THE ROLES OF CIRCULATING CYTOKINES AND RIGHT VENTRICULAR ADAPTATION IN PRE-CAPILLARY PULMONARY HYPERTENSION

PhD thesis

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Budapest

2025

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List of abbreviations

Akt: protein kinase B

BPA: balloon pulmonary angioplasty

BSA: body surface area

CI: cardiac index

CO: cardiac output

CT: computer tomography

CTEPH: chronic thromboembolic pulmonary hypertension

ERK: extracellular signal-regulated kinase

FC: functional class

FGF: fibroblast growth factor

GC: guanylate cyclase

GFR: glomerular filtration rate

GOT: glutamic-oxaloacetic transaminase

GPT: glutamic-pyruvic transaminase

Hgb: haemoglobin

HIV: human immunodeficiency virus

IGF-I: Insulin-like growth factor-I

IPF: idiopathic pulmonary fibrosis

MEK or MAPK: mitogen-activated protein kinase

MRI: magnetic resonance imaging

NA: not applicable

NS: non-significant

NT-proBNP: N-terminal pro-hormone of brain natriuretic peptide

PA: pulmonary artery

PAC: pulmonary arterial compliance

PAH: pulmonary arterial hypertension

s/d/mPAP: systolic/mean/diastolic pulmonary arterial pressure by right heart catheterization

msePAP: mean systolic ejection pulmonary pressure

PaO_2: arterial oxygen pressure

$PaCO_2$: Arterial carbon dioxide pressure

PAWP: pulmonary arterial wedge pressure

PCH: pulmonary capillary haemangiomatosis

PEA: pulmonary endarterectomy

PH: pulmonary hypertension

PI3K: phosphoinositide 3-kinase

PVOD: pulmonary veno-occlusive disease

PVR: pulmonary vascular resistance

RA: right atrium

RAA: right atrial area

RAP: right atrial pressure

RHC: right heart catheterization

RV: right ventricle

RVOT VTI: right ventricular outflow tract velocity-time integral

RVSWI: right ventricular stroke work index

SMAD: specific intracellular signal transduction protein

sPAPe: estimated systolic pulmonary arterial pressure by echocardiography

SV: stroke volume

SVI: stroke volume index

SvO_2: mixed venous oxygen saturation

TAPSE: tricuspid annular plane systolic excursion

TGF-β:transforming growth factor-β

VEGF: vascular endothelial growth factor

Vmax: maximal velocity

6MWD: 6-minute walk distance

1. Introduction

Pulmonary hypertension (PH) is a progressive disease. Pathophysiological changes in pulmonary vessels lead to elevated pressure and an increase in vascular resistance (1-3). Hemodynamic changes and the course of the disease vary in the distinct forms of PH with different pathophysiological backgrounds (4-6). Primarily, based on hemodynamic characteristics, pre-capillary and post-capillary forms of PH are distinguished. In pre-capillary PH, the mean pulmonary arterial pressure (mPAP) and the pulmonary vascular resistance (PVR) is elevated with normal pulmonary arterial wedge pressure (PAWP<15 mmHg). In contrast, in post-capillary PH, pressure elevation occurs in both the arterial and venous sides of pulmonary circulation, and PAWP is increased (>15mmHg) without a rise in PVR (1).

In all forms of pre-capillary PH, arterial remodelling is common to a varying degree, which induces pressure overload of the right ventricle. Furthermore, there can be significant differences in disease severity and clinical course among the PH groups. In addition to the pulmonary arterial hypertension group (PAH), being the most characteristic phenotype of pre-capillary PH, PH associated with lung diseases and/or hypoxia and PH associated with pulmonary artery obstructions, including chronic thromboembolic PH (CTEPH) belong to the form of pre-capillary PH (Table 1). Pathophysiological abnormalities and morphological changes in the pulmonary arteries are heterogeneous in these clinical groups. The remodelling of the arterial wall, vascular obstruction, vasoconstriction, and reduction of the vascular cross-sectional area contribute to the increase in PVR (7-9). Although in everyday clinical practice, there are limited options to characterize the degree of pathological and hemodynamic alterations in pulmonary arteries, their effect on the development of right heart pathology could be more easily followed.

In healthy subjects, the pulmonary circulation is a low-pressure circulatory system even in a wide flow range; the upper limit of normal mPAP is 20 mmHg (10; 11). Under physiological conditions, the right ventricle (RV) works against low pre- and afterload with low stroke work (SW) (12). In pre-capillary PH, the continuously increasing PVR induces RV pressure overload, leading to an adaptation process in the right heart, such as

myocardial hypertrophy and right heart dilatation. A biphasic development of the RV adaptation process can be observed during the progression of the disease. In the adaptive phase, the RV's systolic reserve is still preserved. It is thus able to maintain appropriate circulation against the increasing resistance. In contrast, the maladaptive phase leads to the rapid progression of RV dysfunction and the development of right heart failure. In this process, the development of RV/pulmonary artery (PA) uncoupling is a turning point (13). The mechanism of RV adaptation involves complex processes, which are influenced by different factors, including the aetiology of PH, genetic predispositions, neurohumoral regulation, and immune and inflammatory activation (2;14).

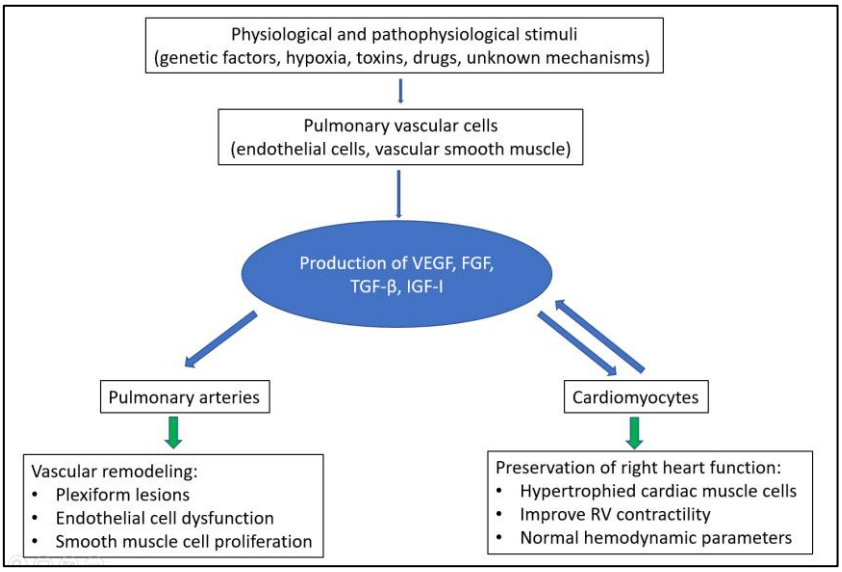

Figure 1. The roles of growth factors in the pathophysiology of pulmonary arterial hypertension. Growth factors are produced in pulmonary vascular cells and cardiomyocytes in response to various stimuli in PH. These factors play a role in developing pulmonary vascular remodelling and the modulation of right ventricular function. Furthermore, the regulatory peptides produced in the pressure-overloaded myocardium also affect the pathophysiological changes. FGF: fibroblast growth factor; IGF-I: Insulin-like growth factor-I; RV: right ventricle; TGF-β:transforming growth factor-β; VEGF: vascular endothelial growth factor. Original work of the author. Reference is found in the main text.

In pre-capillary PH, the treatment goal is to improve the patient's functional capacity, to prevent the right heart failure and to reduce the patient's risk of death based on risk stratification (1), that is, to ensure the maintenance of the adaptive phase of RV function

for as long as possible by reducing PVR. Accordingly, evaluation and monitoring of RV adaptation during the course of the disease is essential. Dimensions and function of the right heart can be followed with routine imaging techniques such as echocardiography and cardiac MRI. The invasive assessment of RV work and RV/AP uncoupling based on the pressure-volume loops is feasible only in experimental studies, but cannot be performed in routine clinical practice. Recently, several authors have suggested using the calculation of RV stroke work (RVSW) from basic hemodynamic parameters to evaluate RV function (15). However, its interpretation in patient care should be clarified.

In the pathophysiology of vascular remodelling, growth factors (GFs), molecules of the inflammatory system and other regulatory peptides play significant roles (9). In addition, some of these peptides have known cardioprotective effects (16); and may also serve as biomarkers in assessing disease progression, following the RV adaptation or therapeutic targets to inhibit vascular remodelling, and preserving myocardial function (Figure 1) (17). However, their precise functions in the development of RV adaptation should be elucidated in more detail.

In this dissertation, we aimed to investigate RV adaptation using hemodynamic parameters such as RVSW and circulating biomarkers and examine their applicability and interpretation in clinical practice.

1.1 Definition and classification of pre-capillary pulmonary hypertension

The invasive right heart catheterization (RHC) is required to verify PH since the definition is based on hemodynamic criteria. Based on the previous 2015 European Society of Cardiology (ESC)/European Respiratory Society (ERS) guideline, the diagnosis of PH could be confirmed if mPAP was above 25 mmHg (18) although the upper limit of normal mPAP is 20 mmHg (11; 10). Since even mildly elevated mPAP is already associated with higher mortality (19; 20), the updated 2022 ESC/ERS guideline defined PH as mPAP > 20 mmHg (1). In addition, in pre-capillary PH, PAWP is not increased, while PVR is elevated (Table 1) (1).

Table 1. Hemodynamic definitions of pulmonary hypertension according to the 2022 European Society of Cardiology/European Respiratory Society guideline (1)

Definitions	Characteristics
Pre-capillary PH	mPAP > 20 mmHg PAWP ≤15 mmHg PVR > 2 WU
Isolated post-capillary PH (IpcPH)	mPAP > 20 mmHg PAWP >15 mmHg PVR ≤ 2 WU
Combined pre- and post-capillary PH (CpcPH)	mPAP > 20 mmHg PAWP >15 mmHg PVR > 2 WU

CpcPH: combined pre-capillary pulmonary hypertension, IpcPH: isolated pre-capillary pulmonary hypertension, mPAP: mean pulmonary arterial pressure, PAWP: pulmonary arterial wedge pressure, PH: pulmonary hypertension, PVR: pulmonary vascular resistance, WU: Wood unit

Table 2. Clinical groups of pre-capillary pulmonary hypertension (1)

PH group 1: Pulmonary arterial hypertension (PAH)
1.1.Idiopathic
1.1.1.Non-responder at vasoreactivity testing
1.1.2. Acute responder at vasoreactivity testing
1.2 Heritable
1.3 Associated with drugs and toxins
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/capillaries (PVOD/PCH) involvement
1.6 Persistent PH of the newborn
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 Other lung disease with mixed restrictive/obstructive pattern
3.4 Hypoventilation syndrome
3.5 Hypoxia without lung disease (e.g. high altitude)
3.6 Developmental lung disorders
PH group 4: PH associated with pulmonary artery obstructions
4.1 Chronic thromboembolic PH
4.2 Other pulmonary artery obstructions

PAH: pulmonary arterial hypertension; PH: pulmonary hypertension; PVOD: pulmonary veno-occlusive disease; PCH: pulmonary capillary haemangiomatosis.

Furthermore, the clinical classification of pre-capillary PH describes categories with similar pathophysiological mechanisms, clinical appearance, and course, hemodynamic characteristics, and therapeutic management (Table 2). During the diagnostic procedure, in addition to RHC, other imaging examinations and diagnostic tests are necessary to accurately define the clinical groups linked with pre-capillary PH (1). Table 3 highlights the most important examinations in groups 1, 3, and 4.

Table 3. Diagnostic steps in the differential diagnosis of pulmonary hypertension in the major clinical groups with pre-capillary disease (1)

Clinical groups	Imaging and functional tests
Group 1. PAH	
IPAH	Diagnostic algorithm of exclusions
	Vasoreactivity testing
PAH associated with:	
Connective tissue disease	Physical examination
	Laboratory tests
	Capillary microscopy
HIV infection	Laboratory tests
Portal hypertension	Laboratory tests
Congenital heart disease	
	Echocardiography
Schistosomiasis	Cardiac MRI
	Laboratory tests
Group 3. PH associated with lung disease and/or hypoxia	Lung function tests
	Chest CT scan
	Polysomnography
Group 4. PH associated with pulmonary artery obstruction	Chest CT angiography
	Ventilation/perfusion scan
	Direct pulmonary angiography

CT: computer tomography; HIV: human immunodeficiency virus; MRI: magnetic resonance imaging; PAH: pulmonary arterial hypertension; PH: pulmonary hypertension.

The degree of arterial remodelling differs in each clinical group with pre-capillary PH. However, its presence and effects on RV adaptation are similar due to the myocardial adaptation mechanisms caused by RV pulsatile and resistive load. In post-capillary PH, the increased filling pressure of the left heart and the intraventricular interdependence also affect the adaptation of the RV, while in pre-capillary PH, these processes are not observed (21). Because of all these common appearances and similar pathomechanism, we analysed RV adaptation in our studies only in pre-capillary PH groups.

1.2. Clinical groups of pre-capillary pulmonary hypertension

The forms of pre-capillary PH show heterogeneous pathophysiological backgrounds, epidemiological characteristics, disease courses, and treatment options.

Pre-capillary PH is a progressive disease in which the development of arterial remodelling is common. Right heart adaptation and right heart failure determine patients' quality of life and life expectancy (25). In early disease, the progression of arterial remodelling does not cause any symptoms, and patients are usually diagnosed when exercise capacity is limited and when symptoms of right heart failure occur. The course of the disease shows inter-individual variability. In the pre-capillary PH groups, our studies focused on patients with PAH, PH associated with lung diseases, and PH due to pulmonary artery obstruction. The main features of these groups are compared in Table 4 and described below (1).

Table 4. Characteristics of the main pre-capillary PH groups (1)

	Pulmonary arterial hypertension	**PH associated with lung disease and/or hypoxia**	**PH associated with pulmonary artery obstruction**
Epidemiology	Rare	Common	Rare
Pathophysiology	Pulmonary arterial remodelling	Hypoxic vasoconstriction Loss of vascular bed Pulmonary arterial remodelling	Obstruction of pulmonary arteries Pulmonary arterial remodelling
Treatment	Specific pulmonary vasodilator therapy Lung transplantation	Treatment of lung disease or the cause of hypoxia In vascular phenotype: specific pulmonary vasodilator therapy Lung transplantation	Pulmonary endarterectomy Balloon pulmonary angioplasty If inoperable or in case of residual PH: specific pulmonary vasodilator therapy Lifelong anticoagulation therapy Lung transplantation

PH: pulmonary hypertension

1.2.1. Pulmonary arterial hypertension

Pulmonary arterial hypertension is an orphan disease. In international reviews, the incidence of PAH is recorded as 1.1-7.6 cases per million, while the prevalence figure is 0.9-26 cases per million (1; 22). Idiopathic, heritable, and anorexigenic-induced PAH make up 52.6% of all PAH cases. The most characteristic PAH patients are women aged between 30 and 60 years (23), but nowadays, the majority of patients are older with co-morbidities (24; 25).

The most characteristic pathophysiological change in pulmonary arteries is the arterial wall remodelling, which is characterized by the thickening of the arterial wall's intimal, medial, and adventitial layers, vascular occlusion by *in situ* thrombi, and the development of plexiform lesions (9; 26). Intimal lesions play a significant role in decreasing the luminal area and consequently elevating PVR. Thickening of the intimal layer is caused predominantly by endothelial and fibroblast cells with a high proportion of undifferentiated cells (27). Intimal remodelling can have different presentations, such as concentric, eccentric thickening, or total occlusion of the arterial lumen. Plexiform lesions are complex, glomeruloid-like vascular formations composed of disorganized, primitive endothelial cells from the remodelled intima layer (26). The medial layer contains predominantly smooth muscle cells, the focus of most pathological studies. The hypertrophy and constriction of smooth muscle tissue contribute to arterial wall thickening, lumen narrowing, and overall PVR elevation. These smooth cells are the targets for specific PAH therapy, as the relaxing and antiproliferative effects of these drugs can cause vasodilatation of pulmonary arteries and improve the hemodynamic status. Previous studies suggested that adventitial thickening also plays a role in developing vascular remodelling in PH. Based on human data, adventitial fractional thickening does not correlate with the severity of hemodynamic parameters in PH, but perivascular inflammatory autoimmune processes are pronounced in this layer (28).

In addition to the development of vascular remodelling, changes in blood flow in the damaged vessels and the formation of *in situ* thrombus can be observed due to neurohumoral inflammatory processes, which lead to a further increase in PVR. Histological examination of explanted lungs in idiopathic PAH showed that *in situ* thrombus formation occurred in 50% of patients (26).

Without effective therapy, the prognosis of PAH is poor, and the mean survival of patients is only 2.8 years (29). However, treatment with modern pulmonary vasodilators extends the survival to 7 years (30).

