcome the concept was re-assessed. In the late 1990s Katzenstein and Myers proposed that the term IPF be reserved for patients with histology consistent with a usual interstitial pneumonia pattern without identifiable cause of their ILD.[4] This concept was later formalised into the diagnostic criteria of the first international consensus statement for IPF in 2001, and has remained a crucial diagnostic criterion of subsequent iterations of the international IPF guidelines.[5-8]

Despite its idiopathic title, IPF is now regarded as a condition resulting from interaction of multiple environmental and genetic risk factors and the physiology of ageing. The pathological process is likely to arise due to environmental stimuli triggering a local and repetitive micro-injury. These micro-injuries initiate aberrant epithelial–fibroblast communication and significant extracellular matrix (ECM) deposition on the lung interstitium, culminating in remodelling of the lung parenchyma with fibrous and non-functioning “scar” tissue.[1,9] Epidemiological data has

consistently demonstrated that men are more commonly affected. The causal mechanism for this remains unclear. Particulate inhalation is implicated in pathogenesis of disease. A history of tobacco smoking (especially >20 pack-year history), coupled with other environmental and occupational exposures including wood and metal dusts, masonry and farming have traditionally been observed more commonly in men.[2] Possible disease triggers such as chronic viral infection and gastro-oesophageal reflux have been investigated in several studies. Although these studies show association with subsets of IPF patients, direct causality is unproven.[2]

There has also been a greater focus on genetic susceptibility to IPF in recent years. The genetic factors influencing IPF susceptibility depend largely on whether a patient has the sporadic form of IPF or familial interstitial pneumonia (FIP).[10] Rare variants of genes encoding telomerase and surfactant in FIP and polymorphisms of the *MUC5B* gene have provided insight to the underlying pathological mechanisms underpinning IPF and led to the discovery that such mutations are significantly higher in the IPF population.[11]

Historically treatment options for IPF were limited, and as such IPF was once thought of as a true "death sentence" owing to its poor prognosis. However, over the last two decades several important discoveries in IPF have has led to a much better understanding of this disease. Clinically this has been reflected in better holistic care and management of symptoms. Scientifically this has led to breakthroughs which have translated to better treatment options and improved patient outcomes.[12] Currently only two anti-fibrotic drugs are commercially available; Nintedanib and Pirfenidone. Both are proven to reduce the rate of progression of IPF while improving progression-free survival.[13,14] Evidence for the use of these therapies is derived from studies which included participants with mild to moderate disease (forced vital capacity 50-80% of predicted). In patients with advanced disease there remains an absence of therapeutic options.[15] Thus, the introduction of Nintedanib and Pirfenidone has placed greater importance for accurate and more timely diagnosis of IPF.

1.1.2 Clinical features and disease course

Clinically, IPF is characterised by progressive breathlessness insidious in nature. Symptoms are non-specific and patients often describe a progressive symptom burden of many months to years before diagnosis.[16] Cough and breathlessness on exertion are the most common presenting complaints.[5] Over time symptoms will inevitably progress. Exertional and resting hypoxemia can develop and may result in secondary pulmonary hypertension.[17]

IPF has a variable clinical course. Some patients progress slowly but inexorably; others display rapid decline in lung function which is mirrored by rapidly progressive symptoms leading to death;

and others suffer a stepwise loss of lung function in between periods of disease stability (Figure 1.1Figure 1.1 Graphical description of clinical courses observed in patients with IPF.

).[18] Some can also fall victim to an acute exacerbation of IPF, which unfortunately caries particularly poor prognosis (described later).[19]

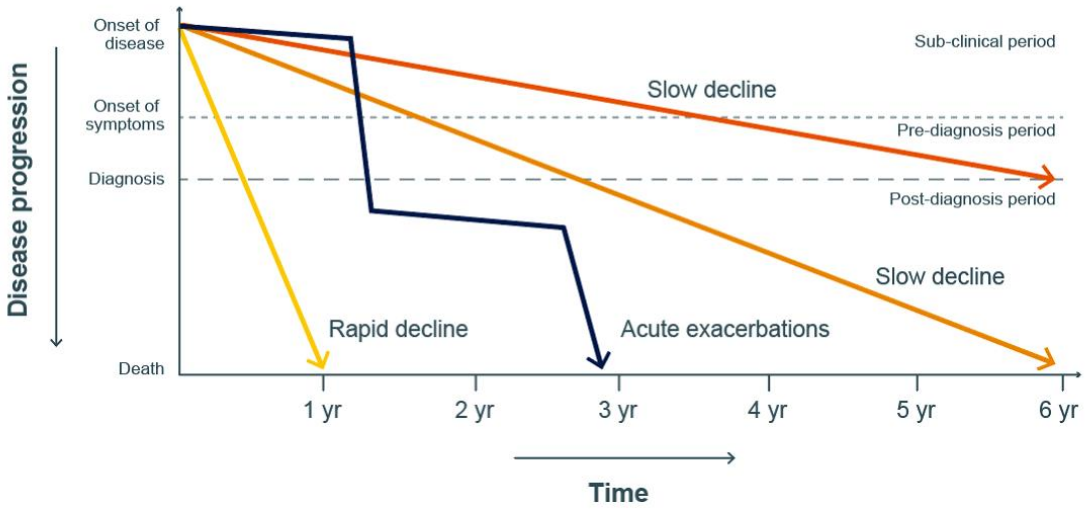

Figure 1.1 Graphical description of clinical courses observed in patients with IPF.
Patient lung function irreversible declines regardless of path of IPF progression. With permission taken from Kim et al (2015).[17]

It is extremely difficult to predict disease course at initial assessment. Careful follow up for monitoring of symptom progression and changes in lung function parameters are essential both to ascertain the nature of the disease and clinical trajectory of each patient, but also to ensure timely intervention with disease modifying therapies if necessary.[8,15]

Physical examination often reveals bilateral 'Velcro-type' crackles on auscultation, in a predominantly mid and lower zone distribution.[1] Clubbing is present in 50% of cases. Additional clinical features of pulmonary hypertension and cor pulmonale may also be present, such as elevated jugular venous pressure, a loud second heart sound and peripheral oedema.[20] The absence of clinical features of connective tissue disease are important negative findings that support the diagnosis of IPF.[20]

Pulmonary function tests (PFTs) are the mainstay for monitoring disease progression.[8] Spirometry typically demonstrates a restrictive defect; a reduced forced vital capacity (FVC) relative to predictive value with an FEV1/FVC ratio >70%. Gas transfer for carbon monoxide (TLCO), a composite measure of pulmonary oxygen absorption is also reduced relative to predicted values.[20] Co-existent pulmonary hypertension and emphysema can lower TLCO disproportionately

to FVC. Additionally, co-existent emphysema, which is observed in a reasonable proportion of patients with IPF, can cause hyper-inflation of the functioning lung parenchyma and can artificially inflate the FVC.[18]

1.1.3 Diagnostic features

IPF is defined either radiographically or histologically by the presence of usual interstitial pneumonia (UIP) pattern. UIP is not exclusive to IPF and can be seen as a clinical manifestation of connective tissue diseases (especially rheumatoid disease), sarcoidosis, chronic fibrosing hypersensitivity pneumonitis, asbestosis and drug toxicities.[21] A thorough and detailed clinical history, examination and downstream investigations are essential to exclude these differentials in order to conclude that disease is truly idiopathic before a diagnosis of IPF can be established.[7]

The typical radiological appearance of UIP is summarised by the presence of honeycombing with or without traction bronchiectasis in a subpleural and basal distribution, and an absence of features considered indicative of an alternative diagnosis.[8,22] If all of these features of UIP are present a diagnosis of IPF can be made in a patient without known cause for UIP. In the majority of cases however, these typical CT features are not present. This is accounted for in the 2018 iteration of the ATS/ERS/JRS/ALAT Clinical Practice Guideline which describes 4 separate CT classifications; definite UIP, probable UIP, indeterminate for UIP and alternative diagnosis (Table 1.1).[8]

Radiological diagnostic criteria for UIP diagnosis			
Definite UIP	**Probable UIP**	**Indeterminate for UIP**	**Alternative diagnosis**
Subpleural / basal distribution	Subpleural / basal distribution	Subpleural / basal distribution	Findings suggestive of non-UIP diagnosis
Honeycombing with / without traction bronchiectasis	Reticular abnormality ± Mild GGO	Subtle reticulation ± Mild GGO	CT features: cysts, mosaicism, predominant GGO, centrilobular nodules, consolidation
Absence of features listed as alternative diagnosis	traction bronchiectasis or bronchiolectasis	CT features and/or distribution not suggestive of specific aetiology	Predominant distribution: Peri-bronchovascular peri-lymphatic Mid / upper zone
	Absence of features listed as alternative diagnosis		Other: pleural plaques, dilated oesophagus, lymph nodes, pleural effusion

Table 1.1 Radiological diagnostic criteria for UIP.
Adapted from ATS/ERS/JRS/ALAT clinical practice statement for IPF 2018.[8]

In cases of diagnostic uncertainty, a surgical lung biopsy is recommended in patients who can tolerate the procedure and in whom establishing diagnosis will alter management. The histological features of UIP are summarised by a patchy dense fibrosis with architectural distortion or honeycombing interspersed with areas of normal lung tissue. a defining feature of

UIP is the presence of fibroblast foci. Briefly, these are fibrotic regions composed mainly of dense collagen aggregates and ECM secreted from fibroblasts and myofibroblasts. As architectural distortion progresses cystic airspaces form and is characterised as honeycombing. Similar to the radiological classification, histological findings are also grouped into discrete categories to guide probability of UIP on histology; definite UIP, probable UIP, indeterminate for UIP and alternative diagnosis (Table 1.2).

Histological diagnostic criteria for UIP diagnosis			
Definite UIP	**Probable UIP**	**Indeterminate for UIP**	**Alternative diagnosis**
Dense fibrosis, architectural distortion (i.e., scarring and/or honeycombing)	Some histologic features present but to an extent that precludes a definite diagnosis of UIP/IPF	Fibrosis ± architectural distortion	Features of other histologic patterns of idiopathic interstitial pneumonia
subpleural and/or paraseptal distribution of fibrosis	Absence of features to suggest an alternative diagnosis	Features of either: 1.Pattern other than UIP 2. UIP secondary to another cause	Histologic findings indicative of other diseases
Patchy areas of fibrosis	Honeycombing only	Some features of definite UIP, but with other features suggesting alternative diagnosis	
Fibroblast foci			
Absence of features to suggest alternative diagnosis			

Table 1.2 Histological diagnostic criteria for UIP diagnosis.
Adapted from ATS/ERS/JRS/ALAT clinical practice statement for IPF 2018.[8]

The specific combination of radiological and histological UIP categorisation is then used to determine the clinical diagnosis; IPF, likely IPF, non-IPF diagnosis (Table 1.3).[8]

IPF suspected		Histology pattern			
		UIP	**Probable UIP**	**Indeterminate for UIP**	**Alternative diagnosis**
HRCT pattern	**UIP**	**IPF**	**IPF**	**IPF**	**Non-IPF diagnosis**
	Probable UIP	**IPF**	**IPF**	**Likely IPF**	**Non-IPF diagnosis**
	Indeterminate for UIP	**IPF**	**Likely IPF**	**Indeterminate for IPF**	**Non-IPF diagnosis**
	Alternative diagnosis	**Likely IPF**	**Non-IPF diagnosis**	**Non-IPF diagnosis**	**Non-IPF diagnosis**

Table 1.3 IPF clinic diagnosis based on HRCT and histological patterns.
Adapted from ATS/ERS/JRS/ALAT clinical practice statement for IPF 2018.[8]

The ATS/ERS/JRS/ALAT clinical practice guideline underwent further update in the 2022 iteration.[23] Inclusion criteria for participants included in the research studies discussed in this thesis were based on the 2018 guideline, and prior to the publication of the latest iteration. In the 2022 iteration, nomenclature of the four HRCT categories is unchanged. However the indeterminate for UIP HRCT pattern is described differently and according to distribution of parenchymal abnormalities (i.e. diffuse distribution without subpleural predominance) in the absence of HRCT features of lung fibrosis suggestive of a specific cause.[23] In the 2018 guideline the indeterminate for UIP HRCT pattern was described according to the presence and absence of specific radiological patterns that did not satisfy definite or probable UIP, or alternative diagnosis. It was indirectly considered an early UIP pattern.[8]

I therefore referenced the 2018 guideline for adjudication of the relevant study cohorts in this thesis.

1.1.4 Management and pharmacological therapies in IPF

1.1.4.1 Holistic management

Establishing a diagnosis of IPF can challenging and requires specialist expertise to exclude other conditions that have a similar clinical and radiological presentation, such as chronic hypersensitivity pneumonitis or ILDs related to autoimmune disease.[19] International guidelines have consistently advocated involvement of an ILD MDT in diagnosis and management.[7,8] Given the insidious nature of symptom patients there is naturally lag between symptom onset and presentation. Referral to a tertiary centre should made in a timely manner as delayed access to a specialist centre is associated with higher risk of death (Figure 1.2).[17]

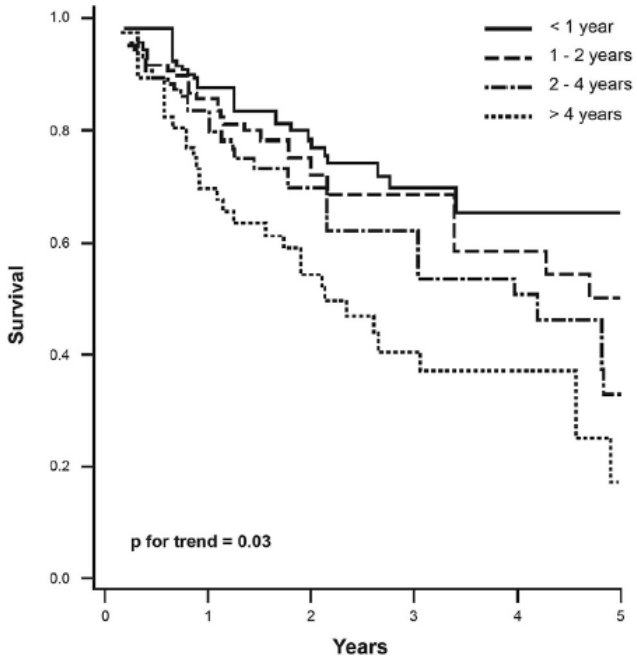

Figure 1.2 Survival from time of evaluation at specialist centre
Kaplan-Meyer curve adjusted for age and FVC across quartiles of delayed access to care. With permission taken from Kim et al (2015).[17]

Despite recent advances in pharmacological management of IPF, drug treatment is only one face of a patient's care in clinical practice. Indeed, like other chronic diseases, the holistic needs of a patient must be addressed through an integrated pathway involving the ILD specialist team. This consists of a consultant physician with ILD expertise, respiratory physiotherapist, nurse specialist and pharmacist. Over time, the health-related requirements of the patient typically increase, and supportive and symptomatic management becomes the priority. Close liaison with the general practitioner through all stages of the disease is essential, and involvement of palliative care teams are helpful, especially during the later stages of illness.[20]

When a patient is first diagnosed, the management strategy adopted depends on the stage of the disease, rate of progression, the health status, and patient preference. Patients presenting with early disease may have demonstrate preserved lung function and under current NICE guidance in the UK, such patients would not qualify for pharmacological intervention.[15] A careful monitoring strategy is advocated for these patients and regular lung function testing to detects advancing disease. A drop in FVC >10% or TLCO >15% is representative of significant disease progression and is associated with greater risk of mortality.[18,24,25] Furthermore, recent data suggest that even small annualised decreases in FVC of 5-10% (within the range of 'measurement noise') are clinically significant and in one clinical trial this correlated with radiographic progression.[26]

1.1.4.2 Important clinical trials in IPF

Pharmacological management of patients with IPF has changed dramatically over the last 2 decades. Numerous Phase 2 or Phase 3 randomised, double-blind controlled trials of potential therapies for IPF have been conducted. The majority of these were declared negative studies, failing to demonstrate the effectiveness of the compound under investigation. However, These have not only enhanced our understanding but importantly have generated a wealth of data to inform the design of future trials, such as enrichment strategies and efficacy end-points.[27] This is especially important in slowly progressive conditions such as IPF, which can which take a long time to manifest or in which relatively few patients experience the event during study follow up. Aligned with this, our understanding of the pathogenesis of IPF has also evolved from that of a predominantly inflammatory pathology to one driven by complex interplay of repeated epithelial cell damage and aberrant wound healing, culminating in fibroblast recruitment, proliferation and differentiation, and excess extracellular matrix formation.[28]

1.1.4.2.1 Anti-inflammatory agents

In 1991 the first randomised double-blind trial for IPF was of prednisone plus placebo versus prednisone plus azathioprine. This trial suggested a potential therapeutic benefit in the prednisone plus azathioprine arm with regards to lung function and survival.[29] This finding supported the previous belief that IPF was a predominantly inflammatory disease. Following on from this, in a separate study, addition of N-acetylcysteine (NAC), a precursor of the antioxidant glutathione, to prednisone and azathioprine was found to further improve pulmonary function tests in patients diagnosed by the historical term fibrosing alveolitis.[30] in the late 1990s and early 2000s triple therapy with prednisone, azathioprine and NAC became widely used as a treatment for IPF based on its potential to counteract the oxidative stress thought to contribute to progression of the disease.

To establish the efficacy and safety of triple therapy the randomised placebo-controlled PANTHER-IPF trial was conducted.[31] The primary endpoint was change in FVC from baseline at week 60. The triple-therapy arm was stopped after 32 weeks after interim analysis showed significantly higher rates of death and hospitalisation in patients treated with triple therapy (prednisone, azathioprine, and NAC) compared to placebo. Whilst the NAC monotherapy and placebo arms completed trial follow up, there was no difference between these groups in terms of change in FVC or mortality.[32] These findings resulted in a recommendation against the use of this triple-therapy and a NAC monotherapy in IPF.

1.1.4.2.2 Interferon gamma and anticoagulants

The INSPIRE trial was a large randomised placebo-controlled trial which assessed the effect on survival of interferon gamma-1b (IFN-1b) versus a placebo in 826 patients with IPF. This followed a preliminary (open label) study which compared IFN-1b plus prednisolone and prednisolone-only therapy. In this study of just 18 patients, the IFN-1b plus prednisolone demonstrated substantial improvement in total lung capacity. The authors concluded that IFN-1b may exert anti-fibrotic properties.[33] However in INSPIRE, at interim analysis there was no difference between treatments and it was therefore terminated early.[34]

Pre-clinical evidence supporting a role for the coagulation cascade in pulmonary fibrosis led to the hypothesis that treating IPF patients with anticoagulation therapy might be of benefit.[35] The ACE-IPF trial was a randomised placebo-controlled study which evaluated the efficacy and safety of warfarin in 145 patients with IPF over 48 weeks. Interim analysis demonstrated higher mortality and a low likelihood of benefit with warfarin versus placebo and the trial was stopped early.[36] This led to recommendation against the use of anticoagulants for directed treatment against IPF.

These findings emphasised a need for the need for large placebo-controlled trials with meaningful endpoints.[27]

1.1.4.2.3 Endothelin receptor antagonists

Endothelin-1 is a mediator of epithelial–mesenchymal transition, a fundamental process in the pathogenesis of IPF whereby epithelial cells differentiate into fibroblast-like cells.[37] It is a potent vasoconstrictor, secreted mainly by endothelial cells and implicated in vascular smooth muscle re-modelling in pulmonary arterial hypertension.[38] Bosentan, a dual endothelin receptor antagonist, investigated as a treatment for IPF in two randomised placebo-controlled trials; BUILD-1 and BUILD-3.

In BUILD-1 there was no difference between Bosentan and placebo with regards to the primary endpoint of change 6-minute walk distance. However, there was a trend favouring Bosentan on time to disease progression or death.[39] Following on from this BUILD-3, a double-blind, placebo-controlled trial was conducted in patients with an IPF diagnosis of less than 3 years duration and evaluated the effect of Bosentan in a subpopulation of patients considered more likely to benefit based on the results of BUILD-1. The primary endpoint was time to FVC decline ≥10% and TLCO ≥15%, acute exacerbation, or mortality.[40] BUILD-3 demonstrated no difference between Bosentan and placebo with respect to the primary endpoint. Similarly, in the randomised placebo-

controlled MUSIC trial, the dual endothelin receptor antagonist Macitentan, showed no benefit with respect to the primary endpoint of FVC decline over 52 weeks.[41]

The ARTEMIS-IPF trial was a further randomised placebo-controlled trial conducted to investigate Ambrisentan, a selective endothelin receptor antagonist, in patients with IPF and minimal honeycombing.[42] Post hoc analysis of BUILD-1 identified improved survival in the subgroup of patients that had undergone surgical lung biopsy to establish IPF diagnosis. The hypothesised rationale for this association was that biopsy was more likely to be done in patients with little or no honeycombing on HRCT, which was assumed to be indicative of less advanced fibrosis. The trial was terminated early after interim analysis showed that there was a low likelihood of efficacy with respect to the primary endpoint. Consensus following these negative studies is that although endothelin receptor antagonists are effective in pulmonary arterial hypertension, they exert no positive benefit in IPF.

1.1.4.2.4 Phosphodiesterase type-5 inhibitors

Sildenafil is a phosphodiesterase-5 inhibitor that causes pulmonary vasodilation via enhanced nitric oxide and cyclic guanosine monophosphate signalling, and improvement in gas exchange in patients with IPF.[43]

STEP-IPF was a randomised, placebo-controlled trial that investigated if Sildenafil improved exercise tolerance, dyspnoea and quality of life in 180 patients with IPF and advanced lung function impairment categorised as TLCO <35%.[44] Although there was no significant difference between Sildenafil and placebo with respect to the primary endpoint of improvement in 6-MWD of ≥20% at week 12, significant benefits from Sildenafil on the secondary endpoints (arterial oxygenation, DLCO, dyspnoea) were observed. Sildenafil failed to meet its primary endpoint, but positive secondary endpoints may indicate patient benefit.

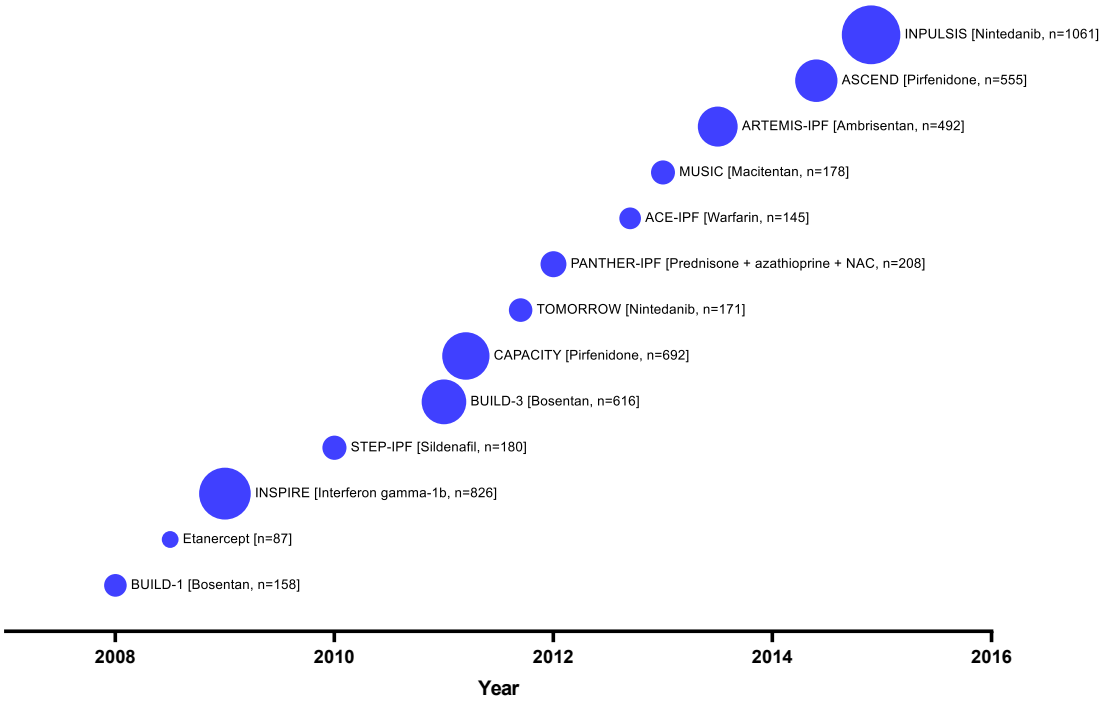

Figure 1.3 Historical placebo-controlled randomised clinical trials in IPF
The size of each dot corresponds to the sample size of the clinical trial.

Phase-II and Phase-III placebo-controlled trials of investigational treatments IPF			
Study	**Treatment**	**Primary endpoint(s)**	**Key findings**
IFIGENIA[45] (n=155) Phase-III, randomised trial	Prednisone + azathioprine + NAC	FVC and DLCO change from baseline at month 12	Significant benefits from triple therapy for both endpoints. Absolute between-group differences for mean change from baseline in VC and DLCO at month 12 were 0.18 L (95% CI: 0.03–0.32; p=0.02) and 0.75 mmol·min−1·kPa−1 (95% CI: 0.27–1.23; p=0.003).
PANTHER-IPF[46] (n=208) Phase-III, randomised trial	Prednisone + azathioprine + NAC	FVC change from baseline at week 60	NAC + prednisone + azathioprine arm terminated due to increased mortality and hospitalisation.
PANTHER-IPF[32] (n=264) Phase-III, randomised trial	NAC	FVC change from baseline at week 60	No significant difference was observed between NAC and a placebo for the primary endpoint (mean changes in FVC of −0.18 L and −0.19 L, respectively; p=0.77).
INSPIRE[34] (n=826) Phase-III, randomised trial	Interferon γ-1b	Survival	Terminated at interim analysis. Showed no significant difference between interferon γ-1b and placebo for primary endpoint (HR 1.15; 95% CI: 0.77–1.71; p=0.50).
ACE-IPF[36] (n=145) Phase-III, randomised trial	Warfarin	Composite: time to death, hospitalisation, or absolute decline in FVC ≥10%	Trial terminated after interim analysis showed higher mortality in warfarin arm: 14 deaths versus 3 in placebo; p=0.005.
BUILD-1[39] (n=158) Phase-III, randomised trial	Bosentan	Change in 6-MWD from baseline at month 12	No difference in 6-MWD between Bosentan and placebo (−52 m vs −34 m, respectively; p=0.23).
BUILD-3[40] (n=616) Phase-III, randomised trial	Bosentan	Time to worsening of IPF: FVC decline ≥10 & DLCO decline ≥15% or acute exacerbation or death	No significant difference observed between Bosentan and placebo for primary endpoint (HR 0.85; 95% CI: 0.66–1.10; p=0.21).
MUSIC[41] (n=178) Phase-II, randomised trial	Macitentan	FVC change from baseline at 12 months	No significant difference observed between Macitentan and placebo (median change of −0.20 L in both groups).
ARTEMIS-IPF[42] (n=492) Phase-III, randomised trial	Ambrisentan	Time to worsening of IPF: death, respiratory hospitalisation, or decline in lung function (FVC decline ≥10 with DLCO ≥5%, or FVC ≥5% with DLCO ≥15%	Trial terminated after interim analysis demonstrated low likelihood of efficacy towards the primary endpoint.
STEP-IPF[44] (n=180) Phase-III, randomised trial	Sildenafil	≥20% increase in 6-MWD at 3 months	No significant difference observed between Sildenafil and placebo for primary endpoint (10% and 7% of patients, respectively; p=0.39).
CAPACITY[47] (n=692) Phase-III, randomised trial	Pirfenidone	Change in FVC from baseline at week 72	Significant benefits with Pirfenidone (2403 mg/day) vs placebo for primary endpoint in CAPACITY-2 (−8.0% vs −12.4%; p=0.001) but not in CAPACITY-1 (−9.0% vs −9.6%; p=0.50).
ASCEND[14] (n=555) Phase-III, randomised trial	Pirfenidone	Change in FVC from baseline at week 52	Significant benefits observed for Pirfenidone versus placebo for the primary endpoint (p<0.001).
TOMORROW[48] (n=171) Phase-II, randomised trial	Nintedanib	Annualised FVC decline	Reduced FVC decline with Nintedanib (150 mg twice daily) versus a placebo (−0.06 L versus −0.19 L; p=0.06 with closed testing procedure.
INPULSIS[13] (n=1061) Phase-III, randomised trial	Nintedanib	Annualised FVC decline	Significant benefits observed for Nintedanib for primary endpoint in INPULSIS-1 (−114.7 mL versus −239.9 mL; p<0.001) and INPULSIS-2 (−113.6 mL versus −207.3 mL; p<0.001).

Table 1.4 Phase-II and Phase-III trials of investigational treatments IPF

List of studies identified by literature review of phase-II and phase-III trials investigational treatments in IPF between 2000 and 2016

1.1.4.3 Antifibrotic therapy

The licencing of 2 novel anti-fibrotic agents, Pirfenidone and Nintedanib which are both proven to slow progression of disease, has been warmly welcomed. Prior to the availability of these agents the 2011 ATS/ERS/JRS/ALAT guidelines recommended best supportive care in conjunction with clinical trial recruitment where possible.[7]

Large multi-centre international trials were conducted to assess the efficacy of these two agents.[13,14] Pirfenidone was the first anti-fibrotic agent to be made available in the UK for patients with mild-moderate IPF. Its precise mechanism is unknown but in early murine models of fibrosis administration of pirfenidone led to a reduction in the production of key profibrotic cytokines including transforming growth factor-β, interleukin-1β and fibroblast growth factor. Reduced lung collagen content and fibrosis scores were demonstrated and fibroblast proliferation attenuated, indicating that pirfenidone acts by inhibiting important fibrogenic pathways.[49,50] Pirfenidone also reduces T-helper cell proliferation and impairs Th-2 cell polarisation via downregulation of the master transcription factor GATA-3.[51]

The history of clinical trials of Pirfenidone in IPF is intriguing. Following successful pre-clinical and phase 2 clinical trials, in 2011 2 concurrent phase 3 clinical trials (CAPACITY 004 and 006) were completed.[47] Unexpectedly, the results of these studies were fairly discordant. CAPACITY 004 demonstrated a significant reduction in FVC decline and improvement in progression-free survival at 72 weeks, compared to placebo. Neither of these endpoints were met in CAPACITY 006, which was concluded as a negative study. Scrutiny of the disparity between study findings revealed (i) a greater degree of airflow limitation in the 006 study cohort and (ii) that difference in FVC decline between the placebo groups was evident. Subsequently, a third phase 3 study (ASCEND) was enriched for patients at greater risk of disease progression. At study completion the primary outcome was positive with a significant reduction in FVC decline and mortality in the intervention group.[14] Pooled analysis of these 3 studies (>1200 patients), Pirfenidone reduced the risk of 1-year all-cause mortality by 48%.[52]

Nintedanib is an intracellular tyrosine kinase inhibitor that binds competitively to receptors to vascular endothelial growth factor, platelet-derived growth factor and fibroblast growth factor, blocking their downstream signalling pathways. These growth factors are implicated in fibrogenic pathways and administration of Nintedanib in murine models of lung fibrosis attenuated inflammation and fibrosis. Inhibition of fibroblast function is understood to be the main mechanism in which Nintedanib augments disease progression in IPF.[53] The anti-inflammatory properties of Nintedanib are less well understood. In bleomycin and silica-induced fibrosis

models, Nintedanib was shown to reduce murine lung neutrophilic and monocytic inflammation.[54,55]

In the Phase 2 trial TOMORROW, efficacy of 4 different doses of Nintedanib were assessed. Patients randomised to high dose Nintedanib demonstrated greater reduction in annualised FVC decline.[48] The INPULSIS phase 3 clinical trials, in which patients were randomised to Nintedanib or placebo, demonstrated positive outcomes with reduction in FVC decline over 52 weeks for those in the intervention arm.[13] Furthermore, pooled analysis of the TOMORROW and INPULSIS studies also inferred reduced risk of exacerbation during the follow-up time.[56]

The positive survival benefit of both Pirfenidone and Nintedanib in these homogenous clinical trial populations has also been replicated in real world clinical cohorts exploring longer-term efficacy.[57-59]

The approval of Pirfenidone and Nintedanib has fuelled IPF drug discovery and development. Although new therapies are likely to be approved for clinical use in the short-to-medium term numerous challenges remain. The lack of animal models that replicate the complexity of human disease and the poor translation of preclinical positive findings to late stage clinical trials are limitations that must be addressed.[60] Furthermore, future clinical trials in IPF will need to confront important challenges, including adoption of appropriate disease stratification, appropriate endpoints and shorter study duration in order to maximise trial effectiveness. Potential agents will also need to answer the need for a more tailored therapeutic approach beyond the pleiotropic anti-fibrotic agents, Nintedanib and Pirfenidone, which now form part of the standard of care.[61]

1.1.5 Acute exacerbations

Patients may also experience a rapid deterioration in symptoms. In most cases this often results in hospital admission and is referred to as an acute exacerbation of IPF (AE-IPF).[62] AE-IPF carries a particularly poor prognosis and accounts for up to 40% of deaths. Short-term mortality is approximately 50% and is higher still in patients admitted to hospital. Those who survive often experience an irreversible stepwise decline in functional status.[63]

Annual incidence is estimated to be 5-15% but this varies depending on the population studied.[17] It predominantly occurs in patients with advanced disease. Diagnosis can be challenging given symptoms are not specific to IPF. AE-IPF is defined as an acute and clinically significant respiratory deterioration <1 month in onset, new air-space opacification and/or consolidation changes on CT imaging superimposed on a background pattern of UIP (Figure 1.4) and absence of other acute

events including pulmonary embolism, pneumothorax and heart failure.[64] This latest consensus statement also recognises the difficulty in excluding infection in this group (bronchoscopy contraindicated in cases of hypoxia) but also questions the necessity of excluding infection given increasing evidence that viral or bacterial infection may act as a trigger of an AE-IPF event.[64,65]

The acute exacerbation is therefore probably best considered as a term that describes a clinical and radiological decline in patients with often advanced disease likely caused by a variety of triggers, the most common of which is likely infection but in a proportion of cases it may truly be idiopathic.

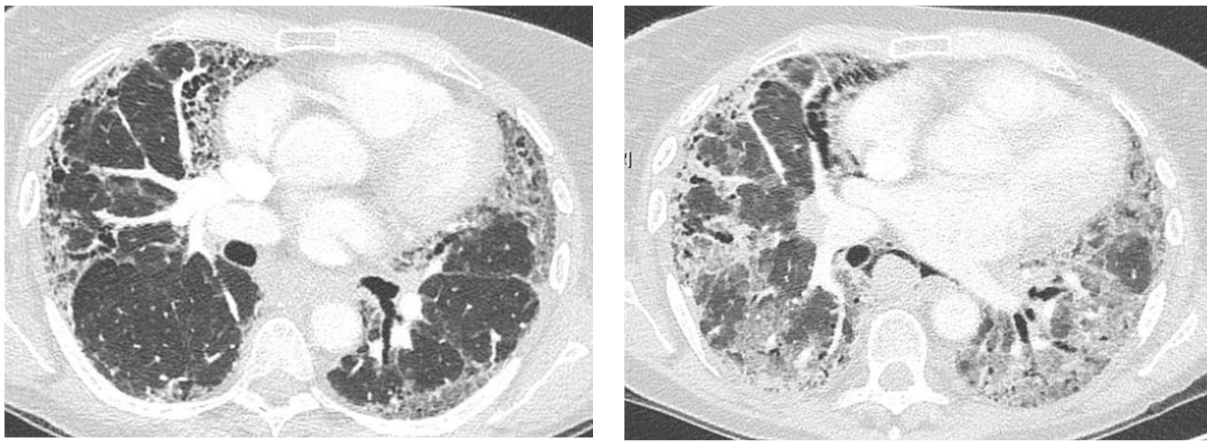

Figure 1.4 Radiological appearance of AE-IPF.
Left: CT pattern demonstrating definite UIP. Right: Image taken 2 months later in same patient demonstrating superimposed GGO and architectural distortion characteristic of AE-IPF. With permission taken from Luppi et al (2015).[66]

Histologically AE-IPF is most commonly described as a diffuse alveolar damage (DAD) pattern superimposed on underlying UIP.[67] DAD is an acute an non-specific form of lung parenchymal injury and represents the histological manifestation of acute lung injury. It is characterised by hyaline membrane deposition and interstitial oedema following alveolar injury.[68] DAD is not pathognomonic of AE-IPF. The causes of DAD are extensive and includes infection, aspiration, drugs, surgery, and transfusion reaction. Histologically therefore, these are indistinguishable from AE-IPF.

The pathogenesis of AE-IPF remains incompletely understood and despite international guidance providing a weak recommendation for the use of glucocorticoids to treat AE-IPF, there are no proven, effective therapies and current approaches are based on small retrospective studies and expert concensus.[69] A recent international survey covering 66 countries confirmed the widespread use of glucocorticoid therapy in the majority of cases (94% cases) but also

demonstrated significant geographical variability in approaches to prevention, diagnosis and treatment of AE-IPF suggesting more insights need to be gained within this area.[70]

1.2 Automated CT quantification of IPF

Accurate prognostication is central to management of patients with IPF. In addition to informing patients with regards to their probable life expectancy, accurate prediction of clinical course can guide appropriate management, such as initiation of antifibrotic therapy, transplantation assessment or palliative care pathways.[71] The currently accepted gold standard means for assessing disease severity in patients with IPF are measurement of FVC and TLCO, and qualitative measure of fibrotic disease on HRCT.[8]

1.2.1 Limitations of conventional measures of disease severity

Although measurements of FVC and TLCO are typically used to assess for changes in lung volumes and respiratory gas exchange that accompany progression of IPF, these methods have significant limitation. PFT results can be confounded by patient effort and mixed obstructive / restrictive lung disease.[72] It has proven difficult to standardise DLCO in multi-centre trials and there remains a lack of agreement for serial change in FVC values to accurately detect significant pulmonary decline.[25] Whilst FVC decline of >10% is a validated measure of disease progression and that is predictive of mortality,[25] longitudinal data demonstrate substantial intra-patient variability further adds to this challenge.[73] FVC in certain diseases may also confound CT correlation and clinical symptoms, given that it is an effort-dependent measure (can be limited by muscle weakness or extra-thoracic restriction). Furthermore, although TLCO correlates largely with the extent of lung disease on CT, TLCO itself is a composite measure of gas transfer. It is influenced not only by the hindrance of a fibrotic interstitium. Haemoglobin concentration and total lung area are important denominators for this metric and pathologies such as emphysema, pulmonary hypertension, cardiac insufficiency, and pulmonary emboli can confound this measure. TLCO measurement is more susceptible to both inter-laboratory and intra-laboratory variability than is FVC.[74]

HRCT is a proven highly sensitive imaging tool for the assessment of macrostructural changes in ILD. It has made possible the ability to visualise nuances between individual ILDs more critically and has become an essential component of initial ILD evaluation and routine case management.[75]

Despite this, disagreement on presence of interstitial abnormalities and quantification of disease severity between independent radiologists is not uncommon.[76] Human interpretation of CTs is

fraught with subjectivity, based not only on the interpreting radiologist's training and experience, but also by the individual's visual perception of the images reviewed. This can result in discordant radiographic interpretations which may ultimately bare implication for diagnosis, disease monitoring, treatment, and prognosis.[75] From a clinical perspective it can be challenging to (i) visually detect and enumerate subtle changes indicative of ILD progression on interval radiology and (ii) determine if subtle changes in PFT metrics are reflective of measurement variability or marginal but genuine deterioration in physiology. Furthermore, in research studies and clinical trials, the ability, and/or inability, to detect subtle nuances in disease using current metrics is critically important in demonstrating accurate and clinically meaningful results.[77]

Although symptom-based scoring systems and tests of exercise capacity are sensitive to change and supplement conventional measure of disease progression, they are in essence composite scores representative of global functional status. These do not specifically provide a direct mechanistic insight into ILD severity. Evolution of other co-existing respiratory co-morbidity, cardiac and peripheral arterial disease, musculoskeletal problems, nutritional status, and cognitive function can confound any interpretation of ILD severity.[78-80]

The technology of HRCT has evolved over the years, most recently with the advent of quantitative HRCT (qCT) analyses using computer-derived algorithmic measurements. Quantification of interstitial lung diseases by automated objective measurement of lung histogram features, regional CT density changes and parenchymal texture of CT imaging through the application of advanced computer algorithms has the potential to standardise interpretation of complex radiographic patterns, minimise inter- and intra-observer variability and develop the role of HRCT in ILD beyond dedicated specialist centres.[75]

1.2.2 Quantification of IPF using lung texture analysis

qCT employs an automated histogram signature mapping technique that utilises density and texture-based analysis of the lung parenchyma. The Hounsfield unit (HU) scale is a measurement of relative densities (attenuation) determined by CT, used to generate images. In the lung parenchyma, CT attenuation, measured in HU, is determined by the relative amounts of air, soft tissue, and blood in each volume element (voxel). Using a Histogram-based method of segmentation, a histogram is computed from all voxels within the image.[81] The CT histogram can thus provide a distribution of HU for the entire lung, permitting calculation of mean lung attenuation, variance, skewness, entropy, and kurtosis. Histogram analysis of individual CT images or specific areas of an image can provide regional information of lung parenchymal status.

Computer-Aided Lung Informatics for Pathology Evaluation and Ratings (CALIPER) is a texture-based algorithm that has been used in multiple research studies of qCT in ILD.[82-86] CALIPER is an image analysis tool that utilises machine learning, 3D histogram signature mapping and regional voxel and morphological analysis to provide quantitative assessment of parenchymal lung disease from HRCT data. This process analyses the histogram signature of each 15x15x15 voxel region and automatically categorises each pixel of a volumetric HRCT into one of seven specific parenchymal features: normal parenchyma, ground-glass opacity, reticular density, honeycombing, and mild, moderate, or severe low-attenuation areas (Figure 1.5 and Figure 1.6).[87]

It was developed by expert consensus thoracic radiologist interpretation of pathologically confirmed training sets. Parenchymal features of voxels were randomly selected from training imaging sets from cases with histopathologically confirmed disease for a variety of lung parenchymal pathologies and control subjects, from the Lung Tissue Research Consortium.[82,88-90] CALIPER detects parenchymal features and provides volumetric quantification, which can be assessed over time with repeat HRCT.[82]

It was originally calibrated using an exemplar cohort of patients with histologically proven IPF.[90] In this retrospective clinical assessment of 55 patients with IPF, CALIPER-measured ILD changes including %ILD, total ILD volume and total reticulation volume were associated with reduced survival in multivariate analysis.[82] Here, each patient's CT scan was uploaded into the CALIPER application, and the proportion of the patient's lungs in each category was calculated. CALIPER data processing steps involve extraction of the lung from the surrounding thoracic structures and segmentation into upper, middle, and lower zones. The parenchymal features are measured per unit volume of the whole lung. Total ILD can then be defined as the volumetric sum of total ground-glass density, reticular abnormalities and honeycombing and percentage ILD measured as the ratio of the sum of total ILD to the CALIPER segmented total lung parenchymal volume.[72,82]

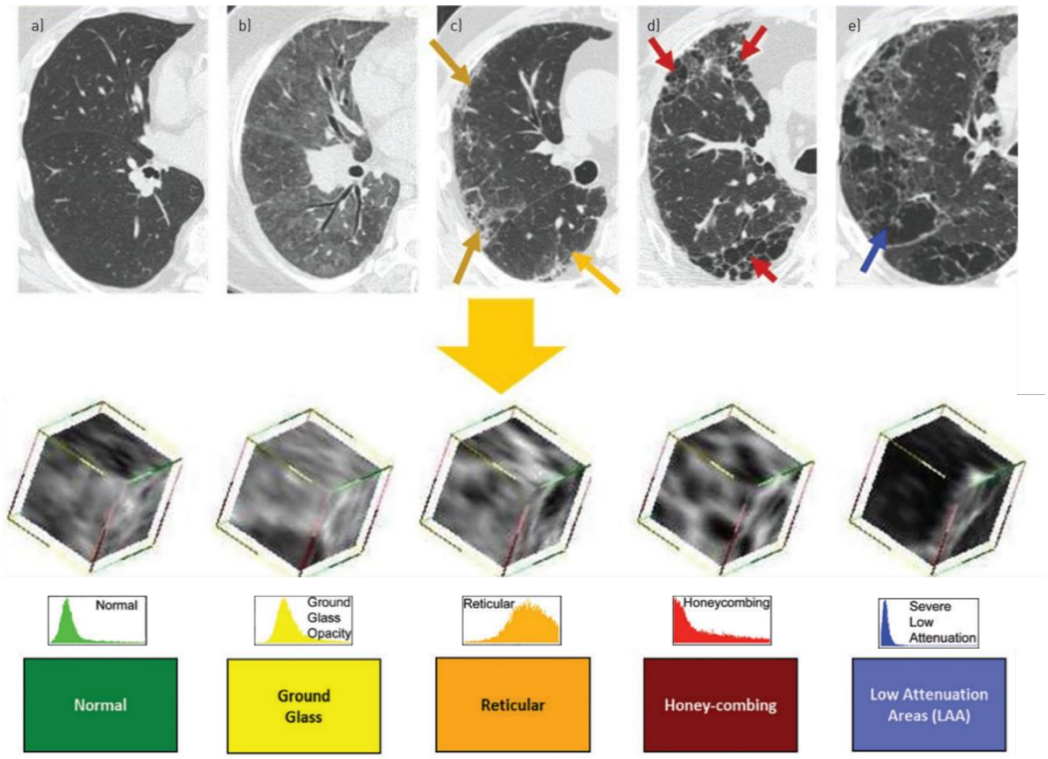

Figure 1.5 CALIPER lung texture analysis

HRCT images demonstrating (a) normal appearance, (b) ground glass, (c) reticulation, (d) honeycombing and e) emphysema. In exemplar datasets, consensus radiologist opinion was used to identify multiple volumes of interest of 15×15×15 pixels corresponding to normal, ground-glass density, reticular abnormalities, honeycombing and emphysema. Adapted with permission from Maldonado.[72]

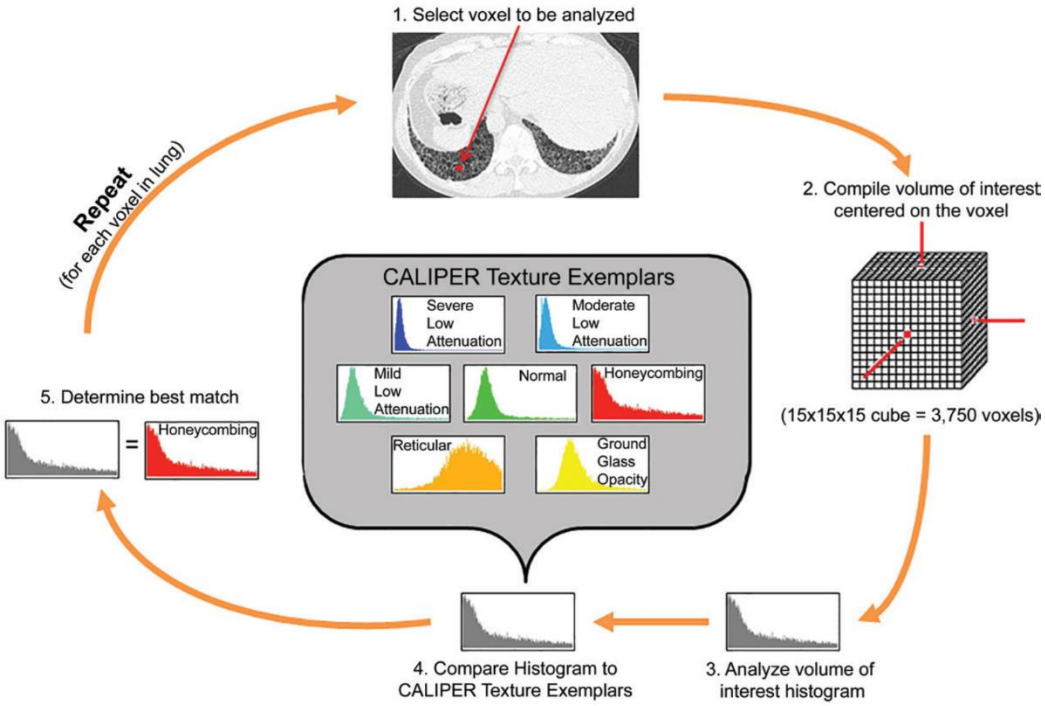

Figure 1.6 CALIPER lung texture algorithm

For each 15x15x15 voxel-sized volume of interest (VOI), histogram-based characteristics are compared to histograms of exemplars of each parenchymal pattern. VOIs are then assigned to the exemplar histogram with least difference. This process is repeated for each voxel until both lungs are classified. Proportions of each feature are measured (%) of lung involvement With permission taken from Chen et al 2020.[91]

Abnormalities of the pulmonary vessels across a variety of pulmonary diseases, including IPF, are commonly seen.[92] Using computer-based image analysis of HRCT, the total volume of the pulmonary blood vessels of varying sizes can now be assessed noninvasively. CALIPER can not only quantify parenchymal patterns, but it can also algorithmically extract and summate pulmonary vascular structures. Pulmonary vessel volume (PVV), a novel non-parenchymal texture-based metric, represents the sum of the volume of pulmonary arteries and veins, after the vessels at the hilum have been excluded (Figure 1.7).[91]

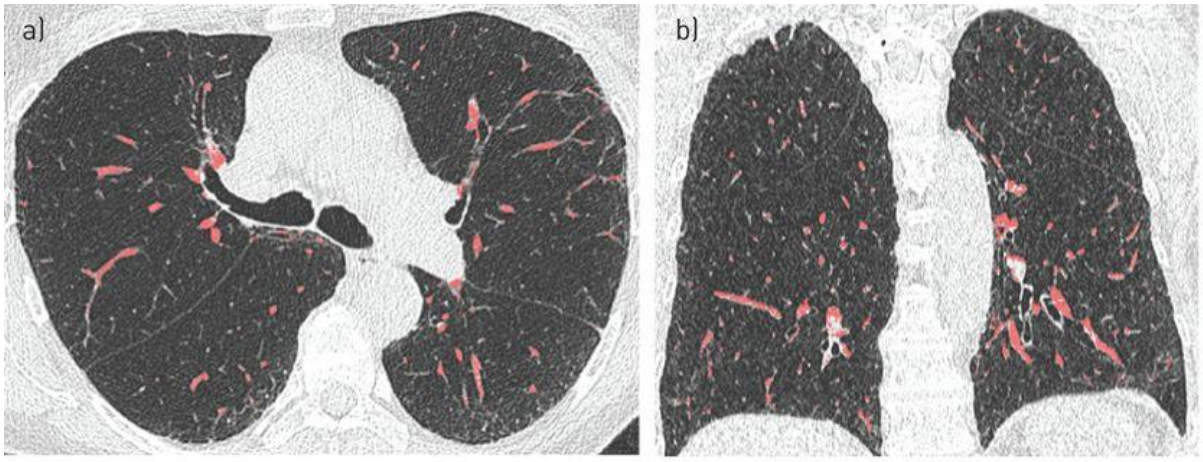

Figure 1.7 CALIPER Pulmonary vessel volume quantification
Case example of delineation of pulmonary arteries and veins (red) as quantified using CALIPER. PVV is expressed as an absolute volume (cm^3) or as a percentage of the total lung volume. Taken with permission from Jacob et al (2017).[93]

1.2.3 Use of CALIPER in IPF studies

IPF has been an interesting area of exploration for the role of qCT in disease monitoring and possible prognostication, given the recognised irreversible and progressive nature of this condition, and the subjective limitation of semi-quantitative CT analysis tools. Jacob et al applied the CALIPER algorithm to CT scans of a cohort of 283 patients with an IPF diagnosis to compare automated computer-based CT analysis with conventional severity measures. In cross-sectional analysis of this cohort Jacob et al compared CALIPER and visual CT scoring to contemporaneous pulmonary function tests. In univariate analysis, CALIPER-derived measurements of ILD extent and PVV demonstrated stronger association with FVC% and TLCO% in comparison to visual CT scoring.[84]

In a separate analysis of the same study cohort, association of CALIPER-derived metrics with mortality was explored.[93] Here, univariate Cox proportional hazard modelling found all visual and

CALIPER-derived interstitial features (GGO, reticulation and honeycombing and total ILD extent) to be predictive of mortality. Interestingly, all interstitial features apart from total ILD extent, measured by CALIPER were slightly more predictive than visual assessment. Multivariate analysis revealed, CALIPER PVV and %honeycombing were independently predictive of mortality.[93]

In a separate study comparing annualised progression of IPF by visual assessment and CALIPER, 66 patients with serial CT imaging had CT features scored visually and with CALIPER.[83] On univariate analysis, changes to CALIPER-derived variables demonstrated stronger linkages to FVC change compared to changes in visual scores. Furthermore, an increase in CALIPER-measured PVV, specifically an increase in middle zone lung vessels, was found to be the strongest radiographic predictor of death and/or 10% FVC decline at 12 months.[83]

In a longitudinal study Sverzellati et al tested whether the application of CALIPER could more sensitively detect radiographic progression of IPF and enhance risk stratification of survival. The authors evaluated CALIPER-rated analysis of HRCTs performed at baseline and annually over a 2 year period, in conjunction with contemporaneous FVC measurements in 58 patients with IPF.[94] The combination of annualised FVC% decline >10% with a >20% relative increase in either CALIPER-total lung fibrosis or CALIPER-PVV improved stratification of survival outcome. The authors demonstrate that quantitative CT can refine current stratification and prognostication of disease.[94]

The utility of CALIPER as a cohort 22nrichent tool for clinical studies has also been explored. In an observational study Jacob et al explored if CALIPER-derived parenchymal and PVV metrics could better predict FVC decline and mortality.[95] Upper and midzone PVV measures were found to be more predictive of FVC and TLCO decline and survival. They also identified that restricting a clinical trial cohort by including only cases with PVV ≥4.4% of total lung volume, it would be possible to reduce the sample size by 26% whilst demonstrating same drug treatment effect. The growing use of PVV in qCT has yet to be fully elucidated but does appear have a promising role.[26]

From a research perspective, CALIPER may provide refined and granular metrics which complement the current conventional measures of pulmonary function testing which provide a global assessment of disease severity in IPF. As such, CALIPER could be more sensitive in capturing important clinical end points such as progression of fibrosis and biomarker associations.

1.3 Mechanisms of disease in IPF

Aberrant repair pathways are believed to contribute to pathogenesis.[1] In healthy subjects, upon lung injury the alveolar epithelium releases chemokines that localise inflammatory cells, including monocytes, macrophages and neutrophils, and secrete pro-fibrotic cytokines, for example Interleukin-1β (IL-1β), transforming growth factor-β (TGF-β), Tumour necrosis factor-α (TNFα). In short, this promotes the differentiation of fibroblasts into myofibroblasts, collagen production and extra-cellular matrix (ECM) deposition necessary for controlled wound healing.[28] At its core, IPF can be thought of as a dysregulated wound healing response in the lung, leading to excessive ECM deposition, and remodelling of the lung parenchyma with fibrotic non-functioning tissue. The result is impaired gas exchange, which manifests clinically as hypoxic respiratory failure.[1]

Wound repair has four distinct stages; a coagulation phase, an inflammatory phase, a fibroblast proliferation phase and finally a remodelling phase whereby normal tissue architecture is restored. Shortly after tissue damage, epithelial cells release inflammatory mediators that initiate a coagulation cascade which triggers clotting and ECM production. Platelet aggregation promotes local vasodilation and permeability, facilitating recruitment of immune cells such as neutrophils, macrophages, and lymphocytes to the site of injury.[28] During this initial leukocyte migration phase, activated macrophages and neutrophils debride the wound and eliminate invading microorganisms. They also secrete proinflammatory cytokines and chemokines, fibroblast proliferation and recruitment. Fibroblasts are recruited from a variety of sources including local mesenchymal cells, bone marrow progenitors and via the process of epithelial-mesenchymal transition whereby epithelial cells differentiate into fibroblast-like cells.[37] Normal wound-healing responses are typically self-limited local reactions. In IPF, however, this wound healing response is widespread and continues over a prolonged period of time.[96]

In the last decade, much research has focused on the contribution of the immune system to tissue repair, regeneration and fibrosis,[97] and has led to a re-evaluation of the role of immune cells in pathogenesis of IPF.[98]

1.3.1 Genetic susceptibility to pulmonary fibrosis

The genetic factors influencing IPF susceptibility depend largely on whether a patient has sporadic IPF or the familial form, termed familial interstitial pneumonia (FIP). A host of common gene variants with modest effect size impart disease risk in patient with the sporadic form of IPF whilst rare variants with large effect size impart greater risk in patient with FIP.[10] Progression of IPF is

thus likely related to interaction of genetic variants and transcriptional changes, ultimately resulting in loss of alveolar epithelial integrity.

1.3.1.1 Rare variants linked to Familial Interstitial Pneumonia

Studies regarding the genetic architecture of sporadic IPF and the currently known mutations involved in the familial form highlight the role of the lung epithelium in development and progression of the disease.[99] Familial Interstitial Pneumonia (FIP) is identified when two or more members of the same biological family are affected. FIP is inherited in an autosomal dominant manner with variable penetrance. It accounts for 2-20% of the overall case burden of Idiopathic interstitial pneumonias.[100]

Several rare genetic variants are implicated in maintenance of telomere length, TERT (Telomerase reverse transcriptase), *TERC* (Telomerase RNA component), *PARN* (Poly(A)-specific ribonuclease) and *RTEL* (Regulator of telomere elongation helicase). Telomerases elongate telomeres, which are repetitive DNA sequences located at the ends of chromosomes. Telomeres shorten with every cell cycle and eventually will reach a critical point resulting in either cellular senescence or DNA damage leading to apoptosis.[101] Researchers have thus questioned whether age-related gene mutations may contribute to the development of pulmonary fibrosis. In an observational cohort, age-adjusted average blood leukocyte telomere length was found to be shorter in patient with IPF versus healthy controls and identified as an independent predictor of reduced survival in IPF.[102] Genetic sequencing of familial cases subsequently identified that approximately 8% were associated with telomerase mutations whilst only 1% of sporadic cases revealed similar mutations.[103] In a separate observational cohort study exploring risk factors associated with progression of radiological abnormalities in familial interstitial lung disease, cases with interstitial lung abnormalities demonstrated significantly shorter peripheral blood mononuclear cell telomere length.[104] Following alveolar epithelial cell injury, short telomeres may compromise the ability of these cells to regenerate damaged areas resulting in activation of DNA damage responses and pro-apoptotic pathways.[10] Shortened telomeres may also partly explain the presence of low-volume and seemingly benign interstitial changes observed on CT in individuals at the extreme of age.[105] As telomeres gradually shorten with age and cellular division, their measure of ‘molecular age’ might complement chronological age as a predictor of survival.[102]

1.3.1.2 Common variants linked to sporadic IPF

Large genome-wide association studies undertaken in 2011 to explore possible genetic risk factors have identified common genetic variants, crucial for epithelial integrity, as risk factors for IPF.[106] These studies have identified the importance of host defence and cellular barrier function in development of IPF.

Mutations in genes encoding surfactant proteins, primarily the gene encoding surfactant protein C (*SFTPC*), which is exclusively expressed by Type 2 alveolar epithelial cells, cause dysfunctional folding and processing of surfactant, deregulated proteostasis and endoplasmic reticulum stress.[106]

The variant rs35705950 of the promoter of the gene encoding mucin 5B (*MUC5B*) is the most common single nucleotide polymorphism (SNP) that increases risk of IPF.[99] MUC5B plays an important role in muco-ciliary clearance. This variant is most frequently observed in Caucasians. One meta-analysis identified a nearly 4-fold risk of development of IPF with this MUC5B promoter SNP.[107] Observational cohort studies have identified association with interstitial lung abnormalities and risk associated with early ILD.[108] Paradoxically, patients with this variant have improved survival compared to those without.[109]

Variants in *TOLLIP*, which encodes an inhibitor of TGF-β pathway and critical regulator of Toll-like receptor mediated innate immune responses, have also been implicated in IPF. Patients with the variant rs5743890_G have a greater risk of mortality.[110]

1.3.2 The role of microbes in IPF

The role of microbes (viral, bacterial, and fungal) in the pathogenesis of IPF has been investigated in several different studies.

IPF has been suggested to have a viral aetiology based on the presence of viral signatures in the lungs of IPF patients, and the observation that patients with IPF demonstrated positive response to treatment with antivirals.[111,112] Viruses detected in IPF patients include members of the Human Herpes Viruses (HHVs) family, such as Epstein-Barr virus (EBV), cytomegalovirus (CMV) and Herpes simplex virus as both initiating and propagating cofactors have been investigated.[2] Analysis of the COMET-IPF study demonstrated a relationship between peripheral blood immune gene expression and BAL microbiome features in IPF.[113]

EBV has received the most attention in relation to IPF. EBV DNA and protein and has been detected in the alveolar epithelium of patients with IPF from BAL and lung tissue samples.[114] A study by Tang *et al* (2003) identified herpesviruses (CMV, EBV, HHV) via PCR in 32 of 33 lung biopsies compared to only 36% of controls.[115] In a separate study, re-arrangement in EBV genome associated with productive EBV replication was found in lung tissue biopsies of 61 percent of EBV DNA-positive IPF patients.[116] HHVs infect epithelial cells and can induce endoplasmic reticulum (ER) stress leading to activation of the unfolded protein response, which may aid viral replication. Epithelial cellular integrity can become compromised leading to apoptosis.[117] There has also been interest in Adenovirus contributing to pulmonary disease due to its ability to upregulate TGF-β1 (a potent pro-fibrotic growth factor) expression in bronchiolar epithelial cells and transformation of lung epithelial cells to express mesenchymal markers. In a recent study by Zhou *et al* (2014), using a murine bleomycin model, intratracheal instillation of high dose adenoviral vectors induced an inflammatory response, lung injury, and pulmonary fibrosis in a dose-dependent manner.[118]

Given that ER stress and the unfolded protein response are features of genetic and sporadic forms of pulmonary fibrosis it is plausible that viral infection can trigger profibrotic responses in genetically susceptible individuals. Viral infection in older age may increase susceptibility to IPF due to the cellular effects of ageing and epigenetic alterations which may alter the ability of AECs to clear infection.[111]

Whilst there is evidence to suggest a role for viral infection in the pathogenesis of IPF, any role of bacteria is much less well established. Bacterial colonisation may also drive fibrosis. A study by Molyneaux et al (2014) measured bacterial DNA within BAL fluid from IPF patients and controls. The authors found that patients with IPF had double the bacterial burden (Haemophilus, Streptococcus, Neisseria, and Veillonella) and patients with greatest load had higher risk of disease progression (decline in FVC >10% over 6 months).[119] One large multicentre, randomised, placebo-controlled study evaluated the prophylactic use of 12 months of Co-trimoxazole as a treatment for IPF. The authors reported that there was no difference in the primary endpoint of change FVC. Post hoc analysis suggested that Co-trimoxazole led to a reduction in infections and mortality.[120] This observation, together with the high mortality associated with bacterial respiratory tract infection in IPF, suggests that bacteria may play a role in driving IPF disease progression.

