University of Sassari

“Genomic profiling by next-generation sequencing of idiopathic pulmonary fibrosis in Sardinia”

by

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SUMMARY OF THE THESIS

Idiopathic Pulmonary Fibrosis is a rare and poorly understood progressive disease of the lungs that rapidly leads to respiratory failure and death if left untreated. Two antifibrotic agents, pirfenidone and nintedanib, were approved in 2014 but can only slow down the disease. Current challenges include the need for new therapeutics and the identification of predictive markers of disease progression and response to treatment.

This thesis is based on original research on the somatic mutational landscape of IPF performed with genomic sequencing tools that are best known in the field of oncology. This orientation in our research methodology is based on current knowledge that IPF and lung cancer share several features both at clinical and biological level, and commits to the hypothesis that the study of cancer-related molecular alterations may prove to be a good place to start when investigating for novel therapeutic approaches for IPF.

We hope that our work may increase our understanding of the disease mechanisms of IPF, and lay ground for further research on expanded patient cohorts.

ABSTRACT

Idiopathic pulmonary fibrosis (IPF) is a devastating disease characterized by progressive scarring of the lung and histopathological features of Usual Interstitial Pneumonia (UIP). The pathogenesis of the condition appears complex, and uncertainties remain regarding clinical and molecular analogies with cancer, with no knowledge on somatic alterations within the fibrotic lung genome.

We aimed to explore the genomic profile of lung tissue from patients with IPF, and to investigate any relevant molecular alterations in cancer-related genes. To do so, we selected surgical lung tissue specimens from 33 patients. The isolated DNA from these samples was analysed by next-generation sequencing (NGS) with a panel that targets 49 oncogenes.

Our research uncovered 87 somatic alterations in a total of 12 oncogenes. The presence of ≥ 3 variants correlated positively with longer survival rates ($p = 0.049$). We also negatively correlated the presence of a KIT gene variant with the presence of a radiological UIP pattern.

To our knowledge, this is the first study that explores the genomic landscape of IPF tissue using NGS technology. Although we detected somatic alterations in several oncogenes, none of them confer sensitivity to known molecular targeted drugs. We observed longer survival in those patients harbouring ≥ 3 mutations. We warrant further, more extended NGS analysis of larger cohorts to provide further insight into the role of somatic mutations in the pathogenesis of IPF.

In Memoriam

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Cantàe.

CONTENTS

LIST OF ACRONYMS AND DEFINITIONS

AIP: acute interstitial pneumonia
ATS: American Thoracic Society
AVATS: awake video-assisted thoracic surgery
ERS: European Respiratory Society
JRS: Japanese Respiratory Society
ALAT: Latin American Thoracic Society
AP1: activator protein 1
ARDS: acute respiratory distress syndrome
BM: basement membrane
CCL: C-C Motif Chemokine Ligand
COP: cryptogenic organizing pneumonia
CTD-ILD: connective tissue disorder-associated ILD
CTGF: connective tissue growth factor
DIP: desquamative interstitial pneumonia
DLCO: diffusion lung capacity of carbon monoxide
ECM: extra cellular matrix
EGFR: epidermal growth factor receptor
EMT: Epithelial to mesenchymal transition
ERK: extracellular signal-regulated kinases
FGF: fibroblast growth factor
FPF: familial pulmonary fibrosis
FVC: forced vital capacity
GTP: guanosine triphosphate hydrolase
GWAS: genome-wide associated studies
HP: hypersensitivity pneumonitis
HRCT: high resolution computed tomography
IGF-1: insulin-like growth factor-1
IIPs: idiopathic interstitial pneumonias
IL: interleukin
ILA: interstitial lung abnormalities
ILD: interstitial lung disease
IPAF: interstitial pneumonia with autoimmune features

IPF: Idiopathic pulmonary fibrosis

JNK: Jun N-terminal kinases

LC: lung cancer

LIP: lymphoid interstitial pneumonia

MAP: mitogen activated protein

MET: mesenchymal-epithelial transition

NGS: next generation sequencing

NSCLC: non-small cell lung cancer

NSIP: nonspecific interstitial pneumonia

PARN: poly(A)-specific ribonuclease

PCR: polymerase chain reaction

PDGF: platelet derived growth factor

PI3K)/AKT: phosphatidylinositol-3-kinase

PPFE: pleuroparenchymal fibroelastosis

RB-ILD: respiratory bronchiolitis-associated interstitial lung disease

ROS: reactive oxygen species

RTEL1: regulator of telomere elongation helicase 1

SCC: squamous cell carcinoma

SCF: mast/stem cell growth factor

SFTPA1: surfactant protein A 1

SFTPA2: surfactant protein A 2

SFTPC: surfactant protein C

SMAD: small mother against decapentaplegic

STS: short telomere syndromes

TERC or hTR: telomerase RNA component

TERT: telomerase reverse transcriptase

TKI: tyrosine kinase inhibitor

TKR: tyrosine kinase receptor

TGF-β: tumour growth factor beta

TNF-α: tumour necrosis factor alpha

TRB: terminal and respiratory bronchiole cells

TRB3: exogenous tribbles homolog 3

UIP: usual interstitial pneumonia

VEGF: vascular endothelial growth factor

WNT: wingless-related integration site

1. INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a chronic condition characterised by progressive, irreversible fibrotic scarring of the lung and respiratory failure. The disease is commonly lethal within a few years after diagnosis and no cure is yet available, although two anti-fibrotic drugs, pirfenidone and nintedanib, can help slow down lung tissue damage.

A major obstacle in preclinical drug development in IPF is the incomplete understanding of the pathogenic mechanisms involved and, consequently, the formulation of safe and effective therapeutic strategies. So far, the phenomenon of epithelial to mesenchymal transition (EMT) is accepted as the main responsible for the abnormal deposition of extra cellular matrix (ECM) by proliferating myofibroblasts. Tumour growth factor beta (TGF-β) production, which is thought to sustain these events, is inhibited by antifibrotic agent pirfenidone, while nintedanib has a broader spectrum of action by reducing the release of profibrotic mediators fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF).

Interestingly, the molecular processes leading to lung fibrosis have been analogized to oncogenic events. Indeed, it has long been acknowledged that lung cancer (LC) and IPF share a few relevant characteristics, such as common risk factors - tobacco smoke, male sex, older age - and progressive behaviour. Moreover, IPF itself is an independent predisposing condition for development of LC, suggesting that the two diseases may hold similar alterations at a molecular level.

In LC, recent advances in molecular and genomic research have led to the identification of novel protein targets for which successful drugs have been developed, causing a profound change in treatment strategies as new agents are released every year. By contrast, the molecular landscape of somatic alterations in IPF is yet to be explored. The few studies available on this topic have so far focused on cases where IPF and LC are concomitant, which could act as a confounding factor when studying the prevalence and role of somatic changes in IPF alone.

To investigate the molecular pathogenesis of pulmonary fibrosis in relation to cancer-related genes, we evaluated the genomic profile of lung tissue from patients with IPF with the use of next generation sequencing (NGS) technology.

2. BACKGROUND AND CONTEXT

2.1 WHAT IS IPF?

The eponym "Diffuse Interstitial Fibrosis of the Lungs" was introduced by American doctors Louis Hamann and Arnold Rich, who in 1935 described a few unusual cases of pulmonary fibrosis with rapid, fatal course similar to acute respiratory distress syndrome (ARDS) at the Johns Hopkins Hospital in Baltimore[1]. Although the clinical features displayed by each case differed from those of the others, the histopathological findings in the lungs at postmortem examination were all of the same nature. In fact, all cases exhibited a peculiar type of widespread inflammation which differed from that of ordinary pneumonia, with relatively few polymorphonuclear leukocytes and filling of alveolar spaces with albumin, fibrin and few red blood cells. This inflammation developed insidiously, in the absence of demonstrable bacteria, whereas there was an extraordinary proliferation of connective tissue resulting in extensive and progressive thickening of the walls of the alveoli, whose lumen became progressively obliterated and replaced with nonfunctional connective tissue. The pathological peculiarities shared by these cases led the researchers to conclude that they must arise from the same underlying morbid process, and that the variation in symptoms upon presentation could be explained by the stage this process had reached at the time the patients presented to their observation. The publications made by Hamman and Rich are crucial because they led to the first worldwide recognition of interstitial lung diseases (ILDs) and prompted physicians to start studying their causes and pathogenic mechanisms, thus representing the first milestone of modern knowledge in the field.

Today, the term "pulmonary fibrosis" loosely indicates a subtype of interstitial lung disease that is characterized by chronic and often progressive scarring of the lungs that can lead to life-threatening respiratory failure. In some cases, pulmonary fibrosis can be related to occupational exposure or is secondary to a systemic disorder, such as connective tissue disease or sarcoidosis. Other times, the disease remains confined to the lung and has unknown causes and is therefore called "idiopathic". In 2001, the American Thoracic Society (ATS) and European Respiratory Society (ERS) released the first joint classification of idiopathic interstitial pneumonias (IIPs), which were defined as seven specific entities: idiopathic pulmonary fibrosis (IPF), nonspecific interstitial pneumonia (NSIP), cryptogenic organizing pneumonia (COP),

acute interstitial pneumonia (AIP), respiratory bronchiolitis-associated interstitial lung disease (RB-ILD), desquamative interstitial pneumonia (DIP), and lymphoid interstitial pneumonia (LIP)[2]. An update was released in 2013 which introduced a group of rarer entities such as pleuroparenchymal fibroelastosis (PPFE), and again in 2015 with the proposed term of interstitial pneumonia with autoimmune features (IPAF)[3-4].