Specific pulmonary vasodilators are therapeutic options that reduce PVR and decrease RV pressure overload; thereby, with hemodynamic improvement, the progression of the disease becomes slower, and patients can have a better life expectancy. The primary effect of vasodilators is the relaxation of smooth muscle tissue in the media layer of the arterial wall, with some also having an antiproliferative effect. One of the main target pathways is nitric oxide (NO)/guanylate cyclase (GC)-mediated vasodilatation. NO is a potent vasodilator in the lung, but it can only be administered by inhalation due to its short half-life. Phosphodiesterase-5- inhibitors (sildenafil, tadalafil) can increase NO availability in pulmonary circulation, resulting in NO-mediated vasodilatation. GS stimulators (e.g. riociguat) directly affect this pathway by increasing the GC level. They can also inhibit the proliferation of smooth muscle and endothelial cells, and they can also inhibit platelet aggregation. Endothelin (ET) plays a prominent role in developing vascular remodelling, having a strong vasoconstriction and proliferatory effect on smooth muscle cells. ET_A and ET_B receptor antagonists (ambrisentan, bosentan, macitentan) inhibit these processes, reducing PVR. The third therapeutic option, prostacyclin (epoprostenol, treprostinil, iloprost), is the most potent vasodilator and, as such, it is the most effective drug for the treatment of PAH, and its analogs are widely used in the therapeutic management of PAH. It can effectively relax smooth muscle cells, has an antiproliferative effect on endothelial and smooth muscle tissue of the arterial wall, and inhibits platelet aggregation via prostanoid receptors. Because prostacyclin has a short half-life (minutes only), the most effective administration forms are parenteral, continuous subcutaneous, or intravenous infusion. Selexipag, an enteral prostacyclin receptor agonist, is a new option in this field. The vasoreactive group is a special group of patients with PAH, who are long-term responders to calcium channel blockers.

1.2.2. Pulmonary hypertension associated with lung disease and/or hypoxia

Pre-capillary PH is not rare in lung disease or conditions with hypoxia, especially in patients with severe lung diseases. The prevalence of PH depends on the severity of the underlying lung condition; for instance, in end-stage chronic obstructive pulmonary

disease, more than 90% of patients have mPAP>20 mmHg, but only 3-5% of patients present with mPAP> 35 mmHg (31). PH is present in up to 86% of patients with interstitial lung disease (ILD), and the severity is affected by the type and stage of the pulmonary condition (32). PH in IPF is associated with advanced disease stages but depends on the severity of parenchymal destruction. The incidence of PH in IPF is 8-15% at diagnosis and 35-45% for patients before lung transplantation (33-35).

Hypoxic vasoconstriction is the main process in this form of PH (36), but in severe presentations, in addition, media remodelling, including smooth muscle cell hypertrophy, is predominant in chronic hypoxia, and this mechanism affects both distal and proximal large arteries via extensive extracellular matrix deposition and wall thickening. This is most pronounced in the vascular phenotype of lung diseases, where the most severe hemodynamic deviations can be detected with PVR > 5 Wood Unit (WU). This phenotype resembles PAH regarding pathology, hemodynamic changes, and clinical characteristics (37-38). Furthermore, in chronic lung diseases, a reduction in the cross-section area of the lung vascular bed also contributes to the increase in PVR (39-41).

The survival of these patients depends on the severity and course of the lung disease. In the international COMPERA registry, the 1-year survival rate is 78%, 43% at 3 years, and 26% at 5 years in patients with group 3 PH (42).

Adequate therapy of the underlying lung disease is essential for this group, and specific vasodilator therapy is not a suitable option in most cases. However, some pulmonary vasodilator treatments, sildenafil or inhaled prostacyclin, should be considered individually in ILD with the vascular phenotype of PH and severe hemodynamic changes (1). In any group of pre-capillary PH, lung transplantation is a therapeutic option if the patient is suitable and there is no contraindication (1).

1.2.3 Pulmonary hypertension associated with pulmonary artery obstruction

CTEPH is the most prevalent form of PH in the group associated with pulmonary artery obstruction. Its incidence ranges from 8 to 40 cases per million of the general population. In contrast, according to a meta-analysis, it occurs in 3.2% of patients surviving acute pulmonary embolism. Still, acute pulmonary embolism had not been diagnosed in many patients before CTEPH was established (8;43).

The survival of patients with CTEPH is crucially affected by the disease's treatability. If the disease primarily affects the proximal arteries and pulmonary endarterectomy can be performed, then the 3-year survival rate is 89%, while in non-operable cases, this falls to 70% (44).

In CTEPH, the mechanical obstruction of proximal and distal arteries by thrombi and extension of secondary arteriopathy show high individual variability since these changes are influenced not only by neurohumoral activation but also by local deviation of blood streams and the shear force by slow and fast blood flows in arterial branches (5; 45). Importantly, the degree of vascular obstruction does not correlate with the severity of PVR. In some cases, a large central obstruction causes only chronic thromboembolic disease without PVR elevation, while in other instances, a mild obstruction of the total vascular cross-section results in severe hemodynamic deterioration (8).

In CTEPH, in addition to the fact that patients must be on lifelong anticoagulant treatment, the first-line therapeutic option is pulmonary endarterectomy (PEA) or balloon pulmonary angioplasty (BPA). Due to secondary vascular remodelling, pulmonary vasodilator therapy can be initiated (1) in cases of inoperability or inadequate hemodynamic improvement after surgery and before and after BPA in patients with severe hemodynamic abnormality (1).

1.3. The role of the right atrium and ventricle in pre-capillary pulmonary hypertension

1.3.1. Morphology and function of the right atrium and ventricle

Under physiological conditions, the RV transfers blood from the systemic veins to the low-pressure pulmonary circulation, thus working with low pre- and afterload. Compared to the left ventricle (LV), the RV can perform its functions with an asymmetrical, “crescent” shape, a lower myocardial mass, and a smaller wall thickness. Human studies have shown that the size of the RV cardiomyocytes is 15% smaller than those of the LV and that the myocardium contains 30% more collagen, resulting in better compliance (46; 47). In healthy adult myocardium, the energy supply and adenosine-triphosphate production are mainly (60-90%) provided by fatty acid oxidation, while the rate of glucose metabolism is lower. In a hypoxic state, the rate of anaerobic glycolysis in the

RV cardiomyocytes is higher than in the LV, so the RV is relatively more resistant to ischemia (48). Although coronary perfusion is continuous in the RV under normal conditions during both systole and diastole, coronary flow is more sensitive to changes in perfusion pressure and systemic hypotension. This is compensated to a certain extent by the characteristics of the RV that the oxygen demand of its myocardial mass is lower than that of the LV, which is associated with a better oxygen extraction reserve (49).

In systole, the RV undergoes a peristalsis-like movement. The contraction begins in the inflow tract and then goes toward the apex and infundibulum so that the RV squeezes the blood into the pulmonary artery. During this complex process, longitudinal contraction is much more pronounced than radial or circumferential shortening (50). The RV's work is considerably affected by that of the LV. The close cooperation between the two ventricles is well illustrated by the fact that 20–40% of the RV stroke volume (SV) is accounted for by LV contraction (51).

Various conditions of the RV, such as acute or chronic pressure or volume overload, initiate adaptation processes in the myocardium, resulting in myocardial hypertrophy and dilatation of the chambers. Initially, these processes aim to maintain the systolic and diastolic functions of the RV under conditions where pressure and volume are altered, thereby maintaining normal hemodynamics (Frank-Starling effect) (52). Later, as the insults are still present, the degree of hypertrophy and dilatation becomes more severe, and RV function is progressively impaired, resulting in damage to the pulmonary circulation, reduced functional capacity, and then right heart failure. The above adaptation mechanisms are similar in the LV (53). However, an important difference between the two heart chambers is that when the insults cease (e.g., after lung transplantation in a PAH patient or pulmonary endarterectomy in CTPEH), reverse remodelling is much better in the RV (54; 55). In most cases, the RV can adapt to the changed conditions quickly, and dilation of the chambers is reversed within a short period with the improvement of systolic and diastolic functions (56). This can be explained by the lower degree of fibrosis in the RV myocardium during remodelling compared to the LV (57).

1.3.2. Right ventricular adaptation

Chronic and progressive pressure overload in the right heart occurs in pre-capillary PH. The severity and progression of RV adaptation and right heart failure vary considerably from patient to patient and significantly influence the prognosis of the disease (58-60). Several factors, such as the patient's age, the aetiology of PH, comorbidities, and other genetic and epigenetic factors, influence this individual variability (25; 45; 61).

Animal studies have shown that a sudden pressure overload on the RV by acute pulmonary artery occlusion initiates rapid adaptation processes, during which RV volume and cardiac output (CO) increase (62). An increasingly progressive pressure overload caused by vascular remodelling initiates and maintains this adaptation mechanism. RV remodelling is thus a process in which the trigger mechanism is myocardial stretching due to increases in pulmonary arterial pressure and afterload. Increasing pressure and/or volume overload causes this gradual remodelling of the RV wall, hypertrophy, and a gradual right heart dilation. Parallel to this process, the ventricular septum shifts towards the LV and causes the narrowing and compression of the LV cavity, which also affects LV function, reducing LV filling and impairing systolic function, thereby reducing stroke volume (Figure 2).

Morphological myocardial changes appear during remodelling. During adaptive remodelling, concentric RV hypertrophy is pronounced. At the same time, ventricular dilatation is moderate, which results in a higher myocardial mass/volume ratio and allows the RV to retain its systolic and diastolic functions. During the progression of the disease, the dilatation of RV increases disproportionately, the myocardial hypertrophy becomes eccentric (low myocardial mass/volume ratio), and this maladaptive remodelling leads to rapid deterioration of systolic and diastolic RV functions (59). In addition, tricuspid regurgitation increases, and right atrial (RA) dilatation induced by the high-pressure gradient becomes apparent. The degree of tricuspid regurgitation significantly correlates with poor life expectancy (63). The highly raised RA pressure causes a systemic circulatory backflow barrier, leading to right heart failure (Figure 2) (17).

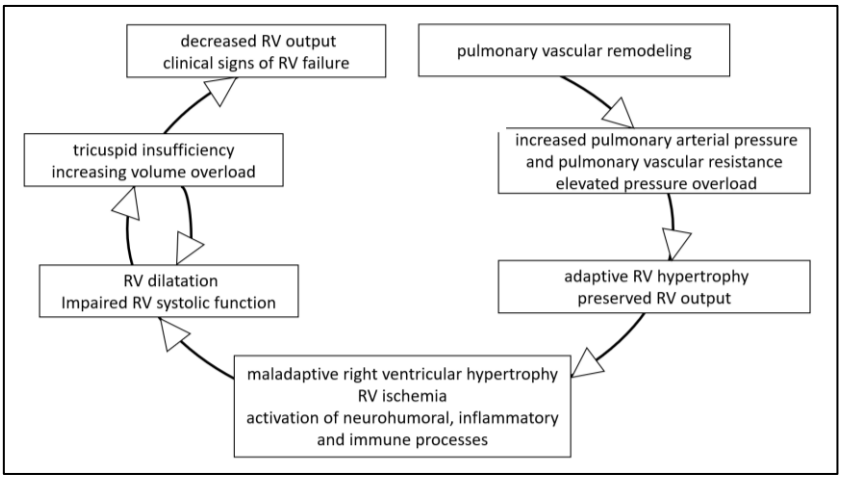

Figure 2. The effects of increased pulmonary vascular resistance on the RV in PAH

A schematic presentation of the viscous circle induced by increased pulmonary arterial vascular resistance on the morphology and function of the right ventricle in PAH. RV: right ventricle. Original work of the author. Reference is found in the main text.

Adaptive and maladaptive remodelling phases are considered interrelated, consecutive processes. A critical point in disease progression is when the function of the RV reaches its peak, and maladaptive processes result in a decline in its performance (64; 65).

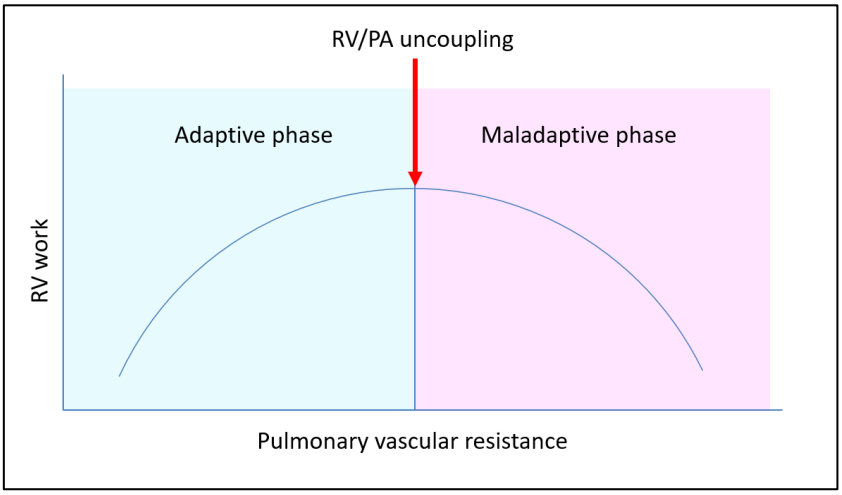

Figure 3. Scheme of RV adaptation in pre-capillary pulmonary hypertension

Changes in the right ventricle work in the adaptive and maladaptive phases in relation to the increase in pulmonary vascular resistance, which characterizes the disease's progression. In the adaptive phase, the rate of RV work increases, while it decreases in the maladaptive phase. The turning point may be RV/PA uncoupling. RV: right ventricle; PA: pulmonary artery. Original work of the author.

A further increase in afterload and/or a decrease in systolic reserve disrupts the cooperation between the RV and the pulmonary artery (PA) unit, leading to RV/PA uncoupling. The timing of RV/PA uncoupling is crucial, as it marks the beginning of the rapid deterioration of RV function (Figure 3) (2; 13).

The high individual difference in vascular remodelling and right heart adaptation is related to the etiology of PH, the pathophysiological mechanisms (pressure and/or volume load), the patient's age, comorbidities, and genetic background. In Eisenmenger's syndrome, the co-existing pressure and volume overloads induce adaptive remodelling in the RV, mainly when it develops in the neonate and the fetal-type myocardial structure is maintained. As a result, the RV functionality is preserved with less severe hemodynamical deterioration in the pulmonary circulation. Consequently, patients with PAH associated with congenital heart disease have the best life expectancy of the overall PAH population (63). Conversely, in other groups of PAH (IPAH and PAH associated with connective tissue disease), maladaptive remodelling develops earlier and progresses more quickly, RV function deteriorates rapidly, and the life expectancy of patients is shorter (30).

Due to this great individual variability, a better understanding and follow-up of the right heart function gives valuable information to provide the best patient management and individually tailored therapy.

1.3.3. Clinical assessment of right ventricular adaptation

A complex evaluation of RV adaptation requires imaging and hemodynamic studies to examine RV myocardial mass, the size of the chambers, RV systolic and diastolic functions, and hemodynamic parameters (Figure 4) (66).

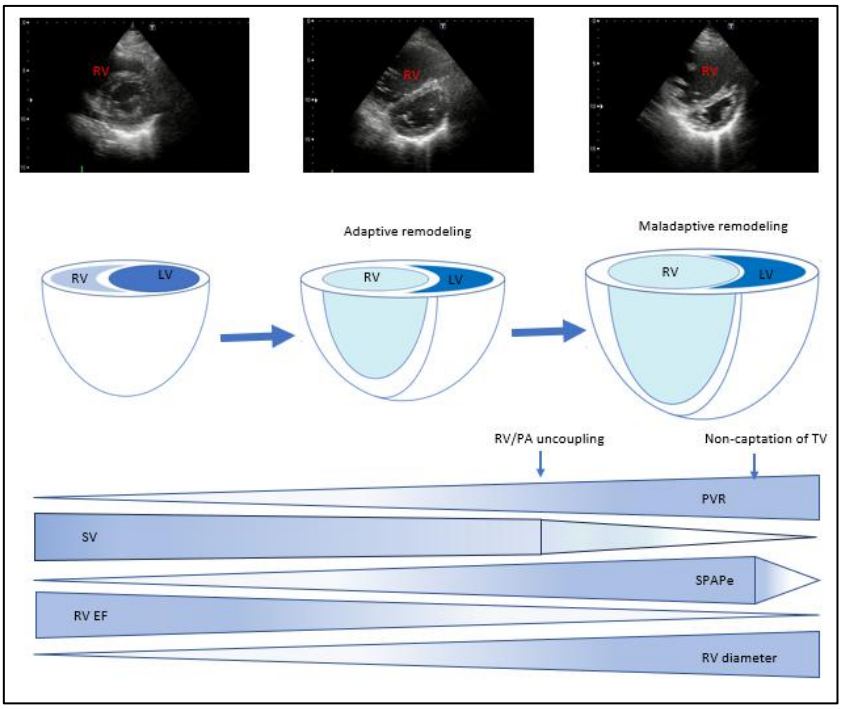

Figure 4. Changes in functional and hemodynamic parameters during right ventricular adaptation

During disease progression, PVR and pulmonary arterial pressure continuously increase. In response, right ventricular remodelling develops, which can appear in both adaptive and maladaptive forms, and in later stages, severe right ventricular dysfunction occurs. LV: left ventricle, PVR: pulmonary vascular resistance, RV: right ventricle, sPAPe: estimated systolic pulmonary arterial pressure by echocardiography, SV: stroke volume, TV: tricuspid valve. Adapted from original work of the author. Reference is found in the main text.

RHC is necessary to diagnose PH, and this also helps in management, including the assessment for lung transplantation and the evaluation of discrepancies between the functional state of a patient and the findings of non-invasive tests. Besides mPAP, PAWP, and PVR, which are essential for the diagnosis, other hemodynamic parameters can also aid the assessment of disease severity and the degree of RV adaptation. Higher right atrial pressure (RAP) and low cardiac index (CI), already present at the time of diagnosis, are signs of severe right heart dysfunction and decompensation suggestive of poor prognosis (67). In PAH, these parameters guide the choice of therapy, as, in conjunction with other functional and laboratory parameters, they can determine the risk of mortality (1). Blood samples taken from the pulmonary artery during a RHC and oxygen saturation in mixed

venous blood reflect both the hemodynamic status and tissue oxygenation and correlate with the prognosis of the disease (68).

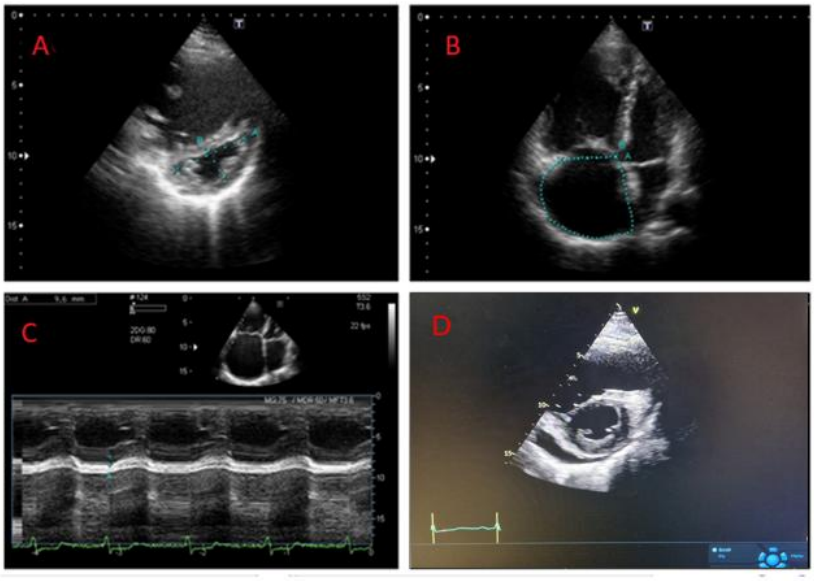

Figure 5. The most important echocardiographic parameters characteristic of the right ventricle

A – D-sign, displacement of the ventricular septum towards the LV. The eccentricity index is the ratio of the left ventricle short-axis diameter perpendicular to the septum to the left ventricle short-axis diameter parallel to the septum; B – Right atrial area (cm^2); C – TAPSE – tricuspid annular plane systolic excursion is the systolic forward movement of the tricuspid lateral annulus in mm, a parameter characterizing systolic right ventricular function; D – pericardial effusion. Original figure of the author

Echocardiography is a routine examination at the diagnosis and follow-up of pre-capillary PH (1; 69). During the diagnostic workup, it is used to determine the probability of PH using the maximum velocity of tricuspid regurgitation (1). However, many other parameters identify the RV function. At the follow-up of pre-capillary PH, echocardiography is used to assess the dimension of the right heart. This requires the measurement of the transverse and longitudinal diameters of the RV, as well as the wall thickness. The eccentricity index shows the ventricular septal displacement (D-sign) level towards the LV caused by the increase in RV pressure (Figure 5A). The size of the RA area is an important parameter in judging right heart overload. The presence of RA

dysfunction in PAH is an independent predictor of mortality and hospitalization (Figure 5B) (70), with an RA area larger than 27 cm^2 indicating a high risk of death (71).

The evaluation of the systolic function of the RV by echocardiography can be challenging, as the two-dimensional (2D) measurement of end-diastolic and end-systolic volumes required to determine the ejection fraction is difficult due to the complex shape of the ventricle. The parameter that characterizes the systolic function is tricuspid annular plane systolic excursion (TAPSE), which shows the systolic longitudinal RV shortening and correlates with global systolic RV function on radionuclide angiography (Figure 5C) (72). A decrease in TAPSE (<18mm) is a good indicator of a high risk of mortality (73), and a change in TAPSE in PAH is also a useful parameter for monitoring the effectiveness of therapy (74). Other parameters that can be used to assess RV systolic function are fractional area change (FAC) and the RV Tei index. The measurement of the RV systolic and diastolic area is required to determine FAC, which is not easy to obtain with routine 2D imaging, and changes in FAC do not significantly follow the effect of therapy (53); thus the clinical use of FAC is not widespread. Tei index is a parameter for the evaluation of the combined systolic and diastolic functions of the RV, calculated from the values of ejection time (ET), isovolumetric relaxation (IVRT), and contraction times (IVCT) (Tei index = (IVRT + IVCT) / ET). In PAH, this value is a predictor of poor survival (Tei index> 0.64) (71).

The appearance of pericardial fluid in pulmonary hypertension results from elevated RA pressure, decreased lymphatic and subepicardial venous outflow due to poor hemodynamics, and right heart failure (Figure 5D). The presence of pericardial fluid warrants a poor prognosis (75), and the extent and rate of fluid change are good indicators of disease progression (76).

Regarding the findings of the more recent echocardiography methods, the RV ejection fraction determined by 3D echocardiography and the longitudinal RV strain have predictive values in PH, and these parameters also reflect ventricular adaptation (77). However, they are not used regularly in patient monitoring, primarily because of the required high technical expertise and the time-consuming measurements.

Magnetic resonance imaging (MRI) examination is the gold standard for assessing RV adaptation, but its availability and routine application in the follow-up of patients is difficult in clinical practice. Volumetric parameters such as RV end-diastolic and end-

systolic volumes, together with volume indices (RVEDVI, RVESVI) and calculated SV, EF (SV / EDV ratio), are typical characteristics of RV adaptation and have prognostic values (68; 78; 79). Furthermore, MR examination is the most accurate method of assessing the presence of adaptive and maladaptive ventricular remodelling and eccentric and concentric RV hypertrophy (59). Pathological abnormalities in the myocardium caused by the increased pressure load and ischemic and fibrotic lesions in the RV can also be detected by MRI. T1-weighted imaging can detect fibrosis in PAH, characterized by increased extracellular volume in the myocardium, even in the early stages of the disease (80). The degree of myocardial fibrosis demonstrated during T1 mapping correlates well with pulmonary arterial stiffness, which is a good indication of the development of RV adaptation (81; 82).

1.3.4. Evaluation of the biphasic change of right ventricular adaptation and uncoupling

The most accurate way to measure RV hemodynamic changes is to evaluate the RV pressure-volume loop with a conductance catheter during RHC (Figure 6) (3; 69). The figure shows that as the pressure increases, the end-systolic and end-diastolic volumes of the RV increase, while the SV initially only slightly decreases. The SV is also significantly reduced with the progression of PH and the further rise in volumes. Furthermore, the area under the curve, the measure of RV work, increases in the early phase (RV coupled), but then it is reduced in the maladaptive phase (RV uncoupled). The gold standard method for assessing uncoupling is the ratio of RV end-systolic elastance (Ees) and arterial elastance (Ea) as calcaulted form the loops (3; 13; 83)**.** Ees/Ea changes in the initial phase (coupling) and then declines, thus defining uncoupling.

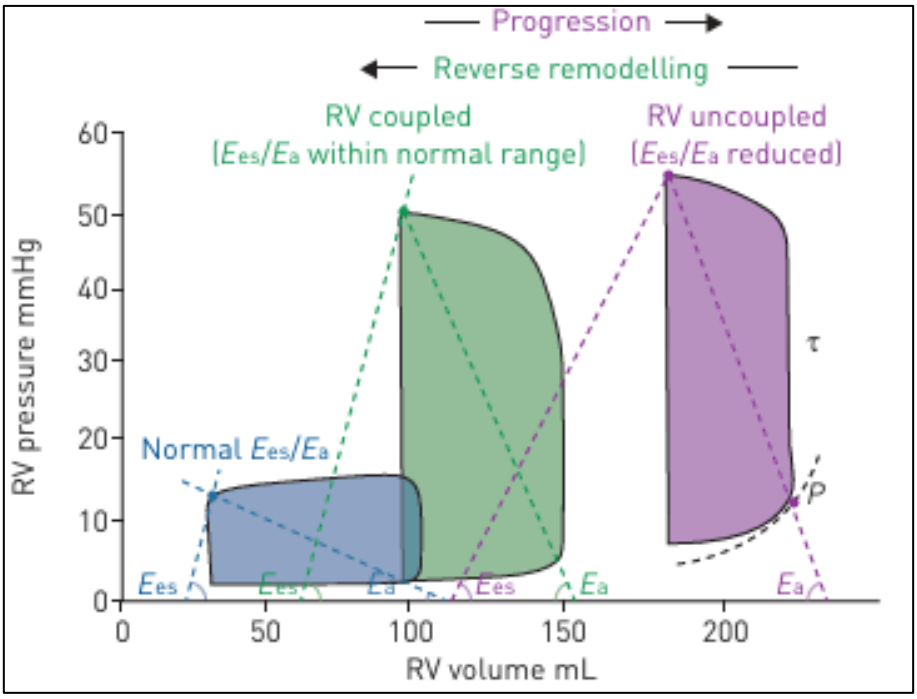

Figure 6. The change in the pressure-volume loop during the progression of pulmonary hypertension (3)

In the early adaptive phase, the RV end-diastolic and end-systolic volume and myocardial work increase as the area under the loop increases, but Ees/Ea stays normal (RV coupled). Later, in the maladaptive phase RV volumes keep increasing, but myocardial work, Ees/Ea, decreases (RV uncoupled). RV: right ventricular,Ea: arterial elastance, Ees: end-systolic elastance

In clinical practice, the routinely used imaging parameters mostly linearly follow the progression of PH. The clinical appearance of biphasic adaptation of the RV is difficult to assess. In response to the progression of the disease and the increase in PVR, the RV becomes more dilated, and systolic function falls, so neither the dimensional parameters nor the ejection fraction, nor other echocardiographic systolic function parameters, such as TAPSE or FAC, follow this biphasic change sensitively enough (3). It is also difficult to evaluate RV/PA uncoupling in clinical routine. Tello and coworkers confirmed that the echocardiographic TAPSE/sPAPe value correlates well with this hemodynamic value (1; 71).

1.3.5. Right ventricular stroke work

Beyond the dimensional, functional, and blood flow parameters, it is also important to evaluate changes in myocardial function to assess adaptation of the RV. Stroke work (SW) that characterizes the work of the myocardium in systole is a well-known parameter; the gold standard measurement method is the determination of the area of the pressure-volume loop (84) (Figure 6.). Right ventricular stroke work (RVSW) describes the active work of the RV myocardium during a contraction, i.e. the ability of the RV to deliver a certain blood volume (stroke volume, SV) into the pulmonary circulation by generating mPAP against a given resistance in the pulmonary arteries (85). In the early phase of PVR increase, the RV myocardium works with systolic reserve that can preserve unchanged pulmonary blood flow. Later, a maladaptive phase develops when systolic adaptation becomes insufficient to maintain normal pulmonary circulation.

In clinical practice, SW cannot be determined precisely, but it can be accurately calculated from the hemodynamic parameters recorded during RHC. The SW can be expressed as the product of the blood volume delivered during systole (SV) and the pressure gradient (RVPgr):

$$RVSW = RVPgr \times SV.$$

At this point, several authors use different formulae and approaches to define RVSW index (RVSWI). In our study on circulating biomarkers, we used the most accepted formula (15):

$$RVSWI = (mPAP-RAP) \times SVI \times 0.0136.$$

According to more recent data, however, a newer approach has been published, which concludes that the pressure gradient can be determined more precisely as the difference between the mean RV pressure or - in the absence of pulmonary stenosis – mean systolic ejection pulmonary pressure (msePAP) and the mean right atrial pressure (RAP as RV end-diastolic pressure equivalent) (85):

$$RVSWI = (msePAP-RAP) \times SVI.$$

In this context, the most accurate approach to the msePAP value is given by the following formula (85):

$$msePAP = 1.25 \times mPAP.$$

Consequently, the RVSW index (RVSWI) may be calculated from these hemodynamic parameters (85; 86):

$$RVSWI = (1.25 \times mPAP - RAP) \times SVI$$

Although it can be hypothesized that RVSWI can characterize the biphasic adaptation of the right ventricle, its clinical usefulness has remained uncertain in the routine clinical management of pre-capillary PH.

1.4. Comorbidities in pre-capillary PH

Generally, PAH used to be most often diagnosed in young female patients. The diagnosis is established following the exclusion algorithm, during which an underlying lung disease, coronary disease, and, in post-capillary PH, left heart diseases are excluded. However, with the accumulation of knowledge and better diagnostic algorithms, the subgroup of elderly patients, who are diagnosed with PAH, is expanding. Although no severe or uncontrolled accompanying disease exists in this subgroup, cardiovascular and pulmonary comorbidities are manifest. This subgroup includes patients with moderate or severe PH who have co-existent left heart failure with preserved ejection fraction, mild lung disease, or metabolic comorbidities, including diabetes. Therefore, based on the current registries, the average age range of PAH patients has recently shifted from 30-40 years to 50-70 years (29; 87).

Patients with PAH and comorbidities (systemic hypertension, diabetes, obesity with a body mass index >30 kg/m^2, ischemic heart disease), which are often associated with left heart disease and myocardial dysfunction were studied using the COMPERA registry (88). The authors found that instead of the risk classification based on the three-strata model already included in the previous guideline, the four-strata model is valid in this subgroup, as well. It can be presumed that in addition to left ventricular dysfunction, these

comorbidities can also damage myocardial function in general and can influence RV function.

1.5. Cytokines in pre-capillary pulmonary hypertension

1.5.1. Cytokines in the development of vascular remodelling and right ventricular adaptation

The pathophysiological background of pre-capillary PH is only partially understood. Although the etiologic and underlying mechanisms are different in some groups of pre-capillary PH, common pathways can still be assumed in the background of the similar nature of vascular remodelling and RV adaptation. Cytokines are signalling proteins produced by tissue residential and immune cells and regulate various biological processes. Several lines of evidence suggest that cytokines, including growth factors, interleukins, and chemokines, are involved in the pathological changes (89).

Growth factors affect the development of vascular remodelling, the proliferation of endothelial cells, smooth muscle cells and fibroblasts, and the formation of plexiform lesions (90) (91). Several GFs are expressed locally in the pulmonary parenchyma and the plexiform lesions. The expression of platelet-derived growth factor (PDGF) and PDGF receptor was elevated in explanted lungs of patients with severe PAH (91) and the appearance of vascular endothelial growth factor (VEGF) and VEGF receptors are confirmed in the plexiform lesions and medial smooth muscle cells of the proximal arteries during lung autopsies (92). Insulin-like growth factor (IGF) is a notable mitogen of vascular smooth cells, and IGF gene depletion leads to reduced proliferation potential in animal models (93). Other studies have proved the elevated circulating levels of these factors in patients and found high transpulmonary gradients of these molecules, suggesting their pulmonary production (94).

The mechanisms behind the abnormal expression of GFs in the lungs in pre-capillary PH are only partially known, and multiple pathways have been implicated. In 80% of hereditary PAH (HPAH), the mutations of the bone morphogenic protein receptor type 2 (BMPR2), a member of TGF (transforming growth factor) superfamily, can be identified, and approximately 20% of sporadic IPAH patients carry this mutation. Epigenetic factors play a role in its penetrance (95). A further 5% of HPAH patients have rare mutations in

other genes of the TGF- superfamily, including activin-like receptor kinase-1 or endoglin. These genetic disorders result in abnormal tissue repair and vascular remodelling caused by the abnormal growth response of pulmonary artery smooth muscle cells and the reduced apoptosis of endothelial cells (96). Interestingly, in the animal model of *Schistosoma* infection, PAH is induced due to the pulmonary activation of TGF-β by bone marrow-derived thrombospondin-1 (97).

Hypoxia is a powerful stimulus for pulmonary vasoconstriction and pulmonary vascular remodelling. The expression of VEGF and FGF (fibroblast growth factor)-2 is increased in endothelial cells by hypoxia-induced signalling molecules (92; 98). Furthermore, the expression of the growth differentiation factor 11, a BMP/TGF-β superfamily member, is induced in pulmonary artery endothelial cells under hypoxia, which plays a crucial role in developing hypoxia-related PH in animal models (99).

Exogenic stimuli including drugs and toxins have been shown to be involved in the development of PAH. Tyrosine kinase inhibitors (TKI) such as imatinib and dasatinib are successfully used to treat chronic myelogenous leukemia. Still, case reports found an increased prevalence of PAH in treated patients, where PAH was (at least partly) reversible in most cases after the cessation of therapy (88; 89). It is presumed that the modulation of PDGF signalling by TKIs is responsible for the development of PAH (100).

Although the exact pathomechanism of vascular remodelling is unclear, the pathogenic role of inflammatory processes may be hypothesized. PAH can be a complication of many immunologic diseases, such as scleroderma, systemic lupus erythematosus, or infectious diseases, such as HIV infection or schistosomiasis. Cytokines, chemokines, and other inflammatory markers form a large group of signal proteins and seem to contribute significantly to PH pathogenesis (89), and the presence of prevascular inflammatory processes can be detected both in animal models and in human studies (101). Stimulation of inflammatory processes in animal models of PH, e.g., overproduction of IL-6, leads to the development of vascular remodelling (79). Interleukin-1β is a key cytokine that is an important mediator of the inflammatory response. Elevated serum levels of IL-1β have been observed in PAH patients and have been shown to correlate with worse outcomes (102). In contrast, inhibiting some inflammatory processes, such as depletion of T helper type 2 cells, can prevent PH (103). The complement cascade is an important element of inflammatory processes and plays a role in developing vascular remodelling and

perivascular inflammation in PH (104; 105). However, more studies involving animal models and human data are needed to consider these cytokines as therapeutic targets in pre-capillary PH.

1.5.2. The role of cytokines in cardioprotection in pre-capillary PH

Experimental and human studies described the involvement of GFs such as VEGF, TGF-β, FGF-2, IGF-I, and PDGF in the development of PAH, but other studies (both in other cardiac disorders and in PAH) suggested that these mediators could exert beneficial effects on cardiac function (17).

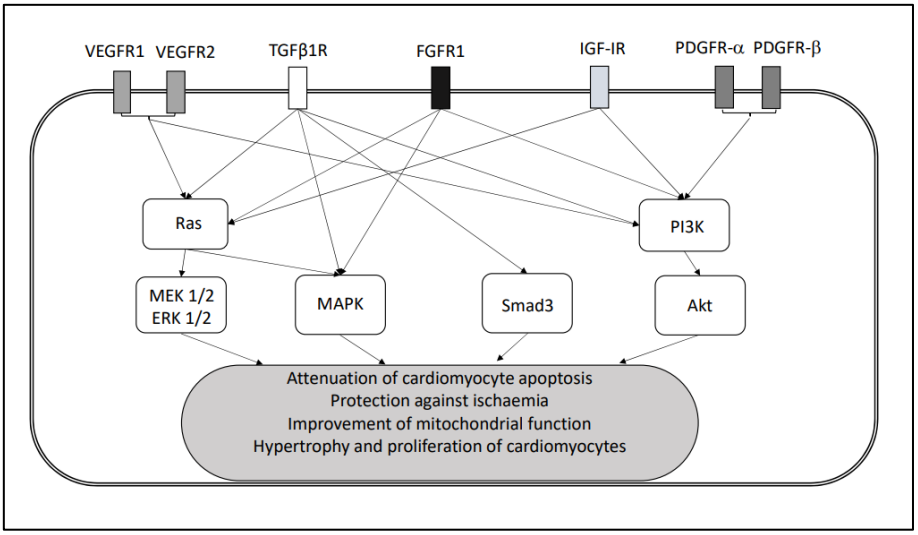

Figure 7. Intracellular pathways of growth factors involved in myocardial protection in pulmonary arterial hypertension

Growth factors use similar intracellular pathways in the adaptation mechanisms of the right ventricular myocardium. These mechanisms support increased myocardial viability against ischemic and metabolic injury and facilitate the development of adaptive myocardial hypertrophy. These processes lead to the preservation of the right ventricular function in the early stage of PAH. VEGFR: vascular endothelial growth factor receptor; TGFβ1R: transforming growth factor - β1 receptor; FGFR: fibroblast growth factor receptor; IGF-IR: Insulin-like growth factor-I receptor; Ras: intracellular signal transduction protein; MEK or MAPK: mitogen-activated protein kinase; SMAD: specific intracellular signal transduction protein; ERK: extracellular signal-regulated kinase; PI3K: phosphoinositide 3-kinase; Akt: protein kinase B. Original work of the author. Reference is found in the main text.

Among their multiple effects, these mediators support hypoxia-induced angiogenesis, restrict hypoxemia-related cardiac injury, promote adaptive hypertrophy and inhibit apoptosis of cardiomyocytes. The major intracellular pathways of GFs involved in the pathophysiology of PH are depicted in Figure 7 (17). However, these mechanisms are not fully elucidated and other cytokines such as apelin and IL-22 are emerging as novel targets. We here describe the cytokines with potential cardioprotective effects in PH, which will be in the focus of the Circulating biomarker study of this thesis.

1.5.2.1. Apelin

Apelin is the endogenous ligand of the apelin receptor (APJ), a member of the G-protein receptor family. The precursor molecule is preproapelin, a 77-amino acid-long peptide, and the active forms with different lengths are derived. Apelin-13, -16, -17, -19, -36. Apelin-13, -17, and -36 exert the most prominent biological functions. Apelin is homologous to angiotensin II; together, they regulate blood pressure and induce myocardial and smooth muscle contraction (106). Apelin and APJ are expressed in various organs, such as the brain's tissues, mammary glands, gastrointestinal (GI) tract, and lungs. In the brain, the hypothalamic secretion of apelin plays a role in regulating fluid and food intake (107). In the mammary gland, apelin messenger ribonucleic acid (mRNA) concentration is elevated during pregnancy and lactation (108). Apelin inhibits histamine release and, in this way, indirectly decreases acid secretion in the stomach (86) and inhibits the glucose-induced insulin secretion in the endocrine cells of the pancreas (109).

Serum and tissue apelin concentrations alter due to hypoxia in the lung, which is regulated by a hypoxia-induced factor (HIF-1α) (83). In an animal model of short-term hypoxic stimulation, apelin mRNA and apelin concentration were elevated in lung tissue (84). Still, chronic hypoxia was associated with decreased pulmonary relative apelin concentration (110).

Apelin has a potent positive inotropic effect (111). It has a direct vasodilatation effect and inhibits angiotensin II-mediated vasoconstriction through an NO-dependent pathway and decreases blood pressure (112); and also plays a role in angiogenesis and regulates energy metabolism and fluid homeostasis (100; 103;104). It was shown in an animal model that it can improve myocardial contraction and reduce cardiac overload by inducing

vasodilation (113). It also has a cardioprotective effect and can inhibit the apoptosis of cardiomyocytes (114). Importantly, in patients with ischaemic left heart failure, a reduced myocardial level of apelin was associated with higher mortality (114).

In left heart failure, the apelin concentration is higher in the left atrial tissue than in the LV tissue, and the atrial apelin concentration correlates with plasma apelin level (115). Furthermore, during the progression of left heart failure, the plasma apelin concentration showed a biphasic change: in the early functional class (FC) I-III stage it increased, but it decreased in the later FC IV stage (107; 108).

Several research groups have investigated the role of apelin in the pathophysiology of PH and right heart failure. It was observed that circulating apelin levels decrease, while others noticed an increase in animal and human studies (106; 109-112). Relevant to PH, it signals via the APJ receptor found on human vascular smooth muscle cells, endothelial cells, and cardiomyocytes (113; 114). Hypoxia, a major factor inducing pulmonary vasoconstriction, triggers apelin expression (116), and signalling pathways known to be involved in the pathomechanism of PAH, such as the BMPR2 and HIF-1, also regulate the expression of apelin and APJ (117) (118). There is some evidence that circulating apelin can be a marker of its cardiac expression. Although pulmonary apelin content is reduced in animals with chronic hypoxic PH, apelin content in the RV is increased, showing a positive correlation to RV pressure. Furthermore, plasma apelin does not correlate with total pulmonary apelin but presents a weak association with RV apelin content (110). The studies suggest that vascular endothelial cells and atrial tissue are the main sources of serum apelin in PH.

In the monocrotaline-induced animal PH model, apelin administration can delay the progression of RV hypertrophy and diastolic dysfunction (117); in the human examination, systemic apelin infusion caused a short-term increase in CO and decrease in PVR in PAH patients (119).

Thus, based on the altered serum concentration of apelin in left heart failure and its altered serum levels in previous studies in PH, a two-phase change in apelin production can be speculated in PH. However, data on circulating apelin levels in patients with PH are limited, and prior research studies on the biomarker role of apelin did not provide consistent results.

1.5.2.2. Interleukin-22 and interleukin-22 receptor

IL22 is a member of the IL10 family and is produced by Th1, Th17, Th22, dendritic cells, and innate lymphoid cells (120). It binds to the heterodimeric IL22 receptor (IL22R) complex. It activates the signal transducer and activator of transcription (STAT) 3 transcriptional factor, which triggers signalling via the Janus kinase/STAT, extracellular signal-regulated kinase, phosphoinositide 3-kinase/protein kinase B pathways (121). The receptor can be found in the skin, lung, small intestine, liver, colon, kidney, and pancreas and is upregulated in response to inflammatory stimuli (122). Conversely, IL22 signalling is involved in neoplasm formations in various cancers, including lung, skin, colorectal, and breast cancers. Furthermore, pathomechanistic steps and disease severity in atopic dermatitis have been linked with IL22 activity (123). On the other hand, as IL22 is expressed by immune cells, it is also involved in adaptive and innate immune processes, such as inducing antimicrobial peptide expression on epithelial surfaces (118). It also protects intestinal epithelial integrity, alleviates inflammation, and prevents intestinal bacterial infection in mice (124).

The decoy receptor, IL22RA2, or IL22 binding protein (IL22BP) inhibits IL22 from binding to IL22R, as it has a much higher affinity for IL22 than IL22R (125). It is expressed in immune cells, dendritic cells, B cells, macrophages (121), and various human tissues, including the placenta, skin, inflamed appendix, lung, gastrointestinal tract, lymph node, thymus, and spleen (126). It provides negative feedback to the effects of IL22 stimulation, and for instance, the downregulation of IL22RA2 leads to the development of colon cancer in mice (127). However, it was also shown that IL22RA2 has a pro-inflammatory role and impairs epithelial barrier function, likely through interaction with IL22, and exacerbates influenza infection and bacterial superinfection in the lungs (128). Furthermore, IL22 was shown to protect against, while IL22RA2 aggravated liver fibrosis and cirrhosis in patients with chronic liver infections (129).

IL22 signalling has also been studied in cardiac conditions. IL22 directly activates the IL22R subunit 1-STAT3 signalling pathway on cardiomyocytes, and IL22 treatment reduces infarct size after ischemia-reperfusion injury (130). Protein expression of the IL22R complex is increased in the cardiac tissue after acute ischemia, and IL22 prevents cardiac rupture by regulating local inflammatory cells and extracellular matrix metabolism (131). Serum IL22 concentration was higher in patients with heart failure of

mixed aetiologies compared to the control group. Interestingly, it showed a negative correlation with the progression of heart failure, and its concentration was lower in the advanced state of heart failure (132).

Regarding PH, IL-22 expression was upregulated in the lung tissue in an experimental model of PH (131), and serum IL22 concentration was elevated, which decreased after treatment with an endothelin receptor antagonist (133) found in a mixed population of patients with PH, with no difference among the clinical groups (133). Importantly, IL22 concentration proved to predict the presence of PH in patients (133). In addition, elevated serum IL22 concentration was reported in patients with scleroderma combined with PAH or ILD compared to patients without lung involvement or control subjects, respectively (134). However, PAH was suspected after echocardiographic examination, and RHC was not done, which suggests a likelihood of false positive diagnoses of PAH in this study. Importantly, IL22R expression was confirmed on pulmonary artery vascular smooth muscle cells (SMCs), and IL22 stimulation led to cell growth (119). Moreover, PH in rats was associated with pulmonary artery SMC IL22 expression (135), suggesting a role of this cytokine in arterial remodelling.

These data imply that IL22 signalling can be a novel, yet less studied pathway in the pathophysiology of PH, however, less is known about the role of IL22RA2 in this process. Crnkovic et al. found that IL22RA2 expression was increased in RV tissue in hypoxia-induced RV remodelling, with expression localized to cardiomyocytes (136). In line with this, the same authors reported a higher serum level of IL22RA2 in patients with IPAH. It was suggested that this phenomenon might be linked with a protective mechanism compensating the inflammatory stimulus by IL22 to prevent the development of fibrosis in RV and promote compensated ventricular remodelling (136). Nonetheless, no data on circulating IL22RA2 concentration are available in other clinical groups of PH, and its dynamics after improvement in the pulmonary haemodynamic of patients is not known.

1.5.2.3. Vascular endothelial growth factor

Members of the VEGF family, including VEGF-A (generally VEGF), -B, -C, -D, -E, and the placental growth factor, play considerable roles in angiogenesis (137). VEGF-A is the most potent angiogenic factor and it is produced in high amounts in the adult lung. The tyrosine kinase VEGF receptors are VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1) and

VEGFR-3 (FLT-4). VEGFR-1 and VEGFR-2 are expressed in vascular endothelial cells, and upon activation, they induce signals for proliferation, migration, and remodelling. Many cell types, including macrophages, platelets, keratinocytes, and renal mesangial cells, express vascular endothelial growth factors. VEGF is involved in a wide range of normal physiological processes. It induces the formation of vascular structures, wound healing, tissue repair, and skeletal muscle development (138; 139).

In PH, the main trigger of VEGF and VEGFR expression is hypoxia (140; 141) and sources include vascular endothelial and myocardial cell. The overexpression of VEGF-A or VEGF-B in the lungs can partially restore endothelium-dependent function and ameliorate PAH in an animal model of chronic hypoxia (141;142). VEGF can be generated in the myocardium in response to myocardial ischemia (141).

VEGF was suggested to be involved in forming plexiform lesions as this cytokine, produced by the modified smooth muscle cells of the plexiform lesions and the medial smooth muscle cells, can activate pulmonary endothelial cells expressing the VEGF receptor (142; 143). In support, VEGFR-2 blockade, combined with chronic hypoxia, resulted in the development of severe PAH in an experimental model due to pre-capillary arterial occlusion induced by proliferating endothelial cells (144).

The VEGFR-1 and VEGFR-2 are expressed both on pulmonary vascular endothelial cells and also on cardiomyocytes, at least in rat models of PH (145), suggesting that VEGF stimulation can induce acute or chronic cardiac effects either by directly acting on cardiomyocytes or by exerting vascular effects. These receptors activate known cytoprotective pathways in myocytes, such as the MEK1/2-Erk1/2-$p90^{rsk}$ (146; 147), which promotes cell survival by increasing the adhesive interactions between cardiomyocytes and extracellular matrix components (148). In an animal model of hypoxia-induced PH, an increased level of VEGF mRNA has been demonstrated in the myocardium, suggesting that VEGF may be one of the factors in the development of hypoxia-induced angiogenesis as shown by an increase in the number of capillaries per myocyte (145). In support, compared to control animals, VEGF mRNA expression was increased in rat RVs with adaptive hypertrophy, but it was unchanged in RV failure (149). Moreover, administering VEGF to the isolated rat heart's recovery solution can improve the myocardium's functional recovery after ischemia-reperfusion injury (150).

Specifically, increased circulating VEGF concentration was measured in patients with IPAH compared to controls (151; 152). In patients, there was a significant positive relationship between plasma VEGF concentrations and the TAPSE, an echocardiographic marker of RV systolic function (152). In other words, patients with better right heart function had higher circulating VEGF values, suggesting a possible role for this mediator in protection against the development of RV failure. In line with this, in an experimental model of LV hypertrophy, the overexpression of VEGF using a viral vector resulted in the preservation of cardiac function (151). Interestingly, the plasma VEGF levels of patients did not change after three months of PAH-targeted therapy (153), although treatment was associated with a decrease in pulmonary vascular resistance and an improvement in CI.

Table 5. Summary of evidence of the involvement of apelin, VEGF and IL22RA2 in pulmonary hypertension

	Apelin	**Ref**	**VEGF**	**Ref**	**IL22RA2**	**Ref**
Regulation	•Hypoxia •HIF-1α •BMPR2	(116) (154)	•Hypoxia	(140) (141)	•Pro-, anti-inflammatory effects	(155)
Sources	•Vascular endothelial cells	(156)	•Vascular endothelial cells	(157) (158)	•Immune cells •Various organs e.g. lungs •RV tissue	(155) (135) (104)
	•Atrial tissue	(115) (156)	•Myocardium	(141)		
Circulating concentration	•Biphasic change – increased in early phase, decreased in advanced phase	(159)	•Increased in IPAH	(153) (152)	•Immune cells •Various organs e.g. lungs •RV tissue	(136) (133) (134)
	•Decreased serum apelin-13 level in severe IPAH	(160)	•Correlation with systolic RV function	(152)		
	•Correlation to atrial apelin level	(115)	•No change after pulmonary vasodilator therapy	(153)		
	•Increased apelin-17 serum level in IPAH	(161)				

Table 5. continued

	Apelin	Ref	VEGF	Ref	IL22RA2	Ref
Vascular remodelling Effect/known intracellular mechanism	• Inhibition of endothelial cells apoptosis and proliferation / PI3-K/AKT pathway	(154) (162)	Formation of plexiform lesions	(27) (143) (142)		
	• NO-dependent vasodilatation/AKT, AMP kinase	(116) (163)				
Right heart adaptation Effect/known intracellular mechanism	• Positive inotropic effect/ phosphorylase C, protein kinase C, Na^+/H^+ exchange, Na^+/Ca^{2+} exchange	(111) (164)	• Direct cytoprotective effect / MEK1/2-Erk1/2-p90rsk	(147)	• Might compensate for myocardial fibrosis • Might promote adaptive myocardial remodelling	(136)
	• attenuated apoptosis of myocardial cells /PI3K/AKT pathway	(165) (166)	• Interactions between cardiomyocytes and extracellular matrix components / p125FAK	(148)		

AKT- protein kinase B; BMPR- bone morfogenetic protein receptor; ERK - extracellular signal-regulated kinases; ERA – endothelin receptor antagonist; HIF – hypoxic induced factor; IL22 – interleukin 22; IL22R- interleukin 22 receptor; IPAH: idiopathic pulmonary arterial hypertension; MEK - mitogen-activated protein kinases; NK cell – natural killer cell; NO - nitric oxide; PI3-K-phosphatidylinositol 3-kinase; RV- right ventricle; TGF: transforming growth factor; VEGF: vascular endothelial growth factor

2. Objectives

2.1. In the Right ventricular work study, we aimed to determine if the right ventricular stroke work index (RVSWI)

1. is associated with pulmonary vascular resistance in a mixed population of patients with and without pre-capillary PH.
2. differs in patients newly diagnosed with pre-capillary PH compared to control subjects.
3. is different in untreated PAH and CTEPH or influenced by the presence of comorbidities which can alter myocardial function.
4. measured at diagnosis predicts clinical response to specific therapies in PAH and CTEPH.

2.2. In the Circulating biomarker study, we aimed to assess the blood concentrations of apelin, IL22RA2 and VEGF, which have potential links to myocardial function, and to determine if

1. these cytokines can distinguish newly diagnosed patients with pre-capillary PH from control subjects.
2. circulating cytokine levels are associated with clinical, hemodynamic and echocardiographic parameters at the diagnosis of pre-capillary PH.
3. changes in blood biomarker concentrations are related to the changes in patients' clinical and echocardiographic parameters following specific treatment.

3. Methods

3.1. Subjects and study design

In our studies, pre-capillary PH patients were recruited, and patients with PH due to left heart disease and PH with unclear and/or multifocal mechanisms were excluded (18). During the diagnostic work-up, patients underwent detailed examinations, including collecting previous medical data, echocardiography, pulmonary angio-CT, lung perfusion/ventilation scans, RHC, lung function tests, blood tests, 6-minute walk test (6MWT) and determination of FC (1). In addition, venous blood samples collected only at diagnosis were stored for cytokine measurements. Lung diseases were diagnosed by a pulmonary specialist of our ILD Team when necessary. Pre-capillary PH was established according to applicable guidelines (1).

In the Right ventricular work study, we collected data from patients undergoing RHC due to suspected PH and treated PH patients who needed re-evaluation of their pulmonary hemodynamic status at the Cardiopulmonary Unit (Department of Pulmonology, Semmelweis University Clinical Centre) between January 2020 and March 2023.

Suspected PH was defined as increased maximal velocity of tricuspid regurgitation (Vmax>3.4 m/s) or moderately elevated maximal velocity (2.9m/s<Vmax<3.4 m/s) with other signs of right heart dysfunction (RV>32 mm, D sign, and TAPSE < 18 mm) on echocardiography.

Based on 2022 ESC/ERS guideline pre-capillary PH was defined as mPAP > 20 mmHg, PAWP ≤ 15 mmHg, and PVR > 2 WU during RHC (1). Patients with postcapillary pulmonary hypertension (PH) and PH associated with lung diseases and/or hypoxia were excluded from the study. PAH was diagnosed after the exclusion of other possible causes (1). CTEPH was defined as pre-capillary PH with thromboembolic origin based on imaging (chest CT and lung perfusion scans). Patients with normal hemodynamic parameters were described as “non-PH”. Patients with abnormal hemodynamic parameters and who did not fulfill PAH or CTEPH criteria were excluded from the study. Due to the possible influence of the changes in intrathoracic pressure and lung compliance on the PVR results, group 3 PH patients were also excluded from this study. In PAH, we evaluated the proportion of specific subgroups. In CTEPH, after a radiologist expert's review of chest CT images, we assessed the extent of thromboembolism and the

involvement of the pulmonary branches of the proximal and distal arteries based on the guideline criteria (1).

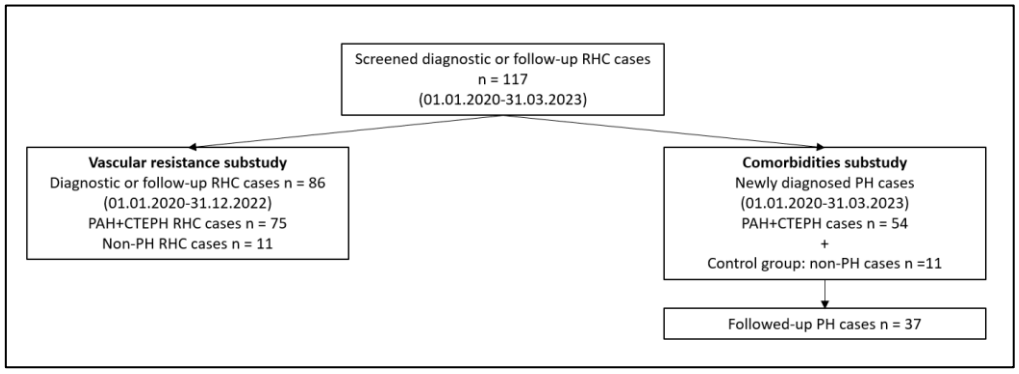

Figure 8. Patient selection algorithm in Right ventricular work study

During the entire study period, diagnostic and follow-up RHC examinations were performed in various PH groups. Non-PH and PH patients with diagnostic and follow-up RHC were included in the Vascular resistance substudy, and some of our patients had multiple RHCs at different times. In the Comorbidities substudy, only newly diagnosed and non-treated PH patients participated in addition to the non-PH control group. CTEPH: chronic thromboembolic pulmonary hypertension, PAH: pulmonary arterial hypertension, PH: pulmonary hypertension, RHC: right heart catheterization

We examined the data in two substudies. In the first Vascular resistance substudy, we aimed to investigate the work of RV at different degrees of right heart afterload regardless of whether PH exists. This analysis included data from a larger population of non-PH, newly diagnosed, or already treated PAH and CTEPH patients who underwent RHC. We evaluated the relationship between RVSWI and PVR, the most important hemodynamic parameter in determining the severity of vascular changes.

In the second, Comorbidities substudy, we included only newly diagnosed, untreated PAH and CTEPH patients. We examined the association of RVSWI with clinical parameters, comorbidities, and mortality risk score at diagnosis. At follow-up, we studied the changes in clinical parameters when patients were divided into low- and high-RVSWI subgroups based on values at diagnosis. The patient selection flow chart is shown in Figure 8.

In the Circulating biomarker study, newly diagnosed pre-capillary PH (PAH, CTEPH, and PH associated with lung disease) patients and non-PH volunteers as control subjects were enrolled at the Cardiopulmonary Unit, Department of Pulmonology, Semmelweis

University, Budapest, Hungary, between June 2017 and September 2020. The 2015 ESC/ERS guidelines confirmed pre-capillary PH i.e. mPAP>25 mmHg and PAWP<15 mmHg (18). For some PH patients, echocardiography, 6MWT, and blood tests were repeated, and venous blood samples were stored during control visits after therapy. Control subjects did not have signs of heart failure and did not have any uncontrolled chronic medical conditions. Medical history was taken, echocardiography and lung function tests were performed, and blood samples were stored for later analysis (Figure 9).

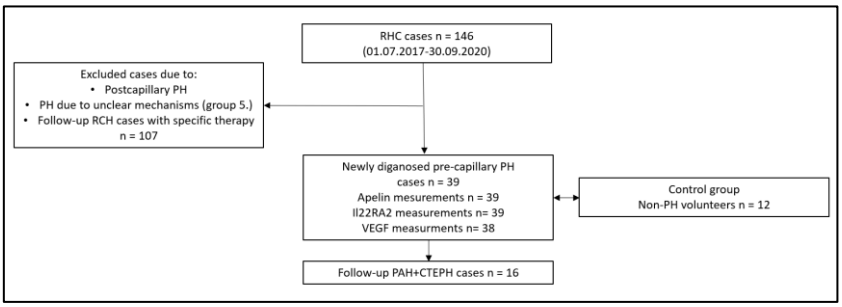

Figure 9. Patient selection algorithm in Circulating biomarker study

RHC examinations were performed in all PH groups with diagnostic and follow-up indications during the study period. Non-PH, post-capillary PH, and pre-capillary PH patients with specific pulmonary vasodilators were excluded. In the follow-up period, 16 PAH and CTEPH patients were examined with specific pulmonary vasodilator therapy. CTEPH: chronic thromboembolic pulmonary hypertension, IL22RA2: interleukin22 receptor agonist, PAH: pulmonary arterial hypertension, PH: pulmonary hypertension, RHC: right heart catheterization, VEGF: vascular endothelial growth factor

3.2. Data collection and measurements

General data collection: We collected demographic data (age, body surface area, sex), results from RHC, echocardiography, and laboratory examinations, and 6MWT and functional classes were also recorded. In addition, mortality risk was evaluated based on the four-strata model at baseline and follow-up (72;73).

Right heart catheterization: The investigation was performed with the patient in a supine position, and the pressure transducer was set to zero at the mid-thoracic level. Routine parameters were registered, including RAP, RV pressure, PAP and PAWP. CO was measured using thermodilution. During the catheterization of IPAH patients,

vasoreactivity tests were done with inhaled iloprost for 15 minutes. Furthermore, the stroke volume (SV = CO/heart rate), SV index (SVI=SV/body surface area), PVR = (mean PAP-PAWP)/CO, and the CI = CO/body surface area were calculated (18; 169). Additional parameters were determined to assess the severity of pulmonary arterial remodelling and systolic RV function, including pulmonary arterial compliance (PAC = SV/(systolic PAP – diastolic PAP)) (167).

In the Circulating biomarker study, RVSWI was calculated as (mean PAP—RAP) x SVI x 0.0136 (168), while in the Right ventricular work study, the RVSWI was determined according to Chemla et al. (RVSWI = (1.25xmPAP - RAP) x SVI) (85).

Echocardiography: Echocardiography was performed with the Mindray DC-70 X-Insight instrument (Shenzhen Mindray Bio-Medical Electronics Co., Shenzen, China). We evaluated the RV outflow tract velocity-tome integral (RVOT VTI) using the pulsatile Doppler curve in the parasternal short-axis view. From the apical 4-chamber view, we measured the RA area (RAA). TAPSE was acquired by placing an M-mode cursor through the tricuspid lateral annulus and measuring the amount of maximal longitudinal motion of the annulus. We calculated the right atrial-ventricular pressure gradient from the peak velocity of TR tracings. We estimated the systolic pulmonary pressure (SPAPe) by adding the RAP to the pressure gradient according to the ASE recommendation based on the size and the change of the inferior vena cava during respiration (1; 169; 170). RV/PA coupling was determined from echocardiographic parameters as a ratio of TAPSE/sPAPe. The cut-off value of 0.32 separates the normal coupling group (TAPSE/sPAPe > 0.32) from the uncoupling group (TAPSE/sPAPe ≤ 0.32) (171; 172)

Assessment of comorbidities: The presence of specific comorbidities that are often linked with left heart disease, especially heart failure with preserved ejection fraction, i.e. arterial hypertension, diabetes mellitus, coronary heart disease, and obesity (defined by a body mass index >30 kg/m^2) were recorded according to Rosenkranz et al. (88). These comorbidities had been diagnosed before the diagnostic workup due to suspected PH.

Blood tests: N-terminal pro-brain natriuretic peptide (NT-proBNP) concentration and arterial blood gases were measured in patients.

Enzyme-linked immune-assay (ELISA) measurements in the Biomarker study: Serum and EDTA plasma were collected from subjects, spun down at 3000 rpm for 10 minutes, and stored at -80°C for later analysis. Serum samples were used to quantify VEGF-A (8) and

apelin. At the same time, IL22RA2 concentration was measured in plasma (15) using commercially available assays according to the manufacturer's instructions (VEGF-A: Human VEGF ELISA kit, Proteintech Europe, Manchester, UK, detection limit: 6.5 pg/ml; apelin (detecting all active forms including apelin-36, apelin-31, apelin-28 and apelin-13): RayBio Human/Mouse/Rat Apelin C-Terminus Enzyme Immunoassay Kit, RayBiotech, Norcross, GA, USA, detection limit: 15.8 ng/ml; IL22RA2: RayBio Human IL-22BP ELISA Kit, RayBiotech, Norcross, GA, USA, detection limit: 1.64 ng/ml).

Lung function tests: All subjects performed spirometry and body plethysmography, and lung diffusing capacity was measured simultaneously (173).

6-minute walk testing: 6MWT was performed in a standard 20-m-long corridor with continuous oxygen saturation and heart rate monitoring. Before and after the test, blood pressure and Borg score were determined (174).

3.3. Statistical analysis

Subject characteristics were analysed using t-test, Mann-Whitney U-test, ANOVA or the one-way Kruskal-Wallis ANOVA with Bonferroni post hoc analysis or chi square test. Data were expressed as mean ± standard deviation. Cytokine concentrations did not show a normal distribution (either before or after log transformation); the Mann-Whitney test, Wilcoxon signed rank test, Kruskal-Wallis test with Dunn's post hoc analysis and Spearman correlation were used to assess the relationship between cytokine concentrations and clinical factors. Data are expressed as median (interquartile range). For data analysis and electronic artwork creation, the GraphPad Prism 9.1 software package was used (GraphPad Software, San Diego, USA). The ability of apelin to identify patients with PH was assessed with the receiver operating characteristic (ROC) curve and optimum cut-off values were chosen using the Youden index (MedCalc version 19., MedCalc software Ltd, Ostend, Belgium). In the Right ventricular work study the Spearman-method was used to assess correlations, and data were analyzed with (SPSS 28.0 (IBM SPSS Statistics, Chicago, USA). Missing data were not used in the calculations. Follow-up time was calculated according to Schemper and Smith (175). $p<0.05$ was considered significant.

4. Results

4.1. Right ventricular work study

4.1.1. Vascular resistance substudy

4.1.1.1. Patient characteristics

Between 01 January 2020 and 31 December 2022, we performed 86 RHC examinations due to suspicion of PH as part of the diagnostic workup, or it was indicated as a follow-up examination in patients already treated with pre-capillary PH.

Table 6. General characteristic and haemodynamic parameters

Variable	Value
Right heart catheterization, N	86
Sex, male/female, N	27/55
Age, years	58.5±14.2
BSA, kg/m^2	1.9±0.3
mPAP, mmHg	43±16
sPAP, mmHg	67±25
dPAP, mmHg	28±13
RAP, mmHg	7±5
PAWP, mmHg	10±4
SVI, mL/m^2/beat	34±9
CI, L/min/m^2	2.6±0.7
PVR, Wood Unit	7.4±4.2
RVSWI, g/m^2/beat	1520±694

BSA: body surface area, CI: cardiac index, N: number, s/m/dPAP: systolic/mean/diastolic pulmonary artery pressure, RAP: right atrial pressure, PVR: pulmonary vascular resistance, PAPW: pulmonary arterial wedge pressure, RAP: right atrial pressure, RVSWI: right ventricular stroke work index, SVI: stroke volume index. Adopted from (176) under the terms of the Creative Commons Attribution Non-Commercial Licence 4.0 (http://creativecommons.org/licenses/by/4.0/).

Significant pre-capillary PH was found in 71 patients (75 incident cases) because RHC was repeated in 4 patients. PAH was diagnosed in 47 cases, and CTEPH in 28 patients. PH was not confirmed in 11 cases (2 men, 9 women, average age 62.6±11.4 years). We used the results of all 86 RHC cases in our subanalysis (Table 6).

4.1.1.2. Association of RVSWI with pulmonary vascular resistance

We found a moderate positive correlation between RVSWI and PVR (Figure 10). However, the curve suggests a biphasic relationship with 10.4 WU as the breaking point, i.e., below 10.4 WU, the relationship is positive. In comparison, above 10.4 WU, there seems to be a negative association between RVSWI and PVR. In addition, coupling could be assessed based on the TAPSE/sPAPe ratio in 84 patients. Only one patient in the subgroups of PVR > 10.4 WU (N=19) presented with coupling (5%), while 38 out of the 63 patients had coupling in the subset with PVR ≤ 10.4 (60%, p<0.001, Fisher's exact test).

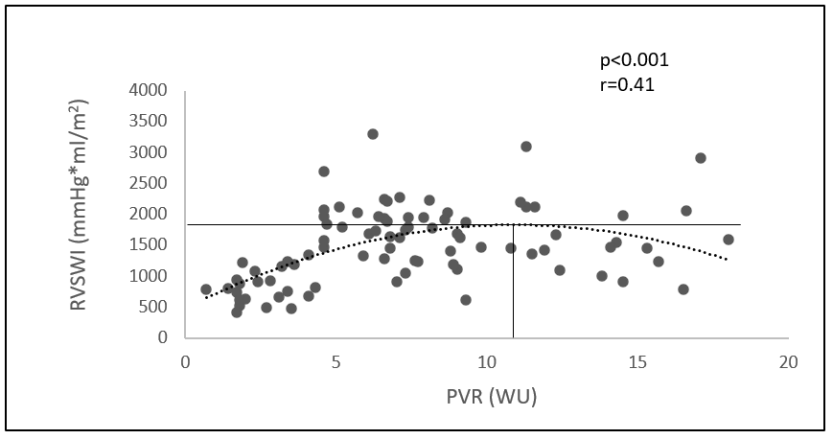

Figure 10. Biphasic relationship between RVSWI and PVR.

We found a significant association between RVSWI and PVR (Spearman's correlation). During the progression of PVR, RVSWI also increases in the left part of the curve. After the inflection point of PVR=10.4, RVSWI decreases when PVR further rises. RVSWI: right ventricle stroke work index, PVR: pulmonary vascular resistance, WU: Wood unit. Adopted from (176) under the terms of the Creative Commons Attribution Non-Commercial Licence 4.0 (http://creativecommons.org/licenses/by/4.0/).

4.1.2. Comorbidities substudy

4.1.2.1. Patient characteristics

One hundred seventeen patients were screened for the study, and 65 were enrolled based on the inclusion and exclusion criteria. Out of the enrolled patients, 30 had PAH (idiopathic: N=20, associated with portal hypertension: N=2, drug-induced: N=1, associated with congenital heart disease: N=5, associated with connective tissue disease:

N=2), 24 had CTEPH (proximal obstruction: N=11, distal obstruction: N=13), and 11 were ascribed to the control group. The median time between diagnosis and the end of clinical observation was 12 months (min-max: 3-17 months). PAH and CTEPH patients had higher haemoglobin and NT-proBNP values compared to control patients ($p<0.05$) (Table 7).

Table 7. Patient characteristics

Variables	Control N=11	PAH+CTEPH N=54	p-value	PAH N=30	CTEPH N=24	p-value
Male %	18 %	40 %	**p<0.05**	27 %	50%	**p<0.05**
Age, years	60±11	59±13	0.47	63±13	58±10	0.10
BSA, kg/m^2	1.9±0.4	1.9±0.2	0.49	1.8±0.2	2.0±0.2	**p<0.05**
6MWD, m	322±181	311±189	0.36	295±171	360±184	0.09
Echocardiographic parameters						
sPAPe, mmHg	39±13	66±20.2	**p<0.001**	65±16.8	68.5±23.5	0.14
RV diam, mm	30.6±3.8	40.6±8.6	**p<0.001**	40.6±8.6	40.4±8.4	0.35
RA area, cm^2	19.4±4.7	25.5±8.1	**p<0.05**	25.4±8.4	25.7±7.3	0.31
TAPSE, mm	23.6±4.2	18.6±5.8	**p<0.05**	19.1±6.4	18.1±4.7	0.56
RVOT VTI, cm	13.4±2.8	12.7±5.4	0.09	14.0±6.2	11.4±3.6	0.14
Laboratory tests						
NT-proBNP, pg/ml	323±117	2344±2959	**p<0.05**	2335±3401	2354±2312	0.48
GFR, ml/min	84±14	72±19	0.07	71±19	73±19	0.43
Hgb, g/L	128±16	152±19	**p<0.05**	151±20	152±17	0.33
GOT, U/L	32±20	27±13	0.14	28±10	27±15	0.11
GPT, U/L	26±17	27±25	0.44	27±19	27±31	0.47
Art. pH	7.43±0.02	7.41±0.04	0.14	7.41±0.03	7.41±0.04	0.31
PaO_2, mmHg	64±12	62±15	0.5	63±16	62±13	0.48
$PaCO_2$, mmHg	35±5	35±7	0.44	35±6	35±7	0.37

Data are presented as mean ± standard deviation. Control vs Total PH and PAH vs. CTEPH groups were compared with T test, Art. PCO_2: Arterial carbon dioxide pressure, Art. PO_2: arterial oxygen pressure, BSA: body surface area, CTEPH: chronic thromboembolic pulmonary hypertension, GFR: glomerular filtration rate, GOT: glutamic-oxaloacetic transaminase, GPT: glutamic-pyruvic transaminase, Hgb: haemoglobin, NT-proBNP: N-terminal prohormone of brain natriuretic peptide, PAH: pulmonary arterial hypertension, sPAPe: estimated pulmonary arterial systolic pressure, RA: right atrium, RV: right ventricle, RVOT VTI: right ventricular outflow tract velocity-time integral TAPSE: tricuspid annular plane systolic excursion, 6MWD: 6-minute walk distance. Adopted from (177) under the terms of the Creative Commons Attribution Non-Commercial Licence 4.0 (http://creativecommons.org/licenses/by/4.0/).

SvO_2 was markedly higher in the PAH group than in CTEPH (Table 10). We did not find vasoreactivity in any IPAH patients. RVSWI was higher in both PAH and CTEPH

patients compared to the control group, but RVSWI showed no difference between the PAH and CTEPH groups (Table 8).

Variables	Control	PAH+ CTEPH	PAH	CTEPH	p
sPAP, mmHg	27.3±6.9	69.7±19.3	68.2±20.2	71.5±17.3	0.13
dPAP, mmHg	9.4±3.8	28.8±9.4	28.5±9.6	29.2±9.1	0.15
mPAP, mmHg	16.3±2.6	43.9±11.6	43.1±12.3	44.8±10.4	0.12
RAP, mmHg	1.7±3.8	7.8±4.7	7.2±4.3	8.5±4.9	0.16
PAWP, mmHg	5.6±3.7	9.5±3.7	9.0±3.8	10.1±3.4	0.27
CI, L/min/m²	2.8±0.6	2.4±0.6	2.5±0.5	2.4±0.7	0.27
PVR, WU	2.2±0.8	8.3±4.1	8.2±4.2	8.4±34	0.39
PAC, mL/mmHg	5.8±4.8	1.7±0.9	1.7±0.9	1.8±1.2	0.43
RVSWI, mmHg*mL/m²	704±140	1408±391	1406±342	1409±470	0.98
SvO_2,%	75.5±5.5	67±10	69.2±10.1	64±9.1	**<0.05**

Data are presented as mean ± standard deviation. Control vs Total PH and PAH vs. CTEPH groups were compared with T-test. CI: cardiac index, CTEPH: chronic thromboembolic PH, p: p-value, PAC: pulmonary arterial compliance, PAH: pulmonary arterial hypertension s/d/mPAP: systolic/mean/diastolic pulmonary artery pressure, PAWP: pulmonary arterial wedge pressure, PVR: pulmonary vascular resistance, RAP: right atrial pressure, RVSWI: right ventricular stroke work index, SVO_2: mixed venous oxygen saturation. Adopted from (177) under the terms of the Creative Commons Attribution Non-Commercial Licence 4.0 (http://creativecommons.org/licenses/by/4.0/).

4.1.2.2. Association of RVSWI with clinical parameters, comorbidities and mortality risk score at diagnosis

We found no correlation between RVSWI and hemodynamic (PVR, PAC, SvO_2), echocardiographic (TAPSE, RV diameter, RAA, RVOT VTI, eccentricity index), and other clinical parameters (age, NT-proBNP, FC, 6MWD) at baseline in all patients with PH or in the subgroup of CTEPH ($p>0.05$ for all parameters, data not shown).

However, in patients with PAH, RVSWI was related to the eccentricity index ($r=0.48$, $p=0.01$). Furthermore, RVSWI showed no difference in patients with coupling and uncoupling (coupling: 1386±426 mmHg*mL/m², N=19 vs. uncoupling: 1421±389 mmHg*mL/m², N=35, $p=0.76$).

Table 9. Subgroup analysis based on the presence of comorbidities at diagnosis

Variables	PH with comorbidities N=31	PH without comorbidities N=23	P-value
Age, years	62±11	55±14	**<0.05**
6MWD, m	284±196	367±146	0.13
NT-proBNP, pg/ml	2418±3613	2249±1693	0.84
Hemodynamic parameters at diagnosis			
sPAP, mmHg	64.8±18	76.2±18.6	**<0.05**
dPAP, mmHg	27.5±9.4	30.6±9.1	0.23
mPAP, mmHg	41.1±11.3	47.6±10.7	**<0.05**
RAP, mmHg	8.9±4.6	6.3±4.3	**<0.05**
PAWP, mmHg	10.4±3.2	8.3±3.9	**<0.05**
CI, L/min/m^2	2.6±0.6	2.2±0.5	**<0.05**
PVR, Wood Unit	6.7±3.5	10.3±3.9	**<0.05**
PAC, mL/mmHg	2.0±1.1	1.4±0.7	0.13
SVO_2,%	68±11	65±8	0.26
RVSWI, mmHg*mL/m^2	1323±378	1513±391	**<0.05**
Echocardiographic parameters at diagnosis			
TAPSE, mm	19.4±6.2	17.6±4.6	0.24
RV diameter, mm	40.1±8.5	41.2±8.6	0.66
RA area, cm^2	25.8±8.2	25.1±7.5	0.76
RVOT VTI, cm	13.8±5.8	11.0±3.7	0.08

Data are presented as mean ± standard deviation. PH with and without comorbidities groups were compared with T-Test. CI: cardiac index, NT-proBNP: N-terminal prohormone of brain natriuretic peptide, PAC: pulmonary arterial compliance, s/d/mPAP: systolic/mean/diastolic pulmonary artery pressure, PVR: pulmonary vascular resistance, RA: right atrium, RAP: right atrial pressure, RV: right ventricular, RVOT VTI: right ventricular velocity time integral, RVSWI: right ventricular stroke work index, SVO_2: mixed venous oxygen saturation, TAPSE: tricuspid annular plane systolic excursion, 6MWD: 6-minute walk distance. Adopted from (177) under the terms of the Creative Commons Attribution Non-Commercial Licence 4.0 (http://creativecommons.org/licenses/by/4.0/).

We examined the association between RVSWI and the presence of comorbidities that potentially modify cardiomyocyte function. Thirty-one patients (CTEPH N=14, PAH=17) had at least one comorbid condition at the time of PH diagnosis, all were well-controlled with adequate treatment (1 comorbidity: N=15, 2-4 comorbidities N=16, Table 9). Comorbidities included systemic hypertension (N=26), diabetes mellitus (N=10), coronary heart disease (N=7), and obesity (N=8). Patients with comorbidities were older and had less severe PH (characterized by lower values for mPAP, PVR and higher CI) than patients without comorbidities. However, RAP and PAWP were higher in the comorbid group than in patients without accompanying diseases. Of note, RVSWI was decreased in comorbid patients compared to patients with no comorbidities (1323±384

vs. 1522±400, mmHg*mL/m^2, p=0.04). Furthermore, patients with PAH and a comorbidity (N=17) had lower RVSWI than PAH patients without comorbidities (N=13, p=0.001), as shown in Figure 11. Still, no difference was noted between comorbid and non-comorbid patients with CTEPH (p=0.37).

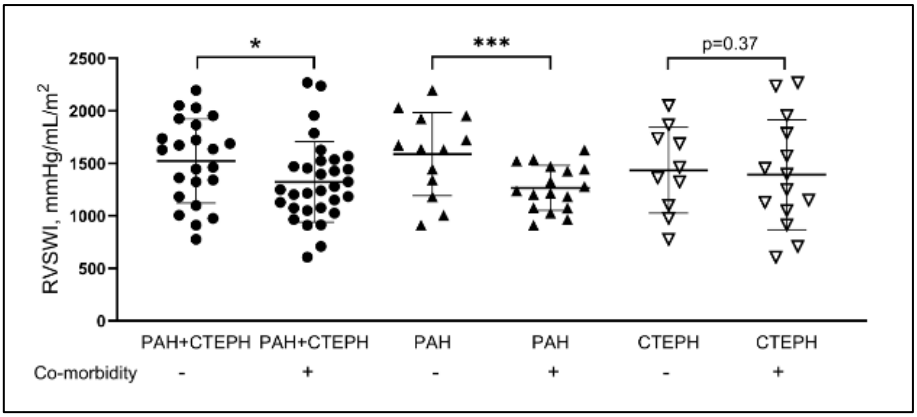

Figure 11. RVSWI at diagnosis between patients with and without comorbidities. Mean with standard deviation is shown, groups are compared with unpaired t-test. *p<0.05, ***p<0.001. CTEPH: chronic thromboembolic pulmonary hypertension, PAH: pulmonary arterial hypertension, RVSWI: right ventricular stroke work index. Adopted from (177) under the terms of the Creative Commons Attribution Non-Commercial Licence 4.0 (http://creativecommons.org/licenses/by/4.0/).

At diagnosis 10, 12, 19, and 13 patients were grouped into having a low, intermediate-low, intermediate-high, and high mortality risk, respectively. RVSWI did not differ in groups with different mortality risk (low: 1368±382 vs. intermediate-low: 1352±408 vs. intermediate-high:1529±416 vs. high:1311±382 mmHg*mL/m^2; p=0.82).

4.1.2.3. Baseline RVSWI and parameters at clinical follow-up

Out of the PAH and CTEPH groups, 37 patients completed clinical visits with follow-up measurements within 12 months after initiating adequate therapies or following surgical interventions. Pharmacological treatments are shown in Table 10. Out of the CTEPH patients, one underwent balloon pulmonary angioplasty, and one patient had a pulmonary endarterectomy before the follow-up visit. The other 17 patients from the study population either did not receive pulmonary vasodilator therapy because of early disease stage (N=5, mPAP: 21-24 mmHg), or they had inadequate compliance to treatment (N=5)

or had received specific therapy for less than six months (N=7), so their control examinations not yet available at the end of the observation period.

Patients were divided by the median RVSWI value (1450 mmHg*mL/m^2) at diagnosis (Table 12). More severe PH with increased PAP but increased CI were found in the high-RVSWI (≥1450 mmHg*mL/m^2) than in the low-RVSWI subgroup (<1450 mmHg*mL/m^2). We analyzed the change in mortality risk score after short-term follow-up (median: 12; range 3-17 months). The improvement in the risk score was higher in the high-RVSWI group than in the low-RVSWI group, and we noted a trend for a higher improvement in NT-proBNP in the high-RVSWI patients (Table 10).

Table 10. Follow-up data of based in the subgroups based on baseline RVSWI values

Variables	Low-RVSWI group N=18	High-RVSWI group N=19	P-value
Baseline mortality risk stratification parameters			
PAH/CTEPH, N	10/8	11/8	1.00
6MWD, m	304±205	331±150	0.83
FC	2.8±0.7	2.8±0.5	0.79
NT-proBNP, ng/ml	2882±2806	2426±3579	0.78
Mortality risk score	2.6±0.9	2.8±0.5	0.79
Baseline haemodynamic parameters			
sPAP, mmHg	69.6±14.8	82.6±14.5	**<0.001**
dPAP, mmHg	27.6±7.0	35.2±8.3	**<0.001**
mPAP, mmHg	43.2±8.4	52.2±8.9	**<0.001**
RAP, mmHg	7.2±3.6	8.0±5.3	0.68
PAWP,mmHg	7.8±3.0	10.1±3.6	0.06
CI, L/min/m^2	2.1±0.4	2.5±0.5	**<0.05**
PVR, Wood Unit	10.2±3.8	9.3±3.8	0.69
SvO_2,%	62.6±9.5	66.2±7.1	0.30
Baseline echocardiographic parameters			
TAPSE, mm	17.2±5.9	17.6±4.9	0.82
RV diameter, mm	37.5±8.7	43.1±8.8	0.06
RA area, cm^2	23.2±7.5	27.9±8.5	0.08
RVOT VTI, cm	11.8±6.2	12.7±3.2	0.69

Table 10 continued Variables	Low-RVSWI group N=18	High-RVSWI group N=19	P-value
Short-term follow-up data using the COMPERA 2.0 stratification parameters			
Therapy at follow-up			
PDE5i, N	5	10	NA
ERA, N	2	0	NA
PDE5i + ERA, N	4	4	NA
sGC stimulator, N	3	3	NA
PGI_2, N	1	0	NA
PDE5i + PGI_2, N	1	0	NA
PDE5i + ERA + PGI_2, N	1	1	NA
Change in outcomes compared to baseline			
Δmortality risk score	0.0±1.0	-0.7±0.6	**<0.05**
Δ6MWD, m	32±196	29±132	0.94
ΔNT-proBNP, pg/ml	56±2010	-1280±2103	0.06
ΔFC	0.6±0.6	-0.7±0.5	0.84

Data are presented as mean ± standard deviation. Groups were compared with unpaired t-test or Mann-Whitney test. CI: cardiac index, ERA: endothelin receptor antagonist, FC: WHO functional class, NA: not applicable. NT-proBNP: N-terminal prohormone of brain natriuretic peptide, PAC: pulmonary arterial compliance, s/d/mPAP: systolic/mean/diastolic pulmonary artery pressure, PDE5i: phophodiesterase-5 inhibitor, PGI_2: prostacyclin, PVR: pulmonary vascular resistance, RA right atrium, RAP: right atrial pressure, RV: right ventricular, RVSWI: right ventricular stroke work index, RVOT VTI: right ventricular velocity time integral, sGC: soluble guanylate cylase, sPAPe: estimated pulmonary arterial systolic pressure, SVO_2: mixed venous oxygen saturation, TAPSE: tricuspid annular plane systolic excursion, 6MWD: 6-minute walk distance. Adopted from (177) under the terms of the Creative Commons Attribution Non-Commercial Licence 4.0 (http://creativecommons.org/licenses/by/4.0/).

4.2. Circulating biomarker study

4.2.1. Subject characteristics

Thirty-nine patients with newly diagnosed pre-capillary PH (PAH, CTEPH, and PH associated with lung disease) and 12 volunteers were enrolled in the study. Sixteen patients were diagnosed with PAH (IPAH n=13, PH associated with portal hypertension N=2, PH due to connective tissue disease N=1), 15 patients had CTEPH, and 8 patients had PH due to lung diseases (ILD N=5, chronic obstructive pulmonary disease N=3). Sixteen patients (PAH N=12, CTEPH N=4) underwent a follow-up study with pulmonary vasodilator therapy.

There was no difference in age, sex, body mass index, smoking habit, and lung function parameters between patients with pre-capillary PH and control subjects (Table 11).

Table 11. Subject characteristics in Circulating biomarker study

Variables	Control	All PH	p	PAH	CTEPH	PH due to lung diseases	p
Number	12	39	NA	16	15	8	NA
Sex,M/F (%)	6 (50)/6 (50)	18(46)/21(54)	0.99	6(38/10(72)	7(47)/8 (53)	4 (50)/4 (50)	0.81
Age, years	57 ± 10	59 ± 13	0.68	59 ± 12	58 ± 14	61 ±14	0.83
BMI, kg/m^2	27.6 ± 4.6	29.1 ± 5.8	0.63	29.5 ± 6.0	29.2 ± 6.0	28.2 ± 5.9	0.87
Non-/Ex-/Smoker, %	58/25/17	72/28/0	0.48	69/31/0	80/20/0	63/37/0	0.63
Haemodynamic variables:							
mPAP, mmHg	NA	48 ± 9	NA	49 ± 8	47 ± 12	50 ± 6	0.82
RAP, mmHg	NA	15 ± 8	NA	14 ± 6	15 ± 6	15 ± 7	0.20
PAWP, mmHg	NA	11 ± 5	NA	11 ± 3	12 ± 6	11 ± 3	0.72
SVI, mL/m^2/beat	NA	26.7 ± 7.7	NA	26.3 ± 7.3	27.0 ± 6.4	27.4 ± 11.2	0.95
CI, L/min/m^2	NA	2.1 ± 0.6	NA	2.0 ± 0.5	2.3 ± 0.7	2.2 ± 0.6	0.31
PVR, Wood Unit	NA	10.5 ± 4.7	NA	10.8 ± 4.3	8.5 ± 4.5	13.3 ± 5.5	0.07
PAC, mL/mmHg	NA	1.26 (0.83-1.62)	NA	1.23 (0.84-2.01)	1.27 (0.94-1.56)	1.25 (0.72-1.36)	0.79
RVSWI, g/m^2/beat	NA	12.40 ± 5.05	NA	12.43 ± 4.34	12.02 ± 5.72	12.99 ± 5.74	0.91
Echocardiographic variables:							
sPAP, mmHg	28 ± 6	72 ± 18	**<0.001**	73 ± 15	67 ± 21	80 ± 19	0.28
RV, mm	27 ± 2	40 ± 7	**<0.001**	42 ± 7	39 ± 7	38 ± 5	0.36
TAPSE, mm	27 ± 5	17 ± 5	**<0.001**	16 ± 5	18 ± 5	18 ± 3	0.33
RA area, cm^2	11.6 ± 2.8	25.0 ± 10.2	**<0.01**	25.5 ± 8.6	26.5 ± 12.6	20.8 ± 7.7	0.47
RVOT VTI, cm	13.9 ± 1.0	10.4 ± 3.7	**<0.05**	9.7 ± 3.7	12.2 ± 3.6	8.6 ± 2.7	0.08
RVOT AT, ms	178 ± 48	72 ± 26	**<0.001**	74 ± 25	72 ± 26	68 ± 30	0.88
Lung function variables:							
FVC %ref	105 ± 12	87 ± 24	0.07	94 ± 19	95 ± 24	66 ± 18**$^{\#\#}$	**<0.01**
FEV_1 %ref	100 ± 9	83 ± 24	0.07	89 ± 15	91 ±28	57 ±15**$^{\#\#}$	**<0.01**
FEV_1/FVC	0.79 ± 0.05	0.76 ± 0.10	0.32	0.76 ± 0.09	0.74 ± 0.05	0.74 ± 0.16	0.22
RV/TLC	0.39 ± 0.05	0.44 ± 0.15	0.36	0.43 ± 0.11	0.41 ± 0.17	0.54 ± 0.16	0.15
K_{CO} %ref	NA	54 ± 23	NA	50 ± 25	66 ± 14	39 ± 20$^{\#}$	**<0.05**
D_{LCO} %ref	NA	64 ± 27	NA	60 ± 29	82 ± 15*	38 ± 12*$^{\#\#\#}$	**<0.001**
Blood tests:							
pH	NA	7.42 ± 0.04	NA	7.42 ± 0.04	7.42 ± 0.04	7.41 ± 0.04	0.70
PaO_2, mmHg	NA	61.1 ± 13.3	NA	60.6 ± 13.9	60.3 ± 12.3	52.2 ± 9.6$^{\#}$	**<0.05**

Table 11. continued

Variables	Control	All PH	p	PAH	CTEPH	PH due to lung diseases	p
$PaCO_2$, mmHg	NA	33.1 ± 6.8	NA	30.9 ± 5.1	32.7 ± 6.9	38.6 ± 7.2*	**<0.05**
NT-proBNP, pg/ml	NA	1489 (600-2778)	NA	1489 (608-2642)	1665 (505-2887)	972 (439-3393)	0.89
Functional categories and tests:							
NYHA I, N	NA	0 (0%)	NA	0 (0%)	0 (0%)	0 (0%)	0.12
NYHA II, N	NA	10 (26%)	NA	3 (19%)	7 (47%)	0 (0%)	
NYHA III, N	NA	28 (72%)	NA	13 (81%)	7 (47%)	8 (100%)	
NYHA, IV, N	NA	1 (2%)	NA	0 (0%)	1 (6%)	0 (0%)	
6MWD, m	NA	339 ± 140	NA	317 ± 142	400 ± 124	261 ± 130	0.06
Circulating cytokine concentrations:							
Serum apelin, ng/mL	952 (782-1261)	1434 (1070-1636)	**<0.05**	1510 (1115-1750)	1313 (936-1493)	1448 (985-1753)	0.60
Serum VEGF, pg/mL	5.78 (3.25-12.89)	7.39 (3.25-10.26)	0.96	7.11 (3.25-9.18)	7.44 (3.25-10.86)	5.32 (3.25-21.00)	0.99
Plasma IL-22RA2, ng/mL	201 (145-668.6)	236.5 (88.7-523.3)	0.83	200.6 (99.8-427.6)	172.8 (34.7-677.8)	328.3 (204.5-535.9)	0.68

Data are presented as mean ± standard deviation or median (interquartile range). Patients and controls are compared with the Fisher exact test, t-test or Mann-Whitney test. Patient subgroups are analysed using the chi-square test, ANOVA with Bonferroni post-hoc test or Kruskal-Wallis test with Dunn's post-hoc test. *p<0.05, **p<0.01 vs. PAH; $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 vs. CTEPH. BMI: body mass index, CI: cardiac index, CTEPH: chronic thromboembolic PH, D_{LCO} : diffusing capacity of the lung for carbon monoxide, F: female, FEV_1: forced expiratory volume in the 1st second, FVC: forced vital capacity, IL-22RA2: interleukin-22RA2, K_{CO}: carbon monoxide transfer coefficient for, M: male, N: number, NA: not applicable, NT-proBNP: N-terminal pro-B-type natriuretic peptide, NYHA FC: New York Heart Association Functional Class, PaO_2/CO_2: arterial partial pressure of O_2/CO_2, PAH: pulmonary arterial hypertension, PAC: pulmonary artery compliance, PH: pulmonary hypertension, s/m/dPAP: systolic/mean/diastolic pulmonary artery pressure, RAP: right atrial pressure, PVR: pulmonary vascular resistance, PCPW: pulmonary capillary wedge pressure, RA: right atrium, ref: reference, RV: right ventricle, RV: residual volume, RVOT AT/VTI: right ventricular outflow tract acceleration time/velocity time integral, RVSWI: right ventricular stroke work index, SVI: stroke volume index, TAPSE: tricuspid annular plane systolic excursion, TLC: total lung capacity, VEGF: vascular endothelial growth factor, 6MWD: six-minute walk distance. Adopted from (178) under the terms of the Creative Commons Attribution Non-Commercial Licence 4.0 (http://creativecommons.org/licenses/by/4.0/).

In patients, RHC results demonstrated moderate-severe increases in mean PAP and PVR with normal or decreased CI. Vasoreactivity was not observed in any IPAH patient. Mild hypoxemia with hypo- or normocapnia was noted in patients, which was most prominent in the group of patients with PH due to lung disease. NT-proBNP levels were increased, and most patients fell into NYHA functional classes III and IV. Hemodynamic and

echocardiographic variables and NT-proBNP concentrations were not different among the subgroups of pre-capillary PH.

Apelin was measured in 63 samples (patients with PH upon enrolment N=38, a control visit N=14; control subjects N=11), and VEGF and IL22RA2 concentrations were quantified in 67 samples (patients with PH upon enrolment N=39, a control visit N=16; control subjects N=12). Half of the detection limit values were assigned to samples with a VEGF concentration below this level (patients: N=21; controls: N=6); all samples' apelin and IL22RA2 concentrations were above the detection limit.

4.2.2. Comparison of circulating cytokine concentrations among the groups

Serum apelin concentrations were higher in patients with pre-capillary PH than in control subjects (Table 11). Using the cut-off value of >1261 ng/mL apelin concentration, patients could be identified with a sensitivity of 66% and a specificity of 82% (ROC area under the curve (AUC)=0.74, p=0.001). Nonetheless, our data did not show a difference in the circulating concentrations of IL22RA2 and VEGF between patients and controls (Table 11), and these mediators could not separate patients from controls (cut-off for IL22RA2: 683.9 ng/mL: ROC AUC=0.52, p=0.96; cut-off for VEGF: 8.49 pg/ml, ROC AUC=0.52, p=0.83) (Figure 12.)

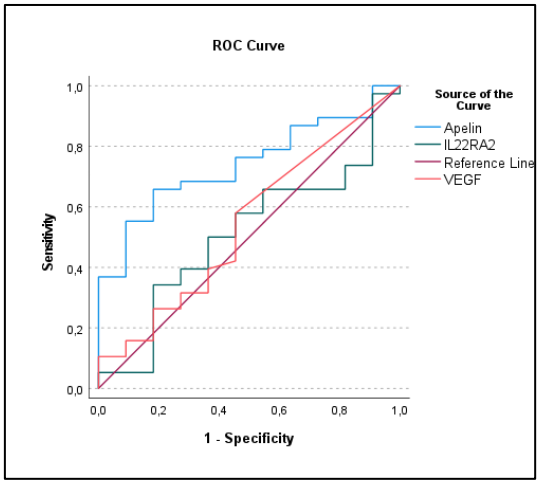

Figure 12. ROC analysis in values of the cytokines between PH and control group.

IL22RA2: interleukin 22 receptor alpha-2, VEGF: vascular endothelial growth factor.

Cytokine concentrations were not different among PH subgroups with different etiology (Table 11).

4.2.3. Correlations between cytokine concentrations

In patients, VEGF concentration showed a moderate positive correlation with both apelin (r=0.33, p=0.04) and IL22RA2 levels (r=0.42, p=0.008), but no association was found between IL22RA2 and apelin concentrations (r=0.14, p=0.41).

4.2.4. Relationship between cytokine concentrations and clinical factors in untreated PH

Plasma IL22RA2 concentrations showed a negative correlation to mean PAP (r=-0.32, p=0.04, Figure 13A) and serum NT-proBNP level (r=-0.42, p=0.01, Figure 13B), but not to other clinical factors (p>0.05). VEGF concentration did correlate with clinical parameters (p>0.05).

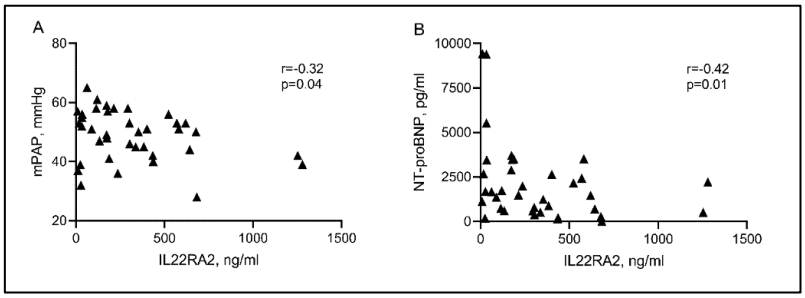

Figure 13. Spearman correlation between plasma IL22RA2 concentrations and mPAP – (panel A) and NT-proBNP (panel B) in patients with PH.

IL22RA2: interleukin 22 receptor subunit alpha2, mPAP: mean pulmonary arterial pressure, NT-proBNP: N-terminal pro-brain natriuretic peptide. Adopted from (178) under the terms of the Creative Commons Attribution Non-Commercial Licence 4.0 (http://creativecommons.org/licenses/by/4.0/).

Serum apelin concentration was positively related to systolic PAP (r=0.33, p=0.04, Figure 14A), RAP (r=0.38, p=0.02), but it showed a negative relationship to SVI (r=-0.58, p<0.001), CI (r=-0.34, p=0.04, Figure 14B), PAC (r=-0.55, p<0.001, Figure 14C) and RVSWI (r=-0.47, p=0.003, Figure 14D).

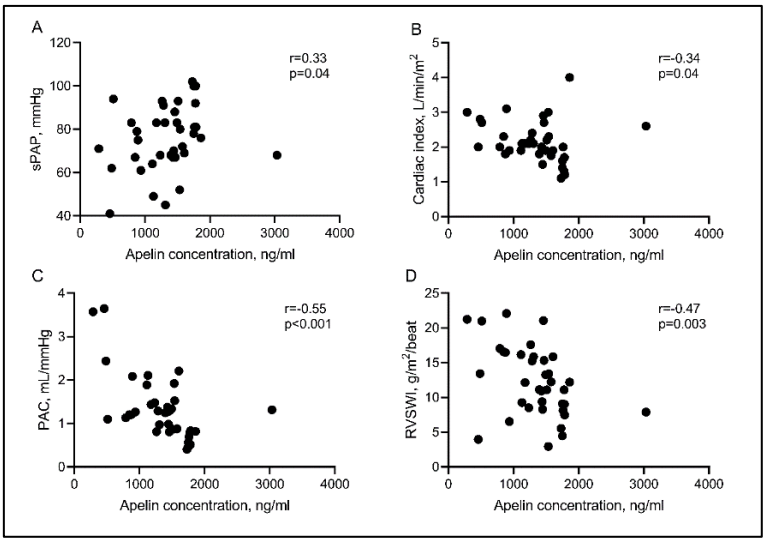

Figure 14. Spearman correlation beween serum apelin concentrations and sPAP (panel A), cardiac index (panel B), PAC (panel C), and RVSWI (panel D) in patients with PH. PAC: pulmonary arterial compliance, sPAP: systolic pulmonary arterial pressure, RVSWI: right ventricle stroke work index. Adopted from (178) under the terms of the Creative Commons Attribution Non-Commercial Licence 4.0 (http://creativecommons.org/licenses/by/4.0/).

4.2.5. Circulating cytokine concentrations in PAH and CTEPH before and after treatment

We also compared cytokine concentrations before and after therapy in 16 patients. 12 patients with IPAH had a second visit after initiating pulmonary vasodilator therapy (phosphodiesterase 5 inhibitor N=12, endothelin receptor antagonist N=5). Furthermore, in 4 patients with CTEPH, data collection was repeated after successful pulmonary endarterectomy, and one patient required additional therapy with a phosphodiesterase-5 inhibitor. The time difference between the two measurements was 7 ± 3 months in IPAH and 15 ± 7 months in CTEPH. There was an improvement in New York Heart Association (NYHA) functional status after therapy, and we found a tendency for an increase in TAPSE, RVOT VTI, and 6MWD (Table 12).

The concentration of circulating VEGF increased after treatment (p=0.001), but the levels of apelin and IL22RA2 were unchanged (Table 12). Interestingly, the change in VEGF concentration showed an inverse correlation to the change in RVOT VTI (r=-0.74, p<0.01), but not to other parameters listed in Table 12 (p>0.05).

Table 12. Clinical variables in patients with IPAH or CTEPH before and after therapy

Variables	Before treatment N=16	After treatment N=16	p-value
Serum apelin, ng/mL [N=14]	1412 (1165-1619)	1343 (1085-1552)	0.95
Serum VEGF, pg/mL [N=16]	7.76 (3.25-8.49)	9.37 (4.16-12.22)	**<0.001**
Plasma IL-22RA2, ng/mL [N=16]	225.2 (143-574.1)	320.7 (109.1-578.4)	0.43
6MWD, m [N=11]	372 ± 143	435 ± 142	0.06
NYHA FC [N=16]	3 (3-3)	1 (1-3)	**<0.001**
NT-proBNP, pg/ml [N=14]	1412 (586-2158)	800 (183-1832)	0.30
sPAP, mmHg [N=16]	69 ± 16	62 ± 18	0.21
RV, mm [N=16]	40 ± 7	38 ± 7	0.33
TAPSE, mm [N=15]	17 ± 5	19 ± 5	0.05
RA area, cm^2 [N=15]	27 ± 14	27 ± 11	0.95
RVOT VTI, cm [N=12]	10.2 ± 3.6	11.7 ± 3.6	0.06
RVOT AT, ms [N=12]	76 ± 30	84 ± 24	0.54

Data are presented as mean ± standard deviation or median (interquartile range). Data are compared with paired t-test or Wilcoxon signed rank test. IL-22RA2: interleukin-22RA2, N: number, NA: not applicable, NT-proBNP: N-terminal pro-B-type natriuretic peptide, NYHA FC: New York Heart Association Functional Class, sPAP: systolic pulmonary artery pressure, right atrium, RV: right ventricle, RVOT AT/VTI: right ventricular outflow tract acceleration time/velocity time integral, TAPSE: tricuspid annular plane systolic excursion, VEGF: vascular endothelial growth factor, 6MWD: six-minute walk distance. Adopted from (178) under the terms of the Creative Commons Attribution Non-Commercial Licence 4.0 (http://creativecommons.org/licenses/by/4.0/).

5. Discussion

In pre-capillary PH, the RV afterload continuously increases with the progression of vascular remodelling and the rise in pulmonary vascular resistance. The disease course is primarily determined by the degree of vascular pathological alterations, such as arterial remodelling, obstruction of arterial branches, and damage to the vascular bed. Still, the clinical progression mostly depends on RV adaptation against the increasing afterload. Routine hemodynamic, echocardiographic, cardiac MRI parameters and serum NT-proBNP concentration are used as biomarkers in clinical practice to follow right heart adaptation (1; 68; 71; 82). These measures characterize the degree of right heart dilatation and RV function, which show linear dynamics during the progression of PH. However, identifying the signals that can be routinely studied in clinical practice and more precisely reflect RV functionality, including its biphasic course of adaptation and the RV/PA uncoupling, is needed. Furthermore, a better understanding of the regulation of RV adaptation and the discovery of new molecular targets are warranted. Thus, we studied in carefully recruited cohorts of patients with pre-capillary PH the circulatory concentrations of cytokines with a potential link to RV function and assessed the clinical usefulness of the calculated RVSWI to predict therapy-related clinical improvement.

Global RV function is most accurately assessed via the construction of pressure-volume loops (3; 83), but this method is cumbersome for clinical use. The work of the RV myocardium and its measurement are discussed by experimental hemodynamic procedures, which allow the calculation of the area under the pressure-volume curve (3). However, it cannot be routinely performed in everyday clinical practice, and the clinical interpretation of the results is equivocal. In our Right ventricular work study, we calculated RVSWI from RHC data according to a previously established method (85) to estimate the active RV work during myocardial contraction (84; 179; 181), and we evaluated this parameter in two different substudies.

In our Vascular resistance substudy, we examined the correlation between RVSWI and PVR in a wide spectrum of data which was collected from patients with no PH and those with pre-capillary PH both with and without specific vasodilator treatment. We found an association between RVSWI and PVR; however, as shown in Figure 11, this relationship seems biphasic rather than simply linear, which corresponds to the known adaptive and maladaptive phases of RV adaptation. In the early stage of the disease, systolic functional

reserve and contractility increase in parallel with the increasing pressure load. At a later stage, when the systolic reserve is exhausted, a further increase in the afterload results in deteriorating RV systolic function (64). The deflection point between the two stages presumably indicates RV/AP uncoupling, after which a gradual decrease in RV work can be observed as PVR increases.

In the Comorbidities substudy, we described increased RVSWI in patients with pre-capillary PH. In recent years, advancements in the management of PH have resulted in improved overall outcomes. Upfront combined treatment regimens (181) and the use of a risk assessment tool (1) are effective, but currently, there is limited data on which baseline parameters can best predict therapeutic response. In this substudy, we used a formula that considered both the steady and pulsatile components of RVSW. Not only does it characterize the active work of the RV myocardium, but it also provides additional information on RV adaptation superior to the volumetric and functional parameters of imaging studies. Because of its complex ability to reflect the dysfunction of the RV, the RVSWI has been proposed as a promising prognostic marker in PH patients.

Previous studies have found that lower RVSWI was associated with decreased survival in patients with PH associated with connective tissue disease (180), familial and idiopathic PAH (182). These studies have examined homogenous groups and proposed that baseline RVSWI might be associated with etiology and predict outcome. In our research, RVSWI was higher in PAH and CTEPH patients compared to the control subjects, on par with previous findings (5; 85). However, we found no difference in RVSWI in PAH and CTEPH groups. The diverse composition of our study group might explain this. Further studies on higher number of patients in the clinical groups might provide more details on the relation of RVSWI to specific patient characteristics at diagnosis.

Progression in PH is difficult to predict, and several factors can adversely affect RV function, such as advanced age and certain comorbidities. Comorbidities, particularly ones that impair the physiological processes of the myocardium due to hypoxia or metabolic disorders, have mainly been investigated regarding LV function (183). In PAH, it has been reported that diabetes has a negative effect on RVSW (184). In our cohort, the presence of co-morbidities (coronary heart disease, hypertension, diabetes, obesity) (88) is associated with older age and less severe PH based on mPAP, PVR and CI values. The

decreased afterload can explain the low RVSWI values in comorbid patients. Interestingly, the difference in RV function confirmed by RVSWI is not reflected by routine echocardiographic parameters showing similarly reduced RV function in patients with and without comorbidities. It is also noteworthy that the difference in the study population only appears in the PAH group, while not in CTEPH. It is assumed that the change in RV work results from complex mechanisms. In addition to the degree of afterload and comorbidities affecting the functioning of the myocardium, differences in the extent of the obstruction in CTEPH and the propagation of the pulse wave also play a role in RVSW. Therefore, mainly in PAH, the RVSWI provides complementary information about the state of the RV in addition to routine echocardiographic parameters, which may impact therapy management and the course of the disease.

Another important aspect of progression in PH is the biphasic nature of RV contractility and its systolic functional reserve. When examining baseline RVSWI, we found no correlation to other echocardiographic, hemodynamic, or clinical parameters or coupling. This could be explained by the previously described biphasic change, where a peak in RVSWI may be assumed during the RV/PA uncoupling. This could explain the lack of correlation between RVSWI and other parameters that mainly change linearly during the progression of PH.

The predictive value of RVSWI in PH is well known. According to a previous study of adult pre-capillary PH patients, higher RVSWI was associated with better outcomes, lower heart failure death rates, and hospitalization (179). In another study of pediatric PAH patients, RVSW was related to functional capacity and mortality (185). The short follow-up period did not permit a detailed analysis of outcomes, including transplant-free survival or death in our cohort; however, we did calculate mortality risk according to current recommendations (1). Although the guideline primarily uses risk determination in the context of PAH, several publications have used the same strategy in CTEPH patients (186-188). Our data show that RVSWI did not correlate with the calculated mortality risk. This seemingly contradictory finding could also be explained by the previously mentioned probable biphasic course of RVSW progression. However, our follow-up analysis revealed an interesting novel finding: patients with RVSWI $\geq$ 1450 mmHg*mL/m^2 showed a greater reduction in mortality risk 1 year after specific treatment. This was accompanied by a greater improvement in functional capacity, as noted by

higher 6MWD values. This phenomenon points to the possible role of RVSWI in guiding the selection and timing of specific therapy in PH, and the potential of this parameter to predict response to specific therapy.

Accumulating evidence shows that cytokines and inflammatory pathways contribute to the development of vascular remodelling, and they can also regulate right heart adaptation in different forms of PH (9;17), implicating that these mediators could serve as disease markers and future therapeutic targets. Importantly, no current therapies used in the management of pre-capillary PH are primarily directed to improve RV function. In our Circulating biomarker study, we assessed three mediators that have been previously linked to the modulation of myocardial function.

Apelin is a paracrine regulatory peptide with a strong positive inotropic and vasodilator effect involved in the pathophysiology of PH. The hypoxia-triggered apelin expression plays a significant role in smooth cell proliferation in pulmonary vessel walls and, thereby, vascular remodelling (115-117). There is some evidence that circulating apelin may be a marker of its cardiac expression and is involved in adaptation. In hypoxic PH, the apelin content of RV is increased, showing a positive correlation to RV pressure (110). In patients with chronic left heart failure, the apelin expression is increased in the left atrium and ventricle, and atrial apelin expression moderately correlates with plasma apelin concentrations, suggesting it may be a source of circulating apelin (115).

Our study proves that circulating apelin increases in treatment-naïve patients with pre-capillary PH. It is a marker of cardiac function, as shown by the link between mediator concentration and stroke volume, CI, and RVSWI. In chronic left heart failure, apelin signalling is initially upregulated in early heart failure with a decrease in later stages (100). This mechanism is also described in animal models of PH with adaptive and maladaptive RV remodelling (141). No comprehensive human studies have examined the serum apelin concentration during the progression of RV dysfunction in PH. Still, it can be assumed that the apelin expression follows a similar pattern in right heart failure. Thus, it can be hypothesized that in patients with pre-capillary PH, apelin production was induced in a compensatory manner to improve and maintain right heart function, as presented by the increased mediator level on diagnosis and at the follow-up visit. Our data extend previous findings on the positive relationship between apelin production and CI observed in a very small cohort of patients with PAH, where apelin expression in RV

tissue was positively related to CO (118). Furthermore, plasma apelin showed a positive relationship to mean PAP and RAP, which implies that pressure overload can induce apelin synthesis in cardiomyocytes and the pulmonary endothelium. Our data on the correlation between apelin concentration and RVSWI suggests that this cytokine could be a candidate marker to follow the biphasic adaptation of the RV during the progression of PH patients. Interestingly, a higher apelin level was associated with reduced pulmonary vascular compliance, representing increased stiffness, implying the possible involvement of this molecule in the development of vascular remodelling as it was described to vessels of the systemic circulation (144).

A growing body of evidence supports the crucial role of inflammatory processes in the processes leading to vascular remodelling and the formation of plexiform lesions in PAH (101; 89). The involvement of IL22 in the pathogenesis of pulmonary hypertension has recently been described. IL22 induces cell growth of pulmonary artery smooth muscle via the activation of IL22 receptor alpha 1 (IL22RA1), and IL22 expression is upregulated in smooth muscle cells from rats with PH due to chronic hypoxia (135). Moreover, using an in vivo model of chronic hypoxia-induced PH with RV hypertrophy, IL22RA2, a decoy receptor for IL22 (189), was identified as a factor associated with adaptive RV remodelling (136). In our study, IL22RA concentration negatively correlates with NT-proBNP and mPAP values. This suggests that as the negative feedback diminishes, the effect of IL22 signalling may be more prominent at later disease stages, and might contribute to the maladaptive RV adaptation. Furthermore, Crnkovic et al. reported an elevated concentration of plasma IL22RA2 in patients with untreated IPAH compared to controls (136). However, our cohort did not find a difference in mediator levels between patients and controls, which could partly be explained by our study's mixed population of patients with pre-capillary PH. In addition, IL22RA2 levels were not different among PH subgroups, albeit the sample sizes of subgroups were limited.

VEGF signalling has a complex effect during the development of vascular remodelling to different stimuli, as different receptor subtypes serve as positive and negative regulators of pulmonary endothelial cell proliferation (190). Kümpers et al. demonstrated that the circulating concentration of VEGF was increased in patients with IPAH; however, they did not show an association with clinical parameters or disease progression (153). We did not find a difference in plasma VEGF levels between patients with PH

compared to controls, which can be due to the different disease etiology and the limited sample size in our cohort. Furthermore, VEGF signalling can also convert cardioprotective function as its production is upregulated in the RV during adaptive hypertrophy, and it can also trigger hypoxia-induced angiogenesis in the myocardium (17). In line with this, we previously showed in a mixed population of treated and treatment-naïve PAH that TAPSE, a measure of RV function, positively affects plasma VEGF concentration (152). In the current study, we extend these findings by observing that the VEGF level increases upon clinical improvement of patients after the initiation of specific treatment in PAH and CTEPH.

We did not find a change in circulating apelin and IL22RA2 concentrations during follow-up compared to the measurements at diagnosis. Treatment with specific pulmonary vasodilator therapy induces vasodilation of the pulmonary artery, resulting in improved hemodynamics and better functional status of patients. However, myocardial reverse remodelling and the improvement in RV function are a consequence of complex processes. We hypothesized that apelin and IL22RA2 are involved in regulating these processes. During the sequential pulmonary vasodilator therapy (set by the current Hungarian regulations), the improvement in RV functional parameters was experienced to some degree at the follow-up visit (Table 12). Still, with up-front combination treatment, as recommended by the latest international guidelines, an earlier change in the concentration of these markers can be expected as reverse remodelling of the RV takes place earlier. However, the magnitude of the change in plasma VEGF level was inversely related to the improvement in RV outflow tract velocity time integral, which is a surrogate marker for right ventricular stroke volume reflecting both RV basal and free wall motion. This might be caused by a compensatory increase in right ventricular VEGF production to promote adaptive hypertrophy, as shown previously in an animal model of PH (149).

Interestingly, baseline VEGF concentration was positively associated with apelin and IL22RA2 levels. This can be explained by the fact that hypoxia can trigger the production of all three cytokines (150; 190; 194), and the expression of VEGF and IL22RA2 can also be induced by perivascular inflammation in the remodelled pulmonary arteries (191).

Our studies have several limitations. In general, the limited sample size of the patient groups in both studies should be acknowledged. However, PAH and CTEPH are rare diseases, and PH is often undiagnosed in patients with lung diseases due to the current

lack of evidence for the use of specific pulmonary vasodilator therapy in this group (1), which restricts the recruitment when a single center is involved. Thus, multi-center prospective studies with higher patient numbers are needed to verify our findings regarding RVSWI and circulatory biomarkers. Furthermore, the retrospective nature of the Right ventricular study, and the relatively short follow-up time did not permit clear conclusions regarding the role of RVSWI in predicting long-term therapeutic response. Regarding the Circulating biomarker investigation, future studies could extend our findings on the diagnostic potential of circulating apelin concentration by recruiting other control groups, including patients with lung disease, or those after pulmonary embolism without PH, or patients with less severe PH. Finally, similar to our previous study (152) despite using a high-sensitivity detection assay, VEGF concentration was below the detection limit in approximately one-third of the samples, which could have influenced the conclusions drawn from these data.

6. Conclusions

1. RVSWI correlates with PVR in a mixed patient population with and without pre-capillary PH, and a biphasic relationship might be assumed.
2. RVSWI is higher in patients with CTEPH and PAH at diagnosis compared to control subjects.
3. RVSWI is not different between patients with PAH and CTEPH at diagnosis, but the presence of comorbidities with possible influence on myocardial function is associated with lower values only in PAH.
4. At follow-up after the initiation of specific pulmonary vasodilator therapies, a baseline RVSWI $\geq$ 1450 mmHg*mL/m^2 is linked with a greater improvement in the mortality risk score and a trend for a larger reduction in NT-proBNP concentration in patients with PAH and CTEPH.
5. At diagnosis, serum apelin concentration is increased in patients with pre-capillary PH, and a biomarker concentration >1261 ng/mL identified patients with 66% sensitivity and 82% specificity. Circulating VEGF and IL22RA levels are not different between patients and controls.
6. In patients, apelin concentration is positively related to sPAP, RAP and CI, while it is negatively correlated with RVSWI. IL22RA2 concentration shows inverse relationships with mPAP and NTproBNP levels. Serum VEGF concentration did not correlate with clinical parameters.
7. VEGF concentration is increased in patients upon clinical improvement, but no change is observed in the circulating levels of apelin or IL22RA2.

7. Summary

The deterioration of RV function determines the survival of patients with pre-capillary pulmonary hypertension. RV adaptation to the increased pulmonary vascular pressure shows great individual variability, however, the process is not fully understood, and its assessment needs better tools. Several cytokines have been implicated to influence myocardial adaptation in PH, but their association to clinical measures is not known. Right ventricular stroke work index, a calculated parameter, has been previously proposed to assess RV work, but its use for clinical practice should be better characterized.

We found that RVSWI correlates with PVR in patients with PAH and CTEPH, and it might be a potential marker to capture the adaptive and maladaptive phases of RV adaptation. Importantly, a decreased RVSWI value at diagnosis can predict an unfavourable therapeutic response to initial pulmonary vasodilator therapies in PAH and CTEPH, suggesting that this parameter might aid decision-making in patient management. Furthermore, we showed that comorbidities with known negative effects on the function of the left ventricle, are also associated with decreased RV function in PAH, as assessed by RVSWI.

The increased concentration of serum apelin, a molecule with a potent positive inotropic effect, can identify patients with PAH, CTEPH and PH due to lung diseases at the diagnosis, and is positively related to disease severity and RV function. IL22RA2 has been recently described to be involved in RV adaption in PAH, and we found that its elevated blood concentration is linked with less severe PH, and a better RV function. Furthermore, serum VEGF concentrations might be suitable to follow the clinical improvement of patients.

Overall, our findings highlight that the hemodynamic parameter of RVSWI and the circulating biomarkers of apelin and IL22RA2 hold potential clinical values in the assessment of cardiac function in pre-capillary PH.

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9. Bibliography of the candidate's publications

9.1. Publications related to the subjects of the dissertation

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10. Acknowledgements

I would like to express my deepest gratitude to my supervisor, Zsófia Lázár, whose dedicated encouragement, guidance, insights, support, and help have been instrumental in my research project.

I would like to sincerely thank the current and former Heads of the Department, Veronika Müller, and György Losonczy, for their encouragement and support. Their leadership has created an environment conducive to research pursuits, and their insights help make my research successful.

Moreover, I would like to express my gratitude to Kristóf Karlócai, who created the opportunity for clinical and research work, paved my path, and deepened my knowledge. Furthermore, I am deeply grateful for Elek Dinya's encouragement and selfless help. His insights have greatly helped to advance scientific ideas.

I also owe a debt of gratitude to my family, whose understanding and patience have sustained me through the challenges of doctoral studies.

Special thanks are also due to the best cardiology assistants for Tímea Baranyi and the clinicians and professionals within the Department whose expertise and collaboration enriched of this research.

I am also grateful to the Hungarian Respiratory Foundation for the research grant it provided, which facilitated the pursuit of this research and contributed significantly to its realization.

Finally, I would like to express my appreciation to all those whose names may not appear here but who have provided encouragement, assistance, and support in various capacities throughout this endeavour.