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THE ROLE OF miRNA IN PRE-CAPILLARY PULMONARY HYPERTENSION: A COMPARISON BETWEEN GROUP 1 AND GROUP 3 PH.

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ABBREVIATIONS

PH	Pulmonary hypertension
PAP	Pulmonary arterial pressure
PAPm	Mean pulmonary arterial pressure
PAPs	Systolic pulmonary arterial pressure
PAPd	Diastolic pulmonary arterial pressure
PVR	Pulmonary vascular resistance
RHC	Right heart catheterization cardiac output (CO)
SvO2	Venous oxygen saturation
PCP	pulmonary capillary wedge pressure
Par	Right atrial pressure
PAC	Pulmonary arterial catheter
CI	Cardiac index
TGP	Transpulmonary pressure gradient
LDPH	Pulmonary hypertension due to lung disease or hypoxia
IPAH	Idiopathic pulmonary hypertension
IPF	Idiopathic pulmonary fibrosis
UIP	Usual interstitial pneumonia
ATS	American Thoracic Society
ERS	European Respiratory Society
ILD	Interstitial lung disease
HRCT	High resolution computed tomography
SLB	Surgical lung biopsy
NSIP	Non-specific interstitial pneumonia
FEV1	Forced Expiratory Volume in the first second
FVC	Forced vital capacity
DLCO	Diffusion capacity for carbon monoxide
6mWT	Six minute walking test
6MWD	Six minute walking distance
EMA	European market authorization
COPD	Chronic obstructive pulmonary disease
GOLD	Global Initiative for Chronic Obstructive Lung Disease
LMICs	low- and middle-income countries
AATD	α-1 antitrypsin deficiency
CTPA	Contrast-enhanced CT scan pulmonary angiography
CLE	Centrilobular emphysema
LTx	Lung transplantation

BNP	Brain natriuretic peptide
CLD-PH	Pulmonary hypertension in chronic lung disease
RCTs	Randomised controlled trials
LTOT	Long term oxygen treatment
CF	Cystic fibrosis
LAM	Lymphangioleiomyomatosis
ARDS	Acute respiratory distress syndrome
BMI	Body max index
CLAD	Chronic lung allograft dysfunction
LVR	Lung volume reduction
IV	Intravenous
SC	Subcutaneous
PVOD	Pulmonary veno-occlusive disease
PCH	Pulmonary capillary hemangiomatosis
PASMC	Pulmonary artery smooth muscle cells
vSMC	Vascular smooth muscle cells
BMPR2 gene	Bone morphogenetic protein receptor type 2
ICAM1	Intercellular adhesion molecule 1
VCAM1	Vascular cell adhesion molecule 1
ECs	Endothelial cells
EndoMT	Endothelial to mesenchymal transition
IL	Interleukin
TNF-α	Tumour necrosis factor-α
TGF B	Transforming growth factor - B
α-SMA	alpha smooth muscle actin
SM22α	Smooth muscle protein 22 alpha
FSP-1	Fibroblast-specific protein 1
MMP	Matrix
SuHx	Sugen-hypoxia rat models
MCT	Monocrotaline
MCTP	Monocrotaline pyrrole
PARP1	Poly(ADP ribose) polymerase 1
PTBP1	Polypyrimidine tract binding protein 1
ET-1	Endothelin 1
PCNA	Proliferating cell nuclear antigen
ASCs	Adipose-derived mesenchymal stem cells

THE ROLE OF miRNA IN PRE-CAPILLARY PULMONARY HYPERTENSION: A COMPARISON BETWEEN GROUP 1 AND GROUP 3 PH.

1. INTRODUCTION

1.1. PULMONARY HYPERTENSION: definition and classification

Pulmonary hypertension (PH) is a pathophysiological disorder involved in multiple clinical conditions and can complicate the majority of cardiovascular and respiratory diseases.

The pulmonary vascular system in healthy adults is a highly compliant, low-pressure, and low-resistance system, capable of accommodating increased blood flow with only minor increases in pulmonary arterial pressure (PAP). However, pulmonary hypertension is a chronic condition that causes an elevation in PAP and pulmonary vascular resistance (PVR) leading to cardiac repercussion as enlargement and hypertrophy of the right ventricle. In early stages, PAP remains normal at rest but rises significantly during exercise. As the condition progresses, PH can occur even at rest. When the right ventricle's adaptive mechanisms of hypertrophy and dilatation are unable to compensate for the increased hemodynamic burden, right heart failure can occur, and prognosis is typically poor. PH can result from respiratory and cardiac disorders, with the most severe forms being thromboembolic PH and primary PH (Chemla et al., 2002).

Recently, the 2022 ESC/ERS guidelines for the diagnosis and treatment of pulmonary hypertension has defined a new hemodynamic cut-off, the current definition for PH is as an increase in mean pulmonary arterial pressure (PAPm) ≥20 mmHg at rest as assessed by right heart catheterization (RHC) (Humbert et al., 2022).

Previous 2015 ESC/ERS guidelines defined pulmonary hypertension as an increase in PAPm ≥25 mmHg at rest, this cut-off value has been revised due to new data assessing the upper limit of normal pulmonary arterial pressure (PAP) in healthy subjects, and evidences on the prognostic relevance of increased PAP (Galie et al., 2015).

Despite the increasing knowledge in echocardiography in PH the right hearth catheterization (RHC) remains the diagnostic gold standard for PH.

It is recommend to perform RHC in patients with unexplained exertional dyspnoea, syncope and/or signs of right ventricular dysfunction assessed for suspected PH using transthoracic echocardiography (which remains the most widely used screening tool), or in those patients affected by lung parenchymal disease with disproportionated dyspnoea and during lung transplant assessment.

1.1.1 PH diagnostic gold standard: right heart catheterization technique

During the diagnostic evaluation of PH it is recommended to perform a right heart catheterization (RHC) that includes a comprehensive assessment of hemodynamic. This assessment involves various parameters, including cardiac output (CO), mixed venous oxygen saturation (SvO2), pulmonary arterial pressure (PAP), pulmonary capillary wedge pressure (PCP), right atrial pressure (RAP), and right ventricular pressure. Calculations such as transpulmonary pressure gradient, diastolic pressure gradient, pulmonary vascular resistance (PVR), and cardiac index (CI) can be derived from these measurements.

To measure the pressures on the right side of the heart, a catheter is inserted into the pulmonary artery. This catheter, commonly known as a Swan-Ganz catheter, was named after its inventors Jeremy Swan and William Ganz in 1970 (Chatterjee, 2009). The pulmonary arterial catheter (Vitulo et al.) is a percutaneous, non-tunneled, multi-lumen, flow-directed central venous catheter. It is inserted through an introducer into a large vein, such as the internal jugular vein (left or right), femoral vein, or antecubital vein. Once the catheter is positioned, the balloon is inflated in the right atrium. With the assistance of blood flow, the

balloon is guided through the right atrioventricular valve into the right ventricle, and finally through the pulmonic semilunar valve into the pulmonary artery (Figure 1).

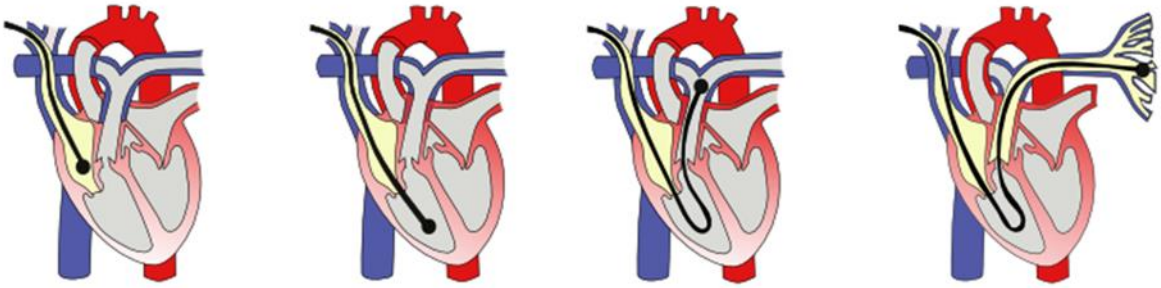

Figure 1. Right heart catheterisation procedure (pcipedia.org)

One important aspect to consider during performing RHC is the position of the pressure transducer, which is essential for correct pressure measurements. To establish uniformity of the pressure transducer setting, the pressure transducer should be set to zero level at the mid-thoracic line with the patient in a supine position, halfway between the anterior sternum and bed surface, which represents the level of the left atrium.

PCP measurement: To measure PCP, it is recommended to inflate the balloon in the right atrium and advance the catheter until it reaches the PCP position. PCP value should be the result of the mean of three measurements at end expiration. The recommendation to measure PCP at end expiration is based on the observation that evaluation should be performed at functional residual volume, where intra- and extra-thoracic pressures are equal. Respiratory movements can significantly affect PCP, as they impact intrathoracic pressure. Notably, these variations are minimal at the end of normal expiration, which is why measuring end-expiratory PCP helps minimize this effect. Indeed, there is minimal influence of lung elastic recoil on pulmonary pressure at functional residual capacity (end expiration), while larger effects of intrathoracic pressure changes are observed in patients with chronic obstructive airway disease or significant obesity (Rosenkranz and Preston, 2015).

Cardiac output and cardiac index: In addition to assessing pulmonary pressures, right heart catheterization (RHC) allows the measurement of cardiac output using the well-established thermodilution method. This method involves a thermistor tip of a Swan-Ganz catheter, which records the decrease in blood temperature in the pulmonary artery after injecting 10 mL of normal saline at room temperature. The change in blood temperature is inversely related to the dilution of the injected solution, enabling the derivation of cardiac output values using computer-generated calculations. The thermodilution technique has proven to be reliable when compared to direct Fick measurement, which requires a direct measurement of oxygen uptake. Alternatively, the indirect Fick technique, which estimates oxygen uptake, can be used to determine cardiac output. In this case, mixed SvO2 is measured with the catheter tip positioned in the right atrium or pulmonary artery, while systemic oxygen saturation is often obtained noninvasively through oximetry. Furthermore cardiac index represents the ratio of cardiac output to body surface area. Although cardiac output varies depending on a patient's weight and size, a normal cardiac index ranges between 2.4 and 4.0 L/min/m^2.

Pulmonary vascular resistance: The determination of pulmonary vascular resistance (PVR) and PVR index plays a critical role in diagnosing pulmonary arterial hypertension (PAH). These parameters are calculated by dividing the transpulmonary pressure gradient (calculated using TPG=PAPm–PCP) by cardiac output. PVR

are included in the 2022 ESC/ERS guidelines, with a value exceeding 2 Wood units being a significant component of the hemodynamic definition of PAH due to its prognostic implications (Humbert et al., 2022).

In summary, the diagnosis of PH relies on the results from haemodynamic parameters directly measured or calculated from RHC. There are a number of best practice recommendations that should be followed when performing RHC to avoid errors in diagnosis and the risk of complications (Rosenkranz and Preston, 2015).

1.1.2. PH: Hemodynamic and clinical classification

Pulmonary hypertension (PH) can be classified into pre-capillary or post-capillary based on specific criteria (Table 1). Pre-capillary PH, is identified by a PCP ≤ 15 mmHg, a mean PAP ≥ 20 mmHg at rest (previous guidelines had a cut-off of ≥ 25 mmHg), and a pulmonary vascular resistance (PVR) > 2 Wood units. Post-capillary PH, is identified by a PCP >15 mmHg, a mean PAP ≥ 20 mmHg at rest (previous guidelines had a cut-off of ≥ 25 mmHg), it can be isolated if PVR ≤2 WU; or combined, if PVR > 2 WU (Humbert et al., 2022).

Table 1. Hemodynamic classficarion (Humbert et al., 2022).

Definition	Hemodynamic features	Clinical group
Pulmonary hypertension	PAP ≥ 20 mmHg	Tutti
Pre-capillary PH	PAP ≥ 20 mmHg PCP ≤15 mmHg PVR > 2 WU	PAH PH due to lung disease PH due to chronic thromboembolic disease PH due to miscellaneous disorders
Post-capillary Isolated post-capillary PH Combined post- and pre-capillary PH	PAPm ≥20 mmHg, PAWP >15 mmHg PVR ≤ 2 WU PVR > 2 WU	PH due to left hearth disease PH due to miscellaneous disorders

The classification system of pulmonary hypertension attempts to guide the clinical approach to pulmonary hypertension by dividing patients into five groups: group 1— pulmonary arterial hypertension (PAH) due to pulmonary vascular disease; group 2—pulmonary hypertension due to left heart disease; group 3—pulmonary hypertension due to lung disease or hypoxia; group 4—pulmonary hypertension due to chronic thromboembolic disease; and group 5—a miscellaneous collection of pulmonary hypertension syndromes caused by a variety of disorders (Table 2). In principle, patients in each of these groups share pathophysiology, prognosis, and therapeutic response; in reality, tremendous heterogeneity exists within each group (Thenappan et al., 2018, Humbert et al., 2022).

Table 2. Clinical classification of pulmonary hypertension (Humbert et al., 2022).

Group 1. Pulmonary arterial hypertension PAH 1.1 Idiopathic 1.1.1 Non-responders at vasoreactivity testing 1.1.2 Acute responders at vasoreactivity testing 1.2 Heritable 1.3 Associated with drugs and toxinsa 1.4 Associated with: 1.4.1 Connective tissue disease 1.4.2 HIV infection 1.4.3 Portal hypertension 1.4.4 Congenital heart disease 1.4.5 Schistosomiasis 1.5 PAH with features of venous/capillary (PVOD/PCH) involvement 1.6 Persistent PH of the newborn
Group 2 PH associated with left heart disease 2.1 Heart failure: 2.1.1 with preserved ejection fraction 2.1.2 with reduced or mildly reduced ejection fraction 2.2 Valvular heart disease 2.3 Congenital/acquired cardiovascular conditions leading to post-capillary PH
Group 3 PH associated with lung diseases and/or hypoxia 3.1 Obstructive lung disease or emphysema 3.2 Restrictive lung disease 3.3 Lung disease with mixed restrictive/obstructive pattern 3.4 Hypoventilation syndromes 3.5 Hypoxia without lung disease (e.g. high altitude) 3.6 Developmental lung disorders

Group 4 PH associated with pulmonary artery obstructions 4.1 Chronic thrombo-embolic PH 4.2 Other pulmonary artery obstructions
Group 5 PH with unclear and/or multifactorial mechanisms 5.1 Haematological disorders 5.2 Systemic disorders 5.3 Metabolic disorders 5.4 Chronic renal failure with or without haemodialysis 5.5 Pulmonary tumour thrombotic microangiopathy 5.6 Fibrosing mediastinitis

Group 1 and group 3 are haemodynamically characterized by pre-capillary PH. The scientific interest in PH is constantly growing, also thanks to the new treatment options that are approved for group 1 and group 4 PH. Unlike group 3, pulmonary hypertension due to lung disease or hypoxia, is still lacking of deep knowledge on pathogenesis and treatment option.

1.2. GROUP 1 - PULMONARY ARTERIAL HYPERTENSION

Pulmonary arterial hypertension (PAH) is a chronic and progressive vasculopathy that primarily affects the pulmonary vasculature, resulting in increased pulmonary vascular resistance. The pathological changes in PAH primarily occur in the pre-capillary pulmonary vasculature, leading to elevated pulmonary vascular pressure, right heart failure and ultimately death if untreated.

PAH incidence settles around 6 cases/million and prevalence around 48–55 cases/million adults. It has been thought to predominantly affect younger individuals, mostly females. However, recent data from the USA and Europe suggest that PAH is now frequently diagnosed in older patients (Humbert et al., 2022).

Group 1 includes patients affected by idiopathic or familial PAH with or without germline mutations. Patients with a diagnosis of PAH should undergo systematic screening for underlying mutations in genes such as BMPR2 (bone morphogenetic protein receptor type 2), and less commonly, ACVRL1 (activin receptor-like kinase type 1), ENG (endoglin), or Smad8. It is important to note that pulmonary veno-occlusive disease and pulmonary capillary hemangiomatosis are distinct conditions within the clinical group 1 (Montani et al., 2013).

Idiopathic PAH refers to a sporadic form of the disease with no family history or identifiable risk factors. BMPR2 mutations have also been found in 11-40% of apparently idiopathic cases without a family history. In fact, the categorization of idiopathic and familial PAH based on BMPR2 mutations is somewhat artificial, as all patients with a BMPR2 mutation have heritable disease. Additionally, BMPR2 mutations have only been identified in 70-80% of families with PAH. Consequently, it has been proposed to replace the term "familial PAH" with "heritable PAH," encompassing idiopathic PAH with germline mutations and familial cases with or without identified mutations (Montani et al., 2013).

1.3. GROUP 3 - CHRONIC LUNG DISEASE AND PULMONARY HYPERTENSION

1.3.1. Idiopathic pulmonary fibrosis

Idiopathic pulmonary fibrosis (Raghu et al.) is the most common form of chronic idiopathic interstitial pneumonia in adults. It was firstly defined in 2000 by ATS/ERS consensus as an anatomopathological or radiological form of usual interstitial pneumonia (UIP) of unknown cause. It is a progressive and irreversible fibrotic disease which solely affects the lungs, leading to a poor prognosis. Clinical manifestations encompass chronic exertional dyspnea, cough, bibasilar inspiratory crackles, and/or digital clubbing. The incidence of IPF tends to rise with advancing age, typically presenting as a gradual onset of dyspnea during the sixth and seventh decades of life. Occurrence of IPF in individuals under 50 years of age is rare, and such cases may subsequently exhibit signs of an underlying connective tissue disorder or familial IPF. IPF is more frequently diagnosed in men compared to women. The primary risk factor is an history of prior cigarette smoking, while other factors that contribute to the risk include gastroesophageal reflux, chronic

viral infections such as Epstein-Barr virus and hepatitis C, as well as a family history of interstitial lung disease (ILD) (Armanios, 2012, Raghu et al., 2018).

Main Thorax HRCT features of IPF: High resolution computed tomography of the thorax in IPF may present specific features including honeycombing, traction bronchiectasis, and traction bronchiolectasis, which could be associated with the concurrent presence of ground-glass opacification and fine reticulation.

Honeycombing is the term to described the presence of clustered air-filled cysts, typically with a diameter ranging from 3 to 10 mm, and with thick and well-defined walls. Honeycombing often appears as multiple layers of subpleural cysts, but it can also manifest as a sub-pleural single layer. Distinguishing honeycombing from paraseptal emphysema or traction bronchiolectasis can sometimes be challenging.

Traction bronchiectasis/bronchiolectasis is a remarkable characteristic of pulmonary fibrosis and include a range of manifestations, from fine irregularities and non-tapering of the bronchial/bronchiolar walls to pronounced distortion and varicosity of the airways. In usual interstitial pneumonia (UIP), traction bronchiectasis is typically peripheral or subpleural and often coexists with honeycomb cysts.

Ground-glass opacification refers to a hazy increase in lung opacity while preserving the bronchial and vascular margins. It is important to differentiate between "pure" ground-glass opacification and ground-glass opacification superimposed on a fine reticular pattern. "Pure" ground-glass opacification is not a typical feature of UIP, and its presence in a patient with IPF should raise the possibility of an acute exacerbation. In contrast, ground-glass opacification superimposed on a fine reticular pattern indicates the presence of fibrosis and can be observed in patients with IPF. The presence of traction bronchiectasis/bronchiolectasis within the latter pattern helps distinguish between these two patterns (Raghu et al., 2018, Sverzellati, 2013).

HRCT Patterns, UIP or not UIP: ATS/ERS consensus depicted four diagnostic categories that incorporate the HRCT features described above. These categories include a “UIP pattern”, “probable UIP pattern”, “indeterminate for UIP pattern”, and “alternative diagnosis” as shown in Table 3.

Table 3. HRTC Patterns (Raghu et al., 2018)

UIP	Probable UIP	Indeterminate for UIP	Alternative diagnosis
-Subpleural and basal predominant; distribution is often heterogeneous* -Honeycombing with or without peripheral traction bronchiectasis or bronchiolectasis	Subpleural and basal predominant; distribution is often heterogeneous Reticular pattern with peripheral traction bronchiectasis or bronchiolectasis May have mild GGO	Subpleural and basal predominant Subtle reticulation; may have mild GGO or distortion (“early UIP pattern”) CT features and/or distribution of lung fibrosis that do not suggest any specific etiology (“truly indeterminate for UIP”)	Findings suggestive of another diagnosis, including: -CT features Cysts Marked mosaic attenuation Predominant GGO Profuse micronodules Centrilobular nodules Nodules Consolidation -Predominant distribution: Peribronchovascular Perilymphatic Upper or mid-lung -Other: Pleural plaques Dilated oesophagus Distal clavicular erosions Extensive lymph node enlargement Pleural effusions, pleural thickening

Definition of abbreviations: CT = computed tomography; GGO = ground-glass opacities; UIP = usual interstitial pneumonia.*Variants of distribution: occasionally diffuse, may be asymmetrical.

A CT scan of the Thorax of probable UIP is shown in Figure 2.

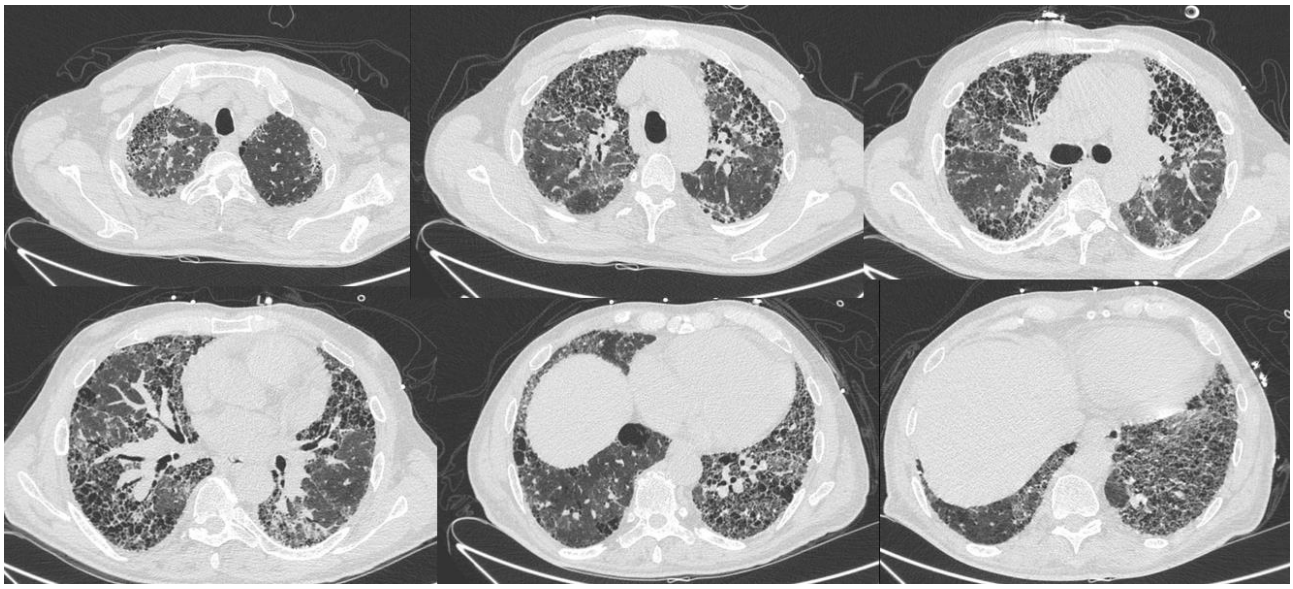

Figure 2. Probable UIP: Subpleural and basal predominant honeycombing; peripheral traction bronchiectasis and bronchiolectasis. Small areas of GGO.

Surgical lung biopsy: In some cases a surgical biopsy is needed to confirm an IPF diagnosis and video-assisted thoracoscopic surgery is the preferred approach in patients who can tolerate single-lung ventilation. Multiple biopsies should be obtained from two to three lobes, because the histologic patterns on SLB specimens obtained from different segments can be discordant (e.g., coexisting UIP pattern and fibrotic NSIP pattern from different lobes). A severe respiratory impairment or the presence of substantial comorbidity increase the surgical risks and a SLB may outweigh the benefits of establishing a secure diagnosis of IPF (Raghu et al., 2018).

Histopathology Features of the UIP Pattern: Usual interstitial pneumonia (UIP) is characterized by typical histopathological feature, the primary diagnostic criteria is the presence of patchy dense fibrosis at low magnification, which leads to remodelling of the lung architecture, often culminates in the development of honeycomb changes, and alternates with areas of less affected lung parenchyma. These histopathological alterations predominantly affect the subpleural and paraseptal regions of the lung. In terms of inflammation, it is typically mild and characterized by a patchy interstitial infiltrate composed of lymphocytes and plasma cells, accompanied by hyperplasia of type 2 pneumocytes and bronchiolar epithelium. The fibrotic regions primarily consist of dense collagen, although scattered subepithelial foci consisting of proliferating fibroblasts and myofibroblasts (known as fibroblast foci) are consistently observed. Microscopic honeycombing is characterized by the presence of cystic fibrotic airspaces that are frequently lined by bronchiolar epithelium and filled with mucus and inflammatory cells. Smooth muscle metaplasia within the interstitium is commonly observed in areas displaying fibrosis and honeycombing. A definitive pathological diagnosis of the UIP pattern can be established when all of the aforementioned features are present, particularly if honeycombing is evident. However, even in the absence of honeycombing, a definite diagnosis of the UIP pattern can still be made if all the other characteristic features are present (Raghu et al., 2018).

Lung function tests in IPF: In patients with idiopathic pulmonary fibrosis (Raghu et al.), pulmonary function tests reveal characteristic impairments, although individuals in the early stages of the disease may still exhibit lung volumes within the normal range. The underlying tissue stiffness and decreased lung compliance result in a restrictive ventilatory defect. Additionally, ventilation and perfusion abnormalities

contribute to a reduction in lung diffusion capacity for carbon monoxide (DLCO). For assessing lung function and stratifying IPF patients, forced vital capacity (FVC) has been established as the most reliable test. However, the interpretation of FVC can be challenging in the presence of concurrent emphysema and resultant lung hyperinflation. Another common complication in IPF is pulmonary hypertension, which can significantly reduce DLCO even when lung volumes remain relatively preserved. Monitoring the decline in FVC over time serves as a primary endpoint in clinical trials for IPF, either alone or in combination with other biomarkers (Bonella et al., 2019).

IPF treatment options: Currently, two anti-fibrotic treatments are recommended in the management of IPF: pirfenidone and nintedanib. Pirfenidone (a synthetic pyridine derivative) is a small molecule for which an anti-fibrotic and anti-inflammatory effect has been demonstrated in vivo and in vitro in animal models by limiting the production of profibrotic growth factors such as TGF-β1. It was evaluated in the CAPACITY 1 and 2 studies, phase III double-blind, pirfenidone versus placebo, lasting 72 weeks, in patients with mild to moderate IPF, defined as an FVC ≥ 50%, a diffusing capacity of the lungs for carbon monoxide (DLCO) ≥ 35%, and a walking distance of at least 150 meters on the 6-minute walk test (6mWT). These studies showed superiority of pirfenidone compared to placebo in terms of decline in FVC, percentage of patients with a decline in FVC ≥ 10%, mean walking distance on the 6MWT, and duration of progression-free survival (PFS) (defined as a decline ≥ 10% in FVC, ≥ 15% decline in DLCO, or death). This superiority was confirmed by the ASCEND study, conducted in patients with a DLCO ≥ 30%. Pirfenidone obtained European market authorization (EMA) in February 2011 for mild to moderate IPF defined as an FVC ≥ 50% and a DLCO ≥ 30%. Nintedanib (nintedanib esilate) is a low molecular weight molecule that acts as an inhibitor of tyrosine kinases (TKI) with anti-receptor activity against platelet-derived growth factor (PDGF-R) alpha and beta, fibroblast growth factor (FGF-R) 1-3, and vascular endothelial growth factor (VEGF-R) 1-3. This therapeutic agent was evaluated in a phase II randomized double-blind trial called TOMORROW. After 12 months, nintedanib significantly limited the decline in forced vital capacity (FVC) compared to placebo. Phase III studies conducted in double-blind, INPULSIS 1 and 2, showed a significantly less annual decline in FVC with nintedanib compared to placebo in patients with mild to moderate idiopathic pulmonary fibrosis (FVC ≥ 50% and DLCO ≥ 30%). Nintedanib received European marketing authorization in January 2015 for mild to moderate idiopathic pulmonary fibrosis, defined as FVC ≥ 50% and DLCO ≥ 30%. Although these two therapeutic agents are not curative, clinical trials demonstrate that they slow the decline in respiratory function, reduce mortality, and seem to increase survival compared to placebo (Maher and Strek, 2019).

1.3.2. Chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease (Lynch et al.) is still a major contributor to poor health and disability worldwide. Research shows that the global prevalence of COPD is between 7.6% and 10.6% among individuals aged 30-79, with a higher burden in low- and middle-income countries. According to the GOLD 2023 guidelines, COPD is a respiratory disease characterized by chronic respiratory symptoms such as dyspnea, cough, expectoration and exacerbations, and is caused by abnormalities in the airways and/or alveoli, which result in persistent and usually progressive airflow obstruction till respiratory failure (Agusti et al., 2023).

Pathogenesis and risk factor: Over the course of a person's lifetime, interactions between genetic and environmental factors accumulate to cause COPD. These interactions interfere with regular lung development and aging processes and cause lung damage.

A significant environmental risk factor for COPD is cigarette smoking. Comparatively to non-smokers, smokers have a higher prevalence of respiratory symptoms, abnormal lung function, an accelerated decline in lung function, and increased mortality from COPD. However, Less than 50% of heavy smokers will experience COPD (Agusti et al., 2023).

Nonsmokers represent an important portion of COPD cases in low- and middle-income countries (LMICs), possibly around 60–70%. The majority of COPD cases worldwide are found in LMICs, so non-smoking risk factors play a significant role in the disease's overall burden. High levels of indoor air pollution are correlated with household air pollution that results from the burning of biomass, such as wood, animal dung, crop residues, and coal. Although this type of pollution has been associated with a higher risk of COPD, it is still unclear the precise role of household air pollution in comparison to other poverty-related exposures (Yang et al., 2022).

Occupational exposures are an often overlooked environmental risk factor for COPD. These exposures include organic and inorganic dust, chemical agents, and fumes, which can contribute to the development of COPD (De Matteis et al., 2019).

Air pollution, consisting of particulate matter, ozone, nitrogen or sulfur oxides, heavy metals, and other greenhouse gases, is a significant global cause of COPD. It is responsible for approximately 50% of the attributable risk of COPD in LMICs. Additionally, air pollution increases the risk of COPD exacerbations, hospitalizations, and mortality (Agusti et al., 2022).

One of the primary genetic risk factors associated with chronic obstructive pulmonary disease (Lynch et al.) is the presence of mutations in the SERPINA1 gene, which result in α-1 antitrypsin deficiency (AATD). AATD is an autosomal codominant disorder caused by various mutations affecting the SERPINA1 gene as α-1 antitrypsin is an important inhibitor of serine proteases that protects lung tissue from proteolytic insult by inhibiting neutrophil elastase, an enzyme with broad substrate specificity. While most of these mutations lead to the production of a functional protein but at lower concentrations, certain mutations can result in dysfunctional or non-functional variants. The main clinical implications of AATD include an elevated risk of COPD and emphysema in adults, as well as liver disease during childhood or later in adulthood (Seixas and Marques, 2021).

Forced Spirometry as diagnostic tool: To establish the diagnosis, spirometry with a forced vital capacity (FVC) manoeuvre and post-bronchodilator measurement is necessary, showing a ratio of forced expiratory volume in 1 second (FEV1) to FVC less than 0.7 (FEV1/FVC <0.7 after bronchodilator). The severity of airflow obstruction, which helps determine the COPD grade in based on FEV1 % values: mild ≥80% predicted, moderate 50-80%, severe 30-49% predicted, very severe <30% predicted (Agusti et al., 2023).

HRCT findings: HRCT of the thorax is recommended for initial COPD characterization, subtyping, and evaluation of other pulmonary and extrapulmonary manifestations. In the evaluation of severe COPD exacerbations, contrast-enhanced CT scan pulmonary angiography (CTPA) can be valuable in ruling out pulmonary embolism as a potential contributing factor to acute respiratory symptom exacerbation. Additionally, CTPA can identify indicators of pulmonary hypertension (PH), such as the presence of peripheral pulmonary vasculature pruning, a frequent complication of COPD (Raoof et al., 2023).

Typical HRCT findings in COPD include bronchial wall thickening and emphysema.

Bronchial wall thickening is indicative of chronic bronchitis and can result from various factors, including airway remodelling, hypertrophy of mucus glands, excessive mucus secretions, hyperplasia of smooth muscle, epithelial thickening, and disrupted alveolar attachments (Reid, 1960). Bronchial wall thickening on CT scan imaging may suggest an asthmatic component of COPD. However, it also may be seen in tobacco users and during COPD exacerbations.

Emphysema is a readily recognizable feature of COPD on CT scan imaging. It may be classified radiologically as centrilobular, panlobular, and paraseptal, and this distinction may suggest the cause of the emphysema (Lynch et al., 2018).

Centrilobular emphysema (CLE) is recognized by the presence of well-defined round lucencies of varying size, sometimes associated with a visible central artery. CLE may be graded as trace (occupying < 0.5% of a lung zone), mild (occupying 0.5%-5% of a zone), moderate (occupying > 5% of any zone), confluent (coalescent lucencies spanning several lobules), or advanced destructive (coalescent lucencies with hyperexpansion of lobules and architectural distortion) (Lynch et al., 2018).

Panlobular emphysema, in contrast to CLE, affects the entire pulmonary lobule, resulting in a diffuse decrease in lung attenuation and reduced vascularity. The terms "confluent emphysema" or "advanced destructive emphysema" are used to describe emphysema related to tobacco use. Panlobular emphysema is primarily associated with α1-antitrypsin deficiency and typically involves all lung lobes, with a lower lung predominance and associated linear abnormalities in the lower lungs (Guest and Hansell, 1992).

Paraseptal emphysema is another subtype of emphysema that is located at the periphery and subpleural regions of the lungs. It manifests as focal areas of lucency, which can be relatively large. Due to its subpleural location, paraseptal emphysema may be associated with pneumothorax. Bullae, which are avascular low-attenuation areas larger than 1 cm in diameter, are commonly observed in paraseptal emphysema. They can cause physiological impairment by compressing adjacent normal lung tissue and, in rare cases, may occupy more than one-third of a hemithorax, leading to giant bullous emphysema or vanishing lung (Raoof et al., 2023).

A CT scan of the Thorax of advanced COPD in α-1 antitrypsin deficiency is shown in Figure 3.

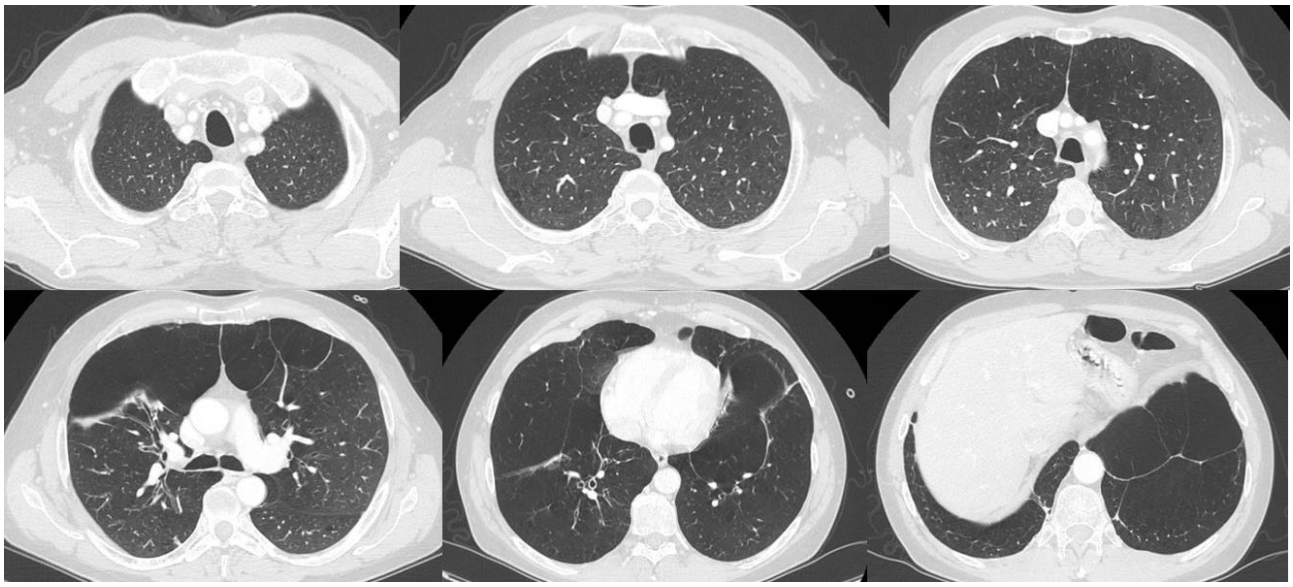

Figure 3. Advanced COPD in α-1 antitrypsin deficiency: Moderate panlobular emphysema at the higher lobes with advanced destructive emphysema at the lower lobes.