Among IIPs, IPF is by far the most widely studied. It is an almost uniformly fatal form of ILD, and unfortunately the most common. It mainly presents in adults over the age of 50, has male predominance and is associated with current or former smoking or other forms of exposure to toxic inhalants. Although most forms present sporadically, IPF can sometimes cluster in families with several members affected by idiopathic or even secondary forms of ILDs. IPF correlates with pathologic findings of usual interstitial pneumonia (UIP), which is a histologic hallmark of the disease so that the terms IPF and UIP are often used interchangeably. UIP is a chronic process that leads to the destruction of lung parenchyma and is considered the prototype of fibrosing interstitial lung diseases. Because of its progressive and irreversible nature, it is easily foreseeable how being able to identify UIP in a patient with respiratory disease yields great prognostic value.

The diagnosis of UIP requires the fulfilment of three main histological criteria:

1. a patchwork involvement of the lung at low magnification, with spatial heterogeneity that alternates areas of abnormal and normal parenchyma that stand side by side without transition zones;
2. distortion of normal background lung architecture by fibrosis and honeycombing; fibrosis is represented by irregular thick zones of collagen deposits that obliterate and destroy alveoli; honeycombing is characterized by enlarged airspaces lined by bronchiolar epithelium and frequently filled by mucus and inflammatory cells; honeycombing areas are usually surrounded by variable degrees of inflammation;
3. presence of few, sparse fibroblastic foci, which are small individual aggregates of spindle-shaped fibroblasts and myofibroblasts that are randomly located in the interstitium; their presence indicates ongoing collagen deposition and an active fibrotic process[5].

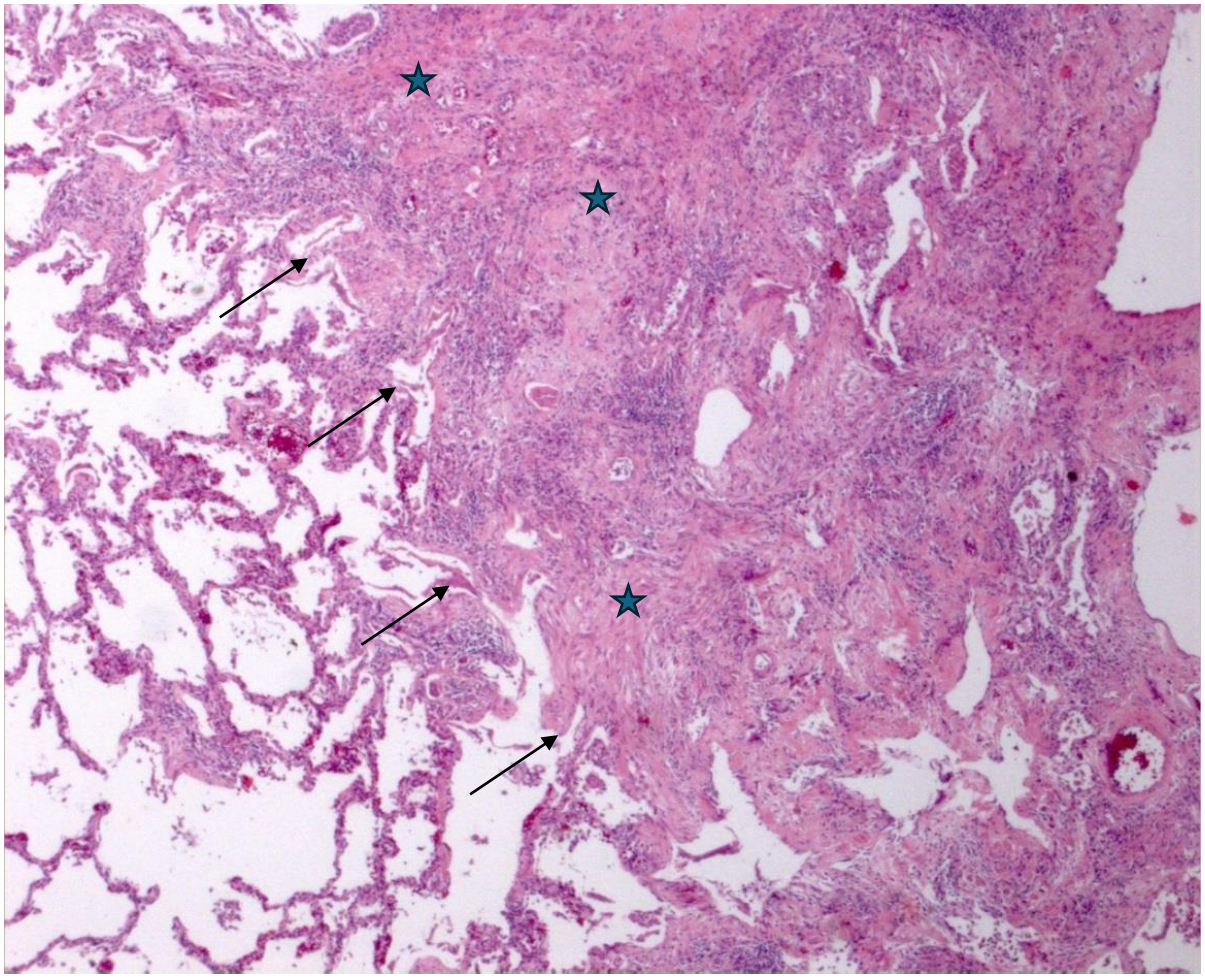


Figure 1. Histological UIP pattern with magnification of an area of advanced fibrosis (arrows) with widely distributed fibroblastic foci (stars); there are normal appearing alveolar walls in the left lower angle and dilated airspaces near the right upper angle.

UIP has been associated with specific radiologic features on high resolution computed tomography (HRCT). In 2018, the Fleischner Society provided the latest diagnostic criteria for the detection of radiological UIP pattern, which consist of fibrotic features of subpleural reticular opacities with peripheral bronchiectasis or bronchioloectasis and radiological honeycombing, all with a subpleural distribution and basal predominance[6]. In the same year, a guideline panel from American Thoracic Society, European Respiratory Society, Japanese Respiratory Society, and Latin American Thoracic Society (ATS/ERS/JRS/ALAT) recommended that a diagnosis of IPF requires the presence of the HRCT pattern of UIP and/or specific combinations of HRCT and histologic patterns, as well as the exclusion of other known causes of ILD[7].

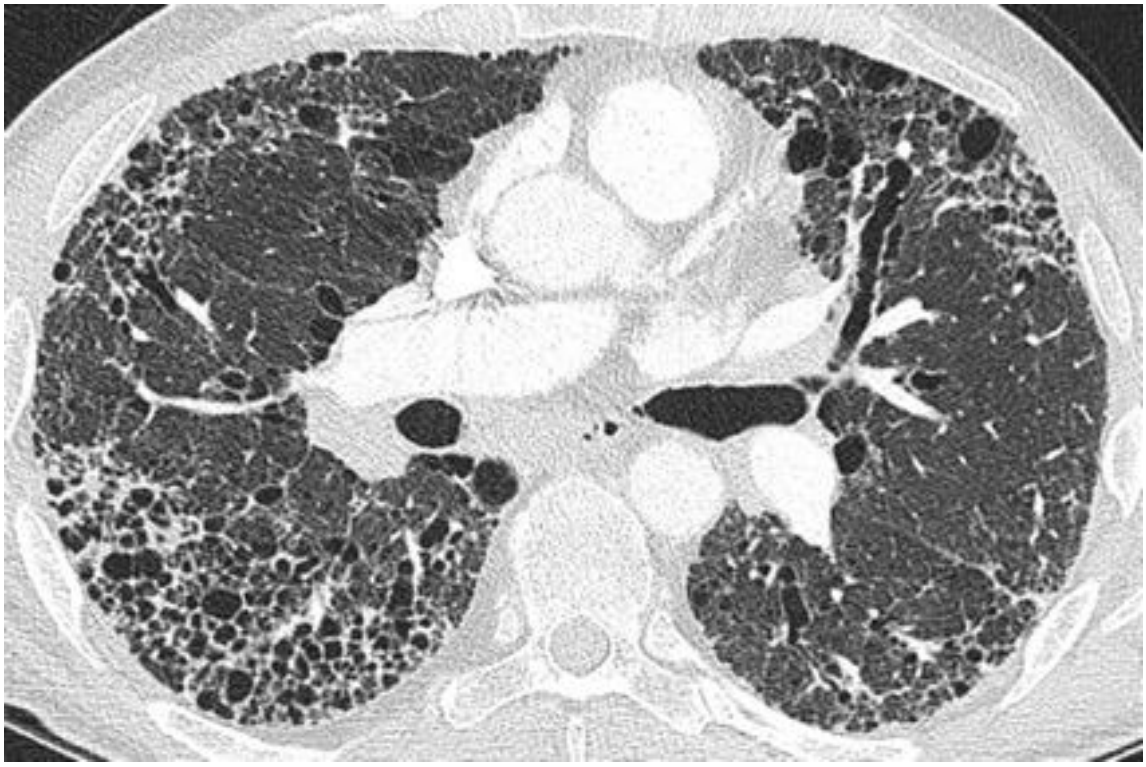


Figure 2. Radiological UIP pattern showing subpleural reticulation, peripheral bronchiectasis and honeycombing.

