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ROLE OF ROS IN THE ENDOTHELIAL-TO-MESENCHYMAL TRANSITION INDUCED BY THE SERA FROM IDIOPATHIC PULMONARY FIBROSIS PATIENTS

Director:
Prof. LEONARDO A. SECHI

Tutor: Prof. GIANFRANCO PINTUS
Co-tutor: Prof. PHAN THI MINH PHUONG

PhD thesis of
PHAN THI HANG GIANG

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ATTESTATION OF AUTHORSHIP

I hereby declare that this submission is my own creation. And It was performed to the best of my knowledge and belief. There is no material previously published or written by another person except were contained by this thesis, except for the citations and acknowledgments that were reported. It also does not contain material of a substantial extent I submitted for the qualification for any other degree to another university, and another institution of higher learning.

Name: Signed:

Phan Thi Hang Giang

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ABBREVIATIONS

IPF	Idiopathic pulmonary fibrosis
IIP	Idiopathic interstitial pneumonia
ILD	Interstitial lung disease
ARDS	Acute respiratory distress syndrome
UIP	Usual interstitial pneumonia
DLCO	The diffusing capacity of the lungs for carbon monoxide
FVC	Forced vital capacity
6MWT	6-minute walk test
HRCT	High-resolution computed tomography
SLB	Surgical lung biopsy
cTBB	Transbronchial cryobiopsy
ANA	Antinuclear antibodies
RF	Rheumatoid factor
ECs	Endothelial cells
AEC	Alveolar epithelial cell
EndMT	Endothelial-to-Mesenchymal Transition
EMT	Epithelial-to-Mesenchymal Transition
PAH	Pulmonary Arterial Hypertension
NOS	Nitric oxide synthase
eNOS	Endothelial NOS
α-SMA	Alpha-smooth muscle actin
VIM	Vimentin
COL I	Type I collagen
COL III	Type III collagens
VE-cadherin (CDH5)	Vascular endothelial cadherin

CD31/PECAM 1	CD31/ Platelet endothelial cell adhesion molecule-1
vWF	Von Willebrand factor
FSP-1	Fibroblast specific protein-1
TGF-β	Transforming growth factor-β
FN	Fibronectin
VEGFR	Vascular epidermal growth factor receptor
p38MAPK	P38 Mitogen-activated protein kinases
ECM	Extracellular matrix
PI3K	Phosphatidylinositol 3-kinase
HIF	Hypoxia-inducible factor
PKC	Protein kinase C
ROS	Reactive oxygen species
NADPH oxidase	Nicotinamide adenine dinucleotide phosphate oxidase
$O^{\cdot -}_2$	Superoxide radical
˙OH •	Hydroxyl radical
H 2 O 2	Hydrogen peroxide
DPI	Diphenyleneiodonium
NAC	N-acetyl cysteine
CHE	Chelerythrine

ABSTRACT

Purpose - This study was performed to investigate whether sera from IPF patients could affect Human Pulmonary Microvascular Endothelial Cells (HPMECs) phenotype and functions, and the molecular mechanism underpinning these effects. For this purpose, we investigate whether IPF sera could induce oxidative stress in HPMECs and whether such a phenomenon could modulate HPMECs proliferation, endothelial-to-mesenchymal transition EndMT. Finally, we focused on identifying the potential relationship between oxidative stress and EndMT on HPMECs in IPF.

Results - Sera of IPF patients markedly increased intracellular ROS level in HPMECs, and diphenyleneiodonium (DPI), a broad-spectrum NOX inhibitor, reduced intracellular ROS levels induced by sera of IPF patients. Besides, sera of patients with IPF increased HPMECs proliferation, and IPF-induced increase of HPMECs proliferation was significantly blunted by the DPI. Interestingly, sera of IPF patients induces Endothelial-to-Mesenchymal Transition in HPMECs, and ROS (NADPH) mediates IPF-induced Endothelial-to-Mesenchymal Transition. Preliminary results show that IPF sera induced the conversion of ECs into myofibroblasts through decreasing the endothelial marker (CDH5, CD3, vWF) and increasing mesenchymal markers (Col 1, α-SMA).

Conclusion - Exposure of HPMECs to oxidant factors present in IPF sera increased disease-associated pathophysiology phenomena such as intracellular ROS levels, cells proliferation, and EndMT. Preliminary data demonstrate the presence of an IPF sera-induced EndMT, indicating this phenotypic shift as an important etiological mechanism of IPF-associated vascular damage and a potential therapeutic target to inhibit obliterative vascular disorder and tissue fibrosis in the future. Reduction of the above-

mentioned phenomena (intracellular ROS level, cells proliferation, EndMT) by DPI suggests ROS (NADPH) mediates IPF-induced EndMT. And the last, the study suggests that the use of antioxidants could be a potential therapeutic tool to prevent the progression of IPF-related complications including fibrosis.

1. INTRODUCTION

1.1. Idiopathic pulmonary fibrosis

Idiopathic pulmonary fibrosis (IPF) is the most common form of idiopathic interstitial pneumonia (IIP) [1]. This disease is the result of direct injury to the lung parenchyma, through an inflammatory response, that causes scarred and thickened (fibrous) pulmonary tissue [2]. IPF is chronic and irreversibly progressive with high mortality rates, where the average life expectancy is only 3–5 years following diagnosis. IPF primarily occurs in males and individuals 60 years and older. IPF patients typically succumb to respiratory failure secondary to loss of respiratory function from extensive fibrotic scarring of the lung parenchyma worsening lung function. Subpleural fibrosis, sub-fibroblastic foci, and microscopic honeycombing are characteristic features of the histopathological hallmarks of IPF. Lung cancer, pulmonary hypertension, acute respiratory distress syndrome (ARDS), and respiratory failure are common complications of IPF [3-6].

Clinical progression is often associated with IPF exacerbations that are complex as indicated by acute episodes of respiratory dysfunction. Most patients have been hospitalized for a long time in the last year of life which the results were unfavorable [7, 8]. Currently, there is no effective therapy to prevent and also control IPF exacerbations [9]. Over the past two decades, drug therapy for IPF has grown significantly. Among them were studied using various immunosuppressant and anti-inflammatory agents, resulting from a randomized clinical trial of a combination of Prednisone, Azathioprine, and N-acetylcysteine (PANTHER-IPF) has shown an increase in harm, which indicates an ineffective approach [10]. In 2014, the FDA approved two IPF treatment drugs: Pirfenidone is an anti-fibrous and anti-inflammatory, Nintedanib is a kinase inhibitor, both of

which can only slow disease progression without therapeutic effect is as expected [11]. A more specific understanding of the underlying pathogenesis of IPF is necessary to enable the development of new therapies for the disease.

Current studies suggest pulmonary blood vessels are negatively affected by the disease, resulting in unstable vascular regeneration. Therefore, regeneration of unstable blood vessels is of great interest in the pathogenesis of IPF, but the role of vascular regeneration in pulmonary fibrosis is not understood clearly [12].

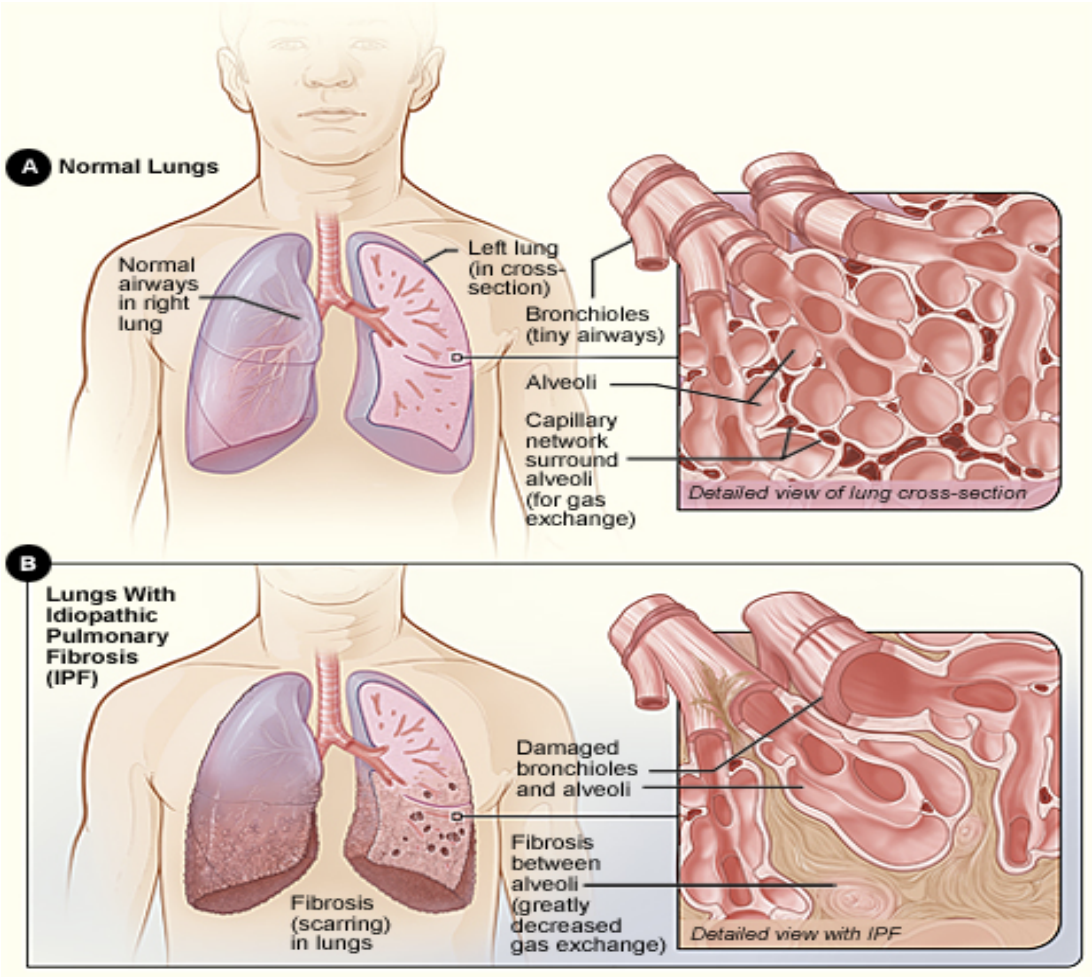

Figure 1.1: A. The structure of normal lungs and normal airways in the human. The image is shown as a detailed cross-sectional view of the airways and air sacs of the lungs. **B. Specific fibrosis (scarring) in fibrosis lungs in the human.** The image inside is an expository look at fibrosis that damages the airways and air sacs [13].

1.1.1. Epidemiology

In interstitial lung disease (ILD), IPF is the most frequent and severe type, which is estimated to be 20% -50% of all cases of ILD [14]. IPF is rare and of low incidence, with only ~40,000 new cases diagnosed each year in Europe [15]. A challenge has been to accurately assess the epidemiology of IPF, in part, because there was no agreed definition of this disease. In 2000 the American Thoracic Society (ATS) and European Respiratory Society (ERS) announced criteria for the diagnosis of IPF based on the radiological and histopathological detection of usual interstitial pneumonia (UIP) and no definite cause [13, 16]. Although the frequency is not high, IPF is a disease with a high direct treatment cost of about 25,000 USD/person/year, higher than breast cancer and many other life-threatening diseases [8].

A study of a USA Medicare population from 2001 to 2011 found a higher rate of IPF at age ≥65 years (from 202.2 cases per 100,000 in 2001 to 494.5 cases per 100,000 in 2011) compared with previous findings (the morbidity rate was 93.7 cases per 100,000 / year). Another Japanese study in Hokkaido, from 2003 to 2007, recorded a prevalence rate of 10 per 100,000 persons per year and an incidence of 2.23 per 100,000 persons per year. All studies noted that prevalence and incidence occurred more frequently in men and increased with age, particularly over 75 years old [17, 18].

The death rate from IPF appears to be steadily increasing across the globe. In 2014, there were an estimated 28,000 to 65,000 deaths in Europe and between 13,000 and 17,000 deaths in the United States [19]. In an American study of the cause of death with IPF, it was found that the most common cause was respiratory failure (60%), the second cause was cardiovascular disease (8.5%), and the next cause was lung cancer (2.9%) [20]. More interestingly, the aforementioned Japanese study found a very

high proportion of the cause of death from IPF exacerbations (40%) which was not noted in other studies [18].

1.1.2. Natural history of IPF

The natural history of IPF is difficult to predict due to variability. Some IPF patients exhibit rapid deterioration of lung function compared to others with slower progression, while some have relatively stable periods with acute impaired pulmonary function which is often fatal [21]. One of the most frightening complications of IPF is an exacerbation. Exacerbation is defined as acute impairment of lung function, worsening of respiratory symptoms, and all evidence of infection and other causes excluded. Every year, about 5% to 10% of patients suffer from exacerbations, with high mortality (85%) [22].

Several factors can predict the risk of death in IPF, including levels of difficulty breathing, the diffusing capacity of the lungs for carbon monoxide (DLCO), Forced vital capacity (FVC), desaturation during 6-minute walk test (6MWT), fibrosis on High-resolution computed tomography (HRCT) and pulmonary hypertension at baseline [13]. Currently, the GAP index is an indicator that has been proposed to predict the course of the disease based on the clinical model, including sex, age, and physiological index (Figure 1.2). Although the GAP index is the most commonly used, the predictive value of the GAP index is still hindered by limited clinical parameters [23]. Therefore, more accurate prognostic tools need to be developed. Diagnosis staging early of this disease improves prognostic outcomes by limiting potentially harmful therapies (glucocorticoids for IPF) and rapidly using effective therapies (anti-fibrotic) in the early stages of the disease [24].

	Predictor		Points
G	Gender		
	Female		0
	Male		1
A	Age, y		
	≤60		0
	61-65		1
	>65		2
P	Physiology		
	FVC, % predicted		
	>75		0
	50-75		1
	<50		2
	DLCO, % predicted		
	>55		0
	36-55		1
	≤35		2
	Cannot perform		3
	Total Possible Points		8
Stage	I	II	III
Points	0-3	4-5	6-8
Mortality			
1-y	5.6	16.2	39.2
2-y	10.9	29.9	62.1
3-y	16.3	42.1	76.8

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Figure 1.2: The GAP index and Staging System [23].

1.1.3. Diagnosis

Following the 2011 guidelines ATS/ERS/JRS/ALAT on the diagnosis and treatment of IPF, diagnosis requires exclusion of other potential agents of pulmonary fibrosis and identification of the form of usual interstitial pneumonia (UIP) by high-resolution computed tomography (HRCT). Diagnosis by surgical lung biopsy (SLB) should be made to determine if the UIP pattern is not clearly defined ("possible" or "unsuitable for UIP") by HRCT [21].

The updated 2018 ATS/ERS/JRS/ALAT Guidelines note that SLB may have an adverse benefit/risk ratio when performed for patients with significant physiological impairment or other medical conditions comorbidities [25]. Transbronchial cryobiopsy (cTBB) may be preferable

to SLB for some patients in these circumstances. A field study of 109 patients, with ILD after cTBB, was performed within 90 days with no noted deaths or exacerbations. A total of 73.4% positive histopathological samples were confirmed [26]. In addition, a multicenter study of 65 patients with ILD in Australia who underwent both cTBB and SLB showed consistent histopathology in 70.8% of cases [27]. Both studies highlight those multidisciplinary discussions are needed to reach a diagnosis and that histopathology is only part of the evidence for the diagnosis of IPF.

Other tests can be supported in the diagnosis of IPF besides imaging and histological tests. To diagnose suspected IPF, analysis of bronchoalveolar lavage fluid can be a suggestion. In addition, serological tests for rheumatoid factor, antinuclear antibodies, and myositis panel can be useful in the diagnosed differentiation of ILDs related to disorders of connective tissue [25, 28].

1.1.4. Management and Treatment

Management of IPF focuses on improving symptoms, increasing overall health status, maintain lung function, facilitating a continuous oxygen supply through oxygen therapy (when needed), mitigating unfavorable events of therapy, and decreasing the rate of occurrence of exacerbations to improve survival [29]. The progression of IPF patients was controlled through exploratory tests of respiratory function, especially Forced vital capacity (FVC) and desaturation during the 6-minute walk test (6MWT). So, the GAP index (sex, age, and physiological index) can be used to evaluate prognosis by assigning a score based on points assigned to the male sex, advanced age, the diffusing capacity of the lungs for carbon monoxide (DLCO), and forced vital capacity (FVC). A high GAP score equates to high mortality. In addition, most specialized centers for Interstitial Lung Disease use the 6-minute walk test to objectively assess

the patient's respiratory function status and screen for respiratory failure due to lack of oxygen [30] [31].

The 2015 guideline of IPF treatment provided by the American Thoracic Society, the European Respiratory Society, the Japanese Respiratory Society, and the Latin American Thoracic Association (ATS/ERS/JRS/ALAT) are resources for the pharmacological management of IPF [32]. In 2018 the JRS published updated clinical guidelines for the treatment of IPF (Figure 1.3) [30].