1.3.3. GROUP 3 PULMONARY HYPERTENSION

Patients with advanced parenchymal and interstitial lung disease who are candidates for lung transplant frequently presents mild pulmonary hypertension (PH).

When to suspect PH in chronic lung disease: In ILD and COPD, PH is generally associated with a lower DLCO, diminished exercise capacity and more impaired gas exchange at rest or during exercise than expected based on ventilatory impairments (Gläser et al., 2013). Non-invasive techniques are useful tool to raise suspicion of PH in chronic lung disease (CLD) including pulmonary function testing, echocardiography, and CT imaging.

Among these modalities, echocardiography is considered the most effective non-invasive method for screening CLD-PH. However, its ability to accurately determine the peak tricuspid regurgitation velocity and estimate the right ventricular systolic pressure is limited in these patients due to a poor acoustic window. As an alternative, other echocardiographic measures such as the right ventricular outflow tract diameter, tricuspid annular plane systolic excursion, and qualitative assessment of right chamber structure and function have been recommended specifically in cases of idiopathic pulmonary fibrosis (Raghu et al.) and chronic obstructive pulmonary disease (Greiner et al., 2014).

In both COPD and IPF, the ratio of the main pulmonary artery diameter to ascending aorta diameter on imaging can serve as an indicator PH. A ratio greater than 1 (within the range of 0.9-1.1) has been suggested as a threshold for predicting PH. Furthermore, combining this pulmonary artery/aorta diameter ratio with other non-invasive measures, such as echocardiographic and physiological variables, enhances the accuracy of PH prediction (Yagi et al., 2017).

The role of RHC in CLD: Right heart catheterization (RHC) remains the gold standard CLD-PH. However, it is important to note that the suspicion of underlying PH does not always necessitate the performance of RHC in patients who already have an established lung disease, especially if there would be no therapeutic or management implications.

RHC should be performed in patients with CLD when significant PH is suspected and the patient's management will likely be influenced by RHC results, including referral for transplantation, inclusion in clinical trials or registries, treatment of unmasked left heart dysfunction, or compassionate use of therapy.

It is worth to perform a RHC if: 1) Clinical worsening, progressive exercise limitation and/or gas exchange abnormalities are not deemed attributable to ventilatory impairment; 2) An accurate prognostic assessment is deemed sufficiently important (Nathan et al., 2019).

The impact of PH in lung disease: PH assessment in CLD appear to be relevant to define prognosis and disease severity. Even before the latest ESC/ERS guidelines for PH it was evident that a mPAP > 20 mmHg was associated to higher mortality in patient affected by ILD. While in COPD, only severe PH presents a worse outcome (Piccari et al., 2022).

In early 2019 it was proposed an adapted definition in CLD patient as follow (Nathan et al., 2019):

1) CLD without PH (mPAP <21 mmHg, or mPAP 21–24 mmHg with PVR <3 WU).

2) CLD with PH (mPAP 21–24 mmHg with PVR ≥3 WU, or mPAP 25–34 mmHg)

3) CLD with severe PH (mPAP ≥35 mmHg, or mPAP ≥25 mmHg with low cardiac index (<2.0 $Lminm^2$)

Group 1 randomized controlled trials (RCTs) for therapies targeting PAH established specific criteria to exclude individuals based on pulmonary function testing. These criteria included the following ranges: total lung capacity below 60-70% of predicted, FEV1 below 55-80% of predicted, or FEV1/ FVC ratio below 50-70%. In previous PAH studies, chest imaging was not employed to exclude patients with lung disease. Consequently, it is plausible that some patients with lung volumes above these inclusion thresholds may have undetected underlying lung conditions. Nonetheless, it should be noted that lung diseases, particularly COPD, are prevalent, and the occurrence of PAH in such patients may not be directly related to these underlying conditions but rather coincidental. The severity spectrum of both pulmonary vascular and parenchymal lung diseases likely exists as a continuum, which often complicates the differentiation between group 1 and group 3 PH (Nathan et al., 2019). Nathan et al. presented a useful chart to help to distinguish group 1 and group 3 PH (Figure 4).

Criteria favouring group 1 (PAH)	Testing	Criteria favouring group 3 (PH due to lung disease)
Extent of lung disease		
Normal or mildly impaired: • FEV_1 >60% pred (COPD) • FVC >70% pred (IPF) • Low diffusion capacity in relation to obstructive/restrictive changes	Pulmonary function testing	Moderate to very severely impaired: • FEV_1 <60% pred (COPD) • FVC <70% pred (IPF) • Diffusion capacity "corresponds" to obstructive/restrictive changes
Absence of or only modest airway or parenchymal abnormalities	High-resolution CT scan¶	Characteristic airway and/or parenchymal abnormalities
Haemodynamic profile		
Moderate-to-severe PH	Right heart catheterisation Echocardiogram	Mild-to-moderate PH
Ancillary testing		
Present	Further PAH risk factors (*e.g.* HIV, connective tissue disease, *BMPR2* mutations, *etc.*)	Absent
Features of exhausted circulatory reserve: • Preserved breathing reserve • Reduced oxygen pulse • Low $CO/\dot{V}O_2$ slope • Mixed venous oxygen saturation at lower limit • No change or decrease in P_{aCO_2} during exercise	Cardiopulmonary exercise test+ (P_{aCO_2} particularly relevant in COPD)	Features of exhausted ventilatory reserve: • Reduced breathing reserve • Normal oxygen pulse • Normal $CO/\dot{V}O_2$ slope • Mixed venous oxygen saturation above lower limit • Increase in P_{aCO_2} during exercise
Predominant haemodynamic profile		Predominant obstructive/restrictive profile

Figure 4. Criteria favouring group 1 versus group 3 pulmonary hypertension(Nathan et al., 2019)

PH in COPD: The prevalence of PH in COPD is in general dependent on the severity of the disease, but also on the definition of PH and the method of diagnostic assessment. Various studies have indicated that approximately 1-5% of patients with advanced COPD and chronic respiratory failure, as well as those eligible for lung volume reduction surgery or lung transplantation (LTx), have a mean pulmonary arterial pressure (mPAP) exceeding 35-40 mmHg, and more in general up to 90% of patients affected by COPD exhibit a PAPm is higher than 20 mmHg at rest (Gredic et al., 2021).

Even during moderate exercise, individuals with COPD may exhibit a significant increase in PAPm, suggesting the impairment of lung vasculature, reduced vascular distensibility, and/or limited vessel recruitment capacity. Within this population, it may exists a subgroup characterized by a distinct "pulmonary vascular COPD phenotype." These individuals typically present with less severe airflow limitation, hypoxemia, significantly reduced diffusing capacity of the lung for carbon monoxide (DLCO), normo- or hypocapnia, and exhibit a cardiovascular exercise limitation profile (Boerrigter et al., 2012).

Interestingly, the vascular lesions in COPD-PH patients are morphologically similar to those in IPAH. It has previously been established that the presence of PH has a stronger association with mortality in COPD than FEV1 or gas exchange variables (Gredic et al., 2021).

Treatment option for COPD-PH: Optimal treatment of the underlying lung disease should be prioritized in accordance with GOLD current guidelines (Agusti et al., 2023). The use of long-term oxygen treatment

(LTOT) appears logical in hypoxemic patients. In stabilized hypoxemic COPD patients, LTOT administered for 15 hours per day was found to prevent the progressive increase in PAPm. Moreover, when LTOT was administered for more than 18 hours per day, a slight decrease in PAPm was observed.

Beneficial haemodynamic effects with long-term PAH therapy, assessed by RHC, have been demonstrated with both sildenafil and bosentan (Valerio et al., 2009, Vitulo et al., 2017). However the effect of PAH-targeted therapy on exercise capacity in patients with COPD-PH is less apparent. Two separate meta-analyses have demonstrated that long-term use of PAH-targeted therapy can improve pulmonary hemodynamics in COPD patients with PH, while the results exploring the effect of PAH-therapy on 6-min walk distance (6MWD), on dyspnoea or quality of life measures were disappointing (Prins et al., 2017, Chen et al., 2015b).

In summary, based on the currently available studies, there is insufficient clear evidence to support the notion that PAH-targeted therapy for COPD-PH leads to improvements in exercise tolerance and symptom relief. The impact of PAH-targeted therapy on pulmonary hemodynamics may not necessarily translate into tangible benefits in terms of enhancing exercise capacity and alleviating symptoms in these patients.

Notably, vasodilator treatment in COPD-PH patients may have a negative impact on gas exchange. In fact, vasodilators can inhibit hypoxic pulmonary vasoconstriction, leading to increased ventilation/perfusion mismatching in COPD. While some studies have shown a deterioration in gas exchange with long-term use of bosentan or sildenafil, others using sildenafil or tadalafil have reported no change in gas exchange (Nathan et al., 2019).

Although preliminary evidence suggests a potential benefit of currently available vasoactive medications in COPD-PH patients with mean pulmonary arterial pressure (mPAP) equal to or greater than 35 mmHg, further research is necessary before recommending PAH therapies for these patients. Larger prospective studies should be conducted, with a focus on this patient population.

PH in IPF: The most part of the available literature about PH and ILD arises from IPF studies. In IPF cases it has been reported an PAPm of 25 mmHg or higher in 8-15% of patients, with a higher prevalence in advanced stages (30-50%) and end-stage disease (>60%) (Shorr et al., 2007).

Longitudinal studies have indicated an average annual increase of approximately 1.8 mmHg in mean pulmonary arterial pressure (mPAP). In late-stage IPF patients, rapid progression of PH has been observed as well (Teramachi et al., 2017). PH in advanced IPF is also associated with an elevated risk of acute exacerbations. Adverse outcomes have been reported in cases where PAPm thresholds reach or exceed 25 mmHg in IPF patients. In fact, the prognosis of IPF with PH is worse than that of IPAH(Nathan et al., 2019).

Treatment option for IPF-PH: In contrast to the results obtained in COPD-PH, the evidence supporting the beneficial effects of LTOT in ILD is less conclusive compared to COPD, and no studies have specifically investigated the impact of LTOT on pulmonary hypertension associated with ILD.

The use of PAH-targeted therapies in patients with idiopathic interstitial pneumonia (IIP) has shown concerning safety signals in certain randomized controlled trials (RCTs). The ARTEMIS study was stopped prematurely after interim analysis revealed that IPF patients treated with ambrisentan had a higher likelihood of disease progression, particularly respiratory-related hospitalizations. The ARTEMIS-IPF trial also failed to show any significant effect of ambrisentan on pulmonary haemodynamics in the subgroup of patients who underwent a second assessment with RHC. As a result, ambrisentan is contraindicated for patients with IPF. Similarly, the RISE-IIP trial investigated the impact of riociguat on the 6MWD in patients with IIP. However, the study was terminated early due to interim findings that indicated increased mortality and a greater risk of serious adverse events among the riociguat-treated group. Consequently, riociguat is contraindicated for patients with IIP-associated PH (Nathan et al., 2017, Raghu et al., 2013).

The impact of PAH-targeted therapy on symptomatic burden in patients with pulmonary hypertension associated with PH-ILD has been examined in two randomized controlled trials (RCTs) and three open-label studies. These studies did not show a significant change in quality of life questionnaires or dyspnea scales (Prins et al., 2017). However, the STEP-IPF study, focused specifically on IPF, demonstrated a positive effect of sildenafil on quality of life when compared to a placebo group (Idiopathic Pulmonary Fibrosis Clinical Research et al., 2010).

In ILD, the acute administration of aerosolised iloprost, inhaled nitric oxide or sildenafil does not worsen ventilation/perfusion relationships. Inhaled treprostinil was recently approved for group 3 PH-ILD based on the results of INCREASE trial and the newer formulation of treprostinil dry powder that comes with a new inhaler was recently approved for both group 1 PAH and group 3 PH-ILD based on BREEZE study

In the context of ILD, the acute administration of aerosolized iloprost, inhaled nitric oxide, or sildenafil does not have a negative impact on ventilation/perfusion relationships. Notably, inhaled treprostinil has been approved for the treatment of group 3 pulmonary hypertension associated with ILD. This approval is based on the positive outcomes observed in the INCREASE trial. Additionally, a new formulation of treprostinil in dry powder form, along with a new inhaler, has recently received approval for both group 1 pulmonary arterial hypertension (PAH) and group 3 PH-ILD, as supported by the BREEZE study (El-Kersh and Jalil, 2023).

On the other hand, the acute administration of IV epoprostenol has been shown to lead to a decline in gas exchange due to increased blood perfusion in non-ventilated alveolar units. However, in longer-term studies, the use of PAH-targeted therapy did not result in a deterioration of gas exchange in patients with ILD (Nathan et al., 2019).

1.4. LUNG TRANSPLANTATION

Lung transplantation (LTx) is a crucial treatment option for patients with advanced lung disease. The field of LTx has been expanding, with a steady increase in transplant volume worldwide over the past three decades, reaching around 4500 transplants per year according to the International Society for Heart and Lung Transplantation (Meyer et al.). Moreover, recipients of lung transplants are experiencing longer survival periods, leading to a growing population of retransplant and posttransplant patients (Chambers et al., 2019).

Rational: Lung transplantation should be considered for adults with chronic, end-stage lung disease who meet all the following general criteria:

1. High (>50%) risk of death from lung disease within 2 years if lung transplantation is not performed.

2. High (>80%) likelihood of 5-year post-transplant survival from a general medical perspective provided that there is adequate graft function (Leard et al., 2021).

General ethical framework and allocation systems: The scarcity of donor lungs worldwide means that the selection of candidates for lung transplant is not only a medical decision but also an ethical one. The ethical principles of utility, justice, and respect for persons should guide the process of candidate selection and organ allocation (Beauchamp and Childress, 2001).

The principle of utility emphasizes maximizing survival when choosing transplant candidates. This means carefully selecting candidates to ensure the best chance of success, as a failed transplant not only affects the individual but also denies another potential recipient the opportunity for a transplant. The goal is to maximize long-term survival and provide overall survival gains for society.

The principle of justice requires that all individuals who could benefit from a lung transplant be given equal consideration and opportunity. Factors such as an individual's value in society, social status, or occupation

should not be used to evaluate transplant candidacy. Similarly, characteristics like race, gender, or socioeconomic position should not be used to disadvantage access to transplants, even if certain groups may have inferior outcomes.

The principle of respect for persons acknowledges a candidate's right to self-determination and autonomy. Transplant centers should provide clear guidelines that explain the criteria for candidate selection and organ allocation, allowing candidates to understand and exercise their rights (Leard et al., 2021).

Timing of referral, evaluation, and listing: The referral process for lung transplant is intricate and, whenever possible, should commence before the need for transplant becomes urgent. Early referral allows candidates to address any modifiable barriers to transplant, such as obesity, malnutrition, medical comorbidities, or inadequate social support.

The latest consensus for the selection of lung transplant candidates proposes specific criteria for referral to lung transplant centre and additional criteria for active listing for each disease susceptible of lung transplant such as COPD, interstitial lung disease, PAH, Cystic fibrosis (CF), non-CF bronchiectasis, lymphangioleiomyomatosis (LAM), ARDS and re-transplant.

A full evaluation includes assessment of lung disease severity, anatomy, nutritional status, degree of frailty, presence and severity of comorbidities, psychosocial circumstances, and health-related behaviors that impact recovery and long-term survival (Leard et al., 2021).

Contraindication and risk factors: The evaluation of transplant candidates must take into account various factors, including medical comorbidities, psychosocial factors, and potential for rehabilitation. Risk factors that increase the likelihood of poor outcomes and absolute contraindication have been identified (Table 4 and Table 5). While it's important to consider the relative risk associated with specific factors like age or obesity, it's also crucial to consider the cumulative impact of multiple risk factors.

Table 4. ABSOLUTE CONTRAINDICATIONS (Leard et al., 2021)	
• Candidates with these conditions are considered too high risk to achieve successful outcomes post lung transplantation. • Factor or condition that significantly increases the risk of an adverse outcome post-transplant and /or would make transplant most likely harmful for a recipient. • Most lung transplant programs should not transplant patients with these risk factors except under very exceptional or extenuating circumstances.	1. Lack of patient willingness or acceptance of transplant 2. Malignancy with high risk of recurrence or death related to cancer 3. Glomerular filtration rate < 40 mL/min/1.73m2 unless being considered for multi-organ transplant 4. Acute coronary syndrome or myocardial infarction within 30 days (excluding demand ischemia) 5. Stroke within 30 days 6. Liver cirrhosis with portal hypertension or synthetic dysfunction unless being considered for multi-organ transplant 7. Acute liver failure 8. Acute renal failure with rising creatinine or on dialysis and low likelihood of recovery 9. Septic shock 10. Active extrapulmonary or disseminated infection 11. Active tuberculosis infection 12. HIV infection with detectable viral load 13. Limited functional status (e.g. non-ambulatory) with poor potential for post-transplant rehabilitation 14. Progressive cognitive impairment 15. Repeated episodes of non-adherence without evidence of improvement (Note: For pediatric patients this is not an absolute contraindication and ongoing assessment of non-adherence should occur as they progress through different developmental stages.) 16. Active substance use or dependence including current tobacco use, vaping, marijuana smoking, or IV drug use 17. Other severe uncontrolled medical condition expected to limit survival after transplant

Table 5. RISK FACTORS WITH HIGH OR SUBSTANTIALLY INCREASED RISK (Leard et al., 2021)	
• Candidates with these conditions may be considered in centers with expertise specific to the condition. • We may not have data to support transplanting patients with these risk factors or there is substantially increased risk based upon the currently available data, and further research is needed to better inform future recommendations. • When more than one of these risk factors are present, they are thought to be possibly multiplicative in terms of increasing risk of adverse outcomes. • Modifiable conditions should be optimized when possible.	1. Age > 70 years 2. Severe coronary artery disease that requires coronary artery bypass grafting at transplant 3. Reduced left ventricular ejection fraction < 40% 4. Significant cerebrovascular disease 5. Severe esophageal dysmotility 6. Untreatable hematologic disorders including bleeding diathesis, thrombophilia, or severe bone marrow dysfunction 7. BMI > 35 kg/m2 8. BMI < 16 kg/m2 9. Limited functional status with potential for post-transplant rehabilitation 10. Psychiatric, psychological or cognitive conditions with potential to interfere with medical adherence without sufficient support systems 11. Unreliable support system or caregiving plan 12. Lack of understanding of disease and / or transplant despite teaching 13. Mycobacterium abscessus infection 14. Lomentospora prolificans infection 15. Burkholderia cenocepacia or gladioli infection 16. Hepatitis B or C infection with detectable viral load and liver fibrosis 17. Chest wall or spinal deformity expected to cause restriction after transplant 18. Extracorporeal life support 19. Retransplant <1 year following initial lung transplant 20. Retransplant for restrictive CLAD 21. Retransplant for AMR as etiology for CLAD

1.4.1. COPD referral and listing criteria (Leard et al., 2021)

Timing of referral:
BODE score 5-6 with additional factor(s) present suggestive of increased risk of mortality (BODE index is shown in Figure 5):
- Frequent acute exacerbations
- Increase in BODE score >1 over past 24 months
- Pulmonary artery to aorta diameter > 1 on CT scan ○ FEV1 20-25% predicted
- Clinical deterioration despite maximal treatment including medication, pulmonary rehabilitation, oxygen therapy, and, as appropriate, nocturnal non-invasive positive pressure ventilation.
- Poor quality of life unacceptable to the patient
- For a patient who is a candidate for bronchoscopic or surgical lung volume reduction (LVR), simultaneous referral for both lung transplant and LVR evaluation is appropriate.

Variables	POINTS of BODE index			
	0	+1	+2	+3
Body max index (BMI)	>21	≤ 21		
FEV1 (Obstruction)	≥65%	50-64%	36-49%	≤ 35
Dyspnea (mMRC)	0-1 Dyspnea only with strenuous exercise or Dyspnea when hurrying or walking up a slight hill	2 Walks slower than people of same age because of dyspnea or stops for breath when walking at own pace	3 Stops for breath after walking 100 yards (91 m) or after a few minutes	4 Too dyspneic to leave house or breathless when dressing
6mWD (Exercise)	≥ 350m	250-349m	150-249m	≤149m

Figure 5. BODE index assessment (Celli et al., 2004)

Timing of listing:
BODE score 7-10
Additional factors that may prompt listing include:

- FEV1 < 20% predicted
- Presence of moderate to severe pulmonary hypertension
- History of severe exacerbations
- Chronic hypercapnia

Notably the assessment of PH in a relevant parameter to take into consideration during lung transplant screening.

1.4.2. IPF referral and listing criteria (Leard et al., 2021)

Timing of referral:
Referral should be made at time of diagnosis, even if a patient is being initiated on therapy, for histopathological UIP or radiographic evidence of a probable or definite UIP pattern.
Abnormal lung function: FVC < 80% predicted or DLCO < 40% predicted.
Any form of pulmonary fibrosis with one of the following in the past 2 years:

- Relative decline in FVC 10%
- Relative decline in DLCO 15%
- Relative decline in FVC 5% in combination with worsening of respiratory symptoms or radiographic progression

Supplemental oxygen requirement either at rest or on exertion.
For patients with familial pulmonary fibrosis, early referral is recommended.

Timing of listing:
Any form of pulmonary fibrosis with one of the following in the past 6 months despite appropriate treatment:

- Absolute decline in FVC > 10%
- Absolute decline in DLCO > 10%
- Absolute decline in FVC > 5% with radiographic progression.

Desaturation to < 88% on 6 minute walk test or > 50 m decline in 6 minute walk test distance in the past 6 months
Pulmonary hypertension on right heart catheterization or 2-dimensional echocardiography (in the absence of diastolic dysfunction)
Hospitalization because of respiratory decline, pneumothorax, or acute exacerbation.

1.4.3. PAH referral and listing criteria (Leard et al., 2021)

Timing of referral:
ESC/ERS intermediate or high risk or REVEAL risk score 8 despite appropriate PAH therapy (REVEAL score is shown in Figure 6)
Significant RV dysfunction despite appropriate PAH therapy
Need for IV or SC prostacyclin therapy
Progressive disease despite appropriate therapy or recent hospitalization for worsening of PAH
Known or suspected high-risk variants such as PVOD/PCH, scleroderma, large and progressive pulmonary artery aneurysms
Signs of secondary liver or kidney dysfunction due to PAH
Potentially life-threatening complications such as recurrent haemoptysis

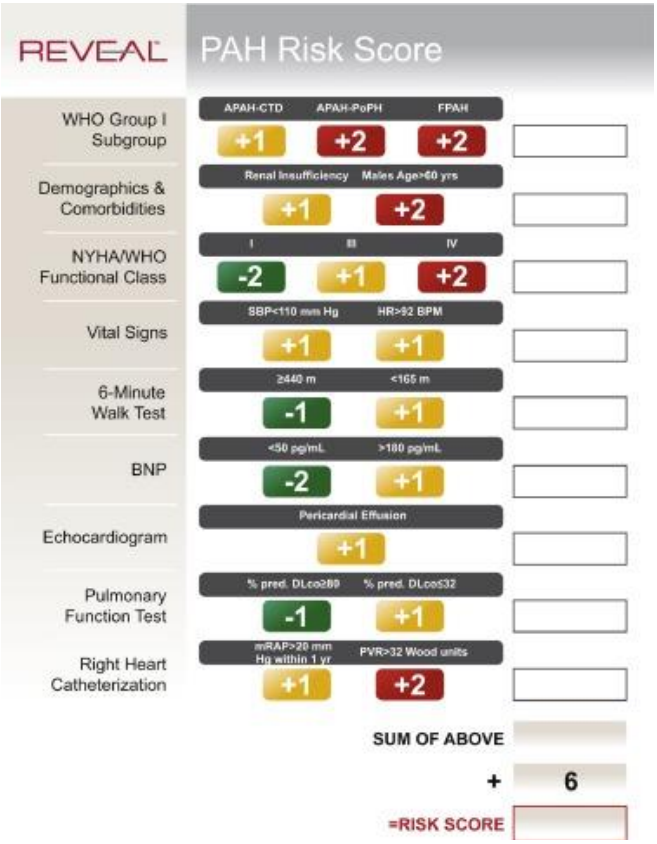

Figure 6. REVEAL score assessment (Frost et al., 2018)

Timing of listing:
ESC/ERS high risk or REVEAL risk score >10 on appropriate PAH therapy, including IV or SC prostacyclin analogues
Progressive hypoxemia, especially in patients with PVOD or PCH
Progressive, but not end-stage, liver or kidney dysfunction due to PAH
Life-threatening haemoptysis

1.5. PH PATHOPHYSIOLOGY

The pulmonary vascular system is typically a highly compliant, low-pressure, and low-resistance system. However, there are several severe clinical conditions that can affect the blood vessels in the lungs, leading to increased resistance and pressure in the pulmonary artery.

In PAH, there is a loss of and obstruction in the pulmonary blood vessels, which causes an elevation in pulmonary arterial pressure and pulmonary vascular resistance (PVR). This results in a gradual failure of the right side of the heart and a decline in overall lung function. In PAH, the remodelling of the pulmonary blood vessels is characterized not only by the accumulation of different types of cells in the walls of the pulmonary arteries (such as smooth muscle cells (PA-SMCs), endothelial cells (ECs), fibroblasts, myofibroblasts, and pericytes), but also by the loss of small arteries and an excessive infiltration of inflammatory cells (such as B- and T-lymphocytes, mast cells, dendritic cells, and macrophages) surrounding the blood vessels (Humbert et al., 2019).

PH can be attributed to lesions mainly occurring in distal muscular-type arteries, ranging in diameter from 500 µm down to 70 µm in humans, as medial hypertrophy/hyperplasia, intimal and adventitial fibrosis, and (in situ) thrombotic lesions and plexiform lesions. Muscular-type arteries are always neighboured by an

airway (bronchiole), and at microscopic anatomy they present a well-defined tunica media that is delimited by the internal and the external elastic lamina. Small pre-capillary pulmonary arteries (arterioles), ranging in diameter from 70 µm down to 20 µm in humans, are also involved in all groups of human and experimental PH, through processes of loss and obliteration, abnormal muscularisation, and perivascular inflammation. The capillary compartment represents the largest vascular surface within the lung is also frequently involved in PH. There is also accumulating evidence supporting the involvement of the post-capillary pulmonary venous vasculature in all PH groups with varying degrees of intensity. In PH due chronic respiratory disease, post-capillary involvement is likely to be explained at least in part by parenchymal destruction and inflammation in patients with lung fibrosis and emphysema (Humbert et al., 2019).

In pulmonary arterial hypertension (PAH), specific vascular abnormalities are commonly observed, including onion-skin lesions, plexiform core lesions, and dilation lesions. However, the exact underlying significance of these characteristic vascular changes in PAH is still not fully understood. Plexiform vasculopathy, a type of lesion seen in PAH, appears to involve systemic blood vessels such as the vasa vasorum and bronchial arteries. These vessels can be found either within the outer layer of the pulmonary arteries (adventitia) or in the connective tissue surrounding the bronchial tubes. In patients with PAH, plexiform lesions seem to represent interconnected structures linking bronchial microvessels with pulmonary arteries and veins (Galambos et al., 2016).

Vascular remodelling

Vascular remodelling is associated with alterations in vasoconstriction, pulmonary artery- endothelial cells (PAECs) and -smooth muscle cells (PASMCs) cell proliferation, inflammation, apoptosis, angiogenesis, and thrombosis, which leads to the muscularization and occlusion of the lumen of pulmonary arteries by the formation of vascular lesions. One of the first triggers for development of PAH is thought to be EC injury triggering the activation of cellular signaling pathways that are not yet completely understood (Figure 7).

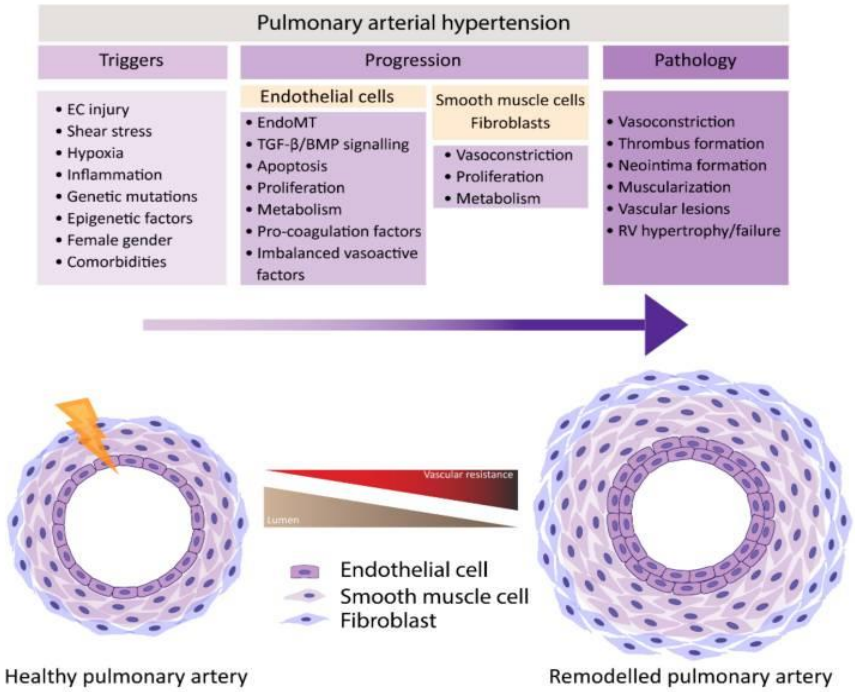

Figure 7. Vascular remodelling in PH (Kurakula et al., 2021)

A. Pulmonary vascular endothelial dysfunction

Despite limited knowledge regarding the exact causes of abnormal vascular remodeling in pulmonary arterial hypertension (PAH), there is a growing body of evidence suggesting that dysfunction of endothelial cells (ECs) serves as one of the initial triggers for initiating this process. In the context of pulmonary arterial hypertension (PAH), the term "pulmonary endothelial dysfunction" is used to describe various impairments in endothelial function. This dysfunction is characterized by a shift towards vasoconstriction rather than vasodilation, as well as a decrease in the anticoagulant properties of the endothelium. It also involves active metabolic changes, the production of reactive oxygen species, increased expression of adhesion molecules (such as E-selectin, intercellular adhesion molecule 1 (ICAM1), and vascular cell adhesion molecule 1 (VCAM1)), and an uncontrolled release of chemokines, cytokines, and growth factors within the local environment. Furthermore, endothelial cells (ECs) undergo a process called endothelial to mesenchymal transition (EndoMT), which contributes to the overall disruption of pulmonary vascular homeostasis. Collectively, these changes lead to impairments in angiogenesis (the formation of new blood vessels) and the mechanisms responsible for pulmonary vascular repair. These alterations play significant roles in the remodeling of the pulmonary vasculature observed in PAH (Humbert et al., 2019).

- **Vasoactivity impairment**

The reduced vasorelaxation observed in pulmonary arterial hypertension (PAH) primarily stems from alterations in the expression of two key vasodilators: nitric oxide (NO) and prostacyclin. NO is a rapidly acting endogenous free radical produced by endothelial NO synthase (eNOS). It plays a crucial role in promoting vasorelaxation in pulmonary artery smooth muscle cells (PASMCs), while also exerting antithrombotic effects and controlling endothelial cell (EC) differentiation and growth (Kurakula et al., 2021).

Prostacyclin, another vasodilator produced by ECs, possesses additional antithrombotic and antiproliferative properties. It is synthesized from arachidonic acid through the action of prostacyclin synthase and cyclo-oxygenase. Decreased levels of prostacyclin are observed in various forms of PAH, contributing to increased pulmonary vasoconstriction, smooth muscle cell proliferation, and coagulation in these patients (Budhiraja et al., 2004).

On the other hand, endothelin-1 (ET-1) is a potent vasoconstrictor primarily synthesized in ECs, with the highest levels found in the lungs. ET-1 exerts its effects by binding to ETA and ETB receptors, which activate signaling pathways in vascular smooth muscle cells (vSMCs) involved in proliferation, vasorelaxation, and vasoconstriction. ETA receptors are predominantly expressed on vSMCs and play a role in vasoconstriction and proliferation of these cells, while ETB receptors are expressed on both vSMCs and pulmonary artery endothelial cells (PAECs), stimulating the release of vasodilators such as NO and prostacyclin, as well as inhibiting apoptosis. The expression of ET-1 and its receptors is increased in the lungs of PAH patients and experimental models of pulmonary hypertension (PH) (Frasch et al., 1999). Moreover, the heightened synthesis of endothelial ET-1, along with an increase in ETA expression in PASMCs, likely contributes to the elevated vasoconstriction and vascular remodeling observed in PAH (Kurakula et al., 2021, Galie et al., 2004).

Finally, it has been observed that the expression of vascular endothelial growth factor (VEGF) and its receptor VEGF receptor 2 (VEGFR2) is increased in endothelial cells (ECs) within plexiform lesions from IPAH patients. The relationship between PAH and elevated VEGF expression is still not well understood. It is hypothesized that in the early stages of PAH, VEGF levels in pulmonary artery endothelial cells (PAECs) are elevated as a protective response. However, as the disease progresses, VEGF continues to promote the growth of PAECs, leading to the formation of plexiform lesions. (Budhiraja et al., 2004).

- **Endothelial to Mesenchymal Transition**

Endothelial-to-mesenchymal transition (EndMT) is a complex biological process characterized by the transformation of endothelial cells (ECs) from their typical endothelial characteristics to mesenchymal phenotypes. During EndMT, ECs start expressing markers associated with mesenchymal cells, including alpha smooth muscle actin and fibroblast-specific protein 1. This transition is often induced by inflammatory responses. In conditions of chronic inflammation, ECs can be persistently activated by various inflammatory stimuli, such as interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), IL-1β, and pathogens. This sustained activation leads to alterations in normal endothelial function and subsequently impairs the endothelial-dependent immune response. This impaired immune response is a characteristic feature of endothelial dysfunction (Cho et al., 2018).

Numerous studies suggest that inflammation-induced EndMT, similar to epithelial-to-mesenchymal transition (EMT), is predominantly regulated by two signaling pathways: the transforming growth factor beta (TGFβ) pathway and the non-TGFβ pathway (Dejana et al., 2017). Among these, TGFβ is the most well-known inducer of EndMT and promotes the upregulation of transcription factors such as SNAIL, SLUG, and zinc finger E-box-binding homeobox 1 (ZEB1). These transcription factors, in turn, contribute to the upregulation of mesenchymal markers including alpha smooth muscle actin (α-SMA), smooth muscle protein 22 alpha (SM22α), calponin, vimentin, type I collagen, fibronectin, fibroblast-specific protein 1 (FSP-1), N-cadherin, matrix metalloproteinase (MMP)-2, and MMP-9 (Perez et al., 2017).

In pulmonary arterial hypertension (PAH), there are additional factors that contribute to endothelial-to-mesenchymal transition (EndMT). Reduced signaling of bone morphogenetic protein receptor 2 (BMPR2) promotes EndMT through the upregulation of High Mobility Group AT-hook 1 (HMGA1) and its target gene SLUG, independent of TGFβ signaling. Furthermore, BMP-7, a protein known for its anti-inflammatory and anti-tumor effects in various diseases, is inhibited by hypoxia-induced EndMT in pulmonary artery endothelial cells (PAECs) both in vivo and in vitro, mediated by the inhibition of the m-TORC1 signaling pathway (Kurakula et al., 2021). Loss of BMPR2 favors EndMT and contributes to a vicious cycle, leading to hyperactivated TGFβ signaling, along with the emergence of myofibroblastic cells (Hiepen et al., 2019). Overall, alterations in TGFβ/BMP signaling are associated with the process of EndMT observed in PAH.

Hypoxia, another defining characteristic of pulmonary arterial hypertension (PAH), triggers EndMT through the influence of hypoxia-inducible transcription factor-1α (HIF-1α) and HIF-2α, both of which are increased in PAH. Endothelial cells in PAH exhibit heightened expression of HIF-2α, resulting in the upregulation of the transcription factor. Furthermore, the effective suppression of hypoxia-induced EndMT has been achieved by knocking down HIF-1α, and partial inhibition of hypoxia-induced EndMT in microvascular endothelial cells (MVECs) has been observed by knocking down its downstream target gene TWIST1 (Zhang et al., 2018). Additionally, microRNAs have also been implicated in EndMT in the context of PAH, alongside transcription factors (Kurakula et al., 2021).

- **Apoptosis**

Various research efforts have been made to unravel the molecular pathways involved in the regulation of pulmonary arterial EC (PAEC) apoptosis. The hypothesis suggests that disrupted responses to vascular endothelial growth factor (VEGF) signaling, combined with hypoxia, initially lead to increased apoptosis in PAECs. This, in turn, gives rise to the emergence of aggressive, apoptosis-resistant, and hyperproliferative ECs that contribute to the formation of intimal lesions (Masri et al., 2007). One possible explanation for the initial rise in PAEC apoptosis is that the loss of signaling through BMPR2 promotes mitochondrial dysfunction, subsequently leading to PAEC apoptosis (Diebold et al., 2015). Notably, White et al. propose an intriguing model in which the pro-apoptotic factor programmed cell death-4 (PDCD4) triggers the cleavage of caspase-3, inducing PAEC apoptosis. Interestingly, their study demonstrates that reducing

PDCD4 levels in vivo, through the overexpression of miRNA-21, prevents the development of PH in SuHx rats (White et al., 2014).

Additionally, there is evidence of elevated levels of Notch1 in the lungs of patients with IPAH and in SuHx rat models. Notch1 plays a role in the pathogenesis of PAH by promoting EC proliferation and inhibiting apoptosis through the downregulation of p21 and the regulation of BCL-2 and survivin expression. Furthermore, the expression of HIF1α promotes Notch signaling in human PAECs. However, in contrast, Miyagawa et al. demonstrated that direct communication between SMCs and ECs activates EC-derived Notch1, leading to epigenetic alterations that regulate Notch1-dependent genes, thereby maintaining endothelial integrity and preventing pulmonary vascular remodeling in a murine model of hypoxia-induced pulmonary hypertension (Miyagawa et al., 2019). Therefore, the role of Notch1 in PAH is complex and controversial, necessitating further research to elucidate the underlying molecular mechanisms (Xu et al., 2022, Kurakula et al., 2021).

B. <u>Accumulation of PA-Smoth Muscle Cell and adventitial fibroblasts</u>

In PAH, the pulmonary arterial microenvironment and the presence of several inherent intrinsic abnormalities and dysregulated signals are known to partly explain the progressive accumulation of resident PA-SMCs and adventitial fibroblasts. As in endothelial cells, DNA damage response pathways are critically implicated in PA-SMC and fibroblast survival in PAH. In both human and experimental PH/PAH the decrease in BRCA1 (breast cancer 1) protein following BMPR2 downregulation is associated with the upregulation of poly(ADP ribose) polymerase 1 (PARP1) in response to the increase in DNA damage insults. The upregulation of PARP1 in PAH PA-SMCs allows them to cope with the environmental stresses by damping DNA damage consequences, adapting their mitochondrial functions into a survival mode (Meloche et al., 2014). In addition, it has been found that tumour necrosis factor-α (TNF-α) inhibits BMPR2 expression and promotes post-translational cleavage via the “a disintegrin and metalloproteases” ADAM10 and ADAM17 in PA-SMCs, favouring BMP-mediated proliferation via alternative activin receptors (Hurst et al., 2017).

In addition, in PA-SMCs, exposure to high glucose levels increases the expression of SMURF1, inhibiting BMP signaling and resembling signaling patterns seen in mutation-negative PAH-derived PA-SMCs. Similarly, depletion of Smad3 in PA-SMCs and pulmonary endothelial cells contributes to increased proliferation and migration. Other potential targets identified include LTB4, IL-6, leptin receptors, CtBP1, TGF-β, PPAR-γ, mTORC1, and FoxO1 pathways. PAH is characterized by dynamic extracellular matrix remodeling, which affects cell behavior, vessel stiffness, and inflammatory cell recruitment. The complex interrelationships between pulmonary arterial cells and perivascular monocytes/macrophages require further investigation for a comprehensive understanding of PAH development (Humbert et al., 2019).

1.6. micro-RNA INVOLVED IN PH

MicroRNAs are small, non-coding endogenous RNA molecules that can regulate gene expression and influence various biological processes. Changes in microRNA expression levels contribute to various cardiovascular disorders, and microRNAs have been shown to play a critical role in PH pathogenesis. In recent years, numerous studies have explored the role of microRNAs in PH, focusing on the expression profiles of microRNAs and their signaling pathways in pulmonary artery smooth muscle cells (PASMCs) or pulmonary artery endothelial cells (PAECs), PH models, and PH patients (Xu et al., 2022).

Mature miRNAs are generated through a multistep process. MiRNA genes are transcribed in the nucleus by RNA polymerase II or III, resulting in primary miRNA transcripts (pri-miRNA) containing hairpin sequences. The RNase III Drosha-DGCR8 complex cleaves pri-miRNAs into precursor miRNAs (pre-miRNAs). Pre-miRNAs are transported to the cytoplasm and converted into double-stranded mature miRNAs by the RNase Dicer with the assistance of the double-stranded RNA-binding protein TRBP. The functional strand of the mature

miRNA is loaded onto Argonaute 2 proteins within the RNA-induced silencing complex. Through complementary binding, miRNAs recognize and bind to the 3'-untranslated regions of their mRNA targets, leading to mRNA destabilization, partial degradation, and inhibition of protein translation. MiRNA pathways are highly conserved and regulate various cellular functions. Over 2,000 human miRNAs have been identified to date, and they are estimated to regulate more than 30% of human genes. The expression patterns of miRNAs are influenced by factors such as developmental stage, age, gender, and environmental conditions, exhibiting spatiotemporal features (Zhou et al., 2015).

In our study we focused on:

- miRNAs involved in pulmonary arterial endothelial disfunction an proliferation: miR21, miR17, miR20a, miR301, miR-126-5p (Chang et al., 2018, Chen et al., 2016, Bertero et al., 2014, Zhou et al., 2015) .
- miRNA involved in accumulation of PA-SMC and adventitial fibroblasts: miR21, miR26a, mi98, miR140, miR-24, miR-103a, miR-92a-1-5p, miR-191-5p (Deng et al., 2016, Kang et al., 2016a, Chen et al., 2018, Kriegel et al., 2012, Wang et al., 2014, Stevens et al., 2016, Watterston et al., 2019, Rothman et al., 2016b).

miRNA-21: MiR-21 is known to be up-regulated in pulmonary arteries from various pulmonary hypertension (PH) models and PH patients. Specifically, human pulmonary artery smooth muscle cells (PASMCs) induced by hypoxia exhibit a significant increase in miR-21 expression, while the expression of its target genes, including programmed cell death protein 4 (PDCD4), Sprouty 2 (SPRY2), and peroxisome proliferator-activated receptor-α (PPARα), decreases (Xu et al., 2022). Furthermore, overexpression of miR-21 in PASMCs promotes cell proliferation and migration, contributing to the development of PH. Conversely, when hypoxia-induced PASMCs are treated with anti-miR-21, cell proliferation and migration are blocked, suggesting that inhibiting miR-21 can prevent and reverse hypoxia-induced PH (Yang et al., 2012). It has also been observed that miR-21 expression is up-regulated by bone morphogenetic protein receptor 2 (BMPR2) signaling under hypoxic conditions. miR-21 directly targets RhoB, suppressing its expression and kinase activity, thereby inhibiting angiogenesis and vasodilation. Moreover, overexpression of miR-21 reduces PDCD4 expression and protects mice from PH in the hypoxia/SU5416 model. Conversely, inhibition of miR-21 expression enhances RhoB expression and kinase activity, exacerbating hypoxia/SU5416-induced PH. Knocking out miR-21 in mice activates the PDCD4/caspase-3 axis in pulmonary arterial endothelial cells (PAECs) and leads to progressive PH. Thus, miR-21 appears to act as a regulatory factor that inhibits the progression of PH in human PAECs (White et al., 2014). To sum up, the apparently contradictory roles of miR-21 in PASMCs and PAECs above are possibly two results from opposite tests obtained in PH models after miR-21 knockdown (Xu et al., 2022).

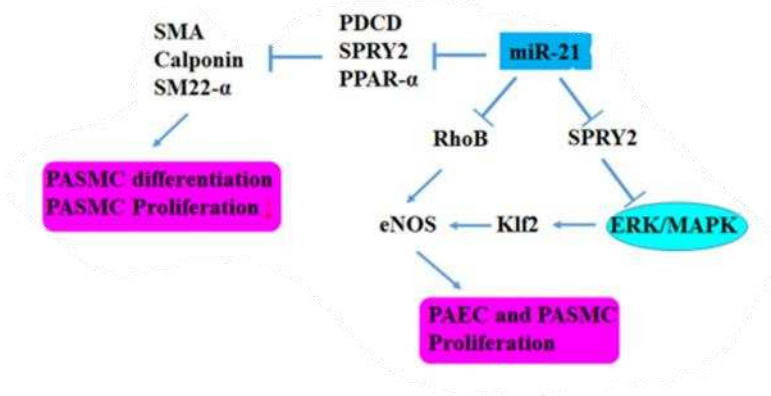

Figure 8. miR-21 pathways in PH. Adapted from Grant et. al (Grant et al., 2013)

miR-17-92 cluster *(miR-17, miR-18a, miR-19a, miR-19b, miR-20a, and miR-92a)*: The miR-17–92 cluster contains six mature miRNAs (miR-17, miR-18a, miR-19a, miR-19b, miR-20a, and miR-92a) that are transcribed as one common primary miRNA until it is processed to mature miRNAs. While the miR-17-92 cluster is known to have oncogenic properties, recent research suggests that it also plays a significant role in lung development and the development of PAH. Studies have reported that miR-17-92 is down-regulated in PASMCs obtained from PAH patients, indicating a potential involvement in the disease (Chen et al., 2015a). Furthermore, the decreased expression of miR-17-92 in PAH patients correlates with reduced levels of α-smooth muscle actin (α-SMA), SM22α, and calponin, suggesting a link between the loss of miR-17-92 expression and a dedifferentiated smooth muscle cell phenotype. Conversely, the overexpression of miR-17-92 restores the differentiated phenotype. Chen et al. also found that miR-17-92 binds to the 3'-untranslated region of PDZ and LIM domain protein 5 (PDLIM5) and inhibits its expression. Additionally, PDLIM5 is crucial for miR-17-92-mediated TGF-β/SMAD signaling and the phenotype of pulmonary arterial smooth muscle cells (Chen et al., 2015a). Additionally, miR-17 was found to be temporarily up-regulated in a mouse model of hypoxia-induced PH. Inhibition of miR-17 was shown to prevent and partially reverse hypoxia-induced PH, likely by inducing the expression of p21, which inhibits the proliferation of PASMCs (Pullamsetti et al., 2012). Finally, miR-17–92 appears to play a role in PAH pathogenesis by two mechanisms: a) in the initial stage of remodelling of blood vessels, up-regulation of miR-17–92 promotes PASMC proliferation, and b) in the later progression stage, reduced miR-17–92 increases PDLIM5 to maintain PASMCs in a dedifferentiated state (Zhou et al., 2015).

miRNA miR-17–92 cluster is also involved in regulation of Pulmonary Artery Endothelial Homeostasis. The expression of miR-17, miR-18, miR-19, and miR-20 is increased during the differentiation of murine embryonic stem cells or the induction of pluripotent stem cells, while miR-92a and the primary miR-17-92 transcript are down-regulated. This suggests that miR-17-92 may play a role in angiogenesis. In endothelial cells, vascular endothelial growth factor (VEGF) induces the expression of miR-17-92, and this miRNA cluster is necessary for endothelial cell proliferation and angiogenesis (Tréguer et al., 2012). A subset of pulmonary arterial hypertension (PAH) patients carries mutations in BMPR2, and the down-regulation of BMPR2 is associated with PAH. Studies by Brock et al. have revealed that two members of the miR-17-92 cluster, miR-17-5p, and miR-20a, directly target BMPR2 in PAECs. They have also demonstrated that interleukin-6 (IL-6), a prohypertensive cytokine, up-regulates the expression of miR-17-92 through the activation of STAT3 (signal transducer and activator of transcription 3). These findings suggest that the inhibition of BMPR2 leads to the up-regulation of miR-17-92, which in turn promotes proliferation and inhibits apoptosis in PAECs, ultimately contributing to the development of PH (Zhou et al., 2015).

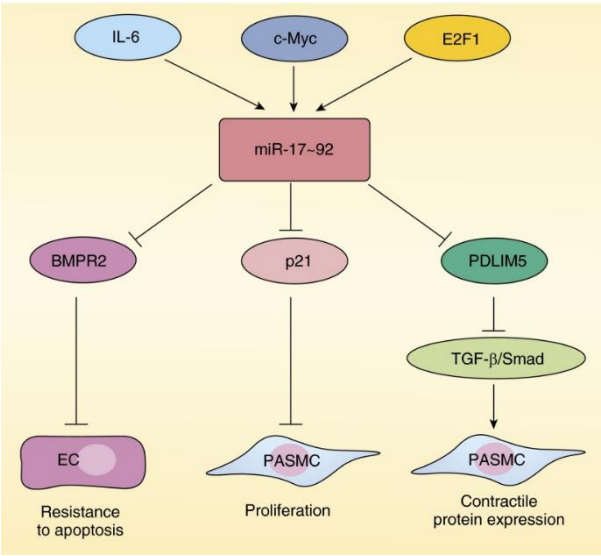

Figure 9. miRNA 17-92 cluster in PH (Zhou et al., 2015)

miR-130/301 family: Bertero et al. initially described the significance of the miR130/301 family and its direct target PPAR in controlling intercellular communication and vasomotor tone in pulmonary vascular cells. Their research demonstrated that this miRNA family also modulates various vasomotor effectors across the vasculature, facilitating communication between different cell types involved in PH. Furthermore, they identified the intricate control of EDN1, where upregulation of miR-130/301 leads to STAT3 activation and vasoconstriction in PASMCs. These findings establish the miR-130/301 family as a master regulator of multiple pathogenic pathways beyond cellular proliferation, specifically relevant to the pulmonary vasculature and PH. Despite the presence of a predicted target sequence in the EDN1 transcript, the apparent paradoxical response of increased EDN1 expression due to miR130/301 is not uncommon in miRNA biology. In certain contexts, miRNAs can induce the expression of their gene targets. Alternatively, indirect regulatory pathways such as the PPAR-AP1 pathway can have a more substantial impact on EDN1 expression compared to direct engagement of the EDN1 transcript by miR-130/301. These complex regulatory features may function as feedback mechanisms, finely tuning the expression of highly interconnected miRNA target genes. However, predicting the specific modulation direction of each target can be challenging, particularly when considering the entire network of miRNA targets involved. (Bertero et al., 2014).

miR-126: Vascular remodeling PAH was believed to primarily affect the distal pulmonary arteries, indicating a localized impact on the lungs and surrounding vasculature. However, emerging evidence suggests that PAH can also lead to peripheral vascular damage, indicating a more systemic involvement in this disease. Potus et al. demonstrated a reduction in capillary density in the peripheral skeletal muscles of PAH patients, indicating a defect in peripheral angiogenesis in PAH. They also identified the molecular pathway involved and highlighted the significant role of microRNAs in this pathological process. Specifically, they found a decrease in the expression of miR-126 in the skeletal muscles of PAH patients. MiR-126 is known to regulate the VEGF/ERK (vascular endothelial growth factor/extracellular-signal-regulated kinase) pathway through its target gene SPRED-1 (Sprouty-related, EVH1 domain-containing protein 1). This finding suggests that miR-126 plays a critical role in regulating angiogenesis in PAH. Furthermore, it was demonstrated that the decreased expression of miR-126 and the reduction in capillary density strongly correlate with the reduced exercise tolerance observed in PAH patients. This suggests a connection between systemic impairment in angiogenesis and the major symptom experienced by PAH patients (Potus et al., 2014).

miR-26a: Accumulated evidence confirmed that microRNA-26 family play critical roles in cardiovascular disease; however, their function in PAH remains largely unknown. Watterston et al. conducted a study suggesting that miR26a plays a crucial role in regulating vascular stability and differentiation (Watterston et al., 2019). They proposed that the normal function of miR26a is to reduce the activation of the Smad1 protein within the endothelium, which indirectly inhibits the differentiation of vascular smooth muscle cells (vSMCs) during early development. Loss of miR26a was found to result in hemorrhage (vascular instability) in vivo, indicating altered vascular differentiation. The decrease in miR26a levels led to increased levels of smad1 mRNA and phospho-Smad1 (pSmad1). The researchers demonstrated that overexpression of smad1, which enhances BMP signaling, also caused hemorrhage. However, when the levels of Smad1 were normalized through a double knockdown of miR26a and smad1, the hemorrhage was rescued, suggesting a direct relationship between miR26a, smad1, and vascular stability. Through the use of an in vivo BMP genetic reporter and pSmad1 staining, the study revealed that the effect of miR26a on smooth muscle differentiation is non-autonomous. BMP signaling was found to be active in embryonic endothelial cells but not in smooth muscle cells. Nonetheless, the increased BMP signaling resulting from the loss of miR26a led to an increase in the number of smooth muscle cells expressing acta2 and promoted a differentiated smooth muscle morphology. Similarly, forced expression of smad1 in endothelial cells resulted in an increase in the number and coverage of smooth muscle cells. Moreover, the smooth muscle phenotypes caused by BMP pathway inhibition could be rescued by the loss of miR26a. Overall, these findings suggest

that miR26a modulates BMP signaling in endothelial cells and indirectly promotes a differentiated smooth muscle phenotype. The study highlights the importance of crosstalk between BMP-responsive endothelium and smooth muscle cells in smooth muscle differentiation (Watterston et al., 2019).

miR-98: Endothelin-1 (ET-1) plays a crucial role in endothelial dysfunction and contributes to the development of PH. In a recent paper Kang et. have speculated that peroxisome proliferator–activated receptor γ (PPARγ) activates specific microRNAs that inhibit ET-1 expression and the proliferation of PAECs. To elucidate the molecular mechanisms underlying the regulation of ET-1 expression by PPARγ, both in vitro and in vivo, they examined PAECs isolated from patients with pulmonary arterial hypertension and observed a reduction in the expression of miR-98, along with increased ET-1 protein levels and PAEC proliferation. In vitro experiments involving hypoxia exposure showed similar results, with decreased miR-98 expression and elevated ET-1 levels and PAEC proliferation. In vivo studies using mice subjected to hypoxia confirmed the downregulation of miR-98 expression and the upregulation of ET-1 and proliferating cell nuclear antigen (PCNA) levels in lung tissue, particularly when combined with treatment using the vascular endothelial growth factor receptor antagonist Sugen5416. A comparative analyses was conducdect on lung tissue from. This comparison mice with endothelial-targeted PPARγ knockout or overexpression revealed decreased miR-98 levels and increased expression of ET-1 and PCNA in lung tissue from mice with endothelial-targeted PPARγ knockout, while increased miR-98 levels and reduced expression of ET-1 and PCNA were observed in mice with endothelial-targeted PPARγ overexpression. In vitro experiments manipulating PPARγ function in PAECs confirmed that alterations in PPARγ were sufficient to regulate the expression of miR-98, ET-1, and PCNA. Furthermore, we investigated the potential therapeutic implications of PPARγ activation using rosiglitazone regimens. The activation of PPARγ attenuated hypoxia-induced PH in vivo and reduced the proliferation of human PAECs in vitro. Notably, PPARγ activation also restored miR-98 levels. In conclusion this study provided evidence that PPARγ regulates the expression of miR-98 to modulate ET-1 expression and PAEC proliferation (Kang et al., 2016b)

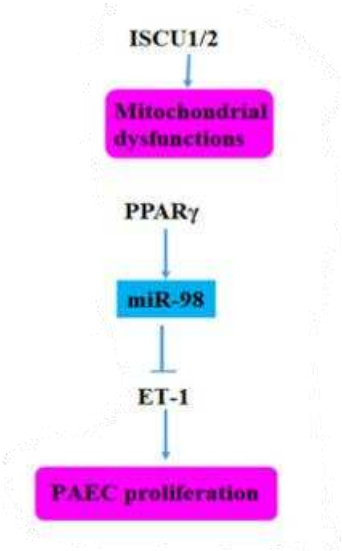

Figure 10. miR-98 pathways in PH. Adapted from Grant et. al (Grant et al., 2013)

miR-24: It was shown that TGFβs and BMPs are able to inhibit vSMC proliferation and migration, and stimulate the expression of contractile vSMC markers, such as smooth-muscle α-actin (SMA), calponin-1 (CNN), and SM22α (SM22) (Misiakos et al., 2001, Lagna et al., 2007). Mutations in genes encoding receptors of TGFβs and BMPs have been associated with vascular disorders such as idiopathic IPAH and hereditary hemorrhagic telangiectasia. In contrast to TGFβs and BMPs, platelet-derived growth factors (PDGFs) are known to induce a transition from a contractile to a synthetic phenotype in vascular smooth muscle cells (vSMCs), characterized by reduced expression of vSMC marker genes and increased proliferation and

migration. In a study by Chan et al., it was demonstrated that PDGF-BB stimulation leads to the induction of miR-24 in vSMCs. MiR-24, in turn, targets the mRNA of Tribbles-like protein-3 (Trb3) and downregulates its expression. Trb3 has been previously identified as a protein that interacts with the type-II BMP receptor (BMPRII), a domain frequently mutated in familial IPAH patients. Trb3 promotes the degradation of Smad ubiquitin-regulatory factor-1 (Smurf1), a negative regulator of BMP and TGFβ Smad-dependent signaling. Consistent with previous findings, the study revealed that PDGF-mediated downregulation of Trb3 through miR-24 in vSMCs resulted in decreased levels of Smad proteins and the expression of contractile genes in vSMCs. Inhibition of miR-24 function prevented vSMCs from transitioning to a synthetic phenotype upon PDGF-BB treatment. Thus, the authors propose that miR-24 plays a crucial role in regulating the crosstalk between the pro-contractile TGFβ/BMP signaling and the pro-synthetic PDGF signaling pathways. (Chan et al., 2010).

miR-140: Expression of miR-140 was found to be significantly decreased in lung tissues of patients with congenital pulmonary hypertension (PH) and pulmonary arterial hypertension (PAH) compared to those without PH (Zhang and Xu, 2016). In patients with congenital PH, higher pulmonary artery pressure and lower miR-140 expression were observed. Correlation analysis revealed a negative correlation between miR-140 expression and pulmonary artery pressure, as well as the expression of proteins related to the Wnt signaling pathway (Wnt-1 and β-catenin). Specifically, under hypoxic conditions, miR-140 expression was downregulated in human pulmonary artery smooth muscle cells (hPASMCs), while Wnt-1 expression was upregulated. Increasing miR-140 expression led to an elevation in the expression of smooth muscle-specific proteins, including α-smooth muscle actin (α-SMA), SM22, and calponin. Conversely, inhibition of miR-140 resulted in increased cell proliferation, enhanced migration, and reduced expression of proteins associated with the contractile phenotype, indicating the importance of miR-140 in maintaining the phenotype of PASMCs. Further investigation demonstrated that miR-140 directly targets the 3'-untranslated region (3'-UTR) of Wnt-1, suppressing phenotypic switching of PASMCs. Apart from the Wnt signaling pathway, miR-140 also targets Dnmt1, reduces SOD2 expression, and inhibits phenotypic switching of hPASMCs (Zhang and Xu, 2016). Furthermore, a study in rats showed that delivering miR-140 to the lungs using liposomes decreased hemodynamic indicators and pulmonary vascular remodeling by suppressing Smurf11 expression (Rothman et al., 2016a). These findings suggest that delivering miR-140 to the lungs through liposomes may hold potential as a therapeutic approach for treating PH (Zhang et al., 2021)

miR-191: In a recent study it was found that miR-191 is upregulated in the circulation of individuals with PH (Wei et al., 2013). In addition, Baglio et al. demonstrated that miR-191 is one of the most abundant miRNAs in Adipose-derived mesenchymal stem cells (ASCs) exosomes (Baglio et al., 2015). Zang et al. investigated coculture of ASCs and monocrotaline pyrrole (MCTP)-treated human PAECs showing that ASCs-exosomes improved proliferation of both control HPAECs and MCTP-treated HPAECs. Moreover, by transfecting ASCs with antagomir the group observed that low exosomal miR-191 expression inhibited HPAECs proliferation whereas the agomir improved. Similar results were observed in vivo using a monocrotaline (MCT)-induced PAH rat model following ASCs transplantation. The transplantation of ASCs attenuated MCT-induced PAH event thought less than the antagomir treated group. These results indicate that ASCs-Exos partly influence HPAEC proliferation through miR-191, potentially impacting vasculature remodelling. Furthermore, it is well-established that BMPR2 signalling plays a critical role in the pathogenesis of PAH. Therefore, Zang et al. explored the involvement of BMPR2 in miR-191 mediated enhancement of growth of HPAECs. Consistently with the previous hypothesis, miR-191 significantly decreased BMPR2 level. In the presence of the miR-191 antagomir, BMPR2 level increased, with corresponding improvement in symptoms of PAH. These findings suggest that BMPR2 is implicated in the miR-191-regulated survival of HPAECs (Zhang et al., 2019).

- **miR-103/107**

Previous studies have shown that miR-103/107 targets HIF-1β. Deng et al reported novel findings in a rat model indicating that miR-103/107 acts as a regulator of pulmonary artery smooth muscle cell (PASMC) proliferation in hypoxia-induced pulmonary arterial hypertension (PAH) by targeting the HIF-1β signalling pathway. They observed significant downregulation of miR-103/107 in remodelled intrapulmonary vessels in rats with hypoxia-induced PAH and in PASMCs exposed to hypoxia. Conversely, the expression levels of HIF-1α and HIF-1β were upregulated. Hypoxia exposure induced substantial PASMC proliferation, which was inhibited by overexpression of miR-103/107 and enhanced by miR-103/107 inhibitors. Gain-of-function and loss-of-function experiments showed an inverse correlation between miR-103/107 expression and HIF-1β levels. However, no significant changes in HIF-1α expression were observed upon treatment with miR-103/107 mimics. The mechanisms by which HIF-1β regulates cell proliferation in this context are only partially understood. One study showed that HIF-1β tightly controls the phosphorylation of AKT, which mediates cell proliferation and survival in cancer cells. Whether a similar mechanism exists in HIF-1β-mediated vascular remodelling in hypoxic pulmonary hypertension requires further investigation. In conclusion, loss of suppression of HIF-1β by miR-103/107 may contribute to excessive PASMC proliferation and vascular remodelling in hypoxic pulmonary hypertension (Deng et al., 2016).

2. THE ROLE OF miRNA IN PRE-CAPILLARY PULMONARY HYPERTENSION: A COMPARISON BETWEEN GROUP 1 AND GROUP 3 PH.

2.1. Rational

Lung transplantation serves as the sole therapeutic approach targeting the enhancement of life expectancy and quality of life for individuals afflicted with advanced parenchymal or vascular lung diseases. The current study aligns with this objective, recognizing the substantial value of lung tissue obtained from explanted lungs for fundamental research purposes. By investigating lung tissue affected by both group 3 and group 1 pulmonary hypertension, it becomes possible to uncover shared pathogenic mechanisms between these two groups and gain a more profound understanding of the underlying mechanisms driving group 3 pulmonary hypertension. Furthermore, delving into the cells responsible for the expression of dysregulated miRNAs offers the potential to identify novel target mechanisms for the clinical management of group 3 pulmonary hypertension, an area currently limited in therapeutic options. Through a comparative analysis of the quantitative expression patterns of each miRNA among distinct groups, it becomes feasible to determine the extent of involvement of specific pathogenic pathways in the various forms of pulmonary hypertension. Consequently, this approach provides guidance for future research, directing focus towards the most relevant mechanisms of interest. In table 6. is shown the panel of miRNA chosen for our study.

Table 6. miRNA panel	
miR-130/301 family	Increase in lung of mice suffering from PH induced by SU5416 Upregulation of miR-130/301 leads to STAT3 activation and vasoconstriction in PASMCs (Bertero et al., 2014)
miR-26a	Promote VSMC differentiation (Watterston et al., 2019)
miR-17-92 cluster **(miR-17, miR-18a, miR-19a, miR-19b, miR-20a and miR-92a)**	miR-17/92 is regulated by IL-6/signal transducer and activator of transcription 3 (STAT3) signaling, and its overexpression blocks BMPR2 signaling, a deficient pathway involved in PH. miR-17/92 has been identified as an enhancer of hypoxic PH by targeting PDZ and LIM domain 5 (PDLIM5) in vivo (Chen et al., 2015a). miR-17/92 overexpression inhibited PDLIM5, consequently enhancing transforming growth factor β (TGF-β)/Smad2/3 activity in PASMCs in vitro.
miR-21	Promote VSMC differentiation Human PASMCs induced by hypoxia exhibit a significant increase in miR-21 expression, while the expression of its target genes, including programmed cell death protein 4 (PDCD4), Sprouty 2 (SPRY2), and peroxisome proliferator-activated receptor-α (PPARα), decreases (Xu et al., 2022).
miR-98	miR-98 was identified to be decreased in the lung tissue of a hypoxic pulmonary hypertension (HPH) rat model and pulmonary artery (PA) smooth muscle cells (PASMCs), which was induced by hypoxia (Kang et al., 2016b).
miR-126	miR-126 is endothelium specific and a critical regulator of angiogenesis: by repressing SPRED-1 and PIK3R2 (two negative regulators of the VEGF signaling pathway) activates both MAPK and PIK3 signaling and promotes angiogenesis (Potus et al., 2014)
miR-140	Expression of miR-140 was found to be significantly decreased in lung tissues of patients with congenital pulmonary hypertension (PH) and pulmonary arterial hypertension (PAH) compared to those without PH (Zhang and Xu, 2016). In rats delivering miR-140 to the lungs using liposomes decreased hemodynamic indicators and pulmonary vascular remodeling by suppressing Smurf11 expression (Rothman et al., 2016a).

miR-24	It was shown that TGFβs and BMPs are able to inhibit vSMC proliferation and migration, and stimulate the expression of contractile vSMC markers, such as smooth-muscle α-actin (SMA), calponin-1 (CNN), and SM22α (SM22) (Misiakos et al., 2001, Lagna et al., 2007) Plays a crucial role in regulating the crosstalk between the pro-contractile TGFβ/BMP signaling and the pro-synthetic PDGF signaling pathways. (Chan et al., 2010).
miR-191	miR-191 resulted upregulated in the circulation of individuals with PH (Wei et al., 2013). miR-191 significantly decreased BMPR2 level. miR-191 antagomir, BMPR2 level increased, with corresponding improvement in symptoms of PAH. (Zhang et al., 2019)
miR-103	Loss of suppression of HIF-1β by miR-103/107 may contribute to excessive PASMC proliferation and vascular remodeling in hypoxic pulmonary hypertension (Deng et al., 2016)

2.2. Study Aim

Primary aim: To determine if specific pathogenic pathways can be identified based on the expression of miRNAs underlying the development of pulmonary arterial hypertension shared between idiopathic pulmonary arterial hypertension and pulmonary arterial hypertension in patients with idiopathic pulmonary fibrosis and chronic obstructive pulmonary disease.

2.3. Study design

We propose a multicentre retrospective study involving the analysis of biological tissues obtained from lung explants of patients diagnosed with idiopathic pulmonary arterial hypertension, and pulmonary hypertension associated to idiopathic pulmonary fibrosis, and chronic obstructive pulmonary disease. These patients underwent right heart catheterization and pulmonary function testing as part of their pre-transplant screening process, thus allowing a correct definition of PH phenotype. The study aims to compare the expression profiles of a selected panel of miRNAs, already described in the literature as involved in the pathogenesis of group 1in the two group of patients (Group 1 and Group 3) in order to unravel differences between these two PH phenotypes and provide preliminary data on pathways that are involved in their pathogenesis. The panel of micro-RNAs includes: : miR-140-5p, miR-24-1-5p, miR-103a-2-5p, miR-126-5p, miR-21-5p, miR-26a-5p, miR-20a-5p, miR-98-5p, miR-301a-5p, miR-17-5p, miR-92a-1-5p, miR-191-5p. These miRNAs are known to play crucial roles in regulating: hypoxic response, endothelial-to-mesenchymal transition, inflammatory activation, and the TGF-beta pathway, which are the primary pathogenic mechanisms/pathways underlying the he development of group 1 pulmonary hypertension. To quantitatively assess the expression of the selected miRNAs in each tissue sample, a customized panel specifically designed for this purpose has been employed. Specifically, small samples of paraffin-embedded explanted lungs, consisting of approximately 20 ultra-thin sections obtained from a tissue block, have been utilized. Following the collection of tissue samples, homogenization and subsequent RNA isolation has been performed to investigate the expression differences of the miRNAs under study, with the aim of identifying the dysregulated miRNAs. The study protocol was approved by the local Ethics Committees of Policlinico Sant'Orsola and Policlinico San Matteo.

2.4. Study population

Tissue samples from lung explants of patients who underwent lung transplantation at IRCCS University Hospital of Bologna and at IRCCS San Matteo Hospital of Pavia from the 1st of January 2010 to the 31th of December 2021 were considered. Among 149 transplant performed in the two transplant centre during the period of interest, we included in this study 27 patients who were submitted to single or double lung transplant with the following indications: idiopathic pulmonary arterial hypertension and idiopathic

pulmonary fibrosis, or chronic obstructive pulmonary disease, both complicated by pulmonary arterial hypertension (defined as PAPm ≥ 25 mmHg) All patients underwent right heart catheterization during pre-transplant screening and had explanted lung samples available for the study.

Thus the study population consist in three study groups:

- Group 1 PAH: Patients who underwent lung transplantation for idiopathic pulmonary arterial hypertension, defined as an increase in mean pulmonary arterial pressure (mPAP ≥ 25 mmHg) and pulmonary vascular resistance (PVR > 3 WU) with pulmonary capillary wedge pressure (PCP) < 15 mmHg on pre-transplant right heart catheterization.
- Group 3 IPF-PH: Patients who underwent lung transplantation for idiopathic pulmonary fibrosis complicated by group 3 pulmonary arterial hypertension, defined as an increase in mPAP (≥ 25 mmHg) and PVR (> 3 WU) with PCP < 15 mmHg on pre-transplant right heart catheterization.
- Group 3 COPD-PH: Patients who underwent lung transplantation for chronic obstructive pulmonary disease complicated by group 3 pulmonary hypertension, defined as an increase in mPAP (≥ 25 mmHg) and PVR (> 3 WU) with PCP < 15 mmHg on pre-transplant right heart catheterization.

The PH cut-off of mPAP was chosen according to the past 2015 ESC/ERS guidelines, in order to include only patients with overt PH and increase the possibility to detect micro-RNAs dysregulation magnifying the molecular model.

The samples of paraffin-embedded explanted lungs underwent an initial re-evaluation using conventional histological methods by a single pathologist to confirm the underlying disease and to identify the most suitable tissue portions for the study, specifically, areas containing at least 3 pulmonary arterioles.

2.4.1. Inclusion criteria

- Patients who underwent lung transplantation at the AOU IRCCS Policlinico Sant'Orsola in Bologna and the IRCCS Fondazione Policlinico San Matteo in Pavia (satellite center) from January 1, 2010, to December 31, 2021.