So, the definition of IPF requires clinical input in addition to the presence of UIP. In fact, UIP is not exclusive to IPF and it can be found in other lung diseases such as chronic hypersensitivity pneumonitis (HP), connective tissue disorder-associated ILD (CTD-ILD), asbestosis, drug-induced or radiation-induced drug toxicity, all of which should be included in the clinical differential diagnosis. Clinical presentation alone is not specific and insufficient for the diagnosis of IPF, because most patients with ILDs – or indeed potentially any respiratory disease - present with progressive dyspnoea and chronic cough and might have restrictive defects on spirometry. Furthermore, there may be a wider range of possible clinical phenotypes in IPF, and more recent experience indicates that IPF can be diagnosed in younger individuals who have mild or subclinical disease[8].

IPF is considered by all standards a rare disease. As of the latest ERS report published in 2015 on the subject, incidence rates were estimated to be 3–9 cases per 100,000 persons per year in Europe and North America, with some evidence that the number of diagnoses is rising[9]. However, great variability can be observed when looking at epidemiological data produced over time and across countries, and the true incidence of IPF remains vague. A targeted literature review conducted by Maher *et al*. identified 22 studies that provided incidence and/or prevalence data between 2009 and 2020. They reported that the adjusted incidence and prevalence of IPF are estimated to be in the range of 0.09–1.30 and 0.33–4.51 per 10,000 persons, respectively[10]. A systematic literature review published in 2023 by Gupta *et al*., which included 80 studies published between 2015 and 2021, reported a prevalence of IPF ranging from 7 to 42 cases per 100,000 persons, but failed to derive incidence estimates due to worldwide lack of uniformity in the reporting methodology[11].

Indeed, the reported temporal trajectory for IPF incidence could convey several conditioning factors that are not necessarily linked to its "true" aetiological and triggering agents. For a start, there have been important improvements in the diagnostic approach and changes in nomenclature of IPF over the last two decades, that have certainly influenced its reporting system. These changes have not been adopted simultaneously across countries, which means that there are significant variations in IPF incidence reports over time across different geographic regions, particularly where a systematic tracking is not in place. Furthermore, recent years have put more emphasis of non-IPF ILDs. Their similarity with IPF disease behaviour and prognosis, especially when a UIP pattern is present, means that anti-fibrotic treatment can now be offered to people living with different forms of ILD other than IPF[12].

While this is certainly good news for a great number of patients, the priority on addressing disease behaviour rather than its true underlying aetiology may actually yield uncertainty when it comes to data interpretation on trends for IPF incidence.

2.2 IPF PATHOGENESIS AND TREATMENT

However cryptic the figures regarding the number of patients with IPF may be, there is far less doubt on the lethality of the disease: IPF has no known definitive cure and is plagued with bleak survival rates if left untreated. The median prognosis has been reported to range from 2 to 5 years, but the truth is the natural history of the disease is unpredictable, and patients can experience periods of rapid loss of pulmonary function – the so-called "accelerated phase" – and even present for the first time to medical attention when the condition is already terminal with severe respiratory failure[13-14]. These figures may come as unexpected for what is, after all, commonly known and defined as a chronic condition, likewise heart disease, diabetes, or arthritis. Instead, the epidemiological indicators of morbidity and mortality for IPF are far more similar to those of cancer[15]. The obvious conclusion is that, as much as it is considered a rare disease, the social burden of IPF is encumbered by its disabling effects and its life-limiting nature.

The lack of safe and effective therapeutic strategies for IPF is primarily explained by the incomplete understanding of its aetiology and pathogenesis. The road to the diagnostic definition alone of pulmonary fibrosis – or rather, the acknowledgment of IPF as a nosologically unique entity in the hodgepodge of diffuse parenchymal lung disorders - has required decades. Research on the underlying biological mechanisms of the disease has always been running parallel to its diagnostic dilemmas and has been hindered by the scarce applicability to real life context of preclinical animal models. In this regard, intra-tracheal or systemic administration of bleomycin in rodents has been widely used in research to create models of pulmonary fibrosis to reveal the mechanism of fibrogenesis and for evaluation of potential therapies[16]. Bleomycin, which was first discovered in 1962 in Japan, is as an anticancer drug that acts by induction of DNA strand breaks, thereby inducing inflammation and lung scarring[17]. However historically prevalent this prototype may have been, no animal model can fully summarize all aspects of human IPF biology and histopathology. There are

considerable anatomic differences between murine and human lungs, and, most importantly, a different biological response to external insults[18]. Furthermore, the fibrogenic process in the human lung may be operated by different molecular mechanisms than those of the murine model.

Nevertheless, a few important advances in the understanding of the pathogenesis of IPF have been made over the last two decades. Perhaps the first milestone is represented by ultrastructural lung tissue studies conducted in the 1980s and early 1990s, which demonstrated loss of integrity of the alveolar-capillary basement membrane (BM) and the disruption of the epithelial cells in fibrotic lungs. In normal conditions, the microscopic structure of adult lung tissue is represented by a delicate net of thin-walled air sacs called alveoli. The alveolar septum is made up of three components: surface epithelium (composed by type I and type II alveolar epithelial cells or pneumocytes), supporting tissue or interstitium, and alveolar capillaries. Type II pneumocytes compose about 5-7% of the alveolar surface area. They are metabolically highly active cells and synthesize and secrete pulmonary surfactant, chemokines and cytokines that regulate host defence. They also serve as progenitor cells and can regenerate type I cells or even terminal and respiratory bronchiole cells (TRB) after injury. There is a fused BM between capillary endothelium and type I cell epithelium that enables gas exchange through a dense amorphous layer of extracellular matrix, with regional variations in the subepithelium and discontinuities beneath type II cells. Alveolar spaces are patrolled by macrophages, which move from one alveolar space to another and can increase in number during disease[19-21].

Studies on fibrotic lung tissue showed the inability of the alveoli, after some sort of injury to the basement membrane, to recreate a normal epithelium from type I pneumocytes despite the intense hyperplasia of type II pneumocytes. Alveolar spaces are instead infiltrated by proliferating fibroblast, macrophages and inflammatory cells. This upheaval results in the obliteration of adjacent alveoli and distortion of normal architecture which now sees foci of hyperactive and proliferating overlayed fibroblasts. These act as the effector cells of the fibrogenic process by deposition of large amounts of extracellular matrix (ECM) made by collagen fibres and fibronectin[22].

This description of the ultrastructural composition of IPF has long sustained the belief that chronic inflammation has a central role in the disease: an initial injury or inflammatory event

damages the alveolar space integrity causing the loss of type I pneumocytes, the recruitment of interstitial cells and the deposition of ECM, mirroring dysregulated wound repair response. The process is perpetuated in cycles by an unresolved chronic inflammation, causing further deposition of collagen and progression to pulmonary fibrosis. Today, the chronic inflammation model has been largely abandoned because there is a body of evidence coming from both *in vitro* and *in vivo* studies that demonstrates that lung fibrogenesis is a consequence of the activation of specific molecular pathways that result in what is called epithelial-mesenchymal transition (EMT). In this process, there is suppressed expression of E-cadherin, a core transmembrane protein that is required for maintaining the intercellular tight junctions between pneumocytes. The loss of cell-to-cell attachment is followed by profound biochemical changes and epithelial cells undergo phenotypic transition to fully differentiated mesenchymal cells such as fibroblasts and myofibroblast. The mesenchymal phenotypes include motility and migratory capacity, resistance to apoptosis, and increased production of ECM components.

The biological process of EMT has been well acknowledged in several fields of research for some time. EMT is a manifestation of cell plasticity that pertains the ability to change from an epithelial to a mesenchymal phenotype through activation of a certain cell programme. There are three known types of EMT described by the literature, each having a central role in distinct physiological and pathological biological scenarios:

- Type 1 EMT in essential in gastrulation, embryogenesis and tissue differentiation. In this setting, certain epithelia can transition back and forth epithelial and mesenchymal states, to generate secondary epithelia from mesenchymal cells. This type of EMT is not associated with inflammation, fibrosis, or dissemination.

- Type 2 EMT, on the other hand, causes an invasive phenotype and is associated with wound repair, tissue reconstruction and organ fibrosis. This type of EMT is provoked by tissue injury, and its ongoing occurrence depends on a myriad of signalling molecules that are released by locally activated fibroblasts and inflammatory cells. It is characterized by the production of ECM that is composed by different types of collagens, elastins, and other stromal components. While it normally ceases once the inflammation has diminished, type 2 EMT can become dysregulated and result in the fibrotic destruction of the epithelial layers that form the parenchyma. Over the past few decades, research has indicated that type 2 EMT may contribute to organ fibrosis

through the differentiation of epithelial cells into fibroblasts in response to injury to the epithelium. This has been observed not only in the lung, but also in other organs.

- Type 3 EMT, finally, occurs in the context of cancer and is triggered by genetic and epigenetic alterations in genes that are involved in tumour growth and suppression. This programme is hallmarked by excessive epithelial proliferation and the acquisition of an invasive phenotype that leads to systemic metastatic spread of cancerous cells[23].