Whilst active infection in IPF is known to carry a high morbidity and mortality, the effect of latent viral infection or changes in the composition of the lung microbiome remains incompletely understood. It must be noted that most studies implicating microbes in the pathogenesis of IPF are mostly small and retrospective studies. Most demonstrate association but not a causal

relationship with the structurally abnormal IPF lung, in which host defence mechanisms are likely to be defective. It is therefore possible that detection of these microbes reflects defective clearance in a structurally abnormal lung and that they are merely bystanders rather than cofactors implicated in disease pathogenesis. Modern microbiological techniques permit a detailed analysis of lung pathogens. As such, it is to be hoped that the application of these methodologies to large populations, of greatly phenotyped IPF individuals, will help define the role played by viruses and bacteria in the development, progression and acute exacerbations in IPF patients.[121]

1.3.3 Alveolar epithelial cell injury and aberrant wound healing

Healthy pulmonary alveoli are composed of type I alveolar epithelial cells (AEC1), which are situated in close proximity to endothelial cells forming the surface for gas exchange, and type II alveolar epithelial cells (AEC2), which are responsible for the production of phospholipid rich surfactant.[122] The lungs are constantly exposed to insults, of variable magnitude, yet display a remarkable ability to repair and regenerate through a cascade of highly synchronised biological processes.[99]

Selman *et al* first proposed IPF was initiated by aberrant wound healing in response to injury, resulting in accumulation of extracellular tissue.[123] This proposition has subsequently become the prevailing theory. IPF is currently considered an epithelium-driven disease, in which a dysfunctional, ageing lung epithelium is exposed to recurrent microinjury leading to defective attempts at regeneration. This creates imbalance between profibrotic and antifibrotic mediators; favouring fibroproliferation.[9,105]

In normal lungs, loss of AEC1s after injury is followed by differentiation of AEC2s to restore alveolar integrity. This involves several mechanisms including the coagulation cascade, angiogenesis, fibroblast activation and migration and collagen synthesis.[124] Studies have demonstrated that AEC2s secrete cytokines, including TGF-β, Platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), which lead this process. This induces proliferation and activation of fibroblasts, leading to excessive ECM deposition and remodelling of the lung architecture.[125]

In IPF, fibroblastic foci are often seen near denuded, apoptotic, or hyperplastic alveolar epithelium. Fibrosis can develop when the injury is severe or when this process becomes dysregulated (Figure 1.8).

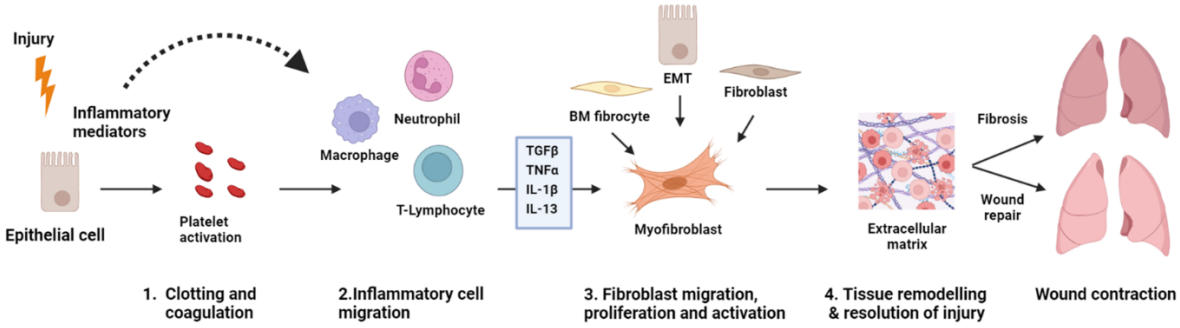

Figure 1.8 Alterations to normal tissue repair contributes to development of IPF.
Adapted from Wynn *et al*.[28]

1.3.4 Inflammatory responses and innate immunity in IPF

While the ILD community agree that IPF does not result from a primary immunopathogenic mechanism, evidence gleaned from animal modelling and human studies suggests that innate and adaptive immune processes can orchestrate existing fibrotic responses.[126]

A predominant mechanism through which innate immune cells adopt fibrosis-promoting properties likely involves the sensing of innate immune agonists by pattern recognition receptors (PRRs). Ligands to PRRs fall into two classes. Those derived from invading microorganisms are known as pathogen-associated molecular patterns (PAMPs), such as bacterial endotoxin Lipopolysaccharide, and those derived from injured cells and tissues known as damage-associated molecular patterns (DAMPs).[127]

In the scenario of IPF, a front-line cell in this process is the AEC II. In healthy innate immune systems these AEC II cells are recognised and phagocytosed through a DAMP-mediated non-inflammatory processes called efferocytosis, allowing the regulated removal of debris, thereby facilitating the resolution of injury and the achievement of repair.[128] A number of mediators are classified as DAMPs. This ranges from intracellular components such as nucleic acids and extracellular/cell wall components to the transformation of inert proteins to signalling molecules.[127] Cell types expressing pattern recognition receptors include innate immune cells such as macrophages and monocytes but also non-immune cells such as epithelial cells and fibroblasts.[128] The activation of these receptors can be protective or harmful depending on the nature of the ligand and the specific receptor.

Neutrophils are innate immune cells that possess several functions through which they might participate in fibrosis including secretion of pro-inflammatory cytokines and reactive oxygen species (ROS).[129] They can be activated through receptors including, Toll-like receptors (TLRs), Fc receptors and various cytokines. Neutrophils are the predominant immune cell isolated from BAL

samples of IPF cases and increasing numbers are associated with worse prognosis.[130] Neutrophils might contribute to fibrosis via their regulation of ECM turnover as Neutrophil elastase (NE), the main proteolytic product of alveolar neutrophils, and is increased in BAL fluid of IPF patients.[131] Its pro-fibrotic roles include myofibroblast differentiation and TGF-β activation.[129] Indeed, NE deficient mice are protected from the fibrosis seen in bleomycin and models.[132]

Neutrophils also secrete Matrix Metalloproteinases (MMPs), such as MMP2, MMP-8 and MMP-9, which are involved in pulmonary fibrosis. The balance between MMPs and their antiproteases (TIMPs) plays a critical role in accumulation or degradation of ECM in pulmonary fibrosis.[133]

An intriguing and newly identified fibrosis-promoting function of neutrophils is generation of extracellular neutrophil traps.[129] These pro-inflammatory collections of chromatin and neutrophils regulate both immune cell function and fibroblast activation.[134] While enhanced detection of intrapulmonary neutrophil extracellular traps has been reported in both the bleomycin model and in some forms of fibrotic ILD, a specific association with IPF has yet to be fully described. Further studies are warranted to understand whether these play a role in IPF pathogenesis.[135]

In summary, neutrophils are innate immune cells that are associated with the production of cytokines and chemokines, presence of injury, regulation of ECM turnover, and generation of NETs. All these functions would be expected to cause fibroblast activation and ECM accumulation. However, because the pathology of UIP is not characterised by neutrophil accumulation their role remains unclear.[135]

Macrophages are key orchestrators of innate immunity and are resident in almost all tissues. They are crucial modulators of innate and adaptive immune responses and possess remarkable plasticity enabling them to adopt different phenotypes in response to injury; pro-inflammatory, anti-inflammatory and pro-repair.[97] Macrophages arise from monocyte lineage and constitute part of the monocyte phagocytic system.[135]Aside from their role as antimicrobial phagocytes, they are also implicated in the pathogenesis of fibrosis.[136] Alveolar macrophages (AMs) have been implicated in the pathogenesis of fibrotic lung diseases and studies have identified AMs represent a potent source of profibrotic cytokines, chemokines, and MMPs (Figure 1.9).[137]

Leukocyte infiltration is a universally recognised hallmark of inflammation. Once recruited to lung tissues , leukocytes can contribute to chronic inflammation and promote fibrogenesis via secretion of various cytokines.[135] Although cytokines are produced locally by epithelial cells and fibroblasts, numerous studies have demonstrated that in IPF patients macrophages express higher levels of these mediators compared to controls.[138] Indeed, the ability of multiple types of immune

and non-immune cells to secrete these cytokines suggests that they play a pivotal role in orchestrating chronic inflammation.[136]

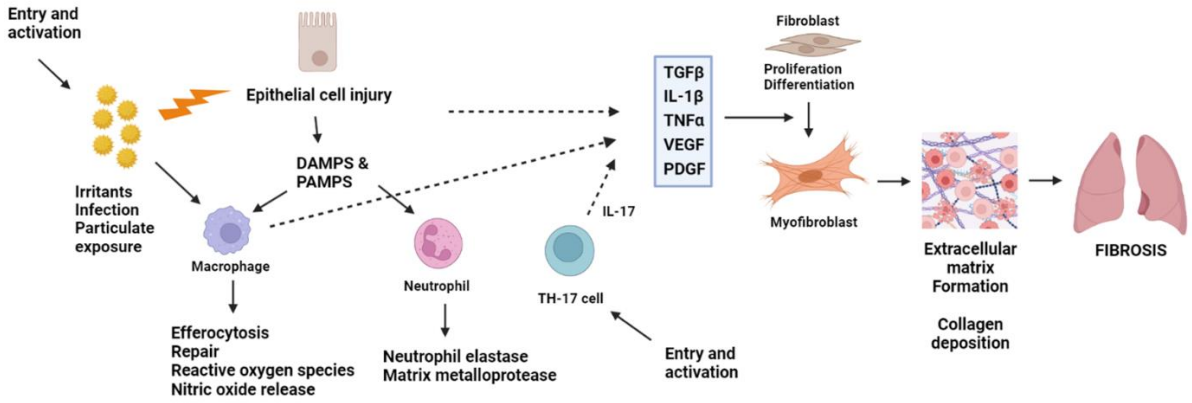

Figure 1.9 Effect of immune cells on myofibroblasts and fibrogenesis.
Exposure to irritants such as tobacco smoke, viruses and bile acids induce alveolar epithelial damage. Release of pro-inflammatory mediators, such as IL-1β, TNFα and ROS enhances recruitment and activation of leukocytes at the site of tissue injury. This in turn stimulates production of TGF-β, which (i) mediates recruitment, proliferation, and activation of fibroblasts into myofibroblasts and together with (ii) induces EMT. This promotes ECM production and fibrogenesis. Macrophages further represent a source of tissue inhibitors of metalloproteinases (TIMPs) that can antagonise MMP-mediated ECM degradation. Neutrophils produce various proteases, particularly neutrophil elastase (NE) and MMPs, which degrade matrix components but can also activate TGF-β through NE and produce TIMPs, thereby promoting ECM accumulation. Modified from Wynn and Kolahian.[28,133]

1.3.5 Adaptive immune responses in IPF

There is increasing awareness that the adaptive immune system plays a role in initiation and/or disease progression IPF. Perturbations in phenotype of T and B cells, auto-antibodies and immune complexes have been identified in IPF patients.[139] Lymphoid aggregates are a recognised pathologic feature of IPF lesions. These aggregates consist of clusters of $CD3^+$ T lymphocytes and dendritic cells which that a situated close to fibroblastic foci and regions of high collagen deposition.[140] The aggregates also display $CD20^+$ B lymphocytes which cluster in the centre of these aggregates.[140] The tertiary lymphoid structures also include mature dendritic and given that it is already known that activated T lymphocytes within the lung retain competency and effector cytokine production, it appears plausible that chronic pulmonary inflammation could be driven by re-activation of T cells by maturing dendritic cells within these IPF lymphoid aggregates.[135,139]

Historically, a Th1/Th2 imbalance was thought to play a modulatory role during the inflammatory phase of pulmonary fibrosis. Systemic depletion of T lymphocytes using anti-CD3 monoclonal antibodies dampened ECM accumulation and fibrosis in a murine model of bleomycin-induced pulmonary fibrosis.[141] Indeed, the Th1 cytokines IFNγ and IL-12 have been shown to attenuate fibrosis.[142] Conversely, the prototypical Th2 cytokines, IL4, IL-5, and IL-13, were shown to

stimulate fibroblast proliferation, collagen production, and fibroblast to myofibroblast differentiation – thereby linking these mediators with fibrogenesis.[143] This lead to the view that Th1 responses are protective, and Th2 responses harmful.[129] However, the negative results of the INSPIRE trial, which assessed if treatment with IFNγ could improve survival in IPF, suggest this view to be too simplistic.[34]

Other studies support a role for IL-17a and Th17 cells by demonstrating that neutralisation of IL-17a delayed the progression and promoted the resolution of pulmonary fibrosis in different murine fibrosis models.[144,145] Th17 cells were first identified as a T-helper cell population that behaved neither as Th1 or Th2 in function. Th17 cells produce cytokines, such as IL-17 and IL-22, which are host-defensive cytokines to many infectious agents but also promote inflammatory pathology in various diseases such as autoimmune conditions.[146] Functions of IL-17 include stimulation of ECM production, collagen deposition, mediation of TGF-β signalling and IL-6 expression.[147] Increased detection of IL-17 in the lung tissue, BAL, and serum,[148] and these human findings are supported by murine studies in which administration of IL-17A is sufficient to induce collagen accumulation and fibrotic lesions.[149] Interestingly, recent work has expanded the concept of IL-17 in fibrosis beyond Th17 lymphocytes. In one study using in an experimental model of hypersensitivity pneumonitis neutrophils and monocytes/macrophages to be a dominant source of IL-17a,[145] however this has not been similarly described in IPF. The exact role of IL-17 in development of IPF is not fully understood and although it appears to be pro-inflammatory and pro-fibrogenic its role in established disease may be less relevant.[129]

The role of regulatory T lymphocytes (Tregs) in pulmonary fibrosis has been gaining acceptance in the recent years. Tregs are crucial for maintaining host tolerance and preventing autoimmunity.[150] Due to their ability to produce both IL-10 and TGF-β, Tregs have the potential to both promote or inhibit fibrosis depending on the context. For example, a now seminal 2009 study reported marked inhibition of functional $CD4^{+}$, $CD25^{high}$, $FoxP3^{+}$ cells in the BAL and peripheral blood of IPF patients, thereby demonstrating a relationship between impaired Tregs and IPF.[151] This immune cell phenotype also correlated with reduced FVC and diffusion capacity. This study showed for the first time, an association between Tregs and IPF. In addition, a population of aberrantly activated Tregs identified by expression of the neuroimmune molecule Semaphorin 7a+ was sufficient to engender increased collagen deposition and TGF-β-induced lung fibrosis.[152] Conversely, a further study found that Treg depletion in mice resulted in an exaggerated fibrotic response in a TGF-β model of lung fibrosis.[153]

From studies conducted thus far, it appears that the function and phenotype of recruited or lung resident Tregs can be either fibrosis-suppressive or fibrosis-stimulatory properties. It is possible

that Tregs play a dual role in fibrosis and that the cytokine milieu in their local microenvironment influences this activity. Collectively, the role of T cells in pulmonary fibrosis seems to be complex and substantially dependent on the subtype of T cells.

The observations that lymphoid aggregates in peri-fibrotic lung tissue coupled with the detection of various auto-antibodies in serum have led to a theory that IPF could be, in part, due to breakdown of immunological self-tolerance to self-antigens derived from injured and ageing airway epithelial cells.[135] In contrast to autoimmune diseases, such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) whereby auto-antibodies and immune complexes can be mechanistically linked to disease, their presence in IPF does not fit with current understanding of disease mechanism. Furthermore, immunosuppressive agents, such as Azathioprine, do not positively alter disease course.[46] It is thus unlikely that abnormalities in the adaptive immune system drive the disease process. However, it is acknowledged that IPF is a heterogenous disease and factors in T and B cell response may play a role in promoting fibrosis in a proportion of cases.

1.4 Monocytes and macrophages in IPF

1.4.1 Monocyte subsets

Human monocytes comprise between 5-10% of peripheral blood leukocytes and are most commonly subdivided in three subsets depending on the level of expression of the LPS co-receptor CD14, the FCγ-RIII receptor CD16 and other surface markers including CD64 (FCγ-RI), CCR2 and CX3CR1.[154] Peripheral blood monocytes are derived from bone marrow from a dividing myeloid progenitor that is shared with platelets, erythrocytes, dendritic cells and granulocytes.[155] Historically, monocytes were thought to represent a bridge linking bone marrow precursors to terminally differentiated tissue macrophages and dendritic cells. However today we appreciate that the majority of resident macrophages, but crucially not all, have an embryonic origin.[156]

In humans, monocytes were initially defined by morphology and later by flow cytometry based on light scatter properties and expression of cell-surface markers CD14 and CD16. The combination of CD14 and CD16 expression on HLA-DR$^+$ cells in humans enabled the classification of three human monocyte subsets (Figure 1.10).[157] CD14^{hi}CD16$^-$ monocytes, commonly referred to as 'classical' monocytes, comprise 80-90% of the circulating monocyte pool. The remaining 10-20% are shared between CD14^{low}CD16$^+$ 'non-classical' monocytes and CD14$^+$CD16$^+$ intermediate monocytes.[154]

$CD14^{hi}CD16^{-}$ monocytes released into peripheral circulation remain in circulation for approximately one day before differentiating into non-classical monocytes (via intermediate monocyte precursors). However, in the event of tissue injury, such as focal lung infection, these monocytes instead traffic along chemokine gradients to re-populate depleted tissue-resident macrophage populations, differentiating into monocyte-derived macrophages (MDMs).[158,159]

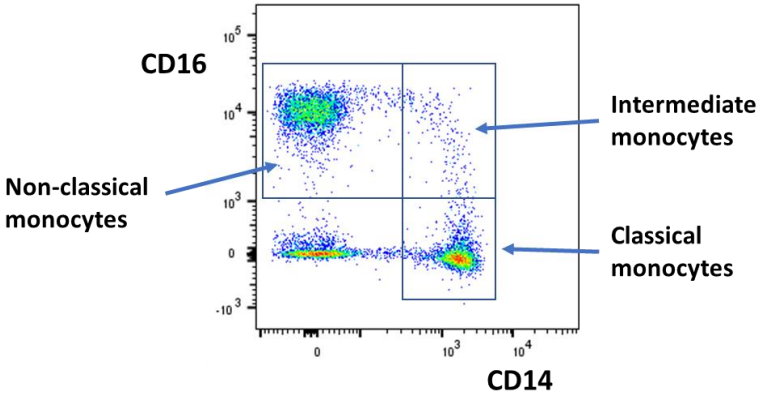

Figure 1.10 Human peripheral blood monocyte subsets.
Illustration of the definition of human monocyte subsets in health based on a typical distribution of events in a CD14 CD16 staining and analysis by flow cytometry. Classical monocyte population 85.1%, Intermediate monocyte population 6.2%, Non-classical monocyte population 8.6%. Note that this qualitative representation does not necessarily accurately depict quantitative measure (A. Achaiah unpublished data).

Significant debate exists regarding the functional repertoires that define each subset and the role each plays in response to injury. Mouse monocytes are more clearly defined and can be broadly divided into two subsets based on Ly6c expression. Transcriptional comparison between mouse and human monocytes correlated $Ly6C^{Hi}$ monocytes with classical $CD14^{hi}CD16^{-}$.[160,161] $Ly6C^{Hi}$ expression is observed in monocytes which are released from bone marrow (BM) in a CCR2-dependent manner and recruited to sites on inflammation and infection. $Ly6C^{Hi}$ monocytes play an active role in homeostasis and continuously survey the endothelium for antigens. Following injury $Ly6C^{Hi}$ monocytes are recruited into the tissue in high numbers whereby local signals trigger the differentiation of the cells into 'inflammatory' macrophages.[158] The monocyte-derived macrophages (MDMs) undergo apoptosis after inflammation subsides or mature into macrophages which assist with repair and resolution processes.[162-164]

$Ly6C^{Low}$ monocytes represent a separate subpopulation. They exhibit crawling behaviour over the epithelium suggesting a surveillance function. They remove cellular debris and particulates and contribute to viral responses, inducing TLR7 pathways and recruiting other inflammatory cells.[155] Gene profiling studies have found that the human equivalent to $Ly6C^{Low}$ monocytes are non-

classical or 'Patrolling' monocytes identified by $CD14^{low}CD16^{+}$ expression. which is characterised by higher MHC class II expression and after stimulation by Toll-like receptor (TLR) ligands by higher TNF production.[154,155,162]

The third human monocyte subset described in recent years express both CD14 and CD16 are termed 'Intermediate monocytes'. These cells express high levels of HLA-DR and are considered important in antigen presentation. This subset is reported to possess high inflammatory potential, producing elevated levels of ROS, secrete cytokines including TNFα, IL-1β and IL-6 upon TLR stimulation and greater phagocytosis capability.[165-167] Increased intermediate monocyte levels have been described in a number of disease states including rheumatoid arthritis, chronic kidney disease and in the immediate aftermath following stroke and acute coronary syndromes.[167-169]

Human monocyte subsets			
Monocyte subset	**Markers**	**Chemokine receptors**	**Function**
Classical: 90% of circulating monocytes	$CD14^{hi}$ $CD16^{-}$ $CD64^{+}$ $CD62L^{+}$ $TNFR1^{+}$ $TNFR2^{lo}$	$CCR2^{hi}$ $CX3CR1^{lo}$	Phagocytic and anti-microbial activity Adhesion Low pro-inflammatory cytokine production
Intermediate: Minor subpopulation of $CD16^{+}$ subset	$CD14^{hi}$ $CD16^{+}$ $CD64^{+}$ $HLA\text{-}DR^{hi}$ $TNFR1^{hi}$ $TNFR2^{+}$	$CCR2^{lo}$ $CX3CR1^{hi}$ $CCR5^{+}$	Pro-inflammatory function Antigen presentation Trans-endothelial migration Actively produces TNF-alpha (in response to LPS), IL-1β and IL-6
Non-Classical: 15-10% of circulating monocytes	$CD14^{lo}$ $CD16^{hi}$ $CD64^{-}$ $TNFR1^{lo}$ $TNFR2^{hi}$	$CCR2^{lo}$ $CX3CR1^{hi}$	Anti-inflammatory, constitutively produces IL-1RA. Trans-endothelial migration Complement and FcR-mediated phagocytosis

Table 1.5 Human monocyte subsets.
Progressively, classical monocytes ($CD14^{Hi}CD16^{-}$) give rise to non-classical monocytes ($CD14^{lo}CD16^{+}$) through an intermediate step of $CD14^{+}CD16^{+}$ monocytes. Classical monocytes can be distinguished from the other two subsets by additional markers, such as CCR2 and CD64 and take part in the host's anti-microbial responses, such as adhesion to the endothelium, migration, and phagocytosis. Intermediate monocytes are characterized by their high expression of CCR5 and HLA-DR molecules and are involved in antigen processing and presentation. Non-classical monocytes express high levels of CX3CR1 and specialise in complement and FcR-mediated phagocytosis, trans-endothelial migration, and anti-viral responses. Adapted from Kapellos et al (2019).[167]

This description of monocytes is likely overly simplistic and more recent research has highlighted the heterogeneity and functional complexity of monocytes. Much of the work based on monocyte subsets has been extrapolated from murine models and accuracy of these functional descriptions has been questioned. However the similarities between mouse and human monocyte subsets indicates a conserved system and the studies conducted in mice are useful for understanding human monocyte biology.[160]

The process by which monocyte subsets evolve is also a topic of debate. Evidence from murine studies suggest that Ly6C^{Low} monocytes represent terminally differentiated cells from the differentiation of Ly6C^{Hi} monocytes in peripheral circulation.[162] A study by Patel *et al* (2017) supported this, demonstrating that developmental trajectory was similar in human monocytes. By using human in vivo deuterium labelling and studying monocyte kinetics, the authors demonstrated classical monocytes emerge first from marrow followed by sequential emergence of intermediate monocytes after 24 hours and non-classical monocytes over 7 days.[158] Other studies however suggest that monocyte differentiation occurs much earlier on during BM development with lineage tracing techniques providing evidence that monocyte progenitors become committed to specific fates prior to entering circulation.[170] It is thus probable that some monocyte progenitors are primed early to differentiate into cells with committed fates whilst other subgroups of monocytes are moulded by their external environment as they mature following BM release.

1.4.2 Macrophage ontogeny and phenotypes

Experiments using parabiosis and genetic modelling, tracing myeloid differentiation in mice have demonstrated that most steady state macrophage populations are seeded during embryogenesis from foetal precursors, with limited contribution from BM-derived monocytic precursors. [171-173]

There are several locations where these tissue-resident macrophages are found under homeostatic conditions in the lung, including the luminal side of the alveoli, the alveolar interstitium, bronchial sub-mucosa and vascular adventitia. The classification of pulmonary macrophages is dictated by their location under steady-state conditions.[173] Alveolar macrophages are established prenatally, derived from the yolk sac and are long-lived cells capable of self-renewal and proliferation following lung injury.[174] They are strategically sited within the airways and alveolar space, can interact with the epithelium and are subjected to exposures such as inhaled particles and manmade xenobiotics contained in the air. As a result they express a characteristic transcriptomic profile that enables them to fulfil specialised homeostatic and innate defence roles in the lung,[175] such as regulating wound healing, clearing apoptotic cells and cellular debris thereby limiting inflammatory responses, and their detrimental effects.[176]

Lung macrophage populations have also been classified according to their activation status. Studies first performed in the 1980s hypothesised that macrophage activity was limited to one of two phenotypes, “M1” or “M2”, and in an in vivo context, was analogous to the emerging Th1/Th2 concept that at the time was dominating T-cell biology.[177]

The administration of LPS or the Th1 cytokine IFNγ to bone marrow-derived macrophages, results in macrophages with enhanced microbicidal activity. Experiments have demonstrated macrophages secrete high levels of pro-inflammatory cytokines, including TNFα, IL-1, IL-6 and IL-23, chemokines such as CCL-2 and nitric oxide, important in host defence and early response to tissue injury.[178,179] These cells were designated M1 and are associated with high expression of receptors such as CD64, CD86 and CCR7. In contrast, administration of IL-4 and IL-13, prototypical Th2 cytokines, resulted in expression of anti-inflammatory cytokines, including IL-10 and TGF-ß, and displayed anti-inflammatory, reparative, and regulatory properties. These macrophages were labelled M2 and were recognised to play a significant role in tissue repair and immune cell modulation.[172,177,178]

Whilst the framework of M1/M2 polarisation has provided a useful system to study in vitro macrophage populations, this paradigm has been met with criticism in recent years. Indeed, in the context of pulmonary fibrosis, the description of macrophages as terminally differentiated M1/M2 cells may lack usefulness because the extent of activation is likely to be dynamic and dependent on the disease context.[98]

Ageing may also influence the cellular origin of the lung macrophage pool at a given time as whilst tissue-resident macrophages are capable of self-renewal, it is unclear if this ability continues through to advanced age. It is possible that these embryonically derived tissue-resident macrophages reach senescence resulting in replenishment by blood-born precursors.[180] In cases where lung injury is chronic or repetitive, as is hypothesised in IPF, tissue resident macrophage populations may become depleted, with reduced capability for self-renewal, and the contribution of monocyte derived macrophages could then become more significant and provide greater bearing on phenotype.

1.4.3 Monocytes and macrophages in inflammation, repair and fibrogenesis

Monocytes and macrophages play a crucial role in acute response to tissue injury, where they are known to produce cytotoxic and pro-inflammatory mediators, and remove invading microorganisms and cellular debris.[181] Following initial injury monocytes are rapidly recruited along chemokine gradients (such as CCR2) to the site of injury where they extravasate into the tissue and exhibit pro-inflammatory characteristics.[172] Pathogen-associated molecular patterns (PAMPs) and or Damage-associated molecular patterns (DAMPs) released by damaged or affected cells are sensed by macrophages. PAMPs and DAMPs activate inflammatory signalling pathways within the macrophages resulting in production of high levels of reactive oxygen species to help

counteract microbial activity, and to stimulate further recruitment of neutrophils, monocytes and other inflammatory cells to the site of injury.[156] Once acute injury has been controlled, macrophages start to display regulatory and reparative activities by suppressing inflammation and initiating wound repair by clearing cellular debris and secreting growth factors and mediators that provide trophic support for local tissue structures.[37]

Macrophages involved in post-inflammatory stages actively secrete factors which promote tissue repair. These include anti-inflammatory molecules such as IL-10 and TGF-ß, growth factors including VEGF and PDGF and matrix metalloproteinases.[137] These factors assist in fibroblast recruitment, proliferation and differentiation into myofibroblasts. As discussed previously, myofibroblasts are major effector cells in repair and secrete structural proteins and other matrix components leading to the deposition of collagen-rich scaffold over which endothelial and mesenchymal cells migrate to regenerate damaged tissue.[182]

Tissue repair is a tightly coordinated sequence of cellular events. Aberrations in this process can lead to delayed tissue regeneration or excessive collagen deposition. Macrophages, both MDM and tissue resident, serve as key players in orchestrating wound healing, undertake dual and opposing roles during different phases of repair, capable of promoting fibrosis and enhancing its clearance. Therefore, imbalance in function or number of these cell populations has been implicated in chronic diseases typified by excessive scar formation.[172,183,184] In support of this, multiple cytokines and chemokines, including TNFα, IL-1ß, CCL-18 and IL-13 that potentiate fibrosis in mouse models are elevated in BAL fluid from IPF patients.[185-187]

Whilst the phenotype of macrophages in IPF cannot be easily categorised, there is compelling evidence that these cells differ in comparison to healthy controls and contribute to progressive fibrotic disease.[183,188] Studies have demonstrated monocyte-derived alveolar macrophages recruited during injury contribute to the development of fibrosis, whilst tissue-resident alveolar macrophages do not.

Using a bleomycin murine model, in 2004 Okuma et al used C-C chemokine receptor 2-deficient (CCR2-/-) mice to explore the relationship between macrophage infiltration and MMP activity in the pathogenesis of pulmonary fibrosis.[189] CCR2 is a crucial receptor expressed by monocytes and critical for of macrophage trafficking. Between days 3-21 CCR2-/- mice BAL fluid demonstrated fewer macrophages than controls. Additionally, immunocytochemical staining demonstrated weaker expression of MMP-2 and MMP-9 in macrophages in BAL fluid from CCR2-/- mice.

Further studies have demonstrated that deletion of CD11b-expressing monocyte-derived macrophages ameliorates bleomycin-induced lung fibrosis. Using flow cytometry analyses,

Misharin et al (2014) characterised myeloid cells at specific time intervals following bleomycin administration.[190] At day 5 the number of alveolar macrophages had significantly decreased, whereas the number of interstitial macrophages and CD11b dendritic cells increased. Conversely, during the fibrotic phase (Day 21), the alveolar macrophage population in bleomycin-treated mice was greater in comparison to control animals, whereas the number of interstitial macrophages returned to control concentrations. The authors identified a new sub-population of alveolar macrophages during the fibrotic phase. These Siglec-F^+ alveolar macrophages expressed CD11b and elevated concentrations of CD11c, CD14, CD36, and CD64. The authors were unable to determine whether this new population of represented an expansion of Tissue-resident or monocyte-derived alveolar macrophages.

However, using a bleomycin murine model in which a monocyte tracking system to distinguish alveolar macrophage ontogeny during injury and over the subsequent life span of the animal, Misharin et al (2017) demonstrated that tissue-resident macrophages predominated within murine lung in steady-state. However, upon inducing injury either by administration of bleomycin or via an adenoviral vector encoding active TGF-β to induce fibrosis, the expanded alveolar macrophage population became heterogenous with monocyte-derived cells. The authors concluded that fibrosis was completely attributable to monocyte-derived alveolar macrophages. These monocyte-derived macrophages persisted for the lifetime of the mouse. When monocytes were depleted prior to administration of bleomycin in separate models, fibrosis was ameliorated, supporting the hypothesis that ontogeny is an important determinant of macrophage function in pulmonary fibrosis.[172,184]

Following this discovery, Reyfman et al performed single-cell RNA sequencing of explanted lungs from individuals receiving transplants for IPF and compared them to biopsy samples from lung transplant donors. Alveolar macrophages from fibrotic lungs expressed higher levels of genes associated with fibrosis, such as IL1RN (encoding IL-1RA). Furthermore, whilst alveolar macrophage populations from lung transplant donors were relatively homogeneous, there was substantial heterogeneity in the populations found in IPF lung explants.[191]

Further single-cell studies implicating aberrant macrophage populations in IPF come from Morse et al and Adams et al. Moore et al conducted single-cell RNA-sequencing of IPF lung explants and analysed differences between lower lobes reflecting late disease, upper lobes reflecting early disease and healthy donor lungs.[192] Discrete macrophage subpopulations in healthy and fibrotic lungs were identified. One expressing monocyte markers, and one highly expressing SPP1 and MERTK. $SPP1^{hi}$ macrophages in fibrotic lower lobes demonstrated highly upregulated SPP1 and MERTK expression in comparison to healthy donor specimens, and therefore potentially

implicating this expanded macrophage subpopulation in proliferation of fibrosis. Previous studies have implicated SPP1 in monocyte/macrophage proliferation.[193]

Adams et al performed single-cell RNA-sequencing analysis using 32 IPF patients and 28 controls observed a similar association. The authors performed an archetypal analysis to sample populations of classical monocytes, inflammatory macrophages, pro-fibrotic macrophages and control-enriched. They identified a gradual transition in features along the IPF-macrophage archetype, summarised by increasing expression profiles of SPP1 and ECM re-modelling genes such as MMP-9. At the terminus of the IPF-macrophage archetype, macrophages expressed CSF-1, suggesting the possibility of an autocrine feedback loop for macrophage differentiation, proliferation, and survival.[194]

Interestingly, single-cell studies exploring COVID-19 (coronavirus disease 2019) also identified pro-fibrotic monocyte-derived alveolar macrophage sub-populations in patients with end-stage disease. Conversely, tissue-resident alveolar macrophages were nearly depleted in COVID-19 patients in comparison to donor lungs from healthy controls.[195]

Thus, in the presence of lung injury, it appears that circulating monocytes recruited to the lung differentiate into macrophages, further augmenting the alveolar macrophage pool. These cells, which originate from bone-marrow precursors and thus from a separate ontogeny to tissue-resident macrophages, could possess a different cytokine repertoire, one that is perhaps more pro-inflammatory and pro-fibrotic.[184]

Findings from clinical studies also support this. In a cross-sectional analysis of an IPF cohort, Fraser et al previously demonstrated monocyte counts (as a proportion of peripheral blood mononuclear cells) are proportionately higher in individuals with IPF compared with aged-matched healthy controls, and correlated with extent of lung fibrosis measured on CT and inversely with forced vital capacity.[196] This is matched by a large scale epidemiological studies demonstrating elevated monocyte counts in peripheral blood correlate with worse survival outcome in patients with fibrotic lung disease.[197] Further prospective studies will need to be conducted to explore if higher monocyte count reflects expanded monocyte sub-populations and if these are associated with adverse clinical outcomes in IPF.

1.4.4 CD64^{hi} monocytes association with type I interferon signalling

Monocytes can also be defined by relative expression of other cell surface receptors including CD64. CD64 is an Fcγ receptor (FcγRI) that demonstrates high affinity for circulating IgG; suggesting a

pivotal role in initiating cellular effector responses.[198,199] CD64 mediates phagocytosis of antibody-bound cells, internalises immune complexes, stimulates inflammatory cytokine production and propagation of pro-inflammatory responses.[200] In health, it is constitutively expressed on classical monocytes and macrophages and can be induced on neutrophils under inflammatory conditions and in sepsis.[201-203]

Phenotyping of CD64 has been explored previously. The differential screening of M1-specific and M2-specific surfaces markers on CD64 human macrophages after stimulation with either IFNγ or LPS (M1) identified that both CD14 and CD64 to be upregulated on human M1-phenotype macrophages. In contrast, exposure to IL-4 (M2), resulted in downregulation of both CD14 and CD64.[204]

Several in vitro, in vivo and ex vivo studies have identified upregulated levels of CD64 on activated monocytes and macrophages in chronic inflammatory diseases. In clinical studies, monocyte CD64 expression has been shown to correlate with indices of disease activity in rheumatoid arthritis,[205] as well as global markers of inflammation such as the C-reactive protein in psoriatic arthritis.[206,207] One of the mechanisms by which CD64 exerts pathogenic responses is thought to be through its interaction with immune complexes that trigger a pro-inflammatory response. The release of cytokines such as Monocyte chemotactic protein-1 and macrophage inflammatory protein-1 following activation of the FcγRI receptor results in recruitment and infiltration of monocytes and immune effector cells that, if dysregulated, can accentuate tissue damage.[208] Its role as a potential biomarker to monitor treatment response in rheumatoid arthritis has also been explored. A study conducted by Matt et al, of patients with active seropositive rheumatoid arthritis, measured CD64 monocyte expression (flow cytometry) and serum immunoglobulin expression pre- and post-treatment with methotrexate and prednisolone. The authors identified that rheumatoid arthritis monocytes expressed greater levels of CD64 and cell surface-bound IgG than healthy control monocytes. Furthermore, patients deemed to have beneficial response to anti-rheumatic treatment demonstrated subsequent reduction CD64 and secretion of immune-complex mediated TNFα.[207]

CD64 is also implicated in development of lupus nephritis. Cross-linking of the Fcγ receptor on monocytes by IgG-containing immune complexes is a key step in immune-complex-mediated nephritis.[208] Here, increased CD64 expression is thought to enhance monocyte chemotactic and inflammatory ability and enhance immune cell recruitment, thereby perpetuating renal injury.[209] Furthermore, it has become increasingly clear that the auto-antibody responses characteristic of Lupus are linked to overproduction of type I interferon signalling.[210,211] Li et al, using ex vivo studies, identified that CD64 expression on circulating $CD14^{+}$ monocytes (MFI using flow cytometry) from

patients with systemic Lupus correlated highly with interferon stimulated gene (ISG) expression (measured with PCR). In vitro, CD64-monocyte expression was inducible by co-culture with type I interferon (IFNα). The authors concluded that CD64 monocyte expression demonstrated utility as a surrogate marker for type I IFN activity, but this has not been further explored in clinical studies.[212]

Interestingly, a small longitudinal analysis of the COMET cohort comparing monocyte profiles in patients with stable and progressive IPF (defined by decline of FVC >10%, TLCO >15%, AEIPF or death) identified greater % CD64-expressing intermediate monocytes were associated with poorer survival outcomes.[213]

Earlier work in our laboratory examined the phenotype of monocytes in IPF.[214] These studies not only identified that $CD14^{hi}$ monocyte counts are proportionally higher in IPF patients vs healthy controls, but also identified increased CD64 monocyte expression (MFI measured by flow cytometry). CD64 expression also correlated with extent of fibrosis measured on CT.[214] Gene set enrichment analysis for differentially expressed genes in a subset of IPF and controls identified the most significantly enriched gene sets were representative of interferon (IFN) signalling. Linked to this, an amplified type I interferon response when stimulated ex vivo with IFN-β_1 was also observed, measured as enhanced transcription of ISGs (qPCR), and the level classical ISGs (MX1) correlated with monocyte CD64 mRNA. These findings imply CD64-expressing monocytes in individuals with IPF are primed to respond to type I interferon stimulation and suggest that increased CD64 expression in IPF is linked to type I interferon signalling.[214]

Interferons are a family of cytokines that serve as a key antiviral host defence. Simply, they 'interfere' with, and restrict viral replication. Interferons were first described over 60 years ago by Isaacs and Lindemann as a key mediator responsible for the phenomenon of viral interference – the ability of infection with a virus to induce resistance to subsequent infection from a different virus.[215-217] Once a virus has invaded a cell, a host defence-mediated response is triggered, which involves induction of this group of a pleiotropic cytokines – which promote cellular resistance to viral infection in both host and adjacent cell populations.[215] Three different interferon subtypes exist based upon ability to bind cell surface receptors; Types I, II and III, and these share a common purpose of modulating the functions of the host immune system and local immune responses to prevent viral replication and propagation of infection.[218]

The type I interferon pathway is triggered by sensing of self- or viral nucleic acids and bacterial pattern recognition molecular patterns (PAMPS). Upon release from the host cell, type I interferon signals via the ubiquitously expressed heterodimeric receptor IFNAR on self and adjacent cells. This activates JAK-STAT signalling pathways and induction of downstream signalling via transcription factors including phosphorylated-signal transducer and activator of transcription (pSTAT).[219] This

results in the transcription of hundreds of specific interferon stimulated genes (ISGs), which propagate immune responses.[218] This results in transcription of ISGs (300+) that directly interfere with key steps of the viral life cycle, and upregulate innate and adaptive immune pathways including pro-inflammatory cytokine production and MHC class I expression (Figure 1.11).[215,220]

Whilst IFN signalling establishes a beneficial mechanism of host defence against acute infection, dysregulation of type I interferon signalling is associated with pathological conditions including autoimmune diseases such as rheumatoid arthritis, SLE and scleroderma – in all of which pulmonary fibrosis is a recognised sequelae.[221,222] Enhanced responsiveness of monocytes to type I interferon is therefore a potentially significant finding in IPF. One consequence of having a primed type I interferon pathway is, when stimulated, a magnified downstream innate and adaptive immune response.[223] In IPF, such dysregulation could result in an over-exaggerated downstream immune responses early on in disease, resulting in widespread and perpetuated alveolar injury, further hampered by the aberrant wound-healing response observed in IPF individuals.[37]

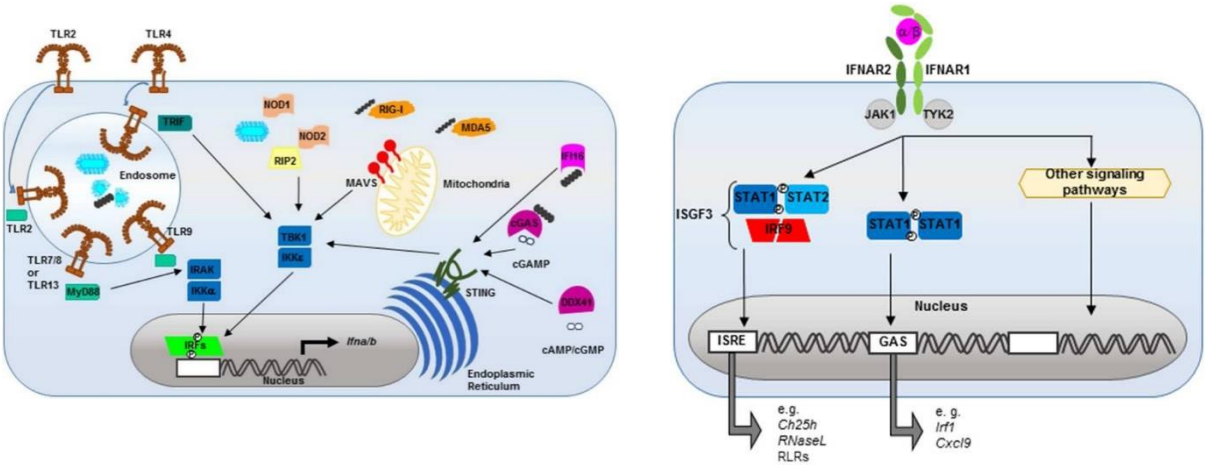

Figure 1.11 Signalling pathways of TI IFN induction
Left: Recognition of bacterial and viral products detected by membrane (TLR2 and 4) and cytosolic receptors (RIG-I, MDA-5, STING and NOD). IRF kinases activate IRFs allowing translocation into the nucleus and IFN expression. Right: Binding of Type I IFN to IFNAR1-2 heterodimer activates JAK1 and TYK2 to phosphorylate STAT transcription factors. Dimerisation of STAT1-2 recruits IRF9 and forms the ISGF3 complex that promotes expression of genes containing the ISRE promoter sequence. With permission taken from Boxx et al (2016).[215]

1.4.5 Observational studies exploring monocytes in IPF

Many landmark findings have been established using animal models, which do not fully recapitulate the histological UIP process that characterises IPF.[224] Many of the novel immune cell populations, surface markers, cytokines and genetic discoveries have correlated with adverse clinical outcomes in IPF. Collectively these have enriched our mechanistic understanding of IPF.

Furthermore, the utility of these as potential biomarkers that could stratify patients of greater risk is commonly described.[225] Despite this, many that have been investigated are expensive, relatively complex, not widely available in clinical settings, and have not been validated in large scale clinical studies. Essentially there remains an unmet need for biomarkers that can easily risk stratify and guide a personalised approach to patient management.[226]

In the COMET study, a relatively small prospective study exploring association of immune cell phenotypes with IPF, Moore et al identified $CD14^{hi}$ monocytes were associated with disease progression.[213] In a cross-sectional study, Fraser et al observed that circulating monocytes in IPF cases displayed a higher M2:M1 ratio profile compared to healthy controls. When monocytes were differentiated to monocyte-derived macrophages these displayed greater pro-repair M2-like features which may contribute to the aberrant repair process in IPF.[196]

At the beginning of my research, Scott et al, published the findings of a large retrospective epidemiological study (involving 7500 patients) in which the authors explored absolute blood monocyte count, measured from standard full blood count analysis. They identified a monocyte count $>0.95x10^3/\mu l$ with adverse clinical outcomes in IPF.[197] Shortly after this Fraser et al reported greater monocyte count, as a proportion of PBMCs, correlated with CT fibrosis score. The association between blood monocyte count with mortality was also observed in analysis of a refined cohort of patients from the Australian IPF registry.[227]

Kreuter et al conducted a similar study using existing data obtained from previous clinical trials in IPF. In pooled data from ASCEND,[14] CAPACITY,[47] and INSPIRE,[34] The authors demonstrated that stratification by monocytes $>0.60x10^3/\mu l$ and $\geq 0.95x10^3/\mu l$ conferred similar 1-year risk of mortality.[228] Therefore it was proposed a cut-off threshold of $>0.60x10^3/\mu l$ could prognosticate for adverse outcome. However, this was not observed in validation studies.[229]

Findings from these large-scale epidemiological studies are providing new knowledge, complementing current basic science and mechanistic understanding of intricate monocyte biology in IPF. Further studies are required to explore the utility of whole blood monocyte count to accurately prognosticate and identify individuals of greater risk. Despite this, these findings are supportive of association between adverse outcome in IPF with absolute monocyte count. Studies directly exploring if higher blood monocyte count reflects expansion of specific monocyte cell populations are limited and are worthy of validation in prospective studies.

1.5 Project aims

My project aims to explore the association between monocytes measured in peripheral blood with progression of mild ILD to IPF, and disease severity of IPF measured by qualitative and quantitative metrics. I will also explore association between neutrophils and lymphocytes with these outcomes.

Hypothesis

I hypothesise that absolute monocyte count predicts progression of fibrosis, decline in lung function and mortality independent of disease severity and extent of fibrosis.

Overall study plan

I will test my hypothesis in the following studies:

1. **Study 1; Monocytes association with IPF progression**
   Explore association between monocyte levels in peripheral blood and (i) progression of mild ILD (the "indeterminate for UIP" CT pattern) to IPF and (ii) progression of established IPF (decline in lung function) in a single centre cohort in the Oxford ILD service.

2. **Study 2; Blood leukocyte association with progression of interstitial lung abnormalities**
   Explore association between blood leukocytes (monocytes, neutrophils, and lymphocytes) with (i) radiological progression and (ii) mortality of pre-fibrotic interstitial lung abnormalities in a single centre cohort of patients identified within the Oxford radiology database.

3. **Study 3; Blood leukocyte association with radiological progression of lung fibrosis in IPF**
   Explore association between blood leukocytes with radiological progression of fibrosis as quantified by automated CT in a single centre cohort in the Oxford ILD service.

Chapter 2 Methods and study cohort descriptions

2.1 Study recruitment and cohort descriptions

2.1.1 Study 1: Monocytes and IPF progression

This was a retrospective cohort study. Consecutive patients were seen in a clinic setting at the Oxford Interstitial lung disease unit between January 2013 and December 2017. Cases included were either diagnosed with IPF or displayed a radiological pattern of Possible UIP, in absence of other known causes and in accordance with the 2011 ATS/ERS/JRS/ALAT clinical practice guideline.[230] Cases were re-categorised in accordance with the 2018 ATS/ERS/JRS/ALAT clinical practice guideline, to identify cases with the indeterminate for UIP CT pattern (iUIP).[8]

In total 176 patients were included. Leukocyte data obtained from full blood count analysis by blood draw either (i) closest to CT or (ii) nearest to first clinic visit were captured. Cases were split into 2 cohorts and analysed separately. **Cohort 1a** comprised of cases demonstrating iUIP on first CT scan. The remainder of cases displaying either probable or definite UIP on first available CT scan were grouped within **Cohort 1b**. Baseline and serial lung function, and CT scan details were documented. Demographic profiles were listed and time intervals between baseline and important clinical outcomes of mortality, relative decline in absolute FVC and change in radiographic appearance between CTs were calculated. Cases were followed up until a censoring date of 1st August 2019.

2.1.2 Study 2: Blood leukocytes and progression of interstitial lung abnormalities

This was a retrospective study reporting association between blood leukocytes and radiographic progression of cases with potentially mild forms of ILD. Subjects that had undergone all-indication CT thorax at Oxford University Hospitals NHS trust were identified from electronic records. Specified search criteria selective for mild fibrotic interstitial lung abnormalities were applied to the local radiology database and a text-based search of CT scan reports was conducted.[22] Subjects were included if they had undergone a thoracic CT and reported as matching search criteria, between January-2015 and December-2020 and were between 45 and 75 years of age. Cases with CT report descriptions of early fibrotic ILA (EF-ILA), traction bronchiectasis or honeycombing on first CT scan were identified.

In total 1259 cases with EF-ILA were included in Study 2. Leukocyte data obtained from full blood count analysis closest to first CT scan was captured. Co-morbidity profiles were obtained by cross referencing international classification of disease (ICD-10) codes to electronic records. The proportion of subjects undergoing at least 1 repeat CT was captured and findings from these scans was recorded. Specifically, outcomes of radiographic progression and mortality were recorded and time to each of these events were calculated. Cases were followed up until a censoring date of 1st April 2021.

2.1.3 Study 3: Blood leukocyte association with radiological progression of lung fibrosis in IPF

This was a retrospective analysis of a cohort of patients who attended the Oxford Interstitial Lung Disease Service between September 2016 and November 2021. All patients had an MDT diagnosis of IPF.[8] 171 cases with a non-contrast, supine, volumetric HRCT scan were included in the study.

I collected Leukocyte data obtained from full blood count analysis closest to first CT scan. Patient demographics (gender, age), co-morbidity profiles, time-interval between HRCT scans, available pulmonary function tests (PFTs) closest to the selected CT scans, and all available CT reports were recorded. The CALIPER algorithm was used to quantify extent of fibrosis and pulmonary vessel structures. Clinical outcome measures including disease progression (extent of CT features, escalating UIP category and time scale of progression) and survival were analysed. Cases were followed up until 1st February 2022.

2.2 Qualitative CT assessment

A thoracic radiologist with a sub-specialty interest in ILD reviewed each patient's cross-sectional imaging at MDT to provide a radiological diagnosis that correlated with the patients' clinical history (Studies 1 and 3). CT interpretation was therefore unblinded. All CT abnormalities were defined using standard Fleischner-based terminology and according to 2018 IPF guidelines.[8,22]

Briefly, 'definite UIP' pattern was defined by the presence of a basal-predominant sub-pleural reticular abnormality and honeycombing with or without traction bronchiectasis. Probable UIP pattern was defined as basal and sub-pleural predominant reticulation and traction bronchiectasis or bronchiolectasis with or without mild ground glass opacification (GGO). 'Indeterminate for UIP'

was defined as subtle reticulation in the presence or absence of mild GGO and with a basal / sub-pleural predominant distribution.[8]

GGO is often referred to as indistinct increase in lung density, not associated with obscuration of the surrounding vessels or bronchial walls. Reticulation is defined as multiple interlacing linear opacities, which represent intralobular septal thickening and interstitial thickening. Traction bronchiectasis represents irregular bronchial and bronchiolar dilatation caused by surrounding retractile pulmonary fibrosis. Here non-tapering airways are often surrounded by abnormal parenchyma (GGO and reticulation). Honeycombing was defined as clustered cystic air spaces, typically 3–10 mm in diameter, with a sub-pleural and basal distribution.[22]

For assessment of progression of fibrosis in subjects with follow-on CTs, these CTs were classified as either 'non-progressive' or 'progressive' based upon comparison to first CT. Cases were defined as 'progressive' if follow-on CT demonstrated either (i) visual (qualitative) increase in volume of disease (i.e. existing parenchymal features) or (ii) progression of UIP pattern. 'Non-progressive' cases were defined as unchanged parenchymal features in follow on CT scan either in terms of (i) extent of these features or (ii) unchanged UIP pattern.

2.3 Quantitative CT analysis

The CALIPER (Computer-Aided Lung Informatics for Pathology Evaluation and Rating) lung texture algorithm (v2.1) was acquired from IMBIO, Minneapolis, USA (www.imbio.com) and installed onto the syngo.via platform (www.siemens-healthineers.com), a multi-modality reading tool integrated into the local hospital trust system ("client-server platform"). Non-contrast, supine, volumetric, high resolution CT scans (0.625mm slice thickness at an interval of 0.625mm) for appropriate subjects were selected and the Digital imaging and communications in medicine (DICOM) formatted images were uploaded into syngo.via from the local picture archiving and communications system (PACS).

Initial data processing steps included (i) lung segmentation from adjacent thoracic and chest wall structures, (ii) separation of right and left lungs and (iii) airway segmentation. Lung segmentation is performed using adaptive density-based morphological approach, and airway segmentation involved density thresholding. Right and left lungs were then segmented into upper, middle, and lower zones (Figure 2.1a). The carina was used as a landmark to identify the lower boundary of the upper zone. The remaining two thirds of each lung were equally subdivided into the middle and lower zones for each lung. Total right and left lung volumes were calculated.

The platform provided data output of DICOM series with a colour-coded overlay reflective of parenchymal abnormality and overall disease extent. At this point I assessed each series. This was used as a quality control check for each scan. Data for each case was exported as a PDF document (Figure A.6) and tabulated in a master database for analysis.

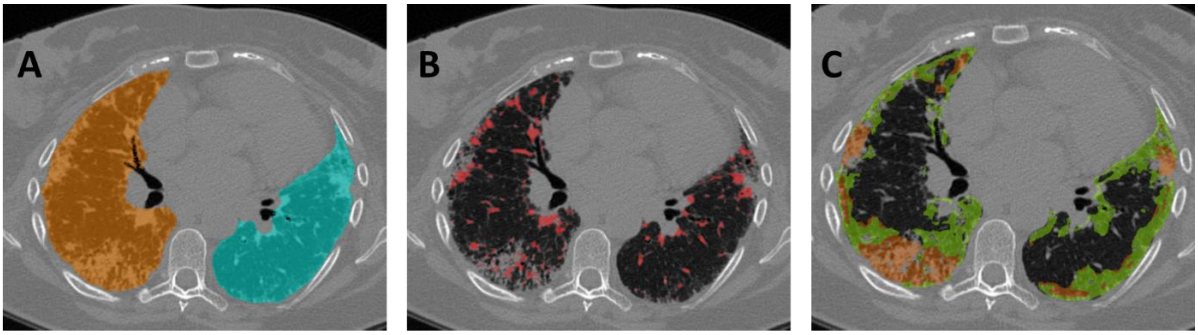

Figure 2.1 Axial slices of CALIPER lung text algorithm
(A) lung labels, (B) pulmonary vessel labels, (C) lung texture analysis map taken from CT scan of exemplar patient with IPF.

2.3.1 Pulmonary vessel quantification

To quantify pulmonary vessel volume (PVV), pulmonary vessels were extracted from lung parenchyma using a multi-scale tubular structure enhancement filter. An algorithm determined likelihood that each voxel was connected to a dense tubular structure representing a blood vessel or blood vessel-related tubular structure (Figure 2.1b).[231] This assessment excluded large vessels at the hilum of the lung. The PVV was calculated as an absolute volume (cm^3) and expressed as a relative percentage of total CALIPER-derived lung volume. Total and regional volumes were calculated. Regional volumes were expressed as upper, middle, and lower zone volume and calculated from sum of respective right and left lung zonal volumes.

2.3.2 Pattern evaluation

After extracting the pulmonary vessels, parenchymal tissue type classification was applied to 15x15x15 voxel volumes of interest using texture analysis and computer-based algorithmic interpretation of volumetric histogram signature mapping features as previously described above (Figure 2.1c).[82] CALIPER evaluation of CT data included classification and quantification of each volume of interest into one of six radiological parenchymal categories: normal lung, hyperlucent, ground glass opacity (GGO), Reticular, honeycombing. Volumes for each parenchymal feature

were expressed as a relative percentage of CALIPER-derived (i) total lung volume or (ii) zonal lung volume. Total lung fibrosis (%) represented the sum of GGO, reticular and honeycomb percentages.[94]

2.4 Lung function tests

Spirometry (FEV1 and FVC) and Transfer Factor of the Lung for Carbon Monoxide (TLco) were captured closest to CT and clinic visit. Values are expressed as absolute (FVC; litres, TLco; mmol/min/kPa) or as percentage of predicted value. All available serial measurements (including date of test) were collated to determine trend. Lung function trends were calculated as relative (%) change from baseline absolute value. Each follow-up FVC and TLco value (absolute) was divided by the baseline value and multiplied by 100.

Where specified, FVC decline is described either as (i) rate of (annualised) decline < or ≥10% or (ii) time to a relative FVC decline ≥10%. Annualised FVC decline was calculated as relative FVC decline (%) divided by time interval (months) between respective FVC measurements, then multiplied by 12. A measured reduction in absolute forced vital capacity (litres) >10% per year was considered clinically significant and representative of physiological decline.[25]

2.5 Ethics approval

All studies were part of a larger study to examine immune factors associated with disease progression in IPF. Ethical approval 14/SC/1060 was obtained from the Health Research Authority and South-Central National Research Ethics Service (appendix).

2.6 Statistical analysis

Data is expressed as absolute values, relative percentages, means (with standard deviation), medians (interquartile range) or by dichotomised value where stated. Tests for normality of data was performed using a D'Agostino & Pearson test and following this the difference between groups was analysed using unpaired t-tests or Mann-Whitney test for respective parametric and non-parametric analysis. Multiple data sets that were not normally distributed were

analysed using Kruskal-Wallis test with Dunn's correction for multiple comparisons. Contingency tests (Fisher's exact test of significance for comparing two groups or Chi Squared for comparing 3 or more groups) were used to assess inter-group differences in categorical data. Reported p values were two-sided and a p value <0.05 was considered significant.

Pearson r correlation was used to assess strength and direction of association between blood leukocyte measurement with continuous measures of disease severity. Where stated Cox proportional hazard models were performed to explore leukocyte associations with clinical outcomes of disease progression or mortality as described in relevant chapters. Models were constructed in either a univariate or multivariate setting. Hazard ratios generated from continuous covariates represents the change in the risk of outcome if the covariate in question changes by one unit. Hazard ratios generated for dichotomised covariates represented the risk of achieving outcome if the covariate is present. Specific time variables, covariates included in multivariate regression models and model outcomes are described in each chapter.

Kaplan-Meier analysis was used for time-to-event analysis to explore association between dichotomised variables with specified clinical outcomes using either categorical or dichotomised variables where stated.

All analyses were performed using GraphPad Prism version 9.00 for windows (La Jolla, California, USA, www.graphpad.com) or SPSS version 26 (IBM Armonk, NY, USA).

In summary, I tested my hypothesis by performing 3 studies. In Study 1, I explored association between blood leukocytes (monocytes, neutrophils, and lymphocytes) with (i) progression of the Indeterminate of Usual interstitial pneumonia (iUIP) CT pattern to IPF and (ii) progression of established IPF. In study 2, I explored blood leukocyte association with progression of early fibrotic-interstitial lung abnormalities (EF-ILA) and in Study 3, I explored leukocyte association with radiological progression of fibrosis per se quantified by automated CT (CALIPER). Patients were enrolled from the Oxford Interstitial lung disease (ILD) service or Oxford Radiology service. Association between leukocytes and disease severity were explored for qualitative CT, quantitative CT and pulmonary function tests. Pearson r correlation was used to assess strength and direction of association between blood leukocyte measurement with continuous measures of disease severity. Disease progression was defined differently in each study as described in Chapters 3, 4 and 5 methods. Cox proportional hazard models were performed to explore leukocyte associations with clinical outcomes of disease progression or all-cause mortality.

Chapter 3 Monocytes and IPF progression

3.1 Introduction

In recent years there has been mounting evidence implicating monocytes in the development of IPF.[98,197] Earlier work in our laboratory identified greater monocytes levels (expressed as % of PBMCs) in patients with IPF compared to healthy controls.[214] The utility of peripheral blood monocyte measurements to prognosticate IPF in retrospective observational cohorts has shown promise.[197,227,228,232] However, there is considerably less literature describing association between monocytes, and other blood leukocyte components, with extent of fibrosis and disease severity in IPF.

I was interested in exploring if this association extended to (i) extent of disease progression during follow up and (ii) if there was an association between monocytes and early / sub-clinical IPF, which as per the 2018 IPF guideline, is described as Indeterminate for UIP.[8]

Indeterminate for UIP (iUIP) is a CT pattern which is best described in the 2018 IPF guideline as subtle reticulation in a basal and sub-pleural distribution with or without accompanying ground glass opacification and crucially an absence of any CT features that could be classified as inconsistent with UIP.[8] Clinically this represents early parenchymal change and is an important finding on CT that identifies individuals which potentially could progress to IPF.[233] In the appropriate clinical context definite and probable UIP CT patterns possess sufficient positive predictive value to diagnose IPF, without the requirement of histological confirmation.[234] However, the Indeterminate for UIP CT pattern lacks sufficient positive predictive value to diagnose IPF. Subsequently, clinical guidelines support the use of lung biopsy in this setting to obtain diagnosis.[8] In up to 30% histology reveals UIP,[235] thus a proportion of cases will progress to a clinical diagnosis of IPF. Large observational cohorts have identified a mortality risk associated with iUIP.[236] Factors associated with progression however are not fully established.

Furthermore, I was also interested in exploring whether any observed association between blood monocytes and adverse clinical outcomes also extended to other peripheral blood leukocytes (neutrophils, and lymphocytes), and indexes derived. Exploration of absolute leukocyte counts and leukocyte indexes with clinical outcomes in IPF is limited.

3.2 Hypothesis and aims

I hypothesised that monocyte counts are elevated in the early stages of IPF. To test this hypothesis, using a clinical IPF database I undertook the following objectives:

I. Determine long-term clinical outcomes of the Indeterminate for UIP CT feature
II. Explore potential drivers for progression of the Indeterminate for UIP to IPF
III. Explore association between blood leukocytes and derived indexes with disease progression and mortality in patients with an already-established diagnosis of IPF

3.3 Methods

3.3.1 Study population

To explore for association between blood leukocytes with iUIP, IPF and disease progression I performed a retrospective analysis of a cohort of patients who attended the Oxford Interstitial Lung Disease Service between January 2013 and December 2017. I collected the following data: patient demographics (gender, age, smoking status, comorbidities), year of diagnosis of IPF (by ILD MDT - 2018 guidelines), all available pulmonary function tests (PFTs) and all available CT reports detailing UIP category and time-interval between CT scans. Clinical outcome measures including disease progression (extent of CT features, escalating UIP category and time scale of progression) and survival were obtained.

To answer my hypotheses, I sub-divided the Study 1 dataset into two separate cohorts based on classification of first available CT; Cohort A (iUIP) or Cohort B (Probable or Definite UIP) and analysed these separately.

3.3.2 Radiological progression

A thoracic radiologist with a sub-specialty interest in ILD reviewed each patient's cross-sectional imaging at MDT to provide a radiological diagnosis that correlated with the patients' clinical history. All CT abnormalities were defined using standard Fleischner-based terminology and according to 2018 IPF guidelines as described in section 2.2.[8,22]

Radiologist reports from all available thoracic CTs for these patients and up to August 2019 were analysed and cross checked against reports from ILD MDT. CT scans that were performed before

publication of the 2018 IPF guideline were reported in accordance with the 2011 IPF guideline.[7] I re-categorised these CTs according to the 2018 IPF guideline to identify those with iUIP pattern.[8]

In both cohorts A and B, follow-on CTs were classified as either 'non-progressive' or 'progressive' based upon comparison to first CT as described in section 2.2.

3.3.3 Pulmonary function tests

Collation of lung function data was previously described in Section 2.4. Briefly, FEV1, FVC, TLco and CPI were captured closest to CT (cohort A) or clinic assessment (cohort B). Annualised lung function trends were calculated from lung function tests recorded closest to first and second CT scan. FVC decline is described as either (i) annualised decline < or ≥10% or (ii) time to relative decline in FVC ≥10%.

3.3.4 Blood leukocyte measurement

Neutrophil, lymphocyte, and monocyte levels performed using standard 'full blood count' analysis within either 4 months of initial CT demonstrating iUIP (Cohort A) or within 4 months of initial ILD assessment (Cohort B) were included. This timeframe was chosen in accordance with previously published studies.[197]

Blood leukocyte measurements were used to derive values for neutrophil-lymphocyte ratio (NLR),[237] monocyte-lymphocyte ratio (MLR),[238] and systemic inflammation response index (SIRI) for the IPF cohort (Cohort B). Briefly, NLR and MLR were calculated the ratio of neutrophil or monocytes (numerator) to lymphocytes (denominator). SIRI was calculated by dividing the product of neutrophil and monocyte values with lymphocyte values.[239]

Leukocyte values were collated as continuous or discrete variables. Discrete variables were categorised by median value, or by high or low absolute blood leukocyte (monocyte; < or ≥0.9x10^3/μl, neutrophils; < or ≥7.5x10^3/μl, lymphocytes; < or ≥1.0x10^3/μl) according to local laboratory reference range. Cases were also dichotomised by median leukocyte index values.