Agent	2015 ATS/ ERS/JRS/ALAT Recommendations	Strength, *Confidence* in Effect Estimates	2018 JRS Recommendations	Strength, *Quality* of Evidence
Anticoagulant (warfarin)	Against use	Strong, Moderate	Not addressed	
Combination prednisone + azathioprine + *N*-acetylcysteine	Against use	Strong, Low	Against use (combination steroids + immunosuppressants)	Strong, Low
Selective endothelin receptor antagonist (ambrisentan)	Against use	Strong, Low	Not addressed	
Imatinib, a tyrosine kinase inhibitor with 1 target	Against use	Strong, Moderate	Not addressed	
Nintedanib, a tyrosine kinase inhibitor with multiple targets	For use	Conditional, Moderate	For use	Weak, Moderate
Pirfenidone	For use	Conditional, Moderate	For use	Weak, Moderate
N-acetylcysteine + pirfenidone	Not addressed		Against use[a]	Weak, Low
Nintedanib + pirfenidone	Not addressed		Withholding judgment at this time	
Dual endothelin receptor antagonists (macitentan, bosentan)	Against use	Conditional, Low	Not addressed	
Phosphodiesterase-5 inhibitor (sildenafil)	Against use	Conditional, Moderate	Not addressed	
Antacid therapy	For use	Conditional, Very low	Not addressed	
N-acetylcysteine monotherapy	Against use	Conditional, Low	Against use[a]	Weak, Low
Corticosteroid monotherapy	Not addressed		Against use	Strong, Very Low

ATS/ERS/JRS/ALAT indicates American Thoracic Society/European Respiratory Society/Japanese Respiratory Society/Latin American Thoracic Association.
[a]May be a reasonable option in a minority of patients.

Figure 1.3: Guideline Recommendations for the Treatment of Idiopathic Pulmonary Fibrosis

Currently, only pirfenidone and nintedanib (a tyrosine kinase inhibitor) are the anti-fibrotic agents approved for use in the treatment of IPF. These two anti-fibrotic drugs have been identified to slow disease progression but have had no notable results in terms of reducing mortality. Therefore, it is advisable to initiate treatment with these two drugs early as further studies have also shown a reduction in IPF exacerbations when these drugs are used therapeutically. However, liver and kidney function tests are recommended to control the use of these two drugs. The most common adverse drug reactions reported for nintedanib were diarrhea, while pirfenidone was photosensitivity, rash, and gastrointestinal disturbances [33].

Our knowledge of the pathophysiology of IPF and prospective therapies for IPF has improved remarkably. However, there is still no measure that truly stops disease progression and no complete cure for this disease. Future therapies should focus on stabilizing the disease, improving symptoms, and improving quality of life with the eventual object of lessening the disease burden. The long-term objective is to discover a new therapeutic cure. Besides, it is necessary to study new effective therapies to treat serious complications of IPF as well as exacerbations of the disease. Develop several clinical trials promising new therapies for the future. The main area of focus on the development of fibrosis pathways in IPF, especially fibroblasts involved in abnormal tissue remodeling, excessive extracellular matrix accumulation, and angiogenesis, all of which are thought to be at the center of this evolving disease.

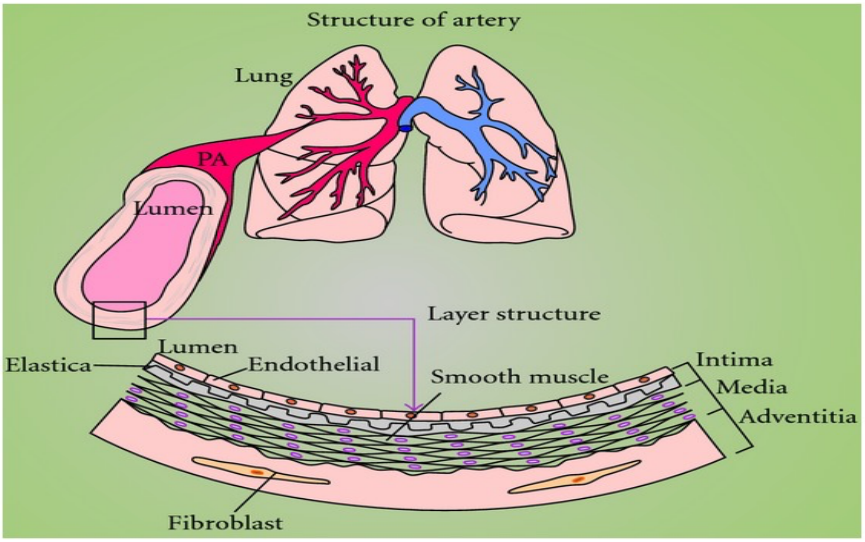
1.2. Endothelial cell and EndMT in IPF

The pathogenesis of IPF is multifactored, including epithelial damage, destruction of lung tissue, and development of mechanisms associated with aging. The combination of these factors induces the release of mediators that lead to migration, proliferation, activation of fibroblasts and anti-

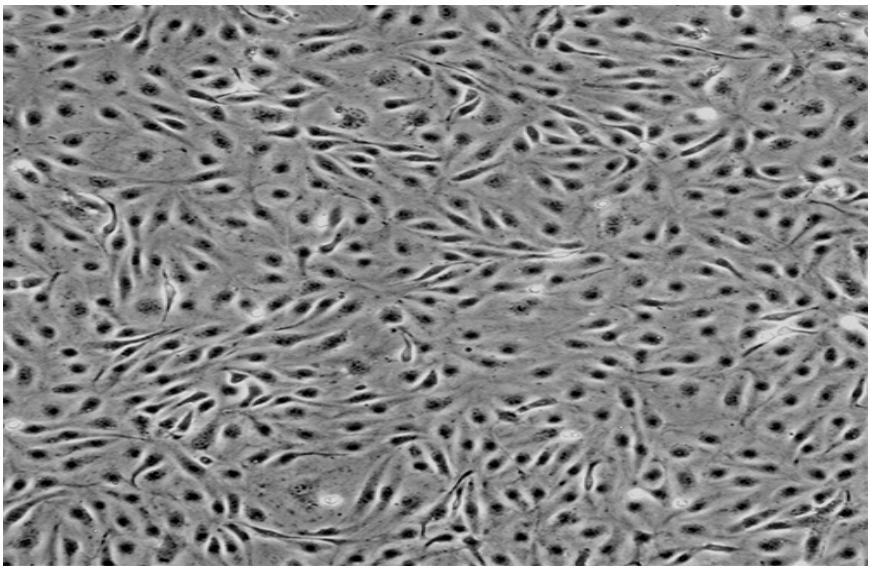
apoptosis fibroblasts, and secretion of extracellular matrix. Released growth factors contribute to the progression of IPF disease [29]. The signs of aging such as genomic instability, mitochondrial dysfunction, and impaired intracellular signaling contribute to the pathogenesis of this disease. In addition, this disease is also the result of complex interactions between genetic and environmental factors, such as smoking, exposure to metal dust and wood, and associated diseases including reflux gastro esophagus, diabetes mellitus, and obstructive sleep apnea [34].

1.2.1. Endothelial cell involvement in IPF

IPF is a complex disease in which many different cell types are present including epithelial cells, fibroblasts, immune cells, and endothelial cells. While apoptosis alveolar epithelial cells (AEC) and fibroblast activation are considered to be the main contributors to the abnormal lung remodeling leading to fibrosis in IPF, the specific role of endothelial cells remains poorly understood and needs further clarification [35, 36]. Endothelial cells (ECs) are cells that line the inner walls of blood vessels. ECs play an important role in ensuring the stability of gas exchange between the alveolar-capillary barriers. They preserve vascular homeostasis by protecting vascular cells from physiological or pathological stimulations. ECs are chronically stimulated by many factors, such as pro-inflammatory cytokines and hypoxia, leading to imbalance homeostasis in ECs and endothelial cell dysfunction. This condition is closely related to vascular disease and fibrosis such as IPF [37].



(A)



(B)

Figure 1.4: Vascular endothelium. (A) a represnetaiton of the tissue and location and (B) the actual histological view of the cell [38]**.**

Two mechanisms of pulmonary fibrosis are involved with endothelial cells. One is the ability of the endothelium to transform into fibroblasts in the Endothelial to Mesenchymal Transition (EndMT) fibroblast creation process. During lung damage, there is an increased amount of fibroblasts to aid in the repair and regeneration of the tissue which produces large amounts of collagen and other extracellular matrix components. The other is a pathway in endothelial cells that results in the production of free

radicals, regulated by three isomers of nitric oxide synthase (NOS), nerve cell NOS, inducible NOS, and endothelial NOS (eNOS). Although nitric oxide normally modulates many biological processes and maintains airway homeostasis [39], at high levels, it can induce harmful inflammation and apoptosis. Several studies suggest that nitric oxide is important for the development of pulmonary fibrosis. The study by Yoshimura et al. showed that overexpression of eNOS reduced bleomycin-induced histological changes, lung collagen accumulation, and mortality in eNOS transgenic mice. They also showed endothelial involvement via a nitric oxide-dependent mechanism in the development of pulmonary fibrosis since overexpression of eNOS mainly occurs in endothelial cells [40].

1.2.2. EndMT in IPF

Regeneration of blood vessels in IPF fibrosis is a controversial research area. In the lungs of IPF patients, there is both the presence of angiogenesis and fibrosis regions. It clearly to indicates that microvascular remodeling is unstable [41]. Vascular redistribution has been demonstrated in several studies to show that areas of normal IPF pulmonary parenchyma have an increase in vascular circulation, while a minimal reduction in blood vessel count is observed in areas affected by fibrosis. The fibrosis center of gravity is almost completely vascular-free [42]. Indeed, endothelial cells from patients with IPF [43] and mouse lung endothelial cells were fibrotic due to bleomycin, which lost integrity of the alveolar-capillary barrier because of increased markers of damage endothelial and endothelial cell death [44, 45]. Notably, endothelial progenitor cells are reduced in IPF patients, impairing the efficiency of endothelial regeneration and vascular repair during injury [46].

During the EndMT process, ECs receive the mesenchymal pattern and exhibit typical markers of fibroblast differentiation, including α-smooth muscle actin (α-SMA), vimentin, N-cadherin, and fibroblast specific

protein-1 (FSP-1/S100A4), type I and III collagen (COL I and COL II), while also reducing endothelial cadherin expression blood vessels (VE-cadherin), CD31/platelet-endothelial cell adhesion molecule (PECAM-1), and von Willebrand factor (vWF), **(**Figure 1.5**)** [47].

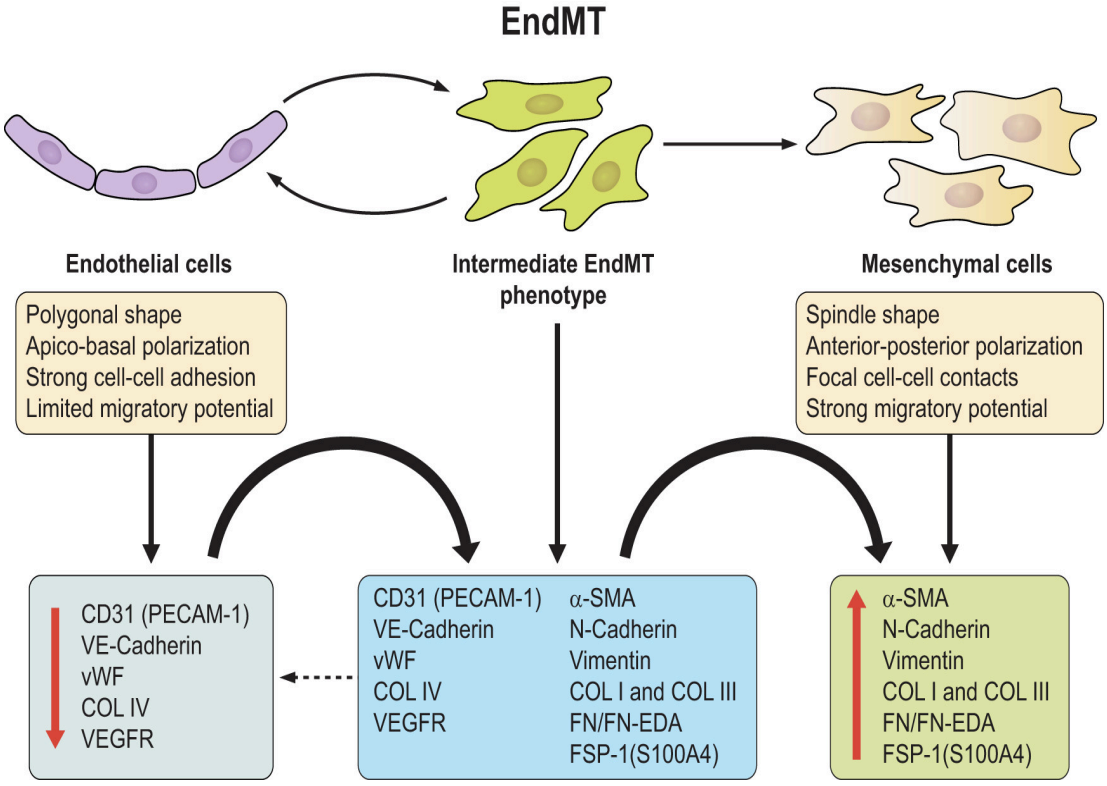


Figure 1.5: The endothelial to mesenchymal transition (EndMT). After activation, the cells exhibited increased production of mesenchymal-specific including smooth muscle active substance α (α-SMA), COL1, COL3, fibronectin (FN), vimentin, N-cadherin. Accompanied by loss of endothelial cell-specific such as CD31/platelet endothelial cell adhesion molecule-1 (CD31/PECAM-1), vascular endothelial cadherin (VE)-cadherin), and von Willebrand factor [48].

The role of EndMT in fibrosis assay has been determined both in vitro and in vivo. EndMT may play an important role in IPF mechanisms with the central role of transforming growth factor-β (TGF-β) in promoting EndMT through a vast network of molecular interactions. EndMT involves molecular and signaling pathways that are stimulated and regulated by

multiple mechanisms basing on the specific cellular and the pathophysiology or/and physiological condition of the cells. First observations of EndMT progression were present during chicken and mouse embryonic development through alterations in morphology and cellular components involved in heart valve development. Results have suggested that valvular cardiac cells arise from a separate population of endocardial endothelial cells that transition into mesenchymal cells [49, 50]. The morphological transition from endothelial cells to mesenchymal cells was also observed during aortic maturing in chick embryos [51, 52]. Then, the other studies determined that EndMT is not limited to embryonic development but can also occur in endothelial cells is involved in the vasculature of adults. The results show that endothelial cells isolated from the adult bovine aorta obtain mesenchymal cell expression such as α-SMA through an initiating and promoted transformation process by TGF-β1. These researchers also confirmed that the hybrid cells expressed both molecular markers that most likely represent an in-between phenotype between endothelial cells and mesenchymal cells/fibroblasts, an indication that altered phenotype is not permanent. The partially reversible conversion was demonstrated after culturing these cells in the absence of TGF-β1 [53].

Many studies describing EndMT are based on evidence of co-expression of ECs markers and mesenchymal markers in lung tissue in animal or human patient models, however, there are a number of major limitations. In response, methods for partial and complete elucidation of the EndMT protocol were improved using endothelial-specific fluorescence transgenic mice [54]. Future studies are needed using samples from IPF patients to clearly understand the molecular mechanisms of IPF.

1.2.3. The role of TGF-β1 in EndMT of IPF

The transforming growth factor β (TGF-β) family of proteins includes some of growth factors that play important roles in many physiological processes such as embryogenesis, cell development, and differentiation, immune system development, immune function, and immune response, inflammation, fibrosis, and wound repair [55, 56].

TGF-β plays a central role in fibrosis in general and IPF in particular as it promotes fibrosis through different signaling pathways. Furthermore, studies have reported TGF-β plays an intimate role in the initiation and progression of EndMT processes [57]. The canonical TGF-β pathway is activated when TGF-β homodimers bind to the transmembrane heterologous TGF-β I/II receptor complex on the cell surface. The phosphorylation-mediated activation of the TGF-β receptor I/II complex results from the binding of TGF-β to the heterodimeric receptor complex. Phosphorylation of the Smad2 and Smad3 proteins in the cytoplasm by the active TGF-β receptor I/II complex results in activated Smad2 and Smad3 forming a complex with Smad4 translocating to the nucleus. Then, inside the nucleus, the Smad2/Smad3/Smad4 complex interacts with Smad-binding factors (SBEs) of TGF-β responsive genes including macromolecules encoding profibrotic extracellular matrix (ECM) and transcription factors, such as Snail1 and 2 and Twist, stimulating their transcription. Smad7 inhibitor is another intracellular Smad protein that displaces TGF-β-induced signaling cascades with an inhibitory effect on activated TGF-β receptors through TGF-β-induced gene expression responses that is a potent negative regulator of TGF-β. In addition, there are signals through non-canonical TGF-β pathways that mediate Smad2/3-independent TGF-β responses. The mitogen-activated protein kinase (MAPK) family of specific protein kinases, serine/threonine, and many other kinases as phosphatidylinositol 3-kinase (PI3K), RhoA, Rac, c-Abl,

and protein kinase C (PKC)-δ, TGF-β can activate all three known MAPK pathways. MAPK pathways include extracellular signal-regulating kinase (ERK), p38 MAPK, and c-Jun NH2 terminal kinase (JNK) which are non-primary TGF-β pathways. Studies demonstrate these pathways lead to decreased transcription of endothelial-specific genes or accrete transcription of mesenchymal-specific genes mediated by EndMT-related transcription factors including Snail1, 2, and Twist1 [58-60] (Figure 1.6).

The roles of both canonical TGF-β and noncanonical pathways in EndMT were confirmed by demonstrating a reduction in the number of mesenchymal cells derived from endothelial cells when TGF-β response was reduced in transgenic mice, as well as by knockout of EndMT using an activated TGF-β inhibitor TGF-β receptor kinase inhibitor or using a molecular inhibitor small amount of TGF-β-mediated intracellular phosphorylation [61-63].

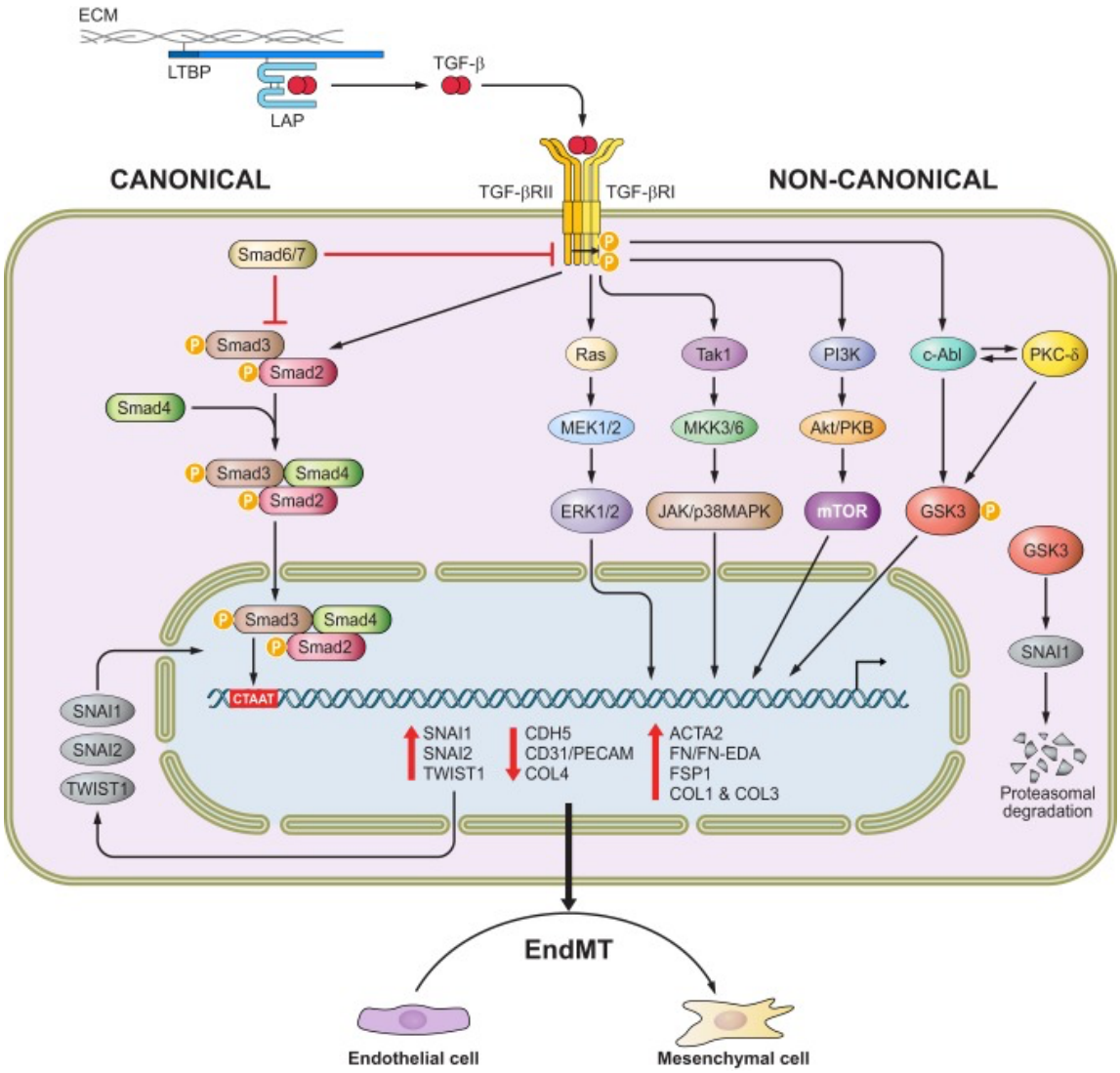


Figure 1.6: Canonical transforming growth factor TGF-β and noncanonical pathways in endothelial to mesenchymal transition (EndMT) [48].

In addition to the aforementioned canonical and non-canonical molecular signaling pathways, additional pathways involved in the regulation of the EndMT include the endothelin (ET)-1, Notch, caveolin (CAV)-1, Wnt, high and weak glucose pathways hypoxia-inducible factor (HIF)-1α. The NOTCH and Wnt morphological pathways are important modulators of EndMT. The role of Notch signaling in EndMT has been described for the first time in vitro in human microvascular endothelial cells and HUVECs [64]. Subsequent studies also showed an important contribution of Notch signaling during EndMT during valvular

development [65, 66]. In addition to NOTCH signaling, the involvement of Wnt signaling in EndMT has been recently examined in fibrosis of the heart, aortic valve, and renal tissues. The in vitro study indicated that during myocardial fibrosis, a significant proportion of α-SMA-positive fibroblasts present in post-myocardial infarction cardiac tissues were derived from cells. Results demonstrate endothelial cells undergo EndMT and express β-catenin-dependent Wnt signaling [67]. Many other investigations have continued to confirm the role of β-catenin in EndMT [68]. CAV-1 has an inhibitory effect due to the entry of TGF-β receptors and their subsequent degradation. The inhibitory effect of CAV-1 in the development of EndMT was confirmed by demonstrating that pulmonary microvascular endothelial cells from CAV1 knockout mice showed high levels of molecular changes leading to EndMT, which were even increased after TGF-β treatment [69]. ET-1 induces TGF-β-induced synergistic stimulation of EndMT associated with the canonical Smad2/3 pathway. Pulmonary microvascular endothelial cells were examined in vitro to demonstrate an interaction between ET-1 and TGF-β1 in the induction of EndMT. Histological analysis in mice treated with TGF-β1 or ET-1 alone expressed EndMT but treatment with TGF-β1 plus ET-1 significantly increased the number of cells undergoing EndMT [70]. Hypoxia induces EndMT through the effect of HIF-1α activation by Snail1. The roles of hypoxia in EndMT in different types of endothelial cells have been determined in vivo in different animal models. Endothelial cell line monitoring in mice indicates a particular green fluorescent protein in endothelial cells confirmed a role for hypoxia in EndMT in the induction of pulmonary arterial hypertension [54]. High glucose levels have been shown to induce EndMT involved in the phosphorylation of extracellular signal-regulating kinase 1/2 (ERK) [71]. In particular, shear stress induces EndMT through many different molecular mechanisms. One mechanism is

started by mechanically induced TGF-β release and the release of TGF-β from the latency-associated peptide (LAP), then by activation of the TGF-β standard pathway. The other mechanisms such as the generation of reactive oxygen species (ROS) and activation of NFκB, next is the activation of NOX1/4 oxidants leading to raised production and accumulation of ROS (Figure 1.7).

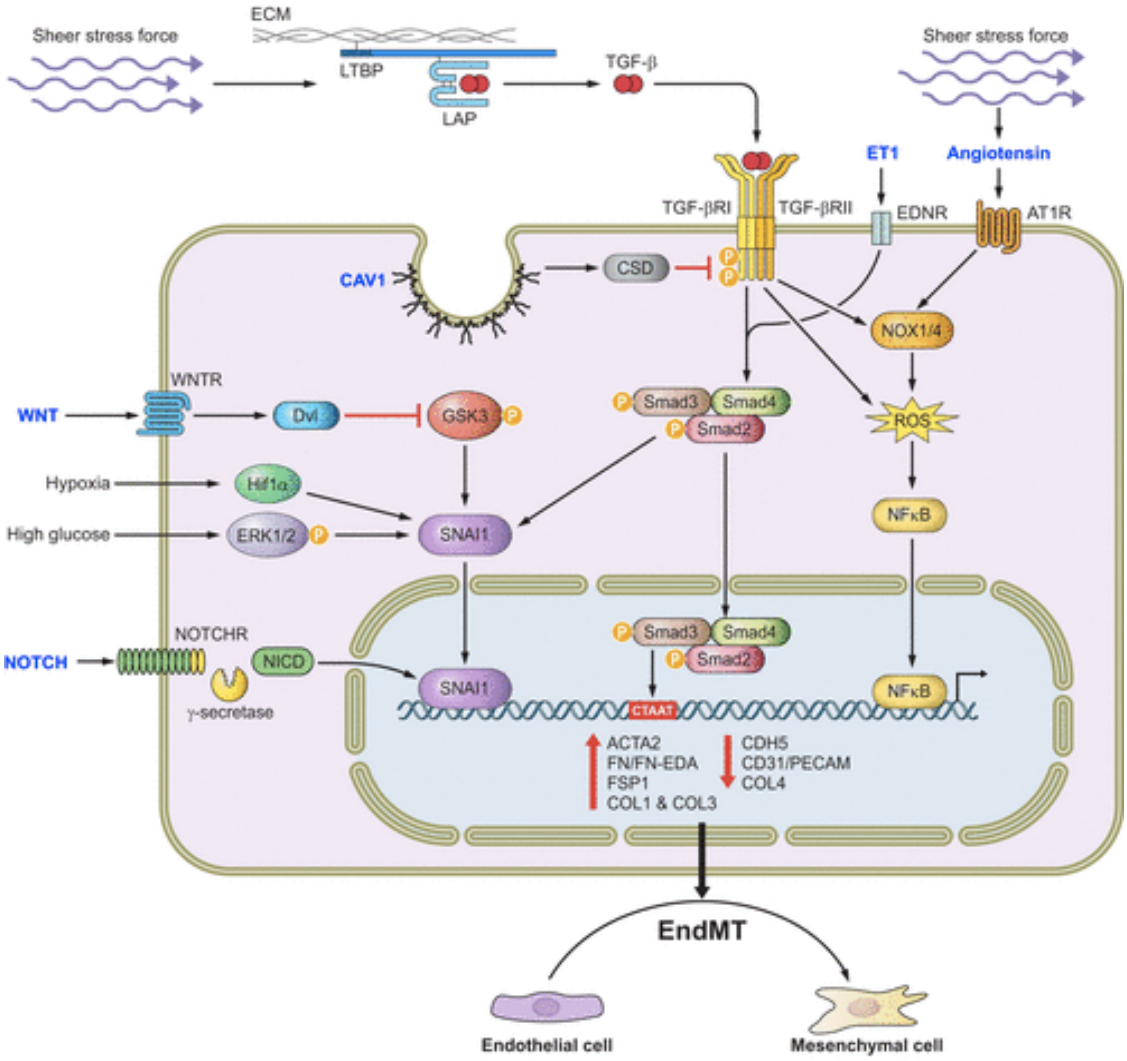


Figure 1.7: Pathways involved in the regulation of endothelial to mesenchymal transition (EndMT) regulation. Molecular signaling pathways in addition to the variable growth factor (TGF-β) center pathways that induce or participate in the EndMT process include endothelin (ET) -1, Notch, caveolin (CAV) -1, Wnt, high glucose and hypoxia inducing factor (HIF) -1α [48].

1.3. Oxidative stress and IPF

In the body, all cells need to use and metabolize oxygen, which leads to the generation of reactive oxygen species (ROS). ROS are free radicals that are naturally formed as byproducts of oxygen metabolism, including superoxide anions (O 2 • -), hydrogen peroxide (H 2 O 2, and hydroxyl radical (OH •), as well as other secondary ROS. ROS are generated by aerobic metabolism in the electron transport chain of the mitochondria; in addition, they can also be generated by various enzyme systems, including xanthine oxidase, lipid peroxidase, cytochrome P450 enzyme, and NADPH oxidase (NOXes) [72]. Endothelial cells, neutrophils, eosinophils, alveolar macrophages, and alveolar epithelial cells of the lung are all primary cells that produce ROS. In the pulmonary vascular system, ROS can be generated from complexes in cell membranes, cellular organelles such as peroxisomes and mitochondria, and in the cytoplasm. Excessive or uncontrolled ROS production due to increased NOX activation or mitochondrial dysfunction, or ROS metabolism compromised by antioxidant systems is often thought to contribute to pathogenesis, that is, oxidative stress, and causes cell damage and cell transformation [73, 74].

1.3.1. Disturbed redox homeostasis in IPF

Similar to other organs in the body, the lungs normally contain multiple antioxidant systems to prevent excessive cellular ROS production, including enzyme-converting ROS (superoxide dismutases [SODs], catalases, peroxiredoxins [PRXs], and glutathione peroxidase), and small molecular weight antioxidants (vitamins and glutathione). In IPF, some of these antioxidant systems have been reported to be altered or impaired [73].

Redox processes are disturbed by increased ROS production and impaired antioxidant mechanisms may contribute to the pathogenesis of IPF (Figure 1.8**).** Over the past decade, it has been discovered that the production of biological ROS serves a wide range of physiological

functions that can be reversed by redox-dependent signals such as proliferation, migration, differentiation, or survival of cells [75, 76]. The increased production of ROS in IPF contributes to the pathogenesis of IPF through the promotion of epithelial cell death and an unstable wound healing response after repeated chronic trauma to the pulmonary epithelium [77]. Fibroblast accumulation with an apoptosis-resistant phenotype, which is associated with impaired Nrf2 induction and increased H 2 O 2 production, is a consequence of an increased oxidative burden [78]. Specific study of Nox-mediated redox imbalance during fibrosis could be important for the development of better therapeutic strategies for fibrotic disorders such as IPF.

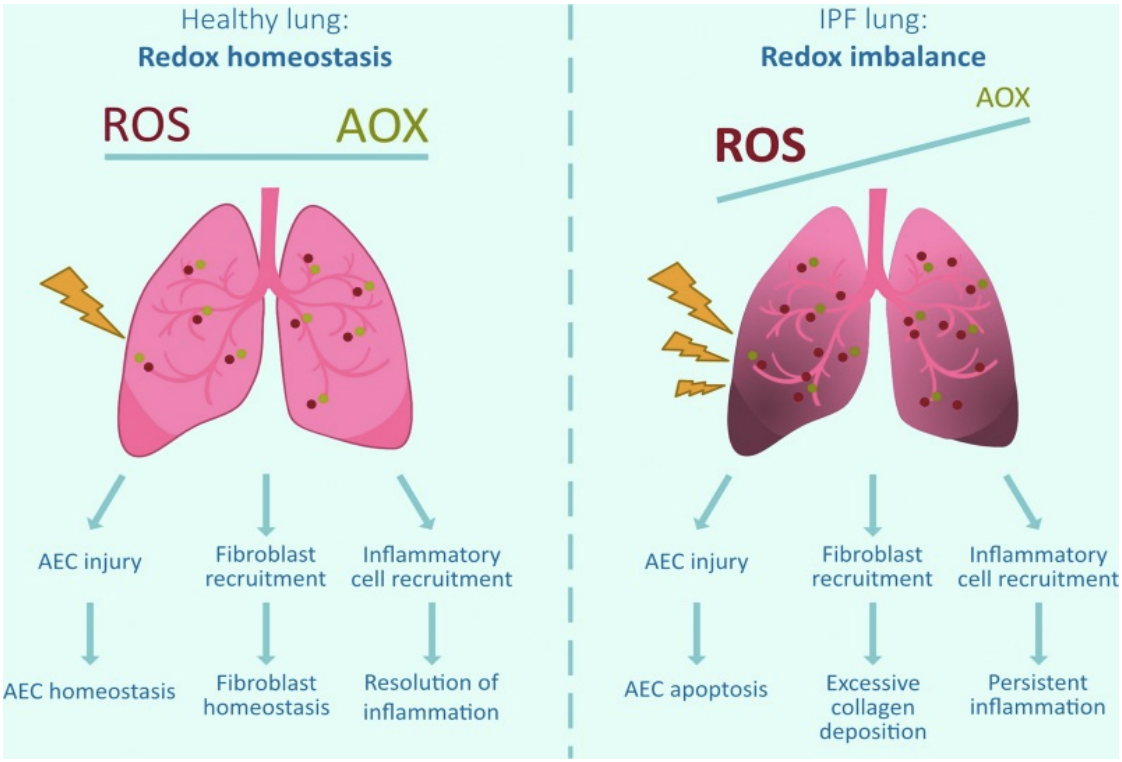


Figure 1.8: **Altered lung redox homeostasis in IPF**. In a healthy lung, there is redox homeostasis, i.e., ROS generated by exogenous or endogenous sources are appropriately counteracted by oxidants. In the IPF lung, there is a redox imbalance as ROS-generating processes are triggered by increased NOX production, dysfunction of synthesis. This redox imbalance is thought to contribute to epithelial cell death, excessive collagen loss, and persistent inflammation, leading to pulmonary fibrosis

and tissue scarring. AEC: alveolar epithelial cells; AOX: antioxidant; NOXes, NADPH oxidase; ROS, reactive oxygen species. [79].

1.3.2. The role of NOX enzymes in IPF

Until now, there has been ample evidence of an association between NOX-derived ROS and IPF [80]. The NOX family are enzymes that include seven NOX members (NOX1– 5 and DUOX1-2) [81, 82]. All NOX/DUOX isoforms contain a homologous COOH-terminal flavoprotein region. The homologous region consists of 2 binding regions of NADPH and flavin adenine dinucleotide (FAD), in which the hydrophobic region at the N-terminus consists of six transmembrane alpha-helices containing two Heme linkages [83] (Figure 1.9). Nox1-4 isoforms are activated by forming a heterodimer with the transmembrane protein p22phox [84]. But unlike NOX1-4, Nox5 and Duox are activated when their intracellular domains contain domains specific to phosphorylation and binding calcium.

Expression and regulation of NOX isoforms are different in different tissues and cells, specifically generating distinct ROS. For example, while NOX4 and DUOX1-2 mainly produce H 2 O 2, NOX1–3 and NOX5 mainly produce O 2 • - [85]. This different modulation (ROS production) not only protects the host, but also the proliferation, differentiation, and movement of cells by signaling pathways dependent on redox in certain fibrotic pathological conditions [86]. Currently, several studies indicate that Nox1, Nox2, and Nox4 are involved in the pathogenesis of IPF by the expression or activation of some altered NOX enzymes in the lungs of IPF patients. Indeed, there is ample evidence demonstrating changes of NOX expression/activation in IPF and their functional contribution to pulmonary fibrosis especially in animal models [87]. Therefore, the structural and functional characteristics of Nox1, Nox2, and Nox4 will be discussed.

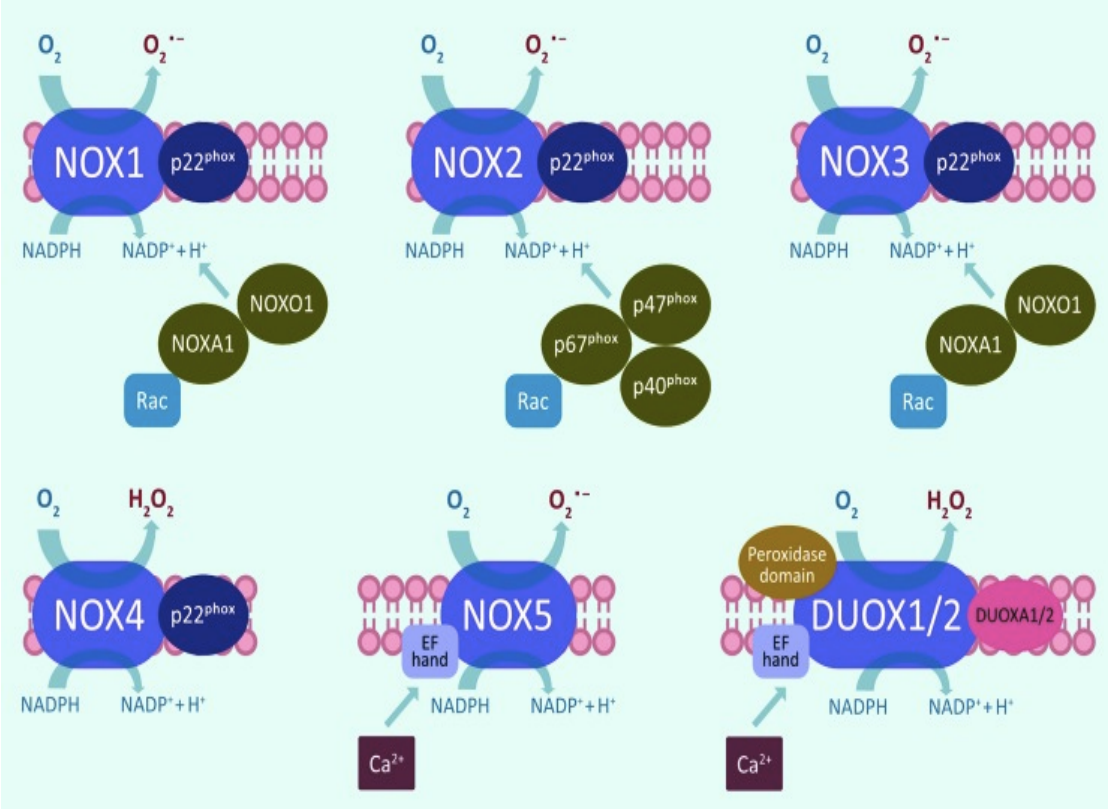


Figure 1.9. Structural overview of NOX family enzymes [79].

NOX1 expression is observed in epithelial and endothelial cells in lung tissues such as human pulmonary artery endothelial cells, bronchial epithelial cells, and pulmonary vascular smooth muscle cells. The increased expression has been reported due to various conditions such as pulmonary hypertension and stimulation of nicotine [88, 89]. At present, the contribution of NOX1 to IPF pathology remains unequivocally unknown.

NOX2 (or Gp91) was the first Nox isoform discovered. Studies report Nox2 had the highest expression in neutrophils and macrophages. In addition, it was expressed in mesenchymal cells, smooth muscle cells, endothelial cells, epithelial cells of the lung but at lower levels. NOX2 has an important role in inflammation [90] and has been shown to promote cell proliferation of endothelial cells [91]. However, the specific role of NOX2

in fibrosis progression of IPF is not yet clear; further studies are needed on this topic.

NOX4 is commonly expressed in many different cell types of the lung including macrophages, smooth muscle cells, endothelial cells, mesenchymal cells [92, 93]. Nox4 is the only Nox isoform capable of releasing mainly H 2 O 2 [94] which strongly induces TGFβ upregulation in the IPF lung epithelial cells and fibroblasts that, in turn, participate in pro-fibrosis [95]. In contrast to other Nox isoforms, Nox4 production is largely dependent on its level expression or after direct transcriptional modification [94] and independent of synaptic proteins of the cell. A common complication of IPF is pulmonary arterial hypertension which may be mediated by increased NOX4 activity. There is evidence that NOX4 expression is present in the thickened arteries of IPF patients [96]. Indeed, in vascular smooth muscle, Nox4-dependent ROS production, cytoskeletal reorganization, and cellular migration are regulated by Nox4/p22phox where they form a multimeric complex with polymerase delta-interacting protein [97]. Vascular smooth muscle cells play an important role in pulmonary perfusion regulation, as they are activated by TGF-β1 to express NOX4 through a SMAD2/3-dependent pathway, resulting in cell proliferation [98]. Pulmonary endothelial cells also show more NOX4 expression at sites of fibrous regions and around fibrous regions [99]. TGF-β is involved in endothelial cell death and in NOX4-dependent induction of ROS production. Despite overexpression, NOX4 prevents endothelial cell apoptosis due to TGFβ induction, but the exact role of NOX4 in these cells remains unclear [100].

1.4. Oxidative stress and EndMT in IPF: is there a link?

There are many factors involved in oxidative stress in IPF. Smoking is one of the factors that stimulate ROS production in inflammatory and other cells through endoplasmic reticulum stress, mitochondrial

dysfunction, and NADPH oxidase (NOX) production, for example, NADPH oxidase-4 (NOX4) [101]. The tight control barrier that prevents plasma secretion into the interstitial and alveolar spaces is an important role in the pulmonary endothelium. Two major Nox isoforms (NOX2 and NOX4) are expressed by pulmonary endothelial cells. The role of Nox4-dependent ROS is reported to be to regulate endothelial cell motility and angiogenesis. Notably, genetic silencing of Nox4 reduced oxidative stress-induced endothelial cell migration and capillary tube formation [102]. Nox4 expression through Nrf2-dependent activation of the Nox4 promoter has been induced by hyperoxia [103]. In addition, Nox2-mediated ROS in artery endothelial cells of the lung has been shown to contribute to impaired angiogenesis in pulmonary hypertension in lambs by the induction of autophagy of NOX2 [104]. In particular, a recent study documented an increase in ROS production and type I collagen deposition along with the proliferation of human pulmonary arterial smooth muscle cells (HPASMCs) induced by the serum of IPF patients. The cytological effects induced by the IPF sera were significantly reduced by diphenyleneiodonium (DPI) treatment, an NADPH oxidase inhibitor when compared with untreated sera [105] (Figure 1.10).

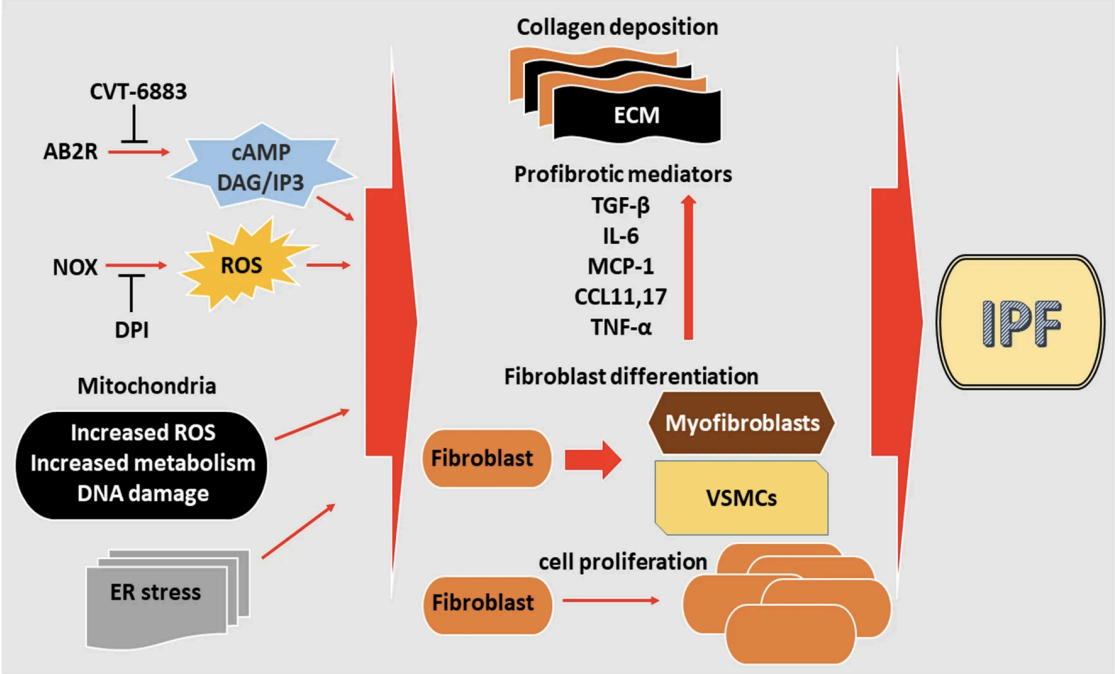


Figure 1.10: Diagram of the cellular and molecular events linked to the pathogenesis of IPF in the pulmonary environment. NOX (NADPH oxidase), ROS (reactive oxygen species), cAMP (cyclic adenosine monophosphate), DAG (diacylglycerol), IP3 (inositol trisphosphate), DPI (diphenyleneiodonium), CVT-6883A 2B-adenosine receptor antagonist, ER endoplasmic reticulum. Red arrows show activation, black lines show inhibition [106].

Endothelial dysfunction has long been reported to be associated with excessive ROS production which induces EMT through stimulation of TGF-β1 signaling. It wasn't until Montorfano et al. studied the role of oxidative stress in EndMT that this finding was reported for the first time in 2014 [107]. Several studies have since investigated a direct relation between oxidative stress and EndMT in PAH. The relationship between oxidative stress, TGF-β, and EndMT signaling has been reported in an in vitro study [107], in which the role of enzymes and co-factors in ROS production and sensitization signals were found to be mediated by oxidation [108]. Their association with EndMT in PAH has also been identified in animal models. Evidence for a pathogenic role of ROS in EndMT, specifically the activation and mediation of intrinsic adaptation to

PAH, has been demonstrated by several redox signaling pathways. For example, Redox-regulated PKGIα, and perhaps others, have yet to be studied. Besides the reports, there is an association between BMPR2 dysfunction and oxidative stress in the PAH that highlights the close connection between signaling pathways involved in the dysfunction of endothelial function and pathogenesis [88, 109]. This also provides a theoretical for a more specific analysis of the relationship between ROS and EndMT in PAH in general (Figure 1.11). Therefore, more studies are useful to understand the role of oxidative stress in the EndMT process of IPF disease. Finally, the EndMT-targeted therapies can be generated to decrease the oxidative stress found in IPF disease, and control disease progression.

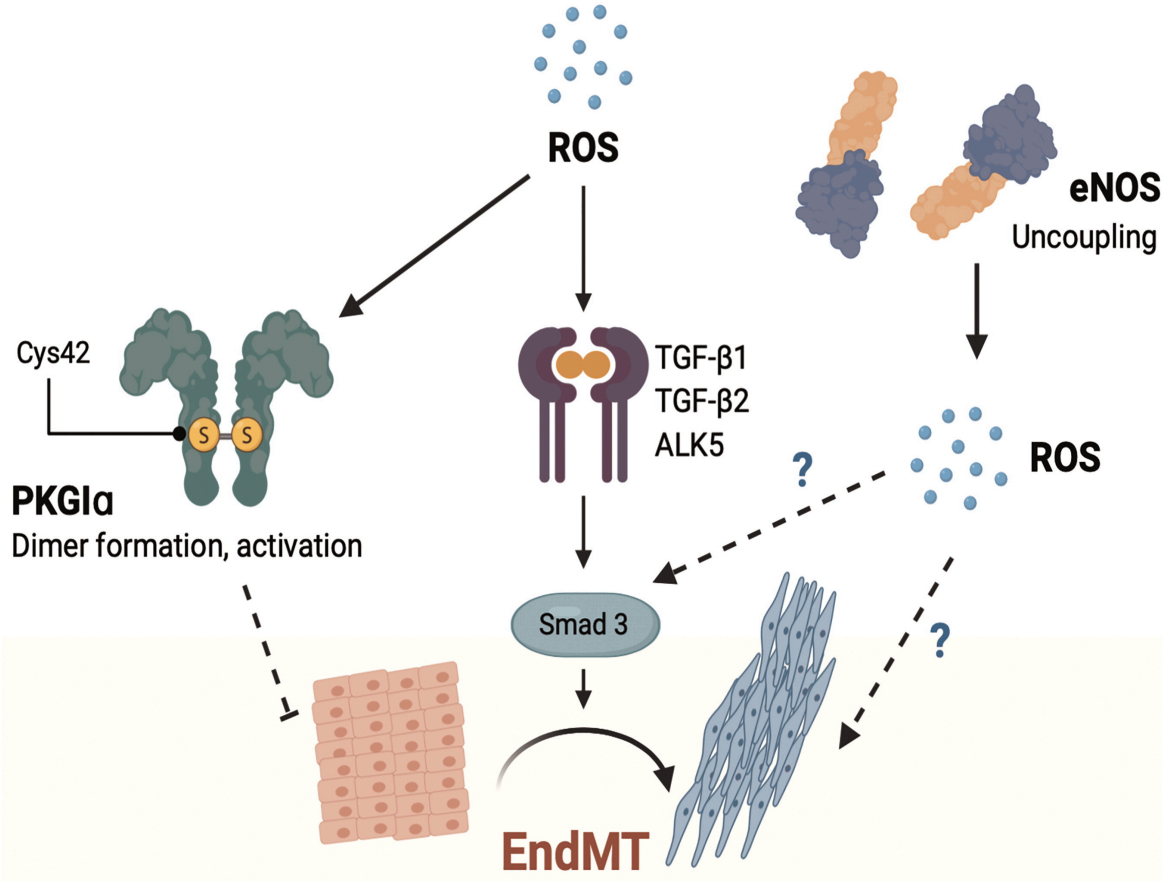


Figure 1.11: A role of ROS in the induction of PH-associated EndMT. Increased production of ROS due to an imbalanced redox state in the pulmonary vasculature in

PH leads to an induction of EndMT through an upregulation of TGF-β1/TGF-β2/ALK5/Smad3 signaling axis as well as a yet to be defined mechanism likely involving eNOS uncoupling. On the contrary, oxidation of PKGIα serves as a protective mechanism to limit PH-associated EndMT. ALK, activin receptor-like kinase; eNOS, endothelial nitric oxide synthase; PKGIα, protein kinase G Iα; ROS, reactive oxygen species [110].

2. RESEARCH OBJECTIVES

Idiopathic pulmonary fibrosis (IPF), the most common form of idiopathic interstitial pneumonia, is a progressive, irreversible, and often fatal disease characterized by an abnormal fibrous response involving several areas of the lung [29]. An unstable tissue structure, including severe collagen secretion and deposition, gradually replaces healthy tissue structure, significantly damaging lung functions and eventually leading to death. The molecular and cellular determinants that activate and maintain abnormal fibrous processes are largely unknown. However, it appears that repeated microtrauma directed to the alveolar epithelium may play a major role. Indeed, the aforementioned process leads to the release of various growth factors and fibrous mediators such as fibroblast growth factor (FGF), transforming growth factor-beta 1 (TGF-β1), which activates fibroblast recruitment, proliferation, and extracellular matrix accumulation in alveolar regions [111].

Despite great scientific efforts in studying the etiology and pathogenesis of IPF, the overall picture of its pathogenic mechanism remains controversial. Specifically, several studies indicate that an initial injury of known or unknown etiology induces unresolved inflammation or alveolar epithelial cell activation. Epithelial and inflammatory cells release cytokines, growth factors that induce fibroblast migration, proliferation, and changes in cell phenotype [111]. Oxidative stress-mediated vascular dysfunction is one of the important features of adverse vascular changes in IPF patients. The presence of large amounts of excess ROS has been supported by numerous studies, which document an aberrant redox state and demonstrate ROS involvement in extracellular matrix deposition, implying that the molecular mechanism remains unclear. Many clinical trials with antioxidants have been performed to inhibit the progression of

IPF [112, 113]. More recently, evidence has also shown that NADPH oxidase (NOX) enzymes induce ROS in IPF. When NOX4 was inactivated, either transcriptionally or pharmacologically, TGF-β1 was unable to promote fibroblast differentiation and collagen secretion [96].

In addition, aberrant microvascular and macrovascular remodeling of the pulmonary vasculature appears to be closely related to the pathogenesis of IPF. Previous studies reported that the injured epithelium and the endothelium can release growth factors, cytokines, and matrix metalloproteinases that induce mesenchymal cell activation and proliferation, ECM deposition, and fibroblast accumulation. Changes in cellular plasticity are associated with the onset and progression of IPF, which can be essentially classified in EMT and EndMT [106]. Experimental evidence suggests that fibroblasts can originate from cellular sources other than fibroblasts. In particular, endothelial cells (ECs) are a potential source of action through EndMT [114]. During this process, ECs lose characteristic cellular features, typical endothelial markers, including VE-cadherin, CD31, von Willebrand factor, and undergo the transition to a mesenchymal phenotype with expression of mesenchymal markers such as α-SMA, vimentin, and collagen type 1. These processes contribute significantly to fibrosis in the affected tissue [115]. The importance of EndMT contributing to fibrosis in the affected tissue, in the process of fibrosis, has been demonstrated to occur in many fibrosis diseases such as chronic kidney disease [116]. However, the large vascular remodeling associated with IPF and the impact of EndMT in fibrosis and their role in the initiation and progression of IPF-associated fibrosis requires further study.