Although all three types of EMTs ultimately partake in the acquisition of cell motility, they clearly happen in very distinct biological contexts and are induced and maintained by different molecular programmes. In IPF, the (myo)fibroblast is believed to play a central role in fibrogenesis through the formation of fibroblastic foci and the deposition of fibrotic matrix compounds, but its origin is debated. On one end, EMT has been well described in IPF through tissue detection of (myo)fibroblasts that appear to have derived from the lung epithelium itself[24]. On the other hand, there is uncertainty as to whether myofibroblasts can also originate from the conversion of resident intrapulmonary mesenchymal elements. This hypothesis derives from *in vitro* evidence that fibroblasts can differentiate into myofibroblasts and avoid apoptosis thanks to the protection provided by TGF-β1[25]. Some studies suggest that myofibroblasts in lung fibrosis may even derive from the differentiation of circulating bone-marrow progenitor cells[26]. These three potential sources of myofibroblasts are not mutually exclusive, but their relative contribution is yet to be exactly determined.

With regards to TGF-β1, its role in IPF is pervasive and transversal and has proven to go well beyond the simple sustaining of fibroblast activity. TGF-β1 is a highly conserved secreted protein member of the transforming growth factor beta superfamily of cytokines, which includes TGF-β1, TGF-β2, and TGF-β3. These peptides are involved in several biological processes thorough regulation of a vast array of cellular activities which include cell growth and proliferation, cell differentiation, and apoptosis. TGF-β1 can be secreted by multiple lineages of immune cells, including macrophages, and mesenchymal (stromal) cells, and can therefore be expressed in virtually every type of tissue . The expression of TGF-β1 is enhanced in the lung tissue of IPF patients, and *in vivo* studies proved that it is secreted by the alveolar macrophages[27]. TGF-β1 acts as a primary inducer of EMT and is involved in several molecular signalling processes that pertain lung fibrogenesis. For example, it promotes epithelial

apoptosis and supresses normal alveolar wall repair, all the while protecting myofibroblasts from apoptosis. It also encourages fibroblast proliferation and leads their differentiation into myofibroblast through the upregulation of other growth factors such as connective tissue growth factor (CTGF) and VEGF[28].

Basic and clinical research studies have shown that TGF-β1 acts through several signalling pathways. These pathways are complex and variously intersected, and their in-depth analysis is fundamental to understand how IPF pathogenesis works. The activated TGF-β receptor complex transduces signals through what are known as canonical and non-canonical pathways:

- The canonical signalling model uses small mother against decapentaplegic (SMAD) 2 and SMAD 3 proteins to transfer signals. SMADs are a family of proteins that are the main transducers for receptors of TGF-β superfamily and are critically important for regulating cell growth and cell cycle progression[29]. In many types of cancer, including lung cancer, defects of SMAD signalling can result in TGF-β resistance, causing dysregulation of cell proliferation[30]. The TGF-β1/SMAD signalling pathway contributes to IPF through regulation of myofibroblast differentiation, induction of EMT through SMAD 2/3-dependent mechanism, and direct fibrogenesis through promotion of collagen synthesis[31-32].

- The SMAD-independent signalling pathways, also referred to as non-canonical, involve a multitude of other intracellular cascades that are directly activated by ligand-occupied receptors to regulate downstream cellular responses. These include the phosphatidylinositol-3-kinase / protein kinase B (PI3K)/AKT and the mitogen activated protein (MAP) kinase pathways, both relevant to IPF pathogenesis[33]. TGF-β1 activates c-Jun N-terminal kinases (JNK) and AKT signalling pathways through PI3K, and directly promotes the production of tissue factor through AP1[34]. The prominent contribution of the PI3K/AKT cascade to IPF has inspired studies that aim to develop antifibrotic drugs that target this cascade. Omipalisib, a potent PI3K/AKT inhibitor, has been shown to slow fibrosis progression in mice and has passed phase I studies for safety.[35-37] The MAP kinase pathway model consist of JNK, p38 and extracellular signal-regulated kinases (ERK), which in turn promote EMT, myofibroblast proliferation and differentiation and protection from apoptosis[38-48]. Another non-canonical pathway involved in TGF-β1-promoted IPF is the Wnt/β-catenin cascade

(Wnt stands for wingless-related integration site), which leads to intracellular accumulation of β-catenin, an inductor of EMT that has been found in fibroblastic foci[49].

Other molecule signalling pathways that have been revealed to be involved in TGF-β-relevant IPF include platelet-derived growth factor (PDGF) through the upregulation of its receptor[50]. Interleukin (IL) 11 may also be an important mediator, and it seems that TGF-β1 increases the expression of its receptor in fibroblasts[51]. Other peptides include exogenous tribbles homolog 3 (TRB3), Fas (a molecule that mediates apoptosis) and insulin-like growth factor-1 (IGF-I)[52-54]. Moreover, TGF-β may promote IPF through epigenetic regulation, as it has been observed that it induces the upregulation of DNA methyltransferase and tet-methylcytosine dioxygenase 3 in fibroblasts[55]. Finally, it was also reported that TGF-β disturbs the oxidative balance through both the inhibition of glutathione production and the stimulation of reactive oxygen species (ROS) production in epithelial cells[56-57].

The centrality of TGF-β1 in lung fibrosis has made researchers put more attention in the role of alveolar macrophages, its secretory cells, in the pathogenesis of the disease[58-61]. Macrophages can activate classic pro-inflammatory pathways and secrete inflammatory cytokines such as tumour necrosis factor alpha (TNF-α). However, macrophages can also assume a pro-fibrotic metabolism. They are involved in wound repair pathways and can secrete fibronectin, pro-fibrotic matrix metalloproteinases and, most importantly, TGF-β. The understanding of all the TGF-β signalling pathways and their mechanism of action in starting and perpetuating the fibrotic process in IPF is fundamental to promote the development of new anti-fibrotic agents and has indeed become a research hotspot not only for IPF, but also for other fibrotic diseases such as myelofibrosis and liver fibrosis[62]. Pirfenidone was the first drug targeting TGF-β signalling pathway approved for the treatment of IPF, although its exact mechanism of action in not fully understood[63]. It is a small molecule that exerts its antifibrotic properties through the downregulation of TGF-β1 production, but it also has antioxidant radical-scavenging activity and reduces the production of inflammatory mediators IL1 and TNF-α. The approval of pirfenidone marked a watershed moment in pulmonary fibrosis therapeutics and was simultaneous with the approval for nintedanib, a potent small molecule inhibitor of the tyrosine kinase receptors (TKR) of PDGF, FGF and VEGF.

2.3 ROLE OF GENETICS IN IPF

The observation that IPF can affect several members of the same family prompted researchers to investigate on the genetic background of the disease. In the early 2000s, the breakthrough introduction of sequencing and genome-wide associated studies (GWAS) allowed the identification of potential genetic factors involved in the development of chronic diseases[64]. This methodology has resulted in a range of applications, such as gaining insight into a disease's underlying biology and pathogenesis, estimating clinical risk predictions, and inferring potential new drug development programmes. With regards to IPF, it is currently estimated that genetic factors contribute to at least one third of the total susceptibility of the disease[65]. However, the interplay between environmental and genetic factors is likely not equally proportionate for all cases.

Amongst common variants, the single nucleotide polymorphism (SNP) rs35705950 of the MUC5B gene has been validated in over 11 independent studies and is the most significant risk variant for both sporadic and familial forms of IPF[66,67]. Mucin 5B is a major component of respiratory tract secretions, and its overexpression conveys aberrant mucociliary clearance and tissue injury. In 2011, a genome-wide linkage study identified a gain-of function allele of rs35705950 in 34% of subjects with familial interstitial pneumonia, 38% of subjects with IPF and 9% of controls[68]. The same allele was later found to have increased frequency also in individuals with ILAs and rheumatoid arthritis-associated ILD[69,70]. Provocatively, although the presence of this variant increases susceptibility to pulmonary fibrosis, it also seems to confer a better prognosis in those patients that harbour it, who have a slower disease progression compared to those not carrying the allele[71].

Other polymorphisms that contribute to the disease are related to wound healing response, regulation of DNA repair at telomeres, and host defence. Gene expression studies have distinguished enhanced patterns pertaining genes involved in developmental programmes and ECM metabolism, as well as genes encoding growth factors, complement, immunoglobulins, and chemokines[72].

The first genetic studies on pulmonary fibrosis focused on families with monogenic patterns of inheritance of the disease. It is now presumed that rare or even private risk gene variants with a high degree of penetrance play an important role in these presentations. These can be

broadly divided into those affecting surfactant-related genes and those pertaining telomere-related genes[73].

The most fully studied risk variants involving surfactant biology are surfactant protein C (SFTPC), A1 (SFTPA1) and A2 (SFTPA2). They have been described in both inherited and sporadic pulmonary fibrosis[74].

There is a large group of mutations involving the SFTPC gene. They result in different classes of alveolar cell dysfunction with a broad and poorly characterized spectrum of manifestations of ILD, ranging from lethal neonatal respiratory distress syndrome, to ILD in childhood or early adulthood[75]. The most common in both children and adults is the I73T substitution, which hampers surfactant secretion of the alveolar type II cells through intracellular trafficking defect of the pro-peptide[76]. Other SFTPC mutations map to the BRICHOS domain, which promotes the correct folding of the protein. SFTPC BRICHOS mutants initiate cell-death signalling pathways through toxic intracellular accumulation of the pro-peptide, resulting in alveolar damage and subsequent scarring[77].