3.3.5 Statistical analysis

Data is expressed as absolute values, relative percentages, means (S.D.), medians (IQR) or by dichotomised value where stated. Normality testing and analysis of inter-group differences conducted as described in section 2.6.

Cox proportional hazard models (univariate and multivariate) were used to assess associations between baseline variables and the study outcomes. Time-dependent effects were included in the model, and proportional hazards assumption was tested for each. Hazard ratios generated for continuous covariates represent the change in the risk of outcome if the covariate in question changes by one unit. Hazard ratios generated for dichotomised co-variates represents the risk of achieving outcome if the co-variate is present.

In Cohort A, Cox proportional hazard (PH) modelling was used to analyse progression of iUIP. This was performed in two settings: (A) assessing all events of iUIP progression on follow-on CT regardless of whether this proceeded to IPF and (B) restricting the patients to only those that did progress to IPF during the follow up period. Univariate and multivariate analyses were performed with the following variables [age, gender, FVC%, smoking status, monocyte, lymphocyte, and neutrophil counts].

For Cohort B, a Cox PH model was employed to test association with blood leukocytes and derived indexes against the clinical outcomes of FVC decline >10%, hospitalisation and survival events.

Kaplan-Meier analysis (Log rank test of significance) was performed to evaluate time-to-event from (Cohort A) first CT to a diagnosis of IPF and (Cohort B) time for cases dichotomised by leukocyte values.

3.4 Results

230 individual patients were identified. 48 (21%) cases with iUIP pattern on first CT were allocated into Cohort A. In 102 (44%) cases initial CT demonstrated probable UIP pattern and 80 (35%) cases demonstrated definite UIP, and these were allocated into Cohort B.

3.4.1 Cohort A: iUIP dataset

Of the 48 patients allocated into cohort A, Mean age (S.D.) was 75.6 (8.2) years; 73% male. 32 patients had at least one follow-on thoracic CT from the first scan. In the 16 cases that did not have a follow-on scan (and therefore could not be categorised into progressors or non-progressors), 13 cases were discharged after mean of 2.1 years as were clinically stable and 3 did not require a second CT scan.

Of the 32 patients that had follow-on scans, 9 (28%) patients showed no change in CT appearance over a mean length of 2.1 years ±0.9 (S.D.) and were classified as non-progressive iUIP. The most frequent indications for follow-on CT in this group was for nodule surveillance (64%) and to identify any further radiographic progression of iUIP (14%). In 23 cases (72%) follow-on CT demonstrated progression. 6 demonstrated an increase in the extent of iUIP but no change in pattern over 3.1 ±0.8 yrs; 11 progressed to probable UIP over 3.8 ±1.6 years and 6 to definite UIP over 4.1 ±2.4 yrs. For the 23 ‘progressors’ the most frequent indication for follow on CT was to investigate worsening symptomatic breathlessness (52%) and decline in lung function parameters (28%). All those with 'definite' or 'probable UIP' were confirmed to have a clinical diagnosis of IPF by our ILD MDT. 5 cases underwent surgical lung biopsy for definitive diagnosis.

Therefore 53% (17 of 32) of our evaluable iUIP cohort (i.e., those with iUIP which had follow-on CTs) or 35% (17 of 48) of all iUIP progressed to a clinical diagnosis of IPF over a mean of 3.9 (1.9) years.

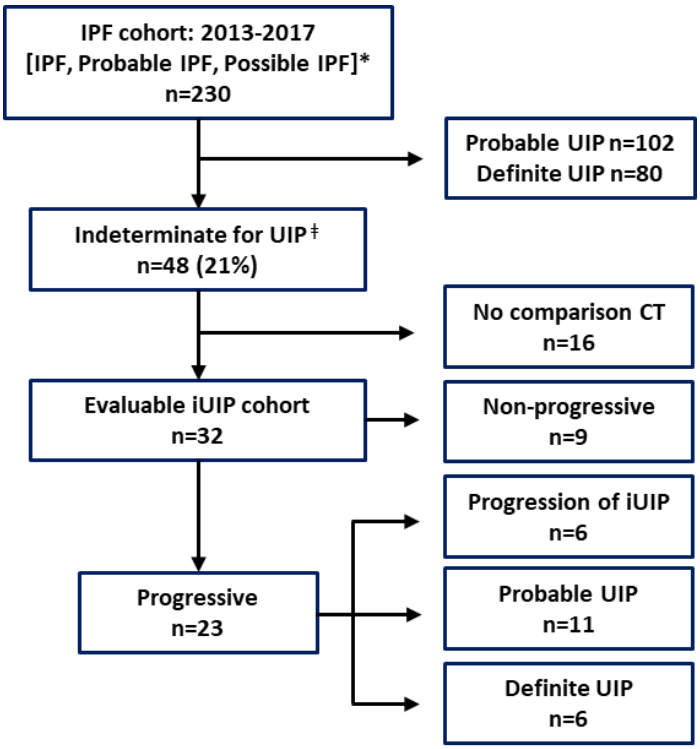

Figure 3.1 Flow diagram of radiographic progression of iUIP (Cohort 1A).
*Clinical diagnosis as per 2011 IPF guideline.[7] ‡; as per 2018 IPF guideline.[8]

Demographic data, physiological indices, comorbidity profiles and imaging features in the progressive and non-progressive iUIP groups are shown in Table 3.1.

Average age at initial CT was slightly lower in the 'progressive iUIP' compared to 'non-progressive' group - 72.3 (±8.6) vs 76.7 (±6.2) years (p=0.28 unpaired t-test). A male predominance was observed across all groups. This was higher in the 'progressive group' compared to the non-progressive group; 75% vs 66%.

Smoking history was higher in the progressive vs non-progressive iUIP groups; 86% vs 67% but not statistically significant; p=0.32 (Fisher's Exact test). In both progressor and non-progressor groups there was a high proportion of additional comorbidities; we observed a higher proportion of respiratory (40% vs 11%) and cardiac co-morbidities (85% vs 66%) in the progressive iUIP group.

A higher proportion of CT scans in the progressive iUIP group displayed ground glass opacification; 18 patients in the progressive arm had at least 1 CT scan demonstrating GGO (78%) vs 3 (33%) in the non-progressive arm (OR 7.2, 95% CI 1.2-31.5, p=0.035). Emphysema was present on CT scan in 4 of 23 cases (17%) with progressive iUIP, none in the non-progressive group.

	Non-progressive iUIP	Progressive iUIP	Odds ratio (95% CI)	P value
Demographics				
n	9	23		
Male	6 (66%)	18 (78%)	1.8 (0.37- 8.34)	P=0.655
Female	3 (33%)	5 (22%)	0.6 (0.12-2.64)	--
Age at first CT showing iUIP	76.7 (±6.2)	72.3 (±8.6)		P=0.277
Smoking status				
Never smoker	3 (33%)	3 (14%)	0.31 (0.06-1.70)	
Ex-Smoker	6 (67%)	19 (86%)	3.2 (0.59-15.9)	P=0.320
Additional Co-morbidity				
n (cases)	9 (100%)	20 (90%)		
Respiratory	1 (11%)	8 (40%)	5.3 (0.61-65.6)	p=0.201
Cardiac	6 (66%)	17 (85%)	1.3 (0.27- 7.52)	p>0.999
Diabetes	4 (44%)	3 (15%)	0.2 (0.05-1.17)	p=0.158
Other	4 (44%)	13 (65%)	2.3 (0.42-9.62)	p=0.422
Lung function parameters				
TLCO (mmol/min/kPa)	5.0 (±0.9)	5.3 (±1.7)	--	p=0.983
%TLCO	77.8 (±18.1)	64.2 (±16.0)	--	P=0.077
FVC (l)	2.90 (±0.7)	3.24 (±1.1)	--	P=0.728
%FVC	102.00 (±21.6)	92.6 (±26.9)	--	P=0.285
FEV1 (l)	2.33 (±0.7)	2.36 (±0.7)	--	P=0.853
%FEV1	98.4 (±18.8)	85.9 (±19.4)	--	P=0.362
CPI Score	67.7 (±18.3)	69.5 (±10.4)	--	P=0.327
Additional CT features				
GGO on first CT	2 (22%)	13 (56%)	2.2 (0.83-5.64)	p=0.146
Emphysema on first CT	0	4 (17%)	--	--
Follow-up time (years)	2.1 (±1.4)	3.4 (±1.7)	--	p=0.034

Table 3.1 Characteristics for iUIP patients, at initial CT when iUIP was identified.
Cases divided into progressive and non-progressive groups. S.D. in parenthesis. GGO; ground glass opacification. CPI; Composite Physiological Index as calculated by Wells et al.[240]

3.4.1.1 Clinical outcomes

In total 12 of 48 iUIP patients (25%) died during follow up. The mean time from initial CT of iUIP to all-cause mortality was 4.6 ±2.9 years. Respiratory-related deaths were confined to the progressive iUIP group; these accounted for 6 of the 9 deaths in this group; 2 to pneumonia and 4 to end-stage IPF. There was a trend to a greater number of hospitalisation events (39% vs 22% [OR 2.25, CI 0.40-12.32) and greater smoking history (86% vs 67% [OR 3.2, CI 0.59-15.9]) in the progressive iUIP group.

I compared lung function (PFT) data corresponding to initial CT scan. I defined the baseline PFT measurements as closest to initial CT scan and compared with measurements after 1 and 2 years (**Error! Reference source not found.**). Baseline FVC and TLCO values were lower in the progressive group compared to the non-progressive group; FVC 92.6% vs 102% (p=0.28), TLCO 62.4% vs 77.8% although this was not significant(p=0.08). At 1 year from initial CT, mean change in FVC for the 'non-progressive' group was -0.03L (±0.26) vs -0.26L (±0.39, p=0.16) in the 'progressive' group.

Mean change in TLCO was +0.89 (±1.20) vs -0.67 (±0.61, p<0.01). In the non-progressive group, there was no significant difference in paired serial values of TLCO (p=0.52) and FVC (p=0.23). For the sub-group of 'progressors' that progressed to 'probable' or 'definite' UIP, the reduction in FVC was -0.33L (±0.42, p=0.20) and TLCO was -0.60 (±0.61, p=0.03 Mann-Whitney).

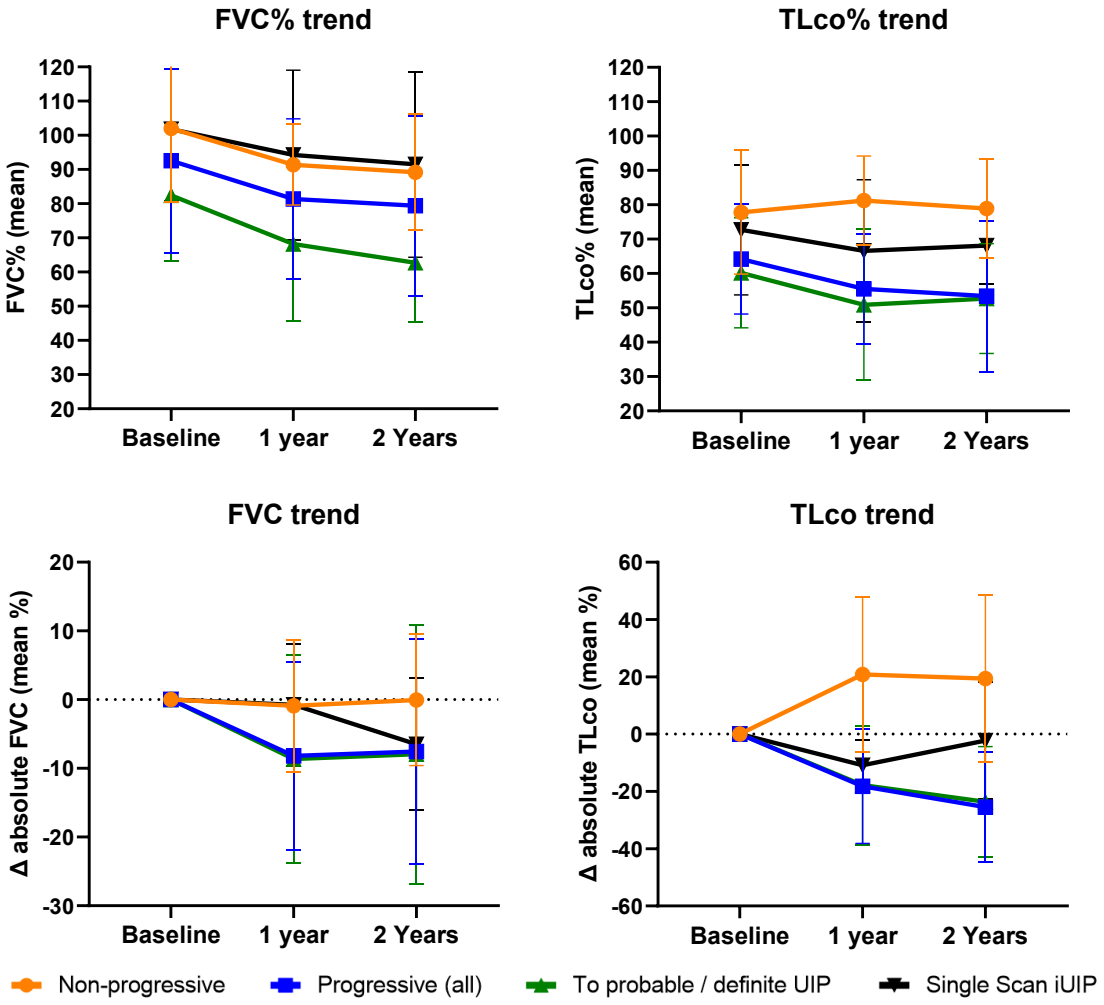

Figure 3.2 Cohort A serial lung function trends over a 2-year period.
Top; mean (SD) FVC % and TLco% values. Bottom; mean relative change (%) in absolute FVC and TLco over a 2-year period.

3.4.1.2 Baseline blood leukocyte parameters

Mean monocyte count <4 months of first CT was slightly higher in the progressive group [0.71 x10^3/µL ±0.31 Vs 0.65 ±0.13, P=0.85]. Mean neutrophil count was slightly higher in the progressor arm 5.35 (±2.60) vs 4.41 (±1.59) although not significant (p=0.57). There was no significant difference in lymphocyte count between the progressor and non-progressors [1.82 (±0.63) vs 1.62 (±0.63), p=0.78].

3.4.1.3 Potential drivers of disease progression to IPF

To explore the factors that drive the progression to IPF, a univariate Cox proportional hazard regression was performed, and hazard ratios were generated in two models. (A) all patients with at least one follow-on scan (n=32) and (B) patients that progressed to IPF during analysis period (n=17).

	Univariate analysis				Multivariate analysis			
Setting (A)	**HR**	**95% CI**	**p value (Likelihood ratio)**	**p value PH assumption**	**HR**	**95% CI**	**p value (Wald)**	**p value PH assumption**
Gender Male vs Female	3.3	0.71-15	0.08	0.52	1.9	0.35-10	0.46	0.92
Smoking (Never vs Ex)	0.65	0.17-2.5	0.54	0.03ǂ	0.10	0.02-0.72	*0.02	0.09
Age at initial CT	0.98	0.92-1.0	0.57	0.57	0.93	0.85-1.0	0.15	0.84
FVC (%) at initial CT	0.98	0.95-1.0	0.07	0.60	0.96	0.92-1.0	*0.05	0.71
Monocytosis (>0.9x10³/µl)	3.9	1.3-12	*0.03	0.40	27	2.0-370	*0.01	0.09
Neutrophilia (>7.5x10³/µl)	43	4.2-430	*0.00065	0.06	35.0	1.7-680	*0.02	0.85
Lymphopenia (<1.0x10³/µl)	2.2	0.28-18	0.49	0.24	0.37	0.01-10	0.56	0.18
Monocytes (x10³/µl)	23.0	1.6-340	*0.03	0.26	--	--	--	--
Neutrophils (x10³/µl)	1.8	1.3-2.3	*<0.0001	0.29	--	--	--	--
Lymphocytes (x10³/µl)	1.6	0.64-4	0.32	0.05	--	--	--	--
Monocytes (median)	3.8	1.2-13	*0.02	0.70	--	--	--	--
Neutrophil (median)	4.1	1.3-12	*0.01	0.85	--	--	--	--
Lymphocyte (median)	1.5	0.51-4.2	0.47	0.002ǂ	--	--	--	--
Setting (B)	**HR**	**95% CI**	**p value (Likelihood ratio)**	**p value PH assumption**	**HR**	**95% CI**	**p value (Wald)**	**p value PH assumption**
Gender Male vs Female	2.3	0.52-10	0.22	0.84	3.5	0.52-24	0.20	0.43
Smoking (Ex- vs never)	0.65	0.17-2.5	0.55	0.084	0.034	0.003-0.42	0.008*	0.37
Age at initial CT	1.0	0.96-1.1	0.42	0.60	0.87	0.78-0.97	0.015*	0.20
FVC (%) at initial CT	1.0	0.97-1	0.81	0.61	0.95	0.89-1.0	0.068	0.23
Monocytosis (>0.9x10³/µl)	3.0	0.95-9.7	0.074	0.67	6700	19-24000	0.003*	0.87
Neutrophilia (>7.5x10³/µl)	20.0	2-200	*0.0053	0.06	180	2.3-14000	0.020*	0.24
Lymphopenia (<1.0x10³/µl	15.0	0.97-250	0.085	0.16	0.007	$4.7x10^{-3}$-1.0	0.052	0.34
Monocytes (x10³/µl)	33.0	1.8-600	*0.019	0.73	--	--	--	--
Neutrophils (x10³/µl)	1.5	1.2-2	*0.0018	0.44	--	--	--	--
Lymphocytes (x10³/µl)	1.4	0.49-3.8	0.55	0.0023ǂ	--	--	--	--
Monocytes (median)	3.9	1.2-13	*0.018	0.88	--	--	--	--
Neutrophil (median)	3.1	1-9.7	*0.039	0.89	--	--	--	--
Lymphocyte (median)	4.0	1.2-13	*0.021	0.99	--	--	--	--

Table 3.2 Unadjusted and adjusted Cox Proportional hazard analysis on patients with iUIP.
Setting (A) including all patients with at least 1 follow-on CT scan (n=32). Setting (B) including only patients progressing to IPF (n=17). Output from smoking status in setting A and lymphocytes (continuous) in setting B violated the proportional hazards assumption of Cox regression in setting and were therefore not used. All values measured within 4 months of initial CT scan. *; p<0.05, ǂ; p value Cox PH assumption <0.05 therefore indicates violation. Setting (A) global significance of multivariate model; Likelihood ratio test p=0.004, PH assumption p=0.18. Setting (B); p=0.004, PH assumption p=0.69.

In both settings (A) and (B), monocytes and neutrophils (continuous and binary variables) were significantly associated with progression of iUIP as described in Table 3.2. Apart from blood leukocytes, no other variables showed significant difference. Due to the small numbers and some high monocyte, lymphocyte, and neutrophil counts, I further checked the sensitivity of HR and their significance in univariate analysis using a more balanced design by splitting the full blood cell (FBC) counts by their median values. Statistical significance and direction of effect in both settings were maintained.

Multivariate analysis using binarised monocyte and neutrophil count (for upper limit of reference range) also preserved their significance. The multivariate model also demonstrated that lower FVC% [HR 0.96 (0.92-1.0), p=0.05] and ex-smoker status [never smoker; HR 0.10 (0.02-0.72), p=0.02] were associated with progression of iUIP.

Cases were re-categorised according to baseline monocyte count as > or <0.90 $x10^3/\mu L$ (the upper limit of local laboratory reference range). Kaplan-Meier analysis of dichotomised monocyte (>0.90 $x10^3/\mu l$ or <0.90 $x10^3/\mu l$) and neutrophil (>7.5 $x10^3/\mu l$ or <7.5 $x10^3/\mu l$) counts demonstrated shorter time to progression of initial CT of iUIP to a clinical diagnosis of IPF. Monocytosis (>0.90 $x10^3/\mu l$); median time to progression 32.5 vs 52.8 months (p=0.03 Log-rank test) and neutrophilia (>7.5 $x10^9/L$); 20.6 vs 52.8 months (p<0.001). See Figure 3.3.

Finally, I examined survival across all 48 cases, according to baseline monocyte count of > or <0.90 $x10^3/\mu l$ and neutrophils of > or <7.5 $x10^3/\mu l$. There was a trend towards shorter survival for monocyte >0.90 (p=0.06) and neutrophils >7.5 (p=0.0002) as described in Figure 3.3.

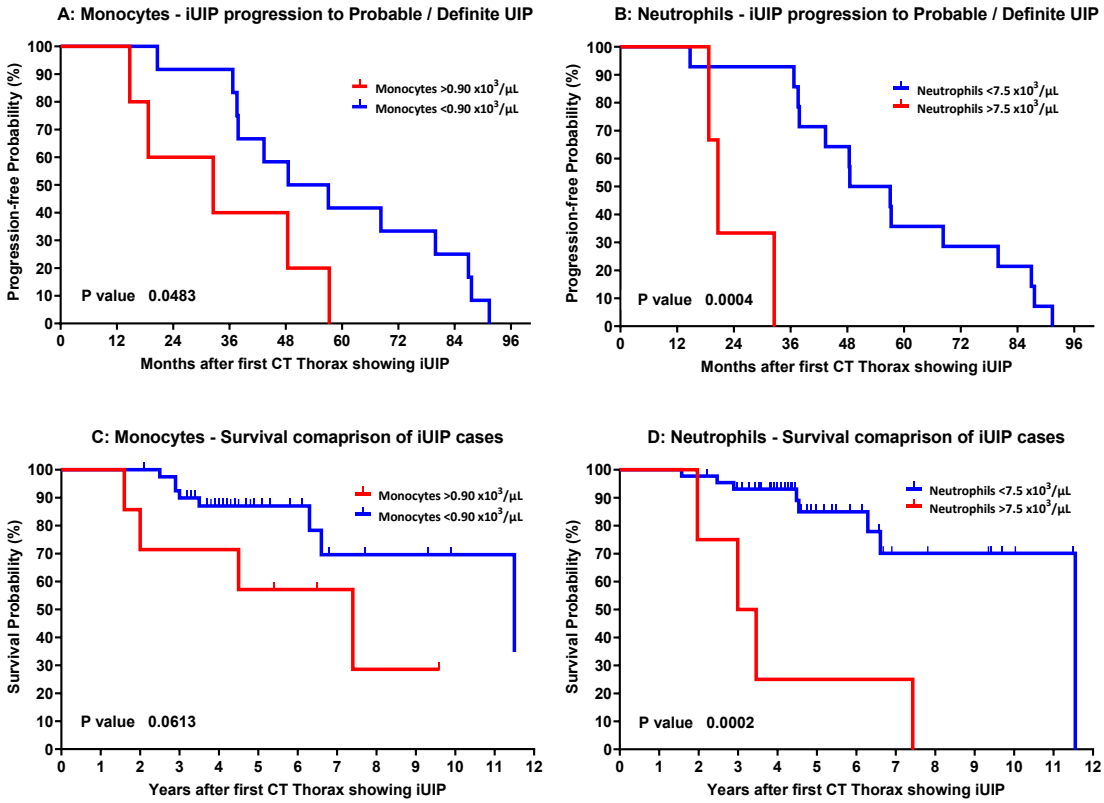

Figure 3.3 Monocyte and neutrophil relationship with progression and survival.
Monocyte count dichotomised < or > 0.90 x10^3/µL. Neutrophils dichotomised < or > 7.5 x10^3/µL. iUIP; Indeterminate for UIP HRCT pattern.

3.4.2 Cohort B: IPF dataset

3.4.2.1 Clinical outcomes

Of the remaining 182 patients, 54 (29.6%) patients did not have an available blood leukocyte measurement within 4 months of initial ILD assessment and were excluded. The final cohort consisted of 128 patients (Figure 3.4).

Baseline demographic data, physiological indices, blood leukocyte levels, comorbidity profiles and imaging features for all evaluable cases are shown in Table 3.3. The distribution of monocyte, neutrophil and lymphocyte values are depicted by histograms available in the appendix. Mean age was 75.2 years ±7.8 and 100 (78%) were male. Median length of follow up in ILD clinic was 31.0 months (16.2-42.4). Median time between blood leukocyte collection and first ILD clinic assessment was 0 days (IQR -31 to 0 days). 70 patients (55%) had blood taken within 7 days of assessment.

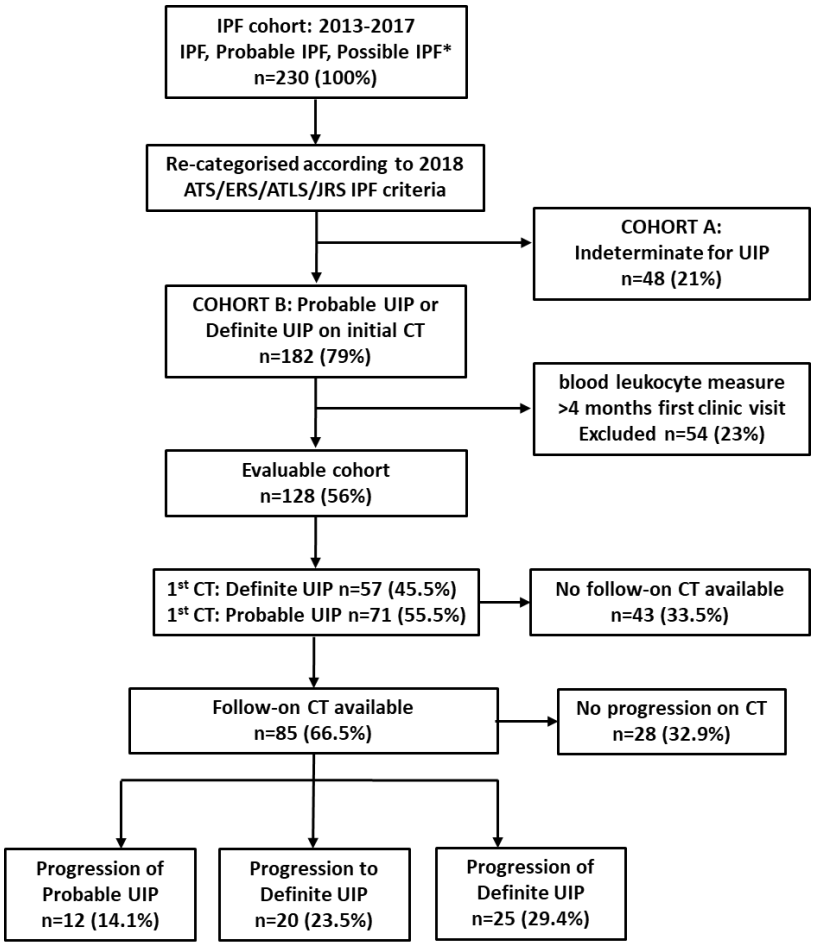

Figure 3.4 Flow diagram of radiographic progression of Cohort 1B.

During follow up 56 deaths (44%) were recorded; median time from first ILD assessment to death was 25.8 months (18.4-39.8) compared to 45.1 months (37.8-56.0) in those alive at time of censoring (01/08/2019). Most frequent cause of death was end-stage IPF (11%); followed by pneumonia (6.3%) and acute exacerbation of IPF (1.6%). Cause of mortality was not identified in 25 cases (19.5%). These cases were all regional referrals with limited available information regarding mortality. All-cause hospitalisation occurred in 25 (19.5%) cases. Time to hospitalisation was 28.3 months (20.1-35.0) for those hospitalised and time to censoring was 41.9 months (35.9-51.8) for those not hospitalised. Most frequent reason for hospitalisation was pneumonia (18 cases).

In 71 cases (55.5%) HRCT prior to initial clinic visit demonstrated probable UIP and 57 (45.5%) definite UIP. Of these, 86 (67.3%) patients underwent at least 1 further HRCT scan. Radiographic progression was observed in 58 (67.4%) cases over a median time interval of 30.8 months (21.5-44.1). 13 (22.4%) patients demonstrated progression in the extent of probable UIP on CT over 32.0 months (21.3-53.7). 20 (57.3%) progressed from probable UIP to definite UIP over 32.4

months (18.1-38.8). The remaining 25 (43.1%) progressed in extent of definite UIP over 30.5 months (23.7-44.4).

Patients categorised by FVC decline					
	All patients (n=128)	**FVC decline <10% / yr (n=62)**	**FVC decline ≥10% / yr (n=53)**	**P value**	**Odds Ratio**
Demographics					
Female (%)	28 (21.9)	13 (21.0)	14 (26.4)	0.516	1.35
Male (%)	100 (78.1)	49 (79.0)	39 (73.6)		0.74
AGE at first clinic visit (SD)	75.22 (7.88)	74.8 (6.9)	74.4 (8.6)		
Smoking status					
Ex-smoker	77 (60.2)	43 (69.4)	26 (49.1)	0.113	0.45
Never	30 (23.4)	12 (19.4)	16 (30.2)		
No data	21 (16.4)	7 (11.2)	11 (20.7)	--	--
GAP index					
1	66 (57.4)	39 (67.2)	26 (54.2)	0.229	0.58
2	39 (33.9)	15 (25.9)	19 (39.6)	0.148	1.87
3	10 (8.7)	4 (6.9)	3 (6.3)	>0.999	0.90
Comorbidity					
Type II diabetes mellitus	23 (18)	12 (19.4)	8 (15.1)	0.626	0.74
Gastro-oesophageal reflux	16 (12.5)	7 (11.3)	8 (15.1)	0.588	1.40
Ischaemic heart disease	20 (15.6)	9 (14.5)	10 (18.9)	0.618	1.37
Atrial fibrillation	9 (7)	2 (3.2)	6 (11.3)	0.141	3.83
COPD	14 (10.9)	5 (8.1)	8 (15.1)	0.255	2.03
Blood leukocytes					
Monocyte (x10^9/l)	0.70 (0.56-0.84)	0.67 (0.56-0.80)	0.70 (0.53-0.81)	0.925	
Lymphocyte (x10^9/l)	1.76 (1.41-2.40)	1.81 (1.56-2.50)	1.65 (1.40-2.32)	0.206	
Neutrophil (x10^9/l)	5.25 (3.96-6.68)	5.23 (3.90-6.31)	5.23 (4.30-7.06)	0.190	
Leukocyte-derived indexes					
NLR	2.77 (1.96-3.85)	2.46 (1.87-3.55)	3.17 (2.09-4.21)	0.049	
MLR	0.37 (0.31-0.50)	0.35 (0.30-0.41)	0.42 (0.30-0.53)	0.151	
SIRI	2.00 (1.22-2.83)	1.71 (1.10-2.49)	2.18 (1.32-2.99)	0.089	
Pulmonary function tests					
%TLCO	57.7 (47.5-69.0)	61.9 (50.9-71.0)	55.5 (47.3-68.4)	0.233	
%FVC	81.0 (68.1-98.3)	85.5 (69.9-98.0)	79.4 (70.1-101.2)	0.667	
CPI Score	68.8 (61.3-76.1)	65.6 (60.9-75.7)	69.19 (62.1-74.5)	0.570	
CT pattern					
Probable UIP	71 (55.5)	38 (61.3)	25 (47.2)	0.138	0.56
Definite UIP	57 (44.5)	24 (38.7)	28 (52.8)	0.138	1.77
Treatment					
Antifibrotic use	56 (43.7%)	25 (40.3%)	31 (58.5%)	0.063	2.08
Corticosteroids use	11 (8.6%)	4 (6.6%)	7 (13.2%)	0.341	2.21
-At baseline visit	1 (0.8%)	0 (0%)	1 (1.8%)	--	--
-During follow up	10 (7.8%)	4 (3.2%)	6 (11.3%)	0.510	1.85

Table 3.3 Characteristics for patients, at the point of initial CT when IPF was first diagnosed.
Characteristics for patients, at initial CT IPF diagnosed; cases divided by FVC decline. OR - odds ratio, GAP - Gender-age-physiology index[241], NLR - neutrophil:lymphocyte ratio, MLR - monocyte:lymphocyte ratio, SIRI; systemic inflammation response index, CPI - Composite Physiological Index as calculated by Wells[240]. Continuous variables expressed as median values (IQR) with exception of age (mean, SD).

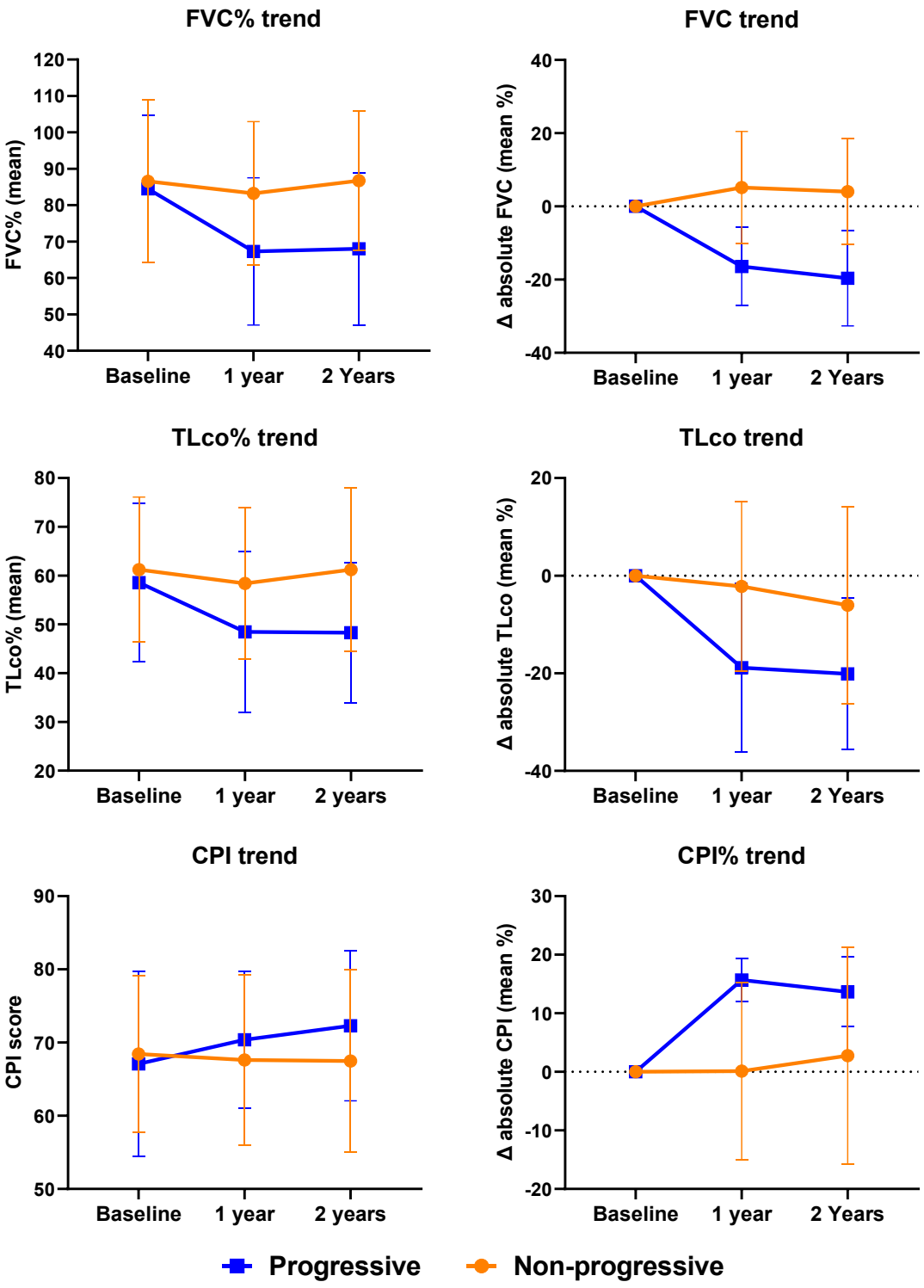

Figure 3.5 Cohort B serial lung function trends over a 2-year period.
Top; FVC, middle TLco, bottom; CPI. Left; %-predicted FVC and TLco value or calculated CPI score trends. Right; mean relative change (%) in absolute FVC and TLco and CPI over 2 year period.

Lung function decline, measured as relative decline in absolute FVC >10% per year, was identified in 53 of the 115 cases (46%) which had available follow up spirometry. 13 cases did not have available follow up spirometry. Mean annualised change across all patients was -8.25% (±17.7).

In total only 11 patients within our cohort received steroids for suspected acute exacerbation. Only 1 patient was prescribed steroids at the time of baseline assessment and prior blood draw.

3.4.2.2 Blood leukocyte associations with clinical outcomes in IPF

Having characterised the cohort, I then explored peripheral blood leukocytes for association between outcomes of FVC decline, hospitalisation events and survival in both univariate and multivariate cox proportional hazard modelling. Regression models were adjusted for age, gender and predicted FVC% measured closest to clinic assessment. I tested the covariates by including time-dependent effects in the model to ensure that for each the proportional hazards assumption was met. Co-variates of absolute monocyte count, SIRI (uni- and multivariate) and MLR (multivariate) violated the proportional hazards assumption for the outcome of lung function decline >10% and were not analysed further (Table 3.4).

Mortality

Univariate analysis demonstrated advancing age, increasing monocyte, neutrophil, MLR, NLR and SIRI were significantly associated with risk of all-cause mortality. Lower FVC% at baseline and lower lymphocyte count were also significantly associated with mortality.

In multivariate analysis, after adjusting for gender, age and baseline predicted FVC (%) absolute neutrophil [HR 1.2, 95% CI 1.10-1.40, p<0.001] and monocyte count [HR 1.4, 1.10-1.80, p=0.013] were independently associated with mortality. There was no association between lymphocyte count and mortality [1.0, 0.70-1.50, p=0.890]. Advancing age [1.1, 1.00-1.10, p=0.005] and lower baseline FVC% [0.97, 0.96-0.99, p=0.001] remained significantly associated with mortality. When dichotomising leukocyte values for limit of reference range only neutrophilia (>7.5 $x10^3/\mu l$) was significantly associated with mortality [2.31, 1.18-4.54, p=0.015]. Lymphopenia (<1.0 $x10^3/\mu l$) demonstrated direction of effect towards mortality, although this was not significant [1.5, 0.52-4.38, p=0.451].

All three leukocyte indexes demonstrated significant hazard towards mortality; MLR [1.32, 1.09-1.60, p=0.005], NLR [1.22, 1.11-1.34, p<0.0001], SIRI [1.06, 1.02-1.09, p=0.001] in adjusted analysis.

To test the postulate that leukocyte index portends worse outcome, I dichotomised the cohort based upon median leukocyte counts. Kaplan-Meier analysis of all three derived indices

demonstrated a significantly shorter median survival time for cases categorised >median leukocyte value. NLR >2.77 (40.7 vs 69.5, p=0.007), MLR >0.37 (40.7 vs 69.5, p=0.019) and SIRI >2.0 (40.7 vs 69.5, p=0.011). See Figure 3.6.

Importantly, the significance of monocyte, neutrophil and all derived indexes towards mortality was preserved when further adjusting the regression models for relative FVC decline (% change from baseline absolute FVC). When adjusting further for hospitalisation, antifibrotic and previous steroid use monocytes and all three derived indexes maintained significant hazard towards death (Table 3.5).

FVC decline

Of the leukocyte variables studied using multivariate analysis, Neutrophilia [HR 3.12, 1.44-6.74, p=0.004] and lymphopenia [3.78, 1.31-10.97, p=0.014] demonstrated greatest risk of FVC decline (as defined as >10% decline of absolute FVC). Neutrophil count [1.40, 1.10-1.70, p=0.001] and NLR [1.31. 1.16-1.48, p<0.0001] were also associated with significant risk of decline. Cases with greater NLR value in theory represents individuals with higher neutrophil (numerator) and lower lymphocyte count (denominator) in this cohort. NLR>2.77 was more predictive of FVC decline than continuous NLR measurement [HR 2.04, 1.12-3.71, p=0.020]. This association was not observed with other leukocyte variables.

Kaplan-Meier analysis demonstrated significantly shorter median time to >10% relative FVC decline for NLR>2.77 (18.9 vs 51.1 months, p=0.01], and SIRI>2.0 (18.7 vs 51.1, p=0.02) and lymphocyte count <1.76 (17.9 vs 51.1, p=0.02). See Figure 3.7.

All-cause Hospitalisation

All-cause hospitalisation events were reported only in 19.5% of cases. In multivariate analysis adjusted for age, gender, and baseline FVC%, lower lymphocyte count [HR 0.41, 0.19-0.87, p=0.021] was significantly associated with greater risk of all-cause hospitalisation events. Neither absolute neutrophil [1.10, 0.94-1.20, p=0.300] or monocyte count [0.98, 0.61-1.60, p=0.920] demonstrated significant association with hospitalisation. Of the three leukocyte indexes measured, only NLR [1.13, 1.02-1.24, p=0.015] demonstrated significant association with hospitalisation (Figure A.3). MLR; [1.07, 0.71-1.79, p=0.757], SIRI; [1.02, 0.96-1.08, p=0.632].

	Univariate analysis				Multivariate analysis			
Death	**HR**	**95% CI**	**p value**	**p value PH assumption**	**HR**	**95% CI**	**p value**	**p value PH assumption**
Gender (Male)	1.4	0.66-2.80	0.401	0.760	0.83	0.38-1.80	0.630	0.970
Age	1.1	1.01-1.10	0.00118	0.630	1.1	1.00-1.10	0.005*	0.930
FVC (%)	0.97	0.96-0.99	0.00011*	0.810	0.97	0.96-0.99	0.001*	0.990
Monocytes (x10³/μl)	1.4	1.10-1.80	0.0033*	0.140	1.4	1.10-1.80	0.013*	0.340
Lymphocytes (x10³/μl)	0.9	0.61-1.30	0.570	0.850	1.0	0.70-1.50	0.890	0.940
Neutrophils (x10³/μl)	1.33	1.20-1.50	0.000003*	0.980	1.2	1.10-1.40	0.0008*	0.940
Monocytosis (>0.90 x10³/μl)	1.5	0.79-2.8	0.220	0.220	1.01	0.50-2.01	0.990	0.291
Lymphopenia (<1.0 x10³/μl)	4.0	1.6-10	0.004*	0.200	1.50	0.52-4.38	0.451	0.279
Neutrophilia (>7.5 x10³/μl)	3.8	2-7.1	0.00004*	0.770	2.31	1.18-4.54	0.015*	0.544
MLR	1.4	1.1-1.7	0.0016*	0.070	1.32	1.09-1.60	0.005*	0.136
MLR >0.37	2.10	1.2-3.6	0.009*	0.348	2.05	1.26-3.74	0.019*	0.583
NLR	1.2	1.1-1.3	0.00001*	0.890	1.22	1.11-1.34	0.00006*	0.691
NLR >2.77	2.19	1.26-3.83	0.006*	0.731	1.81	1.01-3.23	0.046*	0.767
SIRI	1.06	1.03-1.10	0.00005*	0.030‡	1.06	1.02-1.09	0.001*	0.130
SIRI >2.0	2.00	1.15-3.49	0.015*	0.270	1.83	1.02-3.26	0.041*	0.231
Hospitalisation	**HR**	**95% CI**	**p value**	**p value PH assumption**	**HR**	**95% CI**	**p value**	**p value PH assumption**
Gender (Male)	1.5	0.51-4.30	0.480	0.490	1.2	0.41-3.70	0.710	0.340
Age	0.98	0.94-1.00	0.460	0.160	0.99	0.94-1.0	0.660	0.081
FVC (%)	0.99	0.97-1.00	0.170	0.940	0.99	0.97-1.0	0.510	0.980
Monocytes (x10³/μl)	1.00	0.56-1.80	0.990	0.310	0.98	0.61-1.60	0.920	0.300
Lymphocytes (x10³/μl)	0.34	0.16-0.73	0.0056*	0.900	0.41	0.19-0.87	0.021*	0.870
Neutrophils (x10³/μl)	1.1	1.00-1.30	0.049*	0.850	1.1	0.94-1.20	0.300	0.950
Monocytosis (>0.90 x10³/μl)	1.1	0.41-2.9	0.870	0.930	1.03	0.35-2.99	0.956	0.976
Lymphopenia (<1.0 x10³/μl)	1.6	0.38-6.8	0.520	0.22	1.20	0.26-5.49	0.812	0.315
Neutrophilia (>7.5 x10³/μl)	2.3	0.86-6.2	0.097	0.940	1.83	0.59-5.63	0.293	0.969
MLR	1.07	0.72-1.58	0.748	0.480	1.07	0.71-1.79	0.757	0.433
MLR >0.37	3.07	1.22-7.68	0.017*	0.488	3.10	1.22-7.89	0.017*	0.150
NLR	1.13	1.02-1.24	0.014*	0.750	1.11	1.00-1.23	0.045*	0.886
NLR >2.77	4.23	1.59-11.29	0.004*	0.590	4.53	1.64-12.50	0.004*	0.746
SIRI	1.07	0.88-1.31	0.470	0.420	1.02	0.96-1.08	0.632	0.509
SIRI >2.0	2.53	1.06-6.05	0.038*	0.337	2.48	1.02-6.03	0.046*	0.745
FVC decline >10%	**HR**	**95% CI**	**p value**	**p value PH assumption**	**HR**	**95% CI**	**p value**	**p value PH assumption**
Gender (Male)	0.68	0.36-1.30	0.240	0.810	0.48	0.24-0.97	0.040*	0.470
Age	1.0	0.99-1.10	0.160	0.530	1.1	1.0-1.15	0.035*	0.110
FVC (%)	0.99	0.97-1.00	0.065	0.260	0.99	0.97-1.00	0.071	0.630
Monocytes (x10³/μl)	1.1	0.52-2.30	0.810	0.031‡	0.55	0.095-3.2	0.500	0.016‡
Lymphocytes (x10³/μl)	0.76	0.49-1.20	0.230	0.700	0.81	0.52-1.3	0.360	0.850
Neutrophils (x10³/μl)	1.3	1.10-1.60	0.0007*	0.680	1.4	1.1-1.7	0.0011*	0.760
Monocytosis (>0.90 x10³/μl)	1.6	0.8-3.1	0.190	0.120	1.83	0.88-3.82	0.105	0.953
Lymphopenia (<1.0 x10³/μl)	4.5	1.5-13	0.006*	0.630	3.78	1.31-10.97	0.014*	0.669
Neutrophilia (>7.5 x10³/μl)	4.5	2.2-9.3	0.00004*	0.810	3.12	1.44-6.74	0.004*	0.870
MLR	1.2	0.73-1.9	0.530	0.050	1.07	0.70-1.64	0.763	0.757
MLR >0.37	1.70	0.98-2.97	0.062*	0.146	1.91	1.07-3.40	0.029*	0.434
NLR	1.3	1.1-1.4	0.00002*	0.550	1.31	1.16-1.48	0.00002*	0.679
NLR >2.77	2.07	1.18-3.65	0.011*	0.962	2.04	1.12-3.71	0.020*	0.803
SIRI	1.05	0.989-1.11	0.109	0.014‡	1.03	0.97-1.08	0.326	0.578
SIRI >2.0	1.91	1.09-3.43	0.024*	0.742	1.95	1.10-3.47	0.023*	0.549

Table 3.4 Unadjusted and adjusted Cox Proportional hazard analysis in patients with UIP.
MLR; monocyte/lymphocyte ratio, NLR; neutrophil/lymphocyte ratio, SIRI; systemic inflammatory response index. Multivariate models testing blood leukocytes against outcome; these were all tested in combination [absolute monocyte, lymphocyte, and neutrophils]. For models exploring MLR, NLR or SIRI; each was tested individually and in absence of other leukocytes measurements or derived indexes with the covariates gender, age, and baseline FVC% but HR outcome for gender, age, and baseline FVC% are not shown. ‡; covariate violated proportional hazards assumption. All adjusted covariates in each model satisfied the proportional hazard assumption. P values <0.05 are considered significant.

Mortality	HR (95% CI)	p value	p value PH assumption
Leukocytes			
Monocytes (x10^3/μl)	1.57 (1.15-2.13)	0.004*	0.204
Lymphocytes (x10^3/μl)	0.81 (0.52-1.26)	0.353	0.238
Neutrophils (x10^3/μl)	1.21 (1.01-1.43)	0.038*	0.709
Leukocyte ratios			
MLR	1.53 (1.02-1.95)	0.001*	0.123
NLR	1.24 (1.08-1.43)	0.002*	0.984
SIRI	1.08 (1.04-1.13)	0.001*	0.127

Table 3.5 Cox Proportional hazard analysis in Cohort 1B adjusted for steroid exposure

Multivariate Cox proportional hazard analysis for outcomes of survival, and lung function decline adjusted for age, gender, baseline FVC%, FVC decline, hospitalisation, antifibrotic use and steroid use at clinic visit during follow up. For all multivariate models testing contribution of blood leukocytes against outcome these were all tested in combination [absolute monocyte, lymphocyte, and neutrophils] to explore interaction. For multivariate models exploring contribution of the leukocyte derived indexes [MLN, NLR or SIRI] these were tested individually and in absence of other leukocytes measurements or derived indexes. All adjusted covariates in each model satisfied the proportional hazard assumption. P values <0.05 are considered significant. MLR; monocyte/lymphocyte ratio, NLR; neutrophil/lymphocyte ratio, SIRI; systemic inflammatory response index.

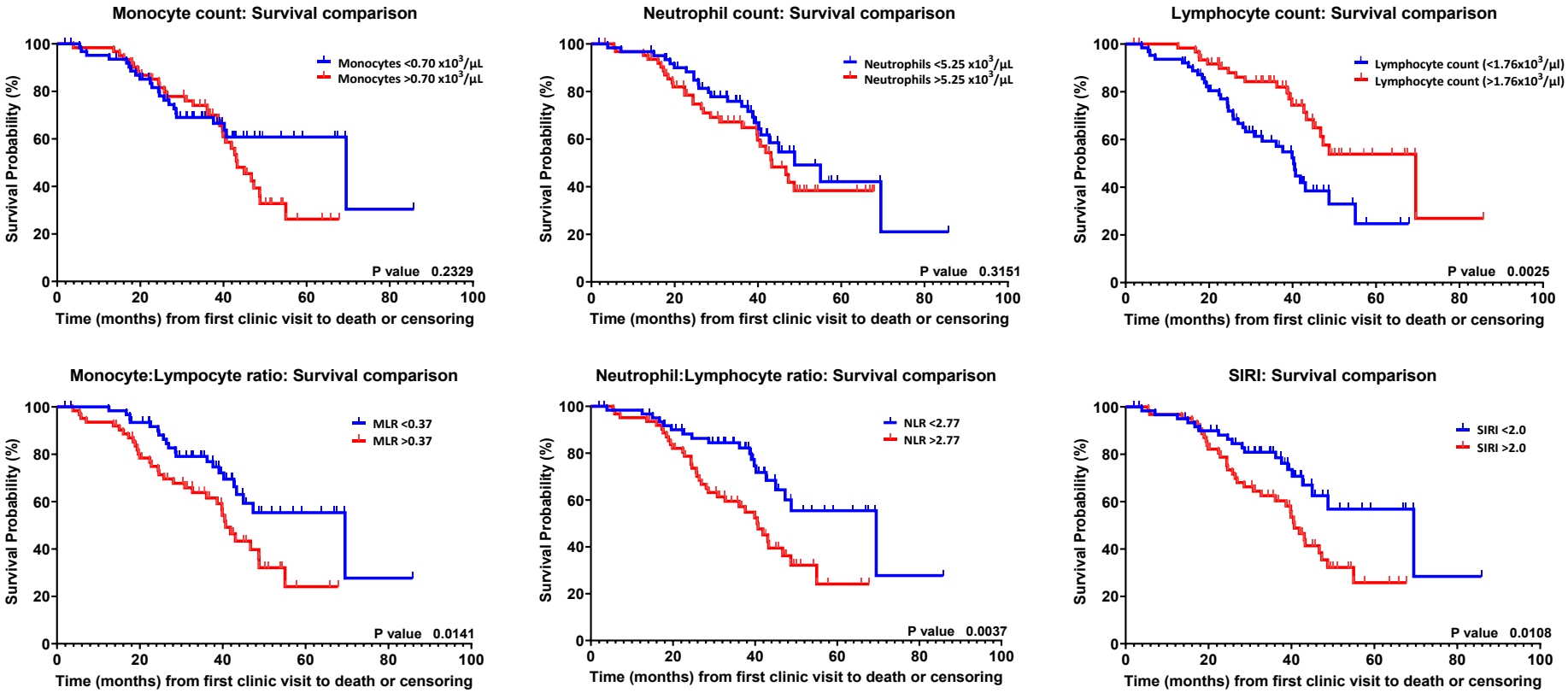

Figure 3.6 Kaplan-Meier curves for time to mortality

Dichotomised for median values for absolute monocyte, neutrophil and lymphocyte counts and derived indexes of monocyte:lymphocyte ratio, neutrophil:lymphocyte ratio and systemic inflammatory response index. All graphs demonstrate as p<0.05 (log rank).

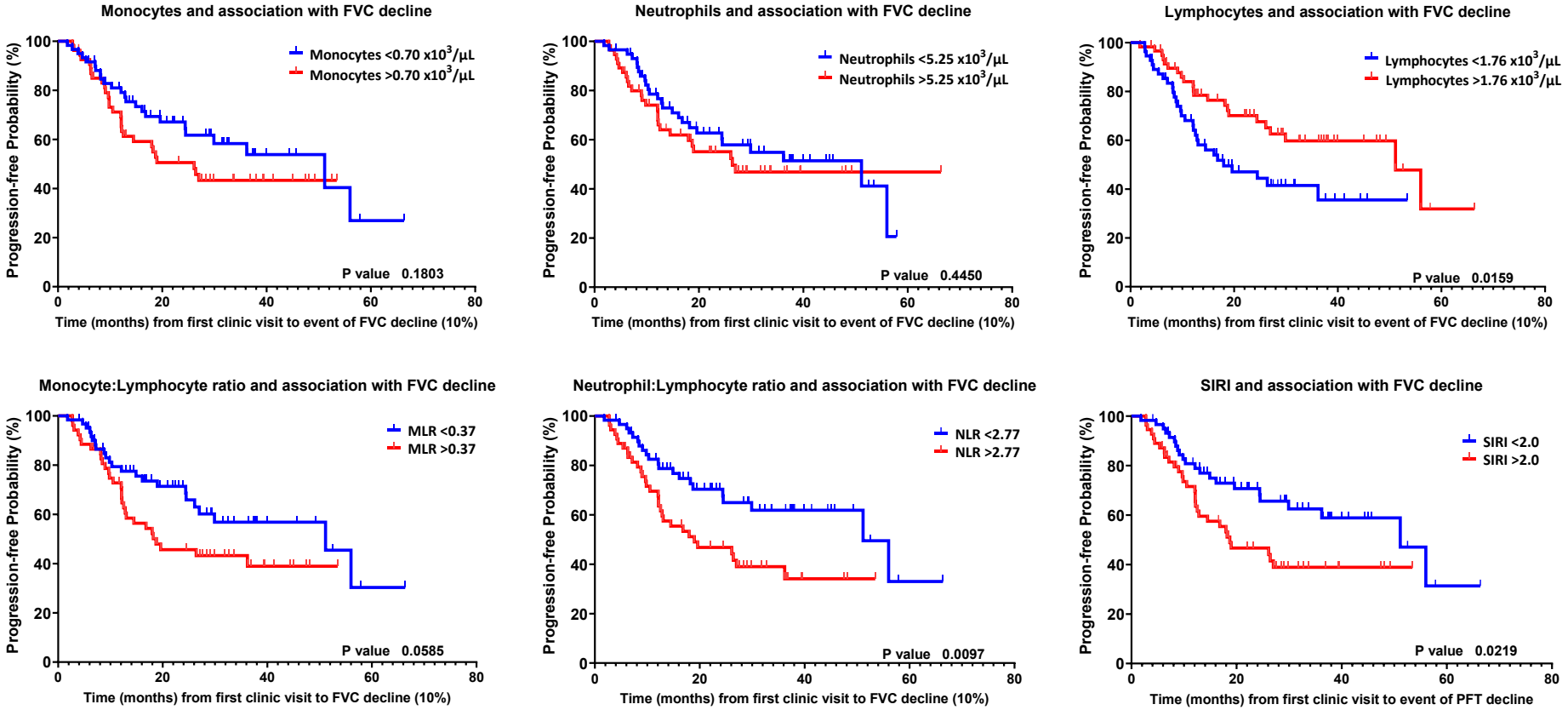

Figure 3.7 Kaplan-Meier curves for time to relative FVC decline of 10%.
Dichotomised for median values for absolute monocyte, neutrophil and lymphocyte counts and derived indexes of monocyte:lymphocyte ratio, neutrophil:lymphocyte ratio and systemic inflammatory response index. All graphs demonstrate as $p<0.05$ (log rank).

3.5 Discussion

Study 1 describes the outcomes of a single centre IPF cohort and demonstrates that peripheral blood monocytes, neutrophils and lymphocytes are independent predictors for adverse outcomes in IPF.

I examined monocyte, neutrophil, and lymphocyte counts, in part, because these are routinely performed in clinic but primarily because of the link between leukocytes and mechanism of disease,[98,130,133] which have been explored in large observational studies. I used the upper limit of local reference range to binarise monocyte counts of >0.90 $x10^3/\mu l$ and neutrophils >7.5 $x10^3/\mu l$, and the lower limit to categorise lymphopenia <1.0 $x10^3/\mu l$, to explore association in cases with extreme leukocyte values.

The findings from analysis of Cohort A confirmed that the iUIP CT pattern is associated with progression to IPF in a considerable proportion of cases. There was a trend towards higher co-morbidity burden and smoking history in those progressing to IPF, and which is comparable to other studies.[104] Monocyte count >0.90 $x10^3/\mu L$ and Neutrophil count >7.5 $x10^3/\mu L$ were associated with greater rate of progression to IPF and all-cause mortality (Figure 3.3). A history of smoking was also associated with radiographic progression of iUIP.

Furthermore, analysis of Cohort B identified strong association between clinical outcomes (mortality, FVC decline and hospitalisation) in IPF with monocyte, neutrophils, and lymphocyte measurements but also with NLR, MLR and SIRI. Absolute neutrophil and NLR were the two covariates demonstrating significant hazard towards all outcomes in univariate analysis. Monocytes demonstrated increased mortality risk in multivariate analysis. NLR was independently associated with all adverse outcomes in multivariate analysis. These observations suggest that higher baseline monocyte and neutrophil counts are associated with mortality risk and disease behaviour in IPF, and perhaps warrant greater attention at initial assessment.

iUIP is a recognised risk factor for IPF. Yet due to the recent evolution of the IPF diagnostic guidelines, there is limited data acknowledging longitudinal outcomes of progression of the iUIP CT pattern to a clinical diagnosis of IPF. When performing a PubMed search using the keywords; “idiopathic pulmonary fibrosis”, “usual interstitial pneumonia”, “indeterminate” and “outcome” (after 2017) I was only able to identify 1 similar study, by Diridollou et al.[242] The authors re-categorised 89 cases of 'possible UIP' CT pattern and 17% of these were re-labelled as iUIP. They did not comment on progression of iUIP or association with blood leukocytes. In a large birth cohort study (Age, Gene/Environment Susceptibility-Reykjavik (AGES), n=5320), Putman et al

observed a 2.52% prevalence of iUIP, and identified a greater risk of mortality compared to individuals with normal CT [1.6, 1.3-2.0, p<0.0001].[243] Neither study examined how iUIP progresses. The greater proportion of iUIP observed in Cohort A (27.3%), and by Diridollou et al (17%), compared with Putman is likely a reflection of iUIP cases within background population that are carried forward to ILD assessment. Differences in symptom burden expediting medical assessment in these cohorts may also account for this, and in part bias these cohorts.

The findings from Cohort B are consistent with previous studies reporting association between monocyte count and IPF prognosis. The landmark study by Scott et al, a large multicentre retrospective cohort study, first demonstrated that monocyte count ≥0.95x10^3/μl is associated with all-cause mortality in IPF.[197] However, their cases were identified by ICD-10 coding of clinical records which may pose limitation to the findings. More recently, in a large retrospective pooled analysis from four phase III randomised, placebo-controlled trials (Ascend, Capacity and Inspire[14,34,47]) comprising 2067 patients, Kreuter et al explored monocyte association with mortality by stratifying patients by monocyte count in increments of 0.20x10^3/μl. The authors demonstrated that stratification by monocytes >0.60x10^3/μl and ≥0.95x10^3/μl conferred similar 1-year risk of mortality, all-cause hospitalisation and disease progression (defined as a composite of >10% absolute decline in FVC% predicted, >50m decline in 6-minute-walk distance, or death).[228] This, therefore, suggested a cut-off threshold of >0.60x10^3/μl could prognosticate for adverse outcome. This threshold was later tested in a validation study involving 2 further IPF cohorts. Although in the derivation cohort, monocytes >0.60x10^3/μl conferred risk of shorter median survival this finding was not observed in the authors' validation cohort.[229] I did not observe this association when dichotomising Cohort 1b by >0.60x10^3/μl. This could be partially explained by inter-cohort differences in baseline physiological status as indicated by greater baseline FVC% and TLCO% in Cohort B. This negative finding may also imply that blood monocyte >0.60x10^3/μl is not sensitive enough to generalise to real-world cohorts.

Although the exact aetiology of IPF remains unknown, several lines of evidence from both animal models and human studies, have implicated innate and adaptive immune response in fibrogenesis.[129,135,136] Recent experimental evidence suggesting migration of monocytes from bone marrow to injured lung and differentiating into pro-fibrotic macrophages.[98,184] Furthermore, translational studies have demonstrated that accumulation of distinct monocyte-derived alveolar macrophage populations are implicated in progression of fibrosis.[191,244,245] Monocyte-derived macrophages, which originate from bone-marrow precursors and thus of a separate ontogeny tissue-resident macrophages, possess a different cytokine repertoire, one that is more pro-inflammatory and pro-fibrotic, which in animals models has been shown to propagate fibrosis.[184] In Fraser et al, my predecessor observed in a separate IPF cohort, that monocytes display a type 1

interferon primed phenotype which could account for more robust and potentially injurious response to the alveolar epithelium when triggered, during for example a viral infection,[214] which could potentially perpetuate ongoing fibrotic processes.