- Diagnosis of idiopathic pulmonary arterial hypertension, idiopathic pulmonary fibrosis, or chronic obstructive pulmonary disease.

- Patients who underwent at least one pre-transplant right heart catheterization in clinically stable conditions, with findings of mPAP ≥ 25 mmHg, PVR > 3 WU, and PCP < 15 mmHg.

- Suitability of lung explant tissue available in the archives of the participating pathology departments: presence of at least 3 pulmonary arterioles in each specimen.

- Acquisition of informed consent.

2.4.2. Exclusion criteria

- Unsuitability of lung tissue for molecular biology investigations (due to preservation issues or deterioration of the necessary arteriolar structures).

- Patients with active neoplastic diseases.

2.5. Data collection

For each included lung transplanted patient who meet the inclusion and exclusion criteria, the following factors had been considered:

- Anthropometric data: age at the time of transplantation, gender, BMI.

- Clinical diagnosis and severity compatible with the indication for lung transplantation.
- Hemodynamic data obtained through right heart catheterization.
- Functional respiratory data.

Based on these data, eligible patients for the study have been identified, and their tissue samples underwent miRNA assay investigations.

2.6. MiRNA assay

We conducted an analysis of thin slices of explanted lungs, which were 10 microns thick, using the miRNeasy FFPE kit. The kit is specifically designed for extracting total RNA from formalin-fixed, paraffin-embedded (FFPE) tissue sections. The RNA obtained from FFPE samples is often fragmented and chemically modified due to the fixation and embedding process. The RNeasy FFPE kit aims to reverse formaldehyde modification without further degrading the RNA.

RNA concentrations and the purity was verified by use of A260/A280 ratios (range 1.7–1.9).

The miRCURY LNA miRNA PCR Assays, in combination with the miRCURY LNA miRNA PCR System, were used to quantify miRNAs with high sensitivity and specificity. These assays utilize miRNA-specific PCR primers, both forward and reverse, which are optimized using LNA technology.Complementary DNA (cDNA) was synthesized with miRCURY LNA RT Kit (Qiagen) at 42 ◦C for 60 min and 95 ◦C for 5 min. Real-time PCR analysis was performed to evaluate miRNAs' expression levels using miRCURY LNA miRNA PCR-specific Detection Probe and miRCURY LNA SYBR Green PCR Kit (Qiagen) with a LightCycler 480 (Roche, Switzerland), according to the manufacturer's recommendations. The so prepared samples have been finally analyzed by the LightCycler® 480 Instrument, a rapid, plate-based, thermal block cycler with integrated real-time. The instrument provides well-to-well temperature homogeneity and inter-well, inter-cycle reproducibility. The arrangement of optical components in the LightCycler® 480 Instrument ensures uniform collection of signals across the plate and makes analysis independent of the sample position on the plate.

The LightCycler® 480 System setup provide a solution for fast and precise qualitative or quantitative detection of nucleic acids. The pre-installed LightCycler® 480 Software provides an intuitive, easy-to-navigate user interface for real-time PCR programming, data capture, and data analysis.

Thermal cycling conditions consisted of initial denaturation at 95 ◦C for 10 min, followed by 45 cycles of 95 ◦C for 10 s followed by 60 ◦C for 1 min. The threshold cycle (Ct) was defined as the fraction cycle number at which fluorescence exceeded the given threshold. Expression levels of the small nuclear RNA RNU6 were used as the normalization control.

Each experimental condition was performed in triplicate. Relative quantifications were calculated with the comparative Ct method.

2.7. Statistical analysis

2.7.1. Variables

Anthropometric variables analysed: age, sex, weight, height.

Hemodynamic variables: mean pulmonary arterial pressure (PAPm), pulmonary capillary pressure (PCP), pulmonary vascular resistance (PVR), cardiac index (CI).

These data were collected from the latest right heart catheterization performed prior to transplantation.

These variables are essential for establishing a diagnosis of pulmonary arterial hypertension and determining its severity.

Respiratory functional variables: forced expiratory volume in one second (FEV1), forced vital capacity (FVC), FEV1/FVC ratio, diffusing capacity of the lungs for carbon monoxide (DLCO).

These data are collected from the latest respiratory function tests conducted prior to transplantation.

These variables are crucial for diagnosing pulmonary fibrosis and chronic obstructive pulmonary disease and determining their severity.

In particular, DLCO value can be evaluated in all groups as an indicator of respiratory impairment.

2.7.2. Confounding factors

Heterogeneity in age, sex, weight, height variables could be a confounding factor in the analysis of miRNA expression, particularly sex and age. The population analysis will be able to define the possible heterogeneity of our sample.

The degradation of miRNAs preserved in formalin could pose a confounding risk factor in the evaluation of quantitative assays. We made the decision to gather data from patients who underwent lung transplantation after 2010 in order to prevent this preservation bias.

2.7.3. Statistics methods

The statistical analysis was conducted using Stata version 17.1 (Stata Corporation, Texas, United States). Continuous variables were presented as means and standard deviations (SD) or medians and ranges. To compare intragroup differences between diseases (specifically, pulmonary hypertension vs fibrosis), we employed the Wilcoxon rank-sum test. This non-parametric test enables us to assess whether two independent samples (unmatched data) are derived from populations with the same distribution.

The relative quantification of miRNAs will be calculated using the comparative ΔCt method with the use of a reference. To predict the target genes and pathways associated with the studied miRNAs, the DIANA miRPath v.2.0 database will be utilized. After processing, the program provides an overview of the pathways regulated by the examined miRNAs and calculates the associated statistical significance value.

3. RESULTS

3.1. Patients characteristics

The following tables display the descriptive statistics for key variables in the study, divided into four panels: Table 7 represents the total sample of 27 participants, Table 8 corresponds to the sample of patients with IPAH, Table 9 includes the sample of patients with IPF, and Table 10 covers the sample of patients with COPD.

In table 7, the average age of the participants is 49.74 years, with a median of 50 years and a standard deviation (SD) of 9.53 years. The binary variable representing gender (0 for female, 1 for male) indicates a higher proportion of males (mean = 0.70) with a balanced distribution (SD = 0.47). PAPm ranges from 25 mmHg to 76 mmHg, with a mean of 42.04 mmHg and a median of 36 mmHg, displaying substantial variability (SD = 14.73 mmHg). The mean value for RAP is 7.00 mmHg, with a standard deviation of 5.09, while PCP has a mean of 9.16 mmHg, with a standard deviation of 2.75. The mean value of PVR is 6.96 WU, with a standard deviation of 3.63. CI has a mean of 2.83 $Lminm^2$, with a relatively low standard deviation of 0.68. The mean value of FEV1L is 1.77 L, with a standard deviation of 0.85. The theoretical FEV1 (FEV1%) exhibits a mean value of 57.08%, with a wide standard deviation of 25.14. FVCL and FVC% have mean values of 2.58L and 66.62%, respectively. FEV1/FVC, shows a mean value of 71.09%, with a standard

deviation 20.80. The mean DLCO% is 38.16, with a significant standard deviation of 24.56. Finally, the mean body mass index (BMI) is 23.45 (range 19.41–27.34).

Table 7. Descriptive statistics - Full Sample								
	Obs.	Mean	Median	SD	p25	p75	Min	Max
Age	27	49.74	50.00	9.53	46.00	59.00	21.00	63.00
Sex	27	0.70	1.00	0.47	0.00	1.00	0.00	1.00
Weight (Kg)	27	71.14	73.00	13.07	60.00	78.00	52.00	105.00
Height (cm)	27	169.33	170.00	7.57	164.00	173.00	153.00	186.00
PAPm (mmHg)	27	42.04	36.00	14.73	30.00	54.00	25.00	76.00
RAP (mmHg)	22	7.00	6.50	5.09	4.00	8.00	1.00	19.00
PCP (mmHg)	25	9.16	10.00	2.75	8.00	11.00	1.00	13.00
PVR (WU)	23	6.96	6.90	3.63	3.86	9.80	2.30	15.60
CI (Lminm^2)	27	2.83	2.70	0.68	2.30	3.20	1.57	4.49
FEV1 (L)	25	1.77	1.68	0.85	0.98	2.46	0.50	3.47
FEV1 (%t)	26	57.08	59.50	25.14	36.00	78.00	18.00	98.00
FVC (L)	25	2.58	2.33	1.15	1.65	3.28	1.02	4.95
FVC (%t)	26	66.62	64.00	24.45	44.00	84.00	32.00	112.00
FEV1FVC (%)	26	71.09	74.50	20.80	63.00	86.00	25.00	97.00
DLCO (%t)	25	38.16	30.00	24.56	16.00	61.00	9.00	89.00
BMI (kg/m^2)	27	24.78	25.18	4.07	21.48	27.34	18.69	33.14

Regarding specific variables for each group:

Table 8. Descriptive statistics – GROUP1 IPAH								
	Obs.	Mean	Median	SD	p25	p75	Min	Max
Age	10	47.90	47.00	8.02	45.00	52.00	32.00	60.00
Sex	10	0.70	1.00	0.48	0.00	1.00	0.00	1.00
Weight (Kg)	10	68.07	68.85	9.06	64.00	76.00	52.00	79.00
Height (cm)	10	170.40	169.00	7.50	164.00	175.00	162.00	183.00
PAPm (mmHg)	10	56.60	54.50	12.31	50.00	62.00	37.00	76.00
RAP (mmHg)	10	9.60	8.00	5.06	7.00	11.00	4.00	19.00
PCP (mmHg)	10	10.10	10.50	1.85	8.00	12.00	7.00	12.00
PVR (WU)	10	9.93	10.00	3.27	7.60	12.10	4.70	15.60
CI (Lminm^2)	10	2.74	2.60	0.61	2.30	3.00	2.10	4.10
FEV1 (L)	9	2.62	2.50	0.60	2.46	2.99	1.58	3.47
FEV1 (%t)	10	80.00	78.50	8.76	75.00	85.00	68.00	98.00
FVC (L)	9	3.71	3.65	0.92	3.12	4.60	2.16	4.95
FVC (%t)	10	91.70	92.00	12.95	79.00	100.00	76.00	112.00
FEV1FVC (%)	10	71.84	73.00	6.53	67.00	75.00	60.40	80.00
DLCO (%t)	9	64.11	69.00	17.74	61.00	73.00	28.00	89.00
BMI (kg/m^2)	10	23.45	23.67	2.94	20.82	26.49	19.41	27.34

Group 1 - IPAH: Table 8 display the descriptive statistics for the Group IPAH patients. The mean PAPm is 56.60 mmHg (median: 54.50 mmHg; SD: 12.31), ranging from 37 mmHg to 76 mmHg. The mean RAP is 9.60 mmHg (median: 8 mmHg; SD: 5.06), with a range of 4 mmHg to 19 mmHg. PCP has a mean of 10.10 mmHg (median: 10.50 mmHg; SD: 1.85), ranging from 7.00 mmHg to 12.00 mmHg. PVR has a mean of 9.93 WU (median: 10.00; SD: 3.27), ranging from 4.70 to 15.60. CI has a mean of 2.74 $Lminm^2$ (median: 2.60; SD: 0.61), ranging from 2.10 to 4.10. FEV1% mean is 80% (median: 78.50; SD: 8.76), ranging from 68% to 98%. FVC% has a mean of 91.70 (median: 92; SD: 12.95), ranging from 76% to 112%. FEV1/FVC has a mean of 71.84% (median: 73; SD: 6.53), ranging from 60.40% to 80%. DLCO% has a mean of 64.11% (median: 69; SD: 17.74), ranging from 28% to 89%. Lastly, the mean BMI is 23.45 (median: 23.67; SD: 2.94), ranging from 19.41 to 27.34.

Table 9. Descriptive statistics – GROUP 3 IPF								
	Obs.	Mean	Median	SD	p25	p75	Min	Max
Age	12	49.42	51.50	11.80	45.50	58.50	21.00	63.00
Sex	12	0.58	1.00	0.51	0.00	1.00	0.00	1.00
Weight (Kg)	12	75.50	75.50	14.28	67.50	81.50	54.00	105.00
Height (cm)	12	167.25	167.50	7.10	162.50	171.50	153.00	178.00
PAPm (mmHg)	12	34.58	35.50	8.62	28.50	36.00	25.00	57.00
RAP (mmHg)	8	5.13	3.50	4.97	1.00	8.00	1.00	15.00
PCP (mmHg)	10	8.10	8.50	3.38	7.00	10.00	1.00	13.00
PVR (WU)	9	4.67	3.64	2.13	3.30	7.00	2.30	7.80
CI ($Lminm^2$)	12	2.97	2.90	0.83	2.50	3.54	1.57	4.49
FEV1 (L)	11	1.53	1.50	0.49	0.98	1.95	0.95	2.44
FEV1 (%t)	11	52.00	47.00	18.68	37.00	61.00	34.00	97.00
FVC (L)	11	1.78	1.73	0.59	1.28	2.04	1.02	3.05
FVC (%t)	11	49.36	44.00	15.82	40.00	55.00	32.00	84.00
FEV1FVC (%)	11	87.00	86.00	8.33	80.00	94.00	72.00	97.00
DLCO (%t)	11	26.18	23.00	14.14	16.00	40.00	9.00	54.00
BMI (kg/m^2)	12	26.92	26.53	4.33	25.29	29.91	18.69	33.14

Group 3 - IPF: Table 9 display the descriptive statistics for the Group IPF patients. The mean PAPm is 34.58 mmHg (median: 35.50 mmHg; SD: 8.62), ranging from 25.00 mmHg to 57.00 mmHg. The mean RAP is 5.13 mmHg (median: 3.50 mmHg; SD: 4.97), with a range of 1.00 mmHg to 15.00 mmHg. PCP has a mean of 8.10 mmHg (median: 8.50 mmHg; SD: 3.38), ranging from 1.00 mmHg to 13.00 mmHg. PVR has a mean of 4.67 WU (median: 3.64; SD: 2.13), ranging from 2.30 to 7.80. CI has a mean of 2.97 $Lminm^2$ (median: 2.90; SD: 0.83), ranging from 1.57 to 4.49. FEV1L mean is 1.53 L (median: 1.50 L; SD: 0.49), ranging from 0.95 L to 2.44 L. FEV1% mean is 52.00 (median: 47.00; SD: 18.68), ranging from 34.00 to 97.00. FVCL mean is 1.78 L (median: 1.73 L; SD: 0.59), with a range of 1.02 L to 3.05 L. FVC% has a mean of 49.36 (median: 44.00; SD: 15.82), ranging from 32.00 to 84.00. FEV1/FVC has a mean of 87.00 (median: 86.00; SD: 8.33), ranging from 72.00 to 97.00. DLCO% has a mean of 26.18% (median: 23.00; SD: 14.14), ranging from 9.00 to 54.00. Lastly, the mean BMI is 26.92 (median: 26.53; SD: 4.33), ranging from 18.69 to 33.14.

Table 10. Descriptive statistics - GROUP 3 COPD								
	Obs.	Mean	Median	SD	p25	p75	Min	Max
Age	5	54.20	56.00	5.45	50.00	59.00	47.00	59.00
Sex	5	1.00	1.00	0.00	1.00	1.00	1.00	1.00
Weight (Kg)	5	66.80	60.00	16.16	59.00	65.00	55.00	95.00
Height (cm)	5	172.20	171.00	8.98	170.00	173.00	161.00	186.00
PAPm (mmHg)	5	30.80	30.00	3.11	29.00	34.00	27.00	34.00
RAP (mmHg)	4	4.25	5.00	2.22	3.00	5.50	1.00	6.00
PCP (mmHg)	5	9.40	10.00	2.61	8.00	10.00	6.00	13.00
PVR (WU)	4	4.69	4.54	0.82	4.12	5.25	3.86	5.80
CI (Lminm^2)	5	2.65	2.60	0.38	2.50	2.79	2.18	3.20
FEV1 (L)	5	0.78	0.87	0.21	0.62	0.90	0.50	1.00
FEV1 (%t)	5	22.40	21.00	4.34	19.00	27.00	18.00	27.00
FVC (L)	5	2.30	2.57	0.83	1.60	2.78	1.29	3.28
FVC (%t)	5	54.40	61.00	12.46	47.00	64.00	36.00	64.00
FEV1FVC (%)	5	34.60	34.00	8.44	31.00	35.00	25.00	48.00
DLCO (%t)	5	17.80	14.00	7.76	12.00	21.00	12.00	30.00
BMI (kg/m^2)	5	22.31	21.72	3.29	20.42	23.15	18.81	27.46

Group 3 – COPD: Table 10 shows the COPD group's descriptive statistics. The mean PAPm for this group is 30.80 mmHg (median: 30.00 mmHg; SD: 3.11), with values ranging from 27.00 mmHg to 34.00 mmHg. The variable RAP has a mean of 4.25 mmHg (median: 5.00 mmHg; SD: 2.22), ranging from 1.00 mmHg to 6.00 mmHg. PCP has a mean of 9.40 mmHg (median: 10.00 mmHg; SD: 2.61), ranging from 6.00 mmHg to 13.00 mmHg. PVR has a mean of 4.69 WU (median: 4.54; SD: 0.82), ranging from 3.86WU to 5.80WU. CI has a mean of 2.65 Lminm^2 (median: 2.60; SD: 0.38), ranging from 2.18 to 3.20. FEV1L has a mean of 0.78 L (median: 0.87 L; SD: 0.21), ranging from 0.50 L to 1.00 L. FEV1% has a mean of 22.40% (median: 21.00; SD: 4.34), with values ranging from 18.00% to 27.00%. FVCL has a mean of 2.30 L (median: 2.57 L; SD: 0.83), ranging from 1.29 L to 3.28 L. FVC% has a mean of 54.40 (median: 61.00; SD: 12.46), ranging from 36.00 to 64.00. FEV1/FVC has a mean of 34.60 (median: 34.00; SD: 8.44), with values ranging from 25.00 to 48.00. DLCO% has a mean of 17.80 (median: 14.00; SD: 7.76), ranging from 12.00 to 30.00. Lastly, the mean BMI for group 3 – COPD is 22.31 (median: 21.72; SD: 3.29), ranging from 18.81 to 27.46.

In order to understand if there is any statistical difference intra (Within) a specific group of patients , we implemented the Wilcoxon test. Table 11 presents Wilcoxon tests (Wilcoxon, 1945) to determine whether our variables of interest (Age; Sex; Weight kg; Height cm; PAPm mmHg; RAP mmHg; PCP mmHg; PVR UW; CI Lminm^2; FEV1L; FEV1%; FVCL; FVC%; FEV1/FVC; BMI; and DLCO%) are different across the patience affected by IPF vs IPAH; and patience affected by IPF+COPD vs IPAH. The null hypothesis for the Wilcoxon test (non-parametric test) was that in both groups there was the same distribution of these variables.

In most cases, the statistics for the Wilcoxon tests are not statistically significant for age; sex; weight; height; PCP mmHg; CI Lminm^2; FEV1FVC; and BMI, suggesting that these measures are not different across the different groups. Notably, some differences are detectable in the variables of PAPm; RAP; PVR; FEV1L; FEV1%, FVCL; FVC% and DLCO% across the diverse groups , but this was expected due to the different disease phenotypes.

Table 11. Wilcoxon tests on diffente groups (IPF vs. IPAH, and (IPF + COPD) vs. IPAH).						
	Fibrosi vs IPAH			(IPF+COPD) vs IPAH		
	Z	p-value	Diff. in means	Z	p-value	Diff. in means
Age	0.86	0.39	1.52	1.26	0.21	2.92
Sex	-0.55	0.58	-0.12	0.03	0.97	0.01
Weight (kg)	1.32	0.19	7.43	0.68	0.50	4.87
Height (cm)	-0.69	0.49	-3.15	-0.33	0.74	-1.69
PAPm (mmHg)	-3.51	0.00	-22.02***	-3.93	0.00	-23.13***
RAP (mmHg)	-1.97	0.05	-4.48**	-2.46	0.01	-4.77**
PCP (mmHg)	-1.53	0.13	-2.00	-1.40	0.16	-1.57
PVR (UW)	-2.94	0.00	-5.26***	-3.38	0.00	-5.26***
CI (Lminm^2)	0.76	0.45	0.23	0.55	0.58	0.14
FEV1 (L)	-3.30	0.00	-1.09***	-3.74	0.00	-1.33***
FEV1 (%t)	-3.21	0.00	-28.00***	-3.72	0.00	-37.25***
FVC (L)	-3.53	0.00	-1.93***	-3.62	0.00	-1.77***
FVC (%t)	-3.59	0.00	-42.34***	-4.01	0.00	-40.76***
FEV1FVC	3.18	0.00	15.16***	1.06	0.29	-1.22
BMI (kg/m^2)	1.88	0.06	3.47*	1.18	0.24	2.11
DLCO (%t)	-3.38	0.00	-37.93***	-3.74	0.00	-40.55***
*p < 0.1. **p < 0.05. ***p < 0.01.						

3.2. Differential expression of miRNAs

Figure 11 depicts the level of all analysed micro-RNAs in the three groups of patients. The most relevant findings are represented by:

a. the expression levels of miR-21-5p which resulted significantly downregulated in IPAH vs IPF (unpaired t-test p<0.05)
b. the expression levels of miR-26a-5p which resulted downregulated in IPF + COPD vs IPAH (unpaired t-test p<0.05).

The expression of all other micro-RNAs (miR-140-5p, miR-24-1-5p, miR-103a-2-5p, miR-126-5p, miR-20a-5p, miR-98-5p, miR-301a-5p, miR-17-5p, miR-92a-1-5p, miR-191-5p 5) showed a consistent variability and did not vary significantly among the groups, even if in some cases a non-significant trend towards higher expression among PAH patients was detected, namely miR20 and miR17.

Additionally, we can speculate that the three groups appear to share a fairly similar expression of miR-98, miR-92 and miR-301.

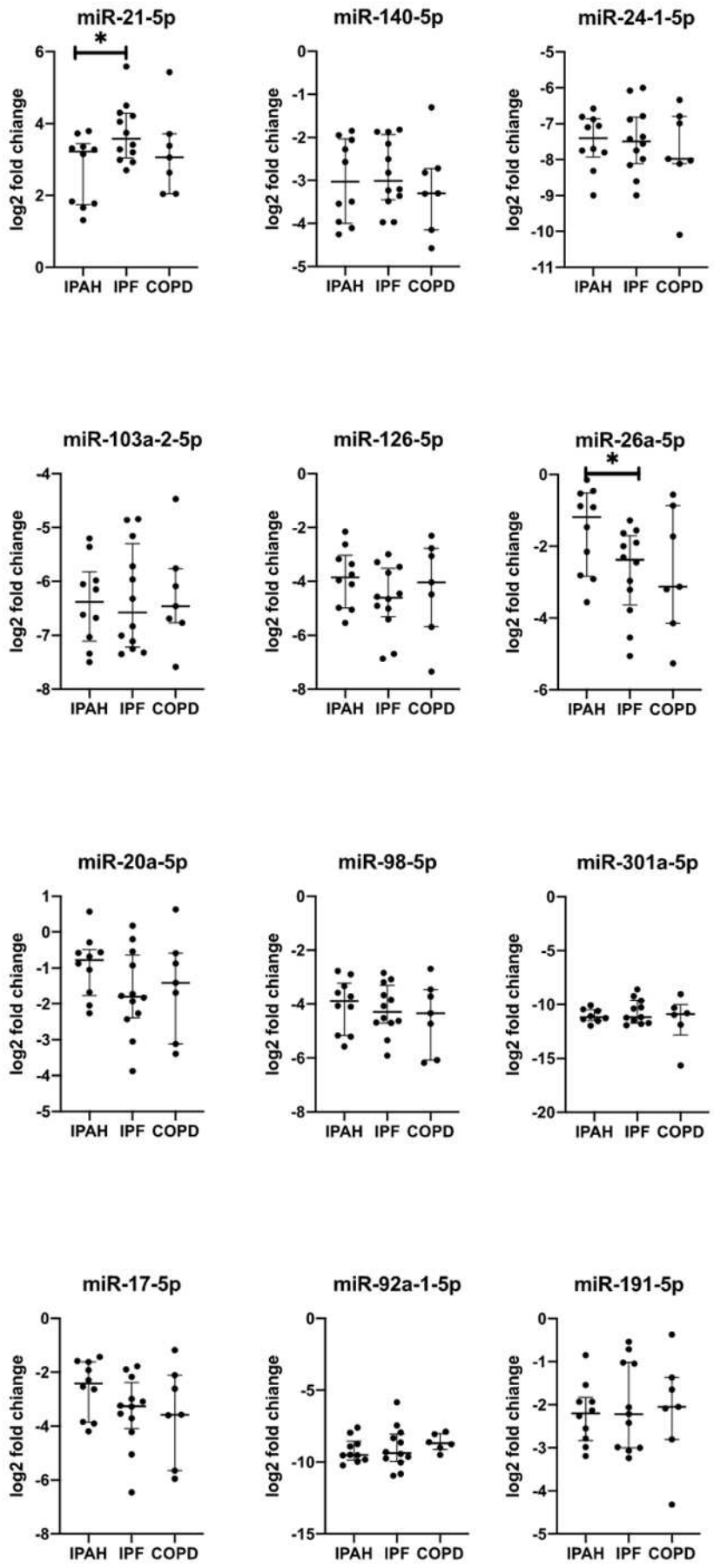

Figure 11. Differential expression of miRNAs

4. DISCUSSION

To our knowledge, this is the first study to explore the specific expression patterns of a panel of specific micro-RNAs previously involved in PAH pathogenesis in lung tissue obtained from group 3 pulmonary hypertension performing a comparison with group 1 patients .

Despite the latest ESC/ERS guidelines defining a new cutoff for the diagnosis of pulmonary hypertension with a value of PAPm ≥ 20 mmHg, the previous cutoff of PAPm ≥ 25 mmHg was employed in this study to enhance the detection of potential histopathological alterations in the pulmonary arterioles (Galie et al., 2015, Humbert et al., 2022). Furthermore, considering that group 3 pulmonary hypertension typically exhibits lower PAPm values compared to group 1, the choice of a higher cutoff allows for a more meaningful comparison between the three study groups.

Indeed, the analysis of continuous variables revealed a statistically significant difference in PAPm values between patients (IPAH vs IPF&COPD $p<0.05$), specifically higher values were found IPAH (mean PAPm: 56.60 mmHg; median: 54.50 mmHg; SD 12.31) in comparison to group 3 pulmonary hypertension associated with IPF (mean PAPm: 34.58 mmHg; median: 35.50 mmHg; SD: 8.62) and COPD (mean PAPm: 30.80 mmHg; median: 30.00 mmHg; SD: 3.11). These findings are consistent with the study's lung transplant context, where patients are highly selected for their age and disease severity.

Among the other variables analysed, a statistically significant difference was also observed in the spirometric values of FEV1 and FVC, both in absolute values and as a percentage of predicted values ($p<0.05$). This difference reflects the variations in underlying diseases. Specifically, the study of lung volumes in group 1 pulmonary hypertension provides values within the normal range, unless there is a mild restrictive syndrome caused by cardiomegaly.

It is intriguing to observe the statistically significant difference in DLCO values as a percentage of predicted values between patients with IPAH compared to those in group 3, with higher mean values in group 1 (mean DLCO%: 64.11; median: 69%; SD: 24.56) than in patients with IPF (mean DLCO%: 26.18; median: 23.00; SD: 14.14), and COPD (mean DLCO%: 17.80; median: 14.00%; SD: 7.76). DLCO represents the capacity of gases to pass from the alveoli through the alveolar epithelium and capillary endothelium to red blood cells. Indeed, the diffusion capacity of carbon monoxide (DLCO) is influenced not only by the area and thickness of the blood-gas barrier but also by the blood volume in the pulmonary capillaries. Considering the analysed pathologies, the discrepancy in DLCO values may be attributed to the selection criteria used for listing patients for lung transplantation. In IPAH DLCO in usually mildly impaired, while the presence of PH in patient with parenchymal disease constitutes ad additional factor to DLCO decline.

To generate specific expression levels of miRNAs, we chose 10 microns thick of formalin-fixed, paraffin-embedded (FFPE) slices from explanted lung of patient who underwent lung transplantation for IPAH , COPD and IPF complicated by PH. To optimize the results we chose samples with at least 3 arterioles and we elaborated the samples using miRNeasy FFPE kit. Subsequentially a miRCURY LNA miRNA PCR Assays, in combination with the miRCURY LNA miRNA PCR System, were used to quantify miRNAs with high sensitivity and specificity.

The miRNAs panel was elaborated on literature and new hypothesis, the selected panel of MIRNA regarded miRNAs involved in pulmonary arterial endothelial disfunction an proliferation: miR21, miR17, miR20a, miR301, miR-126-5p (Chang et al., 2018, Chen et al., 2016, Bertero et al., 2014); miRNA involved in accumulation of PA-Smoth Muscle Cell and adventitial fibroblasts (miR21, miR26a, mi98, miR140, miR-24, miR-103a, miR-92a-1-5p, miR-191-5p) (Deng et al., 2016, Kang et al., 2016, Chen et al., 2018, Kriegel et al., 2012, Wang et al., 2014, Stevens et al., 2016, Watterston et al., 2019, Rothman et al., 2016).

The data we generated by using this approach reveals that only the expression patterns of miR-21-5p and miR-26a-5p differed significantly between IPAH and PH associated with parenchymal disease. Remarkably,

significantly lower miR-21-5p levels and significantly higher miR-26a-5p levels were found in IPAH samples vs IPF or vs both parenchymal lung diseases respectively.

Interestingly, we found no significant differences in the expression of the several other miRNA includes in our panel, known from literature to be involved in PAH pathogenesis, thus suggesting analogous regulation of pathogenic pathways in group 1 and 3 PH patients .

Recent evidences indicate miRNAs may play a relevant role in the diagnostic setting and have the potential to be used as biomarkers. Given that the expression of these factors is similar, deeper studies should be performed at peripheral levels in order to rule out whether their dysregulation in plasma, that has been described in PAH patients, co-exists also in group 3 PH patients. If so, group 3 PH could also in future adopt treatment strategies that are at the moment limited to PAH patients.

It has to be noted, however, that the miRNA expression in small and selected sample of pathological tissue cannot mirror whole anatomopathological events in parenchymal disease that could interfere with PH therapy, like parenchymal disruption and pulmonary blood vessel loss. As it's know that this kind of treatments may amplify the shunt effect (Nathan et al., 2019).

Two factors, as already stated, have been found dysregulated in group 3 with respect to PAH. The first is miR-21-5p that is known to be involved in several pathophysiological pathways occurring in different diseases, however its role in pulmonary hypertension is still not fully clarified, with data showing contradictory results. This study could add another small piece of a puzzle on the role of miR-21 in PH.

MiR-21 is known to be up-regulated in pulmonary arteries from various pulmonary hypertension (PH) models and PH patients. Specifically, human pulmonary artery smooth muscle cells (PASMCs) induced by hypoxia exhibit a significant increase in miR-21 expression, while the expression of its target genes, including programmed cell death protein 4 (PDCD4), Sprouty 2 (SPRY2), and peroxisome proliferator-activated receptor-α (PPARα), is decreased due to its inhibitory effect on gene expression (Xu et al., 2022).

Furthermore, overexpression of miR-21 in PASMCs promotes cell proliferation and migration, contributing to the development of PH. Conversely, when hypoxia-induced PASMCs are treated with anti-miR-21, cell proliferation and migration are blocked, suggesting that inhibiting miR-21 can prevent and reverse hypoxia-induced PH (Yang et al., 2012).

It has also been observed that miR-21 expression is up-regulated by bone morphogenetic protein receptor 2 (BMPR2) signalling under hypoxic conditions. miR-21 directly targets RhoB, suppressing its expression and kinase activity, thereby inhibiting angiogenesis and vasodilation.

Moreover, overexpression of miR-21 reduces PDCD4 expression and protects mice from PH in the hypoxia/SU5416 model. Conversely, inhibition of miR-21 expression enhances RhoB expression and kinase activity, exacerbating hypoxia/SU5416-induced PH. Knocking out miR-21 in mice activates the PDCD4/caspase-3 axis in pulmonary arterial endothelial cells (PAECs) and leads to progressive PH. Thus, miR-21 seems to represent a protective factor that inhibits the progression of PH in human PAECs (White et al., 2014).

Our study has demonstrated that miR-21 is down-regulated in patients with IPAH compared to patients with IPF and pulmonary hypertension.

This result could be explained by a greater hypoxic insult in patients with group 3 pulmonary hypertension compared to patients with IPAH, where hypoxia plays a secondary and promoting role in the context of the underlying vascular disease.

In fact, miR-21 has been shown to be increased up to threefold in human PASMCs in response to hypoxic stimulation. However, it has also been demonstrated that miR-21 has a direct effect on vascular endothelial cells, exerting a protective role on the development of pulmonary hypertension.

This ambivalent role of miR-21 in relation to the findings of our study suggests that the higher expression of miR-21 in patients with group 3 PH may be justified by a greater hypoxic stimulus. Furthermore, the protective role on pulmonary vascular endothelium could account for the significantly lower values of PAPm observed in patients with group 3 PH compared to those with IPAH. An ambivalent role of miR21 has also been shown in other pathologic contexts including lung fibrogenic disorders, heart and kidney fibrosis. In the context of pulmonary fibrosis miR21 can be found significantly up-regulated, with a role that is highly controversial. On one side, miR-21 is able to contain acute lung injuries but, on the other side, is also able to promote differentiation of fibroblasts to myofibroblasts, their proliferation and the process of EndoMT. Its expression in the lung of patients with interstitial lung diseases and chronic lung rejection has been found upregulated as well as its plasma levels, so that it has been suggested as possible biomarker of disease, and therapeutic strategies aiming at its inhibition have been studied (Di Carlo et al., 2016, Bozzini et al., 2021, Yan et al., 2023). Given these data, the significance of a dysregulation of miIR21 in IPAH with respect to IPF-PH patients can have two possible explanation: increased protective response to hypoxia at the vascular level or an increased pathogenic expression in the context of fibrotic tissue. At the moment, data available are not able to solve this issue, an aid should come from in situ hybridization studies that are planned on our samples, which will unravel the specific cellular source of this micro-RNA. Whatever is the role of this factor in IPF-PH, however it should be assessed in future studies the possible role of miR21 as biomarker of group 3 PH, assessing its plasma levels in IPF-PH patients with respect to other patients (IPF without PH or IPAH) .

Another result of our study highlights a statistically significant difference in the expression of miR-26, which is up-regulated in patients with IPAH compared to patients with group 3 pulmonary hypertension, as well as those with IPF and COPD.

miR26 has recently been identified as a mediator in SMCs differentiation, Watterston et al. suggested that the normal function of miR26a is to reduce Smad1 protein activation via BMP-signalling within the endothelium, and indirectly inhibit vascular SMC differentiation in early development, highlighting the role of BMP signaling through Smad1 in regulating vSMC plasticity.

Disruption of BMP signaling can affect both EC and vSMCs. Abnormal vSMC phenotypic switching contributes to the development of vascular diseases (Watterston et al., 2019).

The results obtained from our study are consistent with the hypothesis proposed by Watterson et al. Based on the available literature, we can speculate that the overexpression of miR-26 in patients with IPAH may be correlated with impaired differentiation of pulmonary arterial smooth muscle cells (PASMCs), thus contributing to inhibited PASMC differentiation and the development of pulmonary vascular pathology.

Limitation of the study

Our study presents novel findings on the expression levels of miRNAs in the context of pulmonary hypertension (PH), specifically comparing group 3 PH with group 1 PH. However, it is important to acknowledge that the data presented here are based on a limited study population, and further confirmation is required through larger cohort studies. The limited number of participants in this study is attributed to the absence of hemodynamic data, as right heart catheterization is not consistently performed during lung transplant screening in lung parenchymal disease. Additionally, the analysis of miRNA expression levels was conducted on formalin-fixed, paraffin-embedded (FFPE) slices from explanted lungs, which may be influenced by other pathophysiological mechanisms within the lung parenchyma, particularly in the case of IPF where endothelial-to-mesenchymal transition phenomena is observed in the

fibrotic process. This latter limitation will be addressed in subsequent experiments, specifically investigating the cell types responsible for miRNA expression through in-situ hybridization techniques.

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