Surfactant apolipoproteins SFTPA1 and SFTPA2 are secreted by alveolar type II and bronchiolar cells and regulate surfactant composition and innate immunity[78]. SFTPA2 variants were first identified in families with a Mendelian inheritance pattern of pulmonary fibrosis, with subsequent further reports of Familial Pulmonary Fibrosis (FPF)-associated variants in both SFTPA1 and SFTPA2[79,80]. A recent whole genome sequencing study reported a 1.3% prevalence of SFTPA2 variants in an unselected cohort of IPF, suggesting that SFTPA2 could be and underrecognized contributor of sporadic cases of IPF[81,82].

Telomeres are non-coding regions of repeat DNA sequences that are found at the ends of linear chromosomes and that are lost at each cell division, resulting in progressive shortening over time. Their role is to maintain chromosomal integrity and stability and protect genetic data. To prevent the loss of coding regions, enzymes called telomerase elongate the chromosome ends by adding nucleotide repeats after each cell division, an activity that is mostly evident at the embryonic state. Premature shortening of the telomere regions has been found in accelerated degenerative diseases and in cancer[83].

In IPF, mutations in genes related to telomere maintenance and integrity have been identified in up to 25% of familial cases and in 1-3% of sporadic cases. Conversely to the precocious manifestation of surfactant-protein abnormalities, pulmonary fibrosis secondary to

telomere shortening usually presents in adults in the setting of FPF or, more rarely, in sporadic cases[84-86]. The genes that are most frequently mutated are:

- Telomerase reverse transcriptase (TERT);
- Telomerase RNA Component (TERC or hTR);
- Regulator Of Telomere Elongation Helicase 1 (RTEL1);
- Poly(A)-Specific Ribonuclease (PARN).

Mutations in TERT or TERC genes are reported in 8-15% of cases of FPF and are in fact a major cause of monogenic inheritance of the disease. Interestingly, mutations in TERT/TERC genes are frequently associated with a spectrum of extrapulmonary involvement and accelerated ageing manifestations known as Short Telomere Syndromes (STS). In addition to PF, which is the most frequent manifestation, other commonly affected organs include skin, gastrointestinal tract and bone marrow with varying phenotypes[86-88].

RTEL1 mutations have been reported in 5-9% of familial ILD with a heterogeneous pulmonary phenotype ranging from UIP to PPFE. There is a lower prevalence of extrapulmonary manifestations compared to TERC/TER mutations, such as haematological abnormalities and liver disease[89-91].

PARN mutations have been associated with FPF and rare sporadic cases, but the phenotype is heterogeneous and poorly described. As with RTEL1, extrapulmonary manifestations are less frequent compared to TERT/TERC mutations[90,92,93].

Interestingly, telomere dysfunction has been linked to sporadic IPF cases regardless of the presence of telomere risk gene variants, a fact that suggests the role of non-hereditary factors such as oxidative stress and inhalants exposure[88].

2.4 IPF AND CANCER

Patients affected by IPF display phenotypic features and traits that are highly reminiscent of those observed in lung cancer. For a start, they share relevant predisposing risk factors, namely tobacco exposure, male sex and older age. These characteristics, however, are not specific of either disease and are, in fact, extremely common features of the majority of patients with respiratory affections. The single most impacting common trait of LC and IPF is perhaps their progressive nature and poor prognosis. In addition, the risk of developing LC in patients who already have a diagnosis of IPF is significantly increased even after adjustment for other risk factors, with prevalence figures as high as 48%[94]. With more IPF patients living longer thanks to the introduction of anti-fibrotic treatments, these statistics are expected to increase. Finally, patients who present with concurrent IPF and LC have a much worse prognosis than the single diseases combined, which evokes a unique and specific pattern of initiation of cancer in the fibrotic lung[95].

In the previous chapters, we have explained how the pathogenesis of IPF entails an aberrant tissue injury response that perpetuates the deposition of collagen through a dysregulated crosstalk between epithelial and mesenchymal cells, and on the central role of TGF-β as a profibrotic molecule. A very interesting investigation regards the so-called TGF-β paradox, a phenomenon that has already been described in oncology. In a large variety of cells, TGF-β generally acts as a tumour suppressor and mediates antiproliferative effects, and has an antagonistic relationship with pro-inflammatory cytokines such as TNF-α and IL-1. However, TGF-β has a dichotomous nature and can assume a pro-inflammatory role in a different context. This two-faced characteristic of TGF-β is commonly seen in autoimmune diseases, where TGF-β is an IL-17 inducer in the presence of IL-6[96]. In early stages of cancer, TGF-β can switch its function and promote tumorigenesis owing to the induction of EMT-permissive microenvironment. Thanks to EMT, epithelial cells adopt typical mesenchymal cells phenotype, including reduced cell-to-cell adhesion, immune surveillance evasion, and invasive and metastatic properties. As we have anticipated in the pathogenesis section, this dual nature of TGF-β plays a central role in IPF by influencing EMT, myofibroblast differentiation and fibrogenesis[97]. In conclusion, TGF-β-driven EMT is certainly a pathogenic event shared by fibrogenesis and tumorigenesis.

4. PURPOSE OF THE THESIS

Based on the evidence collected over the last decades, it appears clear that the pathogenesis of IPF entails several mechanisms that are shared by cancerous process, a connection that we believe represents good research material when investigating on IPF.

The scope of this thesis is to demonstrate that the advances in research technology that have been beneficial in the field of lung cancer, can also help researchers to improve our understanding of the molecular background of IPF, and to provide insight on new clinical correlations and potential therapeutic perspectives.

To do so, we applied NGS technology to a study population of IPF patients and described somatic mutations in their fibrotic lung tissue samples. As a secondary purpose, we performed a survival analysis and sought to find clinical associations with the gene variants identified. Our results inspire a new approach to the study of IPF.

4. MATERIALS AND METHODS

4.1 PATIENTS

We retrospectively collected formalin-fixed and paraffin-embedded (FFPE) tissue sections from 33 patients with histologic-based diagnosis of IPF who underwent awake video-assisted thoracic surgery (AVATS) between July 26th 2016 and December 29th 2018 at Ospedale Brotzu in Cagliari, Italy. In all cases the surgical parenchymal biopsy was performed as part of ILD diagnostic work-up. All patients received a diagnosis of IPF through confirmation of histopathological features of UIP pattern as per ATS/ERS/JRS/ALAT Clinical Practice Guideline 2011[14].

Demographic, clinical and radiological information at the time of disease onset were collected from a surgical database. Clinical data included:

- smoking exposure;
- body mass index (BMI);
- Charlson Comorbidity Index (CCI): the CCI is a score that is used to predict 10-year mortality for a patient who may have a range of comorbidities for a total of 17 categories, namely cardiovascular disease, diabetes, or malignancies[98];
- pulmonary function testing at diagnosis, which comprised % predicted of forced vital capacity (FVC) and % predicted of diffusing capacity of carbon monoxide (DLCO).

HRCT imaging results were included as per the 2011 inter-society consensus guidelines[14].

Survival data were obtained from electronical records and analysed up to and including December 31st 2023.

4.2 SAMPLE PREPARATION AND NGS

Genomic DNA was isolated from formalin-fixed and paraffin-embedded (FFPE) lung tissue sections using the GeneRead DNA FFPE Kit (Qiagen, Hilden, Germany) as per

manufacturer's instructions. DNA concentrations were assessed by Qubit 2.0 Fluorometer with Qubit dsDNA HS (High Sensitivity) Assay Kit (Life Technologies, Carlsbad, CA, USA). NGS analyses were performed using the Ion GeneStudio S5 System with the Oncomine Focus Assay that includes a total of 49 cancer-related genes arranged in two primer pools. Libraries were generated starting from 10 ng of DNA per primer pool for a total of 20 ng of input DNA using the Ion AmpliSeq Library Kit Plus, barcoded with Ion Xpress Barcode Adapters (Life Technologies) and purified with Agencourt Ampure XP Beads (Beckman Coulter Life Sciences, Indianapolis, USA). The obtained polymerase chain reaction (PCR) amplicons were diluted to a final concentration of 70 pM and pooled together; emulsion PCR and Chip (Ion 540) loading steps were performed by the Ion Chef Instrument. Sequencing of libraries was done with the Ion S5™ System (Thermofisher). Sequencing data were processed with the Ion Torrent platform-specific pipeline software (Torrent Suite, V5.10.2). Ion Reporter™ Software V5.16 and Integrative Genome Viewer software (http://www.broadinstitute.org/igv) were used for variant annotation and reads visualizations, respectively.

4.3 BIOSTATISTICAL ANALYSIS

The variants retained from the NGS experiments were further investigated against VarSome (https://varsome.com) and COSMIC v92 database (Catalogue of Somatic Mutations in Cancer; https://cancer.sanger.ac.uk/cosmic). The clinical significance of all identified variants was examined using the standards and guidelines for the interpretation of sequence variants recommended by the American College of Medical Genetics and Genomics' (ACMG) Laboratory Quality Assurance Committee and the Association for Molecular Pathology (AMP).

Qualitative variables were reported as frequencies and percentage. Quantitative variables were expressed as mean ± SD if normally distributed and as median and interquartile range if not. The normality of the distribution was determined by Shapiro-Wilk test. Univariate and multivariate regression analyses were performed to assess the potential effect of demographic, clinical and genetics variables on survival. Survival analysis was conducted with Kaplan-Meier approach and the differences between subgroups were assessed by log-rank test. Statistical analysis was performed with R version 4.2.2 (The R Foundation for Statistical Computing, Vienna, Austria). All the results were considered significant for a $p< 0.05$.