In analysis of Cohort 1b I also identified a significant association between blood neutrophils and lymphocytes with mortality, FVC decline and hospitalisation. These observational findings could also be explained by current scientific understanding of neutrophil function. Akin to monocytes, neutrophils are recruited to areas of inflammation and are also implicated in fibrosis.[135] Similar to macrophages, they can shape their environment by secreting proteases, oxidants, cytokines, and chemokines.[246] Neutrophils are also a substantial source of matrix metalloproteinases (MMP) which are involved in collagen deposition and ECM formation.[133] Neutrophilic bronchoalveolar lavage (BAL) specimens taken from patients with IPF has been associated with early mortality.[130] The enzyme neutrophil elastase (NE) has previously shown to be elevated in BAL sampling from IPF patients.[131] More recent findings using murine models suggest that NE promotes activation of the TGF-ß pathway, fibroblast proliferation and myofibroblast differentiation.[247] An intriguing and newly identified fibrosis-promoting function of neutrophils is generation of neutrophil extracellular traps (NETs).[129] These pro-inflammatory collections of chromatin and neutrophils regulate both immune cell function and fibroblast activation.[134] Whilst a specific association with IPF has yet to be fully described, enhanced detection of intra-pulmonary NETs has been reported in bleomycin models and in non-IPF fibrotic ILD studies.[135]

The association between lower blood lymphocyte count and adverse outcomes in Cohort 1b is fascinating. Previous studies have identified association between low blood lymphocyte count, lymphocyte dysfunction and reduced FVC in IPF patients.[248] In a meta-analysis to explore lymphocyte expression patterns in IPF Schott et al explored peripheral blood mononuclear cell expression data obtained from the Gene Expression Omnibus.[248] The authors identified significantly reduced lymphocytic gene expression in patients with IPF. Gene expression correlated with TLco and high expression was significantly associated with survival. Bronchoalveolar lavage fluid from IPF patients is also enriched for several T lymphocyte populations.[136] Relative to samples obtained from healthy individuals, lymphoid aggregates are a recognised pathologic feature of IPF lesions.[135]

The association between low blood lymphocyte count and adverse outcome in IPF could be explained in part by lymphocyte dysfunction and the sequestering of lymphocytes into sites of inflammation, such as the fibrotic lung. Nuovo et al demonstrated greater immunohistochemical staining for CD4+, CD8+, IL-17, and CCR6 in IPF lung specimens relative to healthy controls.[148] In a similar study Daniil et al utilised quantitative immunohistochemistry to measure extent of

lymphoid infiltrates in IPF specimens. Interestingly they identified negative correlation between CD8+ staining and FVC and TLCO%.[249] Todd et al previously described the presence of lymphocyte aggregates on histological samples obtained from surgical lung biopsy in 16 IPF patients. All later received lung transplantation. Paired analysis revealed CD3+ lymphocyte aggregates were present in greater numbers in advanced disease (explant tissue) compared to early disease (surgical lung biopsy) implying cellular inflammation continues as fibrosis progresses.[140] Recently, Th17 lymphocytes isolated from peripheral blood of patients with IPF have been identified as a source of TGF-β (transforming growth factor).[250] TGF-β is a key mediator of fibrogenesis, inducing collagen deposition by fibroblasts.[136]

Despite these interesting findings, the PANTHER clinical trial demonstrated poorer clinical outcome in patients randomised to the lymphocyte-modulating agent Azathioprine, suggesting that the relationship between inflammation, fibrosis and clinical behaviour of IPF remains incompletely understood.[46]

The observations that multiple blood leukocytes are implicated in IPF justifies the investigation of leukocyte derived indexes in this cohort. NLR significance was preserved across all multivariate analysis identifying this as an independent risk factor in this IPF cohort, suggesting that neutrophil activation and lymphocyte exhaustion could be relevant. In the study by Fraser et al monocytes in IPF patients showed type I interferon primed phenotype, and could account for more the potentially injurious response to the alveolar epithelium.[214] My preliminary findings from chapter 3 also support this, and this study provides impetus to investigate the possibility that neutrophils and lymphocytes could also be involved.

NLR, a recognised surrogate marker of immune response, indirectly evaluates inflammatory status and is a recognised correlate of disease severity, hospitalisation and mortality.[251,252] NLR has also been shown to predict outcome in ILDs with associated auto-immune rheumatic diseases such as systemic sclerosis and dermatomyositis.[253,254] MLR and SIRI have been primarily used to prognosticate in cancer studies.[239,255]

Recently one retrospective cross-sectional study demonstrated NLR, MLR and SIRI measurements to be greater in patients with IPF versus healthy controls,[256] suggesting not only multiple leukocytes are implicated in disease activity, but also that ratios of absolute leukocyte counts could potentially serve as useful surrogate measures of real-time immunological status and disease activity and prognostication of IPF. In a retrospective multicentre study evaluating NLR, Mikolasch et al confirmed NLR is an independent predictor of 6-month mortality across multiple IPF cohorts. The authors also demonstrated that incorporation of NLR strengthened ability of the GAP prediction model to risk stratify.[257] Furthermore, in post-hoc analysis of the ACEND and

CAPACITY studies, Nathan et al reported that serial increase in NLR over 12 months was associated with increased mortality risk.[258] Thus, the positive association between these composite index measures of absolute leukocytes could reflect a 'real-time' measure of inflammation, that could be driving disease progression in patients with IPF.

In Cohort 1b all three leukocyte-derived indexes were associated with mortality. Importantly, NLR was also associated with shorter time to >10% FVC decline and hospitalisation. Despite multiple studies demonstrating association for absolute monocyte count with mortality none have successfully demonstrated direct association of monocyte count with relative FVC change,[228,229,259] which is the accepted clinical metric for regular monitoring of disease progression.[25]

NLR could potentially reflect the injurious nature of the immune pathobiology of IPF and thus serve as a predictor of aggressive FVC decline and facilitate decisions on risk stratification and monitoring frequency to detect progression and enable timely treatment intervention. Furthermore Kaplan-Meier analysis, dichotomising cases by median leukocyte index appeared to show greater between-group difference (< or > median leukocyte value) in comparison to absolute leukocyte count which further supports this argument (Figure 3.6, Figure 3.7 and Appendix Figure A.3).

Mortality and FVC decline reported in this study are higher than reported in other studies, most likely because of the long duration of follow up. Also, study recruitment started before antifibrotic prescribing became available in the UK and, not unexpectedly in this cohort, baseline FVC% was lower in patients that died.

Several limitations should be considered. The single-centre and retrospective nature of this study limits interpretation of the data to association rather than causality.

Monocyte, neutrophil and lymphocyte levels were measured at one point (nearest to the CT scan or at clinic assessment). This is in keeping with work from other much larger studies but there is a risk that the values are not representative of the steady state values, particularly in a necessarily small cohort. In future studies, it will be useful to have repeated samples to determine if the neutrophil, lymphocyte and monocyte values are representative for the patient, and reduce bias towards the possibility of levels linked to an infective episode, for example.[258]

The small sample size limits the power of this data and has contributed to some of the large hazard ratios and confidence intervals observed from Cohort A analysis. Therefore, these findings are primarily indicative rather than definitive signals. However, strong statistical analyses including proportional hazards modelling of time-dependent covariates by multivariate analyses suggest that blood monocytes, but also neutrophils correlate with adverse outcomes in IPF.

The differences in statistical significance observed here between neutrophils, lymphocytes and monocytes in our multivariate analysis is probably a question of sensitivity. The HRs generated by this modelling should be interpreted as association between these immune cells and progression, which is statistically significant. Therefore, as a single point biomarker for progression of fibrosis, in established IPF (as opposed to early disease), neutrophils and lymphocytes may be more sensitive than monocytes. Even more sensitive is NLR, which may well prove to be the biomarker for disease progression.

In Cohort 1a, not all follow-on CT scans included in analysis were undertaken in a uniform timeframe. With exception of the lung nodule surveillance imaging most serial CTs were performed according to clinical indication and where there was concern for objective deterioration. A greater proportion of repeat CT scans were performed to investigate symptomatic change in the 'progressor' group, and this may have introduced bias towards detection of progression. Follow-up time was also shorter in the non-progressor group which may have introduced bias.

Furthermore, the median length of follow up in Cohort 1b was 31.0 months which demonstrates the longer-term prognostic value of monocytes, neutrophils, and derived indexes. The effect of co-morbidity burden, which is not insignificant in this and other IPF cohorts, on observed association between blood leukocytes and study outcomes is unknown. Anti-fibrotic treatment and steroid therapy may have affected blood leukocyte measurement in Study 1B, however when adjusting for these covariates using multivariate regression significance was preserved (Table 3.5).

3.6 Conclusion

In summary, analysis of this cohort demonstrates association between peripheral blood leukocytes with accelerated lung function decline, hospitalisation, and all-cause mortality. Specifically, I highlight the association of monocyte, neutrophil and NLR towards these adverse outcomes in this cohort. Peripheral blood leukocyte measurements taken from full blood count analysis could be considered a real-time and simplified reflection of the complex immunopathological events in IPF.

As IPF remains an unpredictable disease, peripheral blood leukocyte measurement could assist clinicians to risk stratify patients and facilitate a more personalised approach to treatment. Utilised in the clinical setting these could assist clinicians to risk stratify patients with greater accuracy and facilitating a personalised approach to treatment. Additionally, these could be used

to enrich research studies for important clinical endpoints. Further prospective studies will be required to validate their use in prognosticating for IPF.

Chapter 4 Blood leukocyte association with progression of interstitial lung abnormalities

4.1 Introduction

In Study 1, I demonstrated that higher blood monocyte and neutrophil counts were associated with progression of the radiographic pattern indeterminate for UIP (iUIP) to probable or definite UIP and to a clinical diagnosis of IPF (cohort A). Patients included in Study 1 were assessed in the ILD clinic and iUIP was defined as a discrete category in the radiological UIP classification of IPF.[8] However, iUIP can also be described purely by its parenchymal appearance, which is classified within the broad spectrum of Interstitial lung abnormalities (ILAs). ILA is an umbrella term for subtle non-dependent mild parenchymal abnormalities occupying >5% of lung volume on CT scan in patients in whom ILD was not necessarily suspected.[260] Typically ILAs can range from cystic appearance and emphysema, ground glass opacities through to fibrotic features including reticular opacities, traction bronchiectasis and honeycombing.[22,261]

Commonly, but not exclusively, ILAs are detected in patients undergoing CT investigation for mild and undifferentiated respiratory symptoms although a proportion are detected coincidentally in asymptomatic individuals undergoing CT for non-ILD investigations. Thus, it can be difficult to determine ILA prevalence in the general population. Historically these have been reported on CT scans as incidental findings of uncertain clinical significance, age-related changes of no significance or simply not reported.[262] Once considered benign entities, there is now growing evidence that their presence on CT has prognostic implication and birth cohort and lung cancer screening studies have reported prevalence of 2-10%,[243,263-267] and a higher risk of mortality.[236,267,268] ILAs have been shown to be associated with symptoms including breathlessness, reduction in lung function and exercise capacity,[267,269] and genetic abnormalities common to familial interstitial pneumonia and idiopathic pulmonary fibrosis (IPF).[109,270]

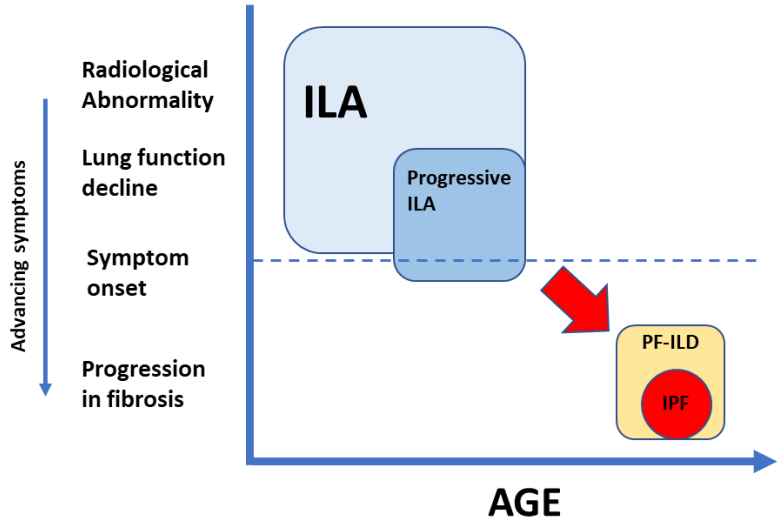

Figure 4.1 Schematic demonstrating progression from ILA to pulmonary fibrosis
ILA; Interstitial lung abnormality, IPF; Idiopathic pulmonary fibrosis, PF-ILD; progressive fibrotic ILD. Adapted from Hunninghake et al (2019) and Walsh et al (2018).[233,263]

A proportion of ILAs will progress to IPF, yet despite this ILA prevalence exceeds that of IPF by considerable margin (Figure 4.1).[233] Therefore identifying cases at risk of progression is an important clinical priority. Future implementation of lung cancer screening and greater use of CT imaging for other diagnostic purposes are likely to increase detection of ILA and pose resource implication for ILD services.[260] In recognition of this the Fleischner society have issued guidance which emphasises risk stratification for follow-up evaluation and the importance of identifying, and subcategorising for, the subpleural fibrotic ILA, which not only carries greater mortality risk and is also considered a precursor to clinical ILD diagnoses.[271,272] As such this is recognised as iUIP in the 2018 IPF clinical guideline.[8] Although earlier detection of fibrotic ILAs could lead to a shorter lag time to ILD diagnosis, facilitating timely treatment intervention and favourable patient outcome,[273] perhaps more pressing is a need for an easily accessible test to stratify patients to those who are more likely to progress and therefore require follow up.

This study explores the association between peripheral blood leukocytes and derived indexes with radiographic progression and mortality in a local cohort with early fibrotic ILA (EF-ILA).

4.2 Hypothesis and aims

I hypothesised that monocyte, neutrophil, lymphocyte counts, and derived indexes MLR, NLR and SIRI were associated with (i) progression of fibrosis and (ii) mortality in a cohort of subjects with evidence of early fibrotic ILA (EF-ILA).

To test this hypothesis, using a radiology database, I undertook the following objectives:

I. Estimate prevalence of EF-ILA in a specified geographical region in patients undergoing all indication thoracic CT scan
II. Explore association between blood leukocytes and radiographic progression of EF-ILA
III. Explore association between leukocytes and all-cause mortality in subjects with EF-ILA

4.3 Methods

4.3.1 Study population

Using the UK National Health Service-based Clinical Record Interactive Search (CRIS) database of the Oxford University Hospitals NHS trust (estimated catchment population of 800,000, Oxfordshire, UK), I examined available CT reports for all thorax-protocolised CT scans performed between January-2015 and December-2020 in subjects aged between 45 and 75 years.

Demographic data collated included age, gender, and co-morbidity profiles. Comorbidities for each case were searched for using the International classification of disease (ICD-10) coding.[274] Specific ICD-10 codes, pertaining to respiratory and non-respiratory diagnoses were cross-referenced with electronic health records. Similarly, ILD clinic attendance, date thereof and date of mortality obtained and time intervals between these events and first CT date demonstrating EF-ILA were deduced.

Total number of patients that underwent a thorax-protocolised CT during the same time period (January-2015 and December-2020) was used as a denominator to estimate EF-ILA prevalence in cases that underwent all-induction thoracic CT.

4.3.2 Search Criteria and data collation

I defined early-fibrotic ILA (EF-ILA) as reticulation in absence of traction bronchiectasis and honeycomb formation. Case identification was conducted in two phases. In phase 1, search criteria were selective for the EF- ILA radiological pattern in a ‘catch-all’ fashion, to maximise the initial search results of potential EF-ILA cases. Search criteria were as follows:

1.["reticulation" or "interstitial"] **AND**

2.["sub-pleural" or "basal" or "lower zone" or "Possible UIP"] **AND**

3.[Age range: 45-75]

Search criteria were defined with close guidance from our lead thoracic radiologist based upon the vocabulary tendencies of our thoracic radiologists when reporting CTs. I defined upper limit of age as 75 years.[275] Reticular abnormalities are common in older individuals and have sometimes been regarded as part of the normal spectrum of senescent lung.[276] Reporting findings as such could downgrade clinical significance and introduce ambiguity to this dataset.[277] Possible UIP was included in search criteria as this term can also include reticulation in absence of traction bronchiectasis and distinct absence of honeycombing,[7] and the initial search date preceded

introduction of the 2018 IPF guideline. Fibrotic ILAs with basal and peripheral predominance are considered to possess greater risk of progression and mortality. As such this distribution was also incorporated into the search criteria in an "OR" rather than "AND" fashion. This was to maximise the initial search results, and also because it has previously been documented that under-reporting of ILA has previously been described.[262]

In phase 2, I screened the preliminary search results for additional radiographic features – ground glass opacities (GGO), traction bronchiectasis, honeycombing in acknowledgment that multiple parenchymal features can co-exist on CT.[22,271] Cases with reticulation +/- GGO, but without traction bronchiectasis and honeycombing were termed 'early fibrotic ILA'. Those with traction bronchiectasis and honeycombing were classified as cases with traction bronchiectasis and/or honeycombing and not classified as EF-ILA. I considered these representatives of established fibrosis and/or UIP pattern fibrosis, and not ILAs. In a proportion of cases identified from the preliminary key word search, I later found on screening that CT reports were detailing negative / absence of specific radiological patterns; these were defined as a 'Nil ILA' reference cohort. And were separated from the EF-ILA cohort.

4.3.3 CT reporting and assessment of progression

CT scans were assessed using text-based searching of CT reports using the search criteria described in section 4.3.2. Radiographic progression was recorded as a binary event based on presence of new or increase in pre-existing parenchymal features as described in section 2.2. It was captured based upon text-based searching of CT report descriptions. Progression was adjudicated based upon perusal of all CT reports of repeat CTs using pre-defined criteria. Progression was defined as (i) increase in either extent of identified early fibrotic features (reticulation and or GGO), (ii) new emergence of traction bronchiectasis, and/or (iii) new emergence of honeycombing on follow on CT. In cases not demonstrating radiographic progression, this was defined as unchanged pre-existing parenchymal features and absence of new features.

4.3.4 Blood leukocyte measurement

As described in Study 1, blood leukocyte counts (monocyte, neutrophil, and lymphocytes) closest to the first CT scan demonstrating EF-ILA were recorded and leukocyte indexes were calculated.

4.3.5 Statistical analysis

Data is expressed as absolute values, relative percentages, means (S.D.), medians (IQR) or by dichotomised value where stated. Normality testing and analysis of inter-group differences conducted as described in section 2.6.

Cox proportional hazard models were used to determine hazard ratios (HRs) to progression and all-cause mortality (separate models). Time to progression was taken as time interval between first and follow-on CT. Time to mortality is measured from first CT scan. In both models, age, gender, monocytes, neutrophils, and lymphocytes levels obtained at a time point closest to the CT scan were included. Leukocyte index and coefficient of variation (CoV) values for each case were calculated from available counts for monocyte, neutrophil, and lymphocytes (and derived indexes) within the 1 year up to first CT to account for within-group variance in these measures.

ILA categories were included in regression models and where stated hazard ratios represent either absolute floating risk or expressed relative to the "nil ILA" category (reference category). Time-to-event analysis was performed between dichotomised leukocyte groups by Kaplan-Meier analysis.

4.4 Results

4.4.1 CT-based patient categorisation and demographics

170,197 CT scans were performed that included any thoracic CT protocol between January 2015 and December 2020 in 40,711 patients. 2735 cases satisfied the starting search criteria. 355 cases did not demonstrate ILD or ILA and were used as a non-ILA reference cohort. 762 cases also demonstrated additional traction bronchiectasis and / or honeycombing present on their first CT. 490 cases demonstrated traction bronchiectasis only, 272 demonstrated honeycombing +/- traction bronchiectasis. These cases were partitioned from the remaining cohort to identify those with EF-ILA.

1259 (3.1% of 40,711) cases demonstrated reticulation +/- GGO, without traction bronchiectasis or honeycombing. 430 also had emphysema and 88 also had non-emphysematous cysts. Therefore, 3.1% of subjects (1259 of the starting cohort of 40,711) requiring thoracic CT between 2015-2020 demonstrated 'early fibrotic ILA'. Mean age of the EF-ILA was 65.4 (±7.32); male; 735 (47.8%).

Demographic profiles are listed in Table 4.1 and a flow chart of ILA features and progression listed in Figure 4.2. Comorbidity profiles were identified by cross referencing electronic health records for specific ICD-10 codes. Comorbidities are representative of positive search events occurring at any time during the patient's history and therefore not specifically at time of first CT. 3217 CT scans (80.7% of scans with available information) were reported by a thoracic radiologist. Median time between blood draw and first CT demonstrating EF-ILA was 0.10 months (IQR -0.39 to 1.11).

343 cases with EF-ILA on first CT were seen in ILD clinic. Of these 198 had undergone repeat CT thorax prior to clinic attendance and of these 48% (95 of 198) demonstrated progression on repeat CT. The mean time from first CT to first ILD clinic attendance was 3.1 years.

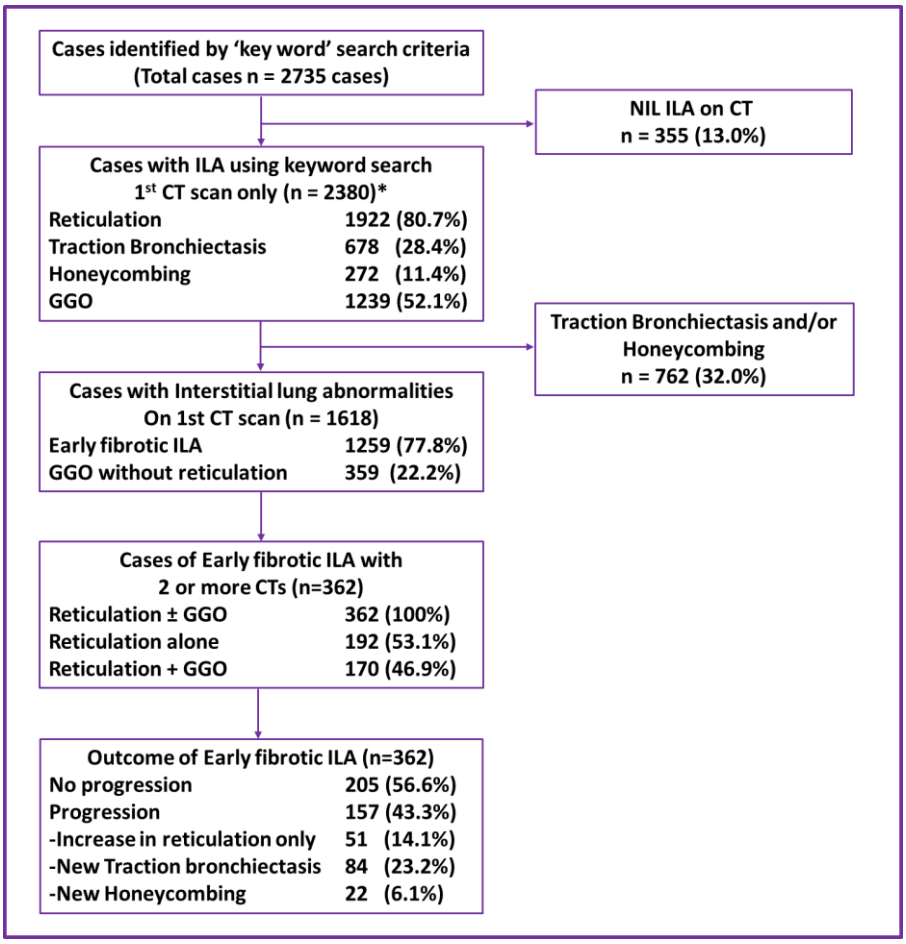

Figure 4.2 Flow diagram of ILA features and radiological progression of EF- ILA

* A proportion of first CTs demonstrated 2 or more ILA features simultaneously

Patient characteristics	Nil ILA	EF-ILA	ILA with Traction bronchiectasis or honeycomb	P Value
Demographics				
Female (%)	152 (42.8%)	803 (52.2%)	474 (62.2%)*	<0.001
Male	203 (57.2%)	735 (47.8%)	288 (37.8%)*	<0.001
AGE at first CT (SD)	65.5 (57.02-70.75)*	67.3 (61.07-71.61)	68.54 (63.32-72.22)	<0.001
Comorbidity				
COPD / Emphysema	52 (14.6%)	306 (19.9%)	175 (23.0%)	0.004
Pneumonia	70 (19.7%)	344 (22.4%)	215 (28.2%)*	<0.001
Lung cancer	30 (8.5%)	183 (11.9%)	43 (5.6%)*	<0.001
Pulmonary hypertension	13 (3.7%)	68 (4.4%)	61 (8.0%)*	<0.001
T2DM	57 (16.1%)	259 (16.8%)	143 (18.8%)	0.342
Hypertension	172 (48.5%)	664 (43.2%)	359 (47.1%)	0.048
IHD	66 (18.6%)	289 (18.8%)	178 (23.4%)	0.028
Cardiomyopathy	115 (32.4%)*	412 (26.8%)	232 (30.4%)	0.016
Blood leukocytes				
Time from CT to Blood test (months)	0.11 (-0.23 to 1.24)	0.10 (-0.39 to 1.11)	-0.10 (-0.76 to 0.72)*	<0.001
Monocyte (x10^3/μl)	0.58 (0.46-0.78)	0.62 (0.49-0.79)	0.67 (0.51-0.83)*	<0.001
Neutrophil (x10^3/μl)	4.71 (3.39-6.55)	4.49 (3.36-5.98)	5.00 (3.84-6.45)*	<0.001
Lymphocyte (x10^3/μl)	1.63 (1.22-2.15)	1.69 (1.25-2.28)	1.63 (1.23-2.2)	0.357
MLR	0.35 (0.25-0.54)	0.36 (0.26-0.51)	0.39 (0.28-0.54)*	0.012
NLR	2.58 (1.8-4.53)	2.54 (1.77-4.03)	2.91 (2.04-4.43)*	<0.001
SIRI	1.64 (0.93-3.06)	1.61 (0.97-2.76)	1.88 (1.21-3.09)*	<0.001
ILD clinic attendance	--	343 (27.2%)	502 (65.9%)	
Length of follow up (months)	--	20.8 (7.93-35.67)	22.95 (9-39.83)	0.254
Time from 1st CT to ILD clinic visit	--	16.9 (3.51-31.72)	17.89 (4.18-29.61)	0.548

Table 4.1 Patient characteristics

Demographic and blood leukocyte profiles of all patients, nil ILA, early and established interstitial lung disease. MLR; monocyte:lymphocyte ratio. NLR; Neutrophil:lymphocyte ratio. SIRI; systemic inflammation response. Kruskal-Wallis test was undertaken to assess the presence of significant differences between the three groups. Corrections for all multi-wise comparisons stated were performed using Dunn's method. Mann-Whitney test was used to analyse difference between 2 groups. Contingency tests (Fisher's exact test of significance or Chi Squared with Bonferonni adjustment) were used to assess inter-group differences in categorical data. * denotes subset whose column proportions differ significantly from other subsets.

4.4.2 Radiological progression of EF-ILA on follow-on CT

Of the 1259 cases with 'early fibrotic ILA', 362 patients underwent at least one follow-on CT scan, and therefore allowing examination of radiological change. The remaining 897 patients did not undergo repeat CT. Median time interval between CTs was 0.83 years (inter-quartile range 0.32-1.95). Progression in type or extent of ILA was observed in 157 cases (43.4%). Of these, increase in reticulation was observed in 51 cases (14.1% of 362). Progression with emergence of traction bronchiectasis (excluding honeycombing) was observed in 84 cases and (23.2%) and honeycombing (with or without traction bronchiectasis) in 22 (6.1%). The remaining 205 (56.6%) cases did not demonstrate progression of EF-ILA.

Median time interval between CT scans in cases demonstrating progression was 1.24 years (IQR 0.53-2.36, maximum 5.20) vs 0.59 years (0.27–1.19, maximum 5.05) in cases demonstrating no

progression (p<0.001). Time interval between CT scans displaying progression of reticulation [0.83 years (0.30-1.98)] was shorter in comparison to new traction bronchiectasis [1.54 years (0.77-2.71)] and honeycombing [1.58 years (0.80-3.12)] on repeat CT. Of the 157 cases demonstrating progression, 62 cases (39.4%) demonstrated progression on interval CT in within 1 year, 43 (27.4%) demonstrated progression between 1-2 year, 29 (18.5%) demonstrated progression within 2-3 years and the remaining 23 patients (14.6%) demonstrated progression between 3 and 5 years (Table A3).

Multivariate Cox regression analysis, that was adjusted for age and gender, identified greater risk of mortality in the 157 cases of EF-ILA demonstrating radiographic progression [HR 1.92, 95%CI 1.51-3.21, p=0.013]. Age was also significantly associated with progression [HR 1.10, 1.05-1.16, p<0.001]. As described in Table A4.

4.4.3 Imaging features and ILA mortality

Death was reported in 448 cases (16.40%) in the six years of analysis. Mean time from first CT to death was 19.8 ±16.5 months vs 35.6 ±20.6 in those that survived (p<0.0001). In cases with the early fibrotic ILA, death was reported in 183 cases. Mean time from first CT to death was 19.0 ±16.6 months vs 32.7 ±20.5 in those that survived.

Kaplan-Meier analysis demonstrated shorter survival time from first CT in the EF-ILA cohort in comparison to the Nil ILA cohort. Survival time was shortest in subjects displaying evidence of honeycombing on first CT (Figure 4.3a, p<0.0001). Furthermore, there was also a trend of shorter survival time in cases of EF-ILA that also demonstrated co-existing GGO or emphysema patterns on CT (Figure 4.3b, p<0.0001).

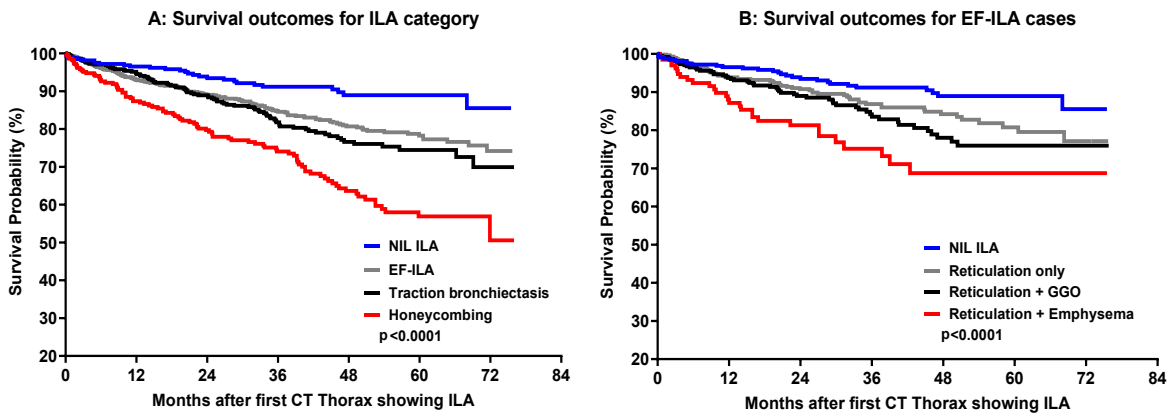

Figure 4.3 Kaplan-Meier survival probability for (A) all cases and (B) cases with EF-ILA

Association between specific ILA features noted on first CT and mortality was explored using multivariate Cox regression (Table 4.2). As expected, traction bronchiectasis [HR 2.09, 95%CI 1.36-3.20, p=0.0001] and honeycombing [HR 3.65, 2.38-5.60, p<0.0001] were significantly associated with mortality relative to the Nil ILA reference category. Cases with the early fibrotic ILA also demonstrated significant mortality risk [HR 1.87, 1.25-2.78, p=0.002]. This risk was slightly higher in cases of EF-ILA that also demonstrated GGO [HR 2.03, 1.29-3.19, p=0.002] in comparison to cases of EF-ILA without GGO [HR 1.80, 1.19-2.72, p=0.005]. Mortality risk of EF-ILA was preserved when adjusting for the respiratory comorbidities; lung cancer, pneumonia, and COPD/emphysema (Table A5).

	n (%)	Death (%)	HR (95% CI)	P value
Covariate				
Age	--	--	1.02 (1.01-1.04)	0.010*
Gender (Male)	1486 (54.3%)	268 (18.0%)	1.11 (0.91-1.36)	0.295
Nil ILA (reference)	355 (12.9%)	43 (12.1%)	--	--
Early fibrotic ILA	1259 (46.0%)	183 (14.5%)	1.87 (1.25-2.78)	0.002*
TBx without Honeycombing	490 (17.9%)	86 (17.6%)	2.09 (1.36-3.20)	0.0001*
Honeycombing +/- TBx	272 (9.9%)	87 (32.0%)	3.65 (2.38-5.60)	<0.0001*

Table 4.2 Multivariate Cox regression examining association of ILA features with mortality
Multivariate Cox regression examining association of ILA features on first CT scan with mortality. Hazard ratios (HR) for ILA categories representative of risk relative to Nil ILA reference category. TBx; Traction bronchiectasis, EF-ILA; Early-fibrotic ILA, p<0.05.

4.4.4 Blood leukocyte association with ILA progression and mortality

I next explored association between peripheral blood leukocytes and their derived leukocyte indexes against radiographic progression and mortality using multivariate Cox proportional hazards models (**Error! Reference source not found.**). Nearest available blood measurement of monocyte, neutrophil, and lymphocyte to first CT scan were obtained from standardised hospital 'full blood count' measurements. Median time interval between CT and nearest blood sampling was 1 day (IQR -13 to 30). All models included age, gender, and the leukocyte values (or their derived indexes) contemporaneous with first CT scan. Histograms demonstrating leukocyte distribution (closest value to CT) are displayed in Appendix Figure A.4.

	EF-ILA Mortality		EF-ILA progression	
Model	**HR (95% CI)**	**P value**	**HR (95% CI)**	**P value**
Blood leukocytes				
Age	1.03 (1.01-1.06)	0.005	1.03 (1.00-1.06)	0.027
Gender	1.04 (0.76-1.42)	0.811	0.92 (0.67-1.27)	0.609
Monocytes (x10^3/μl)	1.12 (1.01-1.36)	0.003	1.79 (1.05-2.86)	0.030
Neutrophils (x10^3/μl)	1.13 (1.07-1.19)	<0.001	1.11 (1.02-1.29)	0.009
Lymphocytes (x10^3/μl)	0.97 (0.85-1.09)	0.574	0.99 (0.94-1.04)	0.596
MLR				
-Age	1.03 (1.01-1.06)	0.006	1.02 (0.99-1.05)	0.113
-Gender	1.00 (0.74-1.36)	0.995	0.92 (0.67-1.27)	0.624
-MLR	1.16 (1.02-1.31)	0.025	2.28 (1.33-3.87)	0.002
NLR				
-Age	1.03 (1.01-1.06)	0.007	1.02 (0.99-1.05)	0.122
-Gender	0.98 (0.72-1.34)	0.910	0.96 (0.70-1.32)	0.814
-NLR	1.07 (1.05-1.09)	<0.0001	1.07 (1.01-1.14)	0.024
SIRI				
-Age	1.04 (1.01-1.06)	0.003	1.03 (0.99-1.05)	0.079
-Gender	1.02 (0.75-1.38)	0.924	0.96 (0.69-1.31)	0.789
-SIRI	1.06 (1.04-1.08)	<0.0001	1.09 (1.04-1.14)	0.0002

Table 4.3 Multivariate cox regression for mortality and progression of EF-ILA

Multivariate cox regression examining association between blood leukocyte and (i) mortality in "early fibrotic" ILA (n=1259) and (ii) radiological progression in "early fibrotic" ILA cohort with available repeat CT for comparison (n=362). Leukocyte contribution to mortality and progression of EF-ILA explore in separate models to leukocyte ratios. MLR; monocyte:lymphocyte ratio. NLR; Neutrophil:lymphocyte ratio. SIRI; [(Monocytes x Neutrophils) ÷ Lymphocytes].

Of the 362 cases of EF-ILA that underwent a repeat CT scan, there was a trend of higher median monocyte, neutrophil and lower lymphocyte count in the 157 cases demonstrating progression, although this was not statistically significant (Table A6). Monocyte count [HR 1.79 (1.05-2.86), p=0.030] and Neutrophil count [HR 1.11, 1.02-1.29, p=0.009], MLR [HR 2.28, 1.33-3.87, p=0.002], NLR [HR 1.07, 1.01-1.14, p=0.024] and SIRI [HR 1.09, 1.04-1.14, p=0.0002] were significantly associated with radiographic progression of the early fibrotic ILA on multivariate Cox regression analysis. (Table 4.3). Monocyte, neutrophil and all their derived indexes were significantly associated with all-cause mortality. Advancing age was also significantly associated with EF-ILA progression and mortality.

In separate regression models I also included additional respiratory comorbidities (pneumonia, Lung cancer and COPD) to adjust for an effect these may have on association between leukocytes and ILA progression and mortality. Not unsurprisingly, pneumonia and lung cancer demonstrated significant association with mortality but interestingly not associated EF-ILA progression (Table A6). Despite addition of these covariates, monocytes remained significantly associated with radiological progression of EF-ILA [HR 1.72, 1.10-2.69, p=0.018]. Although neutrophil count continued to demonstrate direction of effect towards progression this was no longer significant [HR 1.05, 0.98-1.12, p=0.154].

I also explored for any effect variation in serial leukocyte measurement may have on clinical outcomes. Co-efficient of variation (CoV) calculated from serial measurements of each leukocyte / index was also included, to adjust for any effect that natural variation in longitudinal measurement of these leukocyte parameters. CoV was calculated from serial blood sampling over the 1 year prior to the CT for each leukocyte variable. Monocytes, Neutrophils, NLR and SIRI maintained significant association with mortality. In all leukocyte measurements, greater monocyte, neutrophil and lymphocyte CoV measurements were significantly associated with EF-ILA progression and mortality. Although leukocyte CoV measurements were not significantly different between the EF-ILA progressors and non-progressors, monocytes maintained significant hazard towards progression of EF-ILA when adjusted for CoV (Appendix Table A7 and Table A8) in Cox PH multivariate models.

4.5 Discussion

In Study 1, I observed association between monocytes, neutrophils, and derived indexes with IPF progression and mortality. Similarly in Study 2, I wanted to explore if this association also extended to patients with ILA suggesting early features of fibrosis, but not meeting diagnostic criteria of IPF.

This study demonstrates that in an unselected cohort of patients undergoing thoracic CT scanning for all indications, in a 6-year period, 3.1% of patients showed evidence of 'early fibrotic ILA'. In a subset of this cohort which underwent at least one more CT scan during this 6-year period, 43% progressed either in extent of existing disease or demonstrated new traction bronchiectasis or honeycombing. Monocytes, monocyte:lymphocyte ratio, neutrophil:lymphocyte ratio and SIRI were associated with progression in a multivariate analysis which included analysis of age and gender.

Importantly, these findings are comparable to ILA prevalence observed in large population-based cohorts,[243,267,278] and lung cancer screening cohorts.[265,266,279] ILA ranged between 3-10% in these cohorts. I used a definition of 'early fibrotic ILA'. This included reticulation and GGO but not traction bronchiectasis and honeycombing and probably encompasses the 'indeterminate for UIP' feature as defined by the 2018 IPF guidelines.[8] However, as I was unable to assess CT distribution of these ILA in all cases for this relatively large cohort, the terminology of early fibrotic ILA was used.

Advancing age was significantly associated with mortality and progression across all multivariate analyses incorporating absolute leukocytes. Reticular abnormalities are common in older individuals and have previously been regarded as part of the normal spectrum of senescent lung.[276] Reporting findings as 'age-related' could downgrade clinical significance and introduce ambiguity,[277] and I therefore restricted inclusion to individuals aged 45-75 at time of CT.[275,276] Comparable to other studies, gender was roughly of equal proportions across all cases in the EF-ILA group.[243,267] However, and not unexpectedly, a greater proportion of male subjects demonstrated progression with new traction bronchiectasis or honeycomb formation in this cohort, however this was not statistically significant (Appendix Table A9).

The AGES[243] and Framingham[267] population-based studies demonstrated ILA progression in 43% and 64% of cases, with associated risk of mortality which is also similar to the Study 1 cohort. In AGES, prevalence of indeterminate for UIP (iUIP) was estimated at 3.9%. iUIP was associated with mortality risk in univariate analysis [HR 1.6, p<0.0001] but not significantly in multivariate analysis [HR 1.2, p=0.07]. In this Study 2 cohort, with a much higher number of cases, multivariate analysis showed that early fibrotic ILA is associated with all-cause mortality [HR 1.87, p=0.002].

Importantly, this data and that of others,[236] demonstrates radiographic progression is observed in a proportion of cases, but crucially not all. It remains challenging to predict those cases that will progress to established fibrotic ILD. The clinical implications of this are becoming increasingly recognised in the ILD community and to address this, the Fleischner society recently proposed a schema to facilitate triage, management and follow up of ILAs.[271] This includes sub-categorising cases according to radiological findings of ILA distribution on CT and presence (or absence) of ILAs indicative of the early stages of established fibrosis.

ILA assessment in individuals with a history of familial interstitial lung disease has identified association with particulate exposures, age, positive smoking history and shorter telomere length and MUC5b risk allele.[104,280] Associations among ageing-related biomarkers and ILA have been explored,[281] however collectively these are costly and are not commercially available for large scale use. Furthermore, with future implementation of routine lung cancer screening pathways and greater use of thoracic CT for other diagnostic purposes it is anticipated that ILA detection will increase. Patient follow-up could have a huge implication on clinical resources. Peripheral blood leukocyte measure is available as part of routine full blood count analysis and integrating this simple and cheap measure into risk stratification and ILA management could be worthy of future consideration.

In chapter 3, I discussed Study 1 observations of association between monocytes, neutrophils, and lymphocytes in adverse outcomes in a cohort of iUIP and IPF patients from. I also discussed

current mechanistic understandings of leukocyte immunobiology from translational studies that support these associations. Briefly, (i) migration of monocytes from bone marrow to injured lung, and then differentiating into macrophages with a pro-fibrotic phenotype,[184] and (ii) translational studies implicating distinct monocyte-derived alveolar macrophage populations in fibrogenesis.[191,244] Neutrophils are recruited to areas of inflammation,[135] and can alter their microenvironment by secreting proteases, oxidants, pro-inflammatory cytokines, matrix metalloproteinases (MMP) culminating in collagen deposition and Extra-cellular matrix formation.[133,246] Neutrophil elastases (NE) are elevated in IPF BAL samples,[131] and experimental data using murine models suggest that NE activates transforming growth factor-ß (TGF-ß) pathway and fibroblast proliferation.[247]

These Study 1 observations are also comparable to my observations here in Study 2, and in particular the association between blood monocyte count with mortality and EF-ILA progression. Thus, the similar observations among these two cohorts could imply that the immuno-pathobiological action of monocytes, and other blood leukocytes, that are observed in IPF is potentially already active at an earlier stage of fibrogenesis, before onset of significant clinical symptoms.

The study by Scott et al, a large multicentre retrospective cohort study, first demonstrated that blood monocyte count $\geq 0.95 \times 10^3/\mu l$ were associated with all-cause mortality in IPF and non-IPF fibrotic lung disease.[197] Since then other retrospective clinical studies in IPF cohorts have documented similar findings.[227-229] Furthermore, Kim et al explored for similar associations in a retrospective pooled analysis of multiple ILA cohorts comprising 7396 subjects.[282]

Kim reported association between higher absolute monocyte count with reduced baseline FVC% and ILA progression across 4 population-based cohorts; MESA,[283,284] AGES,[236,285] COPDGene,[286] and ECLIPSE.[279] The authors also demonstrated, using LPS-driven stimulations assays of whole blood samples of MESA study participants, greater monocyte expression of tissue factor in the ILA cohort. The latter, a monocyte surface marker, is implicated in inflammation, coagulation, and wound healing responses.[287] There are subtle, but important differences between the findings of Kim et al and my findings from Study 2. At least two cohorts (COPDGene and ECLIPSE) are COPD-focused and therefore smoking burden is higher. Only within the AGES cohort are potential fibrotic radiological appearances captured. The other 3 cohorts predominantly report CT high attenuation areas. Therefore, Kim et al describes monocyte association to generic ILA descriptions. They are unable to discuss if their observed association between monocytes and ILA progression is limited to specific ILAs, such as those with early evidence of fibrosis. However,

monocyte count was associated with ILA progression over 5 years in AGES [Odds ratio 1.2, 95%CI 1.0-1.3], and descriptors of progression were similar to Study 2.[236,282]

In Study 2 inclusion criteria were purposely biased towards selection of cases with CT features potentially compatible with early fibrosis, thereby enriched for patients potentially at risk of progressing to pulmonary fibrosis.[271] Furthermore I was able to describe extent of EF-ILA progression with finer detail, by (i) capturing the proportion of cases with new CT features representative of established pulmonary fibrosis and (ii) report association between absolute monocyte count (and other leukocyte parameters) with progression and all-cause mortality. I also adopted a different approach to data analysis, employing Cox proportional hazards modelling to take account of time until events occurred, in comparison to logistic regression used by Kim et al.

The single-centre and retrospective nature of this study limits data interpretation to association between CT features with blood leukocytes and not causality. There are several other limitations that should be considered when interpreting these results. I was unable to quantify extent of disease and extent of progression. Categorisation was based on, and limited by, qualitative information extracted from radiology reports. I was not able to quantify individual ILA extent beyond a binary classification of present/absent or changed/unchanged features which may have contributed to the rate of progression observed. Scoring EF-ILA extent and progression with superior resolution may have yielded more accurate association between EF-ILA and blood leukocyte profiles as has been demonstrated in other studies.[104,288] Similarly, I was unable to capture indication for CT and I could not account for clinical symptoms reported (or not reported) by patients and lung function was not available. Each of these would have been interesting to explore. Therefore, interpretation of these findings is limited to association. Furthermore, the recently updated definition of ILA has not been universally adopted across clinical studies (including this one), which makes inter-cohort comparisons challenging.[273]

Although the time between first CT showing EF-ILA and closest blood draw was small in this cohort, time of blood draw could not be controlled for. Diurnal variation of leukocytes can be affected by sleep pattern, the physiological demands of exercise and stress.[289-291] Thus it is possible that single isolated leukocyte values used in regression analysis of this cohort (and others) are not entirely representative of mean trends and solitary values could potentially over or under-estimate effect, and introduce bias to these findings. I therefore calculated coefficient of variation for leukocyte parameters for each subject from available blood samples in the 1 year prior to CT. In a separate analysis I attempted to account for any contribution of variation in serial leukocyte measurements by including coefficient of variation values for each case into regression models (Appendix Table A7). Monocytes, but not neutrophils, remained significantly associated

with EF-ILA progression. Interestingly, greater monocyte, neutrophil and lymphocyte CoV measurements were associated with greater risk of EF-ILA progression.

Patients in this cohort were selected because of their need for a thoracic CT, so the true prevalence in the population is unknown, only in those who required a thoracic CT. In the group where I assessed progression, the interval between the first and last CT was longer in those who progressed compared to those who did not. It could be argued that those that did not progress may have done so over a longer period of follow up. The 'Nil ILA' group was identified by description of negative findings of key words from CT reports (e.g. "no reticulation"). This of course represented a smaller proportion of patients with normal CT scans and may have introduced bias to EF-ILA mortality risk calculation. However, selection criteria were identical and demographic and comorbidity profiles were comparable between Nil ILA and EF-ILA group. Furthermore, demographic profiles of the Nil-ILA group are also comparable to the 'Nil ILA' cohorts of other longitudinal studies.[243]

A proportion of our EF-ILA cohort were subsequently seen in ILD clinic. Mean time from first CT scan demonstrating EF-ILA to ILD clinic attendance was +3.1 years. As the ILD clinic was a first-attender clinic, it is likely (but not verified) that these were patients who became symptomatic or demonstrated progression after a follow-on CT scan and did not have a prior diagnosis if ILD. There is therefore possibility of prior undiagnosed ILD. Under-reporting of ILA has previously been described.[262] As such I cannot exclude the possibility that a degree of misclassification may have occurred. However, 80.7% of CT scans were reported by post-radiology fellowship specialist thoracic radiologists who attend interstitial lung disease multi-disciplinary team meetings and are based at a single centre which might mitigate inter-observer difference. Furthermore, excellent inter-observer correlation between thoracic radiologists at this institution in reporting ILD features ($r=0.91$; $p<0.001$) has previously been described.[292] The remaining 19.3% of CTs were reported by 1 of 14 Oxford-based post training radiologists. All radiologists collectively agree on descriptive reporting phrases and there are regular local discrepancy meetings to check on accuracy of reporting.

ICD-10 coding was used to identify comorbidity during the 6 years of study follow up, but without coding dates we were unable to align comorbidity events to 1st CT scan date. I chose not to exclude cases of lung malignancy from the EF-ILA and Nil ILA cohorts.[268,293] This may have introduced bias, however lung cancer staging, treatments, and data on cause of death were unknown. I therefore included these cases in analysis and classified mortality as all-cause. In separate multi-variate analysis lung cancer, and pneumonia, were independently associated with EF-ILA but monocyte count remained independently associated with EF-ILA progression (Table

A7). Smoking history was poorly captured using 'ICD-10 coding'. I therefore elected not to include this information in our multivariate analysis (to avoid imputing missing data) but acknowledge that patients who smoked may show a greater rate of ILA progression.[282]

4.6 Conclusion

3.1% of subjects requiring thoracic CT during a 6-year period demonstrated EF-ILA. Monocyte levels, and blood leukocyte-derived indexes were associated with radiological progression in this cohort and could indicate which patients may require closer follow up.

Notwithstanding these limitations, this study, in a large cohort with high proportion of specialist thoracic-radiologist reporting, demonstrates that monocyte levels, MLR, NLR and SIRI are associated with progression in early fibrotic ILA. Further prospective studies could help determine if these parameters could be used to help prioritise patients who might benefit from follow up.

Chapter 5 Blood leukocyte association with radiological progression of lung fibrosis in IPF

5.1 Introduction

Accurate clinical assessment is central to management of patients with IPF. In addition to informing patients to their probable life expectancy, the accurate prediction of clinical course can guide appropriate management, such as initiation of antifibrotic therapy, transplantation assessment or palliative care pathways.[71] The currently accepted gold standard for assessing disease severity in patients with IPF is measurement of FVC and TLCO, and assessment of UIP category on CT scan.[8]

The Computer-Aided Lung Informatics for Pathology Evaluation and Rating (CALIPER), is an automated software application that uses quantitative CT algorithms to characterise and quantify lung parenchymal abnormalities on volumetric high resolution CT (HRCT).[82] Its application as a research tool to adjudicate ILD cohorts has steadily gained popularity. Many studies conducted using IPF cohorts have demonstrated not only is CALIPER more predictive of mortality in comparison to conventional measures of FVC% and visual CT assessment,[93] but also that it can enhance risk stratification, and facilitate cohort enrichment for important trial end points.[94,95] Collectively these findings suggest CALIPER can strengthen prediction models for adverse outcomes in IPF.

In Study 1, I demonstrated that higher blood monocyte and neutrophil counts were associated with shorter time for progression of iUIP (cohort A), shorter survival time and shorter time to relative FVC decline >10% (cohort B). In these cohorts radiographic progression was classified as a binary event, describing change of radiographic features or UIP category. However, this classification could not enumerate any change in fibrosis on interval CT.

Utilising CALIPER to quantify extent of parenchymal abnormalities on CT, I explored association between blood leukocytes and progression of fibrosis in a separate IPF cohort. Specifically, I was curious as to how association of CALIPER fibrosis scores with blood leukocytes compared to association with gold standard metrics of FVC and TLCO.

5.2 Hypothesis and aims

I hypothesised that progression of fibrosis in IPF patients, measured using CALIPER correlated with blood monocyte and neutrophils. To test this hypothesis, I undertook the following objectives:

I. Determine baseline measure of CALIPER-measured fibrosis and Pulmonary vessel volume (PVV) in IPF patients
II. Determine change in CALIPER-measured fibrosis in cases undergoing repeat CT
III. Explore association between blood leukocytes with baseline and change in CALIPER-measured fibrosis and PVV scores.

5.3 Methods

5.3.1 Study population

To explore for association between blood leukocytes and CALIPER-derived measurements of disease severity I performed a retrospective analysis of a cohort of patients who attended the Oxford Interstitial Lung Disease Service between September 2016 and November 2021. All patients had an MDT diagnosis of IPF. Patients with a non-contrast, supine, volumetric HRCT scan were included.

I collected the following data: patient demographics (gender, age), time-interval between HRCT scans, available pulmonary function tests (PFTs) closest to the selected CT scans, and all available CT reports detailing UIP category.

5.3.2 Qualitative CT assessment

Qualitative assessment of baseline and follow-on CTs has been previously described in chapter 2 (section 2.2). Briefly, a thoracic radiologist with a sub-specialty interest in ILD reviewed each patient's CT imaging at MDT. All CT abnormalities were defined using standard Fleischner-based terminology and according to 2018 IPF guidelines.[8,22] Radiologist reports from all available thoracic CTs for these patients and up to August 2019 were analysed. CT scans pertaining to ILD MDT reports or radiology reports detailing consolidation were excluded as these would affect accuracy of CALIPER evaluation.

5.3.3 CALIPER evaluation

Data processing, pulmonary vessel quantification and pattern evaluation have been previously described in methods section 2.3.

Pulmonary vessel volume (PVV) was calculated as an absolute volume (cm^3) or expressed as a relative percentage of total lung volume. Total and regional lung volumes were calculated. Regional volumes were expressed as upper, middle, and lower zone volume and calculated from sum of respective right and left lung regional volumes. In cases that underwent a follow-on CT, annualised change (time between CT1 and CT2) in PVV is expressed as either absolute change in PVV cm^3 (CT2 - CT1), or absolute change in PVV% per year.

Pulmonary vessel volume (PVV) was also explored in a separate cohort of patients undergoing volumetric CT for routine follow up assessment of pre-existing lung nodules. This was to provide a meaningful comparison of PVV assessment in cases without fibrotic lung disease (Figure A.7).

Volumes for each parenchymal feature were calculated as per section 2.3.2 and expressed as a relative percentage of CALIPER-derived (i) total lung volume or (ii) zonal lung volume. Total lung fibrosis (TLF) score represented the sum of GGO, reticular and honeycomb percentages.[94] Where stated, TLF was also expressed as TLF% per litre of CALIPER-measured total lung volume.

In cases that underwent a second CT, change in fibrosis is expressed as the absolute change in total fibrosis per litre lung volume (CT2 TLF/litre – CT1 TLF/litre). TLF/litre was used as this is a standardised measure to account for difference in lung volume on serial scans with progression in fibrosis. As described above, annualised change in TLF/litre was also calculated.

5.3.4 Pulmonary function tests

Collation of lung function data was previously described in Section 2.4. Briefly, FEV1, FVC, TLco and CPI were captured closest to CT. To draw meaningful comparison to CT trends, annualised lung function trends were deduced from tests recorded closest to first and second CT scan. A measured reduction in absolute forced vital capacity (litres) >10% per year was considered clinically significant and representative of physiological decline.[25]

5.3.5 Blood leukocyte measurement

Blood leukocyte data was captured as previously described in studies 1 and 2. Briefly, Neutrophil, lymphocyte, and monocyte levels captured using standard 'full blood count' analysis within 4 months of initial CT. Indexes MLR, NLR and SIRI were calculated. Leukocyte values were collated as either continuous or discrete variables, dichotomised by median value or by high or low absolute value.

5.3.6 Statistical analysis

As described in section 2.6, data is expressed as absolute values, relative percentages, means (with S.D.), medians (IQR) or by dichotomised value where stated.

Pearson correlation was used to explore association between blood leukocytes (and derived indexes) with (i) baseline and (ii) annualised CALIPER-derived metrics of fibrosis and PVV and pulmonary function tests. Association between blood leukocytes and sub-divisions of CALIPER metrics according to regional measurement (upper, middle, and lower zone) were also explored. Correlation was also used to explore relationship directly between baseline and annualised change in CALIPER-derived metrics of fibrosis and PVV with FVC and TLCO.

Multivariate Cox PH models were used to explore association between blood leukocytes (and derived indexes) against the outcomes of (i) >10% increase in CALIPER TLF/litre of lung volume, (ii) >7.8% increase in CALIPER TLF/litre (lower limit of upper tertile) and (iii) FVC decline >10% between CT1 and CT2. Harrell's Concordance index (C-index) was used to assess model strength. The C-index describes how well a model can discriminate between two survival distributions.[294] Receiver operating characteristic (ROC) analysis was used to compare accuracy of different CALIPER metrics of disease severity for outcomes of disease progression and all-cause mortality.

Finally, to provide a comparison to studies 1 and 2, Kaplan-Meier analysis (Log rank test of significance) was used to evaluate time to all-cause mortality from first CT for cases dichotomised by leukocyte values. A censoring date of 1st January 2022 was applied.

5.4 Results

274 cases were identified in Cohort 3 with an MDT diagnosis of IPF. Of these, 171 cases (62.4%) had at least 1 CALIPER-compatible HRCT (Figure 5.1). Of the eligible 171 patients, mean age (S.D.) was 74.9 years (7.9). 154 patients (90.8%) were male. Median length of follow up from first CT scan was 41.6 (18.1-52.0) months and maximum length of follow up was 76.1 months. Demographic data, physiological and CALIPER-derived indices, comorbidity profiles and blood leukocyte data are shown in Table 5.1.

As would be anticipated, lung function values were generally worse in the Definite UIP group. CALIPER PVV and TLF% were significantly higher in the definite UIP group. Of the CALIPER parenchymal features scored at CT1, upper zone fibrosis, total reticulation, and total honeycombing % were significantly higher in the definite UIP group.

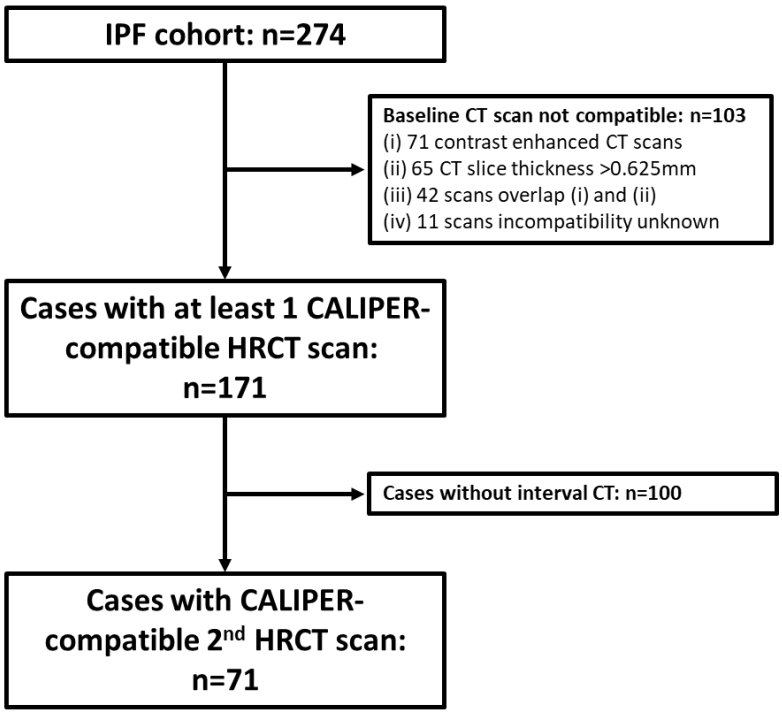

Figure 5.1 Flow diagram illustrating Study 3 case selection

71 (43.3%) patients had at least one follow-on CALIPER compatible HRCT. Median time between blood draw and 1st CT was 0.58 months (-0.52 to 3.34). Median time interval between 1st CT scan and nearest lung function test was 0.23 months (-1.45 to 2.33). 69 patients (40.4%) were receiving

antifibrotics at time of first CT scan. Median duration of antifibrotic treatment was 52.0 months (23.4-67.1). When cases were separated according to UIP categorisation (Probable or Definite UIP) there was no significant difference in demographics, comorbidities, blood leukocytes or antifibrotic prescribing.

For comparison purposes PVV analysis was also conducted in a non-fibrotic lung nodule follow up cohort. PVV was significantly greater in the IPF group (Study 3) compared to the lung nodule group with available CALIPER compatible CT (Appendix Figure A.7).

	Qualitative UIP categorisation			
	All patients (n=171)	**Probable UIP (n=83)**	**Definite UIP (n=88)**	**p value**
Demographics				
Age at CT	75.1 (70.1-81.1)	70.8 (70.6-79.1)	77.1 (69.8-81.7)	0.370
Female	17 (9.9)	7 (8.40)	10 (11.4)	0.613
Male	154 (90.1)	76 (91.6)	78 (88.6)	
Smoking status				
Never smoker	48 (27.5)	20 (24.1)	28 (31.8)	0.636
Ex-smoker	91 (52.3)	39 (47.0)	51 (60.0)	
No data	35 (20.2)	24 (28.9)	9 (10.2)	
CALIPER Parenchymal features				
TOTAL lung fibrosis (%)	14 (8-22)	11 (7-21)	15 (9-25)	**0.027**
Upper Zone fibrosis (%)	6 (1.5-13.3)	4.5 (1-13)	7 (3-14.5)	**0.036**
Middle Zone fibrosis (%)	10 (3.8-21)	8.5 (3.5-18.5)	10.5 (4-22)	0.312
Lower Zone fibrosis (%)	25 (12-48.3)	20.8 (11.5-45)	30.5 (13.5-55)	0.084
TOTAL Low attenuation area (%)	1 (0-2)	0 (0-2)	1 (0-3)	0.083
TOTAL Ground Glass (%)	5 (3-13)	5 (2-13)	5 (3-12)	0.475
TOTAL Reticular (%)	5 (3-9)	5 (2-7)	7 (4-10)	**0.002**
TOTAL Honeycombing (%)	1 (0-1)	0 (0-0)	1 (0-2)	**<0.001**
CALIPER Pulmonary vessel volume				
TOTAL PVV (cm^3)	160 (128-201)	143 (121-179)	177 (144-207)	**0.001**
Upper Zone PVV (cm^3)	19 (13-25)	16 (44896)	21 (14-28)	**<0.001**
Middle Zone PVV (cm^3)	52 (42-66)	49 (41-61)	59 (43-73)	**0.003**
Lower Zone PVV (cm^3)	47 (35-65)	43 (30-62)	52 (38-68)	**0.045**
Total PVV (%)	4.02 (3.02-5.21)	3.74 (2.84-4.7)	4.62 (3.39-5.57)	**0.002**
Upper Zone PVV (%)	0.46 (0.33-0.69)	0.41 (0.29-0.56)	0.55 (0.38-0.78)	**<0.001**
Middle Zone PVV (%)	1.29 (1.06-1.76)	1.19 (0.93-1.57)	1.45 (1.12-2.02)	**0.006**
Lower Zone PVV (%)	1.21 (0.82-1.69)	1.13 (0.76-1.53)	1.35 (0.94-1.77)	**0.023**
Lung function				
FEV1 (l)	2.19 (1.83-2.68)	2.25 (1.91-2.78)	2.1 (1.74-2.57)	0.108
%FEV1	81.2 (70.9-92.2)	81.88 (72.3-92)	80 (66.47-92.38)	0.255
FVC (l)	2.8 (2.28-3.365)	2.84 (2.36-3.46)	2.62 (2.24-3.16)	0.074
%FVC	76.57 (66.6-91.15)	77.00 (68.8-94.4)	75.1 (62.55-90.95)	0.155
FEV1:FVC (%)	80.6 (75.0-86.1)	79.4 (74.4-85.0)	82.1 (75.9-86.1)	0.079
TLCO cor (SI)	4.56 (3.49-5.48)	5.04 (3.8-5.77)	4.22 (3.3-5.31)	**0.003**
%TLCO	56.85 (49.5-67)	61.00 (52-71)	53.7 (44.4-63.15)	**0.002**
CPI Score	67.48 (61.05-73.12)	65.86 (60.35-70.02)	69.12 (62.59-74.92)	**0.014**
Blood leukocytes				
Monocyte ($x10^3/\mu l$)	0.67 (0.58-0.84)	0.71 (0.55-0.85)	0.68 (0.59-0.82)	0.567
Neutrophil ($x10^3/\mu l$)	4.90 (3.85-6.53)	5.25 (3.96-6.79)	4.67 (3.65-6.17)	0.059
Lymphocyte ($x10^3/\mu l$)	1.62 (1.31-2.23)	1.67 (1.3-2.17)	1.62 (1.31-2.23)	0.865
MLR	0.37 (0.32-0.55)	0.41 (0.33-0.52)	0.38 (0.31-0.57)	0.758
NLR	2.70 (2.08-4.08)	2.93 (2.27-4.18)	2.73 (1.95-3.65)	0.091
SIRI	1.79 (1.4-3.08)	2.08 (1.42-3.07)	1.76 (1.25-3.09)	0.124
Comorbidity				
Reflux	54 (39.1)	21 (36.2)	33 (41.8)	0.596
PHT	11 (8)	4 (6.9)	7 (8.9)	0.759
IHD	28 (20.3)	16 (27.6)	12 (15.2)	0.089
Cardiomyopathy	6 (5.8)	3 (7)	3 (5)	0.692
Arrhythmia	9 (6.5)	4 (6.9)	5 (6.3)	0.999
Hypertension	29 (21)	15 (25.9)	14 (17.7)	0.292
PE	3 (2.2)	0 (0)	3 (3.8)	0.262
COPD Emphysema	8 (7.7)	3 (7)	5 (8.3)	0.999
T2DM	15 (10.9)	8 (13.8)	7 (8.9)	0.413
Antifibrotics				
Antifibrotic use	69 (40.3)	28 (33.7)	41 (46.5)	0.607

Table 5.1 Baseline characteristics at point of first CT

Cases presented in totality and subdivided for visual assessment of UIP pattern. NLR; neutrophil:lymphocyte ratio, MLR; monocyte:lymphocyte ratio, SIRI; systemic inflammation response index, CPI; Composite Physiological Index as calculated by Wells.[240] Continuous variables expressed as median values (IQR).