EndMT can trigger enhanced fibroblast proliferation and is implicated as a novel mechanism for the generation of activated fibroblasts. As reported above, ROS mediates TGF-β differentiation of fibroblasts. This in turn causes increased production of fibrous type I and type III collagen and another extracellular matrix (ECM) proteins and activates the

expression of smooth muscle α-actin (α-SMA), a process that is strongly correlated with EndMT [117] [114].

The objective of this study was to investigate whether oxidative stress and EndMT could be part of the molecular machinery that causes vascular damage in IPF patients. Therefore, we conducted this study with the following purposes:

1. To investigate whether IPF sera can induce oxidative stress in HPMECs.
2. To investigate whether IPF sera can affect HPMECs survival.
3. To investigate whether IPF sera can induce EndMT in HPMECs.
4. To identify the potential relationship between Oxidative stress and EndMT on HPMECs in IPF.

3. MATERIALS AND METHODS

3.1. Ethical approval

The Ethics Committee from the University of Sassari, Italy approved all study procedures. Informed consent was acquired from patients for all study samples.

3.2. Patients

All IPF patients in the study were diagnosed according to evidence-based guidelines for the diagnosis and management of IPF per the document "An official ATS/ERS/ JRS/ALAT statement: idiopathic pulmonary fibrosis: evidence-based guidelines for diagnosis and management," American Journal of Respiratory and Critical Care Medicine [25].

Inclusion Criteria: High-resolution computed tomography (HRCT) images and lung biopsies of patients diagnosed at the University Hospital of Sassari were used to confirm sample eligibility and examined by four experienced physicians (two radiologists and two pathologists). The definitive diagnosis for each registered patient was approved based on multidisciplinary discussions with experienced interstitial lung disease specialists in the Department of Respiratory and Radiological Pathology of the University of Sassari. In addition to a definitive diagnosis of IPF, all patients enrolled in the study met the appropriate pulmonary function criteria: a predicted forced vital capacity (FVC) of at least 50% and the percentage diffusing carbon monoxide diffusing capacity (DLCO) predicted at least 35%.

Exclusion criteria: Patients were excluded from the study with the following medical conditions: current exacerbation of IPF, comorbidities such as malignancy, bleeding tendency, and liver dysfunction - severe

kidney disease with alanine transaminase or aspartate transaminase levels more than twice the upper limit of normal, or serum creatinine levels above the upper limit of normal, use of immunosuppressants, antifibrotic drugs, interferon, d-penicillamine, and colchicine, or oral corticosteroids at a dose of ≥ 15 mg/day or equivalent for the previous three months, and currently pregnant or nursing.

Healthy donors: Healthy donors with suitable age and sex were selected through postings and enrolling after a passed screen questionnaire to exclude the presence of any autoimmune or potential blood vessel disease.

Serum samples from patients with IPF and Healthy donors were collected at the Hospital of Sassari University. Demographics and clinical characteristics of IPF patients and healthy donors (HD) are summarized in table 4.1, respectively.

Blood samples were taken from each patient and processed within 2 hours of collection. Samples were stored at -80^{0}C until being experimented.

Serum extraction.

Serum was separated from whole blood samples from the IPF patients and healthy donors (HD) for the study. Within 2 hours from collection, the whole blood tube was subjected to centrifugation at a speed of 2,500 RPM for 10 minutes at a temperature of 4^{0}C. The supernatant was recovered and subsequently subjected to further centrifugation of 12,500 RPM for 10 minutes at a temperature of 4^{0}C again. The supernatant representing the serum was stored at - 80^{0}C until the moment of use.

3.3. Cell line

Human Pulmonary Microvascular Endothelial Cells (HPMECs) were provided by Innoprot (Spain). These cells were isolated in the ScienCell Research Laboratories (Carlsbad, CA, USA) from the human lungs of

healthy individuals. HPMECs were treated with sera from IPF patients and controls (Healthy donors) to compare.

3.4. Cell culture and Treatment

Endothelial cell medium (ECM) of Innoprot, S.L, Spain was used to culture the HPMECs and supplemented with 5% Fetal Bovine Serum (FBS), 1% of Endothelial Cell Growth Supplement (ECGS), and 1% Penicillin/Streptomycin solution (P/S solution) in the growth medium. The cells were maintained culture at 37°C and 5% CO_2 in a humid environment in an incubator.

Cells that reached the experimental concentration were seeded in a 96-well plate (BD Falcon). They were treated for experiments in a basic medium containing 5% serum of the study subjects. Depend on the protein content of different subjects was normalized. To investigate the role of NADPH oxidase in the effects of cells induce by IPF serum, in experiments, cells were pretreated for 1 hour with 5μM of the flavin-oxidase inhibitor diphenyleneiodonium (DPI), NOX inhibitor, or 2.5μM Chelerythrine (CHE), or 5mM N-acetyl cysteine (NAC) [118].

3.5. Measurement of Intracellular ROS

By using the 2 ′, 7'-dichlorodihydrofluorescein diacetate (H2DCF-DA) molecular ROS probe for assessed the level of intracellular ROS, as in previous similar studies but with correction [119]. In the cell, esterases cleave the acetate groups on H2DCF-DA, and the reduced form of the probe (H2DCF) is retained. Intracellular ROS oxidize the reduced form of the probe (H2DCF), yielding the fluorescent product (DCF). Fluorescence was measured using a Tecan GENios Plus microplate reader, Tecan, Switzerland, in a dark light condition. The excitation and emission wavelengths used for fluorescence quantification were 485nm and 535nm, respectively.

For measurements of intracellular ROS, 10^5 HPMECs cells

concentration per well were seeded in black 96-well plates, Costar, Corning, Inc, NY, and incubated with their growth medium alone for 24 hours at 37°C with an atmosphere of 5% CO2/95% air. After 24 hours, growth mediums were removed, HPMEC cells were pre-incubated for 30 minutes with PBS 1X containing 1μM H_2DCFDA, then they were washed with PBS and processed as described above. After 15 minutes, cells were treated with 5% sera of IPF and HD. Fluorescence was measured using a Tecan GENios Plus microplate reader, Tecan, Switzerland, under photoprotected conditions. The excitation and emission wavelengths used for fluorescence quantification were 485nm and 535nm. Fluorescence variation was kinetically measured in 5 hours. All fluorescence measurements were adjusted for background fluorescence and protein concentration. Using untreated cells as a control sample compare with treated cells. Intracellular ROS levels results were evaluated by comparing five measurements and expressed as mean ± SD of relative fluorescence unit value (RFU).

3.6. Measurement of intracellular ROS with NADPH inhibitors

For these measurements similar to above, 105 HPMEC cells per well were seeded in black 96-well plates, Costar, Corning, Inc, NY, and incubated with their growth medium for 24 hours at 37°C with an atmosphere of 5% CO2/95% air. After 24 hours, cells were pretreated with the broad NOX inhibitor, diphenylene iodonium (DPI) 5μM for one hour. Then, the culture medium was removed, HPMEC cells were pre-incubated for 30 minutes with PBS 1X containing 1μM H2DCFDA, washed with 1X PBS, and then processed as described above. After 15 minutes, cells were treated with 5% sera of IPF and HD, and the fluorescence was measured using a Tecan GENios Plus microplate reader, Tecan, Switzerland, under photoprotected conditions. The excitation and emission wavelengths used

for fluorescence quantification were 485 nm and 535 nm, respectively. Treatment-induced fluorescence variation was kinetically measured over a 5-hour period. All fluorescence measurements were corrected for background fluorescence and protein concentration. Using untreated cells as control, intracellular ROS levels results were evaluated by comparing five measurements and expressed as mean ± SD of relative fluorescence unit value (RFU).

3.7. Cell Viability Assay

Cell viability was evaluated in 96-well plates (BD Falcon) by using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT reagent) assay (Promega, Madison, WI, USA). Yellow MTT reagent enters the cells and passes into the mitochondria where mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding reduced purple MTT formazan crystals, which are insoluble in aqueous solutions. Cell viability was evaluated by the purple MTT formazan crystals formation, which occurs only when mitochondrial enzymes are active because their conversion can only directly occur to the number of cells that survive. Formazan crystals were dissolved in acidified isopropanol to crease a purple solution, and the obtained purple solution was determined spectrophotometrically at 570 nm. The last, increase in cell number resulted in a large amount of purple formazan MTT crystals being formed and increased absorbance at 570 nm [120, 121].

In our experimental conditions, after 24 hours of treatment, 20 µL of MTT solution (2 mg/mL) in M199 medium was added to the cells and incubated at 37 °C in a cell culture incubator for 4 hours. At the end of the incubation stage, the solution was removed and purple MTT formazan crystals product washed twice with 1X PBS. The purple formazan product crystals were then solubilized by acidic isopropanol (0.04 N HCl in absolute isopropanol). Finally, plates were analyzed at 570 nm using a

GENios plus microplate reader (Tecan). Results were calculated as the means ± SD of five measurements and expressed as a percentage of untreated control cells.

3.8. Protein extraction for ELISA (Lysis buffer method)

10^5 HPMECs cells concentration per well were seeded in white 12-wells plates (Costar, NY) and incubated with a growth medium for 24 hours at 37°C with an atmosphere of 5% CO_2/95% air. Then, HPMEC cells were pre-treated for experiments in a basic medium containing 5% serum of the study subjects (IPF and HD). Serums of different study subjects (IPF and HD) were normalized depending on protein content. To evaluate the role of ROS (NADPH oxidase) in the serum-induced cellular effects of IPF, in select experiments, cells were pretreated for 1 hour with 5μM of the flavin-oxidase inhibitor diphenyleneiodonium (DPI), a NOX inhibitor. This is the experiment that we did for 3 days for extraction of the proteins used for the ELISA kits, following the steps as below:

- Trypsinize cells.
- Block trypsin by adding 5 ml of could clture media containing (5-10 %FBS)
- Centrifuge the cell at 1100 rpm for 5 min, then discards the supernatant.
- Add 1 ml of cold PBS – centrifuge at Centrifuge the cell at 1100 rpm for 5 min - discard the supernatant.
- Repeat step 4 for 2-3 times (make sure no culture media is present).
- Lysis Buffer method
- Lysis Buffer: Triton Lysis Buffer (TLB) cold + protease and phosphatase inhibitors.

Lysis Buffer Cocktail	
Triton lysis buffer	1 ml
PMSF	17 µl
1M NaF	50 µl
100mM NaVO3	17 µl
Protease inhibitor	20 µl

• Add 100 µl of lysis buffer to the cell pellet coming from point 5 and perform 4 Freezing-Thawing cycles as describe below:

- 8 minutes freezing at -80°C
- 2 minutes at 37°C in the water bath
- Vortex 30-60 seconds.

• Centrifuge 10 min at max speed at 4°C (14000 rpm)

• Collect the supernatant and discard the pellet (you may aliquot the supernatant is needed "e.g 2 aliquots 50 ml)

• Store sample at - 80°C.

The concentration of proteins in the solution is determined by the Bradford Reagent (Sigma).

3.9. Quantification of the proteins by Bradford.

The protein concentration in the solution was determined by the Bradford experiment, following the manufacturer's protocol, Sigma, St Louis, MO.

The Bradford Reagent was used to evaluate the concentration of proteins in the solution, and the procedure depends on the formation of a complex between the dye (Brilliant Blue G) and proteins in the solution. The protein-dye complex causes a change in the absorption maximum of the dye, from 465 to 595 nm, the quantity of absorption is equivalent to the protein present. Bradford Reagent requires to do no dilution and is appropriate for micro, multi-well plate, and standard experiments. The

concentration range of protein is 0.1-1.4 mg/ml, using bovine serum albumin (BSA) as the standard protein.

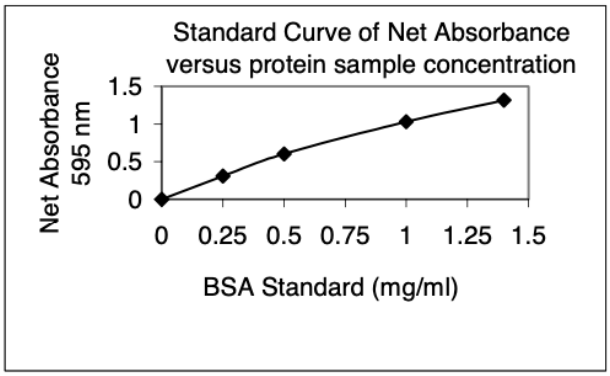


Figure 3.1: Standard Curve

96 Well Plate Assay Protocol (5 µl of a 0.1-1.4 mg/ml protein sample is used). This assay is performed in a 96 well plate. With this assay it is possible to quickly assay multiple protein samples, while using a small sample volume (5 µl). It is also possible to automate your protein determination with this multiwell plate assay. After gently mix the Bradford Reagent in the bottle and bring it to room temperature. Prepare protein standards in buffer ranging from 0.1-1.4 mg/ml using a BSA standard or an equivalent protein standard. Using 5 µl of the protein standards, and 5 µl of buffer for the blank wells. Prepare the unknown sample(s) with an approximate concentration between 0.1-1.4 mg/ml. To each well being used, add 250 µl of the Bradford Reagent and mix on a shaker for approximately 30 seconds. Let the samples be incubated at room temperature for 5 to 45 minutes. Then measure the absorbance at 595 nm. The protein-dye complex is stable for up to 60 minutes. The absorbance of the samples must be recorded before the 60-minute time limit and within 10 minutes of each other. Determine the protein concentration of the unknown sample(s) by comparing the Net A595 values against the standard curve.

3.10. Identifications of endothelial markers and mesenchymal markers by ELISA

Protein levels of both endothelial markers (VE-cadherin, CD31, von Willebrand factor) and mesenchymal markers (α-SMA, Col1) were determined by ELISA kits (Elabscience cat.). This ELISA kit uses the Sandwich-ELISA principle. The micro-ELISA plate in this kit has been pre-coated with an antibody specific to Human-specific protein. Standards and study samples are added to wells of the ELISA micro-plate to combine with the specific antibody. Then, a biotinylated detection antibody specific for Human-specific protein and Avidin-Horseradish Peroxidase (HRP) conjugate are added to each micro-plate well and incubated. After the free components are removed away by washing, the substrate solution is put on each well. But only those wells that contain Human-specific protein (VE-cadherin, CD31, von Willebrand factor, α-SMA, Col1), the biotinylated detection antibody, and Avidin-HRP conjugate will appear fluorescent. The RLU (relative light unit) value is evaluated by the machine Chemiluminescence immunoassay analyzer. The RLU value is obviously associated with the concentration of Human-specific protein. The concentration of Human-specific protein (VE-cadherin, CD31, von Willebrand factor, α-SMA, Col1) in the study samples can be identified by comparing the RLU value of the study samples to the standard curve.

After treatment, HPMEC cells extract were washed and recovered in the protein extraction buffer. Then, normalizing samples for protein concentration, 100 µL of cell extract (protein) was incubated for 90 minutes at 37 °C, standard proteins with a different concentration were also added in separate wells for the standard curve calculated. Following incubation, 100 µL of biotinylated detection Ab buffer was added and incubated for 1 hour at 37 °C and were then aspirated and washed 3 times

with 1X PBS buffer. Continue incubation with 100 µL of HRP conjugate for 30 min at 37 °C and wells also were aspirated and washed 5 times before adding 90 µL of substrate reagent. The substrate reagent was incubated for 15 min at 37 °C and then the reaction was blocked with 50 µL of stop solution. At last, the absorbance at 450 nm was measured and protein concentration determined following the manufacturer's instruction. [105].

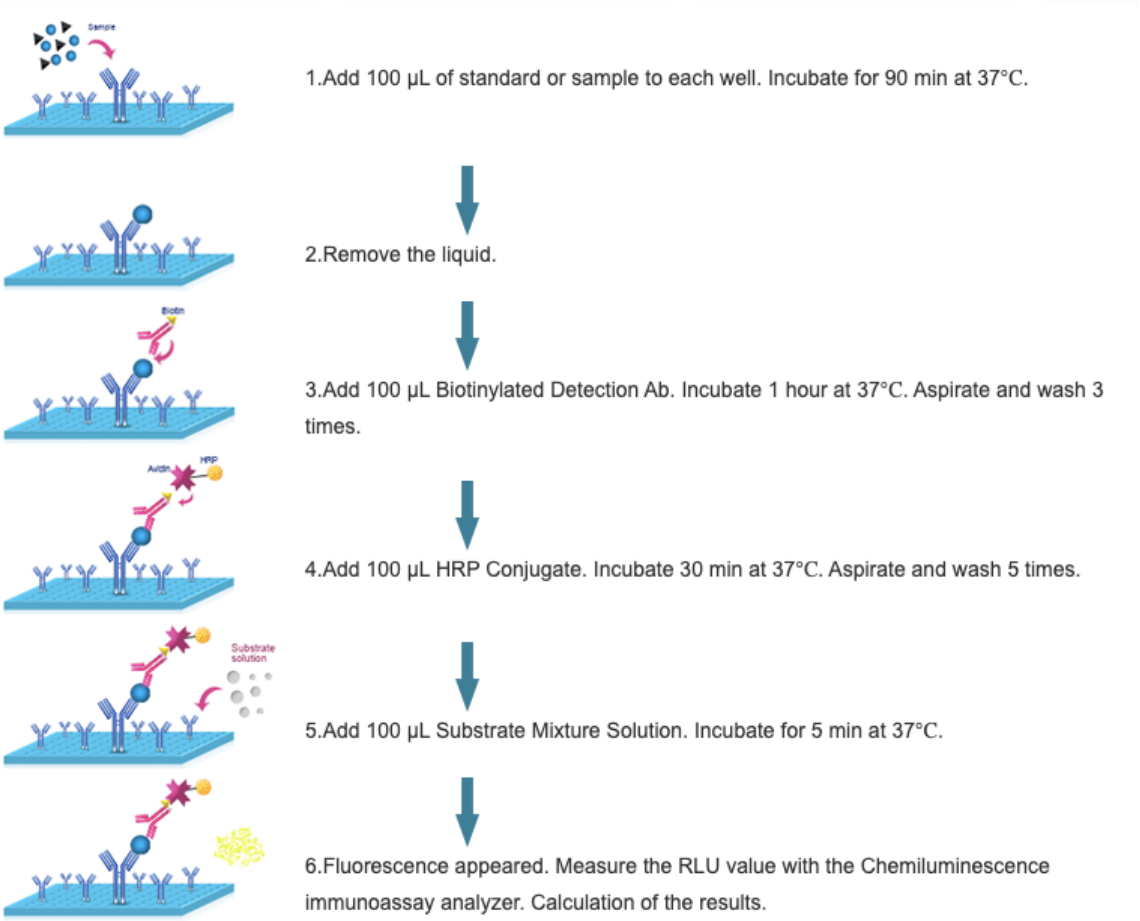


Figure 3.2: ELISA Assay procedures

3.11. Statistical Analysis

Data are shown for individual subjects in absolute values and presented as mean ± SD. One-way ANOVA followed by a post hoc Newman-Keuls Multiple Comparison Test was used to detect differences between more than two groups. Differences were determined to be statistically significant as $p < 0.05$. Statistical analysis was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

4. RESULTS

4.1. Patient demographics and clinical characteristics

Table 4.1. Demographics and clinical characteristics of IPF patients.

All most IPF patients enrolled in this study were older than 70 years and male. Patients with IPF were slightly older than the healthy donors (74 ± 5 and 71 ±2,4, respectively; p = 0.09).

Subjects' characteristics	IPF patients/T0, n = 10	Healthy donors (HD), n = 9	P value
Age, mean (SD)	74 (5)	71 (2,4)	0,09
Male, n (%)	8 (80 %)	8 (88,9 %)	0.54
Former smokers, n (%)	7 (70 %)	8 (88,9 %)	0.34
FVC, % predicted, mean (SD)	75 % (0,14)		
DLCO, % predicted, mean (SD)	54 (0,08)		

The studied samples were collected at the time of admission to the hospital in untreated IPF patients (T0). Healthy donor (HD) samples were collected from healthy blood donors following the criteria of this study. At the time of admission (T0), IPF patients have confirmed the spirometry values, such as FVC was 75 ± 0.14% of the predicted value and DLCO was 54 ± 0.08% of the predicted value.

4.2. Sera of IPF patients demonstrate increased intracellular ROS levels in HPMECs.

The first study determined whether serum from IPF patients could increase intracellular ROS levels. HPMECs were treated with IPF serum and HD serum for comparison. Before stimulation, HPMECs were loaded with the ROS molecular probe 2′,7′-dichlorodihydrofluorescein diacetate, H2 -DCFDA, (in the cell, this probe can be oxidized in the presence of ROS, yielding a fluorescent compound, DCF) and then cultured in basal medium containing 5% (V/V) of sera from IPF patients or HD. The change in intracellular ROS levels was determined kinetically over the course of 5 hours **(**Figure 4.1**).** The graph shows the maximum intracellular ROS levels plateaued at the 2 hours mark and was used for comparison. Fluorescence data were normalized for protein content and expressed in relative fluorescence units (RFU). As depicted in the graph, sera of IPF patients caused a significant increase in intracellular ROS levels in HPMEC compared with that from HD sera (Figure 4.2). Our study demonstrates that serum from IPF patients induces abnormal intracellular ROS production in HPMECs.

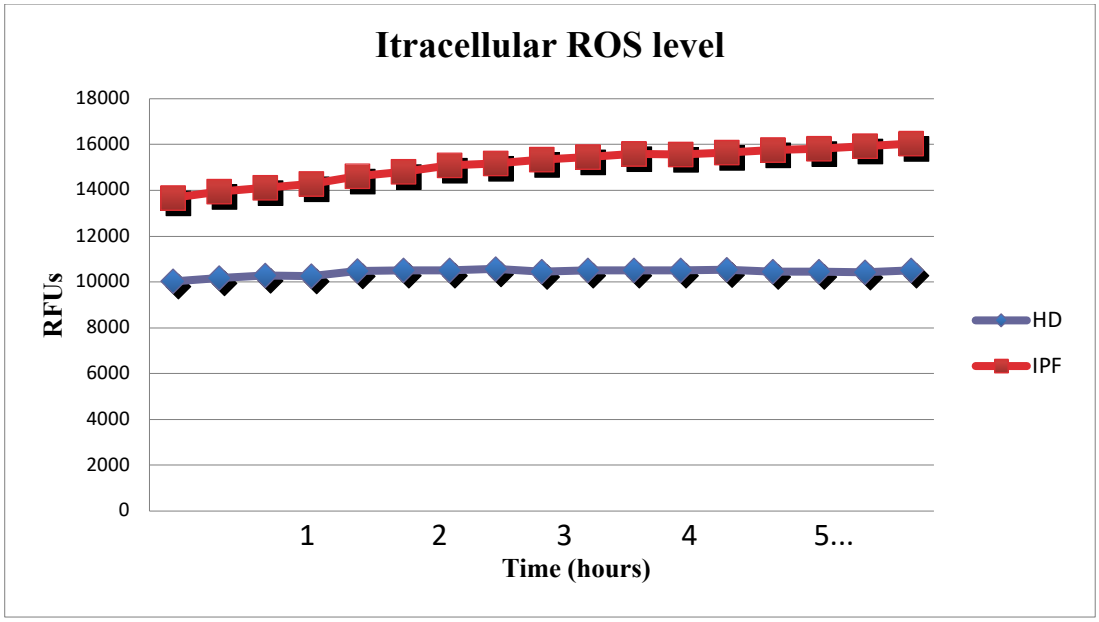


Figure 4.1. Effect of IPF sera on HPMEC production of intracellular ROS levels. Before stimulation, subconfluent HPMECs were loaded with 1µM H_2 -DCFDA and then cultured in a basal medium containing 5% of sera from IPF patients and HD. Variations in intracellular ROS levels were kinetically determined in a 5-hour time-course experiment. Figure report the representative data of one randomly chosen sample.

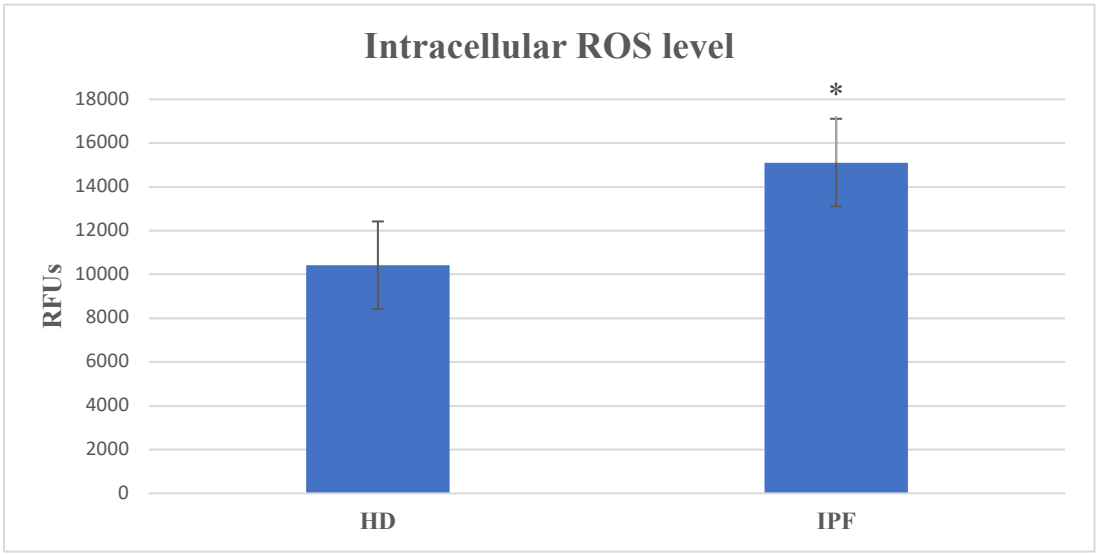


Figure 4.2. Effect of IPF sera on HPMEC production of intracellular ROS levels after 2 hours. Before stimulation, subconfluent HPMECs were loaded with 1µM H_2 -DCFDA and then cultured in a basal medium containing 5% of

sera from IPF patients and HD. Variations in intracellular ROS levels were kinetically determined in a 5-hour time-course experiment, and values at 2 hours were applied for comparison. Fluorescence data were identified by Relative Fluorescence Units (RFU). HD: cells stimulated for 2 hours with 5% sera from healthy subjects (n=10). IPF: cells stimulated with 5% sera from IPF patients (n=10). *, significantly different from control group (HD). ($p < 0.05$)

4.3. DPI reduced intracellular ROS levels induced by sera of IPF patients

NADPH oxidases (NOX) appear to be the most important ligand-mediated source of intracellular ROS in IPF. Therefore, we examined the possible involvement of NOX in the IPF serum-induced increase of intracellular ROS. Diphenyleneiodonium (DPI), a broad-spectrum NOX inhibitor, was used to evaluate the potential relationship between the NOX family and serum-induced ROS. First, HPMECs were treated with DPI 5μM for 1 hour, then with 5% serum from IPF patients and healthy donors (HD). The change in intracellular ROS levels was determined kinetically in a 5-hour time-progression experiment (figure 4.3). The data of fluorescence are shown for protein content and expressed as relative fluorescence units (RFUs).

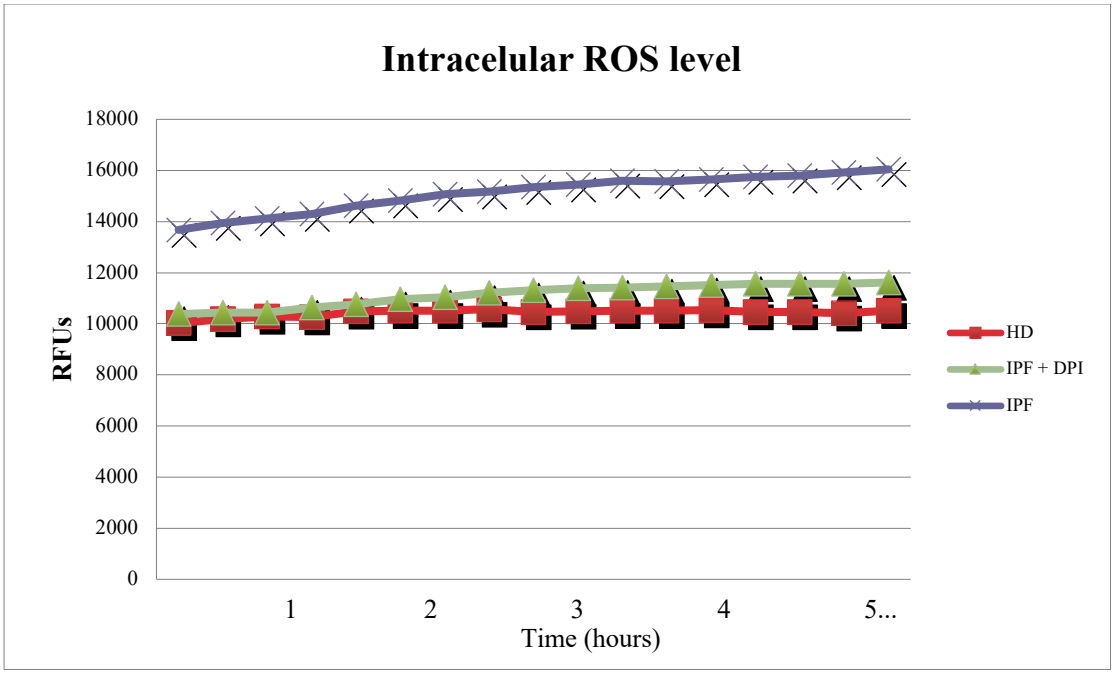


Figure 4.3. Effect of Diphenyleneiodonium on IPF-induced production of intracellular ROS levels in HPMECs. Before stimulation, subconfluent HPMECs were loaded with 1μM H2 -DCFDA, added with 5 μM Diphenyleneiodonium (DPI), and then cultured in a basal medium containing 5% of sera from IPF patients (IPF) and health donor (HD). Variations in intracellular ROS levels were kinetically determined in a 5-hour time-course experiment. Figure report the representative data of one randomly chosen sample

HPMECs were treated with DPI 5μM for 1 hour then cells were exposed to IPF and HD patient serum. The results of serum-treated HPMECs indicate that DPI-pretreated cells have decreased levels of intracellular ROS compared with DPI-non pretreated cells. In particular, the increase in intracellular ROS induced by IPF serum with DPI was significantly reduced compared with IPF serum without DPI treatment, with the value indicating that the significance is reported $p < 0.01$ (Figure 4.4). This result suggests that the IPF-induced increase in intracellular ROS was significantly attenuated by DPI in the observation of our experiment. These results suggest an implication of NOX in ROS generation observed in these cells exposed to IPF serum.

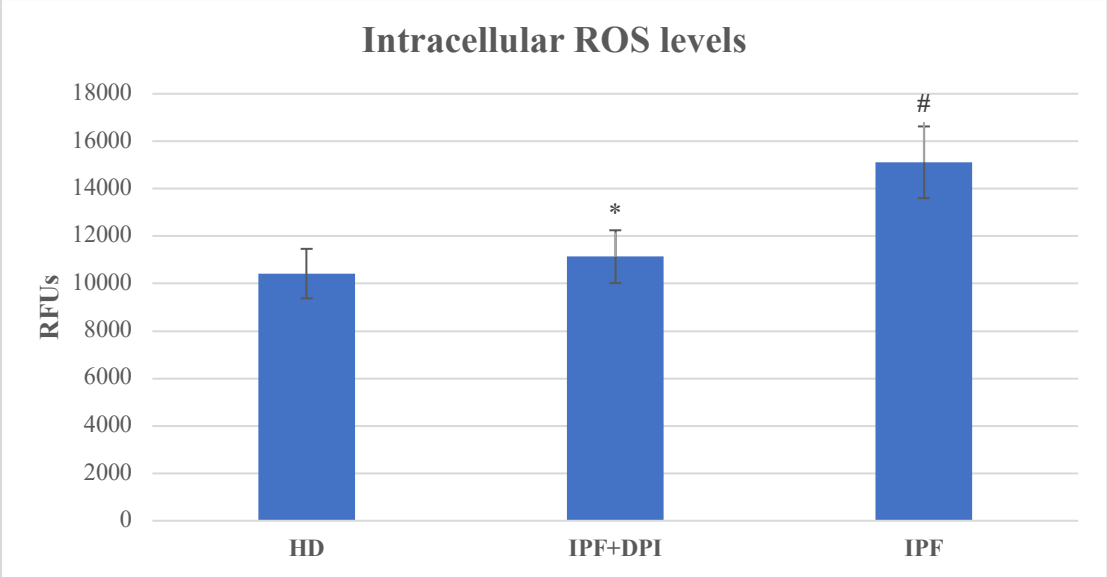


Figure 4.4. Effect of Diphenyleneiodonium (DPI) on IPF-induced production of intracellular ROS levels in HPMECs after 2 hours.

HD: cells stimulated with 5% sera from healthy subjects (n=9). IPF: cells stimulated with 5% sera from IPF patients (n=10). IPF + DPI: cell pretreated for 1 hour with 5 μM DPI and then stimulated with 5% sera from IPF. Values are shown as mean ± SD, #, significantly different from HD; *, significantly different from IPF. ($p < 0.05$)

4.4. Sera of IPF patients increased HPMEC cell proliferation

ROS can be an important modulator of both physiological and pathological cell proliferation depending on the concentration. To understand whether intracellular ROS can affect HPMEC proliferation, we assessed the rate of cell proliferation after cells were exposed to IPF and HD serum. The method employed is based on using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT reagent) assay (Promega, Madison, WI, USA). The yellow MTT reagent enters into the cell and in the mitochondria where mitochondrial dehydrogenases cleave the tetrazolium ring, yielding purple MTT formazan crystals, which can only be directly related to the number of viable cells. Cell proliferation was assessed with MTT after 48 hours of treatment. And in our study results, serum from IPF patients

significantly increased cell proliferation in HPMEC compared with HD serum (Figure 4.5).