5. RESULTS

Demographic, clinical and radiological characteristics of all 33 cases are shown in Table 1. The descriptive statistics are shown in Table 2.

Table 1: Characteristics of the study population.

Case	Sex	Age	% FVC	% DLCO	BMI	Smoke	CCI	HRCT pattern
1	M	52	88	60	25,7	Yes	5	UIP possible
2	F	70	N/A	N/A	N/A	N/A	N/A	N/A
3	M	62	119	81	29,4	Yes	3	Indeterminate for UIP
4	M	65	108	77	N/A	N/A	N/A	N/A
5	M	55	94	49	31,2	Yes	2	Alternative diagnosis
6	M	78	121	72	28,0	Yes	4	UIP possible
7	F	63	81	81	24,0	No	3	UIP definite
8	M	72	81	36	27,2	Yes	5	UIP definite
9	F	64	72	55	24,9	N/A	2	N/A
10	M	68	108	86	31,3	Yes	4	UIP definite
11	F	75	102	59	34,7	Yes	6	UIP definite
12	M	70	85	61	28,0	Yes	7	UIP definite
13	M	74	86	54	32,6	Yes	5	Alternative diagnosis
14	M	58	47	55	18,8	Yes	9	Indeterminate for UIP
15	F	61	58	N/A	22,9	Yes	4	N/A
16	F	73	75	64	25,3	Yes	5	UIP definite
17	M	78	46	34	32,7	Yes	4	UIP definite
18	M	78	80	71	26,7	Yes	7	N/A
19	M	66	138	71	25,5	No	4	UIP definite
20	M	73	N/A	N/A	26,8	No	4	N/A
21	M	61	79	53	35,4	Yes	3	Alternative diagnosis
22	M	71	38	38	27,7	Yes	9	UIP possible
23	M	68	72	49	34,7	Yes	3	N/A
24	F	70	100	88	25,1	No	6	Indeterminate for UIP
25	M	55	N/A	N/A	26,4	Yes	2	Alternative diagnosis
26	M	64	63	54	24,5	Yes	3	UIP definite
27	M	73	94	67	29,4	Yes	8	UIP definite
28	F	73	94	85	23,1	No	5	Indeterminate for UIP
29	M	75	97	56	24,1	Yes	5	UIP definite
30	M	73	65	47	34,5	Yes	4	Alternative diagnosis
31	M	69	61	35	29,1	Yes	8	UIP definite
32	M	60	N/A	N/A	32,5	Yes	3	UIP possible
33	M	69	N/A	N/A	N/A	N/A	N/A	N/A

M = male; F= female; FVC = forced vital capacity; DLCO = diffusing lung capacity of carbon monoxide; BMI = body mass index; CCI = Charlson comorbidity index; UIP = usual interstitial pneumonia; HRCT = high resolution computed tomography; N/A = not acquired.

Table 2: Descriptive statistics.

Characteristics	**n. of patients (%)**
Median age at disease onset (range), y n=33	69 (52,8-78,6)
Male Sex n=33	25 (75,8)
Smoking exposure n=30	24 (80)
median BMI, index n=30	27,45
Charlson Comorbidity Index n=30	4,83
median FVC, % predicted value n=28	83
median DLCO, % predicted value n=27	58
HRCT UIP pattern n=25	16 (64)

FVC = forced vital capacity; DLCO = diffusing lung capacity of carbon monoxide; BMI = body mass index; UIP = usual interstitial pneumonia; HRCT = high resolution computed tomography.

75,8% patients were of male sex. The median age at disease onset was 69 years (range 52,8-78,6). There was exposure to tobacco smoke in 24 (80%) cases (n=30). The median BMI was 27,45 (n= 30). The average CCI was 4,83 (n=30). Median FVC at diagnosis was 83% of predicted value (n=28). Median diffusing capacity of the lungs for carbon monoxide (DLCO) was 58% of predicted value (n=27). HRCT findings at onset showed either a possible or definite UIP pattern in 16 (64%) cases. The pattern was indeterminate for UIP in 4 cases and suggested an alternative diagnosis in 5 cases. There was no data available for the remaining 8 cases. All patients had a final diagnosis of IPF based on histopathological findings of UIP pattern, which was determined in all samples.

We were able to retrieve a total of 87 somatic alterations from the NGS analysis, including repeats of 20 point mutation variants occurring in 12 genes (ALK, CKD4, FGFR1, FGFR3, FGFR4, KIT, MET, MTOR, MYC, PIK3CA, RET and SMO). No somatic mutation that confers susceptibility to approved targeted drugs for lung cancer and no gene fusions were identified in the samples. The gene variants identified for each case are listed in Table 3.

Table 3: NGS analysis.

Case n.	ALK				CDK4	FGFR1	FGFR3			FGFR4		KIT	MET	MTOR	MYC		PIK3CA		RET	SMO
	p.Asp1529Glu	p.Ile1461Val	p.Gly1619Glu	p.Thr680Ile	p.Gly47Arg	p.Val771Met	p.Ser84Leu	p.Val117Ile	p.Ala261Thr	p.Pro136Leu	p.Ser688Phe	p.Met541Leu	p.Asn375Ser	p.Thr1977Ile	p.Asn26Ser	p.Glu269Lys	p.Gly359Asp	p.Gln374Ter	p.Ala919Val	p.Asp473Asn
1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0
2	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	1	1	0	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0
4	1	1	0	0	0	0	0	0	0	1	0	1	0	0	1	0	0	0	0	0
5	0	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0
6	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
7	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
8	0	1	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0
9	0	1	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0
10	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
11	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
12	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
13	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
14	1	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0
15	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
16	0	1	0	0	0	0	0	0	1	1	1	0	0	0	1	0	0	0	0	1
17	1	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0
18	1	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0
19	1	1	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0
20	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
21	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
22	0	1	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0
23	0	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0
24	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
25	1	1	1	1	1	1	0	0	0	1	0	1	0	1	0	1	1	1	1	0
26	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
27	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
28	1	1	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0
29	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
30	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
31	1	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0
32	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
33	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0

All samples carried ad least one somatic alteration. 15 cases were found to harbour 3 or more mutations (5.4 on average), while the remaining 18 cases showed only 1 or 2 mutations (1.3 mutations on average). Across all samples, the most frequently mutated genes were ALK, with 33 variants, followed by FGFR4 with 32 variants, KIT with 11 variants, and MYC with 7 variants identified.

The descriptive statistics of each mutation are shown in Table 4. Pathogenic/likely pathogenic genetic variants are shown in bold.

Table 4: Gene panel statistics.

Gene	Variant	Number of cases
ALK	p.Asp1529Glu	20
	p.Ile1461Val	33
	p.Gly1619Glu	1
	p.Thr680Ile	**2**
CDK4	p.Gly47Arg	1
FGFR1	**p.Val771Met**	**1**
FGFR3	**p.Ser84Leu**	**1**
	p.Val117Ile	**1**
	p.Ala261Thr	**2**
FGFR4	p.Pro136Leu	31
	p.Ser688Phe	**1**
KIT	p.Met541Leu	11
MET	**p.Asn375Ser**	**1**
MTOR	**p.Thr1977Ile**	**1**
MYC	**p.Asn26Ser**	**6**
	p.Glu269Lys	**1**
PIK3CA	**p.Gly359Asp**	**1**
	p.Gln374Ter	**1**
RET	**p.Ala919Val**	**1**
SMO	**p.Asp473Asn**	**1**

Table 5 includes survival data for each patient as of 31st December 2023.

Table 5: Survival data.

Case n.	Survival (weeks)	Case n.	Survival (weeks)
1	140	19	388
2	265	20	304
3	305	21	179
4	300	22	285
5	45	23	300
6	72	24	119
7	229	25	322
8	350	26	209
9	290	27	222
10	315	28	197
11	376	29	293
12	387	30	383
13	349	31	358
14	292	32	149
15	284	33	141
16	211		
17	283		
18	219		

The median survival time was 285 weeks (range 45-388 weeks).

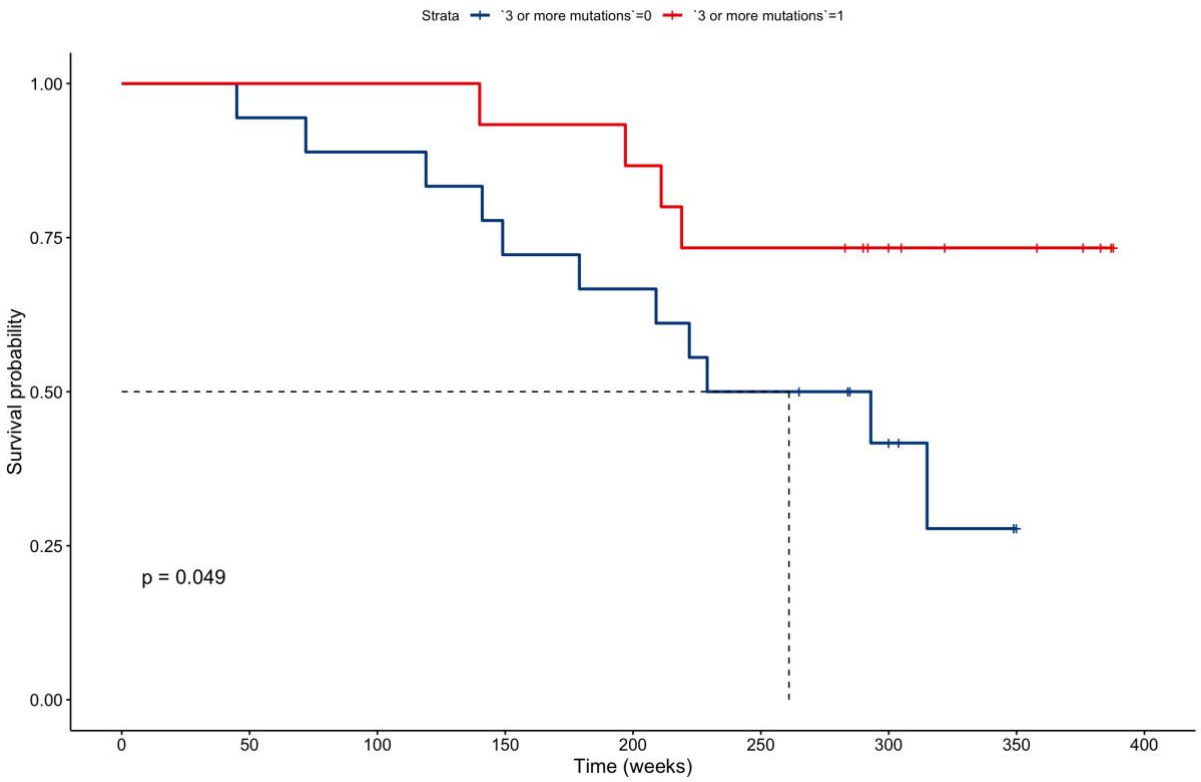
Univariate analysis showed significant effect on survival for %FVC, %DLCO and presence of ≥3 somatic mutations, as shown in bold in Table 6. The multivariate model built using these variables showed significant effect on survival only for the presence of ≥3 somatic mutations (p = 0.0378).

Table 6: Univariate and multivariate analyses against survival.

Variable	UNIVARIATE ANALYSIS			MULTIVARIATE ANALYSIS		
	Coefficient	SE	P value	Coefficient	SE	P value
Age	4.07	2.31	0.088			
Male sex	21.67	36.1	0.553			
Smoke	1	45.32	0.983			
CCI	11.02	8.87	0.225			
BMI	2.33	4.23	0.585			
%FVC	**1.31**	**0.496**	**0.0137**	0.84	0.6	0.18
%DLCO	**3.49**	**0.97**	**0.00135**	1.92	1.28	0.15
UIP pattern	23.24	42.72	0.592			
≥ 3 mutations	**68.4**	**30.06**	**0.0299**	**66.04**	**29.97**	**0.0378**

%FVC = forced vital capacity; %DLCO = diffusing lung capacity of carbon monoxide; BMI = body mass index; CCI = Charlson comorbidity index; UIP = usual interstitial pneumonia; SE = standard error.

Survival analysis confirmed a positive correlation with the presence of ≥3 mutations (p=0.049), as depicted in the chart below.



We explored any relationship between the presence of ≥ 3 mutations and relevant patients' baseline characteristics such as age, gender, smoking exposure, and presence of UIP pattern (see Table 7) and did not find any significant correlation.

Table 7: Univariate analysis for the presence of ≥ 3 mutations.

	UNIVARIATE ANALYSIS		
Variable	coefficient	Standard error	P value
Age	-0.001	0.01	0.92
Male sex	0.01	0.2	0.94
Smoke	-0.1	0.2	0.68
UIP pattern	-0.1	0.2	0.59

As for the presence of any association between the variants identified and the patients' characteristics, we tested variants ALK p.Asp1529Glu, KIT p.Met541Leu, and MYC p.Asn26Ser, detected in 20, 11 and 6 cases respectively (see Tables 8-10). We excluded all the other variants because they were present in either most samples or in 1-2 cases only. We found a significant negative correlation between variant KIT p.Met541Leu and the presence of radiological UIP pattern (coefficient -0.43, p value = 0.02).

Table 8: Univariate analysis for the presence of ALK p.Asp1529Glu (n=20).

	UNIVARIATE ANALYSIS		
Variable	coefficient	Standard error	P value
Age	0.001	0.01	0.91
Male sex	0.07	0.2	0.73
Smoke	0.27	0.23	0.27
UIP pattern	-0.1	0.2	0.63

Table 9: Univariate analysis for the presence of KIT p.Met541Leu (n=11).

Variable	UNIVARIATE ANALYSIS		
	coefficient	Standard error	P value
Age	-0.01	0.01	0.25
Male sex	0.15	0.19	0.42
Smoke	0.23	0.22	0.31
UIP pattern	**-0.43**	**0.17**	**0.02**

Table 10: Univariate analysis for the presence of MYC p.Asn26Ser (n=6).

Variable	UNIVARIATE ANALYSIS		
	coefficient	Standard error	P value
Age	0.004	0.004	0.4
Male sex	-0.11	0.07	0.1
Smoke	-0.17	0.09	0.07
UIP pattern	-0.11	0.08	0.19

6. DISCUSSION

IPF is a disorder with a complex pathogenesis of unknown origin. Its prognosis is poor, and although two antifibrotic drugs were approved 10 years ago to slow down the disease, there is still great need for new therapeutics. Our work explored the application of novel sequencing technologies adopted from the field of oncology to research on IPF. We described the somatic mutation landscape of 33 fibrotic lung tissue samples using an oncogene panel. Statistical analysis on our results pointed to a positive association between the presence of at least 3 variants and longer survival. Furthermore, we found a negative correlation between a mutation in the KIT gene and the presence of radiological UIP pattern at baseline.

Our results confirm that the application of NGS to complex, non-cancerous diseases such as IPF may prove to be an innovative yet successful approach when investigating on underlying molecular pathways and new interventional perspectives. The idea that IPF might be a cancer-like disease is part of an ongoing debate that has been fuelled by recent evidence that shows significant mechanistic overlap between the two conditions. This adds up to their more obvious phenotypical similarities, such as the patients' background characteristics and risk factors, and their mutual aggressive nature. It has long been acknowledged that patients with IPF are also significantly more at risk to develop lung tumours. Nevertheless, the bond between the two diseases is manifest even from the earliest stages of fibrosis. Interstitial lung abnormalities (ILAs) are asymptomatic radiographic changes that are largely underreported, but that were found to be a precursor to IPF and other forms of progressive pulmonary fibrosis. A large study conducted on participants in the National Lung Cancer Trial found that ILAs are an independent risk factor for lung cancer that can be incorporated into risk score models[99]. This finding is fundamental because it suggests the existence of a common underlying mechanisms that drives both diseases form their earliest stages. This theory is in opposition to the assumption that cancer develops more favourably in IPF because of the structural upheaval caused by the disease, as is the case, for instance, with hepatocellular carcinoma and liver cirrhosis where the incidence of cancer increases as the chronic liver disease advances[100]. Another important consideration springs from the notorious effects of antifibrotic drugs on lung cancer. Nintedanib, a tyrosine kinase inhibitor (TKI) that acts on the pathways regulated by VEGF, FGF and PDGF, exerts significant suppression on cancer growth and was already prescribed in combination with cytotoxic agents for the treatment of LC long before being investigated as a potential treatment for lung fibrosis. Pirfenidone as well, whose mechanism

of action is less understood, has shown antiproliferative activity and has recently demonstrated to reduce the risk of lung cancer in patients with IPF[101]. Taken together, these observations suggest that we need to expand our knowledge on the common pathophysiological events that sustain IPF and LC. This research could work in a two-way fashion by providing novel therapeutic perspectives for both diseases.

A few years back, we reviewed current evidence regarding the main drugs involved in IPF and lung cancer treatment and their mutual implications[102]. We reported previous examples of agents with curative effects on both conditions, such as bevacizumab, a monoclonal antibody targeting VEGF developed for lung cancer which has shown a protective effect against acute accelerated phase in patients with concomitant IPF and LC compared to traditional chemotherapy[103]. Paclitaxel, a chemotherapy drug that inhibits cell replication through blockage of cellular cytoskeletal dynamics, has shown to downregulate the canonical SMAD cascade of TGF-β and suppress EMT in rats, although it has never been repurposed as a potential antifibrotic agent in real life studies[104]. We also focused on the antifibrotic and profibrotic properties of TKIs, a large group of molecules which comprise both nintedanib, which is approved for the treatment of both IPF and LC, and EGFR-TKIs such as erlotinib and alectinib which, conversely, can *induce* interstitial lung disease[105,106].

New genomic sequencing technologies such as NGS, which first became available in year 2005, have revolutionized cancer research and cracked a window open on its biology, resulting in the detection of biologically significant mutations that physicians now use to tailor treatment to each patient[107]. NGS was developed after the traditional Sanger sequencing method and evolved from the need to process large DNA samples in a cost-effective way. The concept behind Sanger and NGS technology is similar: fluorescent nucleotides are added by a polymerase onto a growing nucleic acid strand, and each nucleotide is identified (read) by its fluorescent tag. However, while the Sanger method can read a single DNA fragment at a time, NGS is characterized by the ability to sequence millions of fragments in a single run, thereby producing an enormous volume of data at a fast, affordable rate. The advent of NGS has deeply impacted several disciplines of medicine and biology. It has, in fact, marked the transition to the genomic era by enabling the assembly of human genome reference databases and thus providing a fundamental step forward in personalized medicine. In advanced lung cancer, for which treatment options have historically been very slim, NGS allowed the discovery of activating molecular alterations and the introduction of several new biological therapies,

starting with the landmark studies on epidermal growth factor receptor (EGFR)[108]. To date, the list of small-molecule inhibitors approved for lung cancer has grown to include several other targeted therapies, with more drugs currently under investigation (clinicaltrial.gov)[109].

While somatic mutations have traditionally been studied in cancer, healthy tissue is not exempt from harbouring various degrees of genomic alterations, which accumulate with age at different rates in different cell lines[110]. In this regard, extensive heterogeneity has been observed amongst tissues, with the highest rates of clonal expansion detected in the oesophagus and endometrium[111]. Compared to other tissues, the lung epithelium is directly exposed to the external environment and can have widespread acquired somatic mutations, especially in smokers. A whole-genome sequencing analysis identified driver mutations in 4-14% of bronchial cells of non-smokers, and in ≥ 25% of cells in current smokers, who also showed increased mutation burden and cell-to-cell heterogeneity without overt changes in the morphology of the respiratory epithelium[112]. Nevertheless, very few studies exist examining the potential role of somatic polymorphisms in non-malignant complex diseases, let alone in healthy individuals. In 2003, Andersson and Bozinovski hypothesized that acquired somatic mutations such as EGFR and p53 could contribute to the molecular pathogenesis of chronic obstructive pulmonary disease by altering signal transduction pathways that control key inflammation, host defence and steroid response pathways[113]. In IPF, the presence of somatic alterations was first observed at the microsatellite level in cytologic sputum specimens in the early 2000'[114-115]. In a recent large-scale study conducted by the Lung Tissue Research Consortium, IPF patients not only were found to have significantly elevated mutational burden, but this correlated with pathologic cell type proportion and metrics of disease severity, suggesting somatic mutations may indeed contribute to disease phenotype[116]. A very small number of studies exist on the exploration of somatic mutations with NGS in fibrotic tissue samples, although previous research has detected somatic alterations in cancer-related genes in patients with concomitant IPF and LC. In 2020, Otsubo and colleagues uncovered somatic mutations in paired tumour and tumour-adjacent fibrosing lung tissue specimens by targeted NGS[117]. Their results showed that the areas of fibrosis and LC had a similar tumour mutational burden, but found no significant overlap between the cancerous and the juxta fibrosing tissue in terms of specific molecular variants. This finding was confirmed in a later study by Yoneshima et al., who again found that somatic alterations were rarely shared between tumour and corresponding IPF tissue[118]. Another interesting Japanese single case report identified 5 cancer-related mutations in IPF and 4 more distinct mutations in the tumour region. The authors

then hypothesized that IPF-associated LC may not be a result of the accumulation of somatic mutations[119]. This evidence contradicts the theory that the aberrant proliferative substrate in IPF should promote the accumulation of somatic alterations and subsequent clonal selection of malignant cells, which is in keeping with what we discussed above starting from evidence regarding ILAs and risk of LC. However subtle this difference might seem, it indeed points to the conclusion that UIP is *not* a pre-cancerous lesion, but rather that IPF and LC are associated because they are linked at a deeper level and probably share some sort of pathogenic event. Indeed, this information makes the higher incidence of cancer in IPF patients more complicated to interpret, as lung tumours that develop in this population also have unique characteristics. In the general population, lung cancers are more frequently adenocarcinomas and have an upper-lobe predominance. Conversely, most studies indicate that squamous cell carcinoma (SCC) is the most frequently seen type of LC in IPF and, as opposed to typical SCCs in non-IPF patients, the lesions are mainly peripheral and located in the lower lobes, near areas of honeycombing. In addition, patients with concomitant IPF and LC have higher morbidity and mortality[120].

All this said, it is evident that NGS is a potentially extraordinary tool for investigating the yet mysterious pathogenetic background of IPF at a molecular level. To our knowledge, ours is the first NGS study conducted on lung tissue specimens of IPF without the presence of juxta cancerous tissue. Our results uncovered a total of 87 somatic mutations, which included repeat mutations of 20 single nucleotide variants across 12 oncogenes. None of the variants that we identified are known to confer susceptibility to molecular targeted drugs, a finding that is in keeping with the above mentioned NGS studies on lung tissue with concomitant IPF and LC. All 49 genes included in our panel are involved in oncogenic processes that sustain cellular proliferation and survival. While 14 of the variants identified in our research are considered “pathogenic” or “likely pathogenic” according to current database classification, their mechanistic role in IPF remains undetermined. The most frequent mutations occurred in ALK, FGFR4, KIT, and MYC genes.

ALK gene encodes a TKR protein which is thought to play a significant part in neuronal development, although its exact function in unknown. ALK gene alterations are associated with several cancerous diseases including non-small cell lung cancer (NSCLC), and several ALK-inhibitors have been developed to treat advanced ALK-mutated cases[121]. Interestingly, it has been shown that the MAP/ERK cascade, whose role we have discussed above in IPF, is critical for the survival of tumours treated with ALK-inhibitors[122].

FGFR4 encodes a FGF TKR that regulates cell proliferation, differentiation and migration. Its signalling complex includes PI3K/AKT, which is also one of the non-canonical pathways involved in TGF-β-dependent lung fibrogenesis. FGFR4 has been implicated in several types of cancers such as liver, breast and colorectal cancer. Because of the sequence homology of the FGFR family, research on FGFR-inhibitors has been limited by their toxicity profiles[123].

KIT encodes another TKR called *c-kit* that is activated by the mast/stem cell growth factor (SCF). Activating mutations of KIT, which is a known EMT inducer and a key component of the PI3K/AKT pathway, are associated with various neoplasms[124]. Imatinib, a TKI specific for the tyrosine kinase domain of *c-kit* that has been used for the treatment of leukaemia for over 20 years, was the first TKI with anti-fibrotic effects[125]. In fact, imatinib is a potent suppressor of lung fibroblast-myofibroblast differentiation and proliferation, as well as an inhibitor of ECM production through downregulation of PDGF and TGF-β signalling. The safety and efficacy of imatinib were investigated in the first clinical trial on the use of a TKI in IPF, but the results showed no benefit with respect to disease progression[126-127]. Another molecular target agent of the PI3K/AKT cascade, omipalisib, has shown to reduce lung fibrosis in mice and is expected to proceed to further experiments[128]. In our study we identified KIT exon 10 variant M541L in 11 patients. This somatic mutation has been investigated in several cancerous diseases and as a potential predictive factor of good response to TKI therapy, with conflicting reports. The real clinical significance of this variant has been debated and larger studies have shown that its frequency in some tumours does not differ from that of the general population, and it is currently classified as a benign variant[129].

MYC gene is a regulator of EMT and is frequently overexpressed in several cancers. Upregulated MYC expression was observed in a mouse model of IPF, where it promoted fibroblast proliferation and differentiation through a mechanism that involves the microRNA miR-9-5p and the TATA box binding protein-like 1 (TBPL1) expression[130].

While all our specimens included at least 1 alteration in one of the genes included in the panel, the number of mutations varied significantly across samples. 18 tissue samples were found to harbour only 1 or 2 mutations. The other 15 cases showed a higher number of mutations with an average of 5.4 mutations per sample. Our results showed that the patients harbouring at least 3 variants had significantly longer survival rates after adjustment for age, gender, smoking status, CCI, baseline respiratory functional parameters, and presence of radiological UIP pattern. Although no explanation can yet be given for this association, we can try to formulate a hypothesis on the role of somatic mutations in predicting response to

antifibrotic drugs, and postulate that the patients presenting with more mutations may be more likely to respond to antifibrotic treatment, as both pirfenidone and nintedanib are known to participate in oncogenic molecular pathways. Unfortunately, the lack of information regarding follow-up visits and, particularly, the choices of treatment made for our patients, hinders our ability to investigate further on this matter, although it certainly stimulates us to follow this track and expand our future investigations.

Among all variables, none of the patients' baseline clinical characteristics correlated with survival, including lung function. This appears in contrast with larger cohort studies, which show that the presence of mild functional impairment at baseline is predictive of better survival rates[131-135]. However, if we consider the traditional FVC threshold of 50% predicted and a DLCO threshold of 35% predicted to separate mild-to-moderate IPF from those with severe disease, as commonly accepted in most RCTs, our cohort was indeed predominantly composed by patients with mild functional impairment[136]. In this regard, our findings would need to be validated in more heterogeneous groups of patients.

Among all samples, our statistical analysis did not highlight any correlation between relevant clinical characteristics and any specific gene variant or the presence of ≥ 3 mutations. The only exception was a significantly lower incidence of UIP pattern in the samples carrying the KIT p.Met541Leu variant (coefficient 0.17, p value = 0.02). As we discussed above, KIT p.Met541Leu is currently classified as a benign somatic mutation. Its protective role against the presence of a HRCT UIP pattern at baseline will need further research, although we must highlight that this feature did not correlate with survival in our study.

The main limitation of our research, apart from the small number of patients and the missing gaps on clinical information that we have already manifested above, is the absence of a control group and thereby the lack of opportunity to check for the presence of somatic genomic variation in non-IPF patients. However, this remains the first study on the somatic genomic landscape of IPF based on surgical tissue samples that do not include adjacent cancer. We believe that this unprecedented information contributes significantly to the research on IPF and we intend to pursue this track by broadening both our sample and the NGS gene panel tested. We hope that further validation studies can stimulate interest on this new approach and, most importantly, help us contribute to providing new ideas for advancing clinical trial designs on IPF.

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