5.4.1 Relationship between lung function, CALIPER metrics and leukocytes

Relationship between pulmonary function (FVC, TLCO and CPI), CALIPER PVV, parenchymal, and fibrosis scores at baseline was explored using Pearson correlation and is summarised in Table 5.2.

Significant negative correlations were observed between baseline FVC% and TLCO% with TLF%, upper, middle, and lower zone fibrosis and TLF%/litre and positive correlation between CPI and the same parameters. Stronger correlation with FVC% was observed when CALIPER fibrosis was expressed as TLF%/Litre (r=-0.430, p<0.0001) in comparison to TLF% (r=-0.392, p<0.0001). PVV% correlated with FVC% (r=-0.448, p<0.0001), TLCO% (r=-0.473, p<0.0001) and CPI (r=0.262, p<0.0001). CALIPER GGO% and reticulation% negatively correlated with FVC% and TLCO%. Honeycombing% significantly correlated with CPI but not FVC% and TLCO%.

CALIPER and function testing	FVC%		TLCO%		CPI		PVV (cm^3)	
Baseline metrics	**r**	**p Value**	**r**	**p Value**	**r**	**p Value**	**r**	**p Value**
Lung volume								
CALIPER lung volume (L)	**0.634**	**<0.0001**	**0.411**	**<0.0001**	**-0.112**	**0.156**	**0.085**	**0.278**
CALIPER parenchymal features								
CALIPER Total Fibrosis (%)	**-0.392**	**<0.001**	**-0.461**	**<0.001**	**0.275**	**<0.001**	**0.381**	**<0.001**
Upper zone Fibrosis (%)	**-0.290**	**<0.001**	**-0.344**	**<0.001**	**0.195**	**0.013**	**0.246**	**0.002**
Middle zone Fibrosis (%)	**-0.363**	**<0.001**	**-0.426**	**<0.001**	**0.233**	**0.003**	**0.343**	**<0.001**
Lower zone Fibrosis (%)	**-0.423**	**<0.001**	**-0.431**	**<0.001**	**0.208**	**0.008**	**0.346**	**<0.001**
CALIPER Total Fibrosis/litre (%/L)	**-0.430**	**<0.001**	**-0.444**	**<0.001**	**0.246**	**0.002**	**0.276**	**<0.001**
GGO (%)	**-0.286**	**<0.001**	**-0.316**	**<0.001**	**0.175**	**0.005**	**0.201**	**0.001**
Reticulation (%)	**-0.306**	**<0.001**	**-0.407**	**<0.001**	**0.285**	**<0.001**	**0.379**	**<0.001**
Honeycomb (%)	-0.039	0.534	-0.087	0.173	**0.134**	**0.033**	0.006	0.919
Low attenuation areas (%)	0.217	0.061	0.107	0.177	0.066	0.404	0.014	0.869
CALIPER PVV scores								
PVV (cm^3)	-0.068	0.284	**-0.322**	**<0.001**	**0.253**	**<0.001**	--	--
Upper zone PVV (cm^3)	0.003	0.973	**-0.242**	**0.002**	**0.242**	**0.002**	**--**	**--**
Middle zone PVV (cm^3)	-0.107	0.172	**-0.275**	**0.001**	**0.206**	**0.001**	**--**	**--**
Lower zone PVV (cm^3)	0.098	0.208	**-0.169**	**0.033**	**0.169**	**0.033**	--	--
PVV (% of total lung volume)	**-0.448**	**<0.001**	**-0.473**	**<0.001**	**0.262**	**<0.001**	**0.734**	**<0.001**
Upper zone PVV %	**-0.329**	**<0.001**	**-0.439**	**<0.001**	**0.291**	**<0.001**	**0.804**	**<0.001**
Middle zone PVV %	**-0.442**	**<0.001**	**-0.451**	**<0.001**	**0.254**	**0.001**	**0.776**	**<0.001**
Lower zone PVV %	**-0.306**	**<0.001**	**-0.400**	**<0.001**	**0.237**	**0.003**	**0.804**	**<0.001**

Table 5.2 Correlation of lung function and CALIPER baseline parameters
Pearson (r) correlation matrix of lung function and CALIPER baseline parameters. n=171 cases. PVV; pulmonary vessel volume. GGO; ground glass opacification.

I then explored association between lung function and CALIPER metrics with blood leukocytes measured closest to first CT. A correlation matrix is listed in Table 5.3. Generally, stronger correlation was observed between blood leukocytes and CALIPER expressed as a standardised

measurement per unit of lung rather than an absolute measurement. Significant correlation was observed only with absolute neutrophil and lymphocyte counts. Monocytes, MLR, NLR and SIRI did not demonstrate significant association with CALIPER measured fibrosis and PVV or lung function in this cohort. Neutrophil levels showed significant positive correlation with Total fibrosis (%/L) and PVV (%) and upper and middle zone sub-divisions. Neutrophil level demonstrated a weaker, but significant, negative correlation with FVC% but not TLCO or CPI.

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	Monocytes		Neutrophils		Lymphocytes		MLR		NLR		SIRI	
	r	p value	r	p value	r	p value	r	p value	r	p value	r	p value
CALIPER fibrosis score												
CALIPER Total Fibrosis (%)	0.084	0.282	0.086	0.274	0.147	0.061	-0.046	0.558	-0.044	0.491	-0.011	0.881
Upper zone Fibrosis (%)	0.087	0.267	0.134	0.089	0.077	0.328	-0.003	0.970	0.061	0.441	0.061	0.441
Middle zone Fibrosis (%)	0.001	0.991	**0.189**	**0.015**	0.141	0.070	-0.106	0.175	0.021	0.782	-0.003	0.969
Lower zone Fibrosis (%)	0.109	0.165	0.072	0.358	**0.187**	**0.016**	-0.111	0.154	-0.101	0.195	-0.062	0.433
CALIPER Total fibrosis / litre Lung (%/L)	0.040	0.606	**0.208**	**0.007**	0.136	0.082	-0.083	0.289	0.027	0.730	0.010	0.897
Upper zone Fibrosis (%/L)	-0.042	0.590	**0.223**	**0.004**	0.085	0.275	-0.066	0.401	0.093	0.234	0.039	0.622
Middle zone Fibrosis (%/L)	-0.020	0.802	**0.226**	**0.003**	0.145	0.063	-0.123	0.115	0.031	0.696	0.001	0.996
Lower Zone Fibrosis (%/L)	0.091	0.245	0.141	0.071	**0.191**	**0.014**	-0.124	0.112	-0.066	0.401	-0.038	0.626
CALIPER PVV score												
PVV (cm^3)	0.087	0.276	0.074	0.348	-0.082	0.299	0.085	0.283	0.039	0.622	0.067	0.397
Upper zone PVV (cm^3)	0.076	0.337	0.013	0.875	0.030	0.704	-0.027	0.731	-0.090	0.252	-0.054	0.497
Middle zone PVV (cm^3)	0.130	0.099	0.054	0.497	0.073	0.356	-0.042	0.594	-0.116	0.142	-0.055	0.492
Lower zone PVV (cm^3)	0.007	0.927	-0.083	0.295	**-0.156**	**0.049**	0.091	0.249	-0.056	0.476	-0.034	0.666
PVV % Total lung volume	0.110	0.164	**0.259**	**0.001**	0.019	0.809	0.037	0.639	0.122	0.121	0.133	0.091
Upper zone PVV %	0.093	0.241	**0.210**	**0.007**	0.072	0.366	-0.019	0.803	0.061	0.436	0.081	0.304
Middle zone PVV %	0.125	0.113	**0.277**	**0.0004**	0.108	0.171	-0.029	0.709	0.075	0.338	0.099	0.206
Lower zone PVV %	0.062	0.430	0.132	0.094	-0.099	0.209	0.106	0.181	0.099	0.213	0.103	0.192
Lung function tests												
FVC%	-0.096	0.101	**-0.127**	**0.029**	-0.014	0.814	-0.083	0.159	-0.091	0.119	-0.115	0.049
TLCO%	-0.104	0.080	-0.104	0.079	-0.085	0.155	-0.013	0.833	-0.043	0.468	-0.089	0.133
CPI	0.103	0.081	0.091	0.127	0.022	0.708	0.062	0.298	0.057	0.334	0.115	0.053

Table 5.3 Correlation of blood leukocytes with CALIPER and lung function parameters

Pearson (r) correlation matrix of blood leukocytes and derived indexes with CALIPER parameters and lung function. n=171 cases. PVV; pulmonary vessel volume, MLR; monocyte:lymphocyte ratio, NLR; neutrophil:lymphocyte ratio, SIRI; systemic inflammatory response index. n=171.

5.4.2 Qualitative CT, lung function and CALIPER assessment of disease progression

I first explored disease progression by qualitative assessment of HRCTs from MDT and CT reports. Baseline values and change in lung function and CALIPER metrics in relation to qualitative CT assessment of disease progression are summarised in Table 5.4.

Of the 71 cases which had a CALIPER-compatible follow-on CT 45 patients (63.4%) demonstrated progression. 21 cases demonstrated progression of pre-existing probable UIP, 5 cases demonstrated emergence of honeycombing on follow-on CT and therefore progressed to definite UIP and 19 cases demonstrated progression of pre-existing definite UIP. I examined this to provide a clinically established view on disease progression. The remaining 26 cases (36.6%) did not demonstrate evidence of progression on visual assessment. Time between first and second CT scan was shorter in the group demonstrating stable and non-progressive features on second CT. Median time 17.0 months (6.4-24.0) vs 34.8 (21.9-48.7) in the progression group, $p<0.0001$.

In cases demonstrating qualitative progression at follow-on CT, total, upper, and middle zone fibrosis, reticulation score and upper and middle zone PVV (%) were significantly higher compared to those demonstrating stable disease. There was a trend of greater annualised change in CALIPER fibrosis scores in the 45 cases demonstrating visual evidence of radiographic progression, however this was not statistically significant. Median annualised change in TLF was 1.62 %/Ltr/yr (-0.54 to 6.89) in stable cases and 3.45 (1.07-9.9) in cases displaying progression ($p=0.097$). Median annualised FVC decline was -3.01%/yr (-9.52 to 2.83) in cases with stable CT appearances and -4.84 (-10.9 to -0.49) in cases with progression on CT ($p=0.410$). Leukocyte counts and baseline lung function were not significantly different between the two groups.

I then explored if there was association between annualised change in CALIPER fibrosis, volume and PVV with annualised change in absolute FVC, TLCO and CPI (Table 5.5). Significant positive correlation was observed between Δ CALIPER Lung volume (%)/yr and ΔTLCO%/yr ($r=0.336$, $p=0.006$), but not ΔFVC%/yr ($r=0.185$, $p=0.138$). Significant negative correlation was observed between ΔTLCO%/yr and Δ total, upper, and middle zone PVV (%/yr). There was no correlation between ΔCALIPER fibrosis with lung function change, or between annualised PVV change in with FVC.

I next explored for association between blood leukocytes measured within 4 months of first CALIPER CT with annualised metrics of CALIPER and lung function as described above (Table 5.6). Correlation between leukocytes and annualised CALIPER scores and lung function were weaker. The only significant observation was ΔTLF (%/yr) and MLR ($r=0.245$, $p=0.038$).

Qualitative assessment of change between CT1 and CT2	Stable (n=26)	Progression (n=45)	P value
Progression of Probable UIP	--	21 (29.6%)	
Progression to definite UIP	--	5 (7.0%)	
Progression of definite UIP	--	19 (28.8%)	
CALIPER Parenchymal features			
Baseline			
Low attenuation areas (%)	1 (0-5)	1 (0-2)	0.309
Ground Glass (%)	4 (2-9)	5 (3-12)	0.060
Reticular (%)	3 (2-7)	6 (4-8)	**0.009**
Honeycombing (%)	0 (0-2)	0 (0-1)	0.871
Total lung fibrosis (%)	8 (4-19)	14 (8-20)	**0.017**
Upper Zone fibrosis (%)	3 (0.5-7)	7.3 (2.3-13.3)	**0.017**
Middle Zone fibrosis (%)	4.5 (1-13.5)	10 (5.8-19.5)	**0.033**
Lower Zone fibrosis (%)	16 (8.5-37.5)	27 (16.5-45)	0.105
Total Lung fibrosis / litre (%/L)	2.11 (0.75-5.14)	3.51 (2.02-5.11)	**0.021**
Annualised change			
Δ% Lung Volume / yr	-3.16 (-16.56-3.75)	-6.23 (-10.39 to -0.45)	0.827
Absolute change in Fibrosis / year (%/yr)	1.62 (-0.54-6.89)	3.45 (1.07-9.9)	0.097
Absolute change in Fibrosis / litre / year (%/L/yr)	0.36 (-0.19-2.05)	1.17 (0.24-4.1)	0.135
UZ absolute change (%/L/yr)	0 (-0.27-6.87)	2.05 (0-11.26)	0.504
MZ absolute change (%/L/yr)	0 (-2.12-6.64)	3.45 (-0.2-7.18)	0.312
LZ absolute change (%/L/yr)	1.07 (-2.05-12.6)	5.73 (-0.4-10.34)	0.607
CALIPER Pulmonary vessel volume			
Baseline			
TOTAL PVV (cm^3)	150 (134-219)	171 (140-200)	0.699
Total PVV % Lung Vol	3.66 (2.67-4.62)	4.06 (3.11-5.12)	0.100
Upper Zone PVV (%)	0.31 (0.23-0.5)	0.52 (0.41-0.72)	**<0.001**
Middle Zone PVV (%)	1.09 (0.72-1.53)	1.34 (1.13-1.8)	**0.016**
Lower Zone PVV (%)	1.2 (0.82-1.7)	1.17 (0.88-1.67)	0.608
Annualised change			
TOTAL Absolute Change in PVV/yr	-11.55 (-33.4-7.83)	3.7 (-2.45-13.8)	0.013
TOTAL Absolute Change in %PVV/yr	0.01 (-0.46-0.76)	0.31 (-0.02-0.73)	0.101
UZ Absolute Change in %PVV/yr	0.01 (-0.04-0.13)	0.04 (-0.01-0.09)	0.781
MZ Absolute Change in %PVV/yr	0 (-0.09-0.22)	0.11 (-0.01-0.34)	0.135
LZ Absolute Change in %PVV/yr	-0.09 (-0.18-0.16)	0.06 (-0.1-0.2)	0.312
Lung function			
Baseline			
%FVC	80.6 (69.5-96.3)	75.15 (68.51-88.45)	0.395
%TLCO	63.2 (51.1-72.6)	58 (50.57-68.44)	0.402
CPI Score	65.75 (58.68-73.12)	65.72 (59.73-70.02)	0.970
Annualised changed			
ΔFVC%/yr	-3.01 (-9.52-2.83)	-4.84 (-10.9 to -0.49)	0.410
ΔTLCO%/yr	-11.72 (-30.21-1.24)	-7.79 (-19.26 to -1.8)	0.790
ΔCPI/yr	4.84 (-6.32-7.79)	2.34 (0.70-6.99)	0.727
Blood leukocytes			
Monocyte ($x10^3/\mu l$)	0.66 (0.55-0.8)	0.67 (0.56-0.81)	0.838
Neutrophil ($x10^3/\mu l$)	4.75 (3.86-5.8)	5.09 (3.87-6.46)	0.651
Lymphocyte ($x10^3/\mu l$)	1.58 (1.46-2)	1.87 (1.3-2.36)	0.642
MLR	0.38 (0.33-0.51)	0.37 (0.27-0.53)	0.548
NLR	2.71 (2.06-3.61)	2.69 (2.04-3.71)	0.928
SIRI	1.73 (1.44-2.72)	1.79 (1.23-2.91)	0.910
Antifibrotics			
Antifibrotic use	11 (42.4%)	25 (55.6%)	0.120

Table 5.4 Cases categorised by visual (MDT) assessment of repeat CT

Comparison of baseline parameters and annualised changes in lung function and CALIPER metrics.

Annualised change in CALIPER metric between CT1 and CT2	ΔFVC%/yr		ΔTLCO%/yr		ΔCPI/yr	
	r	p Value	r	p Value	r	p Value
Δ CALIPER Lung volume %/yr	0.185	0.138	**0.336**	**0.006**	-0.004	0.970
Change in Fibrosis (%/yr)						
Total Fibrosis (CT2%-CT1%/yr)	-0.064	0.605	-0.065	0.599	0.184	0.133
Upper zone Fibrosis (%/yr)	-0.144	0.241	-0.069	0.580	0.048	0.699
Middle zone Fibrosis (%/yr)	-0.100	0.416	-0.166	0.180	**0.275**	**0.023**
Lower zone Fibrosis (%/yr)	-0.085	0.489	-0.183	0.138	0.175	0.153
Change in Fibrosis (%/Ltr/yr)						
Total Fibrosis (CT2%/Ltr – CT1%/Ltr)/yr	-0.034	0.784	-0.181	0.147	0.121	0.329
Upper zone Fibrosis (%/Ltr/yr)	-0.181	0.137	-0.126	0.311	0.034	0.784
Middle zone Fibrosis (%/Ltr/yr)	-0.154	0.208	-0.200	0.105	0.164	0.180
Lower zone Fibrosis (%/Ltr/yr)	-0.159	0.191	-0.227	0.065	0.094	0.048
Change in PVV (cm^3/yr)						
Total PVV (cm^3/yr)	-0.118	0.339	**-0.369**	**0.002**	0.069	0.577
Upper zone PVV (cm^3/yr)	-0.001	0.994	-0.193	0.118	0.231	0.058
Middle zone PVV (cm^3/yr)	-0.122	0.320	**-0.400**	**<0.0001**	0.208	0.088
Lower zone PVV (cm^3/yr)	-0.127	0.301	-0.199	0.106	-0.109	0.376
Change in PVV% (%/yr)						
Total PVV (%/yr)	-0.185	0.131	**-0.389**	**0.001**	0.048	0.700
Upper zone PVV (%/yr)	-0.115	0.352	**-0.360**	**0.003**	0.281	**0.020**
Middle zone PVV (%/yr)	-0.183	0.135	**-0.405**	**<0.0001**	0.193	0.115
Lower zone PVV (% /yr)	-0.063	0.607	-0.173	0.161	-0.219	0.073

Table 5.5 Correlation matrix (r) of annualised lung function and CALIPER parameters
n=71 cases. PVV; pulmonary vessel volume. Ltr; litre, Yr; year.

Annualised change in CALIPER metric	Monocytes		Neutrophils		Lymphocytes		MLR		NLR		SIRI	
Between CT1 and CT2	**r**	**P value**	**r**	**P value**	**r**	**P value**	**r**	**P value**	**r**	**P value**	**r**	**P value**
Δ CALIPER Lung volume (%)/yr	0.136	0.256	0.120	0.315	0.202	0.090	-0.116	0.333	-0.028	0.816	-0.006	0.961
Change in Fibrosis (CT2%–CT1%)/yr												
Total Fibrosis (%/yr)	0.122	0.309	0.108	0.366	-0.127	0.287	**0.245**	**0.038**	0.147	0.218	0.214	0.072
Upper zone Fibrosis (%/yr)	0.006	0.962	0.089	0.455	-0.134	0.260	0.123	0.302	0.104	0.382	0.126	0.293
Middle zone Fibrosis (%/yr)	0.054	0.650	-0.048	0.690	-0.033	0.783	0.079	0.512	-0.033	0.785	0.001	0.994
Lower zone Fibrosis (%/yr)	-0.048	0.692	-0.084	0.484	-0.050	0.679	0.036	0.767	-0.027	0.822	-0.041	0.732
Change in Fibrosis (CT2%/Ltr-CT1%/Ltr)/yr												
Total Fibrosis (CT2%/Ltr-CT1%/Ltr)/yr	0.013	0.916	0.088	0.458	-0.163	0.172	0.205	0.084	0.152	0.204	0.178	0.136
Upper zone Fibrosis (%/Ltr/yr)	-0.061	0.608	0.057	0.633	-0.132	0.269	0.078	0.517	0.088	0.463	0.079	0.507
Middle zone Fibrosis (%/Ltr/yr)	-0.036	0.765	-0.029	0.806	-0.081	0.497	0.052	0.664	-0.005	0.695	-0.002	0.983
Lower zone Fibrosis (%/Ltr/yr)	-0.108	0.366	-0.076	0.523	-0.095	0.425	0.020	0.864	-0.015	0.904	-0.045	0.707
Change in absolute PVV (cm^3 / yr)												
Total PVV (cm^3/yr)	-0.081	0.498	-0.147	0.217	-0.164	0.168	0.125	0.297	-0.011	0.930	-0.018	0.882
Upper zone PVV (cm^3/yr)	-0.057	0.632	-0.065	0.588	-0.110	0.359	0.069	0.565	0.020	0.865	-0.007	0.955
Middle zone PVV (cm^3/yr)	-0.051	0.668	-0.128	0.284	-0.187	0.117	0.168	0.157	0.018	0.882	0.019	0.875
Lower zone PVV (cm^3/yr)	-0.044	0.716	-0.127	0.289	-0.057	0.636	0.032	0.791	-0.068	0.572	-0.062	0.605
Change in PVV% / yr (CT2% – CT1% / yr)												
Total PVV (%/yr)	-0.152	0.204	-0.139	0.243	-0.174	0.145	0.088	0.461	0.003	0.980	-0.033	0.785
Upper zone PVV (%/yr)	-0.097	0.415	-0.118	0.322	-0.137	0.251	0.102	0.396	0.029	0.810	-0.010	0.933
Middle zone PVV (%/yr)	-0.125	0.294	-0.120	0.316	-0.169	0.157	0.106	0.374	0.024	0.842	-0.009	0.943
Lower zone PVV (%/yr)	-0.116	0.331	-0.119	0.319	-0.096	0.425	0.026	0.827	-0.029	0.808	-0.054	0.655
Change in lung function												
ΔFVC%/yr	0.182	0.102	0.113	0.312	0.099	0.378	0.005	0.962	-0.028	0.801	0.049	0.656
ΔTLCO%/yr	0.004	0.970	0.261	0.019	-0.085	0.452	0.074	0.514	0.168	0.137	0.0152	0.179
ΔCPI/yr	-0.001	0.999	-0.034	0.763	-0.100	0.374	0.181	0.106	0.167	0.136	0.138	0.218

Table 5.6 Correlation matrix (r) of annualised parameters with blood leukocytes

n=71 cases. PVV; pulmonary vessel volume. Ltr; litre, Yr; year. MLR; monocyte:lymphocyte ratio, NLR; neutrophil:lymphocyte ratio, SIRI; systemic inflammatory response index.

5.4.3 Multivariate analysis of disease progression

Multivariate cox proportional hazard regression was used to explore independent association between blood leukocytes and disease progression. I explored this for outcomes of (i) increase in CALIPER fibrosis score and (ii) FVC decline as a meaningful comparator.

I first performed a Receiver operating characteristics (ROC) analysis to identify the optimum CALIPER metric for measuring progression of fibrosis. Absolute change in Fibrosis per litre (%/Ltr) consistently outscored other CALIPER variables for the clinically important outcomes of (i) annualised FVC decline >10% (ROC 0.610), (ii) qualitative radiographic progression (0.690) and (iii) mortality (0.591). I chose this covariate to model outcome of change in CALIPER fibrosis and I defined a cut off as either (Setting A) the upper tertile of the cohort >7.8%/Ltr and (Setting B) an arbitrary unit increase of >10%/Ltr. ROC analysis is summarised in Table 5.7.

Receiver operating characteristics for outcomes in Study 3 cohort	Qualitative progression on CT	FVC decline >10%/yr	Mortality
CALIPER metric of disease progression			
Δ% Lung Volume (CALIPER)	0.418	0.361	0.395
Absolute change in Fibrosis (%)	0.675	0.575	0.581
Absolute change in Fibrosis per litre (%/Ltr)	0.690	0.610	0.640
Absolute change in PVV (cm^3)	0.646	0.551	0.556
Absolute change in PVV (%)	0.660	0.607	0.639
Absolute change in FVC (Between CT1 and CT2)	0.404	0.096	0.440

Table 5.7 Receiver operating characteristics analysis of Study 3
CALIPER metrics of fibrosis score and PVV compared with FVC change for the clinically important outcomes of (i) visual evidence of progression of fibrosis on CT, (ii) FVC Decline >10% / yr and (iii) mortality in cases that underwent follow-on CT.

I explored multivariate analysis for the outcome of FVC decline >10% as I wanted to compare any observed CALIPER fibrosis association to leukocytes with the standard measure of disease progression used in the clinical setting.[25] I could not use the exact combination of covariates to model the outcome of FVC decline >10% due to major co-linearity between relative change in CALIPER-measured lung volume (Δ% Lung Volume) and FVC decline. I therefore used (Setting C) the same combination of covariates to model FVC decline as in Study 1b (chapter 3), thereby allowing inter-cohort comparison.

In Settings A and B, multivariate models included blood leukocytes and were adjusted for age, gender, Δ% Lung Volume, baseline total lung fibrosis (%/L), baseline PVV (%) and Low-attenuation

areas (%). In setting C, blood leukocytes and were adjusted for age, gender, and baseline FVC%. High C-Index scores were observed (0.90-0.93) across all models, implying good model fit.

In Setting A, neutrophil count [HR 1.81, 95%CI 1.10-2.99, p=0.020] was significantly associated with an increase in CALIPER Fibrosis >7.8%/Ltr (Table 5.8). Lower lymphocyte level was significantly associated with progression [0.26, 0.08-0.91, p=0.034] however lymphopenia was not (Table 5.9). Interestingly female gender was significantly associated with progression [male gender HR 0.03, 0.01-0.056, p=0.019]. The CALIPER-derived measures of %reduction in lung volume [0.86, 0.80-0.93, p<0.001], baseline TLF%/Ltr [1.12, 1.05-1.20, p=0.001] and PVV% [1.03, 1.01-1.05, p=0.014] were also independently associated with progression of fibrosis on repeat CT.

Similarly in both Settings A and B, there was a trend of higher monocyte count with progression of fibrosis but in both settings, this was not statistically significant. In both settings A and B, neutrophil count >7.5x10^3/μl, demonstrated significant association with progression, however in both settings the hazard ratios generated from the multivariate analysis were exceptionally high and this likely reflects the relatively small number of cases with this degree of neutrophil count (Table 5.9). All leukocyte derived indexes were significantly associated with progression.

Multivariate Cox regression	HR	95%CI	p Value
Setting A: Increase in CALIPER Fibrosis > 7.8% / Ltr			
-Age at CT	0.96	0.88-1.05	0.404
-Male	0.03	0.01-0.56	**0.019**
-Δ% Lung Volume	0.86	0.80-0.93	**<0.001**
-Total lung fibrosis (%/Ltr) on 1st CT	1.12	1.05-1.20	**0.001**
-Total PVV (%)	1.03	1.01-1.05	**0.014**
-Low attenuation areas LAA (%)	1.06	0.94-1.20	0.318
-Monocyte (x10^3/μl)	1.35	0.10-18.38	0.820
-Neutrophil (x10^3/μl)	1.81	1.10-2.99	**0.020**
-Lymphocyte (x10^3/μl)	0.26	0.08-0.90	**0.034**
Harrell's Index of concordance = 0.91			
Setting B: Increase in CALIPER Fibrosis >10% / Ltr			
-Age at CT	0.93	0.84-1.04	0.208
-Male	0.02	0.02-0.46	**0.016**
-Δ% Lung Volume	0.82	0.73-0.92	**<0.001**
-Total lung fibrosis (%/Ltr) on 1st CT	1.13	1.03-1.25	**0.013**
-Total PVV (%)	1.04	1.01-1.07	**0.037**
-Low attenuation areas LAA (%)	1.10	0.97-1.25	0.142
-Monocyte (x10^3/μl)	2.37	0.09-62.20	0.604
-Neutrophil (x10^3/μl)	2.66	1.35-5.25	**0.005**
-Lymphocyte (x10^3/μl)	0.30	0.07-1.24	0.096
Harrell's Index of concordance = 0.93			
Setting C: Decline in FVC >10%			
-Age at CT	1.01	0.92-1.11	0.856
-Male	0.40	0.07-2.31	0.308
-%FVC	0.96	0.92-0.99	**0.049**
-Monocyte (x10^3/μl)	0.96	0.03-28.51	0.979
-Neutrophil (x10^3/μl)	1.17	0.91-1.52	0.222
-Lymphocyte (x10^3/μl)	0.71	0.30-1.71	0.448
Harrell's Index of concordance = 0.90			

Table 5.8 Multivariate Cox regression for increase in fibrosis and FVC decline

Hazard ratios in multivariate model generated for outcomes of increase in fibrosis on follow on CT scan in three settings: (A) Increase in fibrosis > 7.8 % / Ltr, (B) increase in fibrosis > 10% / Ltr and (C) relative decline in absolute FVC >10%. In Settings (A) and (B) blood leukocyte covariates (monocyte, neutrophil and lymphocyte levels) adjusted for age, gender, % decline in CALIPER lung volume, Total lung fibrosis (%/Ltr) on 1st CT, PVV (Pulmonary vessel volume) and LAA%. In setting (C) blood leukocytes adjusted for age, gender and FVC% as per analyses for Cohort 1B. HR; hazard ratio.

In setting C, I explored for association between leukocytes, and their derived indexes, with the outcome of FVC decline >10%. Lower baseline FVC% was predictive of FVC decline [HR 0.96, 0.92-0.99, p=0.049]. Monocytes, neutrophils, and lymphocytes, expressed as continuous variables in this multivariate model were not predictive of FVC decline. Only neutrophils >7.5x10^3/μl [HR 9.34, 2.05-42.60, p=0.004] demonstrated significant association with FVC decline.

Multivariate Cox regression		HR	95%CI	p Value
Setting A: Increase in Fibrosis >7.8 % / Ltr				
Model A1	-Monocyte >0.67‡	0.93	0.22-4.02	0.926
	-Neutrophil >4.90‡	2.89	0.58-14.51	0.196
	-Lymphocytes >1.62‡	0.08	0.02-0.37	**0.001**
Model A2	-Monocyte >0.90Φ	0.20	0.02-1.91	0.163
	-Neutrophil >7.5Φ	1947.61	20.5-1.8x10^5	**0.001**
	-Lymphocyte <1.0Φ	0.05	0.01-12.71	0.291
Model A3	MLR	75.88	3.8-1515	**0.005**
Model A4	NLR	1.44	1.1-1.9	**0.009**
Model A5	SIRI	1.76	1.19-2.6	**0.005**
Setting B: Increase in Fibrosis >10.0 % / Ltr				
Model B1	-Monocyte >0.67‡	0.72	0.13-3.98	0.705
	-Neutrophil >4.90‡	10.76	1.38-83.71	**0.023**
	-Lymphocytes >1.62‡	0.07	0.01-0.37	**0.002**
Model B2	-Monocyte >0.90Φ	0.12	0.01-2.41	0.165
	-Neutrophil >7.5Φ	26x10^3	9.1-74x10^6	**0.012**
	-Lymphocyte <1.0Φ	0.01	0-54.09	0.306
Model B3	MLR	122.04	3.76-3957.09	**0.007**
Model B4	NLR	1.57	1.12-2.22	**0.010**
Model B5	SIRI	2.06	1.25-3.38	**0.004**
Setting C: Decline in FVC >10%				
Model C1	-Monocyte >0.67‡	1.00	0.03-3.81	0.997
	-Neutrophil >4.90‡	2.57	0.06-11.11	0.207
	-Lymphocytes >1.62‡	0.47	0.14-1.61	0.230
Model C2	-Monocyte >0.90Φ	0.51	0.09-2.98	0.453
	-Neutrophil >7.5Φ	9.34	2.05-42.60	**0.004**
	-Lymphocyte <1.0Φ	0.38	0.03-5.17	0.470
Model C3	MLR	4.12	0.3550.3	0.258
Model C4	NLR	1.12	0.95-1.31	0.174
Model C5	SIRI	1.20	0.93-1.54	0.154

Table 5.9 Multivariate Cox regression for risk of disease progression for blood leukocyte
Hazard ratios in multivariate model generated for outcomes of increase in fibrosis on follow on CT scan in two settings: (A) Increase in fibrosis > 7.8 % / Ltr and (B) increase in fibrosis > 10% / Ltr. Blood leukocyte listed as monocyte, neutrophil and lymphocyte levels (combined in same model) or solely as leukocyte index (MLR, NLR or SIRI) and adjusted for age, gender, % drop in CALIPER lung volume, Total lung fibrosis (%/Ltr) on 1st CT, PVV (Pulmonary vessel volume) and LAA% (not shown). In setting (C) blood leukocytes adjusted for age, gender and FVC% as per analyses for Cohort 1b. ‡; monocyte, neutrophil and lymphocyte median covariates included in same model, Φ; monocyte, neutrophil and lymphocyte dichotomised by reference range limit included in same model.

5.4.4 Association between blood leukocytes and mortality

During the follow up period 60 all-cause deaths (35.1%) were reported. Median time from first CT to death was 41.6 months (18.1-52.0). In the 71 cases that had a CALIPER-compatible second CT all-cause mortality was observed in 25 cases (35.2%). Of these, 17 cases demonstrated radiological progression (visual assessment) on follow-on CT, 8 cases did not. Median survival time was comparable between these two groups; 41.5 months (29.5-50.9) vs 46.1 (33.2-60.7, p=0.101).

Kaplan-Meier analysis was used to explore association between dichotomised leukocyte variables with time to mortality. Akin to Study 1, cases were dichotomised by median values and reference range limits. (Figure 5.2). Significantly shorter survival times were observed for cases dichotomised by >median monocyte (log rank p=0.033) and neutrophil count (p=0.018), but not lymphocyte count (p=0.110). Shorter survival times were also observed for >median TLF (>3.30%/L, p<0.001), PVV (>4.02%, p<0.001) and FVC (>76.6%, p<0.001).

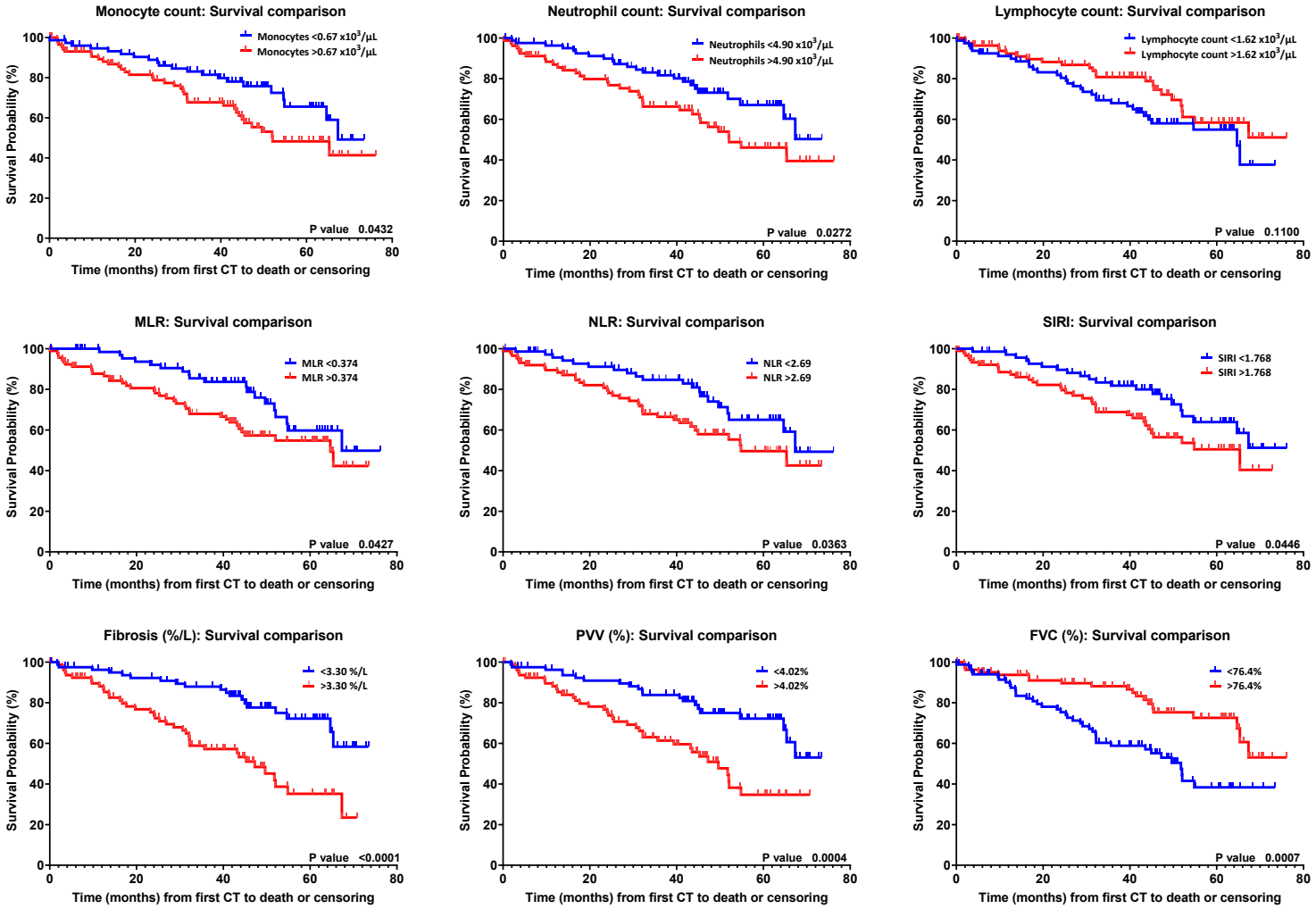


Figure 5.2 Kaplan-Meier curves for mortality in Study 3 cohort
Dichotomised by median values of absolute monocyte, neutrophil and lymphocyte (top), indexes (middle) and baseline disease severity (bottom)

5.5 Discussion

The limitations of lung function measurement and qualitative CT have been previously described.[75] CALIPER provides an objective assessment of lung fibrosis and can enumerate the extent of fibrotic change on CT with greater resolution over the tiered descriptions of UIP classification and qualitative nature of visual CT reporting.[84] From a research perspective, use of CALIPER could enhance the accuracy of capturing important clinical end points such as progression of fibrosis and biomarker associations. The purpose of this study was to explore association between blood leukocytes with (i) extent of lung fibrosis and (ii) progression of fibrosis, measured objectively using the CALIPER quantitative CT algorithm.

I observed significant correlation between lung fibrosis score and PVV with FVC%, TLCO% and CPI. Similarly, significant correlation was observed between baseline lung function with regional (upper, middle, and lower zone) fibrosis and PVV scores. Stronger correlation was observed between lung function and standardised measurements of CALIPER fibrosis (%/L) and PVV (%).

Neutrophil count correlated with total, upper and middle zone fibrosis (%/L) and PVV (%) scores. Neutrophil count also negatively correlated with FVC%, although this association was weaker. Weak correlation was observed between lymphocyte count with lower zone fibrosis and PVV. No other leukocyte associations were observed at baseline in this cohort. Multivariate analysis demonstrated that higher neutrophil count and lower lymphocyte counts were independently associated with an increase in CALIPER measured fibrosis (Table 5.8) in this cohort. Greater fibrosis and PVV measurements at first CT and greater relative reduction in lung volume at repeat CT (Δ% Lung Volume) were also predictive of progression of fibrosis in this cohort.

The calculation of fibrosis score varies between studies that have utilised CALIPER to explore structure-function relationships in ILD pathologies.[84,94] As per Sverzellati et al, I chose to define total lung fibrosis as the sum of GGO%, reticulation% and Honeycombing%.[94] Specifically, I elected to include %GGO. Although GGO is less specific of fibrosis in comparison to other CT features, the appearance can be considered reflective of interstitial thickening observed in early fibrosis.[295,296] This cohort consisted of relatively stable cases, and all CALIPER analyses were performed using thin section series from non-contrast CTs.[94]

Fibrosis scores obtained from analysis of this cohort are comparable to other IPF and non-IPF study cohorts previously explored using CALIPER. In 2016 Jacob et al demonstrated that CALIPER-derived estimates of ILD extent correlated with FVC% and TLCO%,[84] and shortly after this the same authors demonstrated linkage between PVV and lung function.[83] In those studies, reported

CALIPER fibrosis scores and parenchymal features were slightly higher in comparison to this cohort, although FVC% and TLCO% were also lower, thus probably reflective of more advanced disease in those study cohorts. Low attenuation area score was also substantially higher in Jacob et al, and smoking history proportionally greater. Collectively these differences likely represent inter-cohort variation.

Strength of correlation between baseline lung function and CALIPER fibrosis scores are also comparable to other studies.[297] However, in this cohort I observed stronger regional association between FVC% and TLCO% with CALIPER lower Zone Fibrosis (%) and upper and middle zone PVV (%). Similarly, Jacob et al also reported association regarding sub-divisional PVV score and comment on ability of this regional metric to predict mortality.[95] These associations are likely explained by the basal and peripheral predominance of fibrosis in IPF and progression of fibrosis apically.[8]

Generally, association between annualised measurement of lung function change and CALIPER fibrosis and PVV change were much weaker. There was a weak but significant association between annualised change in total, upper and middle zone PVV (Δ%/yr) with ΔTLCO%/yr (r=-0.405 to -0.360). Other studies comparing annualised lung function and CALIPER fibrosis scores also report weaker association between annualised changes in comparison to respective baseline metrics.[297,298]

The focus of this study to was explore association between leukocytes with progression of fibrosis. I undertook a multivariate analysis to explore leukocyte association with a defined value of progression. I used total lung fibrosis/litre of lung volume to measure progression of fibrosis instead of TLF%. CALIPER utilises total lung volume (denominator) to calculate proportions of each parenchymal abnormality, which I then summate to determine TLF scores. As TLF is expressed as a percentage of total lung, any relative change in lung volume captured at repeat CT could influence measured change in fibrosis score. Therefore, establishing if any change in TLF is reflective of either new fibrosis and/or change in lung volume is potentially challenging and could confound interpretation of disease progression. Lung volume decline would likely not be uniform across all patients in this cohort, therefore I measured TLF %/Litre to normalise for this potential confounder. This is different to Jacob et al, who reported progression as an absolute change in fibrosis.[83] Despite this, ROC analysis consistently demonstrated superior area under the curve characteristics for TLF%/Ltr for the clinically important outcomes of (i) qualitative progression of fibrosis (ii) FVC Decline >10%/yr and (iii) mortality (Table 5.7).

Multivariate analysis identified higher neutrophil and lower lymphocyte counts were independently associated with progression of fibrosis at interval CT. MLR, NLR and SIRI was also

significantly associated with progression in adjusted models. Monocyte count did not display any association with disease progression in this cohort.

Previous studies have demonstrated firm association between monocytes and mortality, however association with disease progression was not as clear in these studies. Statistically significant association was only evident when disease progression was measured as a combined endpoint of physiological change (FVC decline, 6-minute-walk distance reduction), acute exacerbation or mortality.[213,228] Direct association between monocyte count and FVC decline has been explored however appears less convincing, and is discussed only in very few post-hoc analyses.[259]

I explored association with blood leukocytes and FVC decline in Study 1b, and I did not identify an association with monocyte count. However, in that study monocyte count was associated with mortality, and similarly in this cohort, monocyte count >0.67 $x10^3/\mu l$ was associated with shorter median survival (Figure 5.2). Cohorts 1b and 3 share comparable age, baseline FVC%, definite and probable UIP radiology and anti-fibrotic medication use. Blood leukocyte counts were marginally higher in Cohort 1b, however similar trends were observed between neutrophil and lymphocyte counts in both cohorts.

Total lung and sub-divisional measurements of PVV also correlated with neutrophil count. Baseline PVV was significantly higher in cases demonstrating visual evidence of progression at repeat CT scan, it was independently predictive of progression of fibrosis, and annualised change in PVV inversely correlated with TLCO decline. PVV% was also significantly higher in IPF patients compared to non-ILD patients undergoing nodule surveillance CT (Appendix Figure A.7). PVV is an intriguing measurement. Originally CALIPER software was programmed to segment out and exclude vascular structures during classification of CALIPER parenchymal features.[299] However PVV has now proved a consistent predictor of disease severity, progression and mortality in multiple ILD studies.[93,94,300-302]

The mechanism associating greater PVV with adverse clinical outcomes in ILD is not fully understood and several theories have been suggested. Firstly, it is recognised that blood perfusion is reduced in areas of pulmonary fibrosis,[303] but increased in adjacent areas of spared lung unaffected by fibrosis.[304] In 2017 Jacob et al postulated that correlation between ILD extent and vessel calibre may represent regional elevation in pulmonary artery pressures in mildly fibrotic lung or destruction of the capillary bed in more advanced disease leading to neovascularisation and diversion of blood to unaffected lung. The vascular capacitance of spared lung in the upper and middle zones (IPF is a predominantly basal disease) could result in increased volume in more advanced disease.[93] Another possible explanation relates to the reduced compliance (of the fibrosed and stiffened lung parenchyma) and greater negative intrathoracic

pressures necessary to generate adequate inspiratory volumes in fibrotic lungs. This could in turn exert additional "tractional" force on the lung vasculature resulting in dilatation in fibrotic regions in a traction-like phenomenon, akin to traction bronchiectasis.[305] Alternatively PVV may be a surrogate measure of pulmonary hypertension as association between right ventricular systolic pressure has been reported.[93]

My findings are supportive of the original work performed by Jacob et al who demonstrated in a longitudinal study PVV% increase was independently associated with FVC decline,[83] and Chung et al who demonstrated in a cross-sectional IPF study that PVV negatively correlated with TLCO%.[305] These might suggest that the mechanism underlying PVV is not solely related to fibrosis. In Cohort 3, neutrophil count significantly correlated with total and regional PVV and fibrosis scores (Table 5.3). In chapter 3 I discussed evidence linking immuno-pathobiology of neutrophils, and lymphocytes to fibrosis. In these retrospective studies, findings were limited to association. Therefore, I am unable to conclude if the observed association between neutrophils and PVV is a direct interaction between neutrophils and the pulmonary vasculature, or the result of the structural changes caused by fibrosis and destruction of the alveoli to which neutrophils could be contributing. However, this association between neutrophil count, fibrosis and PVV is intriguing.

The trend of lower lymphocytes count with fibrosis and progression is also fascinating and mirrors the findings from Study 1b. No patients in this cohort were receiving immunosuppression therapy during the follow up period. Infection was reported in relatively few patients, however changes to the lung microbiome and the structural changes of the IPF lung may contribute to the inflammatory state and influence adaptive immune responses.[232] The putative role of the adaptive immune system in IPF has been widely published but the precise role of lymphocytes in IPF remains incompletely understood.[136]

The relationship between adverse outcomes with neutrophils, lymphocytes, and monocytes suggests that a systemic inflammatory state exists in IPF. In post-hoc analysis of the ACEND and CAPACITY studies Nathan et al reported that serial increase in NLR over 12 months was associated with mortality.[258] Higher NLR, MLR and SIRI were also associated with progression of fibrosis in this cohort. The lymphocyte count is the denominator of these indexes and the lower counts observed in this cohort would have contributed to higher index scores.

There are several limitations that should be considered, and the results should be interpreted within the limitations of this analysis. The retrospective nature limits the findings of this study to association.

Neutrophils correlated with upper and middle lobe TLF and PVV but not lower lobe involvement. This is intriguing as we know that IPF is a condition with a basal predominance and progresses in an apical manner. Indeed, lower lung zones are where fibrosis is most evident and single-cell RNA-sequencing studies have demonstrated greater immune cell presence in lower zones of IPF lung explants.[192]My findings could be explained by the limitations of this single centre study. Perhaps the association between neutrophils and upper and mid zone TLF and PVV simply represents association of neutrophils with greater disease burden in a cross-sectional manner, and lower zone association which is much more abundant across this population is not statistically identified and an issue of sensitivity.

Total fibrosis score was calculated by the summation of GGO%, reticulation% and honeycombing%.[94] Although GGO can imply fine fibrosis,[296] this radiological pattern is fairly broad and can encompass other differentials such as pulmonary oedema and infection.[295] Despite this very few patients reported a history of infection at time of CT and only 6 cases was cardiomyopathy reported. Extent of traction bronchiectasis was not quantified. I was unable to quantify this adequately from MDT and CT reports and CALIPER is unable to quantify traction bronchiectasis. 29.6% of cases that underwent repeat CT scan demonstrated progression of probable UIP. Increasing traction bronchiectasis is considered a strong visual predictor of mortality in IPF,[26] and measured change in traction bronchiectasis may have complimented the associations observed using CALIPER.

Over 50% of this cohort demonstrated a definite UIP radiological appearance however CALIPER honeycomb scores appeared disproportionately low. A limitation of CALIPER is that it can miscategorise areas of severe honeycombing as low attenuation area.[299] Despite this LAA scores were extremely low (median 1%), a small proportion of patients reported co-existing COPD and median FEV1:FVC ratio was >80%. Patient-related factors are also worthy of consideration. Efforts were made to ensure that only non-contrast and thin-section CT images were analysed. Despite this strict selection, a proportion of patients included in the study were referred to our centre from an external institution. It is possible that CT images transferred from external institutions may have been obtained using CT scanners of different manufacturers and likely using different reconstruction algorithms, which could lead to some variation in the results generated by CALIPER.[87]

CALIPER segmentation and feature quantification is based upon lung density, which in turn is influenced by CT scanning dose, slice thickness and patient inspiratory effort and volume. Thorough coaching of patients for optimal inspiratory effort and timing with scanning protocols is pertinent to reproducibility of results, but challenging in retrospective cohorts.[87] Again, because

of the retrospective nature of this study I was unable to apply a spirometry-gated CT approach which would have adjusted for potential variation in measured CT lung attenuation caused by differences in inspiratory status for study participants.[306] This approach however is also not performed by many other studies exploring IPF cohorts with quantitative CT because of their retrospective nature.[299] This unfortunately limits intercohort comparisons. I included only inspiratory CT scans, specifically avoiding analysis of expiratory CT scans and CALIPER lung volume strongly correlated with absolute FVC in this cohort. To account for these potential confounders, I standardised CALIPER scores per unit lung volume and in ROC analysis this metric outscored other measurements.

Anti-fibrotic treatment may have affected blood leukocyte measurement. 50.7% of patients that underwent a second CT scan were receiving antifibrotics at first CT scan, and this may have biased the observed association between neutrophils, and leukocyte indexes with progression of fibrosis. However, in sensitivity analysis adjusting for antifibrotic use, significance was preserved for neutrophils, NLR and SIRI (Appendix Table A10).

Repeat CT scans were not performed in a uniform time frame and median time between CTs was significantly shorter in patients not demonstrating visual evidence of progression on repeat CT. This may have affected annualised measurements and may have introduced bias into these results. Repeat CTs were also based on clinical indication and concern of clinical deterioration, thus the cohort was likely enriched for capturing cases demonstrating progression.

Cox proportional hazard modelling of FVC decline >10% (setting C) involved a different combination of covariates in comparison to Fibrosis progression (Settings A and B) and the results should be interpreted with this in mind. This could account for the difference observed in between neutrophil and these different clinical outcomes. However, Setting C contains the same covariates as Study 1b which did demonstrate significant association between neutrophils and FVC decline in the Study 1b cohort. Although inter-cohort difference may account for this, another possibility is that CALIPER measurements are more sensitive in capturing disease progression and therefore exposing the association with neutrophils observed here.

5.6 Conclusion

In this cohort, higher neutrophil and low lymphocyte levels correlated with CALIPER fibrosis and PVV scores. Greater neutrophil count and lower lymphocyte levels were also associated with greater risk of radiological progression of fibrosis (within 3 years). Correlation between leukocytes

and FVC decline were generally much weaker and only neutrophil count demonstrated a significant association. Greater monocyte level was associated with mortality in this cohort but not progression of fibrosis or FVC decline. Neutrophil levels could indicate patients at greater risk of progression of fibrosis in IPF.

Chapter 6 Discussion

6.1 Thesis overview

In an extensive review of the literature, at the time of writing, I draw together current understanding of the underlying pathophysiology of IPF. I discuss the evidence from basic science and clinical studies implicating monocyte pathobiology, from which I developed hypotheses (**Chapter 1**). The main aims of this thesis, then, were to explore monocyte interactions, with important clinical outcomes in IPF. Study populations used to explore hypotheses, clinical data capture and statistical methodology are next described in **Chapter 2**. I explored absolute monocyte, neutrophil and lymphocyte (measured from standard full blood count analysis) and leukocyte indexes derived from these. I explored association of these parameters with important clinical outcomes. In **Chapter 3** I explored leukocyte association with FVC decline, radiographic progression, and mortality in a cohort of patients with iUIP or IPF. In **Chapter 4**, I describe a large cohort of patients with radiological evidence of early-fibrotic ILA and explored leukocyte association with mortality and progression to UIP. In **Chapter 5**, using a separate IPF cohort, I explored leukocyte association with quantitative measure of fibrosis and interval progression using the automated CT algorithm, CALIPER. In each chapter of analyses, key findings were independently presented in context of the wider literature, with strengths and limitations.

This chapter summarises the principal findings of all chapters and discusses their implications in context to each other and IPF as a whole. Next, the main strengths and limitations over the overall work presented in this thesis are summarised. Finally, areas for future research are outlined.

6.2 Key findings and their implications

6.2.1 Monocytes, neutrophils and lymphocytes and study outcomes

Large epidemiological studies have explored blood monocyte profiles in patients with IPF.[197,227-229] Collectively, these have been important in bridging the knowledge gap between evidence from basic science studies implicating monocytes immune-pathobiology in IPF with medical understanding of disease behaviour and clinically important and patient-centred outcomes.

In a retrospective ILD cohort (Study 1, Chapter 3) the monocyte association between cases with the indeterminate for UIP pattern on first CT. As neutrophils and lymphocytes populations are also implicated in IPF I explored these leukocytes simultaneously.

Indeterminate for UIP is an important CT appearance that identifies patients at risk of progressing to a diagnosis of radiological UIP, and to IPF, in the appropriate clinical context.[8] Data regarding the prevalence of iUIP is limited, and predominantly comes from retrospective and observational cohort studies. Given the relatively small amount of disease on CT and the relatively low symptom burden associated with this condition, a proportion of cases will not seek medical attention until symptoms have progressed. At this stage CT appearances are likely to have progressed to probable or definite UIP.[8,307]

In the Study 1 cohort, 21% of cases had a first CT compatible with iUIP, with the remainder of patients displaying either probable or definite UIP. The iUIP proportion of this cohort is comparable to other ILD cohorts. Diridollou et al identified iUIP in 17% of cases reviewed retrospectively from their ILD MDT that were re-categorised from possible UIP.[242] Age and gender differences were comparable to the Study 1a cohort. In AGES-Reykjavik, a large observational study involving 5320 participants, Putman et al identified a 2.52% prevalence of iUIP.[236] The lower figure quoted by Putman likely reflects burden (possibly asymptomatic) of iUIP in background population, and representative of an observational birth cohort. Differences in symptom burden and referral for ILD assessment may account for the greater prevalence observed by Diridollou et al and by myself in the Study 1a cohort.

Neither Putman nor Diridollou examined progression of iUIP. Therefore, to explore progression, I conducted a multivariate Cox proportional hazard analysis to identify if monocytes were implicated. In univariate analysis I consistently observed association between higher monocyte and neutrophil counts (values expressed as either continuous, >median or >normal range) for the outcomes of (i) progression of iUIP and (ii) progression of iUIP to probable or definite UIP. In multivariate analysis I identified that monocyte count >0.90x10^3/μl and neutrophil count >7.5 x10^3/μl were significantly associated with greater risk of progression (Table 3.2). Furthermore Kaplan-Meier analysis demonstrated significantly shorter survival time for patients with a monocyte count >0.90x10^3/μl and neutrophil count >7.5 x10^3/μl (Figure 3.3).

Given these observations for monocytes and neutrophils, I was then curious to see if similar associations were present in cases of the Study 1 cohort demonstrating probable or definite UIP at initial CT. Specifically, I explored association of leukocytes for the clinically important outcomes of (i) all-cause mortality, (ii) time to FVC decline >10% and (iii) all-cause hospitalisation events during the follow-up period (Study 1b). I also explored association of indexes derived from

monocytes, neutrophils and lymphocytes in the multivariate modelling used in Study 1b, which have previously been used in pulmonary and non-pulmonary studies to prognosticate for adverse outcomes.[239,308-310]

Multivariate analysis, adjusted for age, male gender, and baseline FVC% demonstrated higher monocyte and neutrophil levels were associated with mortality and cases with a neutrophilia at clinic visit appeared to be at greatest risk of mortality (Table 3.4). Greater neutrophil count was significantly associated with the outcome of FVC decline >10%. Lower lymphocyte count was also associated with greater risk of FVC decline and NLR, which incorporates both of these, demonstrated significant association with FVC decline (Table 3.4 and Figure 3.7).

Multiple large scale epidemiological studies exploring blood monocyte count and mortality in IPF have now been published. In the landmark study conducted by Scott et al, a large retrospective study involving nearly 7500 IPF cases, two key findings were identified. Firstly, Discovery analysis demonstrated Classical monocytes ($CD14^{hi}CD16^{neg}$) occupied a greater proportion of PBMC populations. Second, blood monocyte count >$0.95x10^3$/µl was associated with greater mortality risk in individuals with IPF.[197] This study has since been followed by studies using more refined IPF cohorts demonstrating comparable risk of mortality with lower monocyte values. In pooled analysis of IPF clinical trial data, Kreuter et al identified a monocyte count greater >$0.60x10^3$/µl was associated with mortality risk and disease progression, defined as a composite measure (mortality, hospitalisation, FVC decline and decline in 6 minute walk distance), but not solitary FVC decline.[228] Although the authors discuss the utility of the monocyte count >$0.60x10^3$/µl to prognosticate in IPF, this finding was not validated in real-world IPF cohorts,[229] neither did I observe this association in the 1b cohort or association between monocytes and FVC decline. However, in Study 3 Kaplan-Meier analysis demonstrated monocyte count >$0.67x10^3$/µl (median cohort value) was associated with significantly shorter survival time (Figure 5.2).

Furthermore, a Chinese study that was published after I completed my data analysis also demonstrated that IPF patients with a monocyte count >$0.67x10^3$/µl had significantly higher mortality.[311] The different monocyte cut-offs demonstrating mortality risk among these different cohorts could be due to different timing of monocyte data collection, different follow up durations and subtle differences in study end-points and the collective heterogeneity among these clinical and study cohorts.

Several lines of evidence from both animal models and human studies, have implicated monocytes in fibrosis. Experimental evidence suggesting migration of monocytes from bone marrow to injured lung and differentiating into pro-fibrotic macrophages.[98,184] Furthermore, translational studies have demonstrated that accumulation of distinct monocyte-derived alveolar

macrophage populations are implicated in progression of fibrosis.[191,244,245] Monocyte-derived macrophages, which originate from bone-marrow precursors, could possess a different cytokine repertoire, one that is more pro-inflammatory and pro-fibrotic, and which in animals models has been shown to propagate fibrosis.[184] Fraser et al has previously demonstrated that classical monocytes display a type 1 interferon primed phenotype which correlated CT fibrosis score. This could account for more robust and potentially injurious response to the alveolar epithelium when triggered, during for example a viral infection,[214] which could potentially perpetuate ongoing fibrotic processes, ultimately increasing risk of mortality as observed with complete monocyte count.

Although monocyte association with mortality in Study 1b aligns with findings of multiple clinical studies, association between monocytes and disease progression appears less striking. Disease progression should be considered a broad term and definition varies across clinical studies. Often the definition includes clinically important outcomes including decline in spirometry or gas transfer, radiographic evidence of progression or symptom-based or exercise-based scores, used either alone or in combination.[312] Serial FVC monitoring is considered the gold standard means of identifying disease progression in clinical practice.[25] Yet, in studies conducted thus far association between monocytes and FVC decline appears to be lacking. Composite measures of disease progression are perhaps more likely to capture any association but are potentially vulnerable to effect of confounders of multiple parameters tested.[313]

Fraser et al had previously demonstrated in a cross-sectional study that higher monocyte proportion (of the PBMC population) and greater CD64 expression on classical monocytes correlated with semi-quantitative assessment of fibrosis.[214] Given this observed association I was curious if a similar association was present between blood leukocytes and extent of fibrosis on CT scan.

I adjudicated a separate IPF cohort (Study 3) and analysed CT scans using automated lung texture analysis software (CALIPER), which provided an objective quantification of fibrosis and vessel-related structures. In this cohort, higher monocyte count was associated with greater risk of all-cause mortality (Figure 5.2), but no associations were observed between monocytes and baseline total fibrosis score, or progression of fibrosis on interval CT. Similarly, no associations were observed between monocytes with baseline and annualised changes in lung FVC, TLCO or CPI (Table 5.3 and Table 5.6). Different statistical approaches and inter-cohort differences may partly account for these findings however baseline leukocytes profiles, comorbidity profiles, age and smoking histories were comparable. Regretfully, I was unable to analyse Cohort 1 with CALIPER. This cohort first attended clinic assessment much earlier (2013-2017) than Cohort 3 (2016-2021)

and for the majority of these cases, CT scans were not compatible with CALIPER software. This unfortunately limits comparison between these cohorts.

Use of antifibrotics was comparable between cohorts however duration of use was not adjudicated for in Cohort 1. Pirfenidone suppresses TGF-β, IL-1β and FGF and thereby attenuates fibroblast proliferation, differentiation, collagen synthesis, and ECM deposition.[50] Nintedanib, an intracellular tyrosine kinase inhibitor, binds competitively to VEGF, PDGF and FGF receptors blocking downstream signalling pathways.[53] In vitro studies have demonstrated Nintedanib alters polarisation of M1 and M2 monocyte-derived macrophages.[55] Furthermore, in post-hoc analysis of the INPULSIS and TOMORROW trials, Nintedanib reduced the rate of FVC decline vs placebo, with a greater treatment effect observed in patients with higher monocyte count.[259] In post-hoc analysis of ASCEND and CAPACITY, a serial reduction in monocyte trend was observed in the Pirfenidone arm.[228] The effects of Nintedanib and Pirfenidone on monocyte biology are not fully understood, but further prospective studies exploring theragnostic properties of these agents may enhance our current understanding.

Higher neutrophil count was associated with shorter time to mortality in both IPF cohorts (studies 1 and 3). Neutrophils were independently associated with FVC decline in Cohort 1b and in Study 3 greater neutrophil count was associated with progression of fibrosis but not FVC decline. Neutrophils also correlated positively with baseline total fibrosis score and pulmonary vessel volume (PVV) in Study 3. Intriguingly, in Study 3, correlation with upper and middle lobe involvement but not lower lobe involvement was observed, despite IPF being a condition of lower lobe predominant fibrosis which progresses in an apical manner. Perhaps this represents association of neutrophils with greater disease burden in a cross-sectional manner. Indeed Jacob et al identified that middle zone features were strongly predictive of mortality.[93]

PVV is a volume measurement of pulmonary arteries and pulmonary veins within the lung parenchyma. Although it is not a direct measure of fibrosis, IPF and non-IPF ILD studies have demonstrated strong association between PVV and CALIPER fibrosis scores, impairment in lung function and progression of fibrosis.[83,95,298,300-302] In Study 3 similar associations were observed. Furthermore, neutrophils correlated with total, upper and middle zone PVV and total fibrosis scores (Table 5.3). Although the mechanism(s) implicating PVV with fibrosis are at this stage hypothesised, the injurious and perhaps pro-fibrotic role of neutrophils could be implicated in this process. IPF is predominantly a disease with basal and peripheral distribution, which progresses apically involving the middle and then upper zones. The stronger upper and middle zone associations of neutrophils with PVV, and fibrosis scores, could further provide insight with regards to neutrophil activity at specific stages of the fibrotic process. Further prospective studies

will be required to explore this in greater detail, aligned with superior spatial and computational analysis.

Neutrophil activity in IPF has been heavily explored. Neutrophils recruited to areas of inflammation can shape their environment by secreting proteases, oxidants, cytokines, and chemokines which augment pro-inflammatory reponses.[246] Neutrophils also release metalloproteinases (MMP) which are involved in collagen deposition and ECM formation, therefore propagating fibrosis.[133] Neutrophilic bronchoalveolar lavage (BAL) specimens taken from patients with IPF has been associated with early mortality.[130] The enzyme neutrophil elastase (NE) has previously shown to be elevated in BAL sampling from IPF patients.[131] More recent findings using murine models suggest that NE promotes activation of the TGF-ß pathway, fibroblast proliferation and myofibroblast differentiation.[247] An intriguing and newly identified fibrosis-promoting function of neutrophils is generation of neutrophil extracellular traps (NETs).[129] These pro-inflammatory collections of chromatin and neutrophils regulate both immune cell function and fibroblast activation.[134] Although basic science studies have implicated neutrophils in the pathogenesis of IPF further studies will be required to explore causality, and particularly if high absolute neutrophil counts in blood represent expanded neutrophil sub-populations associated with adverse outcomes.

There was also a general trend of lower blood lymphocyte count with adverse clinical outcomes in IPF. In Study 1b, lymphocyte count below median value ($<1.76x10^3/\mu l$) was significantly associated with shorter survival time (Figure 3.6) and greater rate of FVC decline (Figure 3.7). In multivariate analysis lower lymphocyte counts were associated with hospitalisation and lymphopenia was independently associated with greater risk of FVC decline >10%. In Study 3 I identified lower lymphocyte count was associated with progression of fibrosis.

Previous studies have identified association between low blood lymphocyte count, lymphocyte dysfunction and reduced FVC% in IPF patients.[248] In a meta-analysis to explore lymphocyte expression patterns in IPF, Schott et al identified significantly reduced lymphocyte gene expression in patients with IPF.[248] Gene expression correlated with TLco and high expression was significantly associated with better survival. Bronchoalveolar lavage fluid from IPF patients is also enriched for several T lymphocyte populations,[136] and lymphoid aggregates are a recognised pathologic feature of IPF lesions.[135]

The association between low blood lymphocyte count and adverse outcomes in IPF could be explained in part by lymphocyte dysfunction and the sequestering of lymphocytes into sites of inflammation, such as the fibrotic lung. Histological analysis of surgical lung biopsies and explanted IPF lungs has revealed greater immunohistochemical staining for CD4+, CD8+, IL-17,

and CCR6 in IPF lung specimens.[148] Quantitative immunohistochemistry using IPF biopsies has identified negative correlation between CD8+ staining with FVC and TLCO%.[249] Paired analysis of surgical lung biopsy specimens with explanted lung of patients later proceeding to lung transplantation has revealed CD3+ aggregates are present in greater numbers in advanced disease (explant tissue) implying cellular inflammation continues as fibrosis progresses.[140] Recently, Th17 lymphocytes isolated from peripheral blood of patients with IPF have been identified as a source of TGF-β.[250]

These findings do not provide direct explanation for the lower circulating lymphocyte counts observed. However, phenotypic changes in circulating lymphocytes have been reported, and may partly explain this observation. Changes in circulating CD4+ T cells, with down-regulation of CD28 is associated with adverse outcomes in IPF. CD28 is a T-lymphocyte receptor which provides co-stimulatory signals for T-lymphocyte activation and survival.[135] Repeated cycles of antigen-induced T-cells proliferation can result in loss of surface expression of CD28.[314] IPF patients displaying a greater proportion of $CD4^{+}CD28^{-}$ cells in PBMCs also demonstrate greater production of pro-inflammatory cytokines, and less frequently expressed the regulatory T-cell marker FoxP3.[315] Furthermore, 4 T-cell genes (CD28, ICOS, LCK and ITK) involved in co-stimulatory signalling pathways are part of the 52 gene signature that is associated with a poor disease outcome.[316] In the PANTHER clinical trial patients randomised to the lymphocyte-modulating agent Azathioprine demonstrated poorer clinical outcome.[46]

Given these interesting observations that circulating monocytes, neutrophils and lymphocytes are associated with mortality and disease progression in IPF, I next explored if similar associations existed in individuals with interstitial lung abnormalities (ILA). ILA is an umbrella term describing subtle, non-dependent, mild parenchymal abnormalities detected on CT.[236]

Specifically, I was interested in exploring association between blood leukocytes with fibrotic ILAs. I conducted a text-based search of local CT reports using pre-specified search criteria selective for, as I described, early fibrotic-ILA. I explored association between monocytes, neutrophils, lymphocytes, and indexes derived from these with, with mortality and radiographic progression of EF-ILA. Using multivariate cox proportional hazard regression adjusted for age and gender, I demonstrated EF-ILA present on initial CT and radiographic progression of EF-ILA were both associated with greater mortality risk. In this cohort (which captured early parenchymal change) monocytes and neutrophils were significantly associated with increased risk of all-cause mortality and radiographic progression. Lymphocytes were not associated with progression or mortality.

In Study 1a, I explored leukocyte associations with progression of iUIP, and in this much smaller cohort greater monocyte and neutrophil levels were significantly associated with radiographic

progression. A major difference between Cohort 1a and Cohort 2 is the methodology used to identify cases. Cohort 1 iUIP was specifically identified from cases that had been vetted through the ILD MDT. Cohort 2 was identified by qualitative descriptions of thoracic CT reports. As such I opted to use a separate radiographic description (EF-ILA) to describe Cohort 2. A much smaller proportion of cases in Cohort 2 would have been discussed in ILD. Although this limits inter-cohort comparison, importantly analysis did demonstrate lower mortality risks in EF-ILA in comparison to cases displaying traction bronchiectasis and or honeycombing in both cohorts.

This finding is intriguing as it captures an association between monocytes and neutrophils, but not lymphocytes, with earlier and milder parenchymal abnormalities yet also implicates these leukocytes with progression. Indeed, the immune-pathobiological actions of monocytes and neutrophils that I have discussed above could potentially be active in earlier stages of ILD than anticipated, and before diagnosis can be established.

Future implementation of lung cancer screening and greater use of CT imaging for other diagnostic purposes are likely to increase detection of ILA.[271] Earlier detection of fibrotic ILAs could lead to a shorter lag time to ILD diagnosis, potentially allowing earlier treatment intervention and improved patient outcome.[273] Perhaps more pressing is a need for an easily accessible test to stratify patients according to those more likely to progress and therefore require follow-up. Integrating blood leukocyte measurement into risk stratification could be worthy of future consideration.

6.2.2 Leukocyte indexes and study outcomes

There is growing evidence that indexes derived from neutrophils, monocytes and lymphocytes are more predictive of outcome in chronic inflammatory conditions in comparison to individual cell populations.[317] Among these composites, the neutrophil:lymphocyte ratio (NLR), monocyte:lymphocyte ratio (MLR) and systemic inflammation response index (SIRI) have previously been investigated as markers of disease activity in non-pulmonary,[308,318,319] and pulmonary diseases.[237,320-323] NLR, a recognised surrogate marker of immune response, indirectly evaluates inflammatory status and is a recognised correlate of disease severity, hospitalisation and mortality.[251,252] NLR is also predictive of outcome in ILDs with associated auto-immune features such as systemic sclerosis and dermatomyositis.[253,254] MLR and SIRI have been primarily used to prognosticate in cancer studies,[239,255] and although their role in exploring ILD outcomes is limited, IPF and pulmonary malignancies share similar risk factors and pathogenesis. Indeed

inflammation, tissue injury, aberrant wound healing and remodelling are key to progression of IPF. Therefore, these leukocyte indexes could predict adverse outcomes in IPF.

Given the interesting observations between monocytes, neutrophils, and lymphocytes with clinical outcomes, I examined if similar patterns were observed when exploring outcomes with indexes derived from these leukocytes. In studies 1b, 2 and 3, I modelled NLR, MLR and SIRI against study outcomes.

In Study 1b, NLR was the only leukocyte variable that was significantly associated with all study outcomes (mortality, FVC decline and hospitalisation) in multivariate analyses. Both high neutrophil and low lymphocyte count contributed to higher NLR values, suggesting concurrent neutrophil activation and lymphocyte exhaustion could be relevant. Greater MLR and SIRI values (>median) were also significantly associated with mortality and FVC decline. In Study 2 NLR, MLR and SIRI were significantly associated with radiographic progression of EF-ILA and all-cause mortality. MLR was most predictive leukocyte variable of EF-ILA progression. In Study 3, all three were predictive of progression of fibrosis in multivariate analysis, but intriguingly none of these correlated with extent of fibrosis on baseline HRCT. I did not explore correlation between leukocyte indexes and baseline lung function in Cohort 1a which unfortunately limits comparison.

Recently one retrospective cross-sectional study demonstrated NLR, MLR and SIRI measurements to be greater in patients with IPF versus healthy controls,[256] suggesting not only that multiple leukocytes implicated in disease, but also that ratios of absolute leukocyte counts could potentially facilitate prognostication. In a retrospective multicentre study evaluating NLR, Mikolasch et al demonstrated this was an independent predictor of 6-month mortality across multiple IPF cohorts. The authors also demonstrated that incorporation of NLR strengthened ability of the GAP prediction model to risk stratify.[257] Another study measured NLR in bronchoalveolar lavage (BAL) samples obtained from IPF patients and found that BAL NLR negatively correlated with paired FVC measurement.[324] Furthermore, in post-hoc analysis of the ACEND and CAPACITY studies, Nathan et al reported that serial increase in NLR over 12 months was associated with increased mortality risk.[258] Thus, the positive association between these composite index measures of absolute leukocytes could reflect a 'real-time' measure of inflammation, that could be driving disease progression in patients with IPF.

The predictive ability of NLR may suggest a role for neutrophilic inflammation in the pathogenesis of IPF. It has been recognised for some time that neutrophilic BAL specimens of IPF patients correlates with a poor outcome.[130] Molyneaux et al. have shown that BAL neutrophilia is associated with both increased microbiome burden and progressive IPF,[325] with subtle changes in

the microbiome implicated in the initiation and progression of IPF in the absence of identified infection.[326]

Despite multiple studies demonstrating association between absolute monocyte count with mortality, very few studies have demonstrated direct association between monocyte count with relative FVC decline or CT-measured progression of fibrosis in IPF.[228,229,259] Relatively few studies have explored MLR with important clinical outcomes. In a cross-sectional analysis, Zinellu et al first demonstrated MLR was significantly higher in IPF cases versus age-matched healthy controls, and negatively correlated with FVC%.[256] Bernardinello et al demonstrated MLR was not statistically different in IPF patients categorised as either newly diagnosed (median FVC 80%) versus end-stage IPF (FVC 51%).[327] Although there is strong evidence implicating monocytes in pathogenesis and mortality in IPF, further studies are required to identify if monocytes are predictive of disease progression in IPF.

6.2.3 Biomarker utility of leukocytes and leukocyte indexes

Despite availability of antifibrotic therapies, IPF remains a clinical challenge. The high mortality, symptomatic burden and difficulty in predicting disease course highlight the unmet need for a simple, cost-effective and clinically applicable prognostic tool.[328]

A biomarker is defined as an indicator of normal or pathological processes, or response to an exposure or intervention.[225] Indeed, growing evidence implicates innate and adaptive immune cell populations in IPF. In this thesis I have demonstrated higher blood monocytes and neutrophils are implicated in mortality in IPF and EF-ILA. Monocytes were also implicated in radiographic progression of EF-ILA and neutrophils were associated faster rate of FVC decline and progression of fibrosis. There was also a trend of lower lymphocytes in cases progressing. Thus, leukocytes indexes were also implicated in these findings. Many similar studies reporting leukocyte association with adverse outcomes in IPF, and pre-clinical disease discuss the utility of these to prognosticate at-risk populations. Although testing prognostic ability of these using large prospective studies appears the next logical step a specific role and application will also need to be explored, and any integration into current risk stratification models should be carefully considered. However, Mikolash et al demonstrated that NLR enhanced the predictive ability of GAP scoring,[257] and Kreuter et al similarly demonstrated that inclusion of monocytes improved the predictive ability of GAP to discriminate disease progression, mortality, and hospitalisations.[329] Such integration would certainly appear feasible in prospective cohorts.

Furthermore, specific cut-off values of any chosen leukocyte variables would need to be universally agreed. Scott et al first identified association between IPF mortality risk and blood monocyte count >0.95x10^3/μl.[197] Although dichotomising for the limit of reference range is reasonable, and identifies patients at high risk, analysis of other IPF cohorts has identified monocyte count >0.60x10^3/μl carries similar mortality risk, but importantly is inclusive for a larger proportion of the study cohort.[228] Other studies exploring leukocytes and indexes with study outcomes have dichotomised cases by median counts to explore association which limits generalisability and inter-cohort comparisons. Future studies will need to identify optimal cut off values and categorical ranges for risk stratification generalisable to the wider population. However, these studies have collectively demonstrated that greater monocyte count is associated with adverse clinical outcomes. It could be that higher monocyte levels measured early in disease are likely to be maintained at a higher steady state through the course of the disease and potentially identify high-risk IPF sub-populations earlier than other physiological indicators.[316]

Another potential application of leukocytes could be as a cohort enrichment tool. Given the challenge of recruiting enough patients in a timely fashion to identify drug efficacy in IPF trial settings, cohort enrichment strategies are often employed to create homogenous study populations of patients at greater risk of experiencing clinical events.[61] This not only reduces sample size, but also shortens study recruitment time and length and reduces overall study cost.[126] In this scenario, leukocyte variables could be used to identify patients at greater risk of study end points, and potentially reduce the number of participants enrolled into a study to demonstrate effect.

Whilst there are no universally agreed-upon biomarkers, there has been substantial progress recently to detect blood biomarkers predictive of disease progression in IPF such as markers of epithelial cell dysfunction and ECM remodelling. However, blood biomarkers will need to be validated in larger clinical trials. Furthermore, it is likely that not a single biomarker, but a biomarker signature will most reliably predict disease progression, and to which blood leukocytes could potentially contribute to.[61]

6.3 Strengths and limitations

The specific strengths and limitations of analyses have been presented in the discussion section of each relevant chapter. The overall strengths and limitations of this thesis are summarised here.

6.3.1 Selection of participants into study cohorts

For Studies 1 and 3 patients were included only if their case was reviewed in the ILD MDT. This MDT comprises of specialist ILD physicians, thoracic radiologists and histopathologists with an interest in pulmonary disease. Several studies have reported that MDT diagnosis is associated with higher levels of diagnostic confidence and superior interobserver agreement when compared to individual components of the MDT in isolation.[330,331] In particular key aspects of case history were reviewed, important positive and negative findings noted, key radiographic features and patterns described and indications for exclusion from study identified. Therefore, it is likely that these cohorts represent refined study populations, with accurate radiological and clinical diagnoses. This strengthens the association observed between leukocytes with iUIP and IPF.

In Study 2, I extended my exploration of leucocytes to mild fibrotic interstitial lung abnormalities, which can be considered precursor to ILD. I termed these early fibrotic-ILA. Cases were identified using a text-based keyword search of thoracic radiology reports. By virtue of the methods used to populate this study cohort, and as interstitial lung abnormalities are considered 'pre-ILD' abnormalities, this cohort is less refined. Capturing indication for CT was not possible for this cohort and indeed a proportion with EF-ILA will have been asymptomatic, which may have introduced bias. Although comorbidity profiles were identified from clinical coding, it was also not possible to date this information. Therefore, I was unable to determine if comorbidities were present at time of CT demonstrating EF-ILA or if these events occurred after CT scan. ILAs, in general, appear to be associated with multimorbidity and this too may have biased interpretation of study outcomes,[332] although efforts were made to adjust for comorbidities (see below). Despite these limitations, search criteria for this study (Section 4.3.2) were selective for early fibrotic radiological appearances and the large cohort size compensates for this.

All study participants included across all three cohorts were either enrolled during scheduled clinic visit at the Oxford ILD service or identified from radiological records of the Oxford radiology department. The work presented in this thesis is therefore reflective of heterogenous clinical datasets representative of a single UK ILD centre, which limits the generalisability of the findings. However, they are clinically translatable.

6.3.2 Adjustment of confounding risk factors

A limitation of all studies comprising this thesis were the relatively small sample sizes and the retrospective nature in which studies were conducted. Although the EF-ILA cohort comprised

1259 cases, it was only possible to assess for progression in the 362 subjects that underwent repeat CT. This limited study findings to association and causality was not investigated.

Multivariate risk models were used to explore association between clinical outcomes and blood leukocytes and indexes. Advancing age, male gender, smoking history and disease severity parameters at initial assessment are all considered risk factors for disease progression and mortality.[333] Where feasible multivariate models incorporated these covariates to adjust for any confounding risk factors overall and therefore any effect on study outcome. In addition, and where feasible, robustness of results was tested through sensitivity analysis. For example, the potential effect of steroid and antifibrotics therapy, and additional respiratory comorbidities during study follow up periods were assessed by including these covariates. subsequent multivariate modelling continued to demonstrate leukocyte associations with study outcomes. Despite this, and as previously discussed, study populations were relatively small. This may explain the large hazard ratios observed in some multivariate analyses of Study 1a and 3 and limits control of type I error. However comparable observations between all studies were observed.

Monocyte, neutrophil and lymphocyte counts closest to enrolment were selected for analysis, either closest to first CT or first clinic visit (depending on study protocol). Median time interval between blood draw and enrolment was <1 month across all cohorts; -0.03 months (-1.20 to 0.00), 0.02 (-0.40 to 1.18) and -0.87 (-2.58 to 0.24) for studies 1, 2 and 3 respectively. This relatively short time interval is similar to other clinical studies exploring leukocytes in IPF and allows for meaningful comparison.

Time of blood draw could not be controlled for in retrospective studies. This is a potential limitation to interpretation of study outcomes presented not only in this thesis, but also other similar studies to which I have drawn comparison. Indeed, circadian rhythms are present in many cellular and humoral components of the immune system. Monocytes, granulocytes and lymphocytes exhibit circadian oscillations in blood count and leukocyte recruitment is regulated by circadian expression of pro-migratory factors within endothelial cells and oscillatory patterns of immune cell trafficking and host-pathogen interactions.[334] Consistent with variations in the immune cell number and function, inflammatory diseases display circadian manifestations. Furthermore, diurnal variation in leukocyte counts can be affected sleep pattern, stress and after exercise.[290,291]

Solitary monocyte, neutrophil and lymphocytes for each case were used in regression analyses and I am unable to say if these are representative of longitudinal trends of these leukocytes for each case. In Study 2, coefficient of variation (CoV) for cases with serial leukocyte data was

calculated (Table A8) and incorporated into multivariate analyses and demonstrated comparable associations. However serial blood tests for each case were not taken in a uniform timeframe across the cohort and this may have biased the observed associations between leukocyte CoV and study outcomes. Again, this represents the limitation of retrospective analyses.

6.3.3 Measurement of disease progression

The principal study outcomes explored in this thesis were (i) mortality and (ii) progression of disease. Mortality was defined as all-cause events. Importantly, this allowed for inter-cohort comparison of mortality between studies 1, 2, and 3 and observed leukocyte associations. This also allowed for comparison to other retrospective studies which have explored monocyte, neutrophil, lymphocyte, and leukocyte indexes in IPF cohorts. Although progression to end-stage disease, acute exacerbations and pneumonia are often cited as cause of death in patients with IPF, registry data demonstrates that these account for approximately 50% of accounted deaths.[335] Furthermore, studies have identified ILAs are increasingly accompanied with multimorbidity, which in turn will influence mortality risk.[332] Classifying mortality as all-cause ensures that all mortality events are captured. Observed leukocyte associations with mortality in IPF and EF-ILA studies of this thesis were comparable to other study cohorts.

Mortality in IPF studies is often discussed in parallel to severity and progression of disease. Explorations of study outcomes incorporating disease progression allows for greater understanding of associations, causality, and pathobiology of IPF. Despite this, multiple definitions of progression have been used in clinical studies. Progression has been defined by relative or absolute declines in FVC or TLCO,[17,25] visual assessment of parenchymal CT features,[336] and automated quantification of novel biomarkers.[83] Progression has also been defined as a composite outcome measure of varying combinations of these. In some studies composite measures have also incorporated assessment of exercise capacity.[228] This makes comparison of inter-cohort differences between retrospective studies conducted by the wider ILD community more challenging. Furthermore, I recognise this is a limitation of the retrospective studies that comprise this thesis. In Study 1a radiographic progression of iUIP was assessed by multivariate analysis and in Study 1b progression was defined by time FVC decline >10% taken from serial lung function tests. In Study 2, progression of EF-ILA was defined as radiographic progression. In Study 3, I defined progression as either relative FVC decline or increase in CALIPER-measured fibrosis on interval CT. I also acknowledge that CT scans were reported in an unblinded fashion, and radiologists would have had access to clinical information and study cohorts may have been

subjected to an element of interobserver variation in CT reporting, which may have introduced bias to CT reporting.

Despite differences in these definitions of progression Study 1a and 2 are comparable as both non-IPF cohorts demonstrated similar (and significant) monocyte and neutrophil association with mortality and progression, using similar statistical analyses. I also used the same qualitative scale to describe progression of fibrosis by visual assessment and I used the 2018 radiological UIP classification system to describe CT appearances across all studies.

A further limitation across all studies was that interval CT scans were performed due to clinical indication and therefore undertaken in a non-uniform timeframe. Indeed, there was a trend across all cohorts of greater extent of progression (either qualitatively or quantitatively) in cases with greater time duration between CT scans. This may have biased interpretation of results. Limiting inclusion of data sets by specific CT time intervals would have drastically reduced case numbers used in analysis. The long follow-up period observed for some cases would have captured a greater number of adverse events and will have strengthened the associations observed between leukocytes and adverse outcomes.

All statistical analyses were conducted using Cox Proportional hazard modelling, incorporating time-dependent variables into risk models, and as previously discussed comparable leukocyte signals were observed across cohorts of higher monocytes, neutrophils, and lymphocytes.

6.3.4 Summary

In summary, the main strengths include: the exploration of monocytes, neutrophils and lymphocytes with defined clinical outcomes across multiple patient cohorts with varying degrees of disease severity, selection of cases with robust MDT diagnosis of IPF or careful selection by radiographic pattern of fibrotic ILA. Long follow-up periods will have increased capture of disease progression and mortality events and strength of association between leukocytes and adverse clinical outcomes. Strong statistical analysis facilitated comparison of these analyses to other published studies. The main limitations include: the retrospective nature of the analyses, which introduces bias that cannot be eliminated and cannot establish causal relationships between leukocytes and mortality and progression of IPF. Study populations were relatively small and despite thorough multivariate analyses I cannot exclude residual confounders. The inclusion of all study subjects from a single tertiary hospital network limits the generalisability of the findings. The use of different definitions of disease progression across the studies limits the strength of

associations between leukocytes and disease progression. Therefore, the analyses presented in this thesis provide the best possible estimate of monocyte, neutrophil, lymphocyte association with adverse clinical outcomes of IPF, and precursor states, of the populations studied, but cannot establish causal relationships.

6.4 Future research

6.4.1 Whole blood preservation and neutrophil exploration

Immunophenotyping of PBMCs using Mass cytometry including the enumeration of complex leukocyte subsets from peripheral blood is a powerful tool to stratify patients according to disease phenotype and behaviour. The PBMC fraction of blood cells includes lymphocyte populations, including T cells, B cells and natural killer cells and myeloid cells such as monocytes. PBMCs are isolated from whole blood using density gradient media such as Ficoll which during centrifugation separates the constituents based upon relative densities. During this process granulocytes, plasma and platelets are separated owing to their differing density with respect to PBMCs and as part of the extraction process are discarded.[337] PBMC extraction and preservation of viable cells for future stimulation assays or *in vitro* mechanistic studies is essential and potentially performed at expense of other important cell populations. Thus, PBMCs are not representative of whole blood populations *in vivo*.[338]

Neutrophils represent the largest population of immune cells in peripheral blood. Indeed, these cells serve important functions as immediate responders to bacterial infection, wound healing and formation of fibrotic tissues,[135] and as I have demonstrated, measurement of neutrophils in whole blood are also associated with progression of iUIP to IPF and accelerated FVC decline in IPF. This evidence clearly points to a necessity of including this cell population in future experiments. Neutrophils are also extremely sensitive cells when removed from their in vivo environment, and analysis must be performed rapidly to avoid biased results.[338] The increased ability of modern technologies to capture the complexities of human immune responses is now enabling the assessment of whole blood instead of fractionated PBMCs or purified individual cell types as the principle source of human cells in immunological studies.[339] The cryopreservation assays involve stabilisation and freezing of whole blood immediately after venesection. Cryopreservation of whole blood therefore allows the researcher to enumerate granulocyte populations such as neutrophils that are discarded by PBMC isolation procedures.[339]

Future experimental studies could be conducted using cryopreserved whole blood sampling. As previously discussed, neutrophil populations are implicated in fibrosis. In Study 1, I observed a strong association between neutrophilia and progression to IPF, accelerated FVC decline and mortality in IPF. Using Time of Flight mass cytometry (CyTof), I would be interested to explore if any neutrophil sub-populations are implicated in this. Further experiments would involve familiarisation with and optimising of whole blood cryopreservation assays and designing an antibody panel containing pertinent granulocyte surface markers to gate for neutrophil populations and explore co-interaction with other immune cell populations.

6.4.2 Effect of antifibrotics on serial measurement of monocytes

Many studies exploring blood leukocyte association with IPF have argued for the potential role of these leukocyte (and index) measures as prognostic biomarkers. In essence, the need for a reliable biomarker to both guide personalised IPF patient management, risk-stratification, and cohort enrichment for clinical trials in IPF is becoming more important.[226]

In the generic sense, biomarkers can be described as a parameter "that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes or pharmacologic responses to a therapeutic intervention".[340] Many of those explored are considered either predisposition (genetic) markers, diagnostic, prognostic or therapeutic. Prognostic biomarkers should contribute to quantitative assessment of mechanism or biological pathways relevant to disease progression. Therapeutic biomarkers should provide quantitative assessment of mechanisms or biological pathways targeted by therapy and therefore predict treatment response.[226] Theragnostic markers combine both a prognostic measure, to risk stratifies patients for adverse outcomes, with a therapeutic measure, to identify individuals most likely to respond favourably to targeted drug therapy based on the test results.[341]

Data supporting theragnostic biomarkers mainly comes from post-hoc analyses of retrospective or prospective cohort studies. Recent studies have demonstrated a short-term dynamic change in various putative biomarker in response to therapeutics that could be predictive of a subsequent response to therapy.[342,343]

In chapters 3, 4 and 5 I report association between monocytes, neutrophils, lymphocytes, and adverse clinical outcomes in patients with IPF and ILA. I discussed the potential mechanistic implications of these in pathobiology of IPF, and I also discussed how these findings complement other similar studies published in scientific literature.

The availability of data from previous phase II and phase III IPF drug trials has facilitated large retrospective studies using refined IPF cohorts. As I previously discussed, Kreuter et al analysed data from ASCEND and CAPACITY and identified monocyte count >0.60x10^3/μl was associated with composite disease progression and mortality. In supplementary analysis the authors demonstrated a serial reduction in 3-monthly monocyte counts in the Pirfenidone arm compared to placebo.[228] However they did not comment on association between extent of monocyte count reduction and efficacy of Pirfenidone towards stabilisation of FVC. Using similarly methodology, Tzouvelekis et al explored relationship between monocyte count and outcomes using data from INPULSIS and TOMORROW studies. The authors demonstrated that monocyte count >0.51x10^3/μl (>median) was associated with a faster rate of FVC decline in both Nintedanib and placebo groups.[259]

Nathan et al demonstrated that increase in NLR over the 12 month follow up period in ASCEND and CAPACITY studies was associated with greater FVC decline and mortality in the Placebo group.[258] However, the authors did not discuss if this analysis was conducted in the intervention arm. It remained unclear if antifibrotics influence NLR and what this could mean.

I would like to go one step further and explore if these absolute leukocyte measures possess a theragnostic potential. Specifically, I would like to explore if (i) treatment with antifibrotic agents is accompanied by change in the longitudinal measure of these leukocyte parameters and (ii) if the relative change of such parameters corresponds to any change in measure of disease progression such as FVC decline, quantitative increase in fibrosis on CT or mortality. Ultimately, I would like to explore if any of these leukocyte parameters could be predictive of treatment response. This would most likely be a hypothesis-driven retrospective study utilising existing data from the historic phase II and phase III antifibrotic studies.

6.4.3 Hyperpolarised 129Xenon MRI to explore leukocyte association with alveolar integrity in IPF

As previously discussed, although FVC and TLCO are typically used to assess lung volumes and gas exchange in IPF, accuracy of these global metrics can be confounded by patient effort and mixed obstructive / restrictive lung disease.[72] Longitudinal data demonstrate substantial intra-patient variability, such that marginal changes of FVC can be difficult to distinguish from genuine disease progression.[25,73] Although HRCT is a highly sensitive tool for assessing ILD,[75] interpretation is subjective and fraught with intra-observer variability.[76]

Recently, novel radiographic modalities which sensitively and objectively measure disease have been explored in IPF. Xenon (Xe) is a highly soluble, non-toxic noble gas. Upon inhalation it readily diffuses from the alveoli, across the alveolar interstitium and into the blood. When hyperpolarised, the unique resonance shift of ^{129}Xe within these different compartments; gaseous, aqueous (tissue and plasma (TP)) and red blood cell (RBC) environments provides regional information on diffusion capacity.[344] Therefore ^{129}Xe distribution within these compartments can be measured. Subtle differences in ^{129}Xe absorption spectra can detect changes to local alveolar integrity and regional gas exchange (Figure 6.1).[75,345]

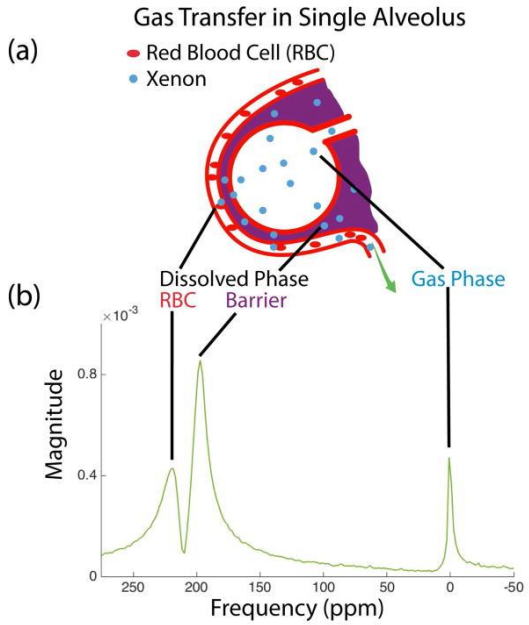


Figure 6.1 Gas-phase, barrier, and RBC peaks in Magnetic resonance spectroscopy.
(A) Diagram of gas-phase ^{129}Xe transfer from alveoli to interstitial barrier tissues and capillary RBCs that comprise the dissolved phased signal. (B) The ^{129}Xe spectrum exhibits three resonances in lung corresponding to ^{129}Xe in airspaces, dissolved in barrier and RBCs. ^{129}Xe dissolves along the barrier tissues (alveolar capillary) and bind to red blood cells. Each compartment presents a different local environment, causing ^{129}Xe to exhibit distinct magnetic resonance frequency shifts. With permission taken from Wang et al.[346]

Cross-sectional and longitudinal studies have demonstrated enhanced barrier uptake and reduced diffusion of ^{129}Xe across the alveolar interstitium in IPF patients compared to healthy controls. Furthermore, reduction in RBC transfer and ratio of RBC/barrier uptake, correlated strongly with FVC and TLCO decline.[346,347] Figure 6.2 demonstrates how ^{129}Xe absorption spectra change in IPF.

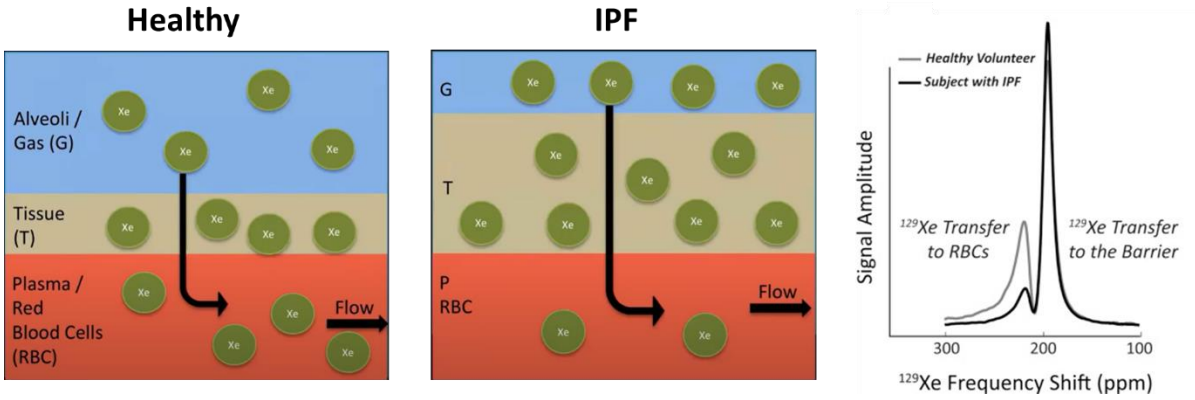


Figure 6.2 Spectral peaks generated from hyperpolarised ^{129}Xe MR spectroscopy.
^{129}Xe diffusion across alveolar interstitium in healthy lung (Left). In IPF, the fibrosed interstitium causes accumulation of ^{129}Xe and reduced diffusion into the red blood cell compartment (Middle). Spectral peaks from a healthy volunteer and IPF patient (Right). In (IPF, the RBC peak is diminished with respect to the tissue/plasma peak, suggesting reduction in gas transfer efficiency of the lung and reduction in RBC:TP ratio. Adapted from Weatherley (left) and taken with permission from Mammarappallil.[75,345]

Other studies have demonstrated that changes in ^{129}Xe MRI metrics, particularly longitudinal change in the RBC:TP ratio, offers more sensitive measurement of disease severity and functional impairment in IPF in comparison to conventional metrics of lung function decline.[75] Therefore, simultaneously measuring structural and functional abnormality using ^{129}Xe MRI, with immune profiling could provide an opportunity to closely explore any relationship between the architectural distortion and physiological abnormalities observed in IPF patients with monocyte activity and phenotype.

In a subset of IPF patients I would like to explore if regional ^{129}Xe MRI (RBC:TP ratio) measures correlate with (i) CD64 expression and type I interferon activity and (ii) peripheral blood leukocyte measurement.

Appendix A Ethics approval

Oxford University Hospitals
NHS Foundation Trust
From the Head of Research Governance
OUH Research & Development
Joint Research Office
OUH Cowley

Tel: 01865 572392
Email: fay.davies@ouh.nhs.uk
Date: 02/10/2018

Confirmation of Trust Management Approval

Re: Comprehensive analysis of immune events as contributor of disease progression in IPF
IRAS Reference: 230547
Research and Development Reference: 13172
Research Ethics Committee Reference: 18/SC/0227
Sponsor: University of Oxford

Dear Dr Ling-Pei Ho,

On behalf of the Oxford University Hospitals NHS Foundation Trust, I am pleased to confirm Trust Management Approval and Indemnity for the above research as outlined in the application received.

Approved Documents

Protocol Version 1.0 dated 16/03/2018 is recognised as the most current to date.

The documents approved for use at this Trust are as listed in the following approval documents:

Health Research Authority Letter dated: 31/07/2018
Research Ethics Full Approval Letter dated: 31/07/2018

Any subsequent, relevant amendments are additionally included to date.

Metrics and Recruitment

First Participant target	Agreed Total recruitment target
Target date: 25/10/2018	Target minimum: 100
You are currently on day: 0	Recruitment Target date 19/04/2023

To comply with national research performance requirements the OUH Trust will monitor and publish recruitment for your study:
1. Performance of the recruitment of the first participant to your study calculated from the time of receipt of a valid research application in R&D;

Signed electronically in Studyline by Mrs Fiona Parker on 02/10/2018 15:14

Non-CTIMP TMA approval template including conditions of approval,
Letter template 4 TMA-01-Appendix 2 V10.1, June 2018 NHSPT4

Figure A.1 Study 1, 2 and 3 research ethics approval

Appendix B Study 1

Outcome	Non-Progressive iUIP	Progressive iUIP	P Value / Odds Ratio	Confidence Interval
Death	1 (11%)	9 (39%)	5.1	0.63-62.63
Mean age at initial iUIP	73.1	72.0 (±12.3)	-	-
Mean age at death	73.5	77.1 (±11.2)	-	-
CT appearance at death				
iUIP	1 (11%)	1 (4%)	-	-
Probable UIP	0 (0%)	4 (17%)	-	-
Definite UIP	0 (0%)	4 (17%)	-	-
Cause of death				
Pneumonia	0 (0%)	2 (9%)	-	-
End-stage IPF	0 (0%)	4 (18%)	-	-
Cancer	1 (11%)	1 (4%)	-	-
Unclear	0 (0%)	2 (9%)	-	-
Follow up				
Length of follow up (years)	2.1 (±1.4)	3.4 (±1.7)	P=0.034	-
F/U - Discharged from clinic	1.8 (±1.2)	3.5 (±2.0)	-	-
Death during follow up	0 (0%)	3 (13%)	-	-
Indication for clinic Discharge				
Stable (clinically / PFTs)	7 (78%)	10 (44%)	-	-
Frail	1 (11%)	1 (4%)	-	-
End-stage disease	-	2 (9%)	-	-
Active follow up	1 (11%)	7 (30%)	-	-
Hospital admission	2 (22%)	9 (39%)	2.25	0.40-12.32
Pneumonia / LRTI	1	4	1.7	0.20- 22.91
Heart failure	0	1	-	-
AEIPF	0	1	-	-
Disease progression	0	1	-	-
Pneumothorax	0	1	-	-
COPD exacerbation	0	1	-	-
Other	1	0	-	-

Table A1 Clinical outcomes of progressor vs non-progressor groups.
Odds ratios of Progressive Vs non-progressive iUIP listed with 95% confidence intervals. P<0.05 considered significant.

Appendix B

	Non-Progressive iUIP		Progressive iUIP		iUIP progression to Probable / Definite UIP		P Value	Odd ratio (95%CI)
n	9		23		17			
Pulmonary function								
TLCO	mmol/min/kPa	% predicted	mmol/min/kPa	% predicted	mmol/min/kPa	% predicted	-	-
TLCO - Baseline	5.0 (±0.9)	77.8 (±18.1)	5.3 (±1.7)	64.2 (±16.0)	5.29 (±1.85)	60.2 (±16.43)	-	-
1 year	6.13 (±1.7)	81.2 (±13.4)	4.69 (±1.6)	55.5 (±16.6)	6.04 (±5.60)	50.9 (±22.9)	-	-
2 years	6.11 (±2.0)	78.94 (±14.5)	4.39 (±2.0)	53.4 (±22.0)	4.40 (±1.50)	52.7 (±16.3)	-	-
3 years	5.43 (±2.1)	74.77 (±10.2)	4.91 (±2.08)	59.1 (±19.9)	-	-	-	-
FVC	Litres	% predicted	Litres	% predicted	Litres	% predicted	-	-
FVC - baseline	2.90 (±0.7)	102.00 (±21.6)	3.24 (±1.1)	92.6 (±26.9)	2.96 (0.85)	82.4 (±19.3)	-	-
1 year	2.95 (±0.6)	91.4 (±11.8)	2.96 (±1.1)	81.4 (±24.0)	2.31 (±1.69)	68.2 (±22.5)	p<0.002	-
2 years	2.70 (±0.4)	89.2 (±17.0)	3.07 (±1.2)	84.4 (±28.7)	2.60 (±0.91)	68.7 (±17.2)	-	-
3 years	2.91 (±0.4)	95.3 (±18.2)	2.96 (±1.1)	83.7 (±22.7)	-	-	-	-
CPI								
CPI Score	67.7 (±18.3)	-	69.5 (±10.4)	-	68.9 (±11.3)	-	-	-
1 year	56.5 (±11.4)	-	70.9 (±9.5)	-	64.7 (±19.5)	-	-	-
2 years	66.1 (±11.5)	-	73.4 (±11.24)	-	68.0 (±88.8)	-	-	-
3 years	62.2 (±9.9)	-	69.6 (±14.9)	-	-	-	-	-
CT scans								
Total CT scans	23		65		53		-	-
Interval scans	14		42		36		-	-
GGO on CT	3 (33%)		18 (78.3%)		14 (82%)		*p=0.03	7.2 (1.20-31.48)
GGOs in total CTs	7 (30.4%)		32 (47.8%)		28 (52%)			
GGOs in First CTs	2 (22.2%)		13 (56.5%)		11 (64%)		p=0.146	2.2 (0.83-5.64)
Emphysema	0		4 (17%)		2 (12%)		p=0.122	0.46 (0.84-24.21)
Indication for CT	Initial CT	Repeat CT	Initial CT	Repeat CT	Initial CT	Repeat CT	-	-
SOB	2 (22%)	1 (7%)	9 (39%)	22 (52%)	8 (47%)	20 (56%)	-	-
? ILD pattern	1 (11%)	0	8 (35%)	3 (7%)	5 (29%)	1 (3%)	-	-
? Progression	0	2 (14%)	0	9 (21%)	0	8 (22%)	-	-
Worsening PFTs	0	1 (7%)	0	3 (7%)	0	3 (8%)	p=0.001	11.4 (1.71-126.8)
? AEIPF	0	0	0	3 (7%)	0	2 (6%)	-	-
Nodule	1 (11%)	9 (64%)	0	1 (2%)	0	1 (3%)	-	-
Cough	0	0	4 (17%)	1 (2%)	3 (18%)	1 (3%)	-	-
Abnormal CXR	5 (55%)	0	3 (13%)	2 (5%)	2 (12%)	2 (6%)	-	-
Weight loss	0	1 (7%)	1 (4%)	3 (7%)	0	3 (8%)	-	-

Table A2 Comparisons Non-Progressive iUIP and Progressive iUIP

Comparisons of pulmonary function tests (from baseline to 3 years), CT features and indication for CT in each sub-category. Comparison of paired absolute or predicted values with t-test; baseline vs 1 year non-progressive vs progressive iUIP groups. Odds ratios listed with 95% confidence intervals. P<0.05 considered significant. Comparisons of progressor vs non-progressor group, *cases with GGO on a CT. OR; odds ratio.

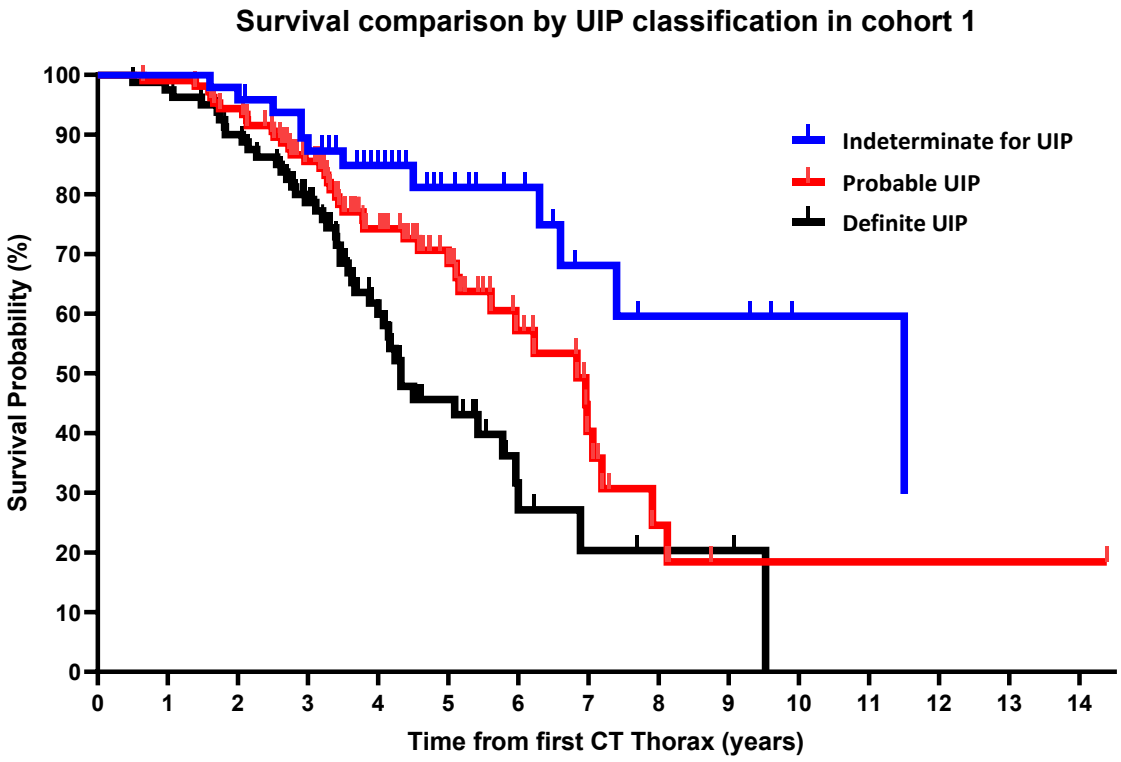


Figure A.2 Kaplan-Meier curves for mortality in Cohort 1

Kaplan-Meier analysis demonstrating survival proportions for all 230 cases of iUIP (n=48, median survival 11.5 years), probable UIP (n=102, 6.83 years, HR 2.01, CI 1.1-3.5) and definite UIP (n=80, 4.32 years, HR 3.3, CI 1.9-5.5) on first CT [p<0.0001].[8]

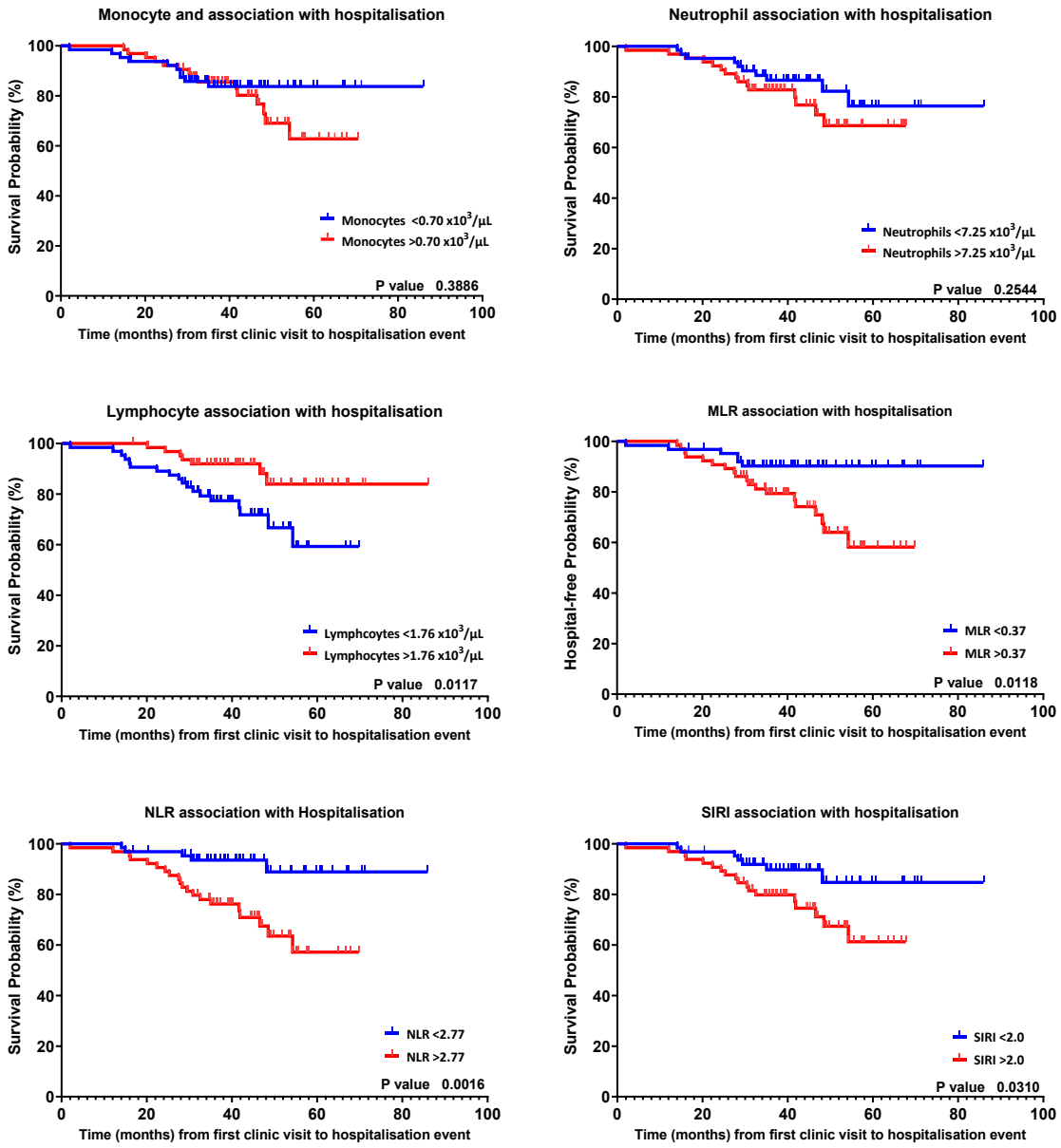


Figure A.3 Kaplan-Meier curves for time to event of hospitalisation

Dichotomised for median values for absolute monocyte, neutrophil and lymphocyte counts and derived indexes of monocyte:lymphocyte ratio, neutrophil:lymphocyte ratio and systemic inflammatory response index. All graphs demonstrate as p<0.05 (logrank).

Appendix C Study 2

	Time interval of repeat CT (n/%)			
	<1 year	1-2 years	2-3 years	3-5 years
Outcome of repeat CT in EF-ILA cohort				
No progression	139 (67.8%)	37 (18.0%)	16 (7.8%)	13 (6.3%)
Progression	62 (39.4%)	43 (27.4%)	29 (18.5%)	23 (14.6%)
-Progression of reticulation	29 (56.0%)	10 (20.0%)	7 (14.0%)	5 (10.0%)
-New Traction bronchiectasis	27 (34.6%)	22 (26.9%)	18 (23.1%)	16 (15.4%)
-New Honeycombing	6 (30.0%)	7 (35.0%)	5 (15.0%)	4 (20.0%)

Table A3 Timeframe for outcome of repeat CT scan
Proportions displayed as n (row %).

	Univariate		Multivariate	
	HR (95% CI)	P Value	HR (95% CI)	P Value
Covariates				
Age	1.02 (1.01-1.04)	<0.0001*	1.10 (1.05-1.16)	<0.001*
Gender	1.15 (0.96-1.37)	0.140	0.83 (0.51-1.37)	0.469
Progression on CT	1.62 (0.97-2.69)	0.063	1.92 (1.51-3.21)	0.013*

Table A4 Cox PH regression for mortality in cases of EF-ILA undergoing repeat CT

	n (%)	Death (%)	Mortality HR (95% CI)	P Value
Covariates				
Age	--	--	1.01 (0.99-1.03)	0.107
Gender (Male)	1486 (54.3%)	268 (18.0%)	1.09 (0.88-1.40)	0.425
Lung cancer	269 (9.8%)	154 (57.2%)	4.98 (3.96.23)	<0.001
Pneumonia	657 (24.0%)	251 (38.2%)	3.05 (2.45-3.81)	<0.001
COPD / Emphysema	569 (20.8%)	165 (28.9%)	1.14 (0.90-1.43)	0.278
Nil ILA (reference)	355 (12.9%)	43 (12.1%)	--	--
-Early fibrotic ILA	1259 (46.0%)	183 (14.5%)	1.52 (1.01-2.27)	0.043
-TBx without Honeycombing	490 (17.9%)	86 (17.6%)	1.70 (1.11-2.62)	0.016
-Honeycombing +/- TBx	272 (9.9%)	87 (32.0%)	3.12 (2.03-4.81)	<0.001

Table A5 Multivariate Cox regression exploring ILA features with mortality
Hazard ratios (HR) for ILA categories representative of risk relative to Nil ILA reference category. TBx; Traction bronchiectasis, EF-ILA; Early-fibrotic ILA. Model adjusted for age, gender, and respiratory co-morbidity (lung cancer, pneumonia, and COPD/emphysema). HRs pertaining to ILA covariates are expressed relative to Nil ILA.

	Mortality		Radiological progression	
	HR (95% CI)	P value	HR (95% CI)	P value
Covariates				
Age	1.02 (1.01-1.03)	0.015	1.03 (1.00-1.06)	0.038
Gender	1.00 (0.82-1.22)	0.977	0.91 (0.66-1.26)	0.573
Pneumonia	2.97 (2.40-3.67)	<0.001	1.38 (0.99-1.32)	0.060
COPD / Emphysema	1.12 (0.90-1.39)	0.294	0.64 (0.44-0.94)	0.023
Lung cancer	4.46 (3.61-5.51)	<0.001	1.27 (0.80-2.00)	0.309
Monocytes (x10^3/μl)	1.15 (0.85-1.55)	0.376	1.72 (1.10-2.69)	0.018
Neutrophils (x10^3/μl)	1.07 (1.04-1.10)	<0.001	1.05 (0.98-1.12)	0.154
Lymphocytes (x10^3/μl)	0.79 (0.70-0.90)	<0.001	0.99 (0.95-1.03)	0.690

Table A6 Multivariate cox regression examining mortality and EF-ILA progression

Multivariate cox regression examining association between blood leukocyte and (i) mortality in "early fibrotic" ILA (n=1259) and (ii) radiological progression in "early fibrotic" ILA cohort with available repeat CT for comparison (n=362). Leukocytes adjusted for age, gender and respiratory comorbidities of COPD, lung cancer and pneumonia.

Leukocytes	EF-ILA no progression	EF-ILA progression	P Value
Leukocytes			
Monocyte (x10^3/μl)	0.60 (0.48-0.79)	0.67 (0.5-0.83)	0.202
Monocyte CoV	0.24 (0.18-0.38)	0.27 (0.18-0.41)	0.608
Neutrophil (x10^3/μl)	4.31 (3-5.86)	4.73 (3.32-6.11)	0.230
Neutrophil CoV	0.30 (0.19-0.46)	0.30 (0.21-0.42)	0.064
Lymphocyte (x10^3/μl)	1.76 (1.28-2.33)	1.65 (1.27-2.27)	0.131
Lymphocyte CoV	0.01 (0-0.17)	0.08 (0-0.21)	0.927
Leukocyte ratios			
MLR	0.33 (0.25-0.47)	0.37 (0.29-0.53)	0.010
MLR CoV	0.30 (0.19-0.44)	0.31 (0.21-0.43)	0.658
NLR	2.53 (1.75-3.33)	2.75 (1.83-4.32)	0.074
NLR CoV	0.42 (0.24-0.61)	0.37 (0.25-0.55)	0.443
SIRI	1.43 (0.82-2.33)	1.75 (1.12-2.98)	0.020
SIRI CoV	0.46 (0.28-0.73)	0.47 (0.3-0.75)	0.990

Table A7 Leukocyte and Coefficient of variation EF-ILA

Leukocyte measurements and Coefficient of variation in cases of EF-ILA demonstration stable features or progression at interval CT scan. Data expressed as median (IQR). CoV; Coefficient of variation.

	Mortality		Radiological progression	
Covariates	**HR (95% CI)**	**P Value**	**HR (95% CI)**	**P Value**
Full blood count				
-Age	1.03 (1.01-1.06)	0.007	1.03 (1.01-1.06)	0.023
-Gender	0.96 (0.71-1.31)	0.797	0.937 (0.68-1.30)	0.700
-Monocytes (x10^3/μl)	1.18 (1.04-1.33)	0.011	1.74 (1.15-2.64)	0.009
-Monocyte CoV	7.37 (3.85-14.3)	<0.0001	7.43 (2.84-19.44)	<0.0001
-Lymphocytes (x10^3/μl)	0.95 (0.84-1.07)	0.395	0.99 (0.95-1.03)	0.652
-Lymphocytes CoV	8.88 (4.72-16.4)	<0.0001	7.50 (2.77-20.31)	<0.0001
-Neutrophils (x10^3/μl)	1.08 (1.04-1.13)	<0.0001	1.05 (0.99-1.12)	0.127
-Neutrophils CoV	4.42 (2.68-7.31)	<0.0001	2.75 (1.28-5.90)	0.009
MLR				
-Age	1.04 (1.01-1.06)	0.003	1.03 (0.99-1.06)	0.065
-Gender	0.983 (0.72-1.34)	0.915	0.90 (0.65-1.24)	0.535
-MLR	1.05 (0.93-1.20)	0.441	2.03 (1.17-3.56)	0.013
-MLR CoV	3.25 (2.42-4.37)	<0.0001	3.08 (1.43-6.68)	0.004
NLR				
-Age	1.04 (1.01-1.06)	0.002	1.03 (0.99-1.06)	0.075
-Gender	0.97 (0.72-1.32)	0.859	0.95 (0.70-1.31)	0.100
-NLR	1.05 (1.03-1.07)	<0.0001	1.08 (1.01-1.56)	0.026
-NLR CoV	2.59 (1.83-3.59)	<0.0001	1.41 (0.79-2.77)	0.317
SIRI				
-Age	1.04 (102-1.07)	0.001	1.03 (0.99-1.06)	0.058
-Gender	1.04 (0.76-1.41)	0.826	0.95 (0.69-1.30)	0.731
-SIRI	1.04 (1.02-1.07)	<0.0001	1.08 (1.03-1.14)	0.001
-SIRI CoV	2.54 (1.93-3.37)	<0.0001	1.62 (1.04-2.54)	0.032

Table A8 Multivariate cox regression exploring leukocyte CoV

Multivariate cox regression examining association between blood leukocyte indexes and (i) mortality in "early fibrotic" ILA (n=1259) and (ii) radiological progression in "early fibrotic" ILA cohort with available repeat CT for comparison (n=362). Covariates in “full blood count”, MLR, NLR and SIRI models adjusted for age, gender, and leukocyte co-efficient of variation (CoV).

	Female	Male	Odds Ratio	p value
Outcome of follow-on CT				
No progression	90 (56.3%)	115 (50.7%)	--	--
Progression all cases	70 (47.3%)	112 (49.3%)	1.25	0.301
Progression of reticulation	26 (32.9%)	25 (22.3%)	0.81	0.607
Reticulation to Probable UIP	30 (40.5%)	54 (50.0%)	1.36	0.278
Reticulation to Definite UIP	7 (8.9%)	17 (15.2%)	1.84	0.268

Table A9 ILA progression categorised by gender (n=362)

Odds ratios assessed for significance using Fishers exact.

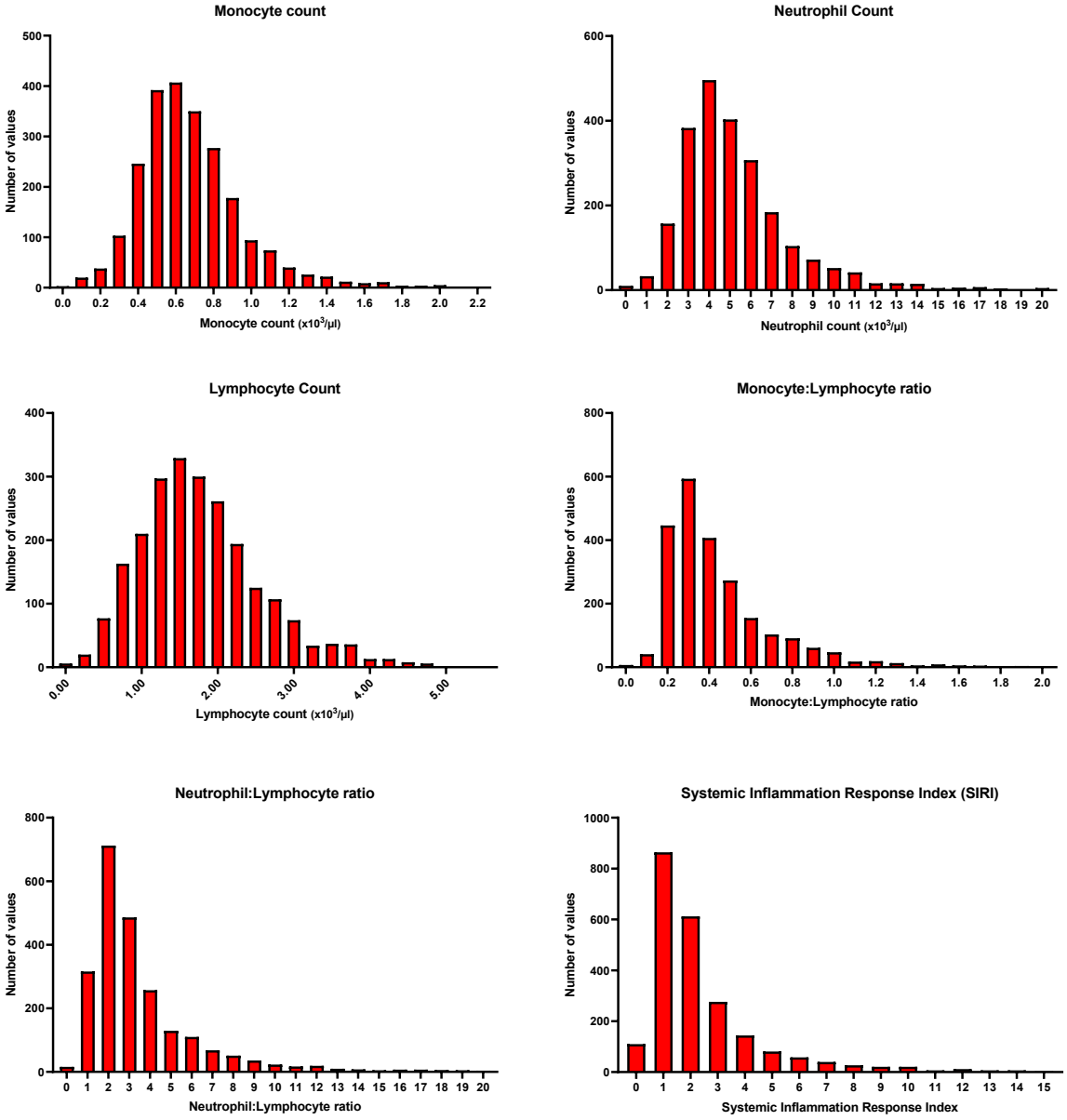


Figure A.4 Histograms demonstrating Leukocyte distribution

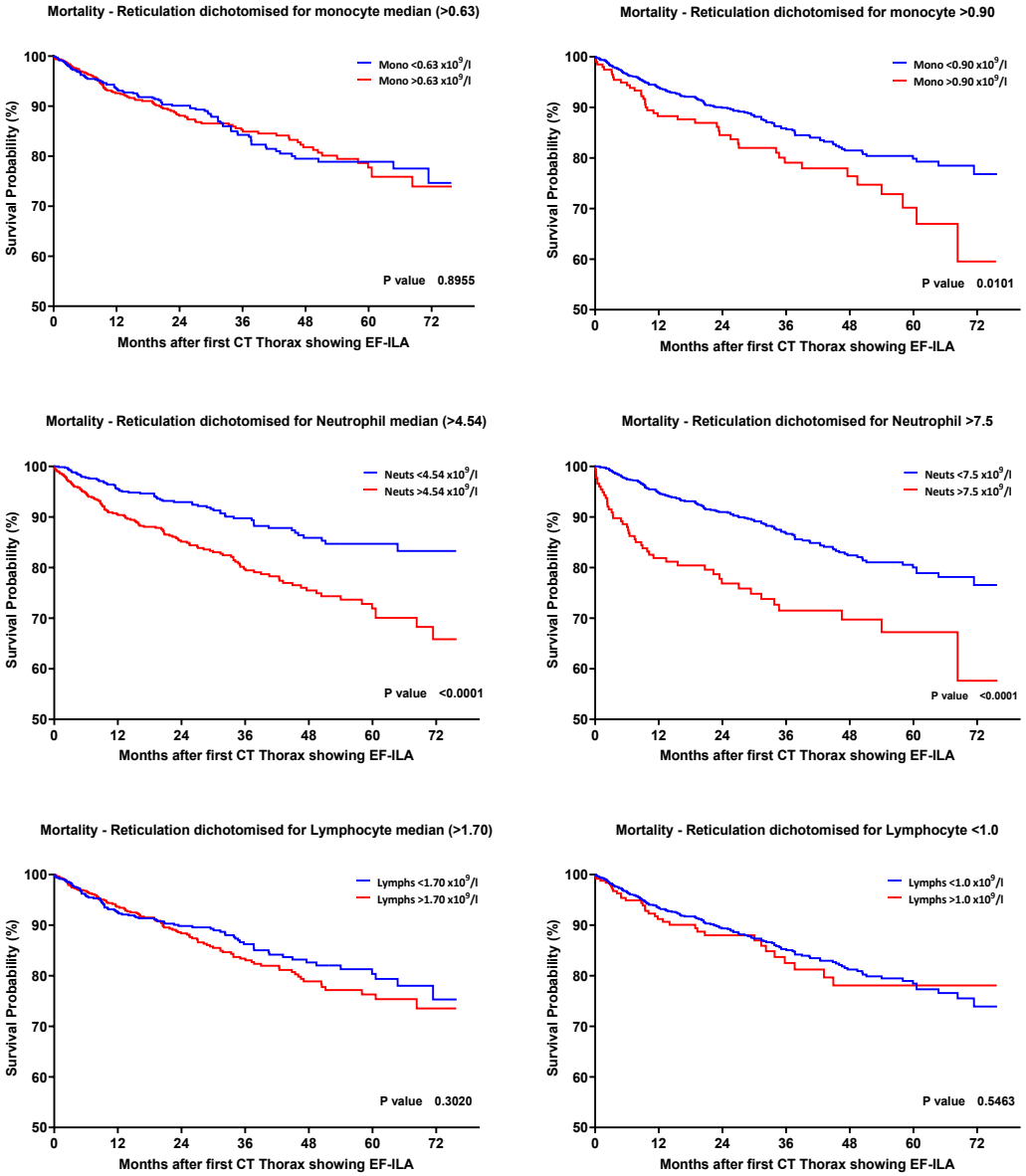


Figure A.5 Survival probability of cases with reticulation on first CT
Blood leukocytes (monocytes, neutrophils, lymphocytes) dichotomised by median values and upper or lower limit of reference range.

Appendix D Study 3

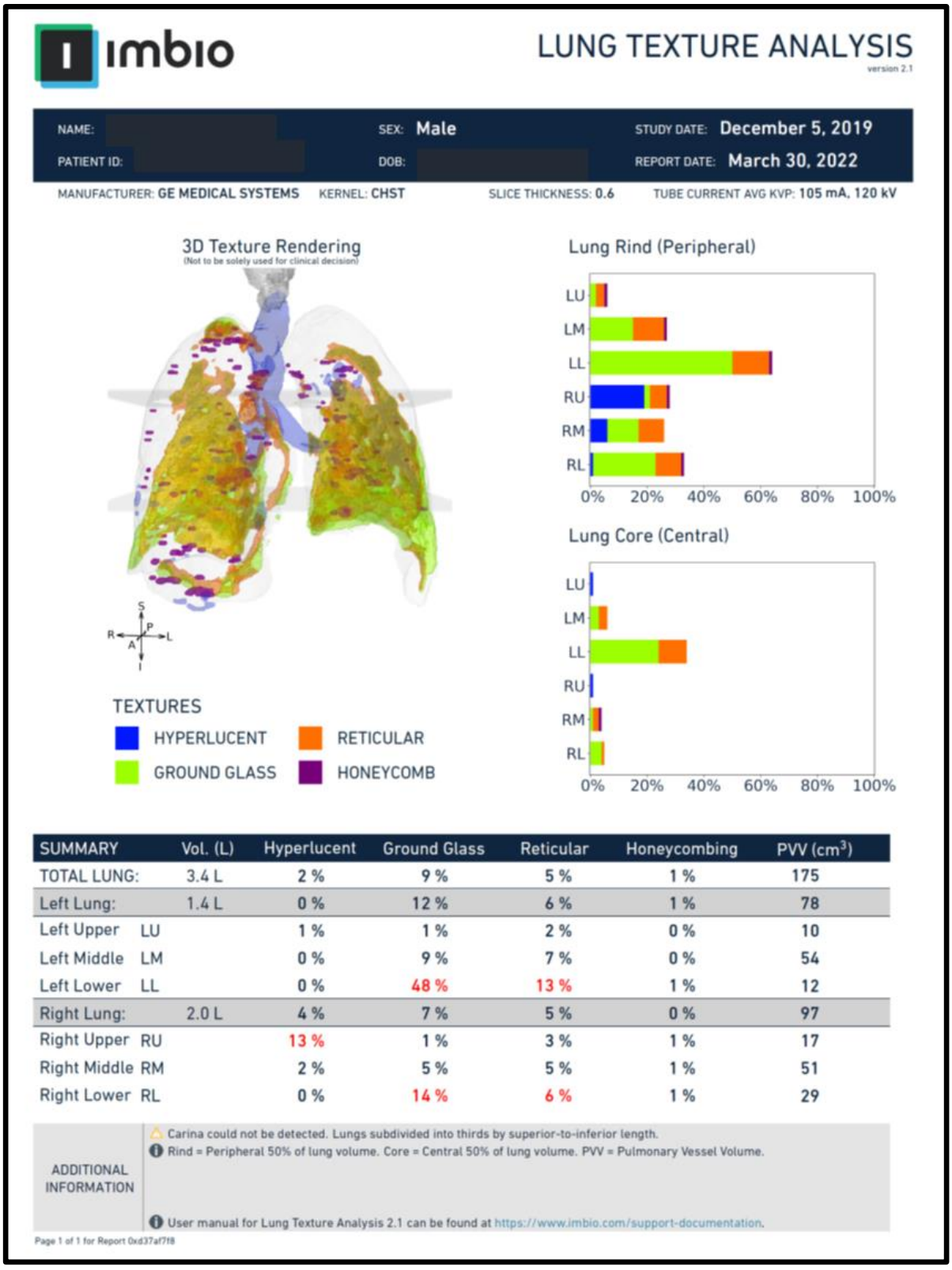


Figure A.6 Specimen LTA CALIPER summary report

Outcome >10%/Ltr increase in fibrosis	**HR (95% CI)**	**p value**
Leukocytes		
Monocytes (x10^3/μl)	3.08 (0.13-72.58)	0.485
Neutrophils (x10^3/μl)	2.23 (1.05-4.71)	0.036
Lymphocytes (x10^3/μl)	0.23 (0.03-1.57)	0.132
Leukocyte ratios		
MLR	76.15 (0.86-6770)	0.058
NLR	1.59 (1.03-2.45)	0.037
SIRI	2.08 (1.11-3.9)	0.022

Table A10 Multivariate analysis of progression of fibrosis adjusted for antifibrotic use

Multivariate Cox proportional hazard analysis for outcomes of progression of fibrosis >10%/Ltr. Adjusted for age, gender, baseline fibrosis score and antifibrotic use at first CT scan. For all multivariate models testing contribution of blood leukocytes against outcome these were all tested in combination [absolute monocyte, lymphocyte, and neutrophils] to explore interaction. For multivariate models exploring contribution of the leukocyte derived indexes [MLN, NLR or SIRI] these were tested individually and in absence of other leukocytes measurements or derived indexes.

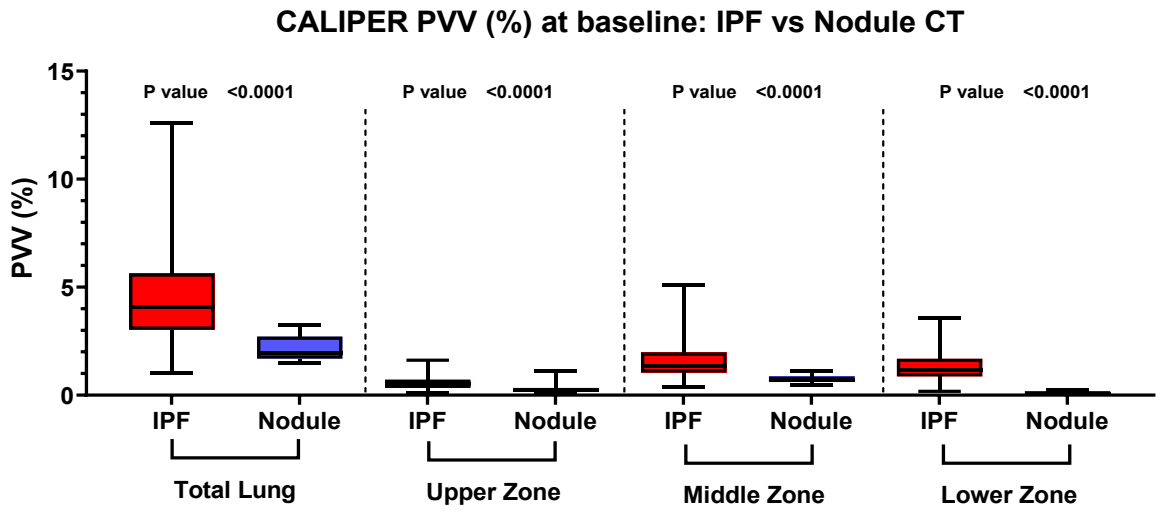


Figure A.7 PVV comparison in IPF and nodule surveillance cases

Total lung PVV and regional PVV compared in n=71 IPF cases and n=30 cases without ILD undergoing nodule surveillance CT thorax (non-contrast volumetric CT). Unpaired student T-test used to compare difference between IPF and nodule groups.

Appendix E Cohort Comparisons

	iUIP COHORT Study 1A	UIP COHORT Study 1B	ILA COHORT Study 2	CALIPER COHORT Study 3	p value
Baseline metrics					
Age (Years)	77.1 (71.3-81.9)	75.9 (70.4-80.9)	67.07 (60.9-71.5)	75.6 (70.0-81.1)	<0.001
Male (%)	36 (75)	100 (78.1)	704 (52.9)	156 (90.2)	<0.001
Time from blood to CT	1.43 (-4.29-18.26)	1.5 (0.15-5.63)	0.02 (-0.4-1.18)	-0.87 (-2.57-0.27)	<0.001
Leukocytes					
Monocyte	0.66 (0.59-0.82)	0.70 (0.57-0.84)	0.62 (0.49-0.79)	0.70 (0.58-0.84)	<0.001
Neutrophil	4.96 (3.64-5.82)	5.25 (3.99-6.66)	4.49 (3.37-6.03)	4.87 (3.85-6.53)	<0.001
Lymphocyte	1.72 (1.43-2.2)	1.76 (1.41-2.38)	1.69 (1.25-2.3)	1.65 (1.31-2.23)	0.383
Lung function					
FVC%	95 (76-116)	81 (68.1-98)	-	76.44 (66.6-91.1)	<0.001
TLCO%	68.0 (59.7-79.0)	57.7 (47.65-70.0)	-	57.0 (49.5-67.2)	<0.001
Clinical outcomes					
ΔFVC%/Yr	-1.6 (-5.9-0.52)	-3.97 (-11.3 to 0.47)	-	-3.02 (-8.2 to -0.18)	0.277
ΔTLCO%/Yr	-5.62 (-10.75--0.94)	-2.02 (-6.24-0.94)	-	3.32 (0.85-8.41)	<0.001
Progression (visual)	23 (71.9)	58 (67.4)	157 (44.4)	48 (64)	<0.001
Progression (>10% TLF)	-	-	-	13 (17.8)	--
CT Interval (months)	36.6 (24.43-49.73)	27 (16.63-38.97)	0.8 (0.31-1.84)	26.4 (16.77-39.98)	<0.001
Mortality	12 (25.0)	56 (43.8)	185 (13.9)	60 (35.9)	<0.001
Time to Mortality (years)	51.9 (41.4-76.6)	42.5 (34.0-54.8)	27.1 (13.9-46.0)	41.5 (17.3-52.0)	<0.001

Table A11 Inter-cohort comparison of characteristics and clinical outcomes
Difference between groups assessed using Kruskal-Wallis test or Chi^2.

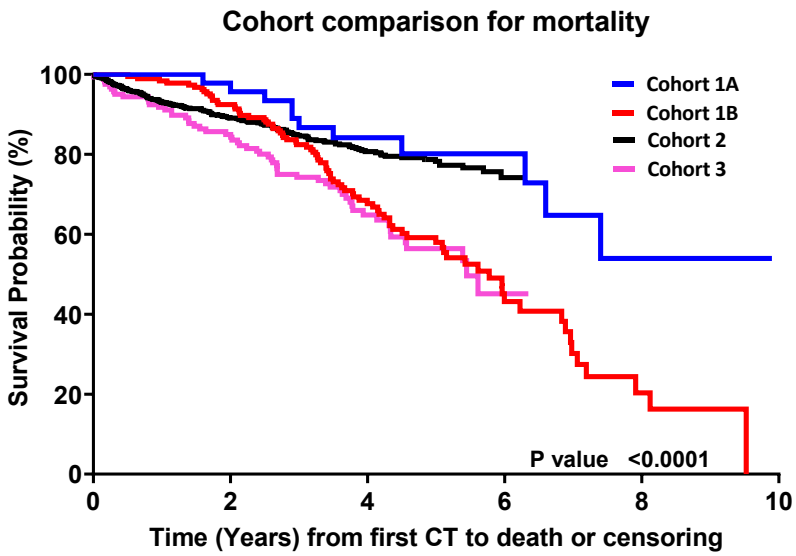


Figure A.8 Kaplan-Meier cohort comparison for mortality

Appendix F Thesis publications

Scientific papers

Open access

heterogeneous group, and many cases were subjected to surgical lung biopsies[3] for clarification of diagnosis. Data accumulated from these biopsies suggest that in a significant number of patients with possible UIP pattern and particularly those with traction bronchiectasis, there is a reasonably good association with a histology diagnosis of UIP.[4–7] As a result, the 2018 guidelines specify that where there is traction bronchiectasis, 'possible UIP CT patterns should be placed into the category of 'probable UIP'. This leaves those with no traction bronchiectasis as 'iUIP' with features described above. Little is known of how radiographic iUIP progresses, although a study showed that up to 30% of cases that were biopsied demonstrated a histology pattern of UIP.[6] This indicates that at least a proportion of patients with iUIP progresses to a clinical diagnosis of IPF. It is not clear which factors are associated with this.[8]

In this retrospective cohort study, we evaluated the radiological and clinical progression of patients with the iUIP CT pattern in one ILD centre, dividing cases into 'non-progressors' and 'progressors' (to 'probable' and 'definite' UIP pattern on CT by 2018 criteria or in extent of fibrosis on CT). We explored the association between blood neutrophil, monocytes and lymphocyte levels near to the point of first CT and patient demographics with progression. We found that 53% of evaluable cases progressed to a CT pattern of 'probable' or 'definite UIP (all with IPF clinical diagnosis) within 4 years (mean/SD of 3.9/1.5 years) of initial CT. Using Cox proportional hazard analysis, we found that neutrophil and monocytes (but not lymphocytes), measured within 3 months of initial CT, significantly correlated with progression of iUIP in extent, and to a diagnosis of IPF.

METHODS

Study design

We first, identified patients with iUIP CT patterns among our lung fibrosis cohort of patients who attended the Oxford Interstitial Lung Disease Service between 2013 and 2017. All patients in this lung fibrosis cohort had a CT pattern of 'possible UIP' or 'UIP' according to 2011. Radiologist reports for all available thoracic CTs for these patients (including first CTs before 2013) and up to August 2019 were reanalysed and cross-checked with reports from ILD multidisciplinary team (MDT) meetings, and recategorised according to the 2018 IPF guideline.[5] Patients with iUIP CT patterns were then grouped as either 'non-progressive' or 'progressive' based on comparison of their first CT (including those prior to attendance at Oxford) to the latest follow-on CT (up to cut-off point of August 2019). We defined 'non-progressive' as no change in CT scan in terms of extent of disease or change in pattern of disease; and 'progressive' if there were either visual (qualitative) increase in extent of disease or progression of CT pattern to 'definite' or 'probable UIP' pattern.

The following data were collected—patient demographics, dates of first CT and follow-on CTs, year of diagnosis of IPF (by ILD MDT meetings, and according to 2018 guidelines), all available pulmonary function tests, comorbidities, neutrophils, lymphocytes and monocyte levels performed using standard hospital 'full blood count' analysis and clinical outcomes including disease progression, survival and years of clinic follow-up. Indications for follow-on CT were also recorded.

Patient and public involvement

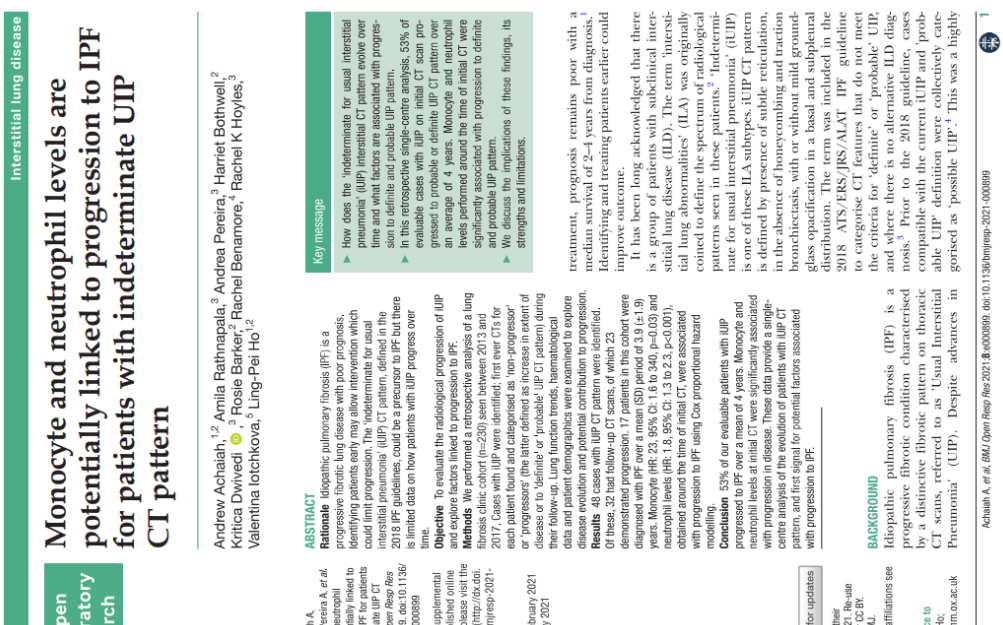
Patient and public were not involved in design, recruitment or conduct of this study.

CT scan and analysis

CT scans were acquired using a 64-detector row CT scanner (LightSpeed VCT XT; GE Medical Systems, Milwaukee, Wisconsin, USA). Images were reconstructed using a high spatial resolution algorithm. All CT abnormalities were defined and analysed using standard Fleischner-based terminology.[9]

Statistical analysis

Where data are expressed as means, SD are shown. Tests for normality of data were performed using a D'Agostino and Pearson test and following this the difference between groups was analysed using Student's t-tests or Mann-Whitney test for parametric and non-parametric analysis, respectively. Contingency tests (Fisher's exact test of significance) were used to assess categorical data. Survival analysis (log-rank test of significance) was performed to evaluate time-to-mortality. For analyses of correlates for progression to IPF (2018 criteria),[2] the Cox proportional hazard modelling to determine HRs was employed, testing their significance in two settings: (A) using data from patients regardless of whether they proceeded to defined events ('definite' or 'probable' UIP pattern on CT or progression in extent of disease) but up to August 2019 (n=32) and (B) restricting the patients to only those that did progress to 'definite' or 'probable' UIP pattern on CT during the period of analysis (n=17). HRs generated for continuous covariates represent the change in the risk of outcome if the covariate in question changes by one unit. HRs generated for dichotomised covariates represents the risk of achieving outcome if the covariate is present. All analyses were performed using Graphpad Prism (V.8) with the exception of Cox proportional hazard modelling which was performed with R Studio (V.3.6.2) statistical programming language packages Survival (V.3.1.8) and Survminer (V.0.4.6) by our statistical team (led by VI). We used the cox.zph function in the R package survival for testing the proportional hazards assumption of a Cox regression. Statistical significance was performed using the likelihood ratio test (preferred compared with Wald test due to our smaller number) as reported by the coxph function at the 95%



BMJ Open Respiratory Research

Interstitial lung disease

Monocyte and neutrophil levels are potentially linked to progression to IPF for patients with indeterminate UIP CT pattern

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ABSTRACT

Rationale Idiopathic pulmonary fibrosis (IPF) is a progressive fibrotic lung disease with poor prognosis. Identifying patients early may allow intervention which could limit progression. The 'indeterminate for usual interstitial pneumonia' (iUIP) CT pattern, defined in the 2018 IPF guidelines, could be a precursor to IPF but there is limited data on how patients with iUIP progress over time.

Objective To evaluate the radiological progression of iUIP and explore factors linked to progression to IPF.

Methods We performed a retrospective analysis of a lung fibrosis clinic cohort (n=230) seen between 2013 and 2017. Cases with iUIP were identified, first ever CTs for each patient found and categorised as 'non-progressor' or 'progressors' (the latter defined as increase in extent of disease or to 'definite' or 'probable' UIP CT pattern) during their follow-up. Lung function trends, haematological data and patient demographics were examined to explore disease evolution and potential contribution to progression.

Results 48 cases with iUIP CT pattern were identified. Of these, 32 had follow-up CT scans, of which 23 progressed to IPF over a mean of 4 years. Monocyte and neutrophil levels at initial CT were significantly associated with progression in disease. These data provide a single-centre analysis of the evolution of patients with iUIP CT pattern, and first signal for potential factors associated with progression to IPF.

Conclusion 53% of our evaluable patients with iUIP progressed to IPF over a mean of 4 years. Monocyte and neutrophil levels at initial CT were significantly associated with progression in disease. These data provide a single-centre analysis of the evolution of patients with iUIP CT pattern, and first signal for potential factors associated with progression to IPF.

Key message

- How does the 'indeterminate for usual interstitial pneumonia' (iUIP) interstitial CT pattern evolve over time and what factors are associated with progression to definite and probable UIP pattern.
- In this retrospective single-centre analysis, 53% of evaluable cases with iUIP on initial CT scan progressed to probable or definite UIP CT pattern over an average of 4 years. Monocyte and neutrophil levels performed around the time of initial CT were significantly associated with progression to definite and probable UIP pattern.
- We discuss the implications of these findings, its strengths and limitations.

BACKGROUND

Idiopathic pulmonary fibrosis (IPF) is a progressive fibrotic condition characterised by a distinctive fibrotic pattern on thoracic CT scans, referred to as 'Usual Interstitial Pneumonia' (UIP). Despite advances in treatment, prognosis remains poor with a median survival of 2–4 years from diagnosis.[1] Identifying and treating patients earlier could improve outcome.

It has been long acknowledged that there is a group of patients with subclinical interstitial lung disease (ILD). The term 'interstitial lung abnormalities' (ILA) was originally coined to define the spectrum of radiological patterns seen in these patients.[2] 'Indeterminate for usual interstitial pneumonia' (iUIP) is one of these ILA subtypes. iUIP CT pattern is defined by presence of subtle reticulation, in the absence of honeycombing and traction bronchiectasis, with or without mild ground-glass opacification in a basal and subpleural distribution. The term was included in the 2018 ATS/ERS/JRS/ALAT IPF guideline to categorise CT features that do not meet the criteria for 'definite' or 'probable' UIP, and where there is no alternative ILD diagnosis.[5] Prior to the 2018 guideline, cases compatible with the current iUIP and 'probable UIP' definition were collectively categorised as 'possible UIP'.[4] This was a highly

Table 1 Characteristics for patients with iUIP who had at least two CT scans (n=32), at the point of initial CT when iUIP was identified

	Non-progressive iUIP	Progressive iUIP	P value or OR	95% CI
n	9	23		
Male	6 (66%)	18 (78%)	1.8	0.37 to 8.34
Female	3 (33%)	5 (22%)	0.6	0.12 to 2.64
Age at first CT showing iUIP (±SD)	76.7 (±8.2)	72.3 (±8.6)	p=0.277	–
Never smoker	3 (33%)	3 (14%)	0.3	0.06 to 1.70
Ex-smoker	6 (67%)	19 (86%)	3.2	0.59 to 15.9
Respiratory comorbidity	1 (11%)	8 (40%)	5.3	0.61 to 65.6
Cardiac comorbidity	6 (66%)	17 (85%)	1.3	0.27 to 7.52
Diabetes mellitus	4 (44%)	3 (15%)	0.2	0.05 to 1.17
TL_{CO} (mmol/min/kPa)	5.0 (±0.9)	5.3 (±1.7)	p=0.983	–
$\%TL_{CO}$	77.8 (±18.1)	64.2 (±16.0)	p=0.077	–
FVC (l)	2.90 (±0.7)	3.24 (±1.1)	p=0.728	–
%FVC	102.00 (±21.6)	92.6 (±26.9)	p=0.285	–
FEV_1 (l)	2.33 (±0.7)	2.36 (±0.7)	p=0.853	–
$\%FEV_1$	98.4 (±18.8)	85.9 (±19.4)	p=0.362	–
CPI score	67.7 (±18.3)	69.5 (±10.4)	p=0.327	–

Data are divided into progressor and non-progressor groups. % in parenthesis is proportion of specified group.
Statistical analysis expressed at p value or OR with 95% CI. Lung function parameters refer to those measured within 3 months of first CT scan. CPI as calculated by Wells *et al*,[8]
CPI, Composite Physiological Index ; FEV_1, forced expiratory volume in one second; FVC, forced vital capacity; iUIP, indeterminate for UIP; TL_{CO} carbon monoxide transfer factor; UIP, usual interstitial pneumonia.

status did violate the proportional hazards assumption of a Cox regression (table 2). Output from smoking status in setting A and lymphocytes in setting B were therefore not used.

In both settings A and B, we found that increased neutrophils and monocytes (both binary and continuous variables) were associated with progression within the follow-up period— monocytes (continuous) β=3.10; HR (95% CI)=23 (1.6–340), p=0.03; neutrophils (continuous) β=0.57; HR (95% CI)=1.8 (1.3–2.3), p<0.001 (table 2; and figure 3A,B). Apart from blood leukocytes, no other variables showed significant difference. Histograms for the distribution of monocyte, neutrophil and

Figure 2 Lung function progression from baseline (within 3 months of initial CT scan) for non-progressors, those who progressed in amount of disease and to 'definite' and 'probable' UIP ('progressors (all)'), and those who progressed to definite and probable UIP only ('progressors (to probable/definite UIP)'). Mean (SD) values are displayed; no statistical analyses were performed. Survival curve for all patients divided into those with iUIP, definite and probable UIP on thoracic CT scan at their first CT scan in the study. FVC, forced vital capacity; iUIP, indeterminate for UIP; TL_{CO} carbon monoxide transfer factor; UIP, usual interstitial pneumonia.

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significance level. Reported p values were two-sided and a p value<0.05 was considered significant.

RESULTS

Progress of iUIP patients from point of initial CTs

Of the 230 individual patients who attended the lung fibrosis ILD clinic between 2013 and 2017 with CT patterns of 'probable', 'possible' or 'definite UIP', 48 (21%) cases with iUIP pattern were found. Thirty-two of these patients had at least one follow-on thoracic CT from the first scan. In the 16 cases that did not have a follow-on scan (and therefore could not be categorised into progressors or non-progressors), 13 cases were discharged after a mean of 2.1 years without a CT, as they were clinically stable and three were deemed not to require a second CT scan clinically.

Of the 32 patients who had follow-on scans, 9 (28%) patients showed no change in CT pattern or extent in disease over a mean (±SD) of 2.1±0.9 years and were classified as non-progressors. The most frequent indications for follow-on CT in this group was for nodule surveillance (64%) and to identify any further radiographic progression of iUIP (14%). Twenty-three (72%) demonstrated progression. Six showed increase in extent of iUIP but no change in pattern over 3.1±0.8 years; 11 progressed to 'probable UIP' over 3.8±1.6 years and 6 to 'definite' UIP over 4.1±2.4 years. For the 23 'progressors' the most frequent indication for follow-on CT was to investigate worsening symptomatic breathlessness (52%) and decline in lung function parameters (28%). All those who progressed to 'definite' and 'probable UIP' were diagnosed clinically, with IPF after discussion in the ILD MDT meetings; five of which underwent surgical lung biopsy to attain definitive diagnosis. Therefore 53% (17 of 32) of our evaluable iUIP cohort (ie, those who had follow-on CTs) or 35% (17 of 48) of all patients with iUIP (if those who did not have a follow-on CT were included) progressed to a clinical diagnosis of IPF over a mean period of 3.9±1.9 years. These findings are summarised in figure 1.

Twelve (25%) of 48 iUIP cases died during follow-up. The mean time from initial CT reporting iUIP to all-cause mortality was 4.6±2.9 years. Respiratory-related deaths were confined to the progressive iUIP group; these accounted for six of the nine deaths in this group. two to pneumonia and four to end-stage IPF. There was a trend to a greater number of hospitalisation events (39% vs 22% (OR: 2.25, 95% CI: 0.40 to 12.32)) and greater smoking history (86% vs 67% (OR: 3.2, 95% CI: 0.59 to 15.9)) in the progressive iUIP group (table 1). Forced vital capacity (FVC) and carbon monoxide transfer factor (TL_{CO}) values at initial CT were not different between the progressor and non-progressor groups. However, at 1 year from initial CT, mean change in FVC for the 'non-progressor' group was −0.03 (±0.26) litres versus −0.26 (±0.39) litres in the 'progressive' group; p=0.16 (figure 2A). Median survival for patients with an initial

Figure 1 Flow diagram of radiographic progression of UIP within the IPF cohort (n=230). Clinical diagnosis 'as per 2011 IPF guideline'[3] as per 2018 IPF guideline.[3] IPF, Idiopathic pulmonary fibrosis; iUIP, indeterminate for UIP; UIP, usual interstitial pneumonia.

CT of iUIP was also significantly better than patients with a first CT demonstrating either 'probable' or 'definite UIP' (figure 2B). Demographic data, physiological indices and comorbidity profiles in the progressor and non-progressor iUIP groups are shown in table 1.

Monocyte and neutrophils but not lymphocyte levels within 3 months of initial CT scan are associated with progression of iUIP to definite or probable UIP CT patterns

Having characterised these subgroups, we explored potential predictor variables for progression to IPF.

A univariate analysis of the following variables (taken at the point of diagnosis (initial CT scan)) age, gender, FVC, smoking status, monocyte count (continuous, median value or dichotomised at > and <0.9×10^9/L), lymphocyte count (continuous, median value < and >1.0×10^9/L) and neutrophil count (continuous, median value, < and >7.5×10^9/L) were undertaken using the Cox proportional hazard modelling method. Dichotomised values were selected from the upper limit of normal range for neutrophils and lymphocytes, and from Scott *et al* paper for monocytes,[10]

We determined the HRs for progression and tested their significance (using likelihood ratio test) in two settings using data from (A) all evaluable cases (n=32) and (B) only patients who progressed to IPF during the period of analysis (n=17). Apart from smoking status none of the variables violated the proportional hazards assumption of a Cox regression in setting A. In setting B lymphocyte count (continuous) did, while smoking

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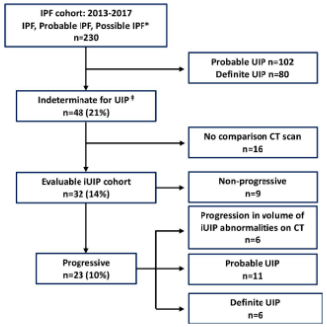

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Table 1 Characteristics for patients with iUIP who had at least two CT scans (n=32), at the point of initial CT when iUIP was identified

	Non-progressive iUIP	Progressive iUIP	P value or OR	95% CI
n	9	23		
Male	6 (66%)	18 (78%)	1.8	0.37 to 8.34
Female	3 (33%)	5 (22%)	0.6	0.12 to 2.64
Age at first CT showing iUIP (±SD)	76.7 (±6.2)	72.3 (±8.6)	p=0.277	–
Never smoker	3 (33%)	3 (14%)	0.3	0.06 to 1.70
Ex-smoker	6 (67%)	19 (86%)	3.2	0.59 to 15.9
Respiratory comorbidity	1 (11%)	8 (40%)	5.3	0.61 to 65.6
Cardiac comorbidity	6 (66%)	17 (85%)	1.3	0.27 to 7.52
Diabetes mellitus	4 (44%)	3 (15%)	0.2	0.05 to 1.17
TL_{CO} (mmol/min/kPa)	5.0 (±0.9)	5.3 (±1.7)	p=0.983	–
%TL_{CO}	77.8 (±18.1)	64.2 (±16.0)	p=0.077	–
FVC (l)	2.90 (±0.7)	3.24 (±1.1)	p=0.728	–
%FVC	102.00 (±21.6)	92.6 (±26.9)	p=0.285	–
FEV_1 (l)	2.33 (±0.7)	2.36 (±0.7)	p=0.853	–
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Statistical analysis expressed at p value or OR with 95% CI. Lung function parameters refer to those measured within 3 months of first CT scan. CPI as calculated by Wells *et al*.[16]
CPI, Composite Physiological Index ; FEV_1, forced expiratory volume in one second; FVC, forced vital capacity; iUIP, indeterminate for UIP; TL_{CO}, carbon monoxide transfer factor; UIP, usual interstitial pneumonia.

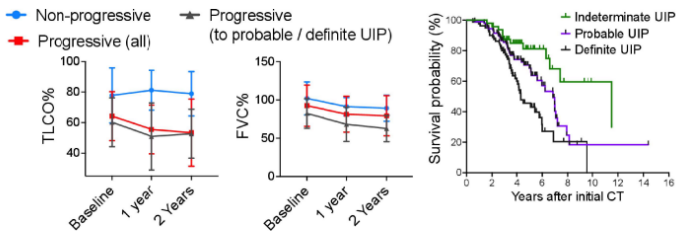

Figure 2 Lung function progression from baseline (within 3 months of initial CT scan) for non-progressors, those who progressed in amount of disease and to 'definite' and 'probable' UIP ('progressors (all)'), and those who progressed to definite and probable UIP only ('progressors (to probable/definite UIP)'), Mean (SD) values are displayed; no statistical analyses were performed. Survival curve for all patients divided into those with iUIP, definite and probable UIP on thoracic CT scan at their first CT scan in the study. FVC, forced vital capacity; iUIP, indeterminate for UIP; TL_{CO}, carbon monoxide transfer factor; UIP, usual interstitial pneumonia.

monocyte levels (HR: 2.9, 95% CI: 0.59 to 14.51, p=0.06 log-rank test, censoring event as death or August 2019 for survival group); and higher neutrophils (HR: 6.95, 95% CI: 0.72 to 66.7, p=0.0002 log-rank test) (figure 3C,D).

DISCUSSION

In this single-centre retrospective analysis of iUIP progression, we observed that among an unselected group of 230 patients followed up for lung fibrosis, the prevalence of those with iUIP CT pattern was 21%. A minimum of 35% (if we included those who did not have a second thoracic CT scan due to lack of need), progressed to IPF within 4 years, with 25% death in the follow-up period. These data suggest that iUIP CT pattern is an important entity, a precursor to IPF in some patients within a few years, and for these patients, there could only be a short period to intervene to prevent progression. Further analyses suggest that increased neutrophils and monocytes levels might identify this group of patients with higher risk of progression to IPF.

The prevalence of iUIP among patients seen in our IPF clinic is similar to the analysis from only one other study on prevalence. Diridollou *et al* recategorised 89 cases with 'possible UIP' CT pattern and found 17% of these were iUIP.[11] In a large birth cohort study (AGES-Reykjavik, N=5320), Putman *et al* observed a 2.52% prevalence of patients with iUIP, and demonstrated that patients with iUIP CT pattern had a greater risk of mortality compared with those without any interstitial lung abnormality (p<0.0001 (HR: 1.6, 95% CI: 1.3 to 2.0)).[12] Neither study examined how patients with iUIP progress in the ensuing years from initial diagnosis.

It is noteworthy from our analysis that there is no statistical difference between the starting FVC and TL_{CO} for the progressor and non-progressor groups of patients with iUIP; though there is a trend towards lower % predicted TL_{CO} (table 1). The calculated CPI score which adjusts for presence of emphysema is, not unexpectedly, also similar between the two groups. However, with univariate (but not multivariate) analysis (table 3), lower FVC at initial CT did (just) correlate with greater likelihood to progression for the individual patient, which is clinically cogent. These data suggest that factors other than severity of disease may be driving the progression of iUIP to IPF.

We chose to examine monocyte, neutrophils and lymphocyte levels, in part, because of the potential utility in clinic due to these being routine performed blood tests but primarily because of the possible link between monocytes and mechanism of disease, as shown in Scott *et al*[10] and our own work.[13] We used monocyte counts of $>0.9\times10^9$/L as Scott *et al* had identified this as the level of monocyte, above which was associated with higher mortality risk in patients with IPF. In Fraser *et al*,[13] we observed that monocytes in patients with IPF showed type 1 interferon primed phenotype which could account for more robust and potentially injurious response to the alveolar epithelium when triggered, during for example, a viral infection. This study provides impetus to investigate the possibility that neutrophils (which were not investigated in Scott or Fraser studies) could also be involved.

There are several limitations to our study. The monocyte, neutrophil and lymphocyte levels were measured at one point (nearest to the CT scan). This is in keeping with work from other much larger studies[10] but there is a risk that the values are not representative of the steady-state values, particularly in a necessarily small cohort as ours. In further studies, it will be useful to have repeated samples over 6–12 months to determine if the neutrophil, lymphocyte and monocyte values are representative for the patient, and reduce bias towards the possibility of levels linked to an infective episode, for example.

The most obvious limitation is the small number of patients. This probably reflects the relatively small proportion of iUIP scans that are referred for clinical assessment without delay. These patients are often asymptomatic in the early stages of their ILD.[14] However, it is becoming increasingly recognised that a proportion of ILAs will progress to clinically significant ILD.[14] Furthermore, identification of ILAs is predicted to increase with implementation of lung cancer screening and increased use of CT for other diagnostic purposes.[15] This may increase reporting of iUIP in the future, making the need for biomarkers that can risk-stratify for progression even more pertinent.

In our retrospective study, not all follow-on CT scans included in analysis were undertaken in a uniform timeframe across the cohort. With exception of the lung nodule surveillance imaging most serial CTs were performed according to clinical indication and where there was concern for objective deterioration. A greater proportion of repeat CT scans were performed to investigate symptomatic change in the 'progressor' group, and this may have introduced bias towards detection of progression. We also acknowledge that length of follow-up is shorter in the non-progressor group, although not significant (p=0.06). However, it has been well documented that initial progression particularly during first 6–12 months is predictive of progression of fibrotic ILDs.[16 17] Therefore a follow-up for 2 years or more, as seen in the majority of our cohort, may be sufficient to identify patients with a progressive phenotype.

The small sample size limits the power of this study and has contributed to some of the large HRs and CIs observed. Therefore, our findings are primarily indicative rather than definitive signals. The values of the HR cannot be interpreted as an absolute numerical risk but rather an indication that the risk exist and that it is statistically significant since the lower limit of the 95% CI is above 1. Consistent findings in proportional hazards modelling by both univariate and multivariate analyses also lend support to the contribution of monocytes and neutrophil levels to progression to IPF. As the numbers of patients with iUIP CT pattern disease are small, a multisite cohort will be required to confirm these findings. Nevertheless, our findings suggest that at least some patients with iUIP CT pattern could be patients with early IPF and their disease progression could be linked to higher levels of monocytes and neutrophils. Further studies could validate the use of blood monocytes and neutrophils as biomarkers for patients with iUIP and were at higher risks of progression to IPF and all-cause mortality.

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Contributors AA conducted analysis, interpreted data and wrote the paper. AR, AP, HB, KD and RosieB collected data. RachelB and RKH discussed clinical diagnosis of patients. VI led statistical analysis and interpretation of data. L-PH conceived project, interpreted data, wrote paper and supervised the study. L-PH acts as the guarantor for this study

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Competing interests None declared.

Patient consent for publication Not applicable.

Ethics approval The study was part of a study to examine the factors associated with disease progression in IPF (ethical approval 14/SC/1060 from the Health Research Authority and South-Central National Research Ethics Service).

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement All data relevant to the study are included in the article or uploaded as supplementary information.

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BMJ Open Respiratory Research

Neutrophil lymphocyte ratio as an indicator for disease progression in Idiopathic Pulmonary Fibrosis

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ABSTRACT

Rationale Idiopathic pulmonary fibrosis (IPF) is a progressive fibrotic lung disease. Patients present at different stages and disease course is varied. Blood monocytes have been linked to all-cause mortality, and neutrophils to progression to IPF in patients with the indeterminate for usual interstitial pneumonia CT pattern.

Objective To determine association between blood monocytes, neutrophils and lymphocytes levels (and their derived indexes), with lung function decline and mortality in IPF.

Methods We performed a retrospective analysis of an IPF cohort (n=128) who had their first clinical visit at the Oxford Interstitial Lung Disease Service between 2013 and 2017. Association between blood monocytes, neutrophils, lymphocytes and derived indexes (within 4 months of visit) and decline in forced vital capacity (FVC) and all-cause mortality were assessed using Cox proportional hazard regression analysis. Kaplan-Meier analysis was used to assess time-to-event for 10% FVC decline and mortality for patients dichotomised to high and low leucocyte counts.

Results Median length of follow-up was 31.0 months (IQR 16.2–42.4); 41.4% demonstrated FVC decline >10% per year and 43.8% died. In multivariate models (incorporating age, gender and initial FVC%), raised neutrophils, lymphopaenia and neutrophil:lymphocyte ratio were associated with FVC decline (p≤0,01); while both monocytes and neutrophil levels (and their derived indexes) were associated with all-cause mortality (p≤0,01). Kaplan-Meier analysis also showed association between neutrophils and its derived indexes but not monocyte, with FVC decline.

Conclusion Blood neutrophil and lymphopaenia are more sensitive than monocytes as prognostic indicators of disease progression in those with established IPF.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Idiopathic pulmonary fibrosis (IPF) is a progressive fibrotic condition. Recently, several human studies have identified association between blood monocyte level and mortality. However, association between other leucocytes components of the 'Full blood count' with adverse outcomes in IPF has not been fully explored.

WHAT THIS STUDY ADDS

⇒ In this study, we compared the association between blood monocytes, neutrophils and lymphocytes, measured near the presentation of disease against the specific outcome of forced vital capacity. Levels of blood neutrophil and lymphocytes but not monocytes were associated with more rapid lung function decline. Blood monocyte levels were associated with all-cause mortality.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE AND/OR POLICY

⇒ These simple and easy to measure components of the full blood count have potential utility as biomarkers for lung function decline in IPF. This should be taken forward by prospective and validation studies.

BACKGROUND

Idiopathic pulmonary fibrosis (IPF) is a distinctive and progressive fibrotic condition characterised by usual interstitial pneumonia (UIP) pattern on thoracic CT. Despite advances in treatment, prognosis remains poor with a median survival of 2–4 years from diagnosis.[1]

The clinical course of IPF remains difficult to predict. Affected individuals display differing patterns of progression.[2] This has created an urgent need for reliable, readily accessible and cost-effective biomarkers to identify individuals at greater risk of progressive disease. Most of the research efforts to date on prognostication in IPF have focused on physiological parameters,[3–6] but there is a need to increase the repertoire of biomarkers to provide a more personalised management strategy for individual patients, better selection of patients for clinical trials and potentially widen clinical trial end points.[7]

Recently, several human studies in immunological drivers of IPF and blood-based leucocyte levels have identified monocytes and neutrophils as potential candidates for

biomarkers of progressive disease. Fraser *et al* showed that monocyte levels were associated with the amount of fibrosis on CT scan,[8] and large cohort studies from Scott linked blood monocyte levels to all-cause mortality,[4] while Kreuter found an association with IPF progression (defined as ≥10% absolute decline in forced vital capacity (FVC%) predicted, ≥50 m decline in 6-min-walk distanc or death) within a year.[9] In a smaller study, we found that neutrophil levels were highly significantly associated with progression of indeterminate UIP to diagnosis of IPF.[10] These findings are supported by increasing understanding of the immune pathogenesis of IPF where innate immune cells like monocytes, monocyte-derived macrophages and neutrophils are likely to have a role in promoting fibroblast activity.[11]

In this study, we compared the association between blood monocytes, neutrophils and lymphocytes, measured near the presentation of disease against the specific outcome of FVC over a longer period of time (median 31 months) and mortality in patients with IPF. We also investigated derived indexes like neutrophil to lymphocyte ratio (NLR), monocyte to lymphocyte ratio (MLR) and Systemic Inflammation Response Index (SIRI; monocyte × neutrophils/lymphocytes) as they have been shown to be more predictive of outcome in some chronic inflammatory conditions in comparison to individual cell levels.[12] Using multivariate Cox proportional hazard regression analysis, we demonstrated that in a model which incorporated age, gender, starting lung function and different individual leucocyte levels, blood neutrophil and lymphocyte but not monocytes levels were associated with lung function decline, while both neutrophils and monocytes were associated with all-cause mortality. We discuss the utility of blood leucocytes measured at the time of initial assessment as a prognostic indicator in IPF, and differences between our findings and recently published studies on monocyte levels and outcome in IPF.

METHODS

Study design

We performed a retrospective analysis of a cohort of patients with IPF, probable IPF and possible IPF (defined according to the 2011, or 2018 IPF diagnostic guidelines),[13 14] who presented to the Oxford Interstitial Lung Disease (ILD) Service between 2013 and 2017. Radiologist reports from all available thoracic CTs (including first CTs before 2013) up to August 2019 were reanalysed and cross-checked against reports from ILD multidisciplinary team meetings (MDT). Those characterised according to the 2011 guidelines were recategorised according to the 2018 IPF guideline and CT scans previously labelled as 'possible UIP', were recategorised as 'probable UIP' or 'indeterminate for UIP'.[14] Patients with a baseline CT scan compatible with 'probable UIP' and 'definite UIP' were included in the study, while those with indeterminate for UIP pattern were excluded (figure 1).

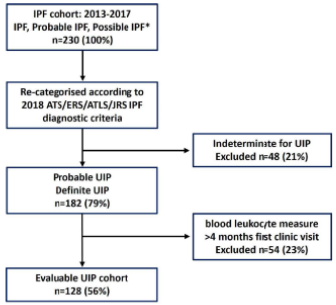

Figure 1 Flow diagram of radiographic progression of UIP within the IPF cohort (n=230). *Clinical diagnosis as per 2011 IPF guideline[13] and as per 2018 IPF guideline.[14] ATLS, Latin American Thoracic Society; ATS, American Thoracic Society; ERS, European Respiratory Society; IPF, Idiopathic pulmonary fibrosis; JRS, Japanese Respiratory Society; UIP, usual interstitial pneumonia.

The following data were collected—patient demographics, date of first and follow-on CTs, date of diagnosis of IPF (MDT), all available pulmonary function tests and additional comorbidities. Neutrophils, lymphocytes and monocyte levels performed using standard 'full blood count' analysis measured within 4 months of presentation to our clinics were included. NLR, MLR and SIRI, the product of neutrophil and monocyte values divided by lymphocyte values,[15] were also calculated.

To characterise our cohort, we compared the initial thoracic CT with the latest in the 5-year follow-up period, in a subset of patient who had follow-on CTs after the initial CT (n=82).

A measured reduction in absolute FVC (litres)>10% per year was considered clinically significant and representative of physiological decline.

CT scan

CT scans were acquired using a 64-detector row CT scanner (LightSpeed VCT XT; GE Medical Systems, Milwaukee, Wisconsin, USA). Images were reconstructed using a high spatial resolution algorithm. A volumetric scan was performed with 0.625 mm slice thickness at an interval of 0.625 mm. All CT abnormalities were defined using standard Fleischner-based terminology.[16] Definitions of CT patterns were according to 2018 IPF guidelines. Briefly, 'definite UIP' pattern was defined by the presence of a basal-predominant subpleural reticular abnormality and honeycombing with or without traction bronchiectasis. Probable UIP pattern was defined as basal

and subpleural predominant reticulation and traction bronchiectasis or bronchiolectasis with or without mild ground glass opacification (GGO). 'Indeterminate for UIP' was defined as subtle reticulation in the presence or absence of mild GGO and with a basal/subpleural predominant distribution.

Patient and public involvement

Patient and public were not involved in design, recruitment, conduct of this study.

Statistical analysis

Data are expressed as absolute values, relative percentages, means (with SD), medians (IQR) or by dichotomised value. Normality tests were performed using a D'Agostino & Pearson test. Difference between groups was analysed using Student's t-tests or Mann-Whitney U test for parametric and non-parametric analysis, respectively. Contingency tests (Fisher's exact test of significance) were used to assess categorical data. Kaplan-Meier analysis (log rank test of significance) was performed to evaluate time-to-event from first clinic assessment for hospitalisation and survival.

We employed a multivariate Cox proportional hazard regression analysis incorporating age, gender, starting lung function and different individual leucocyte levels in the model to test association between blood monocyte, neutrophil and lymphocyte and the clinical outcomes of FVC decline >10% per year or mortality. Neutrophil, monocyte, and lymphocyte counts were analysed in continuous, or dichotomised values either as above or below upper limit of normal range (monocyte <or >0.90x10^9/L, neutrophil; <or >7.5x10^9/L) or lower (lymphocyte; <or > 1.0x10^9/L) or above or below median values for NLR, MLR and SIRI. Time-dependent effects were included in the model, and proportional hazards assumption was tested for each. Hazard ratios generated for continuous covariates represent the change in the risk of outcome if the covariate in question changes by one unit. HRs generated for dichotomised covariates represents the risk of achieving outcome if the covariate is present. Statistical significance was performed using the likelihood ratio test as reported by the coxph function at the 95% significance level. All analyses were performed using Graphpad Prism (V.9) apart from Cox proportional hazard modelling which was performed with R Studio (V.3.6.2) statistical programming language packages Survival (V.3.1.8) and Survminer (V.0.4.6). Reported p values were two sided and a p<0.05 was considered significant.

RESULTS

Cohort characteristics and clinical outcomes

A total of 230 individual patients with a clinical diagnosis of IPF (according to 2011 criteria) were identified. In 48 cases (20.9%), thoracic CT at time of initial ILD assessment was consistent with 'Indeterminate for UIP' pattern and excluded. Of the remaining 182 patients, 54 (29.6%) patients did not have an available blood leucocyte measurement within 4 months of initial ILD assessment and were excluded (figure 1). The final cohort consisted of 128 patients; 55% had 'probable UIP' on CT scanning and 45% had definite UIP.

Baseline demographic data, physiological indices, blood leucocyte levels, comorbidity profiles and imaging features for the final cohort are shown in table 1. Mean age was 75.2±7.8 years, 78% were male. Median length of follow-up was 31.0 months (16.2–42.4). Median time between blood leucocyte collection and first ILD clinic assessment was 0 days (IQR 0–17 days); all blood samples were taken before or after initial visit to clinic, and all within 4 months. Seventy patients (55%) had blood taken within 7 days of assessment.

During follow-up 56 deaths (44%) were recorded; median time from first ILD assessment to death was 25.8 months (18.4–39.8) compared with 45.1 months (37.8–56.0) in those alive at time of censoring (01/08/2019). Most frequent cause of death was end-stage IPF (11%); followed by pneumonia (6.3%) and acute exacerbation of IPF (1.6%). Cause of mortality was not identified in 25 cases (19.5%).

All 128 patients had CT scans; 86 had a follow-on CT within 5 years of the first CT scan. 55.5% of the initial CT scans demonstrated probable UIP and 45%, definite UIP. Radiographic progression was observed in 58 (67.4%) cases over a median time interval of 30.8 months (21.5–44.1)—13 patients demonstrated progression in the extent of probable UIP, 20 progressed from probable UIP to definite UIP and 25 progressed in extent of definite UIP (figure 2).

Lung function decline of FVC >10% of starting FVC, per year, was identified in 46% of the cohort. Mean annualised change across all patients with available readings was −8.25% (±17.7).

Association of neutrophils, monocytes and lymphocytes with FVC decline

Of the leucocyte variables studied using multivariate analysis, neutrophil count >7.5×10^9/L (3.12, 1.44–6.74, p=0.004) was most associated with FVC decline over a median of 31 months (table 2). In addition, continuous neutrophil level, lymphopenia, NLR, age and being female, but not monocytes were also associated with FVC decline of greater than 10% in a year (table 2). NLR was most statistically significantly associated with FVC decline (HR 1.3; 1.16–1.48; p=0.00002).

These data support neutrophil and lymphopaenia as a correlate for progression of fibrosis in IPF.

Association of neutrophils, monocytes and lymphocytes with all-cause mortality

Both neutrophil (HR 1.2, 95% CI 1.10 to 1.40, p<0.001) and monocyte levels (1.4, 95% CI 1.10 to 1.80, p=0.013) were significantly associated with all-cause mortality

Table 1 Characteristics for patients, at the point of initial CT when IPF was first diagnosed; all patients divided on presence of radiographic progression on follow on CT

		Patients categorised by FVC decline			
	All patients (n=128)	FVC decline <10%/year (n=62)	FVC decline ≥10%/year (n=53)	P value	OR
Demographics					
Female (%)	28 (21.9)	13 (21.0)	14 (26.4)	0.516	1.35
Male (%)	100 (78.1)	49 (79.0)	39 (73.6)		0.74
Age at first clinic visit (SD)	75.22 (7.88)	74.8 (6.9)	74.4 (8.6)		
Smoking status					
Ex-smoker	77 (60.2)	43 (69.4)	26 (49.1)	0.113	0.45
Never	30 (23.4)	12 (19.4)	16 (30.2)		
No data	21 (16.4)	7 (11.2)	11 (20.7)	--	--
GAP index					
1	66 (57.4)	39 (67.2)	26 (54.2)	0.229	0.58
2	39 (33.9)	15 (25.9)	19 (39.6)	0.148	1.87
3	10 (8.7)	4 (6.9)	3 (6.3)	>0.999	0.90
Comorbidity					
Type II diabetes mellitus	23 (18)	12 (19.4)	8 (15.1)	0.626	0.74
Gastro-oesophageal reflux	16 (12.5)	7 (11.3)	8 (15.1)	0.588	1.40
Ischaemic heart disease	20 (15.6)	9 (14.5)	10 (18.9)	0.618	1.37
Atrial fibrillation	9 (7)	2 (3.2)	6 (11.3)	0.141	3.83
COPD	14 (10.9)	5 (8.1)	8 (15.1)	0.255	2.03
Blood leucocytes					
Monocyte (×10^9/L)	0.70 (0.56–0.84)	0.67 (0.56–0.80)	0.70 (0.53–0.81)	0.925	
Lymphocyte (×10^9/L)	1.76 (1.41–2.40)	1.81 (1.56–2.50)	1.65 (1.40–2.32)	0.206	
Neutrophil (×10^9/L)	5.25 (3.96–6.68)	5.23 (3.90–6.31)	5.23 (4.30–7.06)	0.190	
Leucocyte-derived indexes					
NLR	2.77 (1.96–3.85)	2.46 (1.87–3.55)	3.17 (2.09–4.21)	0.049	
MLR	0.37 (0.31–0.50)	0.35 (0.30–0.41)	0.42 (0.30–0.53)	0.151	
SIRI	2.00 (1.22–2.83)	1.71 (1.10–2.49)	2.18 (1.32–2.99)	0.089	
Pulmonary function tests					
%TLCO	57.7 (47.5–69.0)	61.9 (50.9–71.0)	55.5 (47.3–68.4)	0.233	
%FVC	81.0 (68.1–98.3)	85.5 (69.9–98.0)	79.4 (70.1–101.2)	0.667	
CPI Score[37]	68.8 (61.3–76.1)	65.6 (60.9–75.7)	69.19 (62.1–74.5)	0.570	
CT pattern					
Probable UIP	71 (55.5)	38 (61.3)	25 (47.2)	0.138	0.56
Definite UIP	57 (44.5)	24 (38.7)	28 (52.8)	0.138	1.77
Antifibrotic use	56 (43.7%)	25 (40.3%)	31 (58.5%)	0.063	2.08
Corticosteroids use	11 (8.6%)	4 (6.6%)	7 (13.2%)	0.341	2.21
At baseline visit	1 (0.8%)	0 (0%)	1 (1.8%)	--	--
During follow-up	10 (7.8%)	4 (3.2%)	6 (11.3%)	0.510	1.85

Continuous variables expressed as median values (IQR) with exception of age (mean, SD).
COPD, Chronic obstructive pulmonary disease; CPI, Composite Physiological Index as calculated by Wells et al; FVC, forced vital capacity; GAP, Gender-Age-Physiology Index; IPF, idiopathic pulmonary fibrosis; MLR, monocyte:lymphocyte ratio; NLR, neutrophil:lymphocyte ratio; SIRI, systemic inflammation response index; TLCO, Transfer factor for carbon monoxide; UIP, usual interstitial pneumonia.

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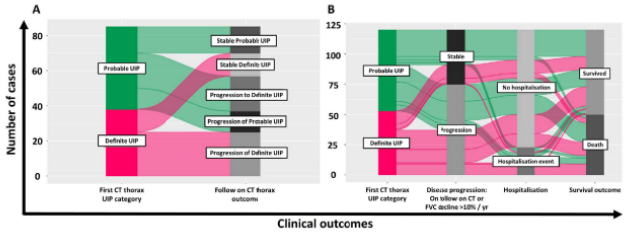

Figure 2 Alluvial plots demonstrating the proportion of patients per clincal outcome measure. (A) Illustrates the proportions of cases demonstrating stable and progressive radiological appearance in patients that underwent follow-on CT scan (n=86). (B) Illustrates the proportion of cases with disease progression (either on follow-on CT and/or by demonstrating FVC >10% decline/year), hospitalisation events and survival. FVC, forced vital capacity; UIP, usual interstitial pneumonia.

(table 2). In addition, male gender, increasing age, FVC, MLR, NLR and SIRI were also associated with mortality within 5 years of measurement.

Greater rate of FVC decline in patients with higher neutrophil levels

Kaplan-Meier analysis also supported the findings from our multi-variate analysis. Neutrophilia, lymphopenia and NLR and SIRI; but not monocytosis or MLR were associated with more rapid lung function decline (figure 3). All leucocyte levels were dichotomised by greater or lower than the upper limit of normal. Using this dichotomy, we did not observe a significant correlate in monocytosis with mortality within 5 years (figure 4). However, neutrophilia and lymphopaenia, and the derived leucocyte indexes correlated with worse survival (figure 4).

DISCUSSION

In this single-centre retrospective study, a strong association was observed between neutrophil levels lymphopaenia and NLR, but not monocyte levels with FVC decline. Instead, monocytes (and neutrophils) were associated with mortality. This finding extends that from Scott and Kreuter,[4 9] who found that monocyte levels were associated with mortality and a composite outcome measure respectively in IPF patients. There were key differences between our study and these previous publications. First, we examined all the three main parameters of the blood leucocyte profile—neutrophils, lymphocytes and monocytes while Scott and Kreuter focused on monocyte levels only. We did this in part, because of our previous findings of correlation between neutrophils and progression of iUIP to definite/probable UIP on CT scans,[10] but also because there is ample evidence that neutrophils are involved in the immune-pathogenesis of IPF (described further below).[11] Although Scott also examined the transcriptome of blood immune cells, these were on RNA from stored peripheral blood mononuclear cells, where the neutrophil component would not have survived storage.

The second major difference were the outcome measures. In our study, we specifically separated disease progression from all-cause mortality. We defined disease progression by relative decline in absolute FVC and/or progression in CT abnormalities to provide a more IPF-specific measure of deterioration in disease state. This complemented all-cause mortality, which in an elderly IPF cohort is likely to have other contributory factors to mortality. By contrast, in the larger studies Scott *et al*[4] only report all-cause mortality and Kreuter *et al*[9] define disease progression as a composite outcome of mortality, lung function decline and decline in 6 min walk test. Our study also had a longer follow-up period (median of 31 months for the cohort with a 5-year recruiting period, as opposed to 1 year for the other studies). As such we were able to calculate annualised FVC decline over a larger time period and identify FVC decline >10%/yr more accurately. Scott's cases were also identified by International Classification of Disease-10 coding of clinical records which may pose limitation to the findings. These differences may account for the differences in findings between these studies.

Thus, while there is corroboration for the association between peripheral monocyte count and all-cause mortality observed by Scott, and with the composite outcome in Kreuter *et al*[9] that included mortality, our study suggests that when divided into outcomes that relate more specifically to disease progression (FVC decline), neutrophil and lymphopaenia appear more sensitive compared with monocytes as a correlate.

Mechanistically, our findings could be explained by experimental evidence implicating neutrophils, monocytes and lymphocytes in immunopathogenesis of IPF. Strong evidence supports monocyte and monocyte-derived macrophages in the aetiopathogenesis lung

Table 2 Multivariate models testing blood leucocytes against outcome

	Multivariate analysis			
Outcome	**HR**	**95% CI**	**P value**	**P value (PH assumption)**
FVC decline >10%/year				
Absolute leucocytes				
Gender (male)	0.48	0.24 to 0.97	0.040*	0.470
Age	1.1	1.0 to 1.15	0.035*	0.110
FVC (%)	0.99	0.97 to 1.00	0.071	0.630
Monocytes (×10^9/L)	0.61	0.11 to 3.74	0.500	0.249
Lymphocytes (×10^9/L)	0.81	0.52 to 1.3	0.360	0.850
Neutrophils (×10^9/L)	1.4	1.1 to 1.7	0.0011*	0.760
Monocytosis (>0.90×10^9/L)	1.83	0.88 to 3.82	0.105	0.953
Lymphopenia (<1.0×10^9/L)	3.78	1.31 to 10.97	0.014*	0.669
Neutrophilia (>7.5×10^9/L)	3.12	1.44 to 6.74	0.004*	0.870
Leucocyte indexes	**Adjusted HR**			
MLR	1.07	0.70 to 1.64	0.763	0.757
MLR >0.37	1.91	1.07 to 3.40	0.029*	0.434
NLR	1.31	1.16 to 1.48	0.00002*	0.679
NLR >2.77	2.04	1.12 to 3.71	0.020*	0.803
SIRI	1.03	0.97 to 1.08	0.326	0.578
SIRI>2.0	1.95	1.10 to 3.47	0.023*	0.549
Mortality				
Absolute leucocytes				
Gender (male)	0.83	0.38 to 1.80	0.630	0.970
Age	1.1	1.00 to 1.10	0.005*	0.930
Baseline FVC (%)	0.97	0.96 to 0.99	0.001*	0.990
Monocytes (×10^9/L)	1.4	1.10 to 1.80	0.013*	0.340
Lymphocytes (×10^9/L)	1.0	0.70 to 1.50	0.890	0.940
Neutrophils (×10^9/L)	1.2	1.10 to 1.40	0.0008*	0.940
Monocytosis (>0.90×10^9/L)	1.01	0.50 to 2.01	0.990	0.291
Lymphopenia (<1.0×10^9/l)	1.50	0.52 to 4.38	0.451	0.279
Neutrophilia (>7.5×10^9/L)	2.31	1.18 to 4.54	0.015*	0.544
Leucocyte indexes	**Adjusted HR**			
MLR	1.32	1.09 to 1.60	0.005*	0.136
MLR >0.37	2.05	1.26 to 3.74	0.019*	0.583
NLR	1.22	1.11 to 1.34	0.00006*	0.691
NLR >2.77	1.81	1.01 to 3.23	0.046*	0.767
SIRI	1.06	1.02 to 1.09	0.001*	0.130
SIRI>2.0	1.83	1.02 to 3.26	0.041*	0.231

Absolute leucocyte counts were tested in combination (absolute monocyte, lymphocyte and neutrophils) to explore interaction and adjusted for the covariates gender, age and baseline FVC%. For multivariate models exploring contribution of the leucocyte derived indexes (MLR, NLR or SIRI); each was tested individually with the covariates gender, age and baseline FVC% but HR outcome for gender, age and baseline FVC% are not shown. All adjusted covariates in each model satisfied the proportional hazard assumption (p<0.05 are considered significant).
FVC, forced vital capacity; MLR, monocyte/lymphocyte ratio; NLR, neutrophil/lymphocyte ratio; SIRI, Systemic Inflammatory Response Index.

fibrosis from animal and human studies.[17 18] The contribution of neutrophils and lymphocytes to the pathogenesis of IPF is becoming increasingly understood. Neutrophils secrete proteases, cytokines and chemokines which can propagate inflammatory responses.[19] Neutrophilic bronchoalveolar lavage (BAL) specimens taken

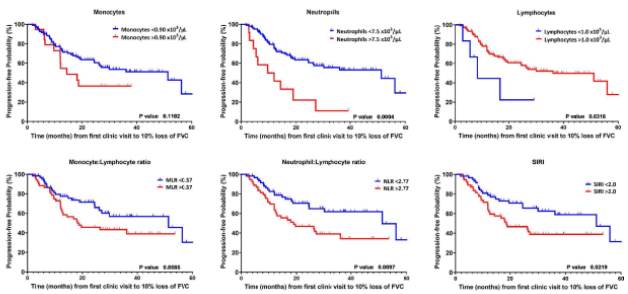

Figure 3 Kaplan-Meier curves for FVC decline dichotomised by normal and abnormal upper limit for leucocytes and by median values for the derived indexes of monocyte:lymphocyte ratio (MLR) (>0.37), neutrophil:lymphocyte ratio (NLR) (>2.77) and Systemic Inflammatory Response Index (SIRI) (>2.00). P value calculated with log rank test. P value calculated with log rank test. FVC, forced vital capacity.

from IPF patients are associated with earlier mortality.[20] Neutrophil elastases (NE) are elevated in IPF BAL samples,[21] and experimental data using murine models implicate NE in activation of the transforming growth factor-ß (TGF-ß) pathway and fibroblast proliferation.[22] Indeed, lymphocytic aggregates are a recognised pathologic feature of IPF lesions.[18] BAL specimens from IPF patients are also enriched for several T lymphocytes populations,[11] and Th17 lymphocytes isolated from peripheral blood of patients with IPF have been identified as a source of TGF-β.[23]

The differences in statistical significance observed here between neutrophils, lymphocytes and monocytes in our multivariate analysis is probably a question of sensitivity. The HRs generated by our modelling should be interpreted as association between these immune cells and progression, which is statistically significant. Therefore, as a single point biomarker for progression of fibrosis, in established IPF (as opposed to very early disease), neutrophils and lymphocytes may be more sensitive than monocytes. Even more sensitive is NLR, which may well prove to be the biomarker for disease progression. NLR is a recognised surrogate marker of immune response, and a recognised correlate of disease severity, hospitalisation and mortality.[24 25] A retrospective cross-sectional study demonstrated NLR, MLR and SIRI measurements to

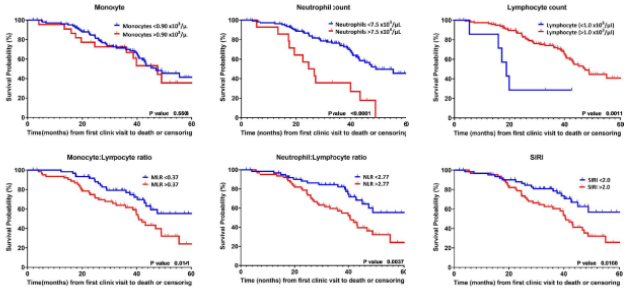

Figure 4 Kaplan-Meier curves for survival dichotomised by normal and abnormal upper limit for leucocytes and by median values for the derived indexes of monocyte:lymphocyte ratio (MLR) (>0.37), neutrophil:lymphocyte ratio (NLR) (>2.77) and Systemic Inflammatory Response Index (SIRI) (>2.00). P value calculated with log rank test.

be greater in patients with IPF versus healthy controls,[26] providing further support for our findings in progression of this disease.

Several limitations should be considered when interpreting the results of this analysis. The most obvious is the relatively small number of patients included, although compensated by more detailed characterisation and follow-up in the patients. The single-centre and retrospective nature of this study should also be taken forward by prospective, intervention and validation studies in a different cohort. Mortality and FVC decline reported in this study are higher than reported in other studies, most likely because of the long duration of follow-up. Also, study recruitment started before antifibrotic prescribing became available in the UK and, not unexpectedly in this cohort, baseline FVC% was lower in patients that died. However, strong points include statistical analyses including proportional hazards modelling of time-dependent covariates by multivariate analyses and longer median length of follow (31.0 months) will have adjusted for this. The effect of comorbidity burden, which is not insignificant in this and other IPF cohorts, on observed association between blood leucocytes and study outcomes is unknown. We also acknowledge that antifibrotic treatment and corticosteroid therapy may have affected blood leucocyte measurement, however, when adjusting for these covariates using multivariate regression significance was preserved (online supplemental table 1). In total only 11 patients within our cohort received Prednisolone (all prescribed for suspected acute exacerbation) during the 5-year follow-up. One patient was prescribed corticosteroids at the time of baseline assessment and prior blood draw. We do not believe this alters our interpretation of the data.

In summary, we report that neutrophil levels and lymphopaenia, and NLR measured from full blood count analysis were significantly associated with, and predictive of, FVC decline in this IPF cohort. Peripheral blood leucocyte measurements taken from full blood count analysis could be utilised to stratify IPF patients to those more likely to progress, and perhaps prioritised for antifibrotic treatment. This readily accessible measurement can also help inform prognosis, and identify patients for therapeutic trials.

Contributors AA conducted analysis, interpreted data and wrote the paper, AR, AP, HB, KD and RBa collected data, RKH and RBe were involved with patient characterisation, VI provided statistical analysis and interpretation of data, L-PH conceived project, interpreted data, wrote paper and supervised the study. L-PH is guarantor of this study.

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Competing interests None declared.

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Not applicable.

Ethics approval The study was part of a study to examine the factors associated with disease progression in IPF (ethical approval 14/SC/1060 from the Health Research Authority and South-Central National Research Ethics Service).

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Data availability statement All data relevant to the study are included in the article or uploaded as online supplemental information.

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Increased monocyte level is a risk factor for radiological progression in patients with early fibrotic interstitial lung abnormality

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Shareable abstract (@ERSpublications)
Monocyte levels are associated with radiological progression of early fibrotic ILA to established interstitial lung disease and could indicate which patients might require closer follow-up
https://bit.ly/3LlS2ff

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Abstract

Background Interstitial lung abnormalities (ILA) are specific spatial patterns on computed tomography (CT) scan potentially compatible with early interstitial lung disease. A proportion will progress; management involves risk stratification and surveillance. Elevated blood monocyte levels have been shown to associate with progression of idiopathic pulmonary fibrosis. The aims of the present study were: 1) to estimate the proportion of "early fibrotic" (EF)-ILAs (reticular±ground-glass opacities, excluding traction bronchiectasis and honeycombing) on CT scans of patients attending all-indications thoracic CTs, and proportion demonstrating radiological progression; and 2) to explore association between peripheral blood leukocyte levels and ILA progression.

Methods We analysed all thoracic CT reports in individuals aged 45–75 years performed between January 2015 and December 2020 in one large teaching hospital (Oxford, UK) to identify patient CT reports consistent with EF-ILA. CT-contemporaneous blood leukocyte counts were examined to explore contribution to progression and all-cause mortality, using multivariate Cox regression.

Results 40 711 patients underwent thoracic CT imaging during this period. 1259 (3.1%) demonstrated the EF-ILA pattern (mean±SD age 65.4±7.32 years; 735 (47.8%) male). EF-ILA was significantly associated with all-cause mortality (hazard ratio 1.87, 95% CI 1.25–2.78; p=0.002). 362 cases underwent at least one follow-on CT. Radiological progression was observed in 157 (43.4%) cases: increase in reticulation n=51, new traction bronchiectasis n=84, honeycombing n=22. Monocyte count, neutrophil count, monocyte:lymphocyte ratio, neutrophil:lymphocyte ratio and "systemic inflammatory response index" were significantly associated with radiological progression.

Conclusion 3.1% of subjects requiring thoracic CT during a 6-year period demonstrated EF-ILA. Monocyte levels and blood leukocyte-derived indexes were associated with radiological progression and could indicate which patients may require closer follow-up.

Introduction

Interstitial lung abnormalities (ILA) refer to specific spatial patterns on computed tomography (CT) scan that are potentially compatible with interstitial lung disease (ILD) in individuals where ILD was not previously suspected [1]. As a proportion of ILAs are detected coincidentally in asymptomatic individuals, it is difficult to determine its prevalence. However, large cohort studies have reported prevalence of 2–10% [2–5] and a higher risk of mortality [2, 6]. ILAs have been shown to be associated with symptoms including breathlessness, reductions in lung function [2] and exercise capacity [7] and genetic abnormalities common to familial interstitial pneumonia and idiopathic pulmonary fibrosis (IPF) [8, 9]. A proportion of ILAs are known to progress to IPF, yet ILA prevalence exceeds that of IPF by a considerable margin. Therefore, identifying cases at risk of progression is an important clinical priority [10].

Future implementation of lung cancer screening and greater use of CT imaging for other diagnostic purposes are likely to increase detection of ILA and pose resource implications for ILD services [11]. A recent position paper on ILA from the Fleischner Society [1] discussed risk stratification, schema for follow-up evaluation and the importance of subcategorising for the subpleural fibrotic ILA, which has greater mortality risk.

Earlier detection of fibrotic ILAs could lead to a shorter lag time to ILD diagnosis, potentially allowing earlier treatment intervention and improved patient outcome [12]. Perhaps more pressing is a need for an easily accessible test to stratify patients according to those who are more likely to progress and therefore require follow-up.

Measurement of peripheral blood leukocytes for prognosis purposes in IPF has gained interest in recent years [13–15]. Furthermore, indexes derived separately from peripheral blood leukocytes have demonstrated correlation with adverse clinical outcomes in ILD [16].

In this study, we examined the prevalence of ILA with early fibrotic features in a population over a 6-year period. Focusing on radiographic appearances, we identified a group of ILAs, which we termed "early fibrotic ILA" (EF-ILA), defined as those with reticular±ground-glass presence and excluding traction bronchiectasis and honeycombing; and questioned whether there is an association between leukocyte profile and outcome, focusing on mortality and radiological progression (in extent, and with emergence of traction bronchiectasis and honeycombing) [17].

Methods

Using the UK National Health Service (NHS)-based Clinical Record Interactive Search database of the Oxford University Hospitals NHS trust (estimated catchment population of 800 000; Oxfordshire, UK), we examined available CT reports for all thorax-protocolised CT scans performed between January 2015 and December 2020. We performed a starting keyword search using criteria selective for parenchymal abnormalities with an early fibrotic pattern: ["reticulation" or "interstitial"] AND ["sub-pleural" or "basal" or "lower zone" or "possible UIP"] AND [age range: 45–75]. Further details are provided in the supplementary methods.

We then screened the preliminary search for additional radiographic features: ground-glass opacities (GGO), traction bronchiectasis and honeycombing, in acknowledgment that multiple parenchymal features can co-exist on CT [1, 18]. Cases with or without GGO, but without traction bronchiectasis and honeycombing were termed EF-ILA. Those with traction bronchiectasis and honeycombing were classified as CTs showing traction bronchiectasis and/or honeycombing. We considered that these were representative of established fibrosis and/or usual interstitial pneumonia (UIP) pattern fibrosis, and not ILAs. In a proportion of cases identified from the preliminary keyword search, we later found on screening that CT reports were detailing negative/absence of specific radiological patterns; these were defined as a "nil-ILA" reference cohort. These cases were separated from the EF-ILA cohort.

Demographic data were collated, including age, gender and comorbidity profiles.

Over this period, patients who had more than one CT scan were identified, and the reports of the earliest and latest CT scans analysed for radiographic progression. Radiographic progression was recorded as a binary event. It was defined as increase in either extent of identified early fibrotic features (reticulation and/or GGO), new emergence of traction bronchiectasis and/or new emergence of honeycombing. In cases not demonstrating radiographic progression, this was defined as unchanged pre-existing parenchymal features and absence of new features. Time interval between first and latest CT was calculated. Mortality was recorded and time from first CT was calculated. For those who survived, a censoring date of 1 April 2021 was used.

Blood leukocyte counts (monocytes, neutrophils and lymphocytes) closest to the CT scan were recorded and monocyte:lymphocyte ratio (MLR), neutrophil:lymphocyte ratio (NLR) and systemic inflammatory response (SIRI: (monocytes×neutrophils) ÷ lymphocytes) indexes were calculated [19].

Statistical analysis

Where relevant, tests for normality of data were performed using a D'Agostino–Pearson test, and following this the difference between groups was analysed using unpaired t-tests or the Mann–Whitney test for parametric and nonparametric analysis, respectively. Contingency tests (Fisher's exact test of significance) were used to assess categorical data.

Cox proportional hazard models were used to determine hazard ratios (HRs) to progression and all-cause mortality (separate models). In both models, age, gender, and monocyte, neutrophil and lymphocyte levels obtained at a time point closest to the CT scan were included. Coefficient of variation values for each case were calculated from available counts for monocytes, neutrophils and lymphocytes (and derived indexes) in the 1 year up to first CT to account for within-group variance in these measures. ILA categories were included in regression models and where stated hazard ratios represent either absolute floating risk or expressed relative to the nil-ILA category (reference category). Hazard ratios generated from continuous covariates represented the change in the risk of outcome if the covariate in question changes by one unit. Statistical significance was performed using the likelihood ratio test.

Reported statistical confidence intervals are at 95%. Two-tailed p-values <0.05 determined statistical significance. All analyses were performed using GraphPad Prism (version 9) or SPSS (version 26; IBM, Armonk, NY, USA).

Ethical approval

The study was part of a study to examine the factors associated with disease progression in IPF (ethical approval 14/SC/1060 from the Health Research Authority and South-Central National Research Ethics Service).

TABLE 1 Demographic and blood leukocyte profiles of patients with no interstitial lung abnormalities (ILA) and early fibrotic (EF)-ILA

	No ILA	EF-ILA
Demographics		
Female	152 (42.8)	657 (52.2)
Male	203 (57.2)	602 (47.8)
Age at first CT (years)	63.4±8.1	65.39±7.32
Comorbidity		
COPD/emphysema	52 (14.6)	306 (19.9)
Pneumonia	70 (19.7)	344 (22.4)
Lung cancer	30 (8.5)	183 (11.9)
Pulmonary hypertension	13 (3.7)	68 (4.4)
T2DM	57 (16.1)	259 (16.8)
Hypertension	172 (48.5)	664 (43.2)
IHD	66 (18.6)	289 (18.8)
Cardiomyopathy	115 (32.4)	412 (26.8)
Blood leukocyte measurements		
Time from CT to nearest blood test (months)		
Mean±SD	0.87±5.92	0.78±6.29
Median (interquartile range)	0.11 (−0.23–1.24)	0.10 (−0.39–1.11)
Monocyte ($\times 10^9$ cells·L^{-1})	0.65±0.29	0.67±0.31
Neutrophil ($\times 10^9$ cells·L^{-1})	5.46±3.30	5.24±3.00
Lymphocyte ($\times 10^9$ cells·L^{-1})	1.76±0.92	1.94±3.60
MLR	0.46±0.39	0.45±0.36
NLR	4.46±5.79	3.92±5.22
SIRI	3.21±5.74	2.75±4.33
Seen in ILD clinic	38 (10.7)	343 (27.2)
Length of follow-up (months)	21.46±21.7	23.4±21.8
Time from first CT to ILD clinic visit (months)	16.75±16.8	37.4±160.9

Data are presented as n (%) or mean±SD, unless otherwise stated. CT: computed tomography; T2DM: type 2 diabetes mellitus; IHD: ischaemic heart disease; MLR: monocyte:lymphocyte ratio; NLR: neutrophil:lymphocyte ratio; SIRI: systemic inflammatory response ((monocytes×neutrophils) ÷ lymphocytes); ILD: interstitial lung disease.

Results

CT-based patient categorisation and demographics

170 197 CT scans were performed, which included any thoracic CT protocol between January 2015 and December 2020 in 40 711 patients. 3987 CT scans (performed in 2735 patients) satisfied the starting search criteria of ["reticulation" or "interstitial"] AND ["sub-pleural" or "basal" or "lower zone" or "possible UIP"] AND [age range: 45–75]. 355 cases did not demonstrate ILD or ILA and were used as a non-ILA reference cohort. 762 cases also demonstrated additional traction bronchiectasis and or honeycombing present on their first CT. 490 cases demonstrated traction bronchiectasis only; 270 demonstrated honeycombing±traction bronchiectasis.

1259 (3.1%) out of 40 711 cases demonstrated reticulation±GGO, without traction bronchiectasis or honeycombing. 430 also had emphysema and 88 also had non-emphysematous cysts. Therefore, 3.1% of subjects (1259 of the starting cohort of 40 711) requiring thoracic CT between 2015 and 2020 demonstrated EF-ILA. Mean±SD age of the EF- ILA group was 65.4±7.32 years; 735 (47.8%) were male.

Demographic profiles are listed in table 1 and a flow chart of ILA features and progression is shown in figure 1. Comorbidity profiles were identified by cross-referencing electronic health records. Comorbidities are representative of the time the search was conducted and not at time of first CT.

3217 CT scans (80.7% of scans with available information) were reported by a thoracic radiologist.

343 cases with EF-ILA on first CT were seen in the ILD clinic. Mean time from CT to clinic attendance was 3.1 years.

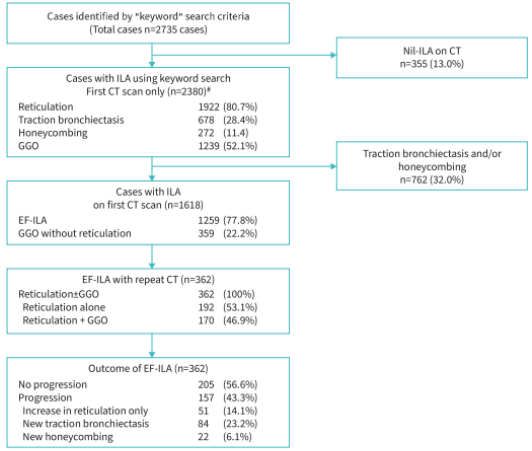

FIGURE 1 Flow diagram of interstitial lung abnormalities (ILA) features and radiological progression of cases with early fibrotic ILA (EF-ILA). Where more than one computed tomography (CT) scan was performed during the observation period, the first CT scan was used as the CT scan for the patient. GGO: ground-glass opacities. #: a proportion of first CT scans demonstrated two or more ILA features simultaneously.

Radiological progression on follow-on CT

Of the 1259 cases with EF-ILA, 362 patients underwent at least one follow-on CT scan, allowing examination of radiological change. Median (interquartile range (IQR)) time interval between CTs was 0.83 (0.32–1.95) years. Progression in type or extent of ILA was observed in 157 (43.4%) cases. Of these, increase in reticulation was observed in 51 (14.1%) out of 362 cases. Progression with emergence of traction bronchiectasis (excluding honeycombing) was observed in 84 (23.2%) cases, and honeycombing (with or without traction bronchiectasis) in 22 (6.1%) cases. 205 (56.6%) did not progress during this time (up to 5 years).

Median time interval between CT scans in cases demonstrating progression was 1.24 years (IQR 0.53–2.36 years, maximum 5.20 years) *versus* 0.59 years (IQR 0.27–1.19 years, maximum 5.05 years) in cases demonstrating no progression ($p<0.001$).

Unsurprisingly, multivariate Cox regression analysis showed that radiographic progression of EF-ILA was associated with mortality (HR 1.92, 95% CI 1.51–3.21; p=0.013).

Imaging features and ILA mortality

Death was reported in 448 (16.40%) cases in the 6 years of analysis. Mean±SD time from first CT to death was 19.8±16.5 months *versus* 35.6±20.6 months in those who survived ($p<0.0001$). In cases with EF-ILA, death was reported in 183 cases. Mean±SD time from first CT to death was 19.0±16.6 months *versus* 32.7±20.5 months in those who survived.

Association between specific ILA features noted on first CT and mortality was explored using multivariate Cox regression (table 2). As expected, traction bronchiectasis (HR 2.09, 95% CI 1.36–3.20; p=0.0001) and honeycombing (HR 3.65, 95% CI 2.38–5.60; $p<0.0001$) were significantly associated with mortality relative to the nil-ILA reference category. Cases with EF-ILA also demonstrated significant mortality risk (HR 1.87, 95% CI 1.25–2.78; p=0.002). This risk was slightly higher in cases of EF-ILA that also demonstrated GGO (HR 2.03, 95% CI 1.29–3.19; p=0.002) in comparison to cases of EF-ILA without GGO (HR 1.80, 95% CI 1.19–2.72; p=0.005). Mortality risk of EF-ILA was preserved when adjusting for the respiratory comorbidities: lung cancer, pneumonia and COPD/emphysema. Lung cancer and pneumonia were also significantly associated with mortality in the EF-ILA group (supplementary table S1).

Blood leukocyte association with radiological progression of ILA and mortality

We explored association between peripheral blood leukocytes and their derived leukocyte indexes against radiographic progression and mortality using multivariate Cox proportional hazards models (table 3). All models included age, gender and the leukocyte values (or their derived indexes) contemporaneous with CT scan. Where stated, leukocyte coefficient of variation of each leukocyte type over the year leading to the CT was included in multivariate models. Nearest available blood measurement of monocytes, neutrophils and lymphocytes to first CT scan were obtained from standardised hospital "full blood count" measurements. Median (IQR) time interval between CT and nearest blood sampling was 1 (13–30) day.

In the 362 cases of EF-ILA that underwent at least two CT scans, monocyte count (HR 1.79, 95% CI 1.05–2.86; p=0.030) and neutrophil count (HR 1.11, 95% CI 1.02–1.29; p=0.009), MLR (HR 2.28, 95% CI 1.33–3.87; p=0.002), NLR (HR 1.07, 95% CI 1.01–1.14; p=0.024) and SIRI (HR 1.09, 95% CI

TABLE 2 Multivariate Cox regression examining association of interstitial lung abnormalities (ILA) features on first computed tomography (CT) scan with mortality

	Patients	Death	HR (95% CI)#	p-value
Age			1.02 (1.01–1.04)	0.010*
Gender (male)	1486 (54.3)	268 (18.0)	1.11 (0.91–1.36)	0.295
Nil-ILA (reference)	355 (12.9)	43 (12.1)		
EF-ILA	1259 (46.0)	183 (14.5)	1.87 (1.25–2.78)	0.002*
Traction bronchiectasis without honeycombing	490 (17.9)	86 (17.6)	2.09 (1.36–3.20)	0.0001*
Honeycombing±traction bronchiectasis	272 (9.9)	87 (32.0)	3.65 (2.38–5.60)	<0.0001*

Data are presented as n (%), unless otherwise stated. Findings were similar when adjusted for respiratory comorbidities (supplementary table S1). HR: hazard ratio. EF-ILA: early fibrotic ILA. #: HR for ILA categories representative of risk relative to nil-ILA reference category. *: p<0.05.

TABLE 3 Multivariate Cox regression examining association between blood leukocytes and 1) mortality in early fibrotic interstitial lung abnormalities (EF-ILA) (n=1259) and 2) radiological progression in the EF-ILA cohort with available repeat computed tomography scans for comparison (n=362)

	Mortality		Radiological progression	
	HR (95% CI)	p-value	HR (95% CI)	p-value
Age	1.03 (1.01–1.06)	0.005*	1.03 (1.00–1.06)	0.027*
Gender	1.04 (0.76–1.42)	0.811	0.92 (0.67–1.27)	0.609
Monocytes#	1.12 (1.01–1.36)	0.003*	1.79 (1.05–2.86)	0.030*
Neutrophils	1.13 (1.07–1.19)	<0.001*	1.11 (1.02–1.29)	0.009*
Lymphocytes	0.97 (0.85–1.09)	0.574	0.99 (0.94–1.04)	0.596

HR: hazard ratio. #: when adjusting for respiratory comorbidities, monocytes remained independently associated with progression (supplementary table S2). *: p<0.05.

1.04–1.14; p=0.0002) were significantly associated with radiographic progression of the EF-ILA on multivariate Cox regression analysis (tables 3 and 4). Higher monocyte count, MLR, NLR and SIRI remained significant when adjusting for respiratory comorbidities (lung cancer, pneumonia and COPD/emphysema) in this cohort. Neutrophil count continued to show similar direction of effect towards progression, but was not significant (supplementary tables S2 and S3).

In the 1259 cases demonstrating EF-ILA on first CT, monocyte count (HR 1.12, 95% CI 1.01–1.36; p=0.003), neutrophil count (HR 1.13, 95% CI 1.07–1.19; $p<0.001$) and all their derived indexes were significantly associated with all-cause mortality: MLR (HR 1.16, 95% CI 1.02–1.31; p=0.025), NLR (HR 1.07, 95% CI 1.05–1.09; $p<0.001$) and SIRI (HR 1.06, 95% CI 1.04–1.08; $p<0.001$) (tables 3 and 4). Mortality risk was preserved in all models adjusting for respiratory comorbidity, except for monocytes (supplementary tables S2 and S3).

In separate regression models, coefficient of variation of longitudinal measurements for each leukocyte/index was also included, to adjust for any effect that variation in longitudinal measurement of these leukocyte parameters may have on clinical outcome. Monocytes maintained significant hazard towards both mortality and progression of EF-ILA (supplementary table S4). Distribution of leukocyte levels and their derived indexes are shown in supplementary figure S1.

TABLE 4 Multivariate Cox regression examining association between blood leukocyte indexes and 1) mortality in early fibrotic interstitial lung abnormalities (EF-ILA) (n=1259) and 2) radiological progression in the EF-ILA cohort with available repeat computed tomography scans for comparison (n=362)

	Mortality		Radiological progression	
	HR (95% CI)	p-value	HR (95% CI)	p-value
MLR				
Age	1.03 (1.01–1.06)	0.006*	1.02 (0.99–1.05)	0.113
Gender	1.00 (0.74–1.36)	0.995	0.92 (0.67–1.27)	0.624
MLR	1.16 (1.02–1.31)	0.025*	2.28 (1.33–3.87)	0.002*
NLR				
Age	1.03 (1.01–1.06)	0.007*	1.02 (0.99–1.05)	0.122
Gender	0.98 (0.72–1.34)	0.910	0.96 (0.70–1.32)	0.814
NLR	1.07 (1.05–1.09)	<0.0001*	1.07 (1.01–1.14)	0.024*
SIRI				
Age	1.04 (1.01–1.06)	0.003*	1.03 (0.99–1.05)	0.079*
Gender	1.02 (0.75–1.38)	0.924	0.96 (0.69–1.31)	0.789
SIRI	1.06 (1.04–1.08)	<0.0001*	1.09 (1.04–1.14)	0.0002*

Each leukocyte index, age and gender is a separate model. Similar findings were observed when leukocyte indexes were adjusted for respiratory comorbidity and when adjusted for coefficient of variation over a year (supplementary tables S3 and S4). MLR: monocyte:lymphocyte ratio; NLR: neutrophil:lymphocyte ratio; SIRI: systemic inflammatory response ((monocytes×neutrophils) ÷ lymphocytes). *: p<0.05.

Discussion

This study shows that in an unselected cohort of patients undergoing thoracic CT scanning for all indications, in a 6-year period, 3.1% of patients showed evidence of EF-ILA. In a subset of patients who had more than one CT scan during this 6-year period, 43% progressed in extent of disease or demonstrated new traction bronchiectasis or honeycombing. Monocytes, MLR, NLR and SIRI were associated with progression in a multivariate analysis which included analysis of age and gender.

Our findings are comparable to ILA prevalence observed in large population-based cohorts [2, 3, 20] and lung cancer screening cohorts [21, 22] in which ILA ranged between 3% and 10%. Our definition of EF-ILA, which includes reticulation and GGO, but not traction bronchiectasis and honeycombing, probably encompassed "indeterminate UIP", as defined in the 2018 IPF guidelines [17]. However, as we are unable to assess CT distribution of these ILAs in all cases for this large cohort, the terminology of EF-ILA was used. Since reticular abnormalities are common in older individuals and have previously been regarded as part of the normal spectrum of senescent lung [23], we limited inclusion to individuals aged 45–75 years at time of CT [24]. Comparable to other studies, gender was roughly of equal proportions across all cases in this cohort in those with EF-ILA, where traction bronchiectasis and honeycombing were excluded [2, 3].

The Age Gene/Environment Susceptibility (AGES) [3] and Framingham [2] population-based studies demonstrated ILA progression in 43% and 64% of cases, respectively, with associated risks of mortality similar to our cohort. In AGES, prevalence of indeterminate for UIP (iUIP) was estimated at 3.9%. iUIP was associated with mortality risk in univariate analysis (HR 1.6, p<0.0001), but nonsignificantly in multivariate analysis (HR 1.2, p=0.07). In our cohort, with a much higher number of cases, multivariate analysis showed that EF-ILA is associated with all-cause mortality (HR 1.87, p=0.002).

Importantly, we and others [6] demonstrate that radiographic progression is not observed in the majority of cases. It remains challenging to predict those cases that will progress to established fibrotic ILD. To address this, the Fleischner Society recently proposed a schema to facilitate triage, management and follow-up of ILAs [1]. This includes subcategorising cases according to ILA distribution on CT and presence (or absence) of ILAs indicative of established fibrosis.

ILA assessment in individuals with a history of familial ILD has identified associations with particulate exposures, age, positive smoking history, shorter telomere length and MUC5b risk allele [25, 26]. Associations among ageing-related biomarkers and ILA have been explored [27]; however, collectively these are costly and are not routinely available for large-scale use. Furthermore, with implementation of routine lung cancer screening pathways and greater use of thoracic CT for other diagnostic purposes, it is anticipated that ILA detection will increase. Patient follow-up could have a huge implication on clinical resources.

Peripheral blood leukocyte measurement is available as part of routine full blood count analysis and integrating this simple and cheap measure into risk stratification and ILA management is worthy of future consideration. This is supported by studies in IPF patients, where elevated peripheral blood monocyte count has been shown to be predictive of disease progression and mortality. The study by Scott *et al.* [13], a large multicentre retrospective cohort study, first demonstrated that monocyte counts $\geqslant 0.95\times10^9$ cells·L^{-1} were associated with all-cause mortality in IPF and non-IPF fibrotic lung disease. Since then, other retrospective clinical studies have documented similar findings [14, 15, 28]. In an analysis of multiple independent cohorts (the Multi-Ethnic Study of Atherosclerosis (MESA), AGES, COPDGene and Evaluation of COPD Longitudinally to Identify Predictive Surrogate End-points (ECLIPSE); n=7396), Kim *et al.* [29] reported association between higher absolute monocyte count and ILA progression.

There are subtle, but important, differences between our study and that of Kim *et al.* [29], who reported the findings of a pooled analysis of four population-based cohorts (two COPD-focused cohorts). In this heterogeneous population, Kim *et al.* do not discuss whether their observed association between monocytes and ILA progression is limited to specific ILAs, such as those with early evidence of fibrosis. In our study, the inclusion criteria were biased towards selection of cases with CT features potentially compatible with early fibrosis, and were thus population-based enriched for patients potentially at risk of progressing to pulmonary fibrosis [1]. In our cohort of progressors, we detail the proportion of cases with new CT features representative of established pulmonary fibrosis and report association between absolute monocyte count (and other leukocyte parameters) with progression and all-cause mortality. In addition, we adopted a different approach to data analysis, employing Cox proportional hazards modelling to take account of time until events occurred.

Mechanistically, our findings could be explained by recent experimental evidence suggesting migration of monocytes from bone marrow to injured lung, then differentiating into macrophages with a pro-fibrotic phenotype [30]. Further support comes from translational studies that implicate distinct monocyte-derived alveolar macrophage populations in progression of fibrosis [31, 32].

Other studies have also implicated neutrophils and lymphocytes in pulmonary fibrosis. Akin to monocytes, neutrophils are recruited to areas of inflammation [33]. Similar to macrophages, they can alter their microenvironment by secreting proteases, oxidants, cytokines and chemokines [34]. Additionally, neutrophils are a substantial source of matrix metalloproteinases which are involved in collagen deposition and extracellular matrix formation [35]. Neutrophilic bronchoalveolar lavage specimens taken from patients with IPF have been associated with early mortality [36].

We have previously demonstrated in a cohort of patients with CT scans demonstrating iUIP that peripheral blood monocyte and neutrophil counts are implicated in progression to IPF [37], and the association of NLR, MLR and SIRI with mortality in IPF [38]. MLR has been used primarily to prognosticate in cancer studies in recognition that host systemic inflammatory responses influence tumour proliferation and disease progression [39]. NLR has been heavily studied as a systemic inflammatory marker [40]. It has been used to prognosticate systemic inflammatory diseases such as rheumatoid arthritis [41], and recently in connective tissue disease-related ILD and IPF [16]. SIRI integrates neutrophils, monocytes and lymphocytes into one composite measure and has shown promise as a prognosticator within oncology [19].

There are several limitations that should be considered when interpreting these results. We were unable to quantify extent of disease and extent of progression. Categorisation was based on qualitative information extracted from radiology reports and does not quantify individual ILA extent, which may contribute to rate of progression. Similarly, we were unable to capture indications for CT and we cannot account for clinical symptoms, which would have been interesting to explore. Therefore, interpretation of our findings is limited to association between CT features with blood leukocytes. The patients were selected because of their need for a thoracic CT, so the true prevalence in the population is unknown, only in those who requires a thoracic CT. In the group where we assessed progression, the interval between the first and last CT was longer in those who progressed compared to those who did not. It could be argued that those who did not progress would do so over a longer period of assessment. The nil-ILA group was identified by description of negative findings of keywords from CT reports (*e.g.* "no reticulation"). We acknowledge that this represents a smaller proportion of patients with normal CT scans and may have introduced bias to EF-ILA mortality risk calculation. However, selection criteria were identical and demographic and comorbidity profiles were comparable between nil-ILA and EF-ILA group. Furthermore, demographic profiles of the nil-ILA group are also comparable to the nil-ILA cohorts of other longitudinal studies [3].

A proportion of our EF-ILA cohort were subsequently seen in our ILD clinic. Mean time from first CT scan demonstrating EF-ILA to ILD clinic attendance was +3.1 years. As the ILD clinic was a first-attender clinic, it is likely (but not verified) that these were patients who became symptomatic or demonstrated progression after a follow-on CT scan and did not have a prior diagnosis of ILD. Therefore, there is a possibility of prior undiagnosed ILD. Under-reporting of ILA has been described previously [42]; as such, we cannot exclude the possibility that a degree of misclassification may have occurred. However, 80.7% of CT scans were reported by post-radiology fellowship specialist thoracic radiologists who attend ILD multidisciplinary team meetings, based at a single centre, which might mitigate interobserver difference. Excellent interobserver correlation between our thoracic radiologists in reporting ILD features (r=0.91; p<0.001) has been described previously [43]. The remaining 19.3% of CTs were reported by one out of 14 Oxford-based post-training radiologists. All radiologists collectively agree on descriptive reporting phrases, and there are regular local discrepancy meetings to check on accuracy of reporting.

World Health Organization International Classification of Diseases version 10 (ICD-10) coding was used to identify comorbidity during the 6 years of study follow-up, but without coding dates we were unable to align comorbidity events to first CT scan date. Smoking history was poorly captured using "ICD-10 coding". Therefore, we elected not to include this information in our multivariate analysis, but acknowledge that patients who smoke may show a greater rate of ILA progression. Finally, the single-centre and retrospective nature of this study should be taken forward by prospective, intervention and validation studies in a different cohort.

Notwithstanding these limitations, our study, in a very large cohort with high proportion of specialist thoracic radiologist reporting, demonstrates that monocyte levels, MLR, NLR and SIRI are associated with

progression in EF-ILA. Further prospective studies will help determine if these parameters could be used to help prioritise patients who might benefit from follow-up.

Provenance: Submitted article, peer reviewed.

Author contributions: A. Achaiah conceived the project, conducted the analysis, interpreted data and wrote the article. P. Lyon performed the interstitial lung abnormality search and collated data. E. Fraser, P. Saunders and R. Hoyles provided guidance to the project. R. Benamore provided guidance to the project and assisted with defining search criteria. L-P. Ho conceived the project, interpreted data, wrote the article and supervised the study.

Conflict of interest: A. Achaiah has nothing to disclose. P. Lyon has nothing to disclose. E. Fraser has nothing to disclose. P. Saunders has received consultancy fees from Trevi Therapeutics and lecture fees from Boehringer Ingelheim, but no other conflict of interest. R. Hoyles has nothing to disclose. R. Benamore has nothing to disclose. L-P. Ho has nothing to disclose.

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Neutrophil levels correlate with quantitative extent and progression of fibrosis in IPF: results of a single-centre cohort study

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ABSTRACT

Background Idiopathic pulmonary fibrosis (IPF) is a progressive fibrotic lung disease with poor prognosis. Clinical studies have demonstrated association between different blood leucocytes and mortality and forced vital capacity (FVC) decline. Here, we question which blood leucocyte levels are specifically associated with progression of fibrosis, measured by accumulation of fibrosis on CT scan using a standardised automated method.

Methods Using the Computer-Aided Lung Informatics for Pathology Evaluation and Rating CT algorithm, we determined the correlation between different blood leucocytes (<4 months from CT) and total lung fibrosis (TLF) scores, pulmonary vessel volume (PVV), FVC% and transfer factor of lung for carbon monoxide% at baseline (n=171) and with progression of fibrosis (n=71), the latter using multivariate Cox regression.

Results Neutrophils (but not monocyte or lymphocytes) correlated with extent of lung fibrosis (TLF/litre) (r=0.208, p=0.007), PVV (r=0.259, p=0.001), FVC% (r=−0.127, p=0.029) at baseline. For the 71 cases with repeat CT; median interval between CTs was 25.9 (16.8–39.9) months. Neutrophil but not monocyte levels are associated with increase in TLF/litre (HR 2.66, 95% CI 1.35 to 5.25, p=0.005).

Conclusion Our study shows that neutrophil rather than monocyte levels correlated with quantifiable increase in fibrosis on imaging of the lungs in IPF, suggesting its relative greater contribution to progression of fibrosis in IPF.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Idiopathic pulmonary fibrosis (IPF) is a progressive fibrotic condition. Recently, several human studies have implicated blood leucocyte levels (monocyte, neutrophil and lymphocyte) with forced vital capacity decline and mortality. However, direct association between leucocytes and progression of fibrosis using quantitative CT analysis has not been explored.

WHAT THIS STUDY ADDS

⇒ This study explored the association between blood monocytes, neutrophils and lymphocytes against increase in fibrosis over time, measured using a quantitative CT algorithm, Computer-Aided Lung Informatics for Pathology Evaluation and Rating. We show that levels of blood neutrophil and lymphocytes but not monocytes were associated with greater risk of progression of fibrosis.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Our study shows that neutrophil rather than monocyte levels correlated with quantifiable increase in fibrosis on imaging of the lungs in IPF, suggesting its relative greater contribution to progression of fibrosis in IPF.

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a progressive fibrotic condition. Animal studies have implicated innate immune cells, particularly monocytes and macrophages in the pathogenesis of IPF.[1] This is supported by several clinical studies which showed that higher blood monocytes levels are associated with mortality in IPF.[2] Blood monocyte levels also correlated with the extent of fibrosis on CT scan,[3] and Kreuter *et al* also found an association with a composite measure of IPF outcome (forced vital capacity (FVC) decline, 6 min walk distance reduction, acute exacerbation and/or mortality).[4] Moreover, we (and others) have shown that the neutrophil:lymphocyte ratio (NLR) is also a predictor of mortality and FVC decline,[5 6] and patients with higher levels of neutrophils were more likely to progress from indeterminate for Usual Interstitial Pneumonia (UIP) CT pattern to UIP pattern and a clinical diagnosis of IPF.[7]

However, mortality and FVC decline may not accurately reflect progression of fibrosis. Death can be due to other causes, for example, cardiovascular diseases, especially in the elderly population of IPF. Although FVC decline of >10% in IPF is the validated measure of disease progression predictive of mortality,[8] and serial FVC change the recommended monitoring variable in international guidelines,[9] this global metric may not accurately reflect regional morphological changes indicative of progression of fibrosis. In fact a recent large study suggests that FVC decline in IPF patients is heterogeneous and may have little correlation with increase in fibrosis in some patients.[10]

The Computer-Aided Lung Informatics for Pathology Evaluation and Rating algorithm (CALIPER) is an automated quantitative CT application which characterises lung parenchymal features on volumetric high-resolution CT (HRCT), thus quantifying abnormal lung.[11] In IPF studies it is highly predictive of mortality,[12] but also enhances risk stratification and cohort enrichment for trial end points.[13 14] Until now, it has not yet been used to explore leucocyte association with progression of fibrosis.

The aim of the study is twofold—(1) to assess if leucocyte levels are linked to progression in fibrosis per se and (2) to explore which leucocytes demonstrate greatest association with fibrosis. We used CALIPER to provide a standardised automated, method of quantifying lung fibrosis.[11]

METHODS

Study design and patients characteristics

We performed a retrospective analysis of a cohort of patients with IPF, who presented to the Oxford Interstitial Lung Disease (ILD) Service between September 2016 and November 2021. All patients with a multidisciplinary team (MDT) diagnosis of IPF,[15] and a CALIPER-compatible HRCT (non-contrast, supine, volumetric HRCT scan) were included.

We asked two main questions—(1) what is the correlation between contemporaneous blood leucocyte levels (within 4 months of the first CT) and baseline amount of lung fibrosis and (2) which blood leucocyte levels (neutrophils, lymphocytes, monocytes and their derived ratios) correlated with progression in amount of lung fibrosis. Association between blood leucocytes and FVC decline and mortality were also examined as comparator end points.

Blood leucocyte measurement

Neutrophil, lymphocyte and monocyte levels captured from standard clinical 'full blood count' analysis within 4 months of initial CT (CT1). NLR, monocyte:lymphocyte ratio (MLR) and Systemic Inflammation Response Index (SIRI) (neutrophil×monocyte/lymphocyte) were calculated as described previously.[5]

CT scans

Non-contrast, supine, volumetric HRCT scan were acquired using a 64-detector row CT scanner. Images were reconstructed using a high spatial resolution algorithm. Non-contrast, volumetric, HRCT scans for appropriate subjects were acquired (0.625 mm slice thickness at an interval of 0.625 mm).

Computer-Aided Lung Informatics for Pathology Evaluation and Rating

Full CALIPER data acquisition and processing are described in online supplemental methods. Briefly, classification of parenchymal features was applied to 15×15×15 voxel volumes of interest (VOI) using texture analysis and computer-based algorithmic interpretation of volumetric histogram signature mapping features.[11] CALIPER evaluation included characterisation and quantification of each VOI into one of five radiological parenchymal categories: normal lung, hyperlucent, ground glass opacity (GGO), reticular opacity and honeycombing. Parenchymal features were expressed as a relative percentage of CALIPER-derived total lung volume. Total lung fibrosis (TLF) represented the sum of GGO, reticular and honeycomb percentages.[14] We included GGO as these had been reported by our radiologists to be found in areas of reticulation or traction bronchiectasis at MDTs and designated as fine fibrosis in keeping with agreed radiological assessments.[16]

Assessment of pulmonary vessel volume (PVV) was also performed prior to pulmonary vessel extraction from lung parenchyma using a multiscale tubular structure enhancement filter.[17] This assessment excluded large vessels at the hilum of the lung. The PVV was calculated as absolute volume (cm^3) and expressed as percentage of total CALIPER-derived lung volume.

The key variables were TLF (the sum of reticulation, GGO and honeycomb, as % of CALIPER-derived lung volume), TLF/litre (TLF expressed as % of lung volume) and PVV. TLF/litre was used to account for difference in lung volume on serial scans with progression in fibrosis.[18] %change in fibrosis is (CT2 TLF/litre–CT1 TLF/litre)/CT1 TLF/litre×100.

Lung function tests

Baseline spirometry (forced expiratory volume in 1 s and FVC), transfer factor of lung for carbon monoxide (TLco) and Composite Physiological Index (CPI)[19] within 3 months of CT were recorded; all as % predicted for age, height and sex. Annualised change in lung function was calculated as the relative (%) change in absolute value divided by time (years) between tests.

Statistical analysis

Pearson correlation was used to explore association between blood leucocytes with baseline CALIPER variables and pulmonary function tests. Cox proportional hazard modelling was employed to test association between each blood leucocyte level (monocytes, lymphocytes and neutrophils) and outcomes of disease progression—(1)≥7.8% increase in TLF/litre (7.8% being the lower limit of upper tertile) (model A), (2)

Table 1 Baseline characteristics for patients, at the point of first CT used for CALIPER analysis.

	All patients (n=171)	Patients for analysis of progression (n=71)
Demographics		
Age at CT in years	75.1 (70.1–81.1)	73.6 (68.3–79.3)
Female, n (% of cohort)	17 (9.9)	6 (8)
Male, n (% of cohort)	154 (90.1)	65 (92)
Smoking status		
Never smoker, n (% of cohort)	47 (27.5)	21 (29.6)
Ex-smoker, n (% of cohort)	89 (52.3)	29 (40.8)
No data, n (% of cohort)	35 (20.2)	21 (29.6)
CALIPER parenchymal features		
Total lung fibrosis or TLF, as % of lung volume	14% (8–22)	13% (7–19)
Low attenuation areas, as % of lung volume	1% (0–2)	1% (0–3)
Total ground glass, as % of lung volume	5% (3–13)	5% (2–10)
Total reticular, as % of lung volume	5% (3–9)	5% (3–8)
Total honeycombing, as % of lung volume	1% (0–1)	0% (0–1)
CALIPER pulmonary vessel volume		
Total PVV in cm^3	160 (128–201)	168 (134–202)
Total PVV as % of lung volume	4.02% (3.02–5.21)	4.60% (3.04–6.62)
Lung function		
FEV1, litre	2.19 (1.83–2.68)	2.34 (1.93–2.83)
%FEV1	81.2% (70.9–92.2)	84.7% (71.53–91.15)
FVC, litre	2.8 (2.28–3.365)	2.91 (2.35–3.56)
%FVC	76.57% (66.6–91.15)	76.44% (68.9–92.37)
FEV1: FVC	80.6 (75.0–86.1)	82.1 (75.1–88.6)
TLCO, SI	4.56 (3.49–5.48)	4.9 (3.87–5.80)
%TLCO	56.85% (49.5–67)	60.05% (51.1–71)
CPI Score	67.48 (61.05–73.12)	65.36 (59.64–71.25)
Blood leucocytes		
Monocyte $\times 10^3/\mu L$	0.67 (0.58–0.84)	0.66 (0.55–0.8)
Neutrophil $\times 10^3/\mu L$	4.90 (3.85–6.53)	4.87 (3.85–6.45)
Lymphocyte $\times 10^3/\mu L$	1.62 (1.31–2.23)	1.64 (1.43–2.27)
MLR	0.37 (0.32–0.55)	0.37 (0.29–0.5)
NLR	2.70 (2.08–4.08)	2.69 (2.06–3.61)
SIRI	1.79 (1.4–3.08)	1.76 (1.27–2.63)
Comorbidity		
Reflux, n (% of cohort)	54 (39.1)	25 (39.7)
PHT, n (% of cohort)	11 (8.0)	4 (6.3)
IHD, n (% of cohort)	28 (20.3)	15 (23.8)
Cardiomyopathy, n (% of cohort)	6 (5.8)	1 (2)
Arrhythmia, n (% of cohort)	9 (6.5)	2 (3.2)
Hypertension, n (% of cohort)	29 (21)	16 (25.4)
PE, n (% of cohort)	3 (2.2)	2 (3.2)
COPD/emphysema, n (% of cohort)	8 (7.7)	4 (8.2)
Type II diabetes mellitus, n (% of cohort)	15 (10.9)	7 (11.1)
Antifibrotics		
Antifibrotic use, n (% of cohort)	69 (40.3)	36 (57.1)

All values are median (IQR) unless stated. CPI as calculated by Wells *et al*.[19] Forty-nine per cent of the cohort had 'probable UIP' as defined by the 2018 American Thoracic Society (ATS) criteria (all had clinical IPF from MDT diagnosis), explaining why some patients do not have honeycomb. Low attenuation areas represent emphysematous areas. CALIPER, Computer-Aided Lung Informatics for Pathology Evaluation and Rating; CO, carbon monoxide; COPD, Chronic obstructive pulmonary disease; CPI, Composite Physiological Index; FEV1, forced expiratory volume in 1s; FVC, forced vital capacity; IHD, Ischaemic heart disease; IPF, idiopathic pulmonary fibrosis; MDT, multidisciplinary team; MLR, monocyte:lymphocyte ratio; NLR, neutrophil:lymphocyte ratio; PHT, pulmonary hypertension; PVV, pulmonary vessel volume; SI, standard international; SIRI, Systemic Inflammation Response Index; TLCO, transfer factor of lung for carbon monoxide.

≥10% increase in TLF/litre (10% as an arbitrary value) (model B) and (3) ≥10% FVC decline between CT1 and CT2 (model C). Median and upper quartiles for model A were also tested in preliminary modelling but no significant correlation was found. Harrell's Concordance index (C-index) was used to assess model strength, describing how well a model can discriminate between two survival distributions.[20] Kaplan-Meier analysis (log rank test) was used to evaluate time to all-cause mortality from first CT. A censoring time of 1 January 2022 was applied.

All analyses were performed using GraphPad Prism V.9.2 (La Jolla, California, USA) or SPSS V.27 (IBM).

RESULTS

Patients

A total of 171 eligible patients were identified. Of these, n=71 had at least one further follow-on HRCT (the CT with the longest interval from the first was selected). Demographics are shown in table 1. Median interval between CT1 and CT2 was 25.9 (16.8–39.9) months. Median time from first CT to death was 26.2 months (12.4–43.4) vs 45.7 (34.6–61.5) in those surviving to censoring date.

Association between blood leucocyte levels and baseline CALIPER and lung function measures

At baseline (CT1, n=171), there were significant correlations between baseline % predicted FVC, TLCO and CPI, and TLF, TLF/litre and PVV (table 2). In terms of correlation between leucocytes and CALIPER variables and lung function, there was significant correlation between neutrophil levels and TLF/litre (r=0.208, p=0.007), total PVV (r=0.259, p=0.001) and FVC (r=−0.127, p=0.029) (table 3). No other leucocyte levels or their derived measures showed significant corelations with CALIPER or lung function at baseline (table 3 and online supplemental table S1).

Association between blood leucocyte levels and progression of fibrosis

Neutrophil count was significantly higher in cases demonstrating progression of fibrosis as defined by ΔTLF>10%/litre (4.87 (3.86–5.66) vs 4.84 (3.85–6.45), p=0.043). No other differences in leucocyte measure between cases with disease progression and stability was identified (online supplemental table 2).

In model A of Cox proportional hazard analysis, neutrophil levels (HR 1.81, 95% CI 1.10 to 2.99, p=0.020) were significantly associated with increase in TLF/litre≥7.8% (table 4). Lower lymphocyte levels were also significantly associated with TLF/litre≥7.8% (HR 0.26, 95% CI 0.08 to 0.91, p=0.034). Similar findings were observed in model B (where outcome was TLF/litre ≥10%), except that lower lymphocyte levels were not significantly associated with progression of >10%/Litre. In both models A and B, there was a trend of higher monocyte levels with progression of fibrosis, but this was not statistically significant.

In model C (where outcome was change by 10% of FVC), no leucocyte variable significantly associated with FVC decline. A lower baseline FVC was predictive of FVC decline (HR 0.96, 95% CI 0.92 to 0.99, p=0.049).

All leucocyte derived indexes were significantly associated with progression in fibrosis (TLF/litre by 7.8% and 10%) in multivariate analysis (online supplemental table 3).

Table 2 Association between lung function and CALIPER parameters (Pearson's correlation) at baseline; n=171 patients.

	FVC%		TLCO%		CPI	
CALIPER baseline metrics	**r**	**P value**	**r**	**P value**	**r**	**P value**
Lung volume (litre)	0.634	**<0.0001**	0.411	**<0.0001**	0.112	0.156
TLF (%)	0.392	**<0.0001**	0.461	**<0.0001**	0.275	**<0.0001**
TLF (/litre)	0.430	**<0.0001**	0.444	**<0.0001**	0.246	**0.002**
Low attenuation areas (% of total lung volume)	0.217	0.061	0.107	0.177	0.066	0.404
GGO (% of total lung volume)	0.286	**<0.0001**	0.316	**<0.0001**	0.175	**0.005**
Reticulation (% of total lung volume)	0.306	**<0.0001**	0.407	**<0.0001**	0.285	**<0.0001**
Honeycomb (% of total lung volume)	0.039	0.534	0.087	0.173	0.134	**0.033**
PVV (cm^3)	0.068	0.284	0.322	**<0.0001**	0.253	**<0.0001**
PVV (%)	0.448	**<0.0001**	0.473	**<0.0001**	0.262	**<0.0001**

Values in bold signifies p<0.05.
CALIPER, Computer-Aided Lung Informatics for Pathology Evaluation and Rating; CPI, Composite Physiological Index; FVC, forced vital capacity; GGO, ground glass opacity; PVV, pulmonary vessel volume; TLCO, transfer factor of lung for carbon monoxide; TLF, total lung fibrosis.

Table 3 Correlation of blood leucocytes with CALIPER parameters and lung function (n=171 patients, Pearson's correlation).

Blood leucocytes	Monocytes		Neutrophils		Lymphocytes	
Baseline metrics	**r**	**P value**	**r**	**P value**	**r**	**P value**
CALIPER scores						
TLF (/litre)	0.040	0.606	0.208	**0.007**	0.136	0.082
PVV (%)	0.110	0.164	0.259	**0.001**	0.019	0.809
Lung function tests						
FVC%	0.096	0.101	0.127	**0.029**	0.014	0.814
TLCO%	0.104	0.080	0.104	0.079	0.085	0.155
CPI	0.103	0.081	0.091	0.127	0.022	0.708

Values in bold signifies p<0.05.
CALIPER, Computer-Aided Lung Informatics for Pathology Evaluation and Rating; CPI, Composite Physiological Index; FVC, forced vital capacity; PVV, pulmonary vessel volume; TLCO, transfer factor of lung for carbon monoxide; TLF, total lung fibrosis.

Table 4 Cox proportional hazard analysis for progression of lung fibrosis.

Multivariate cox regression	HR	95% CI	P value
Model A: Increase in TLF≥7.8%/litre between CT1 and CT2			
Age at CT	0.96	0.88 to 1.05	0.404
Male	0.03	0.01 to 0.56	**0.019**
Change in lung volume (%)	0.86	0.80 to 0.93	**<0.001**
Total lung fibrosis (/litre) on first CT	1.12	1.05 to 1.20	**0.001**
Total PVV (%)	1.03	1.01 to 1.05	**0.014**
Low attenuation areas (%)	1.06	0.94 to 1.20	0.318
Monocyte (×10^3/µL)	1.35	0.10 to 18.38	0.820
Neutrophil (×10^3/µL)	1.81	1.10 to 2.99	**0.020**
Lymphocyte (×10^3/µL)	0.26	0.08 to 0.90	**0.034**
Harrell's Index of concordance=0.91			
Model B: Increase in TLF≥10%/litre between CT1 and CT2			
Age at CT	0.93	0.84 to 1.04	0.208
Male	0.02	0.02 to 0.46	**0.016**
Change in lung volume (%)	0.82	0.73 to 0.92	**<0.001**
Total lung fibrosis (/litre) on first CT	1.13	1.03 to 1.25	**0.013**
Total PVV (%)	1.04	1.01 to 1.07	**0.037**
Low attenuation areas (%) (%)	1.10	0.97 to 1.25	0.142
Monocyte (×10^3/µL)	2.37	0.09 to 62.20	0.604
Neutrophil (×10^3/µL)	2.66	1.35 to 5.25	**0.005**
Lymphocyte (×10^3/µL)	0.30	0.07 to 1.24	0.096
Harrell's Index of concordance=0.93			
Model C: Decline in FVC>10% between CT1 and CT2			
Age at CT	1.01	0.92 to 1.11	0.856
Male	0.40	0.07 to 2.31	0.308
%FVC baseline	0.96	0.92 to 0.99	**0.049**
Monocyte (×10^3/µL)	0.96	0.03 to 28.51	0.979
Neutrophil (×10^3/µL)	1.17	0.91 to 1.52	0.222
Lymphocyte (×10^3/µL)	0.71	0.30 to 1.71	0.448
Harrell's Index of concordance=0.90			

HRs in multivariate model generated for outcomes of increase in fibrosis on follow on CT scan in three models as described in three models—model A—increase in TLF≥7.8 %/litre, (model B) increase in fibrosis≥10%/litre and (model C) relative decline in absolute FVC>10%. Change in lung volume is measured between CT1 and CT2. Leucocyte levels are presented as continuous variables (outcome for dichotomised values are shown in online supplemental table 3).
Values in bold signifies p<0.05.
FVC, forced vital capacity; PVV, pulmonary vessel volume; TLF, total lung fibrosis.

50.7% of patients who underwent repeat CT were receiving antifibrotics at first CT. When adjusted for antifibrotic use and comorbidities, significance was preserved for neutrophils, NLR and SIRI (online supplemental tables S4,S5).

Association between blood leucocyte levels and mortality

During the study period, 60 all-cause deaths (35.1%) were reported in this cohort. Leucocyte levels were dichotomised by median values (figure 1) or normal reference range limits (online supplemental figure S1). Significantly shorter survival times were observed for cases dichotomised by median monocyte count (p=0.033) and neutrophil levels (p=0.0180) (figure 1), and for cases dichotomised by median NLR, MLR and SIRI values (online supplemental figure S1). Shorter survival times were also observed for those with TLF, PVV and FVC greater than the median value (TLF>3.30%/L, p<0.001; PVV>4.02%, p<0.001 and FVC>76.6%, p<0.001) (figure 1).

DISCUSSION

In this study, we showed that when progression of disease is categorised specifically by increase in amount of fibrosis, quantified by an automated quantitative scoring modality, neutrophil levels rather than monocytes were

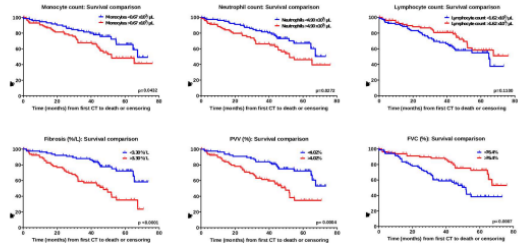

Figure 1 Kaplan-Meier curves for time to mortality for monocyte, neutrophil, lymphocyte levels, TLF, PVV and FVC, all at baseline. Leucocyte levels and CALIPER variables were dichotomised by median value. CALIPER, Computer-Aided Lung Informatics for Pathology Evaluation and Rating; FVC, forced vital capacity; PVV, pulmonary vessel volume; TLF, total lung fibrosis.

the key immune correlate with progression in amount of fibrosis in the lungs. MLR, NLR and SIRI were also associated with progression. Although monocyte count was not associated with disease progression in this cohort, importantly, as with other studies, higher monocyte levels in our cohort were associated with mortality.[4 21] Our findings suggest that neutrophils may be a contributor to active accumulation of fibrosis in IPF, supported by previous findings of high neutrophils in bronchoalveolar lavage (BAL) of IPF patients,[22] and more recent findings of higher NLR in IPF patients with greater rates of FVC decline.[5]

Neutrophils have long been associated with immunopathogenesis of IPF. Neutrophilia in BAL specimens from IPF patients is associated with earlier mortality.[22] Neutrophil elastases (NE) are elevated in IPF BAL samples,[23] and experimental data using murine models suggest that NE activates transforming growth factor-ß pathway and fibroblast proliferation.[24] An intriguing and newly identified fibrosis-promoting function of neutrophils is generation of neutrophil extracellular traps (NETs).[25] These proinflammatory collections of chromatin and neutrophils regulate both immune cell function and fibroblast activation.[26] While a specific association with IPF has yet to be fully described, enhanced detection of intrapulmonary NETs has been reported in bleomycin models and in non-IPF fibrotic ILD studies.[27 28] Here, we are able to link blood neutrophil level measured at baseline CT specifically with progression in amount of fibrosis over time. Further prospective translational studies are required to establish the regulatory role of neutrophils, NET formation over time and cytokine and chemokine activity at different stages of fibrosis.

In addition to TLF change, PVV measures also correlated with neutrophil count. PVV is an intriguing quantitative variable that has proved a consistent predictor of disease severity, progression and mortality in ILD studies.[12 14 29–31] Our findings are supportive of the original work performed by Jacob *et al* who demonstrated that PVV% was independently associated with FVC decline,[32] and Chung *et al* who demonstrated PVV negatively correlated with TLCO%.[33] In our study, PVV was independently associated with progression of fibrosis. The pathological mechanisms linking PVV with adverse ILD outcomes are not fully understood and several theories have been suggested. Blood perfusion is reduced in areas of pulmonary fibrosis,[34] but increased in adjacent areas of unaffected lung.[35] Jacob *et al* postulated that correlation between ILD extent and vessel calibre may represent regional elevation in pulmonary artery pressures in mildly fibrotic lung or destruction of the capillary bed in more advanced disease leading to neovascularisation and diversion of blood to unaffected lung areas.[12] Another possible explanation relates to the negative intrathoracic pressures required of non-compliant fibrotic lungs to generate adequate inspiratory volumes. This could in turn exert additional 'tractional' force on the lung vasculature resulting in dilatation in fibrotic regions in a traction-like phenomenon, akin to traction bronchiectasis.[33]

The trend of lower lymphocytes count with progression of fibrosis mirrors the findings of previous studies.[5 36] Lymphocytic aggregates are a recognised pathological feature of IPF lesions.[27 37] The association between low blood lymphocyte count and adverse outcomes in IPF is currently unknown but could be explained in part by lymphocyte dysfunction,[38] and the sequestering of lymphocytes into sites of inflammation, such as the fibrotic lung. We note that post hoc analysis of the ASCEND and CAPACITY studies Nathan *et al* reported that serial increase in NLR over 12 months was associated with mortality.[39]

In our study, we report association between higher neutrophil count and lower lymphocyte count taken from full blood count analysis with progression of fibrosis scoring on HRCT. To our knowledge, this

specific association has not been reported elsewhere. Other published studies and post hoc analyses have only reported leucocyte association with mortality, hospitalisation and FVC decline.

Our findings should be framed within the following limitations. First, the retrospective nature of the study meant that we selected patients who had repeat CT scans, which were clinically indicated. This introduced a selection bias towards patients who had a clinical reason to have a CT scan—often, this is worsening in disease either over time or acutely, and CT scans would be performed at different/non-uniform time intervals. Unfortunately, we did not have access to blood leucocyte data measured at the time of the second CT scan. Analysing blood leucocytes/ratios at second CT could may have provided additional information, especially with regard to how any relative change in blood count/ratio may differ between patients with progression of fibrosis or stability at second CT scans. Although the leucocyte levels were measured at the time of the first (presenting) CT scans, this group of patients could have a different disease trajectory compared with those who did not have a second CT scan. There is a possibility that neutrophil levels are more likely a driver in those who are clinically deteriorating. The baseline correlation data (n=171 patients), however, are not limited by these factors. Further analysis of prospective validation cohorts would be required to investigate whether neutrophil count, measured in peripheral blood, could identify patients at greater risk of progression of fibrosis in IPF, and consequently facilitate prognostication of the disease.

From the point of fibrosis scoring, the sum of GGO, reticulation and honeycombing was used as the TLF score.[14] Although each scan was reported by a radiologist who 'called' the GGO as fine fibrosis and the overall pattern as probable or definite UIP, other causes such as pulmonary oedema, acute exacerbation and infection could have contributed to this CT finding.[10]

Nevertheless, our study shows that greater neutrophil count was significantly associated with quantifiable increase in fibrosis on imaging of the lungs in IPF. Further studies will be required to validate this finding.

Contributors AA conceived project, conducted analysis, interpreted data and wrote the paper. EF, PS and RKH provided guidance with project. RB provided guidance with project and assisted with defining search criteria. L-PH conceived project, interpreted data, wrote paper and supervised the study. L-PH accepts full responsibility for the work and/or the conduct of the study, had access to the data, and controlled the decision to publish.

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Competing interests AA, EF, RB, L-PH and RKH report no relevant conflict of interests. PS has received consultancy fees from Trevi Therapeutics, and lecture fees from Boehringer Ingelheim, but no other conflict of interest.

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Not applicable.

Ethics approval The study was part of a study examining factors associated with progression of IPF (ethical approval 14/SC/1060 from the Health Research Authority and South-Central National Research Ethics Service).

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