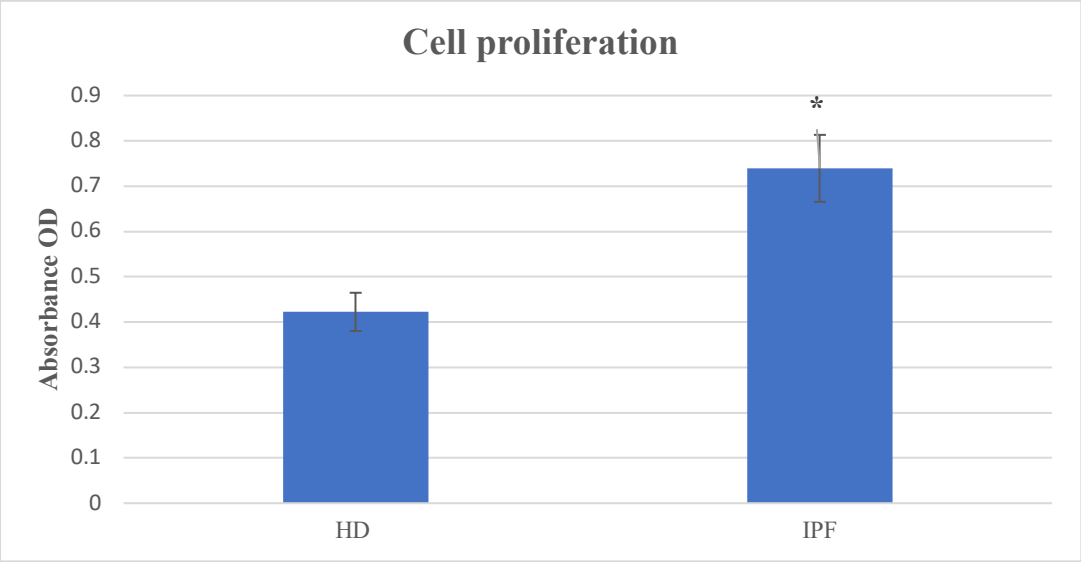


Figure 4.5. Sera of IPF patients increased HPMECs proliferation. Proliferation was assessed with MTT after 48 hours of treatment. Sera from IPF patients significantly increased intracellular ROS levels in HPMECs compared with HD, HD: cells stimulated for 48 hours with 5% sera from healthy subjects (n=9). IPF: cells stimulated with 5% sera from IPF patients (n=10). *, significantly different from Healty Donor (HD). ($p < 0.05$)

By MTT assay, our study demonstrated that IPF-induced increase of HPMEC proliferation was significantly blunted by DPI in our results (Figure 4.6). The results confirm that NOX is implicated in IPF-induced HPMEC proliferation.

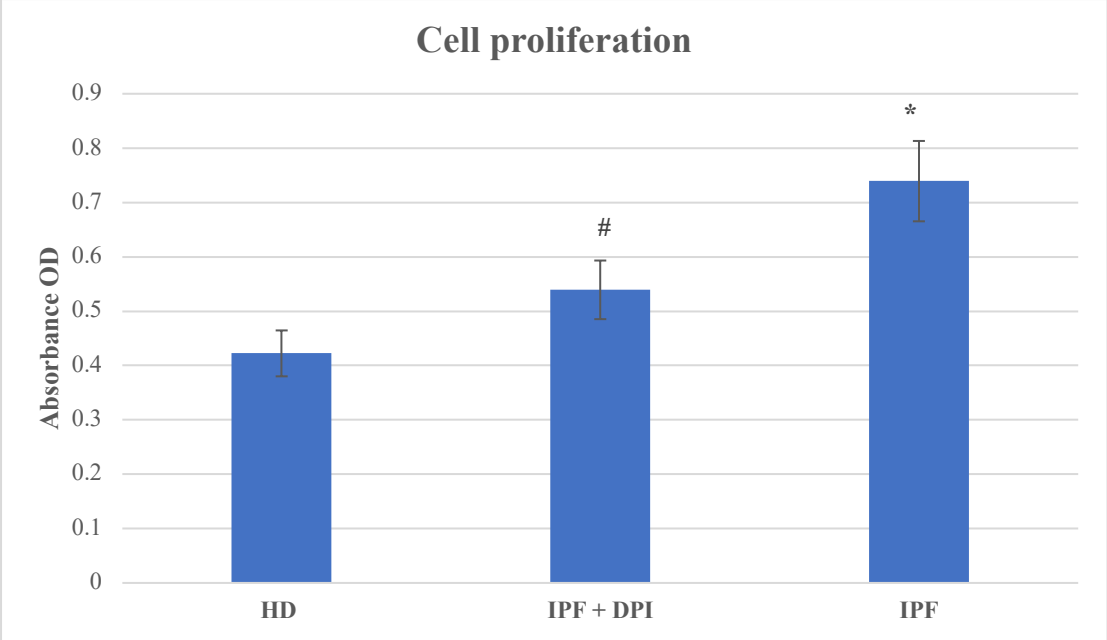


Figure 4.6. DPI reduced HPMECs proliferation induced by sera from IPF patients. HD: cells stimulated for 48 hours with 5% sera from healthy subjects (n=9). IPF + DPI: cell pretreated for 1 hour with 5µM DPI and then stimulated for 48 hours with 5% sera from IPF. IPF: cells stimulated for 48 hours with 5% sera from IPF patients (n=10). Values are shown as mean ± SD, *, significantly different from Healthy Donor HD; #, significantly different from IPF. ($p < 0.05$)

4.5. IPF sera induces Endothelial-to-Mesenchymal Transition (EndMT) in HPMECs.

In pathophysiologies, vascular injury is often associated with endothelial depletion and is characterized by endothelial cells (ECs) with less proliferative potential and, of course, lower angiogenic capacity [3]. Remarkably, as reported in Figure 4.6 above, the serum of IPF patients can increase HPMECs. In this case, the ability of ECs to convert their cellular phenotype to a more fibrous and proliferative phenotype leads to fibrosis [64]. During this transition, known as the Endothelial-Mesenchymal Transition (EndMT), the EC leaves its physiological quiescent state, becomes more proliferative and acquires high mobility and secretes the extracellular matrix [64]. To test whether serum IPF could induce the

conversion of ECs to fibroblasts, HPMECs were exposed to IPF and HD patient serum for 72 h; endothelial and mesenchymal characteristics were then measured using specific markers of both phenotypes.

HPMECs were exposed to 5% of IPF sera and 5% healthy donors sera (Healthy Donor) for 3 days. Protein levels of endothelial markers (vWF, CD31, CDH5) and mesenchymal markers (α-SMA, COL1) were assessed by ELISA. Surprisingly, ELISA results for the HPMECs exposed to IPF serum show protein levels of the endothelial-specific markers (vWF, CD31/PECAM-1, CDH5/VE-Cadherin) were all decreased, whereas protein levels of the mesenchymal-specific markers (α-SMA, Collagen I) were increased (Figure 4.7).

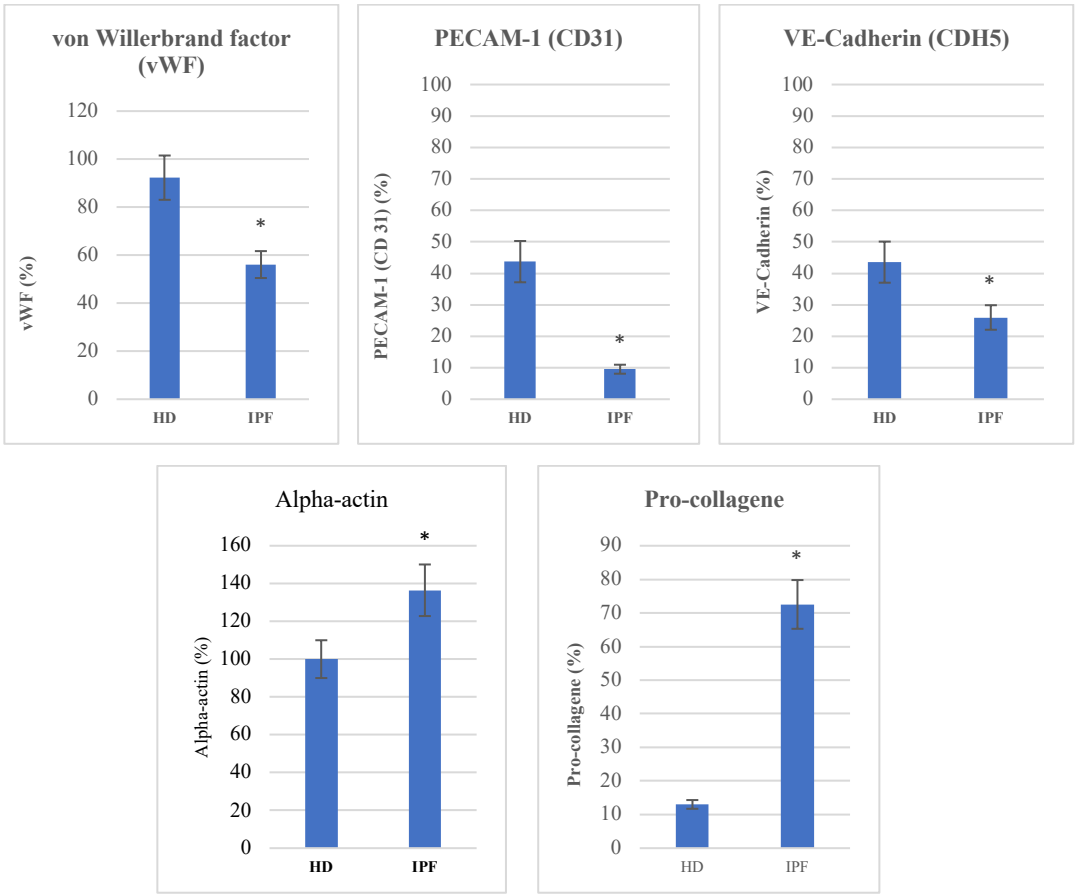


Figure 4.7: IPF induces endothelial-to-mesenchymal-transition (EndMT) in Human Pulmonary Microendothelial Cells (HPMECs). HPMEC cells were exposed to 5% of IPF sera for 3 days. The protein levels of endothelial markers (vWF, CD31, CDH5) and mesenchymal markers (α-Actin, COL1) were assessed by ELISA.

Percentage with respect to the healthy donor (HD) results counted 100%. Values are shown as mean ± SD, n = 5; *, significantly different from Healthy Donor (HD).

4.6. NOX mediates IPF sera-induce EndMT in HPMECs.

Currently, our data confirmed the involvement of oxidative stress in IPF-induced EndMT. To confirm NOX as a major source of EndMT-induced by IPF in HPMECs, we tested whether or not DPI attenuated both IPF-induced ROS intracellular production and EndMT conversion. For this purpose, HPMEC cells were pretreated with 5 μM DPI for 1 hour before being exposed to 5% IPF serum and 5% HD serum for 3 days. Protein levels of endothelial markers (vWF, CD31, CDH5) and mesenchymal markers (α-Actin, COL1) were assessed by ELISA. As shown in figure 4.8, DPI significantly attenuated intracellular ROS levels. In addition, post-treatment with DPI was also found to downregulate IPF-induced EndMT, specifically inhibiting the reduction of endothelial markers (CD31, CDH5, vWF), and simultaneously inhibiting the increased expression of mesenchymal markers (α-SMA, Col1).

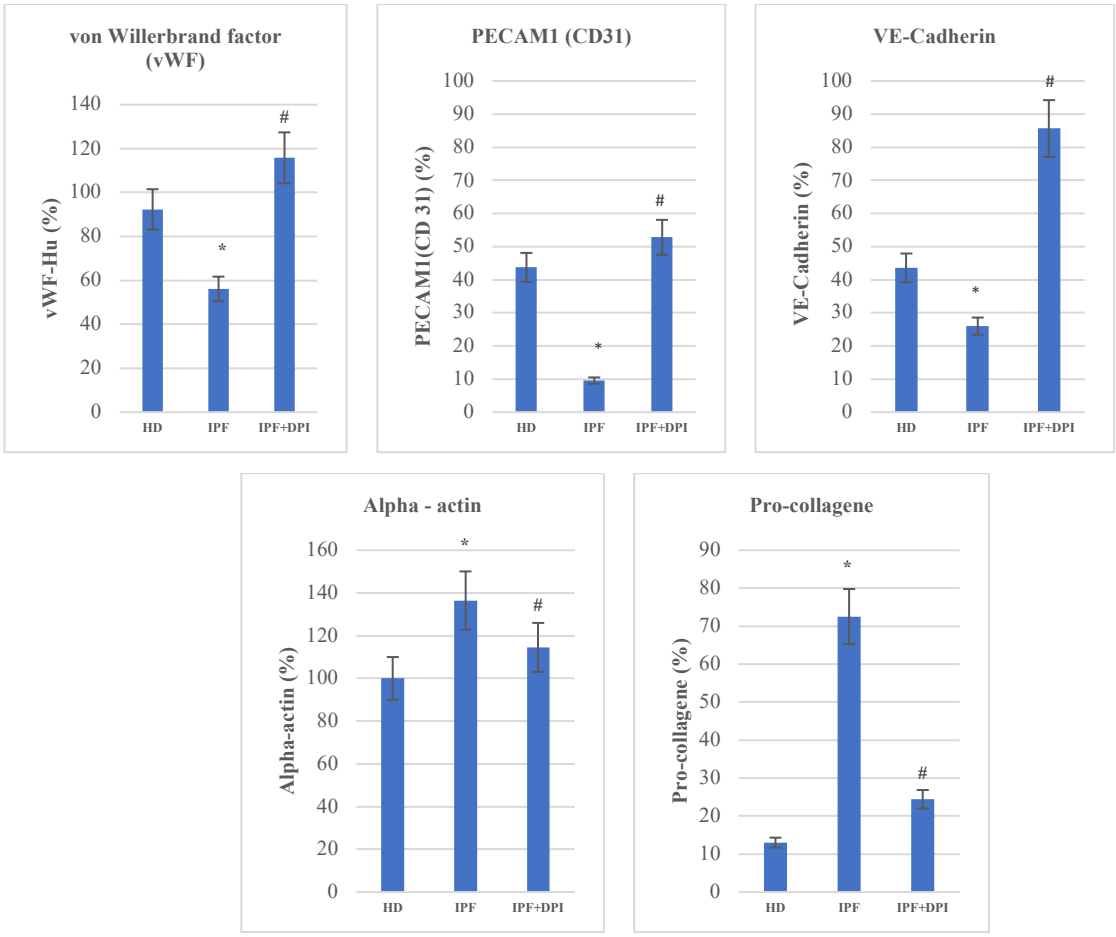


Figure 4.8: NADPH mediates IPF sera-induced EndMT. HPMEC cells were pre-treated with 5 μM DPI for 1 hour, before exposure to 5% of IPF sera and 5% of HD sera for 3 days. The protein levels of endothelial markers (vWF, CD31, CDH5) and mesenchymal markers (α-Actin, COL1) were assessed by ELISA, as detailed in Materials and Methods. Percentage with respect to the healthy donor (HD) results counted 100%. Values are shown as mean ± SD. *, significantly different from HD; #, significantly different from IPF. ($p < 0.05$)

4.7. Involvement of PKC (Protein Kinase C) on IPF sera-induced ROS production and cell viability.

PKC is a pleiotropic enzyme involved in several cellular functions. In this regard, PKC has been reported to be involved in glucose-induced EndMT by modulation ROS production [114] Therefore, we sought to investigate whether PKC could be part of the molecular mechanisms underlying the effect of IPF sera on HPMEC. We reasoned that if IPF sera

activate PKC upstream NADPH, PKC inhibition should block the NOX-mediated ROS generation elicited by IPF sera. To this end, we evaluated the effects of the PKC inhibitor Chelerythrine (CHE) on IPF serum-induced ROS production by treating cells with this inhibitor in the presence of IPF sera and in comparison, using HD sera.

In the same experiment, we used also as a comparison, N-acetyl cysteine (NAC), a well-known broad antioxidant compound [122], and the DPI (Diphenyleneiodonium chloride), an inhibitor of NADPH oxidase and a potent, irreversible, and time-, temperature-dependent iNOS/eNOS inhibitor, as yet reported [105].

Although to a different extent, our results indicate that the effect of Chelerythrine (CHE) on ROS production was similar to the effects of DPI and NAC. (Figure 4.9)

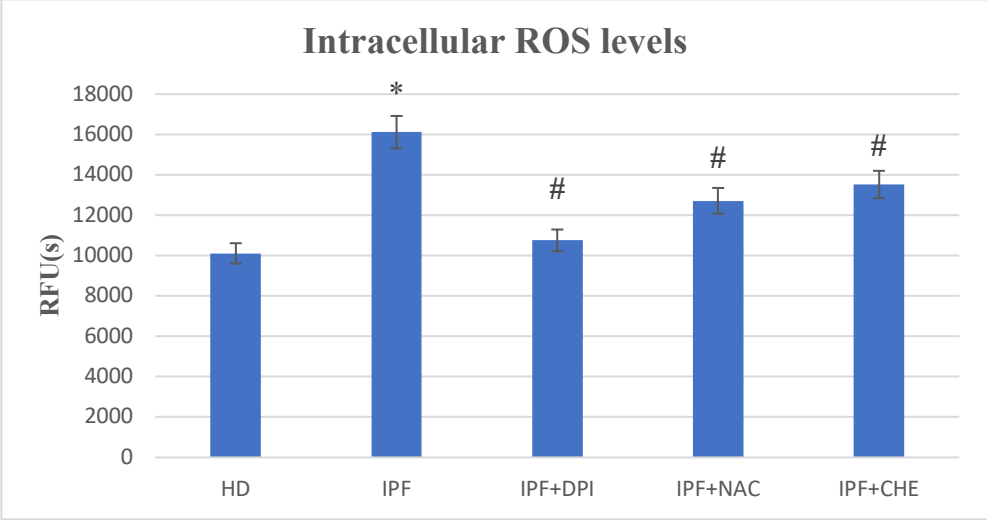


Figure 4.9: Effect of CHE on IPF-induced ROS production. The inhibitory effect of Chelerythrine (CHE) on the decreasing of IPF-induced ROS production. HPMEC cells were pretreated with 2.5µM CHE, 5mM NAC and 5µM DPI for 1 h before being exposed to 5% IPF serum for 3 days. ROS levels were assessed as reported in the Materials and Methods section. The figures represent data obtained at 3 days. Values are expressed as the mean ± SD (n = 5) of the relative fluorescence unit (RFU). *, significantly different from HD; #, significantly different from IPF.

Indeed, CHE was found to effectively abolish IPF sera-induced ROS production. Similarly, CHE was also found to be involved in the related phenomena, such as IPF sera-induced cell proliferation, suggesting that that PKC may be at least in part involved in the IPF-induced cellular events (figure 4.10).

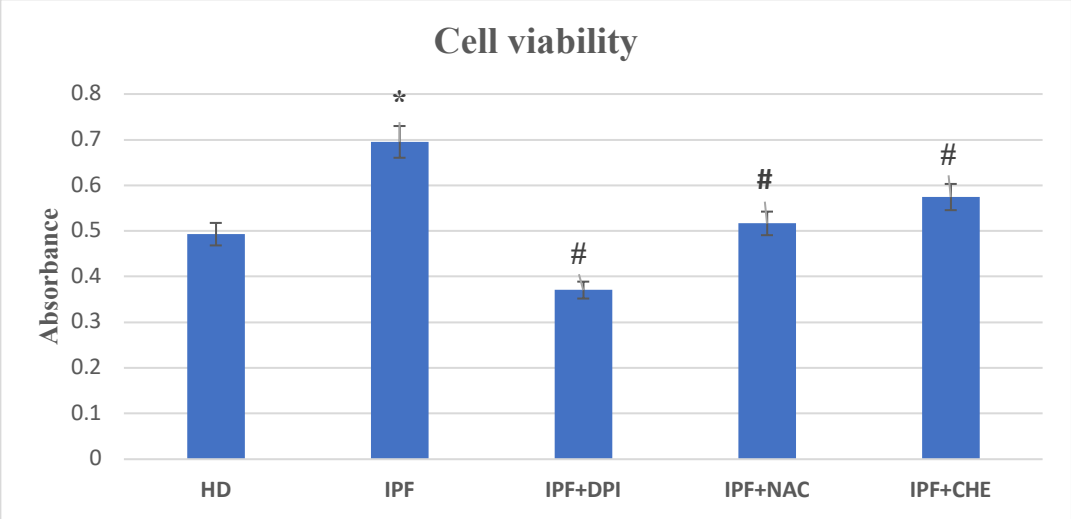


Figure 4.10: Effect of CHE, DPI and NAC on IPF-induced cell viability. HPMEC cells were pretreated with 2.5µM CHE, 5mM NAC, and 5µM DPI for 1 h before being exposed to 5% IPF serum for 24 hours. Cell viability was assessed, as reported in the Materials and Methods section. Results were calculated as mean ± SD (n = 5) and expressed as a percentage of untreated control cells; *, significantly different from HD; #, significantly different from IPF.

5. DISCUSSION

In our study, the enrolled IPF patients participated with no pharmacological treatment, i.e., baseline (T0), and were mainly male (80%) with a mean age of 74 ± 5.1 years. The control group was similar to the study group with the majority of healthy donors consisting of men (88.9%) with an average age of 71± 2.4 (Table 1). At T0, only IPF patients with spirometry values matching the selection criteria were able to participate in this study: FVC was 81.04 ± 26.95% The predicted value and DLCO were 54.17 ± 18.11% of the predicted value.

Recent studies have specifically addressed the importance of oxidative stress in the pathophysiological mechanisms involved in IPF [113, 123]. An extensive systematic review notes oxidative stress markers were identified in various biological samples of IPF patients including sputum, epithelial lining fluid, bronchial lavage fluid, bronchioles, and lung tissue samples. In particular, Paliogiannis et al. performed a meta-analysis of 15 studies in IPF patients, showing either an increase in levels of oxidative stress markers or a decrease in antioxidant markers, independent of the biological sample type [124, 125]. The pathophysiological mechanisms of oxidative stress that facilitate the development of IPF are still not fully understood. However, oxidative stress has been documented to target both the cellular phenotype and promote premature aging, specifically fibroblasts that acquire anti-apoptotic features [126, 127]. The result should lead to changes in the tissue microenvironment that favor regenerative fibrosis in IPF. In line with our hypothesis, we demonstrated for the first time, that serum from IPF patients induced elevated ROS production in HPMECs compared with healthy donor sera (Figure 4.2).

ROS can be generated from different sources in the cells, such as the mitochondrial electron transport chain, xanthine oxidase, cyclooxygenase, and lipoxygenase. Among these sources, NADPH oxidase (NOX) is the most important mediator of ligand-mediated ROS production. NOX are multiunit enzymes that form superoxide by transferring electrons from NADPH to molecular oxygen [128]. To investigate whether the serum IPF-induced ROS in our study derives from the NOX family, we conducted a trial using the generic NOX inhibitor, diphenyleneiodonium (DPI). HPMECs were treated with DPI for one hour prior to treatment with serum from IPF patients. Results indicate DPI inhibits NOX, thus reducing ROS levels in HPMECs. The ROS level produced was lower in the DPI-treated IPF serogroup than in the DPI-untreated IPF serogroup **(**Figure 4.4**)**. The results of our study demonstrate the involvement of NOX in the ROS generation of HPMECs induced by serum IPF. In support of our findings, one study reported an increase in ROS generation from IPF patients' serum in primary human pulmonary artery smooth muscle cells (HPASMCs). IPF serum-induced intracellular ROS levels were significantly reduced when treated with the broad-spectrum NADPH oxidase inhibitor diphenyleneiodonium (DPI) [105].

Although NOX2 was the first isoform of the NOX family identified in endothelial cells, it may be the most important in vascular disease. NOX2 has been reported to be present at lower levels in mesenchymal cells, smooth muscle cells, endothelial cells, and epithelial cells of the lung. NOX2 has been confirmed to be important in inflammation [90]. However, in the lung, the role of NOX4 is more important. Because different factors contribute to oxidative stress, in which cigarette smoking stimulates ROS production through endoplasmic reticulum (ER) stress, disconnection of the mitochondrial enzyme system [101, 129] and production of NOX, especially NADPH oxidase-4 (NOX4), by both inflammatory and lung

cells. The specific mechanisms by which oxidative stress facilitates the development of IPF have not been fully investigated although they have the potential to target both the cellular phenotype and the life cycle promoting senescence and apoptosis. ROS stimulates apoptosis in airway epithelial cells, as well as the production of cytokines and growth factors TGF-β [130], which play important roles in fibroblast differentiation and collagen deposition, leading to fibrosis and reduced antioxidant capacity. Therefore, determining the possible role of NOX2/NOX4 of the ROS family in IPF will be the subject of our investigations in future studies.

The mechanism of fibrosis in pulmonary fibrosis of IPF is a controversial research issue, especially in the vascular remodeling process. Several studies have shown that both angiogenesis and fibrosis are present in the lungs of patients with IPF. This confirms that microvascular tissue remodeling in IPF is unstable [41]. Indeed, during the initiation and progression of IPF, various growth factors and fibroblast mediators are released, such as TGF-β1, which may exert their fibrogenic effects through NADPH oxidase-dependent increase in intracellular ROS levels. The expression of NADPH/NOX4 has been reported to be increased in the pulmonary arteries of IPF patients [96] and in pulmonary fibroblasts of IPF patients where TGF-β1 mediates the differentiation of fibroblasts into myofibroblasts [131]. At the physiological level, intracellular ROS controls cell growth and differentiation, regulates enzyme activity, mediates inflammation, stimulates cytokine production, and eliminates pathogens and molecules strange. On the other hand, if present in excess, ROS destroys cellular components, including lipids and cell membranes, proteins and nucleic acids, and ultimately, cell transformation. Surprisingly, instead of inducing cell death, the observed generation of IPF-ROS was able to highlight it. Consistent with our findings, cell viability assays (assessed by MTT) showed that the IPF-induced increase in HPMECs proliferation was

significantly reduced by the NADPH oxidase inhibitor diphenyleneiodonium (DPI), compared with HPMECs without DPI **(**Figure 4.6**)**. We confirm that NOX, the NADPH family of ROS-generating enzymes, is involved in IPF-induced increase in HPMECs.

IPF is a fibroproliferative disease characterized by abnormal proliferation and secretion of extracellular matrix leading to fibrosis. Of course, all in situ proliferation of fibroblasts and their differentiation have been shown to favor fibrosis progression. Different from fibroblasts, myofibroblasts upregulate the expression of α-SMA and increase the production of extracellular matrix proteins, such as collagen types I, III, V, and VI [132, 133]. Experiments have shown that fibroblasts can originate from cell sources other than fibroblasts. In particular, endothelial cells (ECs) are a potential source of action through EndMT. During this process, ECs express typical markers of fibroblast differentiation and acquire a mesenchymal phenotype (α-SMA, vimentin, and collagens) while also reducing the expression of the vascular endothelial phenotype (VE-cadherin). It has been reported that 16% of fibroblasts express α-SMA and EC-derived type I collagen in the lungs of mice with bleomycin-induced IPF [47]. The importance of EndMT in fibrosis experiments has been demonstrated both in vitro and in vivo. These studies suggest that TGF-β has a central role in promoting EndMT through an extensive network of molecular interactions [134, 135]. Under endothelial to mesenchymal transformation, endothelial cells undergo morphological changes and lose cell surface markers. The first time, our results indicate the presence of EndMT in normal HPMECs exposed to the serum of IPF patients. EndMT indices, three well-established endothelial markers: CD31/PECAM, CDH5/Cadherin, vWF/von Willebrand factor were used, along with the well-known mesenchymal markers α-SMA, collagen I. To confirm the possibility that the EndMT process occurring in IPF promotes an

endothelial cell phenotype towards mesenchymal cell features. We examined the protein levels of selected endothelial and mesenchymal markers. Interestingly, the ELISA results showed the decreasing endothelial markers: cadherin, CD31, and vWF, while the increasing mesenchymal markers: alpha-SMA, Collagen I **(**Figure 4.7**).** The present data confirm the disruption in the presence of molecular markers and demonstrate that IPF promotes the phenotypic transformation of HPMECsThis implies fibroblasts in IPF may originate in HPMECs through the EndMT process.

Notably, recent studies suggest the ability of ROS to either convert epithelial to mesenchymal or mediate the conversion of endothelial cells to fibroblasts [136]. In this regard, we speculate that ROS may be related to IPF-induced EndMT in HPMECs and, at least in part, responsible for IPF-associated fibrosis in vivo. To investigate this aspect, we examined the ability of DPI, a well-known antioxidant compound, to counteract the IPF-induced endothelial changes observed in our study. HPMECs were pretreated with 5μM DPI for one hour and successively exposed to the IPF patient serum for three days. Protein level analysis of selected markers (Figure 4.8) revealed the ability of DPI to suppress IPF-induced cell morphological changes. Indeed, DPI significantly abrogated the expression of IPF-induced EndMT processes that inhibited the reduction of endothelial markers (CDH5, CD3, vWF) and also inhibited the increase of mesenchymal markers (Col 1, α-SMA). As such, IPF-induced EndMT appears to be mediated by NADPH-associated ROS generation. And, in our results, HPMECs pretreatment with the NOX inhibitor DPI was able to decrease EndMT effectively. Choi et al. found increased EndMT in blood vessels due to hypoxic injury in radiation-induced pulmonary fibrosis [137]. In this study, EMT was also observed in alveolar epithelial cells, but only after the appearance of EndMT.

We further deepen our investigation on the cellular IPF-induced effect by assessing the potential involvement of protein kinase c (PKC). PKC is a pleiotropic enzyme involved in several cellular functions. In this regard, PKC has been reported to be involved in glucose-induced EndMT by modulation of ROS production [114]. On these bases, we sought to investigate whether PKC could be part of the molecular mechanisms underlying the effect of IPF sera on HPMEC.

To this end, we evaluated the effects of the PKC inhibitor Chelerythrine (CHE) on IPF serum-induced ROS production and cell proliferation. We used CHE to demonstrate that the mechanism yet studied could be correlated to the involvement of PKC and our results demonstrate our hypothesis since both the IPF-induced ROS production and the effects on cell proliferation were significantly down moduolated by CHE. Interestingly, a comparative experiment performed with, N-acetyl cysteine (NAC), a known antioxidant compound [122], and DPI, a widely employed NOX inhibitor [105] showed that the effect of CHE, by inhibiting PKC, reduced both ROS and cell proliferation in cells exposed to IPF patient serum, suggesting that NADPH effects occurs via PKC in these cells.

To further confirm the potential antifibrotic role of Chelerythrine (CHE) in IPF, further studies are needed to determine the effect of this PKC inhibitor on the IPF-induced EndMT process.

In summary, our work provides further insight into the etiology of IPF-associated fibrotic progression while also opening up new questions and challenges. Specifically, abrogating IPF-associated ROS increase by different means (CHE or DPI) was able to significantly prevent IPF-induced oxidative and its associated effects suggesting an essential role of oxidative stress in triggering fibrosis-related IPF. Therefore, this will be the area of focus for future studies.

6. CONCLUSION

With the intent to investigate the role of ROS in the Endothelial - to - Mesenchymal transition induced by the sera from IPF patients, we obtained several pilot results.

- We demonstrated sera from IPF patients induce an abnormal ROS production in HPMECs. We confirm, NOX, the NADPH family ROS-generating enzymes, is implicated in IPF-induced oxidative stress.
- IPF-induced increase of HPMECs proliferation was significantly blunted by the broad NADPH oxidase inhibitor diphenyleneiodonium (DPI). We confirm NOX is implicated in IPF-induced HPMECs proliferation.
- IPF promotes the phenotype switch of HPMECs, and myofibroblasts in the IPF may originate from ECs through the EndMT process, with the decreasing the endothelial marker(CDH5, CD3, vWF) and increasing mesenchymal markers (Col 1, α-SMA).
- IPF-induced EndMT appears to be mediated by NADPH-associated ROS generation. HPMECs pretreatment with a NOX inhibitor (DPI) was able to decrease EndMT effectively.
- PKC appears to be the one of the signaling key enzyme that mediates IPF sera effects of within the cells - Inhibition of PKC partially block IPF-induced ROS production and cell proliferation.

In summary, our findings indicate an association between EndMT and oxidative stress as an important mechanism in the progression of IPF. This study suggests the use of antioxidants may be an effective therapeutic approach to prevent the progression of IPF-related complications including fibrosis.

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REVIEW

Emerging cellular and molecular determinants of idiopathic pulmonary fibrosis

Thị Hằng Giang Phan[1] · Panagiotis Paliogiannis[2] · Gheyath K. Nasrallah[3,4] · Roberta Giordo[5] · Ali Hussein Eid[6,7,8] · Alessandro Giuseppe Fois[2] · Angelo Zinellu[9] · Arduino Aleksander Mangoni[10] · Gianfranco Pintus[5,9]

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Abstract
Idiopathic pulmonary fibrosis (IPF), the most common form of idiopathic interstitial pneumonia, is a progressive, irreversible, and typically lethal disease characterized by an abnormal fibrotic response involving vast areas of the lungs. Given the poor knowledge of the mechanisms underpinning IPF onset and progression, a better understanding of the cellular processes and molecular pathways involved is essential for the development of effective therapies, currently lacking. Besides a number of established IPF-associated risk factors, such as cigarette smoking, environmental factors, comorbidities, and viral infections, several other processes have been linked with this devastating disease. Apoptosis, senescence, epithelial-mesenchymal transition, endothelial-mesenchymal transition, and epithelial cell migration have been shown to play a key role in IPF-associated tissue remodeling. Moreover, molecules, such as chemokines, cytokines, growth factors, adenosine, glycosaminoglycans, non-coding RNAs, and cellular processes including oxidative stress, mitochondrial dysfunction, endoplasmic reticulum stress, hypoxia, and alternative polyadenylation have been linked with IPF development. Importantly, strategies targeting these processes have been investigated to modulate abnormal cellular phenotypes and maintain tissue homeostasis in the lung. This review provides an update regarding the emerging cellular and molecular mechanisms involved in the onset and progression of IPF.

Keywords Idiopathic pulmonary fibrosis · Molecular pathways · EndMT · Senescence · Apoptosis · Chemokines · Cytokines · EMT · Cell plasticity

Thị Hằng Giang Phan and Panagiotis Paliogiannis have contributed equally to this work.

✉ Gheyath K. Nasrallah
gheyath.nasrallah@qu.edu.qa

✉ Arduino Aleksander Mangoni
arduino.mangoni@flinders.edu.au

✉ Gianfranco Pintus
gpintus@sharjah.ac.ae

1 Department of Immunology and Pathophysiology, University of Medicine and Pharmacy, Hue University, Hue City, Vietnam

2 Department of Medical, Surgical and Experimental Sciences, University of Sassari, 07100 Sassari, Italy

3 Department of Biomedical Sciences, College of Health Sciences Member of QU Health, Qatar University, P.O. Box 2713, Doha, Qatar

4 Biomedical Research Center Qatar University, P.O Box 2713, Doha, Qatar

5 Department of Medical Laboratory Sciences, College of Health Sciences, and Sharjah Institute for Medical Research, University of Sharjah, University City Rd, Sharjah 27272, United Arab Emirates

6 Department of Basic Medical Sciences, College of Medicine, QU Health, Qatar University, PO Box 2713, Doha, Qatar

7 Biomedical and Pharmaceutical Research Unit, QU Health, Qatar University, PO Box 2713, Doha, Qatar

8 Department of Pharmacology and Toxicology, Faculty of Medicine, American University of Beirut, PO Box 11-0236, Beirut, Lebanon

9 Department of Biomedical Sciences, University of Sassari, 07100 Sassari, Italy

10 Department of Clinical Pharmacology, College of Medicine and Public Health, Flinders University, Adelaide, Australia