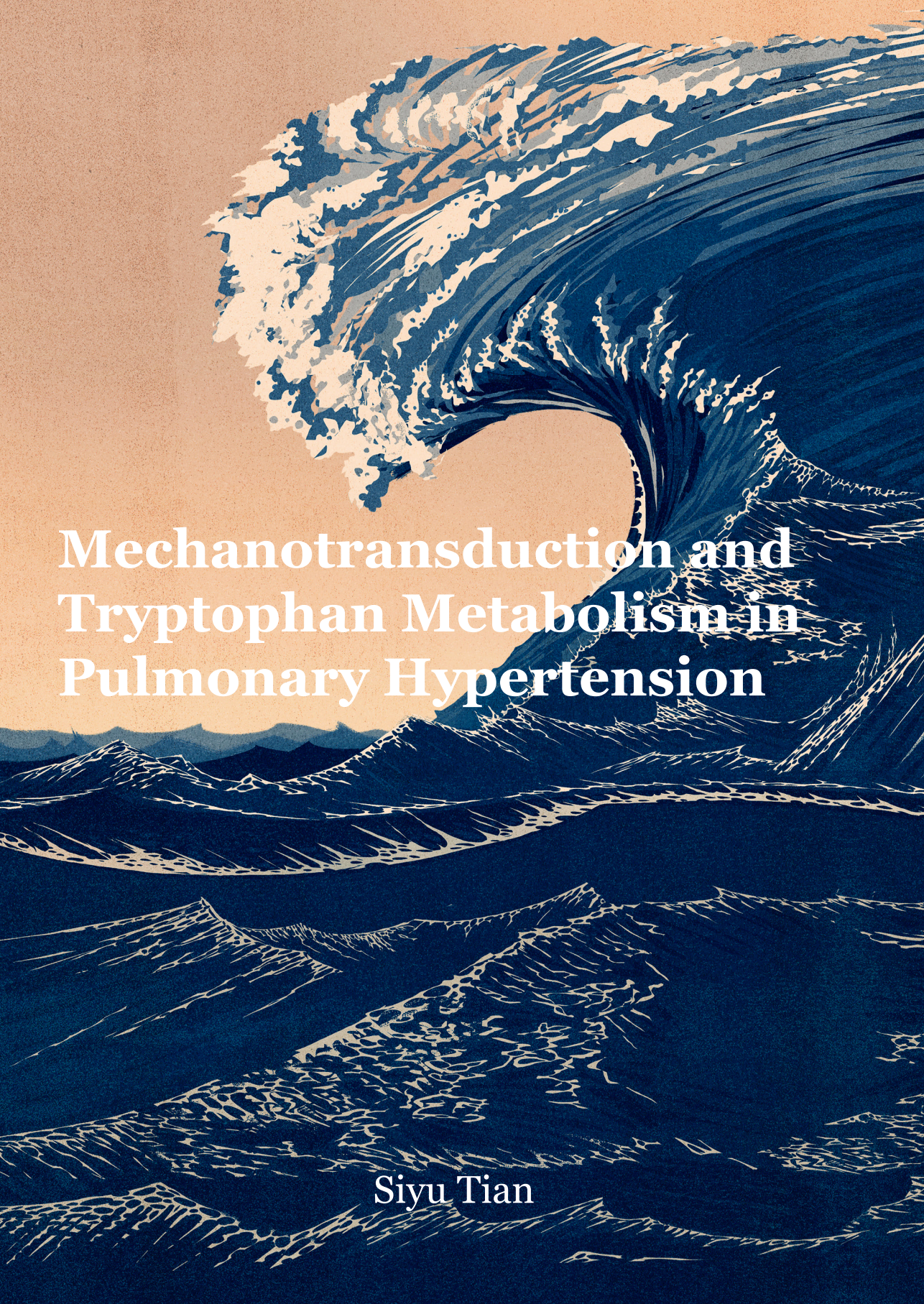


Mechanotransduction and Tryptophan Metabolism in Pulmonary Hypertension

Siyu Tian

Mechanotransduction and Tryptophan Metabolism in Pulmonary Hypertension

Siyu Tian
田思雨

The studies in this thesis were mainly performed in the Division of Experimental Cardiology, Department of Cardiology, Erasmus University Medical Center, Rotterdam, the Netherlands.
The studies in this thesis were financially supported by Royal Netherlands Academy of Science (CVON-2012-08, PHAEDRA), Dutch Heart Foundation (CVON2014-11, RECONNECT), German Center for Cardiovascular Research (DZHK81Z0600207).
The research presented in this thesis was partially supported by the China Scholarship Council (CSC) to Siyu Tian (201708130077).

Financial support by the Dutch Heart Foundation for the publication of this thesis is gratefully acknowledged.

Mechanotransduction and Tryptophan Metabolism in Pulmonary Hypertension

Thesis Erasmus University Medical Center, Rotterdam, The Netherlands.
ISBN: 978-94-6423-939-3
Printed by: ProefschriftMaken NL – www.proefschriftmaken.nl
Cover design: Francesco Bongiorni and Siyu Tian
Layout: Siyu Tian

Mechanotransduction and Tryptophan Metabolism in Pulmonary Hypertension

Mechanotransductie en tryptofaanmetabolisme bij pulmonale hypertensie

Thesis

to obtain the degree of Doctor from the
Erasmus University Rotterdam
by command of the
rector magnificus

Prof. dr. A.L. Bredenoord

and in accordance with the decision of the Doctorate Board.
The public defence shall be held on

Wednesday 21 September 2022 at 10.30 hrs

by

Siyu Tian

born in Hebei, China

Erasmus University Rotterdam

Doctoral Committee:

Promotors:
Prof. dr. D. Merkus
Prof. dr. D.J.G.M. Duncker

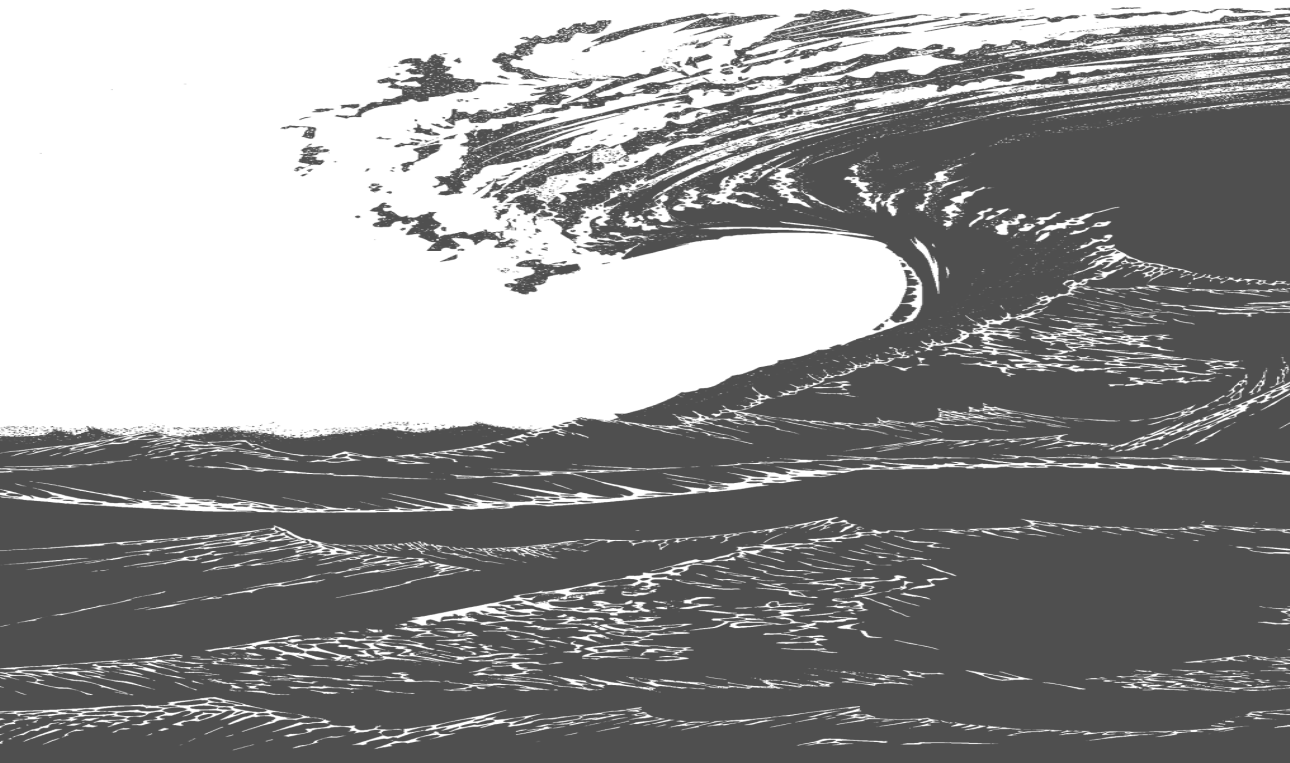
Other members:
Dr. C. Cheng
Prof. dr. R.W. Hendriks
Prof. dr. M.J.T.H. Goumans

合抱之木，生于毫末；九层之台，起于累土；千里之行，始于足下

The tree which fills the arms grew from the tiniest sprout; the tower of nine storeys rose from a (small) heap of earth, and a journey of a thousand miles begins with a single step

-----老子 Laozi (TaoTe Ching)

Contents



Chapter 1

Introduction and outline of this thesis

1. Introduction

1.1 Pulmonary Circulation

The pulmonary circulation consists of a vast network of arteries, veins and lymphatics that functions to provide an efficient gas exchange.[1] The right heart provides the driving pressure for pumping blood through the pulmonary circulation. The pulmonary circulation conducts the entire cardiac output with a remarkably low driving pressure, due to a low vascular resistance, from the pulmonary artery to the left atrium.[2] Conversely, the systemic circulation has a high pressure (mean aortic pressure of 95 mmHg) due to a high vascular resistance.

The pulmonary trunk separates into right and left main pulmonary arteries which subsequently branch into large elastic arteries (diameter >1,000 μm), muscular medium arteries (diameter 100-1,000 μm), small arterioles (diameter <100 μm) and finally to the capillaries which surround the alveoli where the gas exchange takes place.[3] The pulmonary veins collect the oxygenated blood and carry it from the lungs back to the heart. Although the pulmonary vasculature is thin-walled, it structure is complex. Vascular smooth muscle cells, various other contractile cells-including fibroblasts, intermediate cells and pericytes and endothelial cells, nerves and interstitial macrophages populate the vascular wall to varying degrees in each compartment (Figure 1).[4] The endothelium in the pulmonary vasculature is a key player in many processes, including regulation of vascular tone, protection from inflammatory stimuli, maintenance of the barrier function, regulation of metabolic process and angiogenesis.[5]

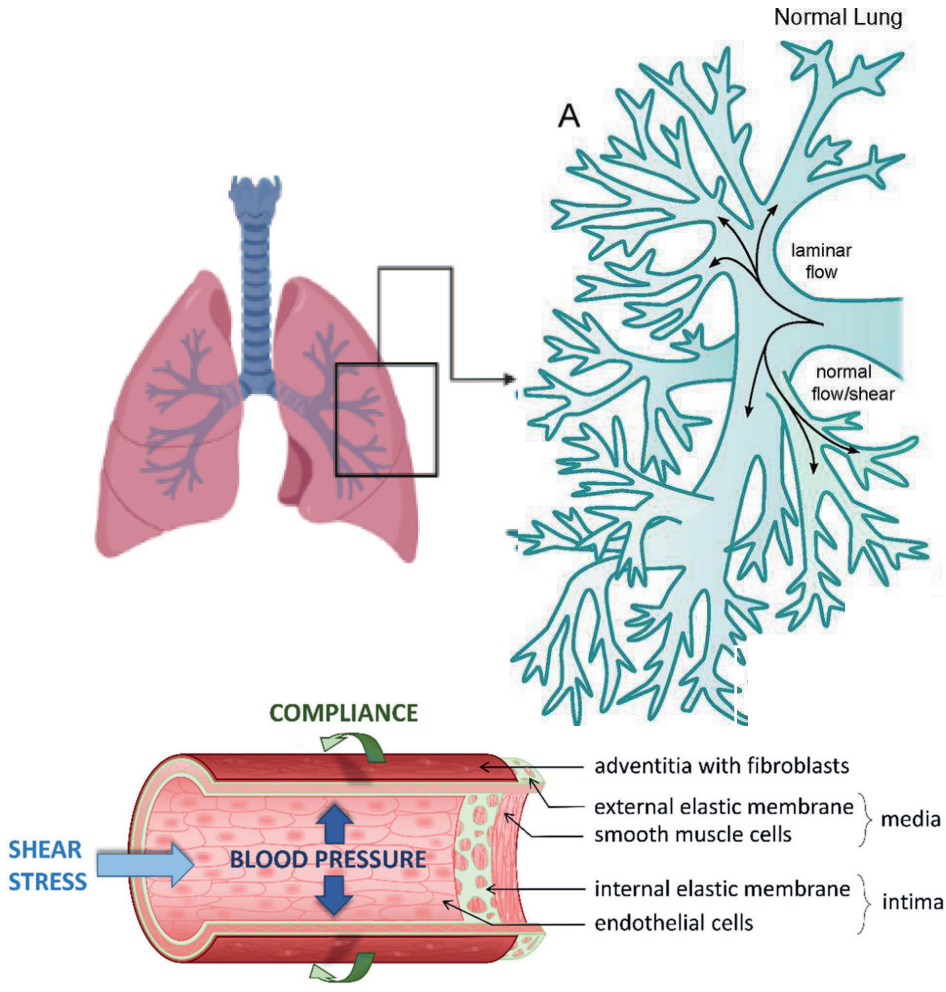


Figure 1. The pulmonary vasculature and hemodynamic forces act on the pulmonary vessels. Section of an artery wall showing that the endothelial cells, smooth muscle cells, and the surrounding adventitia predominantly includes fibroblasts and matrix. (Adjusted from [6, 7])

1.2 Pulmonary hypertension

Pulmonary hypertension (PH) was originally defined as a pulmonary artery pressure (mPAP) above 25 mmHg at rest measured by right heart catheterization during the 1st World Symposium on Pulmonary Hypertension (WSPH) in 1973. The new

definition of pulmonary hypertension in 6th WSPH (Nice, 2018) lowers the threshold of elevation in the mPAP >20 mmHg and includes the need for pulmonary vascular resistance (PVR) ⩾3 WU in the definition of all forms of pre-capillary PH.[8] Pulmonary hypertension is characterized by pulmonary vascular remodeling which could initiate right ventricular (RV) dysfunction and ultimately lead to RV failure and death. PH is classified into 5 groups based on the underlying pathology, the so called world Health Organization (WHO) groups (Table 1). The clinical classification of PH is to categorize PH based on similar pathophysiological mechanisms, clinical presentation, hemodynamic characteristics and therapeutic management.[8] Overall, PH affects approximately 1% of the global population and up to 10% of persons older than 65 years of age, and PH is a life-threatening condition associated with increased mortality regardless of the classification and underlying etiology.[9]

WHO group 1 Pulmonary arterial hypertension (PAH)
Idiopathic PAH
Heritable PAH
Drugs or illegal drugs induced
Congenital heart disease
Other conditions such as HIV infection, chronic liver disease (cirrhosis) and connective tissue disorders (scleroderma, lupus, others)
WHO Group I' (pulmonary veno-occlusive disease and pulmonary capillary haemangiomatosis)
WHO Group I'' (persistent pulmonary hypertension of the newborn)
WHO group 2 Pulmonary hypertension caused by left-sided heart disease
Left ventricular systolic dysfunction
Left ventricular diastolic dysfunction
Valvular heart disease
Specific congenital abnormalities
WHO group 3 Pulmonary hypertension due to lung disease or hypoxia
WHO group 4 Chronic thromboembolic pulmonary hypertension(CTEPH) and other pulmonary artery obstructions
WHO group 5 Pulmonary hypertension with multifactorial mechanisms or uncertain causes

Table 1. WHO clinical classification of pulmonary hypertension.[10]

Pulmonary vascular remodeling is a common pathological change in all PH groups. Pulmonary vascular remodeling in PH, particularly in pulmonary arterial hypertension (PAH), is characterized by proliferation and excessive remodeling in the distal pulmonary arterial bed, resulting in high pulmonary vascular resistance, leading to the high pulmonary arterial pressure and RV afterload.[11] This vascular remodeling includes media hypertrophy and intimal and adventitial thickening, which may be related to an imbalance of different types of cell proliferation, apoptosis and inflammation in the pulmonary vascular wall. One of the hallmarks of vascular remodeling in PAH is plexiform lesions, with varying degrees of endothelial cell proliferation, muscular hypertrophy, intimal fibrosis and complex vascular formations originating from remodeled pulmonary arteries.[12]

Morphological changes like plexiform lesions most commonly form in the distal bifurcation sites of small pulmonary arteries, ranging in diameter from 500 μm down to 70 μm in humans. In all groups of human and experimental PAH remodeling, small pre-capillary pulmonary arterioles ranging in diameter from 70 μm down to 20 μm are also involved through processes of loss and obliteration, abnormal muscularization, and perivascular inflammation.[5] The morphological changes of the pulmonary vascular bed increase the intimal area of the small arteries and arterioles, which reduces the vascular lumen.[13] These pathological changes in the vascular wall are thought to be secondary to the chronic and sustained pulmonary vasoconstriction and further increase the PVR.[14]

Currently available treatments are essentially targeting three signaling pathways that are involved in the regulation of vascular function by the endothelium and smooth muscle cells.[15] The three pathways include the nitric oxide(NO)/cyclic guanosine monophosphate (cGMP) pathway, the endothelin pathway and the prostacyclin pathway (Figure 2).

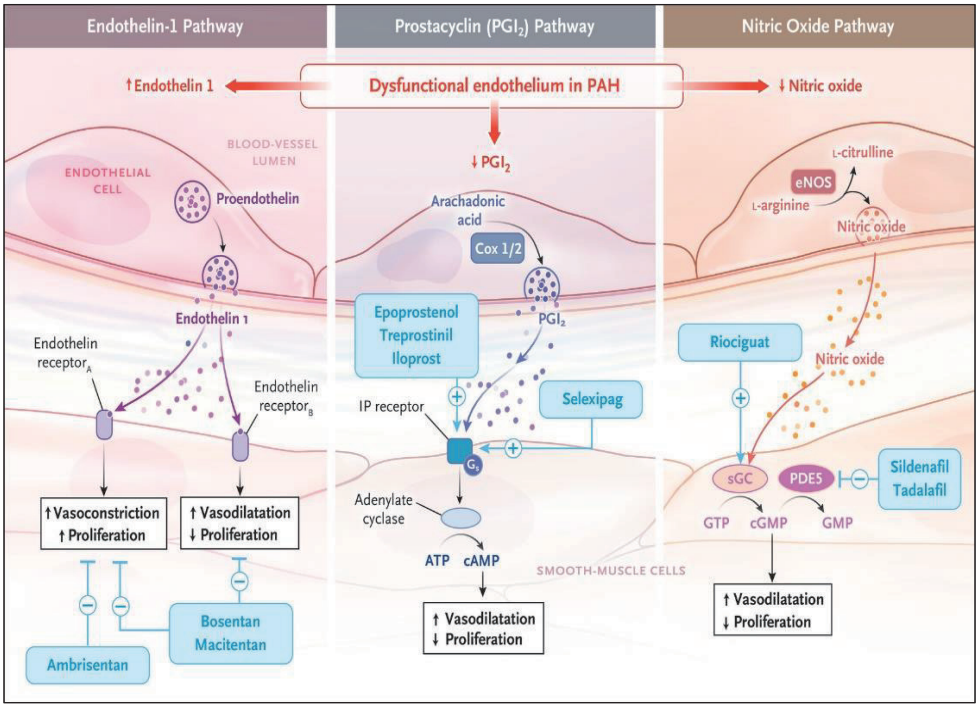


Figure 2. Currently available targeted therapy in PAH and CTEPH from three classic pathways. The three pathway induces the imbalance of vasoconstriction and vasodilators which result the increased pulmonary vascular tone and pulmonary vascular remodeling. (From [16])

The three well-defined pathways are involved in the regulation of vascular tone. 1) Endothelin 1 (ET1) pathway, a potent endogenous vasoconstrictor, which is increased in PAH. ET1 can bind to either endothelin A receptor (ETA) or endothelin B receptor (ETB). Endothelin receptor antagonists, such as bosentan and ambrisentan, block the activity of ET1, resulting in vasodilation.[17] 2) Prostacyclin (PGI2) pathway, a potent pulmonary artery vasodilator, is endogenously produced by endothelial cells.[17] The treatment like treprostinil increases time to clinical worsening and improves symptoms and 6-minute walk distance.[18] 3) NO pathway. The expression and function of the PGI2 and NO pathways are decreased in PAH.[19] Cyclic adenosine monophosphate (cAMP) and cGMP, which are second messengers responsible for vasodilatation and proliferation in these two pathways. The oral

PDE5 inhibitors (sildenafil and tadalafil) inhibit cGMP breakdown in vascular smooth muscle cells and improve exercise capacity and hemodynamics in patients with PAH.[20] Although these treatments are established therapy for pulmonary arterial hypertension (group 1) and CTEPH (group 4),[16] their beneficial effects do not apply to other groups in the WHO classification. Besides, these therapies are principally focusing on pulmonary vasodilation and proliferative/mitogenic properties, but do not specifically target the pulmonary vascular remodeling and inflammation.[21] Therefore, identifying the mechanism of vascular remodeling and developing new therapies is urgently required.

2. Mechanotransduction in pulmonary hypertension

2.1 Mechano-signaling in the pulmonary circulation

Healthy lung microcirculation is a low pressure, high volume system characterized by low blood flow velocity.[14] The normal pulmonary arterial tree has a branching structure and equally divides the blood flow to the alveoli.[7] The endothelium is critical for the development and maintenance of a functional vascular network, and coordinates this process depending upon signals exchanged between different cell types.[5] The pulmonary vessels are continuously subjected to the hemodynamic forces due to the blood flow (Figure 1). Endothelial cells (ECs) are the innermost layer of all blood vessels and are sensitive to different mechanical stimuli generated by blood pressure and flow, including wall stretch and shear stress. The pressure exerted by blood volume produces forces in the direction perpendicular to the vessel wall leading to stretch circumferentially. This is called wall stretch which is mainly studied as a force active in macrovascular vessels.[22] Stretch may be at the very core of the smooth muscle cells (SMCs) differentiation state.[23] Shear stress is the frictional force generated on the vessel wall by blood flow parallel to the flow direction. It mainly acts on endothelial cells resulting in their longitudinal alignment.[6] ECs can directly and principally sense shear stress forces and convert

them into biological signals. ECs respond to changes in shear stress with changes in secretion of the vasoactive mediators such as NO, PGI2, thromboxane, and ET-1.[24] On the cellular level, shear stress is known to influence endothelial morphology and alignment.[25] Besides, the pathological high shear stress induces the ECs dysfunction and SMCs hypertrophy resulting in the proximal vascular stiffening.[24] Altered mechanical forces on the ECs have been proposed to be an important trigger to modulate the vascular remodeling. Upon endothelial cells injury, SMCs directly exposed to high shear stress, further induced the cells apoptosis and arterial remodeling.[26]

2.2 Mechano-signaling in pulmonary hypertension

In PH, the altered hemodynamics forces can result in abnormal blood flow to the lung on the pulmonary endothelial cells.[27] Blood flow in different types of PH can be abnormally high, abnormally low, or non-uniform flow along the vessel wall, resulting in different types of shear stress, i.e. laminar high or low shear stress and disturbed oscillatory shear stress (OSS).[27, 28] The healthy compliant pulmonary arteries play a pivotal role in transiting the pulsatile flow generated by the RV to the nearly steady flow at the capillary level.[29] Proximal large artery stiffness thus influences the local mechanical environment.[30] In PH patients, the chronic pressure overload causes the proximal pulmonary artery (PA) to dilate which increases its stiffness.[7, 31] Increased stiffness and muscularization of the PA impact both static and oscillatory workload of the RV.[30, 32] This reduces RV-PA coupling and contributes to worsening of RV function, eventually resulting in a reduced cardiac output and a lower shear stress in the main pulmonary artery. Yet, stiffening in the proximal pulmonary vasculature can amplify pulse wave transmission to the distal vessels, resulting in a higher pulsatile shear stress, inflammation, and SMCs remodeling, which may explain coupling of the microvascular with macrovascular dysfunction.[30, 33] Furthermore, the proximal PA stiffening aggravates flow

disturbance in the distal PAs resulting in endothelial dysfunction and vessel loss.[7, 34] Endothelial dysfunction is associated with increased pulmonary vasculature cellular proliferation and resistance to apoptosis, which contributes to pulmonary vascular remodeling.[35] Taken together, abnormal shear stress in the whole pulmonary vasculature accelerates the progression of pulmonary vascular remodeling.

In order to investigate the mechano-biological process in PH and to further understand the relation between shear stress and vascular remodeling, it is vital to know the actual shear stress values in the pulmonary vasculature. The shear stress values from different studies are summarized in Table 2. Overall, the time-averaged wall shear stress in the proximal pulmonary vasculature (diameter above 500 μm) decreased with increasing disease severity in pediatric PH patients (age 4–17 years) (from 20 to 6 dynes/cm^2), whereas shear stress increases in the distal small arteries (with a diameter between 100 and 500 μm) (from 20 to 116 dyn/cm^2) and the microvasculature below 100 μm (from 50 to 300 dyn/cm^2).[36] These shear stress values gave us important information to set up the further in vitro experiments or PH animal models.

Table2. Summary of shear stress values in PH patients from different studies.

Mean pulmonary artery(MPA); Right pulmonary artery(RPA); 1 dyn/cm^2 = 0.1 N/m^2; 1 dyn/m^2, = 1.0E-5 Pa

PH Model	Methods	Groups	Measurement Site	Unit	Wall shear stress (WSS)	Ref.
PAH	A combined MRI and computational fluid dynamics (CFD)	n = 5 Control *VS* n=5 PAH patients	Mean WSS in the proximal PAs and distal PAs	dyn/cm^2	Proximal 20.5 ± 4.0 *VS* 4.3 ± 2.8 Distal 14.1 ± 0.7 *VS* 10.1 ± 0.9 WSS was found to be significantly lower in the proximal PAs of PAH patients.	37
PAH	Based on 2D phase contrast MRI	n=12 control *VS* n=11 PAH patients	MPA(2cm proximal to bifurcation)	N/m^2	0.28 ± 0.04 *VS* 0.21 ± 0.06 PAH patients exhibited lower WSS values than control group in proximal PAs	38
PAH	4d flow model; right heart catheter (RHC)	n=6 control *VS* n=9 PAH patients	Mean WSS values in the proximal arterials	pa	MPA 0.47 ± 0.07 *VS* 0.46 ± 0.09	39
PAH	PC-MRI,3D models	n=6 PAH patients	Rest to exercise in proximal WSS and distal WSS	dyn/cm^2	Proximal: 19.8 ± 4.0 to 51.8 ± 6.7 Distal: 13.6 ± 1.4 to 32.8 ± 3.0 Low WSS near branch points in the proximal arteries in PAH.	40
PAH	MRI; 4D flow model	n=19 control *VS* n=17PAH patients	MPA	N/m^2	2.05 *VS* 0.43 Low WSS in MPA of PAH patients.	41
Pediatric PAH patients	MRI to compute time resolved wall shear stress	n=26 Control *VS* n=40 PAH patients	MPA and RPA	dyn/cm^2	WSS(sys)MPA:6.49 ± 1.74 *vs*4.37 ± 2.36 RPA:11.25 ± 2.47 *vs* 7.35 ± 3.74 The WSS is reduced in the proximal pulmonary arteries of pediatric PAH patients.	42
Pediatric PAH patients	phase contrast cardiac magnetic resonance	n=23 Control *vs* n=56 PAH patients	8 points in the MPA lumen	dyn/cm^2	WSS_{max} 6.3 ± 1.9 *vs* 4.2 ± 2.2 Reduced in WSSmax in the PAH of MPA	43

Pediatric PAH patients	CT, MRI, right heart catheterization; 3D computational model	a. control b. moderate PAH c. severe PAH	1. proximal PAs 2.100-500µm distal PAs 3.<500 µm distal small PAs	dyn/cm^2	**1.**20.5 *vs* 15.8 *vs* 6.3 **2.**20 *vs* 52 *vs* 116 **3.**20 *vs* 100 *vs* 200 A decreased WSS in the MPA in PAH. Mean WSS for the distal PAs significantly increased with disease severity.	36
Pediatric patients with PAH	CMR images; MATLAB program	Control (n = 4) *VS* PAH (n = 25) patients	Right pulmonary artery circumferentially averaged	dyn/cm^2	Control −6.6 ± 3.4 PAH −2.2 ± 1.6	44
Suspected secondary PAH	Magnitude images and phase images / 3T MR system	n=12 Non-PAH *VS* n= 5 PAH patients	Pulmonary artery trunk at 1 cm proximal to the bifurcation	N/m^2	WSS(mean) 0.489 ± 0.132 *vs* 0.365 ± 0.035. WSS were significantly lower in the PAH patients.	45
PH	CT, Right heart catheterization, computational fluid dynamics model	n=34 PH patients	arterial bifurcations	dyn/cm^2	Regions with abnormal WSS values (>50 dyn/cm2, i.e. >5 Pa).	46
PH	4D phase-contrast cardiac magnetic resonance imaging	5 Control vs 17 PH patients	In the right side of MPA	N/m^2	Systolic WSS 0.19 ± 0.07 vs 0.13 ± 0.03 In-plane average MPA WSS decreased compared with that in controls.	25
PH	right heart catheterization and MRI phase contrast imaging	n=13 Control *vs* 22 manifest PH patients *vs* 13 latent PH patients	MPA velocity	cm/s	Maximal peak velocities: 77cm/s *vs* 79 *vs* 74	47
PH	phase-contrast magnetic resonance imaging	N=20 PH patients	MPA		Generally, higher WSS is seen in the distal vessels, with occasional high stress concentrations at the left/right arterial bifurcations	48

2.3 Mechanically activated Piezo ion channels in pulmonary hypertension

The most relevant mediators involved in transducing signals from the biomechanical environment to intracellular pathways include integrins, growth factor receptors, G-protein-coupled receptors, mechanosensitive ion channels (e.g. K^+, Ca^{2+}), and cytoskeletal strain responses.[49, 50] Mechanical changes can be converted into chemical signals through mechanosensitive channels in the cell membrane. The mechanosensitive Piezo1/2 channels are non-selective cation channels. In the systemic circulation, Piezo1 has been shown to sense the blood flow by altering the shear-evoked ionic current resulting in calcium influx in endothelial cells.[51] Piezo1 activation in response to changes in flow pattern results in activation of inflammatory signaling and altered NO production.[51, 52] In the pulmonary circulation, Piezo1 is expressed in both pulmonary endothelial cells and smooth muscle cells, where it mediates opposing effects. Piezo1 expression was shown to increase intracellular Ca^{2+} concentration in pulmonary arterial smooth muscle cells (PASMCs) of PH patients, while sustained vasoconstriction is maintained by Ca^{2+} dependent mechanism.[53] Conversely, Piezo1-mediated calcium elevations are involved in flow-induced ATP release and NO formation in pulmonary endothelial cells (PAECs) which would subsequently relax vascular smooth muscle cells and reduce vascular tone.[51, 54]

Piezo2 is associated with sensory neuron biology, touch sensation, and mechanical pain.[55, 56] Piezo2 plays a critical role in the regulation of the Hering-Breuer reflex which prevents over-inflation of the lung, and Piezo2 deletion in vagal, spinal sensory, and dorsal root ganglion neurons led to reduced vagal nerve activity in response to lung inflation, increased tidal volume, and blunted Hering–Breuer reflex.[57, 58] Conditional disruption of Piezo1 or Piezo2 in the ganglia had no effect on blood pressure or baroreceptor reflex, but a double Piezo1/Piezo2 knockout decreased the heart rate and increased systolic blood pressure and blood pressure

variability.[56, 59] In metastatic cancer cells, Piezo2 modulates RhoA-activity, formation of actin-based stress fibers and orientation of focal adhesions, processes that also play a role in endothelial responses to shear stress.[60] Intriguingly, in the pulmonary vasculature Piezo2 expression has recently been shown to be restricted to pulmonary endothelial cells.[61] Whether Piezo2 plays a role in the physiological regulation of PVR and/or whether its altered expression may contribute to the development or progression of pulmonary vascular disease currently remains unclear.

3. Biomarkers of pulmonary hypertension- focus on Tryptophan metabolites

As introduced in section 1 and 2, shear stress and flow are known to influence endothelial cell function. Abnormal shear stress and disturbed flow induced metabolic changes in the endothelial cells that reduce the mitochondrial mass and function.[62] Coupling of mechanical sensing to endothelial phenotypic changes through metabolic signaling may therefore be clinically relevant in pulmonary hypertension.[63, 64] It is known that shear stress can impact the endothelial barrier function, induce inflammatory and metabolic changes with impaired mitochondrial function and fusion in endothelial cells with a BMPR2 mutation.[5, 65, 66] However, the mechanisms connecting cell mechanics and metabolism are still poorly understood.

Nicotinamide adenine dinucleotide (NAD^+) is a coenzyme involved in oxidation-reduction reactions, thereby modulating mitochondrial function and reducing ROS production and inflammation.[67] Laminar shear stress preserves the endothelial homeostasis through an NAD^+ dependent mechanism to promote mitochondrial biogenesis.[68] Furthermore, SIRT1, an NAD^+ dependent deacetylase, has been proposed to function as a master regulator of the mechanical stress response as well as of cellular energy metabolism (glucose metabolism).[69] Altogether, these data suggest that NAD^+ may be a factor in coupling cell mechanics and metabolism.

Mammalian cells can generate NAD^+ de novo from dietary tryptophan (Trp) through the kynurenine pathway (KP), which is initialized by either TDO or IDO.[70] Trp is essential for protein biosynthesis but is also metabolized via different pathways, resulting in metabolites with a wide variety of physiological functions. The main pathway in Trp metabolites is the KP, through which >90% of available Trp is metabolized.[71, 72] The KP, which is shown in Figure 3, produces many biologically active metabolites, including N-methyl-d-aspartate(NMDA) receptor agonist quinolinic acid(QA) and immunosuppressive K metabolites 3-hydroxykynurenine (3-HK) and 3-hydroxyanthranilic acid(3-HA)**.** Apart from the KP, most NAD^+ is recycled from nicotinamide (NAM), nicotinamide riboside(NR) and nicotinamide mononucleotide (NMN) in the salvage pathway to maintain the cellular NAD^+ levels.[73] NAM could be recycled from NAD^+ consumption reactions, including both NAD^+-dependent deacylation and ADP-ribosylation, into NMN by nicotinamide phosphoribosyltransferase (NAMPT), which catalyzes the rate-limiting reaction in the salvage pathway(Figure 3).[70] NAMPT regulates the activity of NAD-dependent enzymes, such as SIRT and poly(ADP-ribose) polymerases(PARPs), and functions as a master regulator of cellular metabolism, and mitochondrial biogenesis.[74] Intriguingly, the NAMPT was shown to be enhanced in PAH patients as well as in animal models of PH.[74] Moreover, it was found that NAMPT-derived NAD^+ promotes TNF-α synthesis and induced PASMCs proliferation, suggesting that the PAH might be a disease of NAD^+ abundancy and NAMPT inhibition may be a potential therapeutic target attenuating the vascular remodeling in the PAH.[74] However, the KP is the sole route for de novo NAD^+ synthesis and some studies highlight the importance of KP in the development and progression of PH which may then also act as potential therapeutic targets in PAH.

The etiology of PAH is incompletely understood; several factors are implicated in its pathogenesis, including genetic predisposition and exposure to toxins and/or

inflammatory mediators.[75] As such, there is increasing interest in the use of biomarkers as a means of diagnosis and screening. The ideal biomarker should be easy to measure and capable of monitoring the course of PAH and its response to the therapy.[75]

Using the KP metabolites as biomarkers for disease dates back to the 1950s, when KP metabolites in urine were used to diagnose the cancer, rheumatoid arthritis, and cardiovascular events.[71, 76, 77] In studies of patients with end-stage renal disease, activation of the KP was associated with increased oxidative stress, inflammation, homeostatic disturbances, and the prevalence of cardiovascular disease.[78] In 2016, findings in a PAH cohort suggested that KP metabolites correlated with pulmonary arterial pressure and pulmonary vascular dysfunction.[79] Tryptophan- KP metabolites are significantly increased in PAH patients' serum, and strongly correlated with mean pulmonary arterial pressure.[80-82] In addition, an elevated kynurenine concentration is associated with immune dysfunction and inflammation as well as with an adverse clinical course.[82] However, although these findings suggest that KP metabolites may be useful biomarkers in PAH, the cause of KP dysfunction in PAH, levels of KP metabolites in treatment-naïve patients and the effect of PAH therapy on KP metabolites have not been assessed.

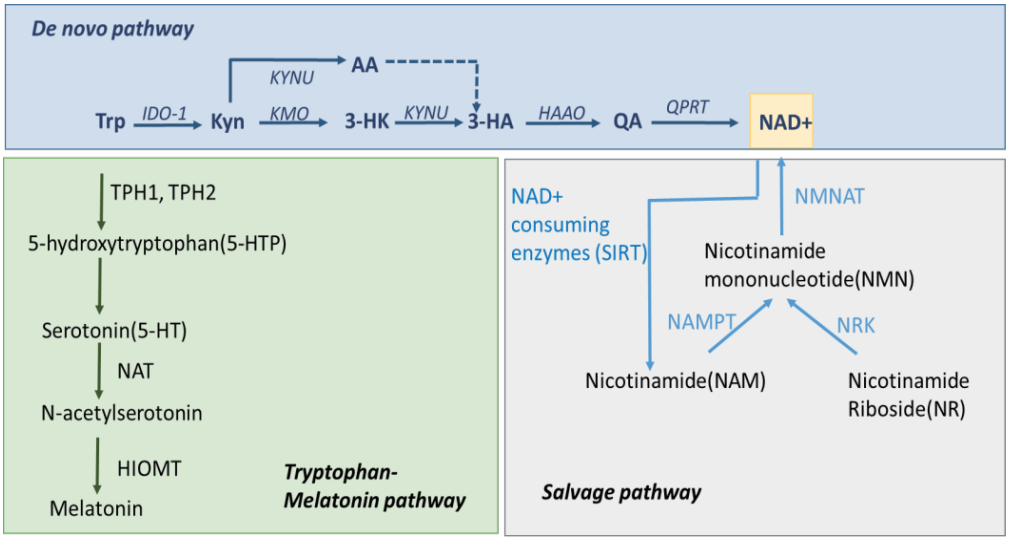


Figure 3. De novo pathway and salvage pathway of NAD^+ and link to tryptophan melatonin pathway. NAD^+ is synthesized through the de novo pathway and the salvage pathway in mammalian cells. Tryptophan is metabolized through the serotonin and melatonin. NMNAT: nicotinamide mononucleotide adenylyltransferase. TPH: tryptophan hydroxylase. (Adjusted from Chapter 4).

Another Trp metabolizing pathway that may have importance in PAH is the serotonin pathway. This pathway encompasses conversion of tryptophan to serotonin (5-HT), via tryptophan hydroxylase(TPH), 5-hydroxytryptophan(5-HTP) and aromatic L-amino acid decarboxylase(Figure 3).[83] The conversion of 5-HT to melatonin is another two-step process and is catalyzed by two rate-limiting enzymes: N-acetyltransferase(NAT) and hydroxyindole-O-methyltransferase (HIOMT).[84]

Melatonin is one of the end products in the tryptophan-serotonin pathway. Melatonin which is secreted from the pineal gland at night, has great potential as a therapeutic drug for preventing inflammation and regulating the vascular reactivity in cardiovascular diseases.[85] Thus, in ischemic heart disease, melatonin treatment significantly reduced the inflammatory cytokines, TNFα, IL6 and TGFβ.[86]

Furthermore, it was reported that melatonin has anti-hypertensive effects in systemic hypertension, by reducing smooth muscle tone and thereby lowering arterial blood pressure.[87, 88] Another study showed that melatonin could decrease LDL and body weight in high-fat diet-induced nonalcoholic fatty liver disease in mice, thereby protecting against metabolic dysfunction.[89]

In PAH, hypoxia is one of the key factors inducing pulmonary vascular remodeling. Melatonin mitigated oxidative injury and restored the NO production in chronically hypoxic rats with pulmonary hypertension.[90] Melatonin also showed antioxidant and vasodilator effects thereby decreasing oxidant stress and improving the pulmonary vascular function in the chronic hypoxic ovine neonate with PAH.[91] Furthermore, melatonin could attenuate endothelial leakage and the formation of inflammasome multiprotein complexes in macrophages in the lungs of PAH mice thereby reducing pulmonary inflammation, which is a critical factor in PAH.[92] Although these studies suggest that melatonin could be beneficial for the treatment of PH patients, at present melatonin metabolism including the endogenous levels of melatonin in treatment-naïve PH patients and their clinical significance are poorly understood.

Aims and outline of this thesis

This thesis aims to investigate new mechanisms underlying pulmonary vascular remodeling as a basis for future targets in the treatment of the PH patients. In Part 1 (**Chapter 2-3**), we summarize the role of mechanotransduction in the development and progression of PH and specifically investigate the potential involvement of mechanotransduction channel Piezo2. In Part 2 (**Chapter 4-5**), we utilized a cohort of PH patients with long-term follow-up to investigate the prognostic values of metabolites of tryptophan through the kynurenine pathway and melatonin pathway. Our approach encompassed evaluation of potential biomarkers in plasma obtained from PAH patients before and after initiation of treatment and analysis of tissues obtained from animal models of PH, in vitro experiments in pulmonary vascular cells.

In **Chapter 2**, we review current knowledge on how changes in vascular stiffness affect mechanical forces acting on the endothelium and smooth muscle cells and how these changes in mechanical forces may contribute to the progression of PH. Mechanical changes can be converted to chemical signals through mechanosensitive channels in the cell membrane. In **Chapter 3**, we investigate the role of two of such mechanosensitive channels, the ion channels Piezo1 and 2, in the pulmonary vasculature. Because expression of particularly Piezo2 appears to be altered in animal models of PH, we performed experiments to identify the role of Piezo2 in the pulmonary vasculature and how altered expression may be involved in development and/or progression of PH. As Piezo2 expression was confined to the pulmonary endothelium, we focus on characterization of the contribution of Piezo2 in shear stress induced Ca^{2+} influx and NO production in pulmonary microvascular endothelial cells, as well as endothelial alignment to flow and endothelial to mesenchymal transition.

To assess alterations in tryptophan metabolism in PAH, in **Chapter 4 and 5** we analyzed samples obtained in the Biopulse study, a cohort of 64 consecutive PH patients (43 PAH, 21 CTEPH) in the Erasmus MC. The patients were diagnosed according to the guidelines by right heart catheterization between May 2012 and October 2016 and they were prospectively followed up until the 1st of January 2019. Ultra-performed liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) was used to determine the tryptophan metabolites, including KP metabolites (**Chapter 4**) and melatonin (**Chapter 5**), from plasma obtained at the time of diagnosis and after initiation of treatment. To further investigate pathogenic mechanisms, lung microvascular endothelial cells, pulmonary artery smooth muscle cells and pulmonary fibroblasts were exposed to different cytokines as well as hypoxia and shear stress, and secretion of kynurenine metabolites was measured in **Chapter 4**.

Our results are summarized and further discussed in **Chapter 6**, which also includes a perspective for future directions.

References:

1. Jain V, Bordes S and Bhardwaj A. Physiology, Pulmonary Circulatory System. 2022.
2. Bevegard BS and Shepherd JT. Regulation of the circulation during exercise in man. *Physiol Rev*. 1967;47:178-213.
3. Chamarthy MR, Kandathil A and Kalva SP. Pulmonary vascular pathophysiology. *Cardiovasc Diagn Ther*. 2018;8:208-213.
4. Townsley MI. Structure and composition of pulmonary arteries, capillaries, and veins. *Compr Physiol*. 2012;2:675-709.
5. Humbert M, Guignabert C, Bonnet S, Dorfmuller P, Klinger JR, Nicolls MR, Olschewski AJ, Pullamsetti SS, Schermuly RT, Stenmark KR and Rabinovitch M. Pathology and pathobiology of pulmonary hypertension: state of the art and research perspectives. *Eur Respir J*. 2019;53.
6. Barbeau S, Gilbert G, Cardouat G, Baudrimont I, Freund-Michel V, Guibert C, Marthan R, Vacher P, Quignard JF and Ducret T. Mechanosensitivity in Pulmonary Circulation: Pathophysiological Relevance of Stretch-Activated Channels in Pulmonary Hypertension. *Biomolecules*. 2021;11.
7. van der Feen DE, Bartelds B, de Boer RA and Berger RMF. Assessment of reversibility in pulmonary arterial hypertension and congenital heart disease. *Heart*. 2019;105:276-282.
8. Simonneau G, Montani D, Celermajer DS, Denton CP, Gatzoulis MA, Krowka M, Williams PG and Souza R. Haemodynamic definitions and updated clinical classification of pulmonary hypertension. *Eur Respir J*. 2019;53.
9. Mandras SA, Mehta HS and Vaidya A. Pulmonary Hypertension: A Brief Guide for Clinicians. *Mayo Clin Proc*. 2020;95:1978-1988.
10. Hoeper MM, Humbert M, Souza R, Idrees M, Kawut SM, Sliwa-Hahnle K, Jing ZC and Gibbs JS. A global view of pulmonary hypertension. *Lancet Respir Med*. 2016;4:306-22.
11. Vazquez ZGS and Klinger JR. Guidelines for the Treatment of Pulmonary Arterial Hypertension. *Lung*. 2020;198:581-596.
12. Jonigk D, Golpon H, Bockmeyer CL, Maegel L, Hoeper MM, Gottlieb J, Nickel N, Hussein K, Maus U, Lehmann U, Janciauskiene S, Welte T, Haverich A, Rische J, Kreipe H and Laenger F. Plexiform lesions in pulmonary arterial hypertension composition, architecture, and microenvironment. *Am J Pathol*. 2011;179:167-79.
13. Chatterjee K, De Marco T and Alpert JS. Pulmonary hypertension: hemodynamic diagnosis and management. *Arch Intern Med*. 2002;162:1925-33.
14. Happe CM, Szulcek R, Voelkel NF and Bogaard HJ. Reconciling paradigms of abnormal pulmonary blood flow and quasi-malignant cellular alterations in pulmonary arterial hypertension. *Vascular pharmacology*. 2016;83:17-25.
15. Humbert M, Lau EM, Montani D, Jais X, Sitbon O and Simonneau G. Advances in therapeutic interventions for patients with pulmonary arterial hypertension. *Circulation*. 2014;130:2189-208.
16. Hassoun PM. Pulmonary Arterial Hypertension. *N Engl J Med*. 2021;385:2361-2376.
17. Ruopp NF and Cockrill BA. Diagnosis and Treatment of Pulmonary Arterial Hypertension: A Review. *JAMA*. 2022;327:1379-1391.
18. Simonneau G, Barst RJ, Galie N, Naeije R, Rich S, Bourge RC, Keogh A, Oudiz R, Frost A, Blackburn SD, Crow JW, Rubin LJ and Treprostinil Study G. Continuous subcutaneous

infusion of treprostinil, a prostacyclin analogue, in patients with pulmonary arterial hypertension: a double-blind, randomized, placebo-controlled trial. *Am J Respir Crit Care Med*. 2002;165:800-4.
19. Sitbon O and Morrell N. Pathways in pulmonary arterial hypertension: the future is here. *Eur Respir Rev*. 2012;21:321-7.
20. Poch D and Mandel J. Pulmonary Hypertension. *Ann Intern Med*. 2021;174:ITC49-ITC64.
21. Sommer N, Ghofrani HA, Pak O, Bonnet S, Provencher S, Sitbon O, Rosenkranz S, Hoeper MM and Kiely DG. Current and future treatments of pulmonary arterial hypertension. *Br J Pharmacol*. 2021;178:6-30.
22. Maringanti R, Meijer E, Brandt MM, Duncker DJ and Cheng C. Contributions of Wall Stretch and Shear Stress to Vascular Regulation: Molecular Mechanisms of Homeostasis and Expansion. In: M. Hecker and D. J. Duncker, eds. *Vascular Mechanobiology in Physiology and Disease* Cham: Springer International Publishing; 2021: 21-46.
23. Lehoux S and Tedgui A. Signal transduction of mechanical stresses in the vascular wall. *Hypertension*. 1998;32:338-45.
24. Li M, Stenmark KR, Shandas R and Tan W. Effects of pathological flow on pulmonary artery endothelial production of vasoactive mediators and growth factors. *J Vasc Res*. 2009;46:561-71.
25. Schafer M, Kheyfets VO, Schroeder JD, Dunning J, Shandas R, Buckner JK, Browning J, Hertzberg J, Hunter KS and Fenster BE. Main pulmonary arterial wall shear stress correlates with invasive hemodynamics and stiffness in pulmonary hypertension. *Pulmonary circulation*. 2016;6:37-45.
26. Fitzgerald TN, Shepherd BR, Asada H, Teso D, Muto A, Fancher T, Pimiento JM, Maloney SP and Dardik A. Laminar shear stress stimulates vascular smooth muscle cell apoptosis via the Akt pathway. *J Cell Physiol*. 2008;216:389-95.
27. Dickinson MG, Bartelds B, Borgdorff MA and Berger RM. The role of disturbed blood flow in the development of pulmonary arterial hypertension: lessons from preclinical animal models. *Am J Physiol Lung Cell Mol Physiol*. 2013;305:L1-14.
28. Chiu JJ and Chien S. Effects of disturbed flow on vascular endothelium: pathophysiological basis and clinical perspectives. *Physiol Rev*. 2011;91:327-87.
29. Sanz J, Kariisa M, Dellegrottaglie S, Prat-Gonzalez S, Garcia MJ, Fuster V and Rajagopalan S. Evaluation of pulmonary artery stiffness in pulmonary hypertension with cardiac magnetic resonance. *JACC Cardiovasc Imaging*. 2009;2:286-95.
30. Dieffenbach PB, Maracle M, Tschumperlin DJ and Fredenburgh LE. Mechanobiological Feedback in Pulmonary Vascular Disease. *Front Physiol*. 2018;9:951.
31. Tan W, Madhavan K, Hunter KS, Park D and Stenmark KR. Vascular stiffening in pulmonary hypertension: cause or consequence? (2013 Grover Conference series). *Pulmonary circulation*. 2014;4:560-80.
32. Saouti N, Westerhof N, Postmus PE and Vonk-Noordegraaf A. The arterial load in pulmonary hypertension. *Eur Respir Rev*. 2010;19:197-203.
33. Kurakula K, Smolders V, Tura-Ceide O, Jukema JW, Quax PHA and Goumans MJ. Endothelial Dysfunction in Pulmonary Hypertension: Cause or Consequence? *Biomedicines*. 2021;9.
34. Yang W, Dong M, Rabinovitch M, Chan FP, Marsden AL and Feinstein JA. Evolution of hemodynamic forces in the pulmonary tree with progressively worsening pulmonary

arterial hypertension in pediatric patients. *Biomechanics and modeling in mechanobiology*. 2019.

35. Lambert M, Capuano V, Olschewski A, Sabourin J, Nagaraj C, Girerd B, Weatherald J, Humbert M and Antigny F. Ion Channels in Pulmonary Hypertension: A Therapeutic Interest? *Int J Mol Sci*. 2018;19.

36. Yang W, Dong M, Rabinovitch M, Chan FP, Marsden AL and Feinstein JA. Evolution of hemodynamic forces in the pulmonary tree with progressively worsening pulmonary arterial hypertension in pediatric patients. *Biomechanics and modeling in mechanobiology*. 2019;18:779-796.

37. Tang BT, Pickard SS, Chan FP, Tsao PS, Taylor CA and Feinstein JA. Wall shear stress is decreased in the pulmonary arteries of patients with pulmonary arterial hypertension: An image-based, computational fluid dynamics study. *Pulmonary circulation*. 2012;2:470-6.

38. Wang HH, Tseng WI, Yu HY, Chang MC and Peng HH. Phase-contrast magnetic resonance imaging for analyzing hemodynamic parameters and wall shear stress of pulmonary arteries in patients with pulmonary arterial hypertension. *MAGMA*. 2019;32:617-627.

39. Odagiri K, Inui N, Hakamata A, Inoue Y, Suda T, Takehara Y, Sakahara H, Sugiyama M, Alley MT, Wakayama T and Watanabe H. Non-invasive evaluation of pulmonary arterial blood flow and wall shear stress in pulmonary arterial hypertension with 3D phase contrast magnetic resonance imaging. *SpringerPlus*. 2016;5:1071.

40. Tang BT, Fonte TA, Chan FP, Tsao PS, Feinstein JA and Taylor CA. Three-dimensional hemodynamics in the human pulmonary arteries under resting and exercise conditions. *Ann Biomed Eng*. 2011;39:347-58.

41. Barker AJ, Roldan-Alzate A, Entezari P, Shah SJ, Chesler NC, Wieben O, Markl M and Francois CJ. Four-dimensional flow assessment of pulmonary artery flow and wall shear stress in adult pulmonary arterial hypertension: results from two institutions. *Magnetic resonance in medicine*. 2015;73:1904-13.

42. Schafer M, Ivy DD, Barker AJ, Kheyfets V, Shandas R, Abman SH, Hunter KS and Truong U. Characterization of CMR-derived haemodynamic data in children with pulmonary arterial hypertension. *European heart journal cardiovascular Imaging*. 2017;18:424-431.

43. Friesen RM, Schafer M, Ivy DD, Abman SH, Stenmark K, Browne LP, Barker AJ, Hunter KS and Truong U. Proximal pulmonary vascular stiffness as a prognostic factor in children with pulmonary arterial hypertension. *European heart journal cardiovascular Imaging*. 2019;20:209-217.

44. Truong U, Fonseca B, Dunning J, Burgett S, Lanning C, Ivy DD, Shandas R, Hunter K and Barker AJ. Wall shear stress measured by phase contrast cardiovascular magnetic resonance in children and adolescents with pulmonary arterial hypertension. *Journal of cardiovascular magnetic resonance : official journal of the Society for Cardiovascular Magnetic Resonance*. 2013;15:81.

45. Terada M, Takehara Y, Isoda H, Uto T, Matsunaga M and Alley M. Low WSS and High OSI Measured by 3D Cine PC MRI Reflect High Pulmonary Artery Pressures in Suspected Secondary Pulmonary Arterial Hypertension. *Magnetic resonance in medical sciences : MRMS : an official journal of Japan Society of Magnetic Resonance in Medicine*. 2016;15:193-202.

46. Piskin S, Patnaik SS, Han D, Bordones AD, Murali S and Finol EA. A canonical correlation analysis of the relationship between clinical attributes and patient-specific hemodynamic indices in adult pulmonary hypertension. *Med Eng Phys*. 2020;77:1-9.
47. Reiter G, Reiter U, Kovacs G, Kainz B, Schmidt K, Maier R, Olschewski H and Rienmueller R. Magnetic resonance-derived 3-dimensional blood flow patterns in the main pulmonary artery as a marker of pulmonary hypertension and a measure of elevated mean pulmonary arterial pressure. *Circ Cardiovasc Imaging*. 2008;1:23-30.
48. Kheyfets V, Thirugnanasambandam M, Rios L, Evans D, Smith T, Schroeder T, Mueller J, Murali S, Lasorda D, Spotti J and Finol E. The role of wall shear stress in the assessment of right ventricle hydraulic workload. *Pulmonary circulation*. 2015;5:90-100.
49. Marchioni A, Tonelli R, Cerri S, Castaniere I, Andrisani D, Gozzi F, Bruzzi G, Manicardi L, Moretti A, Demurtas J, Baroncini S, Andreani A, Cappiello GF, Busani S, Fantini R, Tabbi L, Samarelli AV and Clini E. Pulmonary Stretch and Lung Mechanotransduction: Implications for Progression in the Fibrotic Lung. *Int J Mol Sci*. 2021;22.
50. Duscher D, Maan ZN, Wong VW, Rennert RC, Januszyk M, Rodrigues M, Hu M, Whitmore AJ, Whittam AJ, Longaker MT and Gurtner GC. Mechanotransduction and fibrosis. *J Biomech*. 2014;47:1997-2005.
51. Wang S, Chennupati R, Kaur H, Iring A, Wettschureck N and Offermanns S. Endothelial cation channel PIEZO1 controls blood pressure by mediating flow-induced ATP release. *J Clin Invest*. 2016;126:4527-4536.
52. Albarran-Juarez J, Iring A, Wang S, Joseph S, Grimm M, Strilic B, Wettschureck N, Althoff TF and Offermanns S. Piezo1 and Gq/G11 promote endothelial inflammation depending on flow pattern and integrin activation. *J Exp Med*. 2018;215:2655-2672.
53. Liao J, Lu W, Chen Y, Duan X, Zhang C, Luo X, Lin Z, Chen J, Liu S, Yan H, Chen Y, Feng H, Zhou D, Chen X, Zhang Z, Yang Q, Liu X, Tang H, Li J, Makino A, Yuan JX, Zhong N, Yang K and Wang J. Upregulation of Piezo1 (Piezo Type Mechanosensitive Ion Channel Component 1) Enhances the Intracellular Free Calcium in Pulmonary Arterial Smooth Muscle Cells From Idiopathic Pulmonary Arterial Hypertension Patients. *Hypertension*. 2021;77:1974-1989.
54. Lhomme A, Gilbert G, Pele T, Deweirdt J, Henrion D, Baudrimont I, Campagnac M, Marthan R, Guibert C, Ducret T, Savineau JP and Quignard JF. Stretch-activated Piezo1 Channel in Endothelial Cells Relaxes Mouse Intrapulmonary Arteries. *Am J Respir Cell Mol Biol*. 2019;60:650-658.
55. Dubin AE, Schmidt M, Mathur J, Petrus MJ, Xiao B, Coste B and Patapoutian A. Inflammatory signals enhance piezo2-mediated mechanosensitive currents. *Cell Rep*. 2012;2:511-7.
56. Beech DJ and Kalli AC. Force Sensing by Piezo Channels in Cardiovascular Health and Disease. *Arterioscler Thromb Vasc Biol*. 2019;39:2228-2239.
57. Nonomura K, Woo SH, Chang RB, Gillich A, Qiu Z, Francisco AG, Ranade SS, Liberles SD and Patapoutian A. Piezo2 senses airway stretch and mediates lung inflation-induced apnoea. *Nature*. 2017;541:176-181.
58. Zhong M, Komarova Y, Rehman J and Malik AB. Mechanosensing Piezo channels in tissue homeostasis including their role in lungs. *Pulm Circ*. 2018;8:2045894018767393.
59. Zeng WZ, Marshall KL, Min S, Daou I, Chapleau MW, Abboud FM, Liberles SD and Patapoutian A. PIEZOs mediate neuronal sensing of blood pressure and the baroreceptor reflex. *Science*. 2018;362:464-467.

60. Pardo-Pastor C, Rubio-Moscardo F, Vogel-Gonzalez M, Serra SA, Afthinos A, Mrkonjic S, Destaing O, Abenza JF, Fernandez-Fernandez JM, Trepat X, Albiges-Rizo C, Konstantopoulos K and Valverde MA. Piezo2 channel regulates RhoA and actin cytoskeleton to promote cell mechanobiological responses. *Proc Natl Acad Sci U S A*. 2018;115:1925-1930.
61. Hong J, Arneson D, Umar S, Ruffenach G, Cunningham CM, Ahn IS, Diamante G, Bhetraratana M, Park JF, Said E, Huynh C, Le T, Medzikovic L, Humbert M, Soubrier F, Montani D, Girerd B, Tregouet DA, Channick R, Saggar R, Eghbali M and Yang X. Single-Cell Study of Two Rat Models of Pulmonary Arterial Hypertension Reveals Connections to Human Pathobiology and Drug Repositioning. *American journal of respiratory and critical care medicine*. 2021;203:1006-1022.
62. Chen Z, Peng IC, Cui X, Li YS, Chien S and Shyy JY. Shear stress, SIRT1, and vascular homeostasis. *Proc Natl Acad Sci U S A*. 2010;107:10268-73.
63. Wu D and Birukov K. Endothelial Cell Mechano-Metabolomic Coupling to Disease States in the Lung Microvasculature. *Front Bioeng Biotechnol*. 2019;7:172.
64. Romani P, Valcarcel-Jimenez L, Frezza C and Dupont S. Crosstalk between mechanotransduction and metabolism. *Nature reviews Molecular cell biology*. 2021;22:22-38.
65. Chen NY, S DC, Luo F, Weng T, Le TT, A MH, Philip K, Molina JG, Garcia-Morales LJ, Cao Y, Ko TC, Amione-Guerra J, Al-Jabbari O, Bunge RR, Youker K, Bruckner BA, Hamid R, Davies J, Sinha N and Karmouty-Quintana H. Macrophage bone morphogenic protein receptor 2 depletion in idiopathic pulmonary fibrosis and Group III pulmonary hypertension. *American journal of physiology Lung cellular and molecular physiology*. 2016;311:L238-54.
66. Diebold I, Hennigs JK, Miyagawa K, Li CG, Nickel NP, Kaschwich M, Cao A, Wang L, Reddy S, Chen PI, Nakahira K, Alcazar MA, Hopper RK, Ji L, Feldman BJ and Rabinovitch M. BMPR2 preserves mitochondrial function and DNA during reoxygenation to promote endothelial cell survival and reverse pulmonary hypertension. *Cell Metab*. 2015;21:596-608.
67. Katsyuba E, Mottis A, Zietak M, De Franco F, van der Velpen V, Gariani K, Ryu D, Cialabrini L, Matilainen O, Liscio P, Giacche N, Stokar-Regenscheit N, Legouis D, de Seigneux S, Ivanisevic J, Raffaelli N, Schoonjans K, Pellicciari R and Auwerx J. De novo NAD(+) synthesis enhances mitochondrial function and improves health. *Nature*. 2018;563:354-359.
68. Kim JS, Kim B, Lee H, Thakkar S, Babbitt DM, Eguchi S, Brown MD and Park JY. Shear stress-induced mitochondrial biogenesis decreases the release of microparticles from endothelial cells. *American journal of physiology Heart and circulatory physiology*. 2015;309:H425-33.
69. Somemura S, Kumai T, Yatabe K, Sasaki C, Fujiya H, Niki H and Yudoh K. Physiologic Mechanical Stress Directly Induces Bone Formation by Activating Glucose Transporter 1 (Glut 1) in Osteoblasts, Inducing Signaling via NAD+-Dependent Deacetylase (Sirtuin 1) and Runt-Related Transcription Factor 2 (Runx2). *Int J Mol Sci*. 2021;22.
70. Xie N, Zhang L, Gao W, Huang C, Huber PE, Zhou X, Li C, Shen G and Zou B. NAD(+) metabolism: pathophysiologic mechanisms and therapeutic potential. *Signal Transduct Target Ther*. 2020;5:227.
71. Gal EM and Sherman AD. Synthesis and metabolism of L-kynurenine in rat brain. *J Neurochem*. 1978;30:607-13.
72. Sakurai M, Yamamoto Y, Kanayama N, Hasegawa M, Mouri A, Takemura M, Matsunami H, Miyauchi T, Tokura T, Kimura H, Ito M, Umemura E, Boku AS, Nagashima W,

Tonoike T, Kurita K, Ozaki N, Nabeshima T and Saito K. Serum Metabolic Profiles of the Tryptophan-Kynurenine Pathway in the high risk subjects of major depressive disorder. *Sci Rep*. 2020;10:1961.

73. Braidy N, Berg J, Clement J, Khorshidi F, Poljak A, Jayasena T, Grant R and Sachdev P. Role of Nicotinamide Adenine Dinucleotide and Related Precursors as Therapeutic Targets for Age-Related Degenerative Diseases: Rationale, Biochemistry, Pharmacokinetics, and Outcomes. *Antioxidants & redox signaling*. 2019;30:251-294.

74. Chen J, Sysol JR, Singla S, Zhao S, Yamamura A, Valdez-Jasso D, Abbasi T, Shioura KM, Sahni S, Reddy V, Sridhar A, Gao H, Torres J, Camp SM, Tang H, Ye SQ, Comhair S, Dweik R, Hassoun P, Yuan JX, Garcia JGN and Machado RF. Nicotinamide Phosphoribosyltransferase Promotes Pulmonary Vascular Remodeling and Is a Therapeutic Target in Pulmonary Arterial Hypertension. *Circulation*. 2017;135:1532-1546.

75. Warwick G, Thomas PS and Yates DH. Biomarkers in pulmonary hypertension. *Eur Respir J*. 2008;32:503-12.

76. Tan VX and Guillemin GJ. Kynurenine Pathway Metabolites as Biomarkers for Amyotrophic Lateral Sclerosis. *Front Neurosci*. 2019;13:1013.

77. Kaihara M, Price JM and Takahashi H. The conversion of kynurenic acid to quinaldic acid by humans and rats. *J Biol Chem*. 1956;223:705-8.

78. Pawlak K, Domaniewski T, Mysliwiec M and Pawlak D. The kynurenines are associated with oxidative stress, inflammation and the prevalence of cardiovascular disease in patients with end-stage renal disease. *Atherosclerosis*. 2009;204:309-14.

79. Lewis GD, Ngo D, Hemnes AR, Farrell L, Domos C, Pappagianopoulos PP, Dhakal BP, Souza A, Shi X, Pugh ME, Beloiartsev A, Sinha S, Clish CB and Gerszten RE. Metabolic Profiling of Right Ventricular-Pulmonary Vascular Function Reveals Circulating Biomarkers of Pulmonary Hypertension. *J Am Coll Cardiol*. 2016;67:174-189.

80. Nagy BM, Nagaraj C, Meinitzer A, Sharma N, Papp R, Foris V, Ghanim B, Kwapiszewska G, Kovacs G, Klepetko W, Pieber TR, Mangge H, Olschewski H and Olschewski A. Importance of kynurenine in pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol*. 2017;313:L741-L751.

81. Pawlak K, Mysliwiec M and Pawlak D. Haemostatic system, biochemical profiles, kynurenines and the prevalence of cardiovascular disease in peritoneally dialyzed patients. *Thromb Res*. 2010;125:e40-5.

82. Jasiewicz M, Moniuszko M, Pawlak D, Knapp M, Rusak M, Kazimierczyk R, Musial WJ, Dabrowska M and Kaminski KA. Activity of the kynurenine pathway and its interplay with immunity in patients with pulmonary arterial hypertension. *Heart*. 2016;102:230-7.

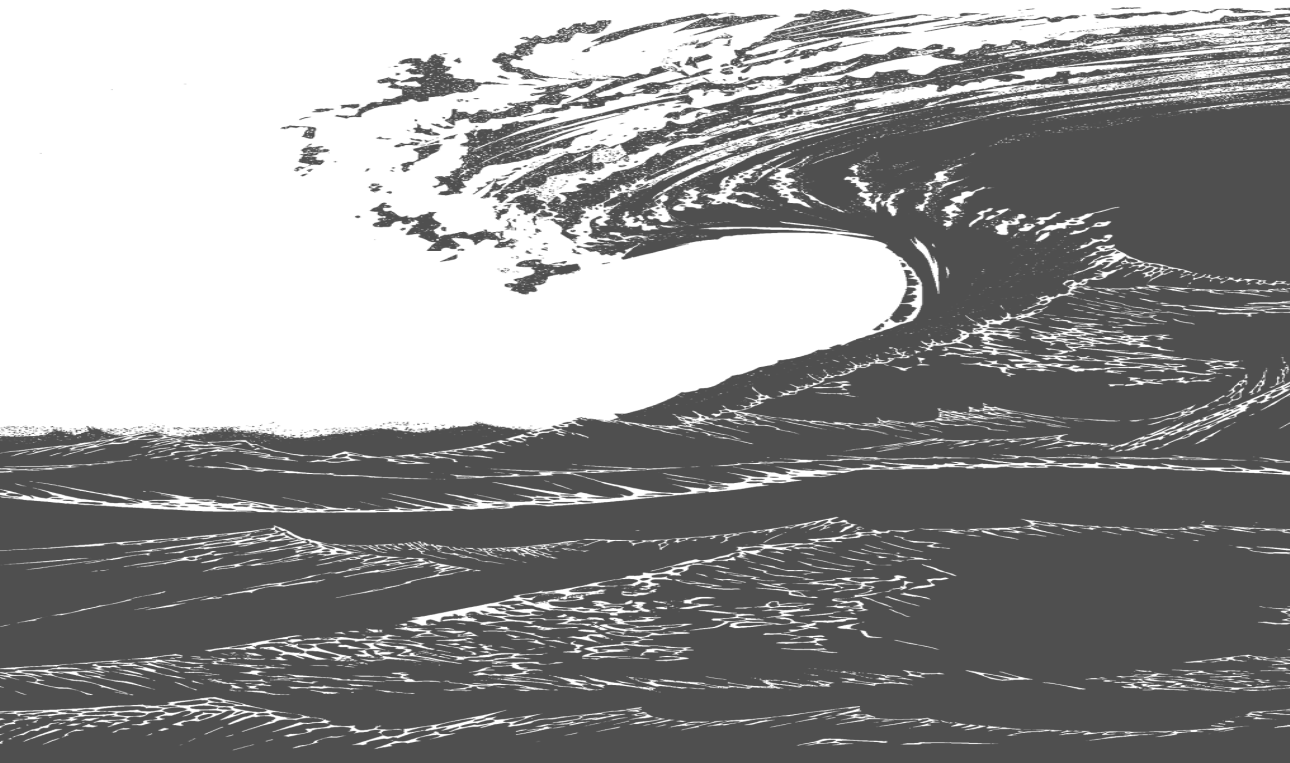
83. MacLean MMR. The serotonin hypothesis in pulmonary hypertension revisited: targets for novel therapies (2017 Grover Conference Series). *Pulm Circ*. 2018;8:2045894018759125.

84. Jena A, Montoya CA, Mullaney JA, Dilger RN, Young W, McNabb WC and Roy NC. Gut-Brain Axis in the Early Postnatal Years of Life: A Developmental Perspective. *Front Integr Neurosci*. 2020;14:44.

85. Wang W and Gao J. Effects of melatonin on protecting against lung injury (Review). *Exp Ther Med*. 2021;21:228.

86. Yeung HM, Hung MW, Lau CF and Fung ML. Cardioprotective effects of melatonin against myocardial injuries induced by chronic intermittent hypoxia in rats. *J Pineal Res*. 2015;58:12-25.

87. Kawashima K, Miwa Y, Fujimoto K, Oohata H, Nishino H and Koike H. Antihypertensive action of melatonin in the spontaneously hypertensive rat. *Clin Exp Hypertens A*. 1987;9:1121-31.
88. Girouard H, Chulak C, Lejossec M, Lamontagne D and de Champlain J. Vasorelaxant effects of the chronic treatment with melatonin on mesenteric artery and aorta of spontaneously hypertensive rats. *J Hypertens*. 2001;19:1369-77.
89. Celinski K, Konturek PC, Slomka M, Cichoz-Lach H, Brzozowski T, Konturek SJ and Korolczuk A. Effects of treatment with melatonin and tryptophan on liver enzymes, parameters of fat metabolism and plasma levels of cytokines in patients with non-alcoholic fatty liver disease--14 months follow up. *J Physiol Pharmacol*. 2014;65:75-82.
90. Hung MW, Yeung HM, Lau CF, Poon AMS, Tipoe GL and Fung ML. Melatonin Attenuates Pulmonary Hypertension in Chronically Hypoxic Rats. *Int J Mol Sci*. 2017;18.
91. Gonzalez-Candia A, Candia AA, Figueroa EG, Feixes E, Gonzalez-Candia C, Aguilar SA, Ebensperger G, Reyes RV, Llanos AJ and Herrera EA. Melatonin long-lasting beneficial effects on pulmonary vascular reactivity and redox balance in chronic hypoxic ovine neonates. *J Pineal Res*. 2020;68:e12613.
92. Zhang J, Lu X, Liu M, Fan H, Zheng H, Zhang S, Rahman N, Wolczynski S, Kretowski A and Li X. Melatonin inhibits inflammasome-associated activation of endothelium and macrophages attenuating pulmonary arterial hypertension. *Cardiovascular research*. 2020;116:2156-2169.



Chapter 2

Mechanosensing and mechanotransduction in Pulmonary Hypertension

Siyu Tian, Jarno J Steenhorst, Kim van der Heiden, Daphne Merkus

Division of Experimental Cardiology, Department of Cardiology, Erasmus MC, The Netherlands
Biomedical Engineering, Department of Cardiology, Erasmus MC, The Netherlands
Institut für Chirurgische Forschung, Walter-Brendel Center, LMU, Germany
German Center for Cardiovascular Research (DZHK), Munich Heart Alliance (MHA), Germany

In book: Vascular Mechanobiology in Physiology and Disease. March 2021

Abstract

Pulmonary hypertension (PH) is a devastating disease with a poor outcome. Progressive remodeling of the pulmonary microvasculature leads to an increase in pulmonary vascular stiffness as well as to an increase in pulmonary vascular resistance. These alterations in mechanical characteristics of the pulmonary vasculature further contribute to the progression of pulmonary vascular disease. This chapter will focus on how changes in vascular stiffness affect mechanical forces acting on the endothelium and smooth muscle cells and how these changes in mechanical forces in turn contribute to the development and progression of pulmonary vascular disease with a focus on the role of mechanosensing and mechanotransduction in these processes.

Keywords: Pulmonary hypertension, mechanosensing, mechanotransduction, extracellular matrix, pulmonary vascular disease

Introduction

Pulmonary hypertension (PH), defined as a pulmonary artery pressure above 20 mmHg is a devastating disease, which, when left untreated, leads to right heart failure and death. PH is divided into 5 categories by the World Health Organization, based on etiology of the disease.[1] The most severe form of PH is pulmonary arterial hypertension (PAH), which originates in the distal pulmonary arterial microvasculature. In other forms of PH, pulmonary arterial pressure is increased secondary to other diseases, such as left heart disease or congenital heart disease, chronic hypoxia, thrombi in the pulmonary vasculature or inflammatory (lung) diseases. In all forms of PH, the progression of pulmonary vascular disease (PVD) involves endothelial dysfunction, vasoconstriction and progressive remodeling of the pulmonary vasculature, which is characterized by increased muscularization of the distal pulmonary arterioles and perivascular fibrosis. Altogether, PVD results in an increased pulmonary vascular resistance (PVR) and increased pulmonary vascular stiffness (decreased pulmonary vascular compliance (PVC)). For all precapillary forms of PH (i.e PH that is not solely due to changes in pulmonary venous pressure), PVR ≥ 3 Woods Units has recently been added to the definition of PH.[1]

Contrary to the systemic vasculature, in which vascular resistance is the main determinant of the afterload of the left ventricle, both the mean pulmonary artery pressure and its pulsatility, and hence both pulmonary vascular resistance and stiffness, contribute to workload of the right ventricle (RV).[2] In the initial phase of the disease, the RV is capable of coping with the increased afterload, initially by increasing its contractility, and in the long term by RV hypertrophy. However, with progression of PVD, the RV can no longer cope with the increased afterload and starts to fail.[3]

Although it is well-known that mechanical forces such as shear stress and wall stress have a direct impact on the endothelium and vascular smooth muscle cells respectively, and could thereby affect vascular remodeling, these mechanical forces acting on the distal pulmonary vasculature of healthy subjects and patients with pulmonary hypertension have only recently been actually measured and modeled.[4] Furthermore. it has only recently been recognized that small changes in vascular stiffness impact the pressure and flow characteristics throughout the entire pulmonary vasculature and thereby alter the mechanical forces acting on both the proximal and distal pulmonary vasculature.[5, 6] It has now become evident that increased pulmonary stiffness not only presents an early disease marker in PH but also contributes to progression of PVD.[5, 6]

This chapter will describe the alterations in mechanical characteristics of the pulmonary vasculature during development and progression of PVD, how these alterations affect mechanical forces acting on the endothelium and smooth muscle cells and how these changes in mechanical forces in turn contribute to the development and progression of PVD with a focus on the role of mechanosensing and mechanotransduction in these processes.

Changes in mechanical characteristics of the pulmonary vasculature in PH

The healthy pulmonary vasculature comprises a system with a low resistance and a high compliance to allow the cardiac output to be pumped through the pulmonary vasculature at low pressure. PVR and PVC are inversely related and are evenly distributed across the vasculature.[7] PVR is calculated as (pulmonary artery pressure -pulmonary capillary wedge pressure)/cardiac output. PVC can be estimated as stroke volume/ (systolic pulmonary artery pressure-diastolic pulmonary artery pressure). This results in a slight overestimation of PVC, as it does not take flow into the distal pulmonary vasculature into account.[8] Contrary to the

systemic vasculature, in which the aorta determines the largest part of compliance, in the healthy pulmonary vasculature, only 15-20% of PVC resides in the proximal pulmonary vasculature, due to the large number of branching vessels.[8, 9]

Pulmonary vascular compliance is important because a considerable part of the energy delivered by the RV disappears in pulsatile power. Thus, the energy required to be delivered by the RV to pump the cardiac output through the pulmonary vasculature, i.e. the hydraulic power, consists of the sum of the mean hydraulic power and the pulsatile (or oscillatory) hydraulic power. Mean hydraulic power is used to propel blood through the pulmonary vasculature and equals the product of mean pulmonary artery pressure and cardiac output. Conversely, pulsatile power is related to pulsatile pressure and is not used for forward movement of flow. In the pulmonary vasculature, pulsatile power is estimated to be 23-33% of total power generated by the RV,[6, 7] and hence contributes significantly to the workload of the RV.

PVD results in narrowing and stiffening of the pulmonary vasculature, thereby increasing PVR and reducing PVC, through increased muscularization and perivascular fibrosis. In early PVD, vascular remodeling mostly impacts PVC, with relatively small changes in PVR. Changes in PVC are even already observed prior to development of overt PH.[10] Furthermore stiffening has been shown to start in the distal pulmonary vasculature, whereas stiffening of more proximal vessels evolves later in disease development.[11] With more severe PH, PVC is low and PVR is high and disease progression only results in minor additional decreases in PVC, that are accompanied by large changes in PVR. Interestingly, it has recently been suggested that not only vascular remodeling, but also rarefaction contributes significantly to the increase in PVR[12] and, given the contribution of the distal pulmonary vasculature, likely also to the decrease in PVC. It has been estimated that in patients with advanced PH, PVR increases 18-fold, and PVC decreases 20-fold.[7]

Because PVR and PVC are inversely related in health and disease, the RC-time of the pulmonary vasculature is constant and the pulmonary pulse pressure is linearly related to mean pulmonary artery pressure. This means that, with increasing disease severity, mean and pulsatile power increase to the same extent, both contributing equally to the increased afterload of the RV.[9] The importance of changes in PVC for progression of PVD are further underscored by several studies showing that PVC is a better predictor of mortality than PVR, not only in PAH, but also in PH associated with congenital heart disease and heart failure.[8] The importance of the pulsatile component of RV afterload is further underscored by the observation that pulmonary arterial impedance, a measure of opposition to pulsatile flow, correlated better with prognosis than PVR in patients with PH.[13, 14]

Changes in mechanical forces acting on the pulmonary vasculature in PH

Decreased PVC results in increased pulse wave velocity, and hence pressure wave reflections from the distal pulmonary vasculature return faster to the heart. Thus, in the healthy pulmonary vasculature, reflected pressure waves reach the proximal pulmonary vasculature in diastole, whereas in the diseased pulmonary vasculature, pressure waves appear during mid- or late systole, thereby further augmenting the pulse pressure in the proximal pulmonary artery and contributing to the increased RV afterload(Figure 1).[15] Furthermore, these returning pressure waves also impact the flow-profile resulting in a shortened time to peak and an enhanced mid and late systolic deceleration.[10] However, as the large PVC also acts to dampen the pressure and flow pulsations that enter the pulmonary microvasculature (i.e. vessels smaller than 100 µm in diameter), the decreased PVC not only impacts the RV, but also results in increased pressure and flow pulsations into and throughout the distal pulmonary vasculature.[5, 6, 10] Computational modeling reveals that time-averaged wall shear stress is lower in the large pulmonary arteries of PAH patients.[16-18] Similarly time-averaged wall shear stress in the proximal pulmonary

vasculature (diameter above 500 µm) decreased with increasing disease severity pediatric PH patients (age 4-17 years) (from 20 to 6 dynes/cm^2) , whereas shear stress increases in the distal small arteries (with a diameter between 100 and 500 µm) (from 20 to 116 dyn/cm^2) and the microvasculature (from ~50 to ~ 300 dyn/cm^2). Furthermore, the oscillatory shear index in the main pulmonary artery increased from 0.13 to 0.20.[4] Conversely, despite the increase in pulmonary artery pressure, wall strain of the main pulmonary artery tended to decrease (from 0.16 to 0.11), but was not different in the left and right pulmonary artery and their branches. Vessel stiffness, as indicated by Young's modulus increased (from 1.26x10^6 to 3.0x 10^6 dyn/cm^2).

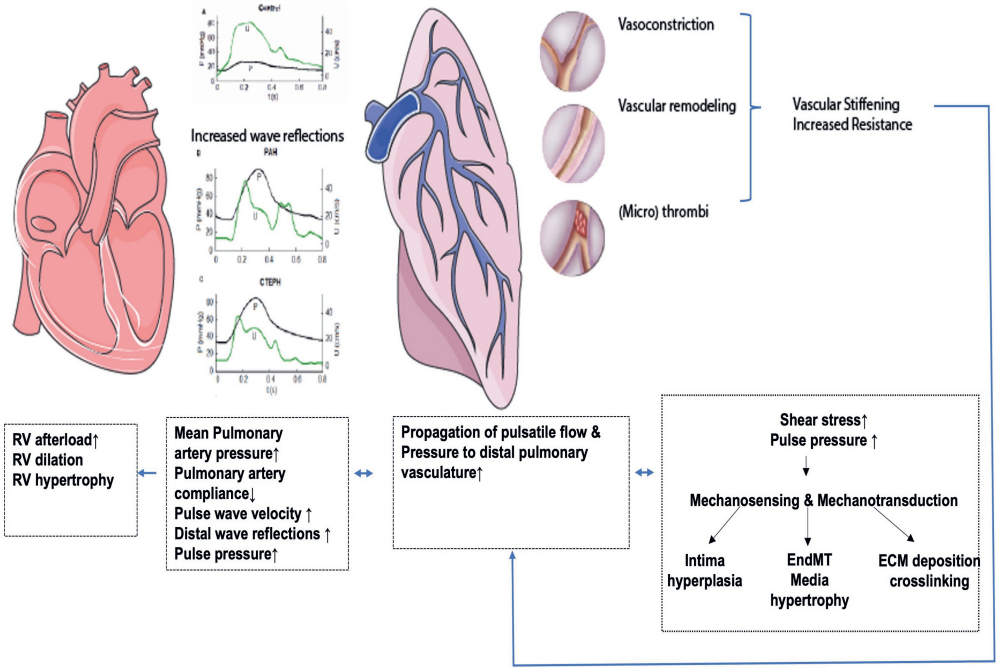


Figure 1. Changes in vascular stiffness initiate alterations in pressure and flow profiles within the pulmonary vasculature that promote pulmonary vascular remodeling, thereby increasing pulmonary vascular resistance and further augmenting right ventricular afterload. Waveforms are from.[15]

Impact of altered mechanical forces on pulmonary vasculature

Altered hemodynamics were already proposed to play an important role in the development of pulmonary microvascular lesions in patients with congenital heart disease in 1957.[19] The response of cells within the pulmonary microvasculature to these altered hemodynamics plays a key role in the (mal)adaptive response of vascular remodeling and lesion formation. Both changes in mechanical forces and the capability to respond to these mechanical forces through mechanosensing and mechanotransduction are altered in PH.[5, 10, 20, 21]

Pulmonary microvascular remodeling in PH encompasses all layers of the vasculature. Thus, endothelial plexiform lesions, (in situ) thrombotic lesions, medial hypertrophy/hyperplasia, muscularization of the distal pulmonary arterioles as well as intimal and adventitial fibrosis contribute to PVD.[22]

Altered mechanotransduction in pulmonary endothelial cells

Particularly PAH is characterized by intimal hyperplasia in the so-called plexiform lesions. Within these lesions, endothelial cells lining the vasculature appear quiescent, whereas the network of channels in the center of the lesions consists of hyperproliferating endothelial cells.[23, 24] These hallmark lesions of PAH are found at the branching point of so-called supernumerary arteries,[23, 24] and have recently found to be associated with broncho-pulmonary anastomoses.[25] The location of these lesions suggests that the altered, hyperproliferative, endothelial phenotype is, at least in part, influenced by mechanical stimuli.

The main mechanical stimulus for endothelial cells, that are directly in contact with the blood flowing through the vasculature is shear stress. Endothelial cells respond to changes in shear stress with changes in secretion of the vasoactive mediators nitric oxide (NO), prostacyclin (PGI2), thromboxane and endothelin (ET-1).[26] Furthermore, endothelial cells exposed to unidirectional shear stress possess the

capability of aligning with the flow direction. Kruppel like factor 2 and 4 (KLF2 and 4) are considered the master regulators of the anti-inflammatory, anti-proliferatory, and anti-thrombotic signaling pathways characteristic of healthy endothelial cells under unidirectional shear stress (Figure 2, see also below). [27]

As outlined above, both average shear stress and the oscillatory shear index are increased in the pulmonary microvasculature in PH [4], due to changes in stiffness of both the proximal and distal pulmonary vasculature. These chronic changes in shear stress are accompanied by endothelial dysfunction in PAH patients.[22] Studies in isolated pulmonary microvascular endothelial cells, exposed to different flow profiles show that pathological high and pathological low flow induce changes in vasoactive pathways (impaired NO-production, enhanced ET-1 production) favoring vasoconstriction, with increased production of reactive oxygen species (ROS)[26], whereas increased pulsatility induces increased mRNA expression of adhesion molecules (E-selectin, MCP-1, intercellular adhesion molecule-1 (ICAM1), vascular cell adhesion molecule 1 (VCAM1)) and release of pro-inflammatory cytokines and chemokines.[28] The resulting vasoconstriction, impaired angiogenesis and defective repair mechanisms contribute to the development and progression of PH. To investigate a role for defective endothelial mechanotransduction in response to shear stress in development of PH, pulmonary arterial endothelial cells (pAECs) and pulmonary microvascular endothelial cells (pMVECs) from healthy control subjects and patients with PAH were exposed to shear stress. Both pAECs and pMVECs align to shear stress. However, when comparing pAECs and pMVECs obtained from healthy lungs with those from patients with PAH, the response of pAECs was similar but the alignment of pMVECs obtained from patients with PAH was delayed from 72 to 120 hours after shear onset, and some cells detached and were washed away, suggesting that the strength of adherence to the fibronectin-coated surface was reduced.[29]

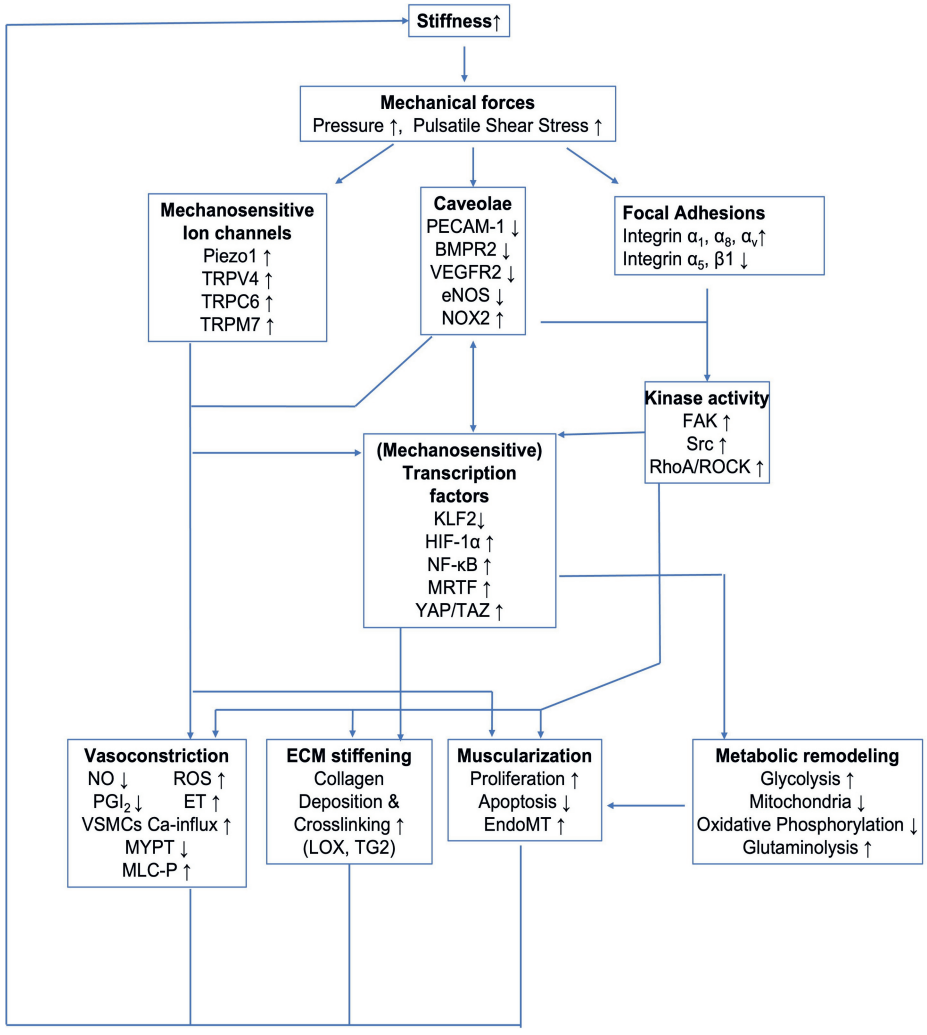


Figure 2. Mechanosensing, involving mechanosensitive ion channels, caveolae and focal adhesions and mechanotransduction, involving both changes in kinase activity as well as transcriptional regulation are altered in pulmonary hypertension, leading to metabolic remodeling vasoconstriction, increased muscularization, and ECM stiffening. These processes constitute a positive feedback loop, culminating in worsening of vascular remodeling and aggravation of pulmonary hypertension.

Altered mechanotransduction in pulmonary vascular smooth muscle cells

In the normal pulmonary circulation, the phenotype of pVSMCs varies throughout the vasculature, and is heterogeneous at a single anatomical site (proximal pulmonary vasculature vs pulmonary microvasculature) and along a specific vascular segment.[30] Within the proximal pulmonary arteries, both fully differentiated pVSMCs as well as less differentiated smooth muscle-like cells have been identified, characterized by α-smooth muscle actin expression. In the distal pulmonary vasculature, pVSMCs exhibit a more uniform, differentiated, phenotype, characterized by expression of smooth muscle myosin heavy chain, h-caldesmon and metavinculin.[30] In the proximal pulmonary vasculature, the main function of the pVSMCs is to provide strength to the vessels to withstand pressure, while maintaining distensibility to accommodate stroke volume with minimal increases in pulse pressure. In the distal pulmonary vasculature, the main function of pVSMCs is to regulate pulmonary vascular tone and resistance.[30] The main mechanical stimulus for pVSMC is stretch, to which they respond with contraction (short-term) as well as proliferation (long-term). Different subtypes of pVSMCs exhibit site-specific and unique responses to pathologic hypertensive stimuli.

In PH, wall thickness of the proximal pulmonary vasculature increases commensurate with the increase in pressure, whereas the increase in media-thickness of the distal pulmonary vasculature precedes (intra-acinar vessels) or follows (hilar arteries) the increase in pressure. Furthermore, muscularization of previously non- or partially muscularized distal pulmonary arterioles occurs in PH.[30] The increased muscularization is only partially derived from proliferation of pre-existing pVSMC. In addition, at the border of muscularized and non-muscularized arterioles, a population of progenitor cells arises which is derived from resident cells, recruited from circulating progenitor cells, or derived from perivascular inflammatory cells, macrophages and/or endothelial cells. These cells migrate

distally and are clonally expanded and differentiated to contribute to muscularization. These processes are regulated by HIF-1α, KLF-4 and PDGF-B.[31, 32]

In addition to medial thickening, α-SMA positive mesenchymal-like cells are increased in obstructive pulmonary intimal lesions. These VSM-like cells are through to be derived from endothelial cells, in a process called endothelial to mesenchymal transition (EndoMT).[33, 34] This process is orchestrated by the transcription factors , Snail, Twist1 and Slug, which are activated in response to low shear stress.[30, 35, 36]

The cross-talk between the different cell-types in the vascular wall influences the pVSMC phenotype. In a co-culture with pulmonary endothelial cells and smooth muscle cells, high shear stress on the endothelium increase smooth muscle actin as well as smooth muscle myosin heavy chain expression in the vascular smooth muscle cells. In the absence of endothelial cells however, high shear stress on the pVSMCs induced a decrease in smooth muscle actin as well as smooth muscle myosin heavy chain.[37]

Mechano-metabolic coupling

Recently, mechanical sensing has been coupled to endothelial phenotypic changes through metabolic signaling.[27] In the lung, very little research in this area has been performed. However, it is known that shear stress can alter substrate utilization and mitochondrial biogenesis and that disturbances in these processes are implicated in PAH(Figure 2).[27] Endothelial cell quiescence is associated with a phenotype of mitochondrial respiration whereas proliferation is associated with a glycolytic phenotype. Unidirectional flow activates KLF2, which increases mitochondria biosynthesis, reduces glycolysis and increases oxidative phosphorylation.[27] Conversely, a reduced mitochondrial mass and upregulation of

glycolysis were shown in endothelial cells under disturbed flow. These changes were mediated by an increase in hypoxia-inducible factor (HIF)-1α, an increase in ROS production and nitric oxide deficiency. Upregulation of NOX4 and the resultant increase in ROS prevent HIF-1α degradation, which in turn activates glycolysis, while impairing the mitochondrial electron transport chain at complex 1 and initiating inflammatory gene-expression.[38]

Glycolysis is further promoted by activation of the hippo-pathway, with Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) as downstream transcription factors. Activation of this pathway occurs in disturbed flow (see below), further linking mechanotransduction to metabolism [27]. Furthermore, glutaminolysis, the conversion of glutamine to glutamate which feeds into the tricarboxilic acid (TCA)-cycle and contributes to synthesis of amino acid, fatty acids and purines and pyrimidines, is stimulated by YAP/TAZ signaling by promoting expression of glutaminase-1.[27] Glutaminolysis is associated with a hyperproliferative phenotype and glutaminolytic reprogramming was observed in vascular lesions of patients and animal models with PAH.[27, 38]

Mechanosensors- caveolae

Also in the pulmonary vasculature, caveolae are thought to be the main platform for sensing and transducing changes in shear stress (Figure 2). Indeed, mutations in the CAV-1 gene have been associated with development of PAH [39] and caveolin-1 is reduced in endothelial cells of patients and animals with PH.[40] It is important to note that bone morphogenetic protein receptor 2 (BMPR2), which is well-known for its role in development of PAH, co-localizes with caveolae.[41] Although endothelial cells from $BMPR2^{+/-}$ mice have higher numbers of caveolae, increased Sarcoma (Src) activity prevents trafficking of the caveolae to the cell membrane.[41]

At the cell-membrane, the caveolin-1 -rich invaginations form a scaffold that brings together several proteins essential in shear sensing and mechanotransduction, including platelet endothelial cell adhesion molecule 1 (PECAM-1), ATP-dependent potassium channels (K_{ATP}), endothelial nitric oxide synthase (eNOS) and NADPH-oxidase 2 (NOX2) (Figure 2).[21, 42, 43] Furthermore, the mechanosensitive cation-channel, transient receptor potential vanilloid 4 (TRPV4), colocalizes with caveoli.[44]

A direct molecular interaction between caveolin-1 and PECAM-1 is required for phosphorylation and activation of PECAM-1.[42] Downstream signaling involves closing of K_{ATP} channels, membrane depolarization, activation of NOX2 and superoxide production [21, 42] as well as phosphorylation of Src, thereby decreasing its activity, resulting in inhibition of ERK1/2-phosphorylation.[29]

Culture of pulmonary endothelial cells on a fibronectin coating under unidirectional shear stress up to 21 dynes/cm^2 increases expression of VE-cadherin, PECAM-1 and vascular endothelial growth factor receptor 2 (VEGFR2) as compared to static conditions.[29] However, in pMVECs from patients with PAH with delayed alignment to shear stress, the caveolin-1 and PECAM-1 were reduced as compared to healthy controls and cells were more prone to detachment from the culture slides. Downstream effectors of PECAM-1 signalling were also altered in that phosphorylation of Src was decreased, thereby increasing its activity, resulting in increased phosphorylation of ERK1/2 in these cells. Conversely, shear-dependent pathways not activated via PECAM-1 (i.e. adenosine mono-phosphate activated kinase (AMPKα) and protein kinase B (AKT)) were not altered.[29]

An acute change in shear stress occurs in vivo with pulmonary embolism, i.e. when a blood clot gets stuck in the pulmonary vasculature. In a series of experiments, Chatterjee and co-workers investigated mechanosensing and mechanotransduction in the lung associated with such embolism and stop of flow.

Their experiments involve a combination of experiments in isolated pulmonary endothelial cells, isolated perfused lung preparation subjected to cessation of flow and in vivo micro-embolization.[21, 43] The cessation of flow was sensed by a complex involving caveolae, PECAM-1 and NOX-2, and resulted in ROS-production. Furthermore, it was shown that this stop of flow resulted in an increased neutrophil influx as well as a pro-angiogenic phenotype shift of the endothelial cells that was consistent with increased VEGF-expression. Both neutrophil influx and VEGF-expression were dependent on PECAM-1 and NOX-2 activity.[42]

Taken together, caveolae serve as scaffolds for several proteins involved in mechanosensing and mechanotransduction in pulmonary endothelial cells. Aberrant signaling involving caveolae has been shown to be present in PH and likely contributes to endothelial dysfunction in PH.

Mechanosensors- mechanosensitive ion-channels

Mechanical changes can be converted to chemical signals through mechanosensitive channels in the cell membrane. In pulmonary endothelial cells, TRPV4, PIEZO1 and inward rectifying potassium channels (Kir, particularly Kir6.x, comprising the pore-forming unit of K_{ATP} channels) induce activation of eNOS in response to shear stress.[5] Both TRPV4 and PIEZO1 are permeable to Ca^{2+}. TRPV4 is activated by ROS from dysfunctional mitochondria, resulting in Ca^{2+} entry and migration and proliferation of pMVECs from rats with PAH. TRPV4 blockade normalized these responses, suggesting a role for TRPV4 in the hyperproliferative lesions in the pulmonary vasculature.[45]

PIEZO1 activation results in an increase in intracellular $Ca^{2+,}$ and activates eNOS as well as Gq/G11, which results in release of ATP from endothelial cells and activation of P2Y2-receptors.[46, 47] PIEZO1 activation causes vasodilation[47] and is involved in

regulation of vascular barrier function.[48] However, development of PH in response to chronic hypoxia is not altered by endothelial deletion of PIEZO1,[47] and PIEZO1 expression is unaltered in endothelial cells from patients with PAH.[49] Hence, a definitive role for these endothelial mechanosensitive channels in development and progression of PH remains to be established.

In contrast, the role of mechanosensitive channels in the pVSMCs in PH is more clear. pVSMC s from mice with chronic hypoxia induced PH showed enhanced Ca^{2+} influx in response to osmotic swelling, which could be blocked by Gd^{3+} as well as GsMTx-4, both blockers of mechanosensitive channels.[50] Similarly, TRPV4, TRPM7 and TRCP6 are upregulated in VSMCs from patients with PAH and contribute to an enhanced Ca^{2+}-influx in response to shear stress.[51] Furthermore, knockdown of TRPV4 attenuated development of PH in response to chronic hypoxia.[52] Since Ca^{2+} is an important factor in both VSMC contraction and proliferation, upregulation of these mechanosensitive channels in pVSMCs likely contributes to development and/or progression of PH (Figure 2).

Given the dual role for upregulation of TRPV4 in development of PH, having detrimental effects in both endothelial and smooth muscle cells, therapeutic targeting of this channel may delay or even prevent progression of PH.

Mechanosensors- integrins and cytoskeleton

Focal adhesions are multi-protein structures that connect the cell's cytoskeleton to the ECM. Within those focal adhesions, clusters of integrins, transmembrane proteins consisting of heterodimers of various α and β subunits, form the interface between the ECM and the cytoskeleton. Currently, 18 α- and 8 β- integrin subunits have been identified, that can form various combinations. In the pulmonary vasculature, α_1- α_5, α_7, α_8 and α_v as well as β_1, β_3 and β_4 were shown to be expressed.

Integrin expression is altered in PH, in that both chronic hypoxia and monocrotaline-induced PH are accompanied by increased expression of α_1, α_8 and α_v, whereas α_5 and β_1 -expression is decreased in the pulmonary vasculature (Figure 2).[53]

Intracellularly, integrins connect via adaptor proteins such as talin and vinculin to the actin structure of the cytoskeleton. Both Src and focal adhesion kinase (FAK) co-localize with focal adhesions and modulate integrin-cytoskeletal links, thereby altering mechanical force transmission.[5] Activation of FAK is Src dependent and FAK-activation is required for mechanosensing in pVSMCs. FAK activation is increased in pVSMCs of PAH patients, where it decreases apoptosis, promotes proliferation and migration.[54] Similarly, FAK activation is increased in pVSMCs of rats with hypoxia-induced PH, but only when these cells were cultured on collagen, and not on fibronectin. These findings are consistent with the elevation of α_1-integrin, which is required for contact with collagen, and the downregulation of α_5, which is required for binding fibronectin in the ECM.[53] Importantly, FAK inhibition can attenuate PH development and pulmonary vascular remodeling in the monocrotaline rat model. Altogether, these data indicate a causal role of FAK-activation in development of PAH.[54]

Small Rho-GTPases, including RhoA are key players in mechanotransduction,[55] activated downstream of integrin signaling (Figure 2). These Rho-GTPases can subsequently alter the dynamics of the actin cytoskeleton and influence cell migration and proliferation. RhoA, and its downstream effector RhoA associated protein kinase (ROCK) have been shown to be altered in animal models of PAH as well as in ECs and VSMCs of patients with PAH. In VSMCs, ROCK enhances phosphorylation (and thereby activation) of the myosin light chain (MLC) as well as of myosin phosphatase (MYPT), thereby inactivating it and reducing MLC dephosphorylation. The resultant phosphorylation of MLC increases the contractile

force exerted by myosin II on actin.[56] In ECs, Rock activation can downregulate eNOS, increase inflammatory markers and is responsible for the cytoskeletal rearrangement in response to shear stress.[5] ROCK inhibition with fasudil attenuates both monocrotaline [57] and hypoxia-induced [56] PH in rats, and decreased PVR in patients with PH.[58]

The activity of Rho-GTPases is regulated by guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs) and Guanine nucleotide dissociation inhibitors (GDIs). GEFs catalyze the exchange of GDP (inactive form) to GTP (active form), GAPs promote intrinsic GTPase activity, leading to inactivation and GDIs extract membrane bound GTPases into the cytosol where they are kept inactivated.[55] Both Src and FAK are implicated in activation of GEFs, and hence in activation of RhoA.[55] Intriguingly, PDE5-inhibition induced increased RhoA phosphorylation and association to its cytosolic inhibitory protein, GDI, in pulmonary arteries, MYPT activation and loss of stress fibers in pVSMCs of rats with PH due to chronic hypoxia.[56]

Hence, RhoA/ROCK activation is required for focal adhesion assembly and regulates stress fiber formation, while ROCK inhibition and vascular smooth muscle specific knockout of ROCK2 protected against development and/or progression of PH.[5] Interestingly, we recently found that the vasodilation in response to ROCK-inhibition was blunted in pulmonary small arteries from swine with chronic thromboembolic PH,[59] suggesting that ROCK activation in the pulmonary vasculature may depend on the type of PH.

Mechanotransduction- Transcriptional regulation

Different transcription factors play a role in translating mechanical forces to changes in gene expression. Yet, different pathways of mechanotransduction

converge on a few transcription factors that were shown to be altered in PH, including KLF2, β-catenin, nuclear factor kapa B (NFκB), myocardin related transcription factor (MRTF), YAP and TAZ (Figure 2).[5]

KLF2 is considered the master transcriptional regulator of the response to uni-directional shear stress that mediates the vasodilatory, anti-inflammatory and antithrombotic properties of endothelium.[27] KLF2 is involved in regulation of eNOS expression and reduced expression of KLF2 is associated with inflammation. Furthermore, KLF2 modulates substrate utilization in endothelial cells and increases mitochondrial biosynthesis.[27] Interestingly, a mutation in KLF2 has recently been implicated in development of PAH.[60] KLF2 is also regulated by the apelin-APJ receptor axis [61], which may allow pharmacological modulation of this mechanosensitive transcription factor.

Activation of β-catenin occurs downstream of RhoA and ROCK activation and subsequently alters Wnt signaling. Although β-catenin activation in response to mechanical stimuli has not been described in the pulmonary vasculature, both endothelial upregulation of β-catenin in pulmonary small arteries of PAH patients and β-catenin downregulation in proliferating smooth muscle cells of animals with hypoxia-induced PH have been shown.[5]

NFκB is transiently activated in endothelial cells in response to physiological levels of unidirectional shear stress, but is highly activated in response to highly pulsatile flow. Its activation is dependent on integrity of the cytoskeleton, involved activation of Toll-like receptor 2 (TLR2) and resulted in an inflammatory endothelial phenotype associated with EndoMT.[10] Both NFκB and TLR2 have been shown to be activated in PAH.[5, 10]

Evidence is accumulating that mechanobiological signalling in the pulmonary vasculature involves activation of the transcription factors YAP and TAZ, which are part of the Hippo pathway. YAP and TAZ translocate from the cytoplasm to the nucleus depending on matrix stiffness.[20, 62] Thus, culture of pVSMCs on a stiff matrix enhances nuclear translocation of YAP and TAZ.[63] YAP and TAZ are increased in lungs of patients and animal models of PAH. In addition to altering metabolism, activation of YAP and TAZ induces activation of microRNA (miR) 130/310, leading to alterations in ECM secretion by pVSMCs. Furthermore, inhibition of YAP and TAZ-signalling reduces expression of lysyloxidase (LOX) as well as transglutaminase (TG2), enzymes involved in crosslinking of ECM proteins,. In addition, inhibition of YAP and TAZ-signalling attenuates pVSMC migration and contraction and the proliferative response of pVSMCs cultured on a stiff matrix.[63] Hence, activation of YAP/TAZ signaling in pVSMCs promotes a shift towards a pro-remodeling phenotype.

Role of extracellular matrix

As outlined above, stiffening of the distal pulmonary vasculature has been proposed as the initiating event of pulmonary vascular remodeling. Vascular stiffness is determined by composition and crosslinking of the extracellular matrix (ECM) in combination with vascular tone and media-thickness. Vascular stiffening in PH was shown to start with disruption of the internal elastic lamina. Loss of elastic fibers and increased deposition of collagen in PH are evidence of ECM remodeling, resulting in a stiffer ECM.[11, 63] The ECM of a normal human pulmonary artery consists of collagens, elastins, laminins, fibronectin, tenascin C, and proteoglycans. In PAH, there is increased collagen deposition and cross-linking in all layers of the vessels (Figure 2).[64]

Collagen deposition is highest in the intima of PAH patients, which is associated with increased expression of different collagen subtypes (Col14A1, Col4A5 and Col18A1) in endothelial cells.[64] pVSMCs in the media and fibroblasts in the vessel adventitia are generally identified as the cellular source for this regional collagen accumulation, although endothelial cells that undergo endoMT likely also contribute.[6] Stiffness is further increased by increased fibronectin and osteopontin, as well as breakdown of the internal elastic lamina and a reduced elastin content in PAH.[64]

The alterations in ECM structure are not only caused by changes in expression its components, but the balance between proteolytic enzymes, such as metalloproteases [a disintegrin and metalloproteinases (ADAMs)], serine elastases, matrix metalloproteinases (MMPs), and their endogenous inhibitors, tissue inhibitors of metalloproteinase (TIMPs), also influences ECM composition. In addition, changes in activity of crosslinking enzymes such as LOX and TG2 also modulate ECM stiffness. The pulmonary arteries from animals as well as from patients with PAH show increased expression of MMPs, ADAMs, serine elastases, LOXs, TG2 and TIMPs.[64] Endothelial cells undergoing EndoMT have been proposed to be the main cells contributing to these changes in ECM composition and crosslinking. Inflammation, disturbed flow, hypoxia and alterations in the TGFβ-BMP balance promote this aberrant signaling.[64] Importantly, prevention of ECM remodeling by inhibition of serine elastase or LOX, as well as through administration of proline analog cis -4-hydroxy-L -proline, an inhibitor of collagen synthesis reduces the progression of PAH in animal models, suggesting that indeed vascular stiffness is a prime determinant of disease progression.[64] Altogether, changes in the balance between collagen deposition and breakdown, increased cross-linking of collagens and enhanced elastin breakdown in the intravascular and

perivascular compartments of the pulmonary arteries all contribute to an increased stiffness of the pulmonary vasculature.

To study the effect of alterations in vascular stiffness on the different cell-types within the pulmonary vasculature, cells were cultured on matrices with varying stiffness. Culturing pAECs and pVSMCs on a stiffer ECM resulted in enhanced proliferation and decreased apoptosis of both cell-types.[11, 63] Furthermore, culture of pAECs and pVSMCs as well as pulmonary arterial adventitial fibroblasts (pAAF) on a stiffer matrix altered expression of genes implicated in hereditary PAH; i.e. matrix stiffening significantly increased Cerebellin2 precursor (CBLN2) and decreased activin receptor-like kinase 1 (ACVRL1), BMPR2, growth differentiation factor 2 (GDF2), and potassium channel subfamily K member (KCNK3) in all three cell types. Also expression of other genes known to be involved in hereditary PAH was altered by ECM stiffening in either one of these cell types. Thus, CAV1 was upregulated in pAECs and pVSMCs, but downregulated in pAAFs,[65] cation-transporting ATPase (ATP13A3) was upregulated in pAECs and pAAFs and Mothers against decapentaplegic homolog 9 (SMAD9) was upregulated in pAECs. Many of these factors modulate expression of miR 130/301; reducing CAV1, BMPR2, GDF2 and ATP13A3 with siRNA resulted in upregulation of miR 130/301, while reducing CBLN2 downregulated expression of miR 130/301 in at least two cell types. Conversely, a miR 130/301 mimetic upregulated CBLN2 and downregulated BMPR2, GDF2, endoglin (ENG) and ATP13A3, while inhibition of miR 130/301 had the opposite effect in all three cell types. Furthermore, these genes were shown to be involved in regulation of genes such as Col1, Col3 and LOX, thereby affecting ECM stiffening. These data suggest an enforcing loop in which miR130/301 plays a key role in linking genes associated with hereditary PAH with genes linked to changes in vascular stiffness.[65]

In accordance with the existence of such an enforcing loop, the effect of stiffening of the matrix on the proliferative response of pVSMCs was further enhanced in pVSMCs with a BMPR2 mutation, that predisposes to PH development, as well as in pVSMC obtained from rodents with hypoxia-induced PH.[11] Furthermore, smooth muscle cells cultured on a stiffer matrix showed increased production of collagen and fibronectin,[11] suggesting a positive feedback loop between ECM stiffening and pVSMC-proliferation, leading to enhanced muscularization of the distal pulmonary vasculature.

Consistent with the reduced circulating levels of prostacyclin in patients with PH,[66] pVSMCs cultured on a stiff ECM had reduced cyclo-oxygenase 2 (COX2)- expression and reduced production of prostacyclin.[11] This effect occurred secondary to upregulation of YAP/TAZ signaling as enhancing YAP/TAZ activity resulted in downregulation of COX-2,[63] [11] while interfering with YAP/TAZ signaling prevented downregulation of COX-2 induced by culture of pVSMCs on a stiff matrix.[63] Importantly, administration of the prostacyclin analogue treprostinil in cell culture attenuated pVSMC proliferation and reduced secretion of the ECM proteins. In addition, treprostinil lowered mRNA expression of collagen 1 and 3 as well as the ECM crosslinking enzyme LOX, and slowed progression of PH development in response to monocrotaline in vivo.[11] Altogether, these findings suggest that prostanoid therapy, that is now applied only late in PVD, could be more beneficial early in the disease, by interfering with mechanosensitive processes in a critical phase of disease progression.

Conclusions and Perspectives

Changes in pulmonary vascular stiffness are now recognized as early drivers of pulmonary vascular disease. Vascular stiffening occurs both in the proximal and distal pulmonary vasculature. Subsequent alterations in pressure and flow patterns

activate mechanosensitive pathways, leading to inflammation, endothelial dysfunction, EndoMT, clonal expansion of progenitor cells into smooth muscle cells, muscularization, metabolic changes predisposing to proliferation and further ECM remodeling, thereby initiating a positive feedback loop leading to progression of pulmonary hypertension. Whether novel therapeutic strategies, intervening within this loop, can ameliorate pulmonary hypertension remains to be established

Conflict of Interest

Support of an Erasmus MC grant (DM) and an DZHK grant (81Z0600207 to DM) is gratefully acknowledged. The authors have no relationships, financial or otherwise, to disclose. Primary data in Figure 1 are from.[15]

References:

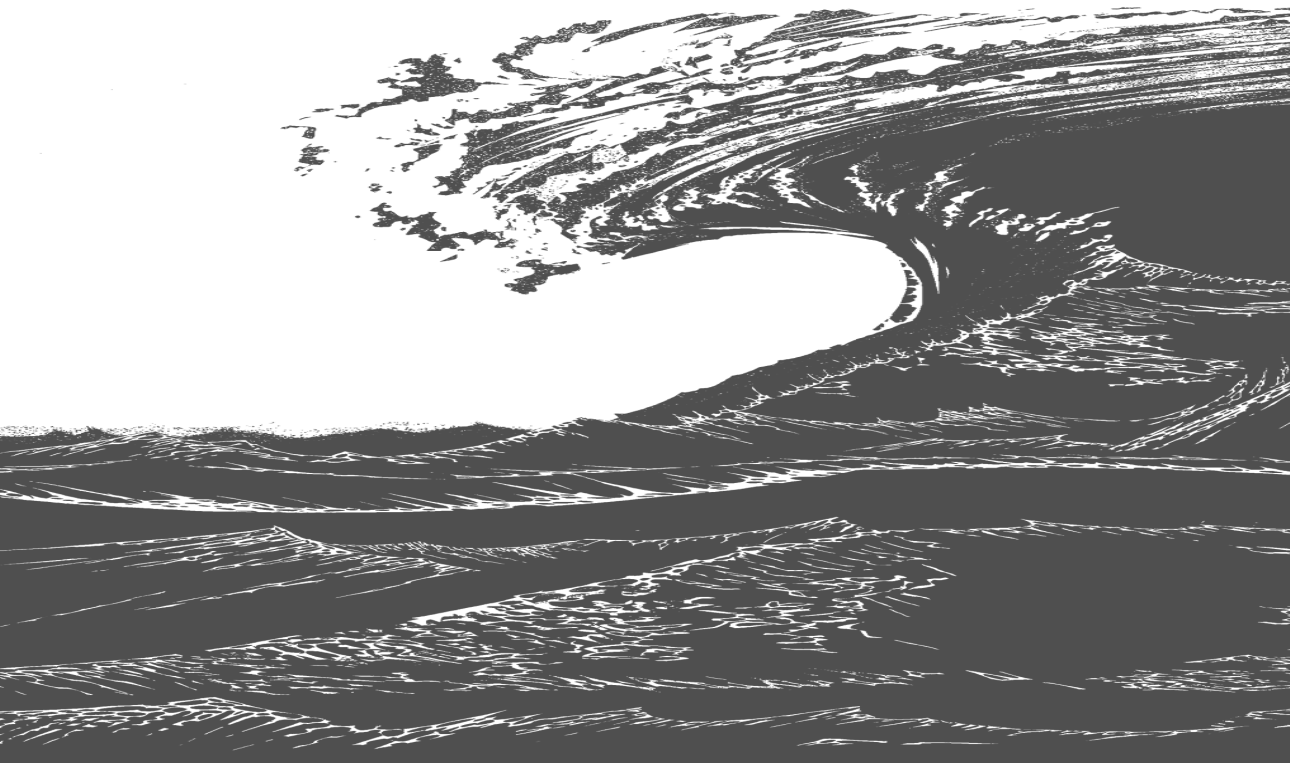
1. Simonneau G, Montani D, Celermajer DS, Denton CP, Gatzoulis MA, Krowka M, Williams PG and Souza R. Haemodynamic definitions and updated clinical classification of pulmonary hypertension. *The European respiratory journal*. 2019;53.
2. Wang Z and Chesler NC. Pulmonary vascular wall stiffness: An important contributor to the increased right ventricular afterload with pulmonary hypertension. *Pulmonary circulation*. 2011;1:212-23.
3. Naeije R, Vanderpool R, Peacock A and Badagliacca R. The Right Heart-Pulmonary Circulation Unit: Physiopathology. *Heart Fail Clin*. 2018;14:237-245.
4. Yang W, Dong M, Rabinovitch M, Chan FP, Marsden AL and Feinstein JA. Evolution of hemodynamic forces in the pulmonary tree with progressively worsening pulmonary arterial hypertension in pediatric patients. *Biomechanics and modeling in mechanobiology*. 2019.
5. Dieffenbach PB, Maracle M, Tschumperlin DJ and Fredenburgh LE. Mechanobiological Feedback in Pulmonary Vascular Disease. *Front Physiol*. 2018;9:951.
6. Bloodworth NC, West JD and Merryman WD. Microvessel mechanobiology in pulmonary arterial hypertension: cause and effect. *Hypertension*. 2015;65:483-9.
7. Saouti N, Westerhof N, Postmus PE and Vonk-Noordegraaf A. The arterial load in pulmonary hypertension. *Eur Respir Rev*. 2010;19:197-203.
8. Thenappan T, Prins KW, Pritzker MR, Scandurra J, Volmers K and Weir EK. The Critical Role of Pulmonary Arterial Compliance in Pulmonary Hypertension. *Ann Am Thorac Soc*. 2016;13:276-84.
9. Saouti N, Westerhof N, Helderman F, Marcus JT, Boonstra A, Postmus PE and Vonk-Noordegraaf A. Right ventricular oscillatory power is a constant fraction of total power irrespective of pulmonary artery pressure. *Am J Respir Crit Care Med*. 2010;182:1315-20.
10. Tan W, Madhavan K, Hunter KS, Park D and Stenmark KR. Vascular stiffening in pulmonary hypertension: cause or consequence? (2013 Grover Conference series). *Pulmonary circulation*. 2014;4:560-80.
11. Liu F, Haeger CM, Dieffenbach PB, Sicard D, Chrobak I, Coronata AM, Suarez Velandia MM, Vitali S, Colas RA, Norris PC, Marinkovic A, Liu X, Ma J, Rose CD, Lee SJ, Comhair SA, Erzurum SC, McDonald JD, Serhan CN, Walsh SR, Tschumperlin DJ and Fredenburgh LE. Distal vessel stiffening is an early and pivotal mechanobiological regulator of vascular remodeling and pulmonary hypertension. *JCI Insight*. 2016;1.
12. Rol N, Timmer EM, Faes TJ, Vonk Noordegraaf A, Grunberg K, Bogaard HJ and Westerhof N. Vascular narrowing in pulmonary arterial hypertension is heterogeneous: rethinking resistance. *Physiol Rep*. 2017;5.
13. Hunter KS, Lee PF, Lanning CJ, Ivy DD, Kirby KS, Claussen LR, Chan KC and Shandas R. Pulmonary vascular input impedance is a combined measure of pulmonary vascular resistance and stiffness and predicts clinical outcomes better than pulmonary vascular resistance alone in pediatric patients with pulmonary hypertension. *Am Heart J*. 2008;155:166-74.
14. Chesler NC, Roldan A, Vanderpool RR and Naeije R. How to measure pulmonary vascular and right ventricular function. *Conf Proc IEEE Eng Med Biol Soc*. 2009;2009:177-80.
15. Su J, Manisty C, Parker KH, Simonsen U, Nielsen-Kudsk JE, Mellemkjaer S, Connolly S, Lim PB, Whinnett ZI, Malik IS, Watson G, Davies JE, Gibbs S, Hughes AD and Howard L.

Wave Intensity Analysis Provides Novel Insights Into Pulmonary Arterial Hypertension and Chronic Thromboembolic Pulmonary Hypertension. *J Am Heart Assoc*. 2017;6.
16. Barker AJ, Roldan-Alzate A, Entezari P, Shah SJ, Chesler NC, Wieben O, Markl M and Francois CJ. Four-dimensional flow assessment of pulmonary artery flow and wall shear stress in adult pulmonary arterial hypertension: results from two institutions. *Magnetic resonance in medicine*. 2015;73:1904-13.
17. Odagiri K, Inui N, Hakamata A, Inoue Y, Suda T, Takehara Y, Sakahara H, Sugiyama M, Alley MT, Wakayama T and Watanabe H. Non-invasive evaluation of pulmonary arterial blood flow and wall shear stress in pulmonary arterial hypertension with 3D phase contrast magnetic resonance imaging. *SpringerPlus*. 2016;5:1071.
18. Zambrano BA, McLean NA, Zhao X, Tan JL, Zhong L, Figueroa CA, Lee LC and Baek S. Image-based computational assessment of vascular wall mechanics and hemodynamics in pulmonary arterial hypertension patients. *J Biomech*. 2018;68:84-92.
19. Heath D and Whitaker W. The small pulmonary blood vessels in atrial septal defect. *Br Heart J*. 1957;19:327-32.
20. Bertero T, Cottrill KA, Lu Y, Haeger CM, Dieffenbach P, Annis S, Hale A, Bhat B, Kaimal V, Zhang YY, Graham BB, Kumar R, Saggar R, Saggar R, Wallace WD, Ross DJ, Black SM, Fratz S, Fineman JR, Vargas SO, Haley KJ, Waxman AB, Chau BN, Fredenburgh LE and Chan SY. Matrix Remodeling Promotes Pulmonary Hypertension through Feedback Mechanoactivation of the YAP/TAZ-miR-130/301 Circuit. *Cell Rep*. 2015;13:1016-32.
21. Chatterjee S. Endothelial Mechanotransduction, Redox Signaling and the Regulation of Vascular Inflammatory Pathways. *Front Physiol*. 2018;9:524.
22. Humbert M, Guignabert C, Bonnet S, Dorfmuller P, Klinger JR, Nicolls MR, Olschewski AJ, Pullamsetti SS, Schermuly RT, Stenmark KR and Rabinovitch M. Pathology and pathobiology of pulmonary hypertension: state of the art and research perspectives. *Eur Respir J*. 2019;53.
23. St Croix CM and Steinhorn RH. New Thoughts about the Origin of Plexiform Lesions. *Am J Respir Crit Care Med*. 2016;193:484-5.
24. Jonigk D, Golpon H, Bockmeyer CL, Maegel L, Hoeper MM, Gottlieb J, Nickel N, Hussein K, Maus U, Lehmann U, Janciauskiene S, Welte T, Haverich A, Rische J, Kreipe H and Laenger F. Plexiform lesions in pulmonary arterial hypertension composition, architecture, and microenvironment. *Am J Pathol*. 2011;179:167-79.
25. Galambos C, Sims-Lucas S, Abman SH and Cool CD. Intrapulmonary Bronchopulmonary Anastomoses and Plexiform Lesions in Idiopathic Pulmonary Arterial Hypertension. *Am J Respir Crit Care Med*. 2016;193:574-6.
26. Li M, Stenmark KR, Shandas R and Tan W. Effects of pathological flow on pulmonary artery endothelial production of vasoactive mediators and growth factors. *J Vasc Res*. 2009;46:561-71.
27. Wu D and Birukov K. Endothelial Cell Mechano-Metabolomic Coupling to Disease States in the Lung Microvasculature. *Front Bioeng Biotechnol*. 2019;7:172.
28. Li M, Scott DE, Shandas R, Stenmark KR and Tan W. High pulsatility flow induces adhesion molecule and cytokine mRNA expression in distal pulmonary artery endothelial cells. *Annals of biomedical engineering*. 2009;37:1082-92.
29. Szulcek R, Happe CM, Rol N, Fontijn RD, Dickhoff C, Hartemink KJ, Grunberg K, Tu L, Timens W, Nossent GD, Paul MA, Leyen TA, Horrevoets AJ, de Man FS, Guignabert C, Yu PB, Vonk-Noordegraaf A, van Nieuw Amerongen GP and Bogaard HJ. Delayed Microvascular

Shear Adaptation in Pulmonary Arterial Hypertension. Role of Platelet Endothelial Cell Adhesion Molecule-1 Cleavage. *American journal of respiratory and critical care medicine*. 2016;193:1410-20.
30. Stenmark KR, Frid MG, Graham BB and Tuder RM. Dynamic and diverse changes in the functional properties of vascular smooth muscle cells in pulmonary hypertension. *Cardiovasc Res*. 2018;114:551-564.
31. Sheikh AQ, Misra A, Rosas IO, Adams RH and Greif DM. Smooth muscle cell progenitors are primed to muscularize in pulmonary hypertension. *Sci Transl Med*. 2015;7:308ra159.
32. Sheikh AQ, Saddouk FZ, Ntokou A, Mazurek R and Greif DM. Cell Autonomous and Non-cell Autonomous Regulation of SMC Progenitors in Pulmonary Hypertension. *Cell Rep*. 2018;23:1152-1165.
33. Deng L, Blanco FJ, Stevens H, Lu R, Caudrillier A, McBride M, McClure JD, Grant J, Thomas M, Frid M, Stenmark K, White K, Seto AG, Morrell NW, Bradshaw AC, MacLean MR and Baker AH. MicroRNA-143 Activation Regulates Smooth Muscle and Endothelial Cell Crosstalk in Pulmonary Arterial Hypertension. *Circulation research*. 2015;117:870-883.
34. Hopper RK, Moonen JR, Diebold I, Cao A, Rhodes CJ, Tojais NF, Hennigs JK, Gu M, Wang L and Rabinovitch M. In Pulmonary Arterial Hypertension, Reduced BMPR2 Promotes Endothelial-to-Mesenchymal Transition via HMGA1 and Its Target Slug. *Circulation*. 2016;133:1783-94.
35. Ranchoux B, Antigny F, Rucker-Martin C, Hautefort A, Pechoux C, Bogaard HJ, Dorfmuller P, Remy S, Lecerf F, Plante S, Chat S, Fadel E, Houssaini A, Anegon I, Adnot S, Simonneau G, Humbert M, Cohen-Kaminsky S and Perros F. Endothelial-to-mesenchymal transition in pulmonary hypertension. *Circulation*. 2015;131:1006-18.
36. Lu X, Gong J, Dennery PA and Yao H. Endothelial-to-mesenchymal transition: Pathogenesis and therapeutic targets for chronic pulmonary and vascular diseases. *Biochem Pharmacol*. 2019;168:100-107.
37. Scott D, Tan Y, Shandas R, Stenmark KR and Tan W. High pulsatility flow stimulates smooth muscle cell hypertrophy and contractile protein expression. *American journal of physiology Lung cellular and molecular physiology*. 2013;304:L70-81.
38. Culley MK and Chan SY. Mitochondrial metabolism in pulmonary hypertension: beyond mountains there are mountains. *J Clin Invest*. 2018;128:3704-3715.
39. Morrell NW, Aldred MA, Chung WK, Elliott CG, Nichols WC, Soubrier F, Trembath RC and Loyd JE. Genetics and genomics of pulmonary arterial hypertension. *Eur Respir J*. 2019;53.
40. Chettimada S, Yang J, Moon HG and Jin Y. Caveolae, caveolin-1 and cavin-1: Emerging roles in pulmonary hypertension. *World J Respirol*. 2015;5:126-134.
41. Prewitt AR, Ghose S, Frump AL, Datta A, Austin ED, Kenworthy AK and de Caestecker MP. Heterozygous null bone morphogenetic protein receptor type 2 mutations promote SRC kinase-dependent caveolar trafficking defects and endothelial dysfunction in pulmonary arterial hypertension. *J Biol Chem*. 2015;290:960-71.
42. Noel J, Wang H, Hong N, Tao JQ, Yu K, Sorokina EM, Debolt K, Heayn M, Rizzo V, Delisser H, Fisher AB and Chatterjee S. PECAM-1 and caveolae form the mechanosensing complex necessary for NOX2 activation and angiogenic signaling with stopped flow in pulmonary endothelium. *Am J Physiol Lung Cell Mol Physiol*. 2013;305:L805-18.

43. Milovanova T, Chatterjee S, Hawkins BJ, Hong N, Sorokina EM, Debolt K, Moore JS, Madesh M and Fisher AB. Caveolae are an essential component of the pathway for endothelial cell signaling associated with abrupt reduction of shear stress. *Biochim Biophys Acta*. 2008;1783:1866-75.
44. Goedicke-Fritz S, Kaistha A, Kacik M, Markert S, Hofmeister A, Busch C, Banfer S, Jacob R, Grgic I and Hoyer J. Evidence for functional and dynamic microcompartmentation of Cav-1/TRPV4/K(Ca) in caveolae of endothelial cells. *Eur J Cell Biol*. 2015;94:391-400.
45. Suresh K, Servinsky L, Jiang H, Bigham Z, Yun X, Kliment C, Huetsch J, Damarla M and Shimoda LA. Reactive oxygen species induced Ca(2+) influx via TRPV4 and microvascular endothelial dysfunction in the SU5416/hypoxia model of pulmonary arterial hypertension. *Am J Physiol Lung Cell Mol Physiol*. 2018;314:L893-L907.
46. Albarran-Juarez J, Iring A, Wang S, Joseph S, Grimm M, Strilic B, Wettschureck N, Althoff TF and Offermanns S. Piezo1 and Gq/G11 promote endothelial inflammation depending on flow pattern and integrin activation. *J Exp Med*. 2018;215:2655-2672.
47. Lhomme A, Gilbert G, Pele T, Deweirdt J, Henrion D, Baudrimont I, Campagnac M, Marthan R, Guibert C, Ducret T, Savineau JP and Quignard JF. Stretch-Activated Piezo1 Channel in Endothelial Cells Relaxes Mouse Intrapulmonary Arteries. *Am J Respir Cell Mol Biol*. 2018.
48. Friedrich EE, Hong Z, Xiong S, Zhong M, Di A, Rehman J, Komarova YA and Malik AB. Endothelial cell Piezo1 mediates pressure-induced lung vascular hyperpermeability via disruption of adherens junctions. *Proc Natl Acad Sci U S A*. 2019;116:12980-12985.
49. Lambert M, Capuano V, Olschewski A, Sabourin J, Nagaraj C, Girerd B, Weatherald J, Humbert M and Antigny F. Ion Channels in Pulmonary Hypertension: A Therapeutic Interest? *Int J Mol Sci*. 2018;19.
50. Ducret T, El Arrouchi J, Courtois A, Quignard JF, Marthan R and Savineau JP. Stretch-activated channels in pulmonary arterial smooth muscle cells from normoxic and chronically hypoxic rats. *Cell Calcium*. 2010;48:251-9.
51. Song S, Yamamura A, Yamamura H, Ayon RJ, Smith KA, Tang H, Makino A and Yuan JX. Flow shear stress enhances intracellular Ca2+ signaling in pulmonary artery smooth muscle cells from patients with pulmonary arterial hypertension. *American journal of physiology Cell physiology*. 2014;307:C373-83.
52. Yang XR, Lin AH, Hughes JM, Flavahan NA, Cao YN, Liedtke W and Sham JS. Upregulation of osmo-mechanosensitive TRPV4 channel facilitates chronic hypoxia-induced myogenic tone and pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol*. 2012;302:L555-68.
53. Umesh A, Paudel O, Cao YN, Myers AC and Sham JS. Alteration of pulmonary artery integrin levels in chronic hypoxia and monocrotaline-induced pulmonary hypertension. *J Vasc Res*. 2011;48:525-37.
54. Paulin R, Meloche J, Courboulin A, Lambert C, Haromy A, Courchesne A, Bonnet P, Provencher S, Michelakis ED and Bonnet S. Targeting cell motility in pulmonary arterial hypertension. *Eur Respir J*. 2014;43:531-44.
55. Burridge K, Monaghan-Benson E and Graham DM. Mechanotransduction: from the cell surface to the nucleus via RhoA. *Philos Trans R Soc Lond B Biol Sci*. 2019;374:20180229.
56. Guilluy C, Sauzeau V, Rolli-Derkinderen M, Guerin P, Sagan C, Pacaud P and Loirand G. Inhibition of RhoA/Rho kinase pathway is involved in the beneficial effect of sildenafil on pulmonary hypertension. *Br J Pharmacol*. 2005;146:1010-8.

57. Abe K, Shimokawa H, Morikawa K, Uwatoku T, Oi K, Matsumoto Y, Hattori T, Nakashima Y, Kaibuchi K, Sueishi K and Takeshit A. Long-term treatment with a Rho-kinase inhibitor improves monocrotaline-induced fatal pulmonary hypertension in rats. *Circ Res*. 2004;94:385-93.
58. Zhang Y and Wu S. Effects of fasudil on pulmonary hypertension in clinical practice. *Pulm Pharmacol Ther*. 2017;46:54-63.
59. Stam K, van Duin RWB, Uitterdijk A, Krabbendam-Peters I, Sorop O, Danser AHJ, Duncker DJ and Merkus D. Pulmonary microvascular remodeling in chronic thrombo-embolic pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol*. 2018.
60. Eichstaedt CA, Song J, Viales RR, Pan Z, Benjamin N, Fischer C, Hoeper MM, Ulrich S, Hinderhofer K and Grunig E. First identification of Kruppel-like factor 2 mutation in heritable pulmonary arterial hypertension. *Clin Sci (Lond)*. 2017;131:689-698.
61. Kim J. Apelin-APJ signaling: a potential therapeutic target for pulmonary arterial hypertension. *Mol Cells*. 2014;37:196-201.
62. Bertero T, Oldham WM, Cottrill KA, Pisano S, Vanderpool RR, Yu Q, Zhao J, Tai Y, Tang Y, Zhang YY, Rehman S, Sugahara M, Qi Z, Gorcsan J, 3rd, Vargas SO, Saggar R, Saggar R, Wallace WD, Ross DJ, Haley KJ, Waxman AB, Parikh VN, De Marco T, Hsue PY, Morris A, Simon MA, Norris KA, Gaggioli C, Loscalzo J, Fessel J and Chan SY. Vascular stiffness mechanoactivates YAP/TAZ-dependent glutaminolysis to drive pulmonary hypertension. *J Clin Invest*. 2016;126:3313-35.
63. Dieffenbach PB, Haeger CM, Coronata AMF, Choi KM, Varelas X, Tschumperlin DJ and Fredenburgh LE. Arterial stiffness induces remodeling phenotypes in pulmonary artery smooth muscle cells via YAP/TAZ-mediated repression of cyclooxygenase-2. *Am J Physiol Lung Cell Mol Physiol*. 2017;313:L628-L647.
64. Thenappan T, Chan SY and Weir EK. Role of Extracellular Matrix in the Pathogenesis of Pulmonary Arterial Hypertension. *Am J Physiol Heart Circ Physiol*. 2018.
65. Bertero T, Handen AL and Chan SY. Factors Associated with Heritable Pulmonary Arterial Hypertension Exert Convergent Actions on the miR-130/301-Vascular Matrix Feedback Loop. *Int J Mol Sci*. 2018;19.
66. Tuder RM, Cool CD, Geraci MW, Wang J, Abman SH, Wright L, Badesch D and Voelkel NF. Prostacyclin synthase expression is decreased in lungs from patients with severe pulmonary hypertension. *Am J Respir Crit Care Med*. 1999;159:1925-32.



Chapter 4

Kynurenine Metabolites Predict Survival in Pulmonary Arterial Hypertension: A role for IL-6/IL-6Rα

Zongye Cai, **Siyu Tian**, Theo Klein, Ly Tu, Laurie W. Geenen, Thomas Koudstaal, Annemien E. van den Bosch, Yolanda B. de Rijke, Irwin K. M. Reiss, Eric Boersma, Claude van der Ley, Martijn Van Faassen, Ido Kema, Dirk J. Duncker, Karin A. Boomars, Karin Tran-Lundmark, Christophe Guignabert, Daphne Merkus

Department of Cardiology, Erasmus MC, University Medical Center, The Netherlands
Department of Clinical Chemistry, Erasmus MC, University Medical Center, Netherlands
INSERM UMR_S 999, Hôpital Marie Lannelongue, Le Plessis-Robinson, France
Université Paris-Saclay, School of Medicine, Le Kremlin-Bicêtre, France
Department of Pediatrics/Neonatology, Sophia Children's Hospital, Erasmus MC, The Netherlands
Department of Clinical Epidemiology, Erasmus MC, The Netherlands.
Laboratory Medicine, University Medical Center Groningen, The Netherlands
Department of Pulmonary Medicine, Erasmus MC, The Netherlands
Department of Experimental Medical Science, Lund University, Sweden
Wallenberg Centre for Molecular Medicine, Lund University, Sweden
Walter Brendel Center of Experimental Medicine (WBex), University Clinic Munich, Germany
German Center for Cardiovascular Research (DZHK), Partner Site Munich, Munich Heart Alliance (MHA), Germany

July 2022 Scientific Reports 12(1)

Abstract

Introduction: Activation of the kynurenine pathway (KP) has been reported in patients with pulmonary arterial hypertension (PAH) undergoing PAH therapy. We aimed to determine KP-metabolism in treatment-naïve PAH patients, investigate its prognostic values, evaluate the effect of PAH therapy on KP-metabolites and identify cytokines responsible for altered KP-metabolism.

Methods: KP-metabolite levels were determined in plasma from PAH patients (median follow-up 42 months) and in rats with monocrotaline- and Sugen/hypoxia-induced PH. Blood sampling of PAH patients was performed at the time of diagnosis, six months and one year after PAH therapy.

Results: KP activation with lower tryptophan, higher kynurenine (Kyn), 3-hydroxykynurenine (3-HK), quinolinic acid (QA), kynurenic acid (KA), and anthranilic acid was observed in treatment-naïve PAH patients compared with controls. A similar KP-metabolite profile was observed in monocrotaline, but not Sugen/hypoxia-induced PAH. Human lung primary cells (microvascular endothelial cells, pulmonary artery smooth muscle cells, and fibroblasts) were exposed to different cytokines *in vitro*. Following exposure to interleukin-6 (IL-6)/IL-6 receptor α (IL-6Rα) complex, all cell types exhibit a similar KP-metabolite profile as observed in PAH patients. PAH therapy partially normalized this profile in survivors after one year. Increased KP-metabolites correlated with higher pulmonary vascular resistance, shorter six-minute walking distance, and worse functional class. High levels of Kyn, 3-HK, QA, and KA measured at the latest time-point were associated with worse long-term survival.

Conclusion: KP-metabolism was activated in treatment-naïve PAH patients, likely mediated through IL-6/IL-6Rα signaling. KP-metabolites predict response to PAH therapy and survival of PAH patients.

Introduction

Pulmonary arterial hypertension (PAH) is characterized by an increase in pulmonary vascular resistance due to pulmonary vascular remodeling. This increases right ventricular afterload, eventually leading to progressive right heart failure.[1] There is currently no therapy reversing pulmonary vascular remodeling. Hence early identification of the disease as well as delineation of novel mechanisms contributing to pulmonary vascular remodeling are still of utmost importance to improve prognosis of patients with PAH.

Recent studies have highlighted the pathophysiological importance of inflammation and mitochondrial dysfunction in the development and progression of PAH.[2-4] Nicotinamide adenine dinucleotide (NAD^+) has been demonstrated to be an important modulator of inflammation and mitochondrial function.[5-10] NAD^+ can be produced from vitamin B3 via the Preiss-Handler pathway and its derivate nicotinamide riboside via the salvage pathway.[10] Interestingly, NAD^+ synthesis via the salvage pathway is enhanced in patients with advanced PAH as well as in rodent models of pulmonary hypertension (PH).[11] A very important pathway that determines NAD^+ levels is the *de novo* synthesis through the kynurenine pathway (KP). This *de novo* NAD^+ synthesis through the KP starts with the conversion of essential amino acid tryptophan (Trp) into kynurenine (Kyn), by indoleamine 2, 3-dioxygenase, followed respectively by 3-hydroxykynurenine (3-HK), 3-hydroxykynurenic acid (3-HA), quinolinic acid (QA), and finally resulting in NAD^+ formation.[12, 13] There are branching points in the KP, as Kyn is also metabolized to kynurenic acid (KA) and anthranilic acid (AA) (Figure 1A).[12, 13] Interestingly, a correlation between circulating Kyn, QA, and AA and pulmonary vascular resistance has been reported in PAH patients.[14] Similarly, increased Kyn levels were observed in other PAH cohorts,[15, 16] and correlated with immune dysregulation and clinical

outcome in a follow-up period of 6 months.[16] Together, these studies highlight the importance of the KP in PAH. However, PAH patients received PAH therapy in these studies and the KP-metabolite profile in treatment-naïve PAH patients as well as the effect of PAH therapy on this profile are currently unknown.

Therefore, we aimed to investigate: 1) KP-metabolite profile in treatment-naïve PAH patients as well as in two rat models of PAH, 2) the effects of PAH therapy on this profile in PAH patients, 3) the prognostic values of KP-metabolites during a long-term follow-up period. Given the potential correlation between KP-metabolism and immunity/ inflammation, we also investigated 4) whether cytokines and hypoxia are able to change the KP-metabolism in human lung cells.

Methods

Study population

The study protocol was approved by the Erasmus MC ethical committee (MEC-2011-392 and 2012-512), and written informed consent was obtained from all PAH patients and healthy volunteers. All procedures were performed in accordance with Declaration of Helsinki. Patients were not involved in the design of the study.

In this prospective observational cohort study, which encompassed forty-three consecutive treatment-naïve adult patients with PAH (mean pulmonary artery pressure ≥ 25 mmHg and pulmonary artery wedge pressure ≤ 15 mmHg) diagnosed by right heart catheterization between May 2012 and October 2016 at Erasmus MC were included. Treatment-naïve was defined as the absence of any history of treatment with approved target medications for PAH, i.e. prostacyclin, endothelin receptor antagonists, or phosphodiesterase type 5 inhibitors, and the diagnosis of PAH was in accordance with definition at time of inclusion.[17, 18] Patients that aged <18 years, that were not treatment-naïve, had an incomplete diagnostic procedure,

or were uncapable of signing informed consent were excluded. Thirty-nine PAH patients were prescribed PAH targeted therapies following diagnosis according to the latest guidelines.[17, 19] PAH patients were prospectively followed till the 1st of January 2019. The primary outcome was defined as all-cause mortality and lung transplantation. Survival status was checked in the Municipal Personal Records database.

A control group consisting of 111 healthy volunteers was selected from the cohort in the study of using 2D speckle tracking echocardiography to evaluate the normal myocardial strain values in healthy subjects. The selected subjects had normal results on physical examination and electrocardiography (ECG), had no (prior) cardiovascular disease or cardiovascular risk factors (hypercholesterolemia, hypertension (blood pressure above 140/90 mmHg at the time of visit), or diabetes mellitus). More details about these PAH and control cohorts have been previously described.[20, 21]

Animal models of PH

Animal experiments were approved by the Ethics Committee of the Université Paris-Saclay (#11484) and carried out in accordance with the Guide for the Care and Use of Laboratory Animals adopted by the National Institute of Health and Medical Research (INSERM).and were performed in accordance with the ARRIVE guidelines. Plasma collected during previous experiments in two well-established rat models of severe PAH was used for this study.[22] Briefly, monocrotaline (MCT)-induced PH (MCT) was established in 4 weeks-old male Wistar rats (Janvier Labs, Saint Berthevin, France), with a single subcutaneous injection of MCT (40 mg/kg, Sigma-Aldrich, Saint-Quentin-Fallavier, France), and evaluated after 3 weeks (MCT-PH n=8, controls n=9). Sugen-hypoxia-induced PH (SuHx) was established in 4 weeks-old male Wistar rats (Janvier Labs, Saint Berthevin, France), by a single

subcutaneous injection of SU5416 (20mg/kg, Sigma-Aldrich, Saint-Quentin-Fallavier, France) combined with exposure to normobaric hypoxia for 3 weeks followed by normoxia for 5 weeks (SuHx-PH n=10, controls n=5).

Human and rat EDTA-plasma samples

At the time of diagnosis (baseline), peripheral venous blood sampling was performed during diagnostic right heart catheterization for PAH patients, while blood sampling was performed at the time of visit for healthy volunteers. Blood sampling was also performed in surviving PAH patients 6 months (±3 months, n=32) and 1 year (±3 months, n=28) after PAH therapy. In the rats, blood sampling was performed under anesthesia (2% isoflurane) prior to excision of the heart and lungs.

In vitro study of cultured human primary lung cells

The effects of cytokines and hypoxia on KP-metabolism were studied in 3 different types of human primary lung cells from healthy donors: microvascular endothelial cells (MVECs, CC-2527, Lonza) from 2 donors, pulmonary artery smooth muscle cells (PASMCs, CC-2581, Lonza) from 2 donors, and lung fibroblast including human lung fibroblasts (CC-2512, Lonza) from 1 donor, and one MRC-5 lung fibroblast cell line (ATCC®CCL-171™). All cells were cultured in the corresponding medium kits. The passage of MVECs, PASMCs, and fibroblasts used in the final experiments were P8, P8, and P7, respectively. Hypoxia with 1% oxygen was achieved in a standard incubator with variable oxygen control (Thermo Fisher Scientific). The concentration of human recombinant cytokines (R&D systems) in the medium was 20 ng/mL for TNF-α (210-TA-020/CF), IL-6 (7270-IL-025/CF), IL-6/IL-6Rα (8954-SR/CF) and TGF-β1 (7754-BH-005/CF). MVECs were exposed to cytokines or hypoxia in basal medium with 0.5% FBS for 24 hours prior to collection of medium. PASMCs and fibroblasts were starved with serum-free medium for 24 hours and

then exposed to cytokines or hypoxia in serum-free medium for 24 hours before collection of medium. The medium was centrifuged at 2,000 g for 20 minutes at 4 °C and the clear supernatant was collected and stored in aliquots at −80°C.

Measurement of KP-metabolites

An in-house developed assay by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) was used to determine the KP-metabolite levels as previously described,[23] using 10 µL sample mixed with 10 µL isotopically labeled internal standard, including deuterated Trp, Kyn, 3-HK, 3-HA, QA, KA, and AA (Buchem BV, Apeldoorn, The Netherlands).

The measurement of NAD^+ in rat lung tissue was performed with an NAD/NADH assay kit (Abcam #65348) according to the manufacturer's instructions. Briefly, 15mg frozen rat lung tissue was washed in cold PBS, homogenized with 400 µl of NAD/NADH extraction buffer, and centrifuged for 5 minutes at 4°C. The supernatant was then collected for NAD/NADH measurement.

Statistical analysis

Normality of continuous data was evaluated by Kolmogorov-Smirnov tests. Continuous variables are presented as mean±standard deviation (SD) or median [interquartile range (IQR)], categoric variables as numbers (percentages). Unpaired t-test or Mann-Whitney test were used to compare differences in continuous variables (e.g. metabolite levels) between 2 groups (e.g. human PAH vs controls). Wilcoxon matched-pairs signed rank test was used to compare differences in KP-metabolites between two time points (baseline vs 6 months, and baseline vs 1 year) in PAH patients. Chi-square test was used to compare the difference in categoric variables (e.g. sex, NYHA) between 2 groups (e.g. survivors vs non-survivors). Spearman correlation coefficients were used to determine correlations between

different KP-metabolites, and correlations between KP-metabolites and baseline characteristics. Logistic regression was conducted to determine whether KP-metabolites were independent predictors that distinguishing PAH patients from healthy controls. Comparisons of survival between groups were performed using the Kaplan-Meier estimator with Breslow-Wilcoxon test and log-rank test. Univariable and multivariate (Corrected for Age, Sex, PAH types (iPAH or APAH), and PAH therapy type (no therapy or mono or double or triple therapy)) Cox proportional hazard regression were used to assess associations between KP-metabolite levels and mortality in PAH patients. Statistical analyses were performed using IBM SPSS software (version 21.0.0.1). $P< 0.05$ was considered statistically significant.

Results

Characteristics of the study population

Baseline characteristics of treatment-naïve PAH patients at the time of diagnosis and healthy controls are summarized in Table 1. During a median follow-up of 42 [interquartile range: 32-58] months, twelve PAH patients (Non-survivors) reached the primary endpoint, the causes included end-stage heart failure (n=5), euthanasia because of end-stage cardiovascular and pulmonary disease (n=2), lung transplantation (n=1), progression of systemic sclerosis (n=1), multi-organ failure (n=1), sudden death presumed cerebral (n=1), and malignancy (n=1). Seven of them reached the endpoint within six months after diagnosis and hence had no follow-up measurements taken. Non-survivors were older, had higher heart rate and shorter 6-minute walking distance than survivors (Table 1).

Table 1. Baseline characteristics of all PAH patients and healthy controls

	Control	PAH		
		All	Survivors	Non-survivors
N	111	43	31	12
Aetiology				
- iPAH, n (%)		15 (35)	14 (45)	1 (8)
- CTD-PAH, n (%)		17 (40)	8 (26)	9 (75)
- CHD-PAH, n (%)		11 (25)	9 (29)	2 (17)
Clinical characteristics				
Age, years old	43±13	53±17 **	49±14	62±19 †
Sex, women n (%)	59 (53)	29 (67)	20 (65)	9 (75)
sBP, mmHg	123 [115-128]	122 [114-132]	123 [116-132]	115 [106-132]
HR, beats/min	68 [62-76]	78 [67-90] **	76 [63-87]	87 [76-99] †
BMI, kg/m^2	23.8±2.9	27.0±6.1 ***	27.6±6.6	25.2±4.5
eGFR, mL/min/1.73m^2	---	66.8±18.4	68.9±16.9	61.3±21.8
hs-CRP, mg/L	---	3.1 [1.5-10.5]	3.1 [1.2-9.0]	3.4 [2.1-22.3]
NYHA, I:II:III:IV	---	1:13:23:6	1:11:16:3	0:2:7:3
6MWD, m	---	337±153	377±136	198±133 ††

Right heart catheterization				
mPAP, mmHg	---	50.5±16.1	50.5±15.9	50.6±17.3
PAWP, mmHg	---	11.8±5.6	11.6±6.0	12.2±4.9
PVR, WU	---	7.1 [5.1-11.8]	7.9 [5.6-12.0]	6.4 [4.2-11.3]
CO, L/min	---	4.7 [3.9-5.5]	4.6 [3.9-5.5]	4.9 [3.9-6.5]
CI, L/min/m^2	---	2.5 [2.2-3.3]	2.5 [2.2-3.2]	2.5 [2.0-3.6]
PAH therapy types at time of censoring				
- No therapy, n (%)		4 (9.3)	2 (6.4)	2 (16.7)
- Mono-therapy, n (%)		9 (20.9)	4 (12.9)	5 (41.6)
- Dual-therapy, n (%)		13 (30.2)	11 (35.5)	2 (16.7)
- Triple-therapy, n (%)		17 (39.5)	14 (45.2)	3 (25.0)

Data are presented as mean ± SD, median [IQR], or numbers (percentages). ***P*<0.01, *** *P* <0.001 PAH versus control. †*P*<0.05, ††*P*<0.01, survivors versus non-survivors. Unpaired T Test, Mann-Whitney U Test, and Chi-Square. PAH: pulmonary arterial hypertension, iPAH: idiopathic PAH, CTD-PAH: connective tissue diseases associated PAH, CHD-PAH: congenital heart diseases associated PAH, sBP: systolic blood pressure, HR: heart rate, BMI: body mass index, eGFR: estimated glomerular filtration rate, hs-CRP: high-sensitivity C-reactive protein, NYHA: New York Heart Association classification, 6MWD: 6-minute walking distance, mPAP: mean pulmonary arterial pressure, PAWP: pulmonary arterial wedge pressure, PVR: pulmonary vascular resistance, CO: cardiac output, CI: cardiac index.

KP-metabolite profile in PAH

At the time of diagnosis (baseline), Trp was significantly lower in treatment-naïve PAH patients compared to controls, while Kyn, 3-HK, QA, KA and AA were significantly higher in treatment-naïve PAH patients and no significant difference in 3-HA was found (Figure 1B). Binary logistic regression analyses showed that KP-metabolites significantly distinguished PAH patients from controls at baseline both in a univariate model and when corrected for age, sex, and body mass index (Table 2). Moreover, manual stepwise logistic regression analyses showed that including the whole panel of altered metabolites in the model predicted better than only one metabolite by significantly increasing the Chi-square of the model (Table 2).

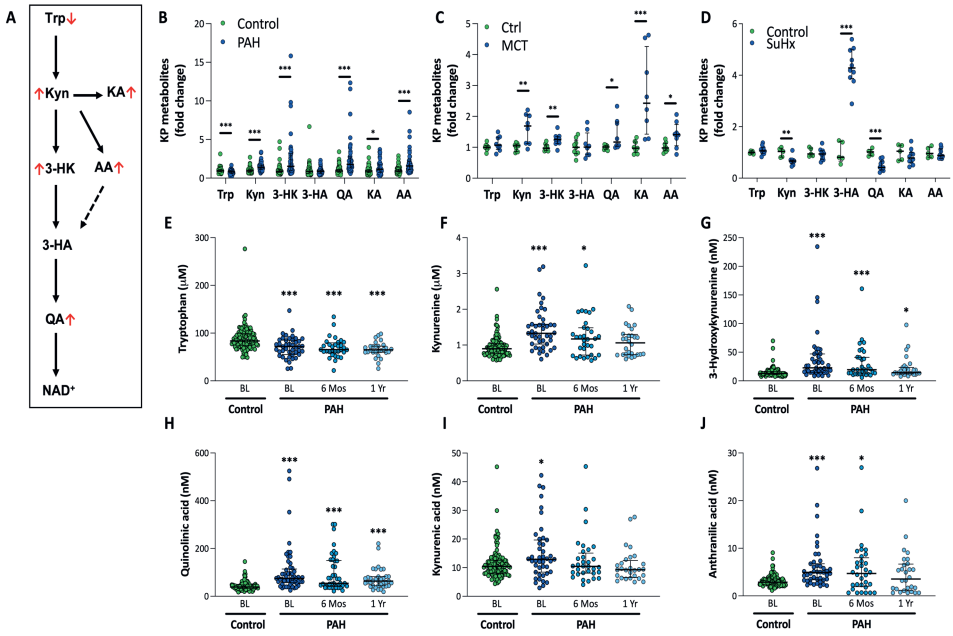


Figure 1. KP metabolite profile in PAH patients and two animal models of PH. **A.** Scheme of *de novo* NAD^+ synthesis through the kynurenine pathway with changes observed in PAH patients shown as red arrows. KP metabolite profiles in: **B.** PAH patients at baseline (n=43) vs Healthy controls (n=111) in the controls), **C.** MCT-PH rats (n=8) vs Control rats (n=9), **D.** SuHx-PH rats (n=10) vs Control rats (n=5). A KP metabolite profile similar to PAH patients was only found in the MCT-PH rats which show a severe inflammatory phenotype, indicating the potential link between inflammation and KP metabolism in PAH. When compared to the controls (n=111), **E.** Tryptophan was significantly lower in PAH at baseline (n=43) and after therapy (6Mos, n=32; 1Yr, n=28), **F & J.** Kynurenine and anthranilic acid were significantly higher in PAH at baseline and 6 months after PAH therapy, **H & I.** 3-hydroxykynurenine and quinolinic acid were significantly higher in PAH at baseline and after therapy, **G.** Kynurenic acid was only higher in PAH at baseline. Data are presented as dot plots with median (IQR). * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs Controls, Mann-Whitney Test. MCT: monocrotaline, SuHx: Sugen plus hypoxia, Trp: tryptophan, Kyn: kynurenine, 3-HK: 3-hydroxykynurenine, 3-HA: 3-hydroxykynurenic acid, QA: quinolinic acid, KA: kynurenic acid, AA: anthranilic acid, NAD: nicotinamide adenine dinucleotide, BL: baseline, Mos: months, Yr: year.

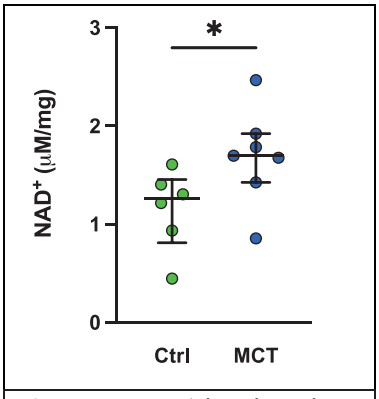


Figure 2. NAD^+ levels in lung tissue are higher in rats with monocrotaline(MCT) induced PAH as compared to Control rats (Ctrl). * *P*<0.05

MCT-PH rats showed a similar KP-metabolite profile as that observed in PAH patients (Figure 1C), which was accompanied by an increase in NAD+ in the lungs from these rats (Figure 2). Conversely, the KP-metabolite profile was different in SuHx-PH rats (Figure 1D). As the MCT-PH rats show the most severe inflammatory phenotype, these results suggest a link between inflammation and KP-metabolism in PAH.

Table 2. Prediction of PAH with each 1 μM decrease for Trp and 1 nM increase in other KP metabolites by binary logistic regression.

	Odds Ratio [95% CI]	*P* value	Chi-Square	*P* value
Univariable				
Trp	1.047 [1.024-1.070]	<0.001	21.083	<0.001
Kyn	1.003 [1.002-1.004]	<0.001	39.370	<0.001
3-HK	1.097 [1.051-1.144]	<0.001	38.390	<0.001
QA	1.053 [1.032-1.075]	<0.001	56.114	<0.001
KA	1.076 [1.023-1.132]	0.005	8.842	0.003
AA	1.924 [1.453-2.550]	<0.001	40.132	<0.001
Whole panel			92.492	<0.001
Adjusted for age, sex and body mass index				
Trp	1.036 [1.011-1.060]	<0.001	35.551	<0.001
Kyn	1.003 [1.002-1.005]	<0.001	57.983	<0.001
3-HK	1.089 [1.043-1.137]	<0.001	53.224	<0.001
QA	1.047 [1.026-1.069]	<0.001	63.944	<0.001
KA	1.090 [1.029-1.155]	0.003	35.368	<0.001
AA	1.870 [1.398-2.502]	<0.001	54.705	<0.001
Whole panel			93.187	<0.001

Trp: Tryptophan, Kyn: Kynurenine, 3-HK: 3-Hydroxykynurenine, QA: Quinolinic acid, KA: Kynurenic acid, AA: Anthranilic acid.

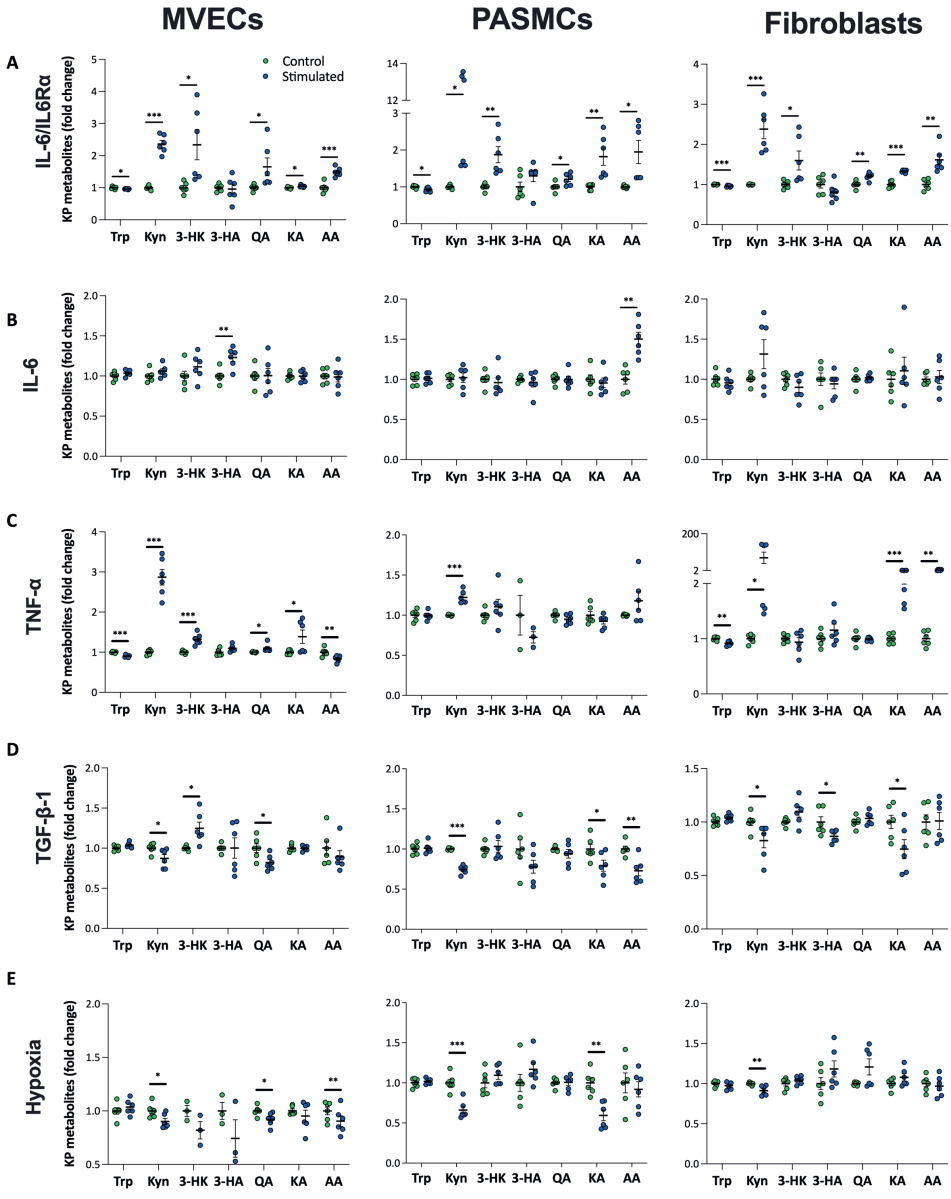


Figure 3. IL-6/IL-6Rα stimulation in vitro mimicked the abnormal KP metabolite profile observed in PAH patients. A. Stimulation with IL-6/IL-6Rα in human lung MVECs (n=6, 2 donors), PASMCs (n=6, 2 donors), and lung fibroblasts (n=6, 2 donors) for 24 hours in vitro induced the abnormal phenotype of KP metabolism that was seen in PAH patients with lower Trp, higher Kyn, 3-HK, QA, KA, and AA,

while unchanged 3-HA. Stimulation with IL-6 **(B),** TNF-α (**C**), TGF-β1 (**D**), or Hypoxia (1% O_2, **E**) for 24 hours in human lung MVECs, PASMCs, or fibroblasts in vitro resulted in different KP metabolite profiles. Data are presented as mean±SEM, fold change to control, *P<0.05, **P<0.01, ***P<0.001, Student T Test. MVECs: microvascular endothelial cells, PASMCs: pulmonary artery smooth muscle cells, IL-6Rα: interleukin-6 receptor α, IL-6: interleukin-6, TNF-α: tumor necrosis factor α, TGF-β1: transforming growth factor beta-1, Trp: tryptophan, Kyn: kynurenine, 3-HK: 3-hydroxy-kynurenine, 3-HA: 3-hydroxykynurenic acid, QA: quinolinic acid, KA: kynurenic acid, AA: anthranilic acid.

IL-6/IL-6Rα contributed to the activation of KP-metabolism in vitro

Stimulation with IL-6/IL-6Rα complex induced KP activation and mimicked the KP-metabolite profile observed in PAH patients in all three cell types, while stimulation with IL-6 alone failed to induce a similar profile (Figure 3A&B). Moreover, the cells responded differently to other cytokines and hypoxia failed to induce a KP profile similar to that seen in PAH patients (Figure 3C-E). Taken together, these results indicate that inflammation, particularly activation of IL-6/IL-6Rα signaling contributed to the KP activation in PAH patients.

Effects of PAH therapy on KP-metabolism

Following six months of PAH therapy, Trp was still significantly lower in PAH patients compared to controls, while only Kyn, 3-HK, QA, and AA were still significantly higher in PAH patients (Figure 1E-J). After one year of PAH therapy, Trp was still significantly lower in PAH patients than in controls, while only 3-HK and QA were still significantly higher in PAH patients (Figure 1E-J). These data suggest KP-metabolite profile partly normalized in PAH patients after PAH therapy.

When baseline KP-metabolite levels were compared between survivors and non-survivors, only Kyn was significantly higher in non-survivors versus survivors (Figure 4D). However, when comparing these levels at the latest measurement available, Kyn, 3-HK, QA, KA, and AA were all significantly higher in non-survivors compared

with survivors (Figure 4, right panels), time between baseline and latest measurement was 9.8±3.8 months and 4.3±5.5 months (mean±SD) for survivors and non-survivors, respectively). In these survivors, Wilcoxon matched-pairs signed rank test showed that Kyn, 3-HK, QA, KA, and AA were significantly decreased after one year but not six months of PAH therapy, indicating that only long-term PAH therapy decreased KP-metabolite levels (Figure 4, left panels).

These data indicate that KP-metabolites could be potential predictors of response to PAH therapy in survivors, and regular monitoring of KP-metabolites may be important to evaluate clinical status of PAH patients.

Correlation of KP-metabolites and disease-severity

Significant correlations between different KP-metabolites were seen in healthy controls and PAH patients at baseline. In healthy controls, KP-metabolites correlated with each other, with one exception (lack of correlation between Trp and QA (Figure 5A)). In PAH patients, Trp was not correlated with any other metabolite, while other metabolites still correlated with each other at baseline as well as after PAH therapy for six months and one year (Figure 5B-D).

Significant correlations of KP-metabolite levels with baseline characteristics were observed both in healthy controls and PAH patients (Figure 5E&F). Importantly, in PAH patients, higher levels of Kyn, 3-HK, 3-HA, and KA correlated with higher pulmonary vascular resistance, while higher levels of Kyn and KA correlated with reduced cardiac output and cardiac index, higher levels of QA correlated with a reduced cardiac index. Higher levels of Kyn, 3-HK,QA,KA and AA correlated with worse functional calss, higher levels of Kyn correlated with high sensitivity C-reactive protein (Figure 5G), further supporting the potential link between

inflammatory and KP-metabolism. C-reactive protein was not different between iPAH and aPAH subgroups.

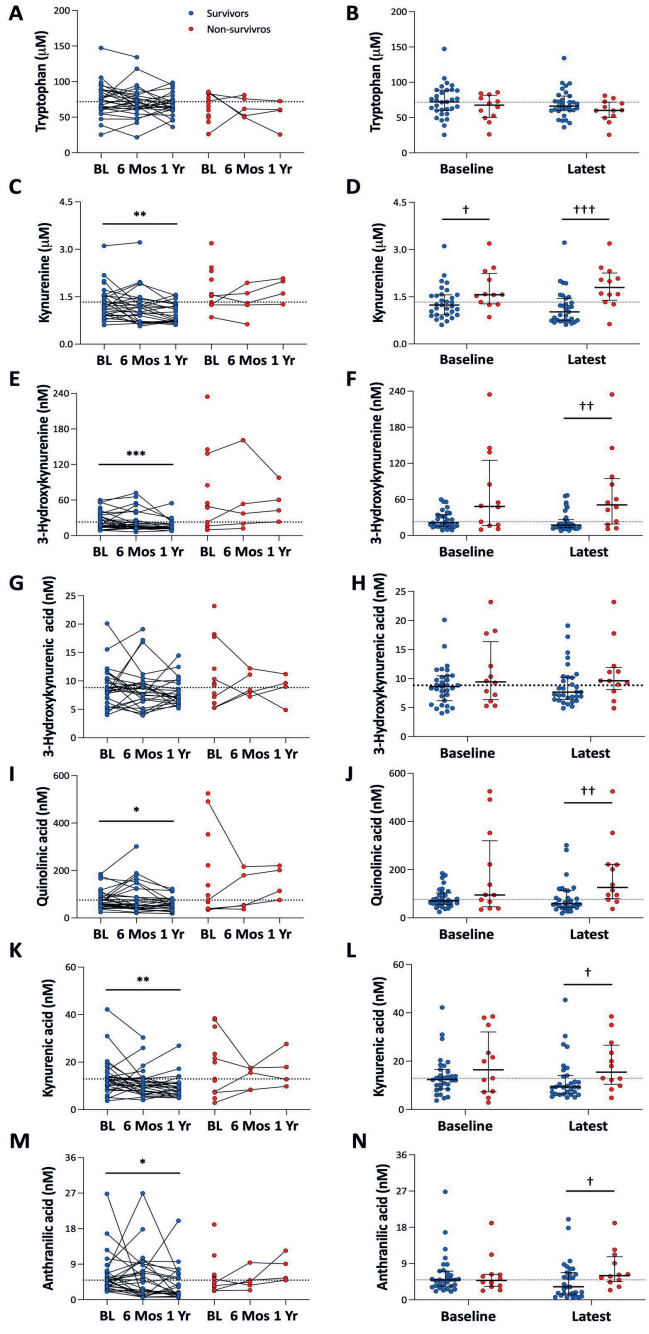


Figure 4. Treatment effects on KP metabolite levels in surviving vs non surviving PAH patients. Wilcoxon matched-pairs signed rank test showed that Kyn (*P*=0.005, **C**), 3-HK (*P*<0.001, **E**), QA (*P*=0.013, **G**), KA (*P*=0.007, **I**) and AA (*P*=0.027, **K**) were significantly decreased in survivors after one year but not six months of PAH therapy. Mixed-effects model showed that this effect was present during the one-year period for Kyn (*P*=0.010, **C**), 3-HK (*P*=0.022, **E**), KA (*P*=0.050, **I**), and with a trend toward significance for QA (*P*=0.082, **G**). Moreover, at baseline, only Kyn was significantly higher in non-survivors when compared with survivors (**D**), while at the latest measurement after PAH therapy, Kyn (**D**), 3-HK (**F**), QA (**H**), KA (**J**) and AA (**L**) were all significantly higher in non-survivors when compared with survivors. Data are presented as dot plots with median (IQR), dash-lines indicate the baseline median value of KP metabolites in all PAH patients. *P*<0.05, ***P*<0.01, ****P*<0.001, Wilcoxon matched-pairs signed rank test. †*P*<0.05, ††*P*<0.01, †††*P*<0.001, Mann-Whitney Test. BL: baseline, Mos: months, Yr: year. Trp: tryptophan, Kyn: kynurenine, 3-HK: 3-hydroxy-kynurenine, QA: quinolinic acid, KA: kynurenic acid, AA: anthranilic acid.

Survival analyses

PAH patients were stratified into two groups based on the median level of KP-metabolites measured at baseline. High levels of Kyn (>1.328 μM), 3-HK (>22.71 nM) or QA (>75.23 nM) predicted worse early survival (Breslow Test), while high Kyn levels also predicted worse long-term survival (Log-rank Test, Figure 6). However, there was no difference between patients with low and high levels of Trp, 3-HA, KA, or AA (Figure 6).

Since survivors had lower levels of KP-metabolites at the latest measurement timepoint, which may be associated with a better response to PAH therapy, we compared the survival curves in PAH patients based on the latest available measurement. Again, high levels of Kyn, 3-HK, or QA predicted worse early survival, ut also worse long-term survival (Figure 6). In addition, patients with high levels of 3-HA (>8.833 nM), KA (>12.92 nM), or AA (>4.944 nM) had worse early survival, and patients with high levels of 3-HA, or KA also had worse long-term survival (Figure 6).

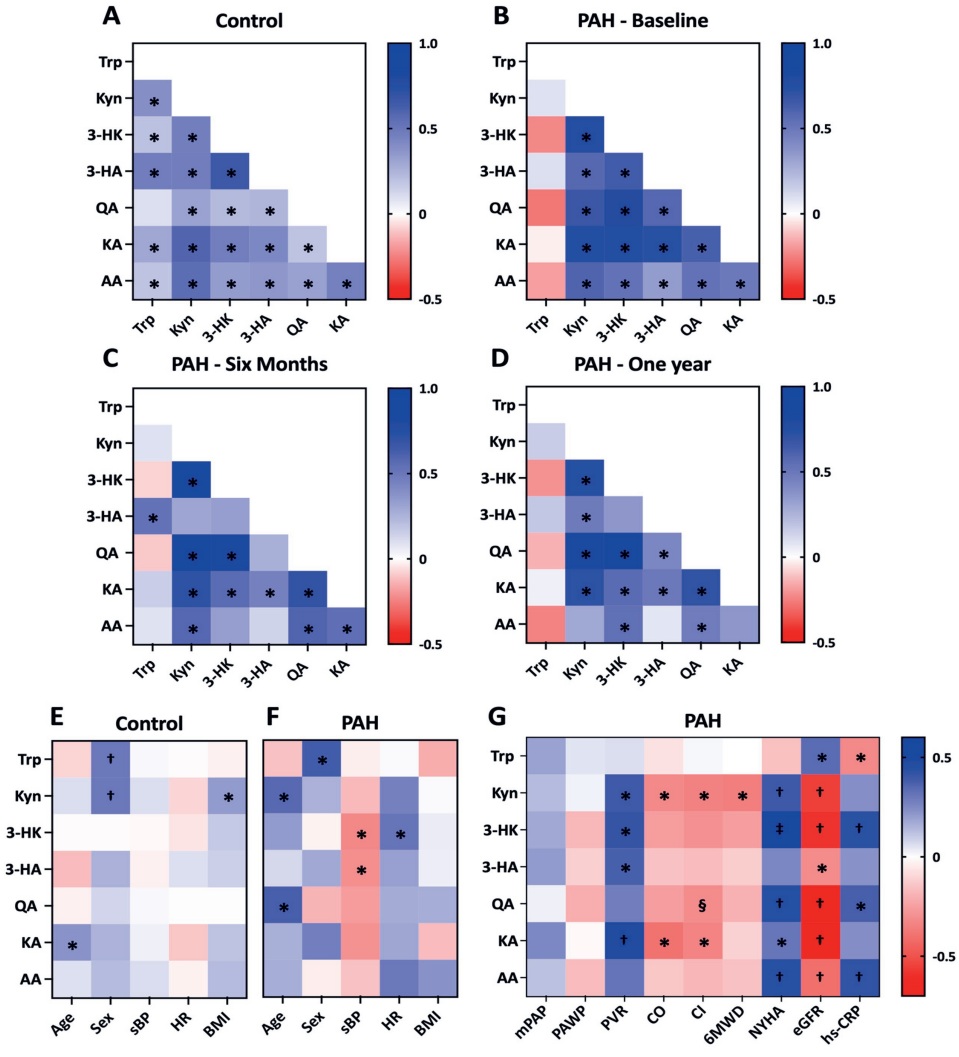


Figure 5. Correlation analysis. Correlation between KP metabolites in healthy controls and PAH patients. A. KP metabolites correlated with each other in healthy controls except for Trp and QA. B. At baseline, Trp lost its correlations with other metabolites, while other metabolites still correlated with each other in PAH patients. Such correlations were generally maintained after PAH therapy for six months (C) and one year (D). Correlations of KP metabolite levels with baseline characteristics. E. In healthy controls, KA was associated with age, Trp and Kyn were associated with Sex, Kyn was associated with BMI. F. In PAH patients, Kyn and QA were associated with age, Trp was associated with Sex, 3-HK and 3-HA were associated with sBP, 3-HK was associated with HR. G. In PAH patients, Kyn, 3-HK, 3-HA, and KA were positively associated with PVR, Kyn, 3-

HK, QA, KA, and AA were positively associated with NYHA, while Kyn was reversely associated with CO, CI and 6MWD, QA was reversely associated with CI, KA was reversely associated with CO and CI. Data are presented as rainbow heat map for Spearman coefficients, *P<0.05, †P<0.01, ‡P<0.001, §P=0.05, Spearman's Rank correlation coefficient. Sex: female=0, male=1; Trp: tryptophan, Kyn: kynurenine, 3-HK: 3-hydroxy-kynurenine, 3-HA: 3-hydroxykynurenic acid, QA: quinolinic acid, KA: kynurenic acid, AA: anthranilic acid, sBP: systolic blood pressure, HR: heart rate, BMI: body mass index, eGFR: estimated glomerular filtration rate, hs-CRP: high-sensitivity C-reactive protein, mPAP: mean pulmonary arterial pressure, PAWP: pulmonary arterial wedge pressure, PVR: pulmonary vascular resistance, CO: cardiac output, CI: cardiac index, 6MWD: 6-minute walking distance, NYHA: New York Heart Association classification.

When considering KP-metabolites as continuous variables, each 1nM increase in Kyn, 3-HK, QA, or KA was associated with an increased hazard ratio of death in both univariate and multivariate analyses, while 3-HA and AA were only associated with an increased hazard ratio in the multivariate model (Table 3). These results indicate that elevations in KP-metabolites are potential predictors of survival for PAH patients, with Kyn being the strongest prognostic biomarker.

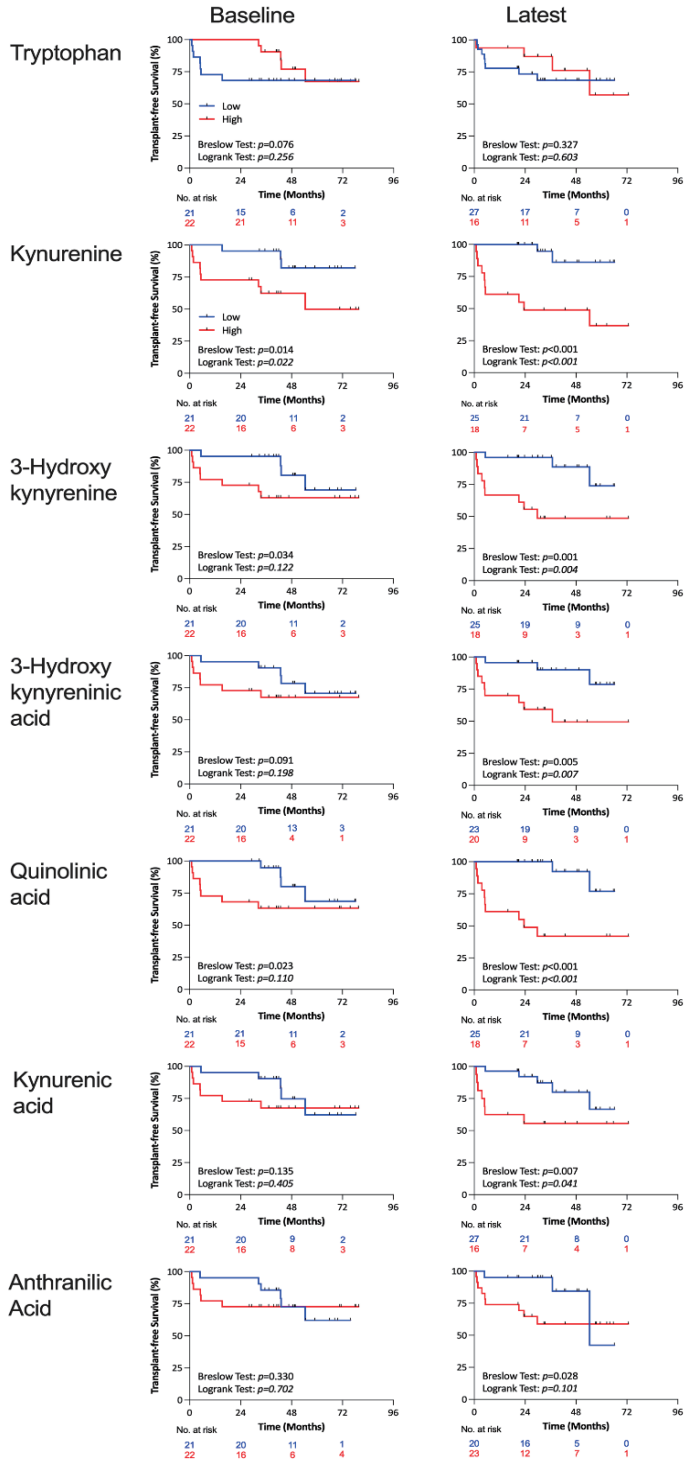

Baseline
Latest
Tryptophan
Breslow Test: p=0.076
Logrank Test: p=0.256
Breslow Test: p=0.327
Logrank Test: p=0.603
Kynurenine
Breslow Test: p=0.014
Logrank Test: p=0.022
Breslow Test: p<0.001
Logrank Test: p<0.001
3-Hydroxy kynyrenine
Breslow Test: p=0.034
Logrank Test: p=0.122
Breslow Test: p=0.001
Logrank Test: p=0.004
3-Hydroxy kynyreninic acid
Breslow Test: p=0.091
Logrank Test: p=0.198
Breslow Test: p=0.005
Logrank Test: p=0.007
Quinolinic acid
Breslow Test: p=0.023
Logrank Test: p=0.110
Breslow Test: p<0.001
Logrank Test: p<0.001
Kynurenic acid
Breslow Test: p=0.135
Logrank Test: p=0.405
Breslow Test: p=0.007
Logrank Test: p=0.041
Anthranilic Acid
Breslow Test: p=0.330
Logrank Test: p=0.702
Breslow Test: p=0.028
Logrank Test: p=0.101
Transplant-free Survival (%)
Time (Months)
No. at risk
Low
High

Figure 6. Survival analyses in PAH patients. PAH patients were stratified into 2 groups according to the median of baseline (left panels) or latest (right panels) KP metabolite levels. Using baseline metabolite levels, PAH patients with high levels of Kynurenine, 3-Hydroxykynurenine, or Quinolinic acid had worse transplant-free survival at short-term than patients with low levels (Breslow Test, *P* all <0.05). Using the latest measurement, patients with high levels of Kynurenine, 3-Hydroxykynurenine, 3-Hydroxykynurenic acid, Quinolinic acid, or Kynurenic acid, had worse short- (Breslow Test, *P* all <0.05) and long-term transplant-free survival (Log-rank Test, *P* all <0.05) than patients with low levels, while patients with high anthranilic acid had worse short-term survival only.

Table 3.Cox proportional hazard analyses for death by 1 unit change in KP-metabolite levels in PAH patients

	Baseline measurement		Latest measurement	
Univariate*	Hazard ratio [95% CI]	*P* value	Hazard ratio [95% CI]	*P* value
Trp	0.982 [0.954-1.010]	0.205	0.976 [0.945-1.007]	0.131
Kyn	1.001 [1.000-1.002]	0.012	1.001 [1.000-1.002]	0.001
3-HK	1.020 [1.009-1.030]	<0.001	1.021 [1.010-1.032]	<0.001
QA	1.006 [1.002-1.010]	0.001	1.007 [1.003-1.011]	0.001
KA	1.045 [0.996-1.096]	0.074	1.054 [1.007-1.104]	0.025
3-HA	1.119 [1.001-1.251]	0.048	1.114 [0.992-1.250]	0.067
AA	1.015 [0.906-1.138]	0.798	1.085 [0.987-1.193]	0.093
Multivariate#				
Trp	0.983 [0.954-1.014]	0.278	0.973 [0.937-1.011]	0.160
Kyn	1.001 [1.000-1.002]	0.022	1.001 [1.001-1.004]	<0.001
3-HK	1.011 [1.003-1.020]	0.007	1.018 [1.007-1.029]	0.001
QA	1.006 [1.001-1.010]	0.010	1.006 [1.002-1.011]	0.006
KA	1.056 [0.998-1.117]	0.058	1.059 [1.004-1.116]	0.034
3-HA	1.124 [0.993-1.272]	0.064	1.166 [1.026-1.325]	0.019
AA	1.015 [0.902-1.142]	0.805	1.150 [1.010-1.310]	0.035

*1 μM decrease for Trp, 1 nM increase for other metabolites. #Corrected for Age, Sex, PAH types (iPAH or APAH), PAH therapy type (no therapy or mono or double or triple therapy).

Kyn: kynurenine, 3-HK: 3-hydroxy-kynurenine, QA: quinolinic acid, KA: kynurenic acid, 3-HA: 3-hydroxykynurenic acid, AA: anthranilic acid.

Discussion

The present study demonstrated that 1) KP-metabolism was activated in treatment-naïve PAH patients compared with healthy controls, 2) the KP-metabolite profile in MCT rats was similar to that in PAH patients and was accompanied by an increase in NAD^+ levels in the rat lungs, 3) exposure of cultured MVECs, PASMCs, and fibroblasts to IL-6/IL-6Rα mimicked the KP-metabolite profile in PAH patients and therefore may contribute to KP activation in PAH patients, 4) PAH therapy normalized KP-metabolite levels in survivors, 5) KP-metabolites correlated with PAH severity at baseline and predicted mortality of PAH patients, particularly when using the latest measurement timepoint.

KP-metabolism is associated with response to PAH therapy and survival

This study is the first to measure the complete KP-metabolite profile in *treatment naïve* PAH patients and to repeat this measurement after six months and one year of PAH therapy. The results showed that PAH therapy normalizes levels of KP-metabolites in survivors after one year of therapy, which may explain why other PAH cohorts, with patients already undergoing PAH therapy, showed unchanged 3-HK, KA, and AA as compared to controls.[14, 16] This suggests that PAH patients in these cohorts responded to PAH therapy. A limitation of our study is that only a single measurement was performed in the control cohort, so the effect of time on KP-metabolite profile in the control cohort could not be assessed.

Our results further demonstrated that KP-metabolites reflect disease severity in PAH patients, with higher levels of KP-metabolites correlating particularly with higher pulmonary vascular resistance, and/or worse NYHA class, and to a lesser extent with reduced cardiac index and shorter 6-minute walking distance. Importantly, survival analysis showed that high levels of KP-metabolites (Kyn, 3-HK, QA, 3-HA, and KA) were strongly associated with worse mortality even after

adjusting for age, sex, PAH type (iPAH or APAH) and PAH therapy type (no therapy or mono or double or triple therapy). Although the model may have been overfitted, as the number of events was relatively small, these results are in accordance with recent studies showing that higher levels of Kyn, 3-HK, and QA correlated with worse functional capacity and mortality in patients with heart failure.[24, 25] Altogether, our data suggest that the KP-metabolite profile can be used as prognostic biomarker for PAH patients. However, detailed analysis of lung tissue obtained from PAH patients should be performed to investigate whether the expression of the enzymes involved in the production of the KP-metabolites is altered in the lungs. Such change in production is suggested by observations in lungs from rats with MCT induced PAH, that show an increase in Kyn and KA, consistent with the increased plasma levels observed in the present study.

In the present study, KP metabolites correlate with eGFR. This is in accordance with studies showing that KP metabolites are higher in patients with acute and chronic kidney disease.[26] However, KP metabolites did predict survival whereas eGFR did not a predict survival in our cohort (HR 0.979 [0.948-1.011, P=0.201), although it has previously been shown that eGFR is a predictor of survival in PAH.[27] Because eGFR did not predict survival in this cohort, and the number of endpoints is relatively low, eGFR was not included in the multivariate analyses to prevent overfitting of the model.

Importantly, as changes in eGFR may also be linked to inflammation,[28] KP metabolites, eGFR and PAH may also be linked through inflammation. Indeed, immune dysregulation, inflammation and hypoxia are important factors that contribute to the development and progression of PAH.[4] The KP-metabolites 3-HK, QA and AA correlated with high-sensitivity C-reactive protein, a biomarker of inflammation and predictor of outcome in PAH.[29] As shown in Figure 1B, these

metabolites also show the largest increase as well as the largest variation between PAH patients. It is therefore likely that inflammation may cause differential alterations of KP enzymes in the lung. It is however, important to note that inflammation can also decrease eGFR. Hence, the association between KP-metabolites and eGFR may also represent a common consequence of inflammation. A link between KP-metabolites and inflammation is further supported by our observation that the MCT-PH rats, a model with an obvious inflammatory phenotype, showed a similar KP-metabolite profile as seen in PAH patients. Furthermore, it has been reported that inflammation in PAH patients is alleviated with PAH therapy in survivors,[29] which coincides with the normalization of KP-metabolite levels.

To further investigate the inflammatory mediator(s) underlying the KP activation, lung microvascular endothelial cells, smooth muscle cells and fibroblasts were exposed to a variety of inflammatory stimuli, such as IL-6, TNF-α, TGF-β and hypoxia. This in vitro study showed that particularly activation of IL-6/IL-6Rα signaling, which plays a prominent role in pulmonary vascular remodeling in PAH,[30] is an important modulator of KP-metabolism. Indeed, IL-6 was elevated in PAH-CTD as well as in a subgroup of iPAH patients in our cohort.[31] However, although 31 patients overlapped between this study and the present study, there was no correlation between KP metabolites and IL-6 (R^2<0.05) except for a modest correlation between IL-6 and AA (R^2= 0.15).

Recently, PASMCs were found to be the local source of increased IL-6 in PAH[32], while increased IL-6Rα has been shown in the MVECs.[30] Although the activation of IL-6/IL-6Rα pathway has been shown in both MCT and SuHx,[30] the KP-metabolite profile differed markedly between human PAH/MCT and SuHx. This difference may in part be explained by the observed reduction in Kyn levels in the different lung

cell types in response to hypoxia. Nevertheless, treatment with tocilizumab, an IL-6R antagonist tested in the TRANSFORM-UK phase-II trial,[33] did not result in significant clinical improvement in PAH patients[34] in spite of one case-report showing that tocilizumab improved symptoms in a patient with PAH associated with Castleman's disease.[35] Since patients recruited in this trial were stable on therapy, it is possible that KP-metabolite levels had normalized, and hence that tocilizumab had no further effects, which might explain the neutral results. It would be interesting to measure the KP-metabolites prior to initiation and post treatment with tocilizumab to identify potential subgroups that might respond to tocilizumab and to further test a causal role of IL-6/IL-6Rα in KP activation.

Other inflammatory factors (IL-1b, IL-8, IL-10, CXCL9, CXCL13 and TGF-β) were measured within the same cohort (31 overlapping patients) and published in.[31] In addition to IL-6, IL-10, TGF-β, CXCL9 and CXCL13 were elevated in iPAH and/or APAH patients. However, only CXCL9 correlated with all KP metabolites (R^2 between 0.20 and 0.44) except Trp (R^2 =0.06). This correlation is in accordance with findings in COPD patients that CXCL9 correlates with Kyn/Trp ratio, as an index of KP activity,[36] and suggests that activation of inflammatory cells plays a role in KP-activation. However, this potential mechanistic link between CXCL9 and KP in PAH remains to be explored in future studies.

Although the KP is best known for its link with inflammation,[6, 13] its metabolites have a wide variety of other functions. For example, Kyn is a vasodilator activating production cAMP and cGMP / soluble guanylyl cyclase in the systemic,[37, 38] coronary,[38] as well as pulmonary circulation.[15] Moreover, acute administration of *exogenous* Kyn reduced right ventricular systolic pressure in rodent models of PH[15]. If vasodilation induced by *endogenous* Kyn would be important in PAH, higher levels of Kyn should be associated with lower systemic blood pressure and/or lower

pulmonary artery pressure. However, although PAH patients had higher levels of Kyn, Kyn correlated with higher PVR with no association with lower systemic or pulmonary artery pressure, and higher levels of Kyn correlated with higher pulmonary artery pressure in previous studies[14, 15]. Therefore, a potential detrimental effect of KP activation that explains its association with worse prognosis in PAH may be mediated through other KP-metabolites.

NAD^+, the end product of KP-metabolism, plays an essential role in regulation of mitochondrial function and has been implicated in aging/longevity[8]. Several studies suggest that NAD^+ depletion is an important contributor to the pathogenesis of age-related diseases and cardiovascular diseases,[8-10, 39] and raising NAD^+ levels has been proposed as a promising therapeutic strategy for diseases such as obesity, renal diseases and heart failure[5, 7, 40, 41] by improving mitochondrial function, and prolonging survival of injured cells or apoptotic cells.[5, 42, 43] In contrast to these diseases, PAH is a disease in which mitochondrial remodeling[3] is associated with excessive survival and proliferation of pulmonary vascular cells.[4] Thus, PAH seems to be associated with NAD^+ abundancy that may contribute to pulmonary vascular remodeling. Indeed, nicotinamide phosphoribosyltransferase (NAMPT), the enzyme responsible for NAD^+ synthesis via the salvage pathway, was increased in the circulation and lung of advanced PAH patients, and NAMPT inhibition has shown therapeutic effects in rodent models of PH by reversing pulmonary vascular remodeling.[11] In our study, higher levels of KP-metabolites also suggest an activation of *de novo* NAD^+ synthesis in PAH patients, particularly since NAD^+ levels were elevated in the lungs of MCT-PH rats. Unfortunately, there was no human lung tissue available to measure NAD^+, and confirm rat data that activation of the KP is indeed associated with higher NAD^+ levels. Nevertheless, available data suggest that KP activation might contribute to pulmonary vascular remodeling of PAH via *de novo* NAD^+ synthesis, future studies should investigate whether

inhibition of *de novo* NAD^+ synthesis, via inhibition of KP activity, may provide a novel therapeutic target for PAH.

Conclusion and clinical relevance

Activation of kynurenine pathway with increased levels of circulating KP-metabolites predict disease severity, response to PAH therapy, and survival in PAH patients. Exposure of lung microvascular endothelial cells, smooth muscle cells and fibroblasts to IL-6/IL-6Rα induced a KP-metabolite profile similar to that observed in PAH patients, suggesting that IL-6/IL-6Rα signaling likely contribute to KP activation in PAH patients. Whether KP activation contributes to pulmonary vascular remodeling via *de novo* NAD^+ synthesis, and may provide a novel treatment strategy for PAH remains to be established.

Declarations

Funding

This work was supported by the China Scholarship Council (201606230252 to ZC & 201708130077 to ST), the China Postdoctoral Science Foundation (2020M680079 and 2021T140598 to ZC), the Netherlands CardioVascular Research Initiative: an initiative with support of the Dutch Heart Foundation (CVON2014-11, RECONNECT, to DM and DJD), and German Center for Cardiovascular Research (DZHK81Z0600207 to DM). Instrumentation support was received from AB Sciex, ltd. for LC-MS/MS analyses performed in this study.

Conflicts of interest/Competing interests The authors declare that they have no conflict of interest

Availability of data and material (data transparency): The datasets used and/or analysed during the current study are available as supplementary material.

Code availability not applicable

Authors' contributions ZC and DM contributed to the conception and design of the work; acquisition, analysis, interpretation of data for the work; drafting and revising the manuscript for the work; final approval of the manuscript to be published. TK, ST, LT, LG, CL, MF, and IK contributed to the acquisition, analysis of data for the work; revising the manuscript for the work; final approval of the manuscript to be published. AB, YR, IR, EB, DD, KB, KL and CG contributed to the interpretation of data for the work, revising of the manuscript for the work; final approval of the manuscript to be published.

Additional declarations for articles in life science journals that report the results of studies involving humans and/or animals

Ethics approval

The (human) study protocol was approved by the Erasmus MC ethical committee and all procedures were performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all PAH patients and healthy volunteers.

All animal procedures were performed conform to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and approved by the respective animal welfare committees of the institute.

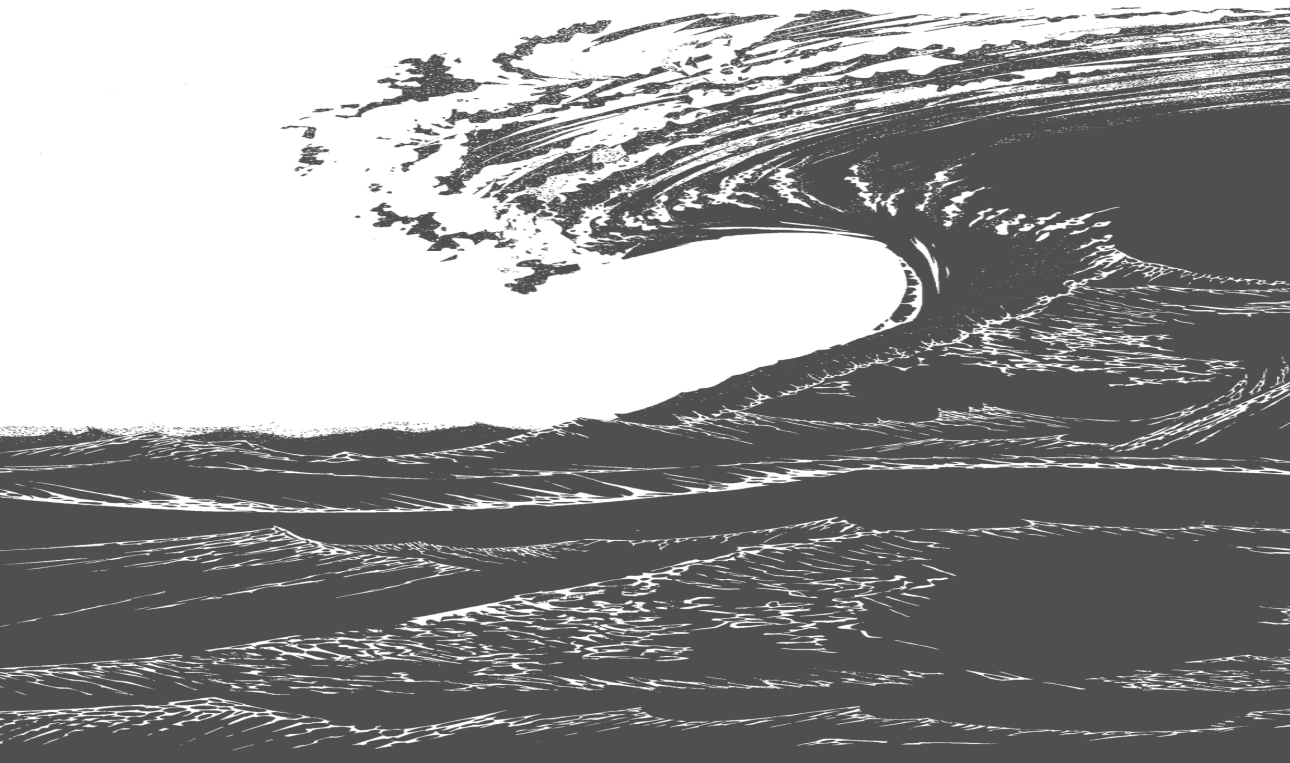
References:

1. Simonneau G, Montani D, Celermajer DS, Denton CP, Gatzoulis MA, Krowka M, Williams PG and Souza R. Haemodynamic definitions and updated clinical classification of pulmonary hypertension. *The European respiratory journal*. 2019;53.
2. Chan SY and Rubin LJ. Metabolic dysfunction in pulmonary hypertension: from basic science to clinical practice. *Eur Respir Rev*. 2017;26.
3. Sutendra G and Michelakis ED. The metabolic basis of pulmonary arterial hypertension. *Cell Metab*. 2014;19:558-73.
4. Humbert M, Guignabert C, Bonnet S, Dorfmuller P, Klinger JR, Nicolls MR, Olschewski AJ, Pullamsetti SS, Schermuly RT, Stenmark KR and Rabinovitch M. Pathology and pathobiology of pulmonary hypertension: state of the art and research perspectives. *Eur Respir J*. 2019;53.
5. Katsyuba E, Mottis A, Zietak M, De Franco F, van der Velpen V, Gariani K, Ryu D, Cialabrini L, Matilainen O, Liscio P, Giacche N, Stokar-Regenscheit N, Legouis D, de Seigneux S, Ivanisevic J, Raffaelli N, Schoonjans K, Pellicciari R and Auwerx J. De novo NAD(+) synthesis enhances mitochondrial function and improves health. *Nature*. 2018;563:354-359.
6. Minhas PS, Liu L, Moon PK, Joshi AU, Dove C, Mhatre S, Contrepois K, Wang Q, Lee BA, Coronado M, Bernstein D, Snyder MP, Migaud M, Majeti R, Mochly-Rosen D, Rabinowitz JD and Andreasson KI. Macrophage de novo NAD(+) synthesis specifies immune function in aging and inflammation. *Nat Immunol*. 2019;20:50-63.
7. Yoshino J, Baur JA and Imai SI. NAD(+) Intermediates: The Biology and Therapeutic Potential of NMN and NR. *Cell Metab*. 2018;27:513-528.
8. Yaku K, Okabe K and Nakagawa T. NAD metabolism: Implications in aging and longevity. *Ageing Res Rev*. 2018;47:1-17.
9. Hershberger KA, Martin AS and Hirschey MD. Role of NAD(+) and mitochondrial sirtuins in cardiac and renal diseases. *Nat Rev Nephrol*. 2017;13:213-225.
10. Verdin E. NAD(+) in aging, metabolism, and neurodegeneration. *Science*. 2015;350:1208-13.
11. Chen J, Sysol JR, Singla S, Zhao S, Yamamura A, Valdez-Jasso D, Abbasi T, Shioura KM, Sahni S, Reddy V, Sridhar A, Gao H, Torres J, Camp SM, Tang H, Ye SQ, Comhair S, Dweik R, Hassoun P, Yuan JX, Garcia JGN and Machado RF. Nicotinamide Phosphoribosyltransferase Promotes Pulmonary Vascular Remodeling and Is a Therapeutic Target in Pulmonary Arterial Hypertension. *Circulation*. 2017;135:1532-1546.
12. Bender DA. Effects of a dietary excess of leucine on the metabolism of tryptophan in the rat: a mechanism for the pellagragenic action of leucine. *Br J Nutr*. 1983;50:25-32.
13. Cervenka I, Agudelo LZ and Ruas JL. Kynurenines: Tryptophan's metabolites in exercise, inflammation, and mental health. *Science*. 2017;357.
14. Lewis GD, Ngo D, Hemnes AR, Farrell L, Domos C, Pappagianopoulos PP, Dhakal BP, Souza A, Shi X, Pugh ME, Beloiartsev A, Sinha S, Clish CB and Gerszten RE. Metabolic Profiling of Right Ventricular-Pulmonary Vascular Function Reveals Circulating Biomarkers of Pulmonary Hypertension. *J Am Coll Cardiol*. 2016;67:174-189.
15. Nagy BM, Nagaraj C, Meinitzer A, Sharma N, Papp R, Foris V, Ghanim B, Kwapiszewska G, Kovacs G, Klepetko W, Pieber TR, Mangge H, Olschewski H and Olschewski A. Importance of kynurenine in pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol*. 2017;313:L741-L751.

16. Jasiewicz M, Moniuszko M, Pawlak D, Knapp M, Rusak M, Kazimierczyk R, Musial WJ, Dabrowska M and Kaminski KA. Activity of the kynurenine pathway and its interplay with immunity in patients with pulmonary arterial hypertension. *Heart*. 2016;102:230-7.
17. Galie N, Hoeper MM, Humbert M, Torbicki A, Vachiery JL, Barbera JA, Beghetti M, Corris P, Gaine S, Gibbs JS, Gomez-Sanchez MA, Jondeau G, Klepetko W, Opitz C, Peacock A, Rubin L, Zellweger M, Simonneau G and Guidelines ESCCfP. Guidelines for the diagnosis and treatment of pulmonary hypertension: the Task Force for the Diagnosis and Treatment of Pulmonary Hypertension of the European Society of Cardiology (ESC) and the European Respiratory Society (ERS), endorsed by the International Society of Heart and Lung Transplantation (ISHLT). *Eur Heart J*. 2009;30:2493-537.
18. Hoeper MM, Bogaard HJ, Condliffe R, Frantz R, Khanna D, Kurzyna M, Langleben D, Manes A, Satoh T, Torres F, Wilkins MR and Badesch DB. Definitions and diagnosis of pulmonary hypertension. *J Am Coll Cardiol*. 2013;62:D42-50.
19. Galie N, Humbert M, Vachiery JL, Gibbs S, Lang I, Torbicki A, Simonneau G, Peacock A, Vonk Noordegraaf A, Beghetti M, Ghofrani A, Gomez Sanchez MA, Hansmann G, Klepetko W, Lancellotti P, Matucci M, McDonagh T, Pierard LA, Trindade PT, Zompatori M, Hoeper M and Group ESCSD. 2015 ESC/ERS Guidelines for the diagnosis and treatment of pulmonary hypertension: The Joint Task Force for the Diagnosis and Treatment of Pulmonary Hypertension of the European Society of Cardiology (ESC) and the European Respiratory Society (ERS): Endorsed by: Association for European Paediatric and Congenital Cardiology (AEPC), International Society for Heart and Lung Transplantation (ISHLT). *Eur Heart J*. 2016;37:67-119.
20. Geenen LW, Baggen VJM, Koudstaal T, Boomars KA, Eindhoven JA, Boersma E, Roos-Hesselink JW and van den Bosch AE. The prognostic value of various biomarkers in adults with pulmonary hypertension; a multi-biomarker approach. *Am Heart J*. 2019;208:91-99.
21. Menting ME, McGhie JS, Koopman LP, Vletter WB, Helbing WA, van den Bosch AE and Roos-Hesselink JW. Normal myocardial strain values using 2D speckle tracking echocardiography in healthy adults aged 20 to 72 years. *Echocardiography*. 2016;33:1665-1675.
22. Tu L, Desroches-Castan A, Mallet C, Guyon L, Cumont A, Phan C, Robert F, Thuillet R, Bordenave J, Sekine A, Huertas A, Ritvos O, Savale L, Feige JJ, Humbert M, Bailly S and Guignabert C. Selective BMP-9 Inhibition Partially Protects Against Experimental Pulmonary Hypertension. *Circ Res*. 2019;124:846-855.
23. Broekhuizen M, Klein T, Hitzerd E, de Rijke YB, Schoenmakers S, Sedlmayr P, Danser AHJ, Merkus D and Reiss IKM. l-Tryptophan-Induced Vasodilation Is Enhanced in Preeclampsia: Studies on Its Uptake and Metabolism in the Human Placenta. *Hypertension*. 2020;76:184-194.
24. Lund A, Nordrehaug JE, Slettom G, Hafstad Solvang SE, Ringdal Pedersen EK, Midttun O, Ulvik A, Ueland PM, Nygard O and Melvaer Giil L. Plasma kynurenines and prognosis in patients with heart failure. *PLoS One*. 2020;15:e0227365.
25. Konishi M, Ebner N, Springer J, Schefold JC, Doehner W, Dschietzig TB, Anker SD and von Haehling S. Impact of Plasma Kynurenine Level on Functional Capacity and Outcome in Heart Failure- Results From Studies Investigating Co-morbidities Aggravating Heart Failure (SICA-HF). *Circ J*. 2016;81:52-61.

26. Zakrocka I and Zaluska W. Kynurenine pathway in kidney diseases. *Pharmacol Rep*. 2022;74:27-39.
27. Chakinala MM, Coyne DW, Benza RL, Frost AE, McGoon MD, Hartline BK, Frantz RP, Selej M, Zhao C, Mink DR and Farber HW. Impact of declining renal function on outcomes in pulmonary arterial hypertension: A REVEAL registry analysis. *J Heart Lung Transplant*. 2018;37:696-705.
28. Mihai S, Codrici E, Popescu ID, Enciu AM, Albulescu L, Necula LG, Mambet C, Anton G and Tanase C. Inflammation-Related Mechanisms in Chronic Kidney Disease Prediction, Progression, and Outcome. *J Immunol Res*. 2018;2018:2180373.
29. Quarck R, Nawrot T, Meyns B and Delcroix M. C-reactive protein: a new predictor of adverse outcome in pulmonary arterial hypertension. *J Am Coll Cardiol*. 2009;53:1211-8.
30. Tamura Y, Phan C, Tu L, Le Hiress M, Thuillet R, Jutant EM, Fadel E, Savale L, Huertas A, Humbert M and Guignabert C. Ectopic upregulation of membrane-bound IL6R drives vascular remodeling in pulmonary arterial hypertension. *J Clin Invest*. 2018;128:1956-1970.
31. Koudstaal T, van Uden D, van Hulst JAC, Heukels P, Bergen IM, Geenen LW, Baggen VJM, van den Bosch AE, van den Toorn LM, Chandoesing PP, Kool M, Boersma E, Hendriks RW and Boomars KA. Plasma markers in pulmonary hypertension subgroups correlate with patient survival. *Respir Res*. 2021;22:137.
32. Simpson CE, Chen JY, Damico RL, Hassoun PM, Martin LJ, Yang J, Nies M, Griffiths M, Vaidya RD, Brandal S, Pauciulo MW, Lutz KA, Coleman AW, Austin ED, Ivy DD, Nichols WC and Everett AD. Cellular sources of IL-6 and associations with clinical phenotypes and outcomes in PAH. *Eur Respir J*. 2020.
33. Hernandez-Sanchez J, Harlow L, Church C, Gaine S, Knightbridge E, Bunclark K, Gor D, Bedding A, Morrell N, Corris P and Toshner M. Clinical trial protocol for TRANSFORM-UK: A therapeutic open-label study of tocilizumab in the treatment of pulmonary arterial hypertension. *Pulm Circ*. 2018;8:2045893217735820.
34. Toshner M, Church C, Morrell N and Corris P. Report: A phase II study of tocilizumab in group 1 pulmonary arterial hypertension. 2018;17 July.
35. Arita Y, Sakata Y, Sudo T, Maeda T, Matsuoka K, Tamai K, Higuchi K, Shioyama W, Nakaoka Y, Kanakura Y and Yamauchi-Takihara K. The efficacy of tocilizumab in a patient with pulmonary arterial hypertension associated with Castleman's disease. *Heart Vessels*. 2010;25:444-7.
36. Naz S, Bhat M, Stahl S, Forsslund H, Skold CM, Wheelock AM and Wheelock CE. Dysregulation of the Tryptophan Pathway Evidences Gender Differences in COPD. *Metabolites*. 2019;9.
37. Sakakibara K, Feng GG, Li J, Akahori T, Yasuda Y, Nakamura E, Hatakeyama N, Fujiwara Y and Kinoshita H. Kynurenine causes vasodilation and hypotension induced by activation of KCNQ-encoded voltage-dependent K(+) channels. *J Pharmacol Sci*. 2015;129:31-7.
38. Wang Y, Liu H, McKenzie G, Witting PK, Stasch JP, Hahn M, Changsirivathanathamrong D, Wu BJ, Ball HJ, Thomas SR, Kapoor V, Celermajer DS, Mellor AL, Keaney JF, Jr., Hunt NH and Stocker R. Kynurenine is an endothelium-derived relaxing factor produced during inflammation. *Nat Med*. 2010;16:279-85.
39. Zhang D, Hu X, Li J, Liu J, Baks-Te Bulte L, Wiersma M, Malik NU, van Marion DMS, Tolouee M, Hoogstra-Berends F, Lanters EAH, van Roon AM, de Vries AAF, Pijnappels DA, de Groot NMS, Henning RH and Brundel B. DNA damage-induced PARP1 activation confers

cardiomyocyte dysfunction through NAD(+) depletion in experimental atrial fibrillation. *Nat Commun*. 2019;10:1307.
40. Walker MA and Tian R. Raising NAD in Heart Failure: Time to Translate? *Circulation*. 2018;137:2274-2277.
41. Canto C, Houtkooper RH, Pirinen E, Youn DY, Oosterveer MH, Cen Y, Fernandez-Marcos PJ, Yamamoto H, Andreux PA, Cettour-Rose P, Gademann K, Rinsch C, Schoonjans K, Sauve AA and Auwerx J. The NAD(+) precursor nicotinamide riboside enhances oxidative metabolism and protects against high-fat diet-induced obesity. *Cell Metab*. 2012;15:838-47.
42. Rajman L, Chwalek K and Sinclair DA. Therapeutic Potential of NAD-Boosting Molecules: The In Vivo Evidence. *Cell Metab*. 2018;27:529-547.
43. Poyan Mehr A, Tran MT, Ralto KM, Leaf DE, Washco V, Messmer J, Lerner A, Kher A, Kim SH, Khoury CC, Herzig SJ, Trovato ME, Simon-Tillaux N, Lynch MR, Thadhani RI, Clish CB, Khabbaz KR, Rhee EP, Waikar SS, Berg AH and Parikh SM. De novo NAD(+) biosynthetic impairment in acute kidney injury in humans. *Nat Med*. 2018;24:1351-1359.



Chapter 5

Plasma melatonin levels predict Survival in pulmonary arterial hypertension

Zongye Cai, Theo Klein, Laurie Geenen, Ly Tu, **Siyu Tian,**
Annemien van den Bosch, Yolanda de Rijke, Irwin Reiss, Eric Boersma,
Dirk Jan Duncker, Karin Boomars, Christophe Guignabert, Daphne Merkus

Department of Cardiology, Erasmus MC, The Netherlands
Department of Clinical Chemistry, Erasmus MC, The Netherlands
INSERM UMR_S 999, Hôpital Marie Lannelongue, Le Plessis-Robinson, France
Université Paris-Saclay, School of Medicine, Le Kremlin-Bicêtre, France
Department of Pediatrics/Neonatology, Sophia Children's Hospital, Erasmus MC, The Netherlands
Department of Clinical Epidemiology, Erasmus MC, The Netherlands
Department of Pulmonary Medicine, Erasmus MC, The Netherlands
Walter Brendel Center of Experimental Medicine (WBex), LMU Munich, Germany
German Center for Cardiovascular Research (DZHK), Partner Site Munich, Munich Heart Alliance (MHA),Germany

Lower plasma melatonin levels predict worse long-term survival in pulmonary arterial hypertension. J Clin Med. 2020, 9(5), 1248.

Abstract

Background: Exogenous melatonin has been reported to be beneficial in the treatment of pulmonary hypertension (PH) in animal models. Multiple mechanisms are involved, with melatonin exerting anti-oxidant and anti-inflammatory effects, as well as inducing vasodilation and cardio-protection. However, endogenous levels of melatonin in treatment-naïve patients with PH and their clinical significance are still unknown.

Method: Plasma samples were collected and the levels of endogenous melatonin were measured by liquid chromatography-tandem mass spectrometry in PH patients (n=64, 43 pulmonary arterial hypertension (PAH) and 21 chronic thromboembolic PH (CTEPH)) and healthy controls (n=111).

Results: Melatonin levels were higher in PH, PAH, and CTEPH patients when compared with controls (Median 118.7 [IQR 108.2-139.9], 118.9 [109.3-147.7], 118.3 [106.8-130.1] versus 108.0 [102.3-115.2] pM, respectively, *P* all <0.001). The mortality was 26% (11/43) in the PAH subgroup during a long-term follow-up of 42 [IQR 32-58] months. Kaplan-Meier analysis showed that, in the PAH subgroup, patients with melatonin levels in the 1st quartile (<109.3 pM) had a worse survival than those in quartile 2-4 (Mean survival times were 46 (95% CI: 30-65) versus 68 (58-77) months, Log-rank, *P*=0.026) with an increased hazard ratio of 3.5 (95% CI: 1.1-11.6, *P*=0.038).

Conclusion: Endogenous melatonin was increased in treatment-naïve patients with PH, and lower levels of melatonin were associated with worse long-term survival in patient with PAH.

Keywords: melatonin, pulmonary hypertension, survival, clinical outcome

Introduction

Pulmonary hypertension (PH) is a severe disease with a wide spectrum of underlying etiologies.[1] Pulmonary arterial hypertension (PAH) and chronic thromboembolic PH (CTEPH) are two subgroups of PH, that both show severe pulmonary vascular remodeling. Current PAH treatment strategies delay disease progression, but curative treatment, reversing microvascular remodeling, has not been established.[2, 3] Therefore, research identifying novel mechanisms of disease progression and identifying potential therapeutic targets are necessary to develop new therapeutic strategies and improve prognosis.

Melatonin is a hormone mainly synthesized by the pineal gland and is well-known for its role in the regulation of circadian rhythm.[4] Over the past decades, an increasing number of studies have demonstrated that exogenous melatonin also exerts protective effects in cardiovascular diseases, [5-7] respiratory diseases,[8] and cancers.[9] It was already shown in 2007 that chronic hypoxia induced PH was associated with the loss of the pulmonary vasorelaxation effect of melatonin, [10] while supplementation of melatonin could prevent chronic hypoxia induced PH via anti-proliferative and anti-inflammatory effects,[11, 12] as well as through inhibiting oxidative stress,[13-15] restoring nitric oxide production,[11] and increasing angiogenesis.[16] These beneficial effects of melatonin were also shown in the rat models of monocrotaline-induced PH, [12, 17, 18] and Sugen-hypoxia-induced PH. [12] In addition, melatonin was also found to be cardio-protective in monocrotaline-induced PH by improving RV- function and inhibiting cardiac fibrosis. [16]

Although animal studies suggest that exogenous melatonin might be beneficial for patients with PH, endogenous melatonin levels in animal models of PH, and in treatment-naïve patients with PH and their clinical significance are still unknown.

In the present study, we therefore tested the hypothesis that lower melatonin levels would be associated with poor prognosis in PH patients. For this purpose, we investigated plasma melatonin levels in two well-established rat models of PH, and in treatment-naïve patients with PH and studied their clinical significance.

Methods

Study population

A total of 64 consecutive treatment-naïve adult patients with PH, including 43 patients with PAH (Group 1) and 21 patients with CTEPH (Group 4), diagnosed by right heart catheterization according to the guidelines between May 2012 and October 2016 were included as PH group in this prospective observational cohort study. [19, 20] Exclusion criteria for PH group were: incomplete diagnostic procedure, not treatment-naïve, not capable of signing informed consent, and other Groups of PH, including some patients from Group 1 PH, and all patients from Group 2, 3, and 5 PH (Figure 1). A healthy control group consisting of 145 self-reported healthy volunteers, without any (prior) cardiovascular diseases and risk factors, was recruited during the same period via an advertisement for healthy subjects, 34 volunteers were excluded from this study because of a blood pressure over 140/90 mmHg at the time of visit. More details about the study design of both cohorts have been previously described. [21, 22] The study protocols were approved by the Erasmus MC Ethical Committee and written informed consent was obtained by all PH patients and healthy volunteers. All procedures were performed in accordance with Declaration of Helsinki.

Follow-up of PH patients

PH patients were prospectively followed-up until 1st of January 2019. All patients were prescribed with specific PAH medications and/or treated with balloon pulmonary angioplasty or pulmonary endarterectomy (CTEPH patients) when

indicated according to the guidelines.[19, 23] The primary endpoint was defined as all-cause mortality. Survival status of all patients was obtained from patients and checked in the Municipal Personal Records database. Patients who did not reach the primary endpoint were censored at the 1st of January 2019.

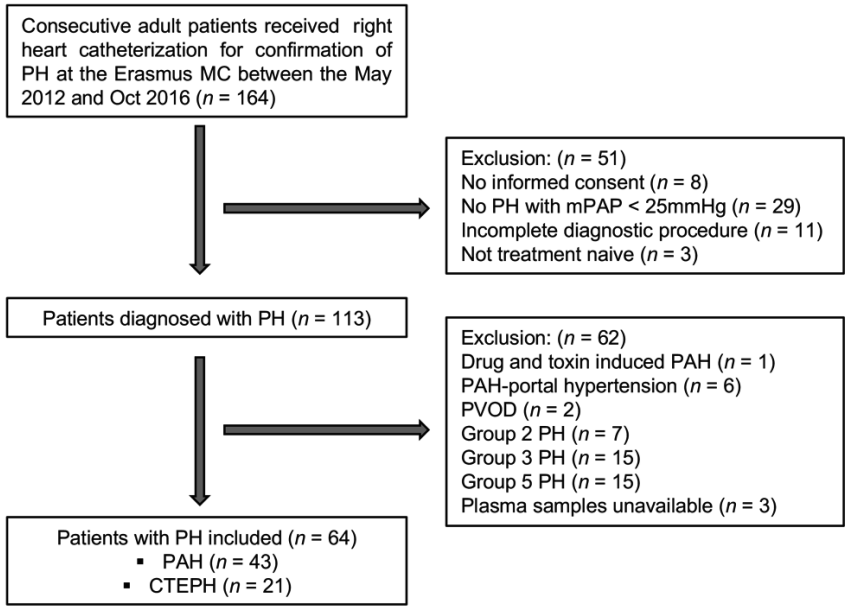


Figure 1. Enrollment scheme of PH patients in the current study. PH: pulmonary hypertension, mPAP: mean pulmonary artery pressure, PAH: pulmonary arterial hypertension, PVOD: pulmonary veno-occlusive disease, CTEPH: chronic thromboembolic pulmonary hypertension.

Animal models of PH

Two well-established rat models of severe PH were used in this study as previously described.[24] In brief, monocrotaline-induced PH model (n=11) was established in 4 weeks-old male Wistar rats (Janvier Labs, Saint Berthevin, France) with a single subcutaneous injection of monocrotaline (40 mg/kg, Sigma-Aldrich, Saint-Quentin-Fallavier, France) for 3 weeks. The Sugen-hypoxia-induced PH model (n=10) was

established in 4 weeks-old Wistar rats (Janvier Labs, Saint Berthevin, France) with a single subcutaneous injection of Sugen (SU5416, 20mg/kg, Sigma-Aldrich, Saint-Quentin-Fallavier, France) combined with exposure to normobaric hypoxia for 3 weeks followed by room air for 5 weeks.

Blood sampling and measurement of melatonin

For PH patients, regular peripheral venous blood sampling was performed during the diagnostic right heart catheterization. For healthy volunteers, regular peripheral venous blood sampling was performed at the time of visit. For animal models of PH, blood sampling was performed before sacrifice. All blood sampling was conducted during daytime between 9:00 and 18:00, in which period the levels of melatonin were reported to be stable [25]. All blood samples were prepared as EDTA-plasma samples, and then frozen and stored in aliquots at −80°C, and thawed only once for use. Melatonin levels were measured using ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). Briefly, 10 µL plasma was mixed with 10 µL deuterated melatonin (Melatonine-D3, Buchem BV, Apeldoorn, The Netherlands) solution as internal isotopically labeled internal standard and subsequently mixed with 80 µL acetonitrile for protein precipitation. After 10 minutes, the samples were cleared by centrifugation and 90 µL supernatant was dried under a stream of nitrogen at 30°C. The residue was reconstituted in 40 µL H_2O (in-house purified using a Milli-Q device) with 0.2 % (v/v) formic acid before quantitative analysis for melatonin using an in-house developed UPLC-MS/MS assay on a Sciex 6500+ QTRAP mass spectrometer (Sciex, Nieuwerkerk ad Ijssel, The Netherlands) hyphenated to a Shimadzu Nexera UPLC system (Shimadzu Benelux, Den Bosch, The Netherlands). 10 µL reconstituted sample was resolved on an Acquity HSS T3 UPLC column (2.1x100 mm, 1.8 µm; Waters, Etten-Leur, The Netherlands) using a gradient of acetonitrile in Milli-Q water, each with 0.2% formic acid (v/v). Melatonin was detected in MRM mode by

the mass transition m/z 233.1/174.1 (DP 56 V, CE 20V) and quantified to a standard calibration curve of 50-20000 pM using the area ratio of melatonin/melatonin-D3.

Statistical analysis

Data were tested for adherence to a normal distribution with the Kolmogorov-Smirnov method. Continuous variables are presented as mean ± standard deviation (SD) or median [interquartile range (IQR)], categoric variables as numbers (percentages), or as otherwise reported.

Group comparisons of continuous variables (eg, melatonin levels, age) were performed using the unpaired t-test or Mann-Whitney test (2 groups, eg, human PH versus controls, Rat models of PH versus controls), and one-way ANOVA or Kruskal-Wallis Test (3 groups, human controls, PAH, and CTEPH). Groups comparisons of categoric variables (eg, sex, NYHA) were performed using the chi-square test. Correlations analysis between melatonin levels and baseline characteristics were determined using the Spearman correlation coefficient. Logistic regression was conducted to determine whether plasma melatonin was an independent risk factor that distinguishes between PH patients and healthy controls. Univariate and multivariate Cox proportional hazard regression were used to assess associations between plasma levels of melatonin and mortality in PAH patients, one PAH patient with a very high melatonin level of 4471 pM was defined as an outlier (> 100 times the IQR), and was excluded to avoid interference. Comparisons of Long-term survival curves between groups in PAH patients were performed using Kaplan-Meier analysis with log-rank (for trend) test.

Statistical analysis was performed using IBM SPSS software (version 21.0.0.1), figures were made using GraphPad Prism (version 8.0.2). A two-sided p value < 0.05 was considered statistically significant.

Results

Baseline characteristics

Baseline characteristics of all treatment-naïve patients with PH, PAH, CTEPH and healthy controls are summarized in Table 1. PH patients were older and had higher heart rate and body mass index than controls. PAH patients showed more severe PH than CTEPH patients.

Levels of plasma melatonin

The median of plasma melatonin levels in healthy volunteers was 108.0 [102.3-115.2] pM, and was higher in treatment-naïve patients with PH (118.7 [108.2-139.9] pM, $p<0.001$), PAH (118.9 [109.3-147.7] pM, $p<0.001$) and CTEPH (118.3 [106.8-130.1] pM, $p<0.01$) (Figure 2A). There was no difference between patients with PAH and CTEPH, and there was no sex difference in either controls or PH patients. In addition, melatonin levels were significantly higher in rat models of monocrotaline-induced PH (148.0 [107.2-175.8] pM, $p<0.01$) and Sugen-hypoxia-induced PH (103.2 [83.7-118.1] pM, $p<0.01$) as compared to the control rats (67.6 [58.9-80.2] pM), and were similar in these two rat models of PH (Figure 2B).

Correlation analysis

In healthy controls, there was a weak association between melatonin and heart rate (r=-0.229, $p=0.016$), while no association was seen between melatonin with age, sex, body mass index, and systolic blood pressure (Table 2).

In patients with PH, melatonin was inversely associated with age (r=-0.368, $p=0.003$) and systolic blood pressure (r=-0.251, $p=0.046$). In the subgroup of patients with PAH, melatonin was also inversely associated with age (r=-0.334, $p=0.029$). No association was seen in patients with CTEPH (Table 2).

Neither in patients with PH, nor in the subgroups of PAH and CTEPH, a correlation was found between melatonin levels with hemodynamic parameters (mean

pulmonary artery pressure, pulmonary artery wedge pressure, pulmonary vascular resistance), or cardiac function (cardiac output, cardiac index), or 6-minute walk distance, or NYHA class (Table 2).

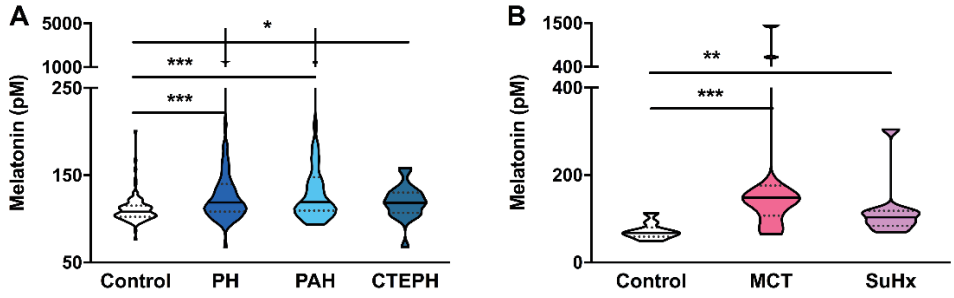


Figure 2. Plasma melatonin was increased in patients with PH and 2 rat models of PH. A. Plasma melatonin was higher in patients with PH (n=64), PAH (n=43), and CTEPH (n=21) than in healthy controls (n=111), but there was no difference between PAH and CTEPH. **B.** Plasma melatonin was higher in 2 rat models of PH, including MCT-induced PH (n=11) and SuHx-induced PH (n=10), than in controls (n=9), but there was no difference between these two models. Distribution of the Data was shown in violin plot with median and interquartile range. *p<0.05, **p<0.01, ***p<0.001, Mann-Whitney Test or Kruskal-Wallis Test. PH: pulmonary hypertension; PAH: pulmonary arterial hypertension; CTEPH: chronic thromboembolic PH; MCT: monocrotaline; SuHx: sugen and hypoxia.

Table 1. Baseline characteristics

	Control	PH		
		Total	PAH	CTEPH
N	111	64	43	21
Etiology				
- iPAH, n (%)			15 (35)	
- CTD-PAH, n (%)			17 (40)	
- CHD-PAH, n (%)			11 (25)	
Age, years old	43±13	55±17***	53±17**	58±18***
Sex, women n (%)	59 (53)	41 (64)	29 (67)	12 (57)
sBP, mmHg	123 [115-128]	127 [115-136]	122 [114-132]	133 [124-141]**,†
HR, beats·min^{-1}	68 [62-76]	78 [65-90]**	78 [67-90]**	71 [61-88]
BMI, kg·m^{-2}	23.8±2.9	28.4±6.3***	27.0±6.1***	31.4±5.7***
mPAP, mmHg	---	46.8±15.7	50.5±16.1	39.3±12.3††
PAWP, mmHg	---	12.4±5.1	11.8±5.6	13.7±3.3
PVR, WU	---	5.8 [3.3-9.8]	7.1 [5.1-11.8]	3.4 [3.0-5.3]††
CO, L·min^{-1}	---	5.0 [4.1-5.9]	4.7 [3.9-5.5]	5.4 [4.7-6.4]†
CI, L·min^{-1}·m^{-2}	---	2.6 [2.3-3.2]	2.5 [2.2-3.3]	2.7 [2.3-3.0]
6MWD, m	---	353±146	337±153	385±130
NYHA, 1:2:3:4	---	1:25:31:7	1:13:23:6	0:12:8:1

Data was present as mean ± SD, median [IQR], or numbers (percentages). **p<0.01, ***p<0.001 versus control; †p<0.05, ††p<0.01 versus PAH. Student T Test, Mann-Whitney U Test, One-way ANOVA, Kruskal-Wallis Test, or Chi-Square Test. PH: pulmonary hypertension; PAH: pulmonary arterial hypertension; CTEPH: chronic thromboembolic PH; iPAH: idiopathic PAH; CTD-PAH: connective tissues diseases associated PAH; CHD-PAH: congenital heart diseases associated PAH; sBP: systolic blood pressure; HR: heart rate; BMI: body mass index; mPAP: mean pulmonary arterial pressure; PAWP: pulmonary arterial wedge pressure; PVR: pulmonary vascular resistance; CO: cardiac output; CI: cardiac index; 6MWD: 6-minute walking distance; NYHA: New York Heart Association classification.

Table 2. Correlations between plasma levels of melatonin and baseline characteristics

	Plasma levels of melatonin							
	Control		PH		PAH		CTEPH	
	r	*p* value	*r*	*p* value	*r*	*p* value	*r*	*p* value
Baseline characteristics								
Age	-0.119	0.212	**-0.368**	**0.003**	**-0.334**	**0.029**	-0.363	0.106
Sex	-0.070	0.466	0.103	0.417	0.112	0.475	0.159	0.491
sBP	-0.178	0.063	**-0.251**	**0.046**	-0.279	0.070	-0.015	0.949
HR	**-0.229**	**0.016**	0.088	0.488	0.155	0.321	-0.182	0.430
BMI	-0.025	0.796	-0.162	0.201	-0.140	0.372	-0.018	0.938
mPAP			0.166	0.191	0.061	0.699	0.403	0.070
PAWP			-0.028	0.841	-0.039	0.820	0.178	0.509
PVR			0.094	0.518	0.097	0.584	0.091	0.737
CO			-0.184	0.160	-0.154	0.351	-0.302	0.184
CI			-0.185	0.158	-0.170	0.301	-0.339	0.133
6MWD			0.103	0.459	0.164	0.340	-0.057	0.823
NYHA			0.029	0.821	0.033	0.832	-0.084	0.717

Significant correlations are shown in bold. PH: pulmonary hypertension; PAH: pulmonary arterial hypertension; CTEPH: chronic thromboembolic PH; sBP: systolic blood pressure; HR: heart rate; BMI: body mass index; mPAP: mean pulmonary arterial pressure; PAWP: pulmonary arterial wedge pressure; PVR: pulmonary vascular resistance; CO: cardiac output; CI: cardiac index; 6MWD: 6-minute walking distance; NYHA: New York Heart Association classification.

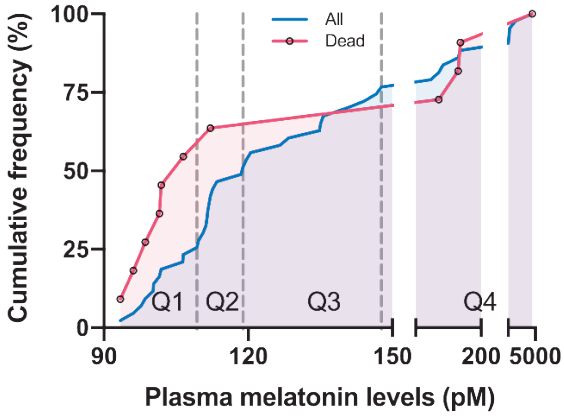


Figure 3. Distribution of mortality in PAH patients. PAH patients were stratified into 4 groups according to the quartiles of melatonin levels in PAH patients: 1st quartile (Q1) < 109.3 pM, 2nd quartile (Q2) from 109.3 to 118.9 pM, 3rd quartile (Q3) from 118.9 to 147.7 pM, 4th quartile (Q4) > 147.7 pM. The mortality per quartile was 55% (6/11), 10% (1/10), 0% (0/12), and 40% (4/10), respectively.

Logistic regression analyses

Logistic regression analysis was performed to determine whether plasma melatonin was an independent risk factor that distinguishes PH patients and controls. Before correction for the potential confounders (age, sex, and body mass index), plasma melatonin distinguished PH patients and controls (Odds Ratio 1.035 [95% CI 1.016-1.055], p<0.001), PAH patients and controls (1.036 [1.016-1.057], p<0.001), CTEPH patients and controls (1.029 [1.002-1.056], p=0.033) (Table 3).

However, after correction for potential confounders, although plasma melatonin still distinguished PH patients and controls, it only distinguished PAH patients but not CTEPH patients and controls (Table 3). These results indicated that plasma melatonin was only an independent risk factor for PAH, but not for CTEPH.

Table 3. Logistic regression analyses of plasma melatonin to distinguish PH patients and controls

		Univariate	Multivariate [#]	
			Model 1	Model 2
PH	Odds Ratio (95% CI)	1.035 (1.016-1.055)	1.048 (1.022-1.074)	1.047 (1.021-1.073)
	p value	<0.001	<0.001	<0.001
PAH	Odds Ratio (95% CI)	1.036 (1.016-1.057)	1.049 (1.022-1.076)	1.047 (1.020-1.074)
	p value	<0.001	<0.001	<0.001
CTEPH	Odds Ratio (95% CI)	1.029 (1.002-1.056)	1.025 (0.989-1.062)	1.025 (0.988-1.062)
	p value	0.033	0.175	0.184

[#]Model 1 was adjusted for age, and body mass index. Model 2 was adjusted for age, sex, and body mass index. CI: confidential interval.

Long-term survival analyses

During a median follow-up time of 42 [32-58] month, 12 patients (11 PAH patients and 1 CTEPH patient) reached the primary endpoint, the observed mortality rates were 19% (12/64) in the total PH group, 26% (11/43) in the PAH subgroup, and 5% (1/21) in the CTEPH subgroup. Long-term survival analysis was performed in the PAH subgroup.

Initially, PAH patients were stratified into 4 groups according to the quartiles of melatonin levels in the PAH subgroup: 1st quartile group < 109.3 pM, 2nd quartile group from 109.3 to 118.9 pM, 3rd quartile group from 118.9 to 147.7 pM, 4th quartile group > 147.7 pM. The mortality in these 4 groups was 55% (6/11), 10% (1/10), 0% (0/12), and 40% (4/10), respectively (Figure 3). No significant difference in the cumulative survival curves was observed among the 4 groups (Log-rank for trend, *p*=0.478, Figure 4A).

Table 4. Cox proportional hazard analysis for death per pM increase in melatonin in PAH patients

Analyses	Hazard ratio (95% CI)	*p* value
Univariate	0.995 (0.981-1.010)	0.546
Multivariate [#]		
Model 1	0.999 (0.992-1.005)	0.653
Model 2	0.998 (0.992-1.005)	0.645

[#]Model 1 was adjusted for age, and body mass index. Model 2 was adjusted for age, sex, and body mass index. CI: confidential interval.

However, patients in the 1st quartile and 4th quartile seemed to have worse survival than others. Therefore, when considering melatonin levels as continuous variable in Cox proportional hazard analysis, there was no significant association between melatonin levels and mortality, without or with adjustment for age, sex, and body mass index (Table 4).

We next undertook a two-group survival comparison based on quartiles of melatonin levels. Kaplan-Meier analyses showed that there was no significant difference between patients with melatonin levels below and above the median (118.9 pM, Log-rank, p=0.449, Figure 4B). Similarly, stratifying patients based on melatonin levels within and below the 4th quartile showed no significant difference in survival (147.7 pM, Log-rank, p=0.122, Figure 4C).

However, patients with melatonin levels in the 1st quartile (<109.3 pM) had worse long-term cumulative survival than patients with melatonin levels in the 2nd to 4th quartile (mean survival times were 46 (95% CI: 30-65) versus 68 (95% CI: 58-77) months, Log-rank, p=0.026, Figure 4D) with a significant increased hazard ratio of 3.529 (95% CI: 1.070-11.642, p=0.038).

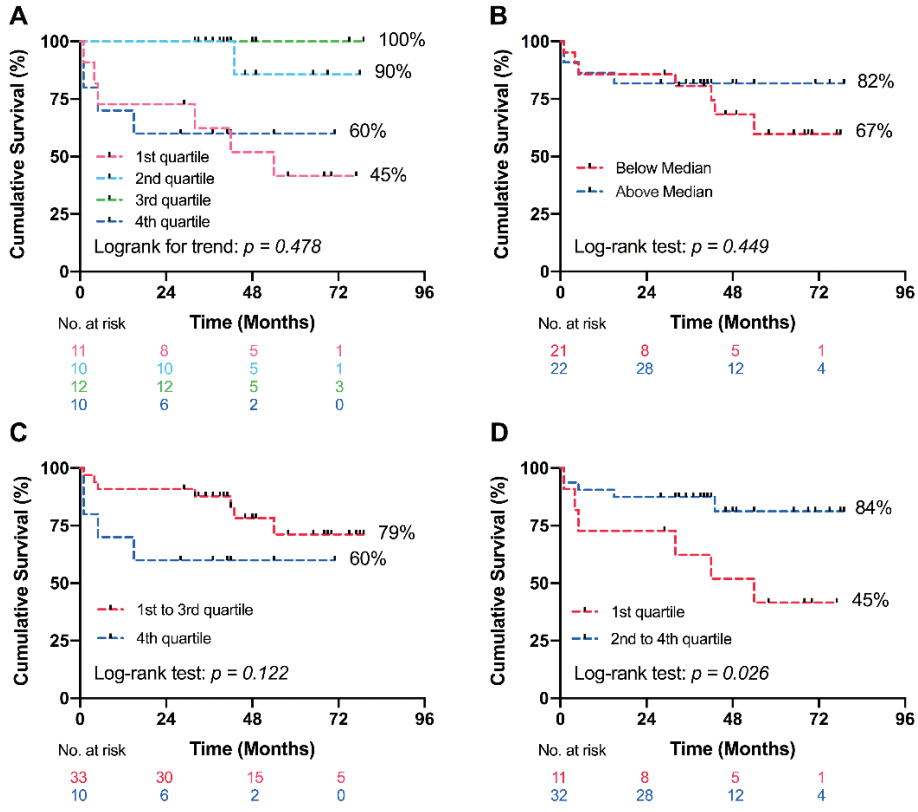


Figure 4. Long-term survival analysis in PAH patients. (A). There were no significant differences in long-term survival among 4 quartiles stratified according to melatonin levels in PAH patients. (B). There was no significant difference in long-term survival between patients with melatonin levels below and above the median (118.9 pM). (C). There was no significant difference in long-term survival between patients with melatonin levels in the 4th quartile (>147.7 pM) as compared to quartile 1-3. (D). Patients with melatonin levels in the 1st quartile (<109.3 pM) had a worse long-term cumulative survival than patients with melatonin levels in quartile 2-4.

When looking at baseline characteristics, patients with melatonin levels in the 1st quartile were older than others, while there was no difference in other characteristics (Table 5). After adjustment for age in the Cox model, the hazard ration of death for low melatonin levels was no longer significant (1.607 (95% CI:

0.402-6.426), p=0.503)), suggesting that age may be a potential confounding variable.

Table 5. Baseline characteristics in PAH patients in and above the 1st quartile of melatonin levels.

	PAH		
	1st Quartile (<109.3 pM)	Quartile 2–4 (≥109.3 pM)	*p* value
N	11	32	
Etiology			
- iPAH, *n* (%)	2 (18)	13 (41)	
- CTD-PAH, *n* (%)	6 (55)	11 (34)	
- CHD-PAH, *n* (%)	3 (27)	8 (25)	
Age, years old	66 ± 13	48 ± 15	0.001
Sex, women *n* (%)	9 (82)	20 (63)	0.213
sBP, mmHg	127 ± 14	123 ± 15	0.466
HR, beats·min $^{-1}$	79 ± 14	80 ± 18	0.965
BMI, kg·m $^{-2}$	27.1 ± 3.9	26.9 ± 6.8	0.931
mPAP, mmHg	47.0 (38.0–65.0)	45.0 (38.8–65.3)	0.880
PAWP, mmHg	13.0 ± 5.1	11.3 ± 5.8	0.450
PVR, WU	5.7 (3.9–11.4)	8.8 (5.6–11.9)	0.316
CO, L·min $^{-1}$	5.1 ± 1.5	4.8 ± 1.4	0.522
CI, L·min $^{-1}$·m $^{-2}$	2.9 ± 0.8	2.6 ± 0.7	0.312
6MWD, m	271 ± 148	356 ± 152	0.172
NYHA, 1:2:3:4	0:4:4:3	1:9:19:3	0.359

Data are presented as mean ± SD, median (IQR), or numbers (percentages). Student T Test, Mann-Whitney U Test, or chi-square test were used for comparison. PAH: pulmonary arterial hypertension; iPAH: idiopathic PAH; CTD-PAH: connective tissues diseases associated PAH; CHD-PAH: congenital heart diseases associated PAH; sBP: systolic blood pressure; HR: heart rate; BMI: body mass index; mPAP: mean pulmonary arterial pressure; PAWP: pulmonary arterial wedge pressure; PVR: pulmonary vascular resistance; CO: cardiac output; CI: cardiac index; 6MWD: 6-min walking distance; NYHA: New York Heart Association classification.

Discussion

The present study demonstrates, for the first time, that plasma melatonin levels predict clinical outcome in patients with PAH. The main findings of the study are that plasma melatonin levels were higher in treatment-naïve patients with PH than in healthy controls, which was supported by the findings in two experimental models of PH. Higher levels of melatonin were an independent risk factor of PAH in logistic regression analysis. However, lower levels of melatonin were predictive of worse long-term survival for PAH patients.

Our study demonstrates that plasma melatonin levels were higher in PH patients when compared with healthy individuals, the finding was also observed in two rat models with monocrotaline- or Sugen-hypoxia-induced PH as compared to control rats, suggesting an increase of melatonin in PH. These data seem to be in contrast with a recent study, showing that melatonin was decreased in serum from PAH patients. [12] An important difference with our study is that the cohort in the study of Zhang *et al* was small (15 PAH patients versus 8 controls), and that the patients were treated with PAH medication whereas our patients were treatment-naïve.

The pathophysiological mechanisms underlying higher levels of melatonin in treatment-naïve patients with PH versus healthy controls are unclear. It has been shown that melatonin levels decline with age in healthy humans. [26] In contrast, such a negative correlation was absent in the healthy controls in our cohort, but was present in PH patients. As PH patients, that were generally older, had even higher levels of melatonin than the younger controls, the difference between PH and controls is unlikely to be caused by the age difference. Melatonin is mainly produced by the pineal gland, [4] and is an important regulator of circadian rhythm. [27, 28] Therefore, an entire 24 h profile of melatonin levels, with knowledge sleeping patterns, is preferable to describe the melatonin levels, with samples taken under a strict light control (<10 lux) because of the strong direct suppressive effect of light

on melatonin synthesis in the pineal gland. However, melatonin production in the pineal gland, which increases at night, is stable during daytime and shows seasonal variation. [25, 29] Since we did not observe a seasonal sampling effect on melatonin levels in either healthy controls or PH patients (data not shown) and higher melatonin levels were also present in two rat models of PH, which were housed in the same facility with identical light–dark cycles, we believe that the increased melatonin represents a feature of disease.

In addition to its synthesis in the pineal gland, the enzymes that convert serotonin into melatonin, serotonin N-acetyltransferase and N-Acetylserotonin O-methyltransferase, were found to be present not only in the pineal gland but also in the plasma [30] and the lung.[31] Melatonin synthesis can be activated by the activation of the sympathetic system and the renin-angiotensin system. [32-35] PH patients and the two rat models of PH have previously been shown to exhibit increased sympathetic activity (consistent with a higher heart rate in PH patients in the present study) as well as activation of the renin-angiotensin system. [36-40] Moreover, serotonin, the precursor of melatonin, is increased in the plasma of patients with PH. [41] These may have contributed to the higher levels of melatonin in the plasma. Although there are differences in pathophysiology between PAH and CTEPH, endothelial dysfunction and pulmonary vascular remodeling are common features for both subgroups.[2] Interestingly, melatonin levels were increased in both PAH and CTEPH patients, but did not correlate with hemodynamic parameters or cardiac functional severity, as evidenced by a lack of correlation with pulmonary artery pressure, pulmonary vascular resistance, cardiac output, 6MWD, and NYHA class. Although melatonin levels were similarly elevated in patients with PAH and CTEPH, melatonin appeared only as an independent risk factor for PAH but not for CETPH in logistic regression. In addition, 11 out of 43 patients with PAH died, whereas all CTEPH patients except one survived. This might be attributed to the

fact that PAH patients showed a more severe PH phenotype with higher pulmonary vascular resistance than CTEPH patients, and indicates that melatonin is not simply a reflection of the disease severity. Importantly, in PAH patients, lower melatonin levels were associated with a worse long-term survival although age may be a confounding factor in this association. Worse survival with lower melatonin levels is in accordance with a recent study showing that lower levels of melatonin in patients with dilated cardiomyopathy correlated with a poor prognosis, worse cardiac function (lower cardiac output) and more cardiac injury (i.e., higher levels of troponin T).[6] Furthermore, several studies show cardiovascular protective effects of exogenous melatonin in both humans and animal models, [5-7, 42, 43] suggesting that higher endogenous melatonin levels may exert a protective effect in PH. Indeed, melatonin induces vasodilation, has anti-proliferative effects as well as antioxidant and anti-inflammatory properties, [11-18, 44] thereby counteracting the vasoconstriction, excessive cell proliferation, increased oxidative stress, and inflammatory infiltration characteristic of PH. [3] We therefore propose that PAH patients with endogenous melatonin in the lowest quartile may have lost the benefits of its protective effects. Importantly, a protective effect of exogenous melatonin is still present in the rat models of PH as utilized in the present study, [12, 17, 18] despite the fact that our study shows that the endogenous levels of melatonin were already increased in these models. Conversely, PAH patients with the highest levels of endogenous melatonin seemed to have a high mortality in the present study. We believe that these high endogenous melatonin levels may be attributed to the hyper-activation of sympathetic system and/or renin-angiotensin system, [32-35] which are present in severe PAH patients and therefore contribute to a poor survival. [40, 45, 46] Therefore, whether exogenous melatonin supplements may be effective as a therapeutic strategy in patients with PH remains to be established.

Conclusion

To our knowledge, this is the first prospective cohort study demonstrating that lower levels of plasma melatonin predicts worse long-term survival in treatment-naïve PAH patients, however, whether exogenous melatonin supplements may be effective as a therapeutic strategy in human PH remains to be established.

Acknowledgements

This work was supported by the China Scholarship Council (201606230252) as well as the Netherlands CardioVascular Research Initiative: an initiative with support of the Dutch Heart Foundation (CVON2014-11, RECONNECT), and German Center for Cardiovascular Research (DZHK81Z0600207). Instrumentation support was received from AB Sciex, ltd. for LC-MS/MS analyses performed in this study.

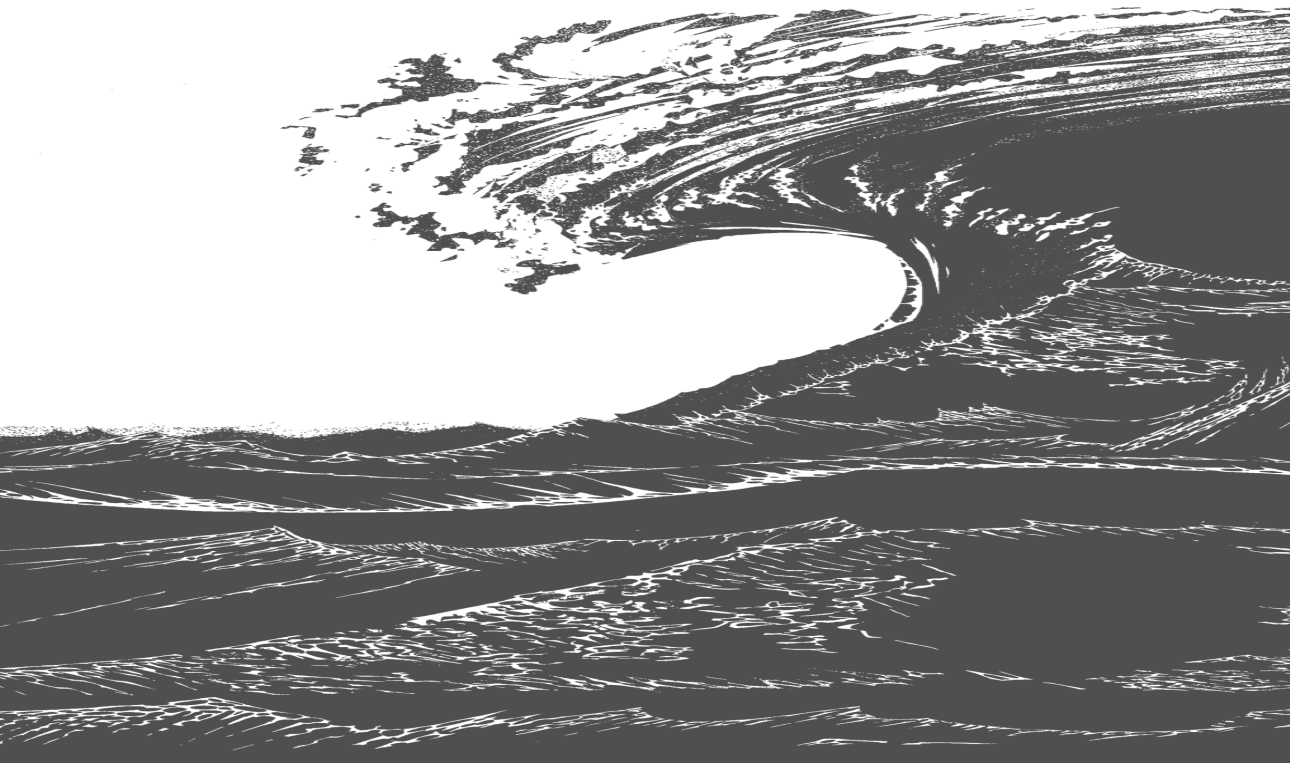
References

1. Simonneau G, Montani D, Celermajer DS, Denton CP, Gatzoulis MA, Krowka M, Williams PG and Souza R. Haemodynamic definitions and updated clinical classification of pulmonary hypertension. *The European respiratory journal*. 2019;53.
2. Humbert M. Pulmonary arterial hypertension and chronic thromboembolic pulmonary hypertension: pathophysiology. *Eur Respir Rev*. 2010;19:59-63.
3. Humbert M, Guignabert C, Bonnet S, Dorfmuller P, Klinger JR, Nicolls MR, Olschewski AJ, Pullamsetti SS, Schermuly RT, Stenmark KR and Rabinovitch M. Pathology and pathobiology of pulmonary hypertension: state of the art and research perspectives. *Eur Respir J*. 2019;53.
4. Claustrat B and Leston J. Melatonin: Physiological effects in humans. *Neurochirurgie*. 2015;61:77-84.
5. Xu L, Su Y, Zhao Y, Sheng X, Tong R, Ying X, Gao L, Ji Q, Gao Y, Yan Y, Yuan A, Wu F, Lan F and Pu J. Melatonin differentially regulates pathological and physiological cardiac hypertrophy: Crucial role of circadian nuclear receptor RORalpha signaling. *J Pineal Res*. 2019;67:e12579.
6. Misaka T, Yoshihisa A, Yokokawa T, Sato T, Oikawa M, Kobayashi A, Yamaki T, Sugimoto K, Kunii H, Nakazato K and Takeishi Y. Plasma levels of melatonin in dilated cardiomyopathy. *J Pineal Res*. 2019;66:e12564.
7. Sun H, Gusdon AM and Qu S. Effects of melatonin on cardiovascular diseases: progress in the past year. *Curr Opin Lipidol*. 2016;27:408-13.
8. Habtemariam S, Daglia M, Sureda A, Selamoglu Z, Gulhan MF and Nabavi SM. Melatonin and Respiratory Diseases: A Review. *Curr Top Med Chem*. 2017;17:467-488.
9. Moradkhani F, Moloudizargari M, Fallah M, Asghari N, Heidari Khoei H and Asghari MH. Immunoregulatory role of melatonin in cancer. *J Cell Physiol*. 2020;235:745-757.
10. Das R, Balonan L, Ballard HJ and Ho S. Chronic hypoxia inhibits the antihypertensive effect of melatonin on pulmonary artery. *International journal of cardiology*. 2008;126:340-5.
11. Jin H, Wang Y, Zhou L, Liu L, Zhang P, Deng W and Yuan Y. Melatonin attenuates hypoxic pulmonary hypertension by inhibiting the inflammation and the proliferation of pulmonary arterial smooth muscle cells. *J Pineal Res*. 2014;57:442-50.
12. Zhang J, Lu X, Liu M, Fan H, Zheng H, Zhang S, Rahman N, Wolczynski S, Kretowski A and Li X. Melatonin inhibits inflammasome-associated activation of endothelium and macrophages attenuating pulmonary arterial hypertension. *Cardiovasc Res*. 2019.
13. Hung MW, Yeung HM, Lau CF, Poon AMS, Tipoe GL and Fung ML. Melatonin Attenuates Pulmonary Hypertension in Chronically Hypoxic Rats. *Int J Mol Sci*. 2017;18.
14. Torres F, Gonzalez-Candia A, Montt C, Ebensperger G, Chubretovic M, Seron-Ferre M, Reyes RV, Llanos AJ and Herrera EA. Melatonin reduces oxidative stress and improves vascular function in pulmonary hypertensive newborn sheep. *J Pineal Res*. 2015;58:362-73.
15. Gonzalez-Candia A, Candia AA, Figueroa EG, Feixes E, Gonzalez-Candia C, Aguilar SA, Ebensperger G, Reyes RV, Llanos AJ and Herrera EA. Melatonin long-lasting beneficial effects on pulmonary vascular reactivity and redox balance in chronic hypoxic ovine neonates. *J Pineal Res*. 2020;68:e12613.
16. Astorga CR, Gonzalez-Candia A, Candia AA, Figueroa EG, Canas D, Ebensperger G, Reyes RV, Llanos AJ and Herrera EA. Melatonin Decreases Pulmonary Vascular Remodeling

and Oxygen Sensitivity in Pulmonary Hypertensive Newborn Lambs. *Front Physiol*. 2018;9:185.
17. Maarman G, Blackhurst D, Thienemann F, Blauwet L, Butrous G, Davies N, Sliwa K and Lecour S. Melatonin as a preventive and curative therapy against pulmonary hypertension. *J Pineal Res*. 2015;59:343-53.
18. Wang R, Zhou S, Wu P, Li M, Ding X, Sun L, Xu X, Zhou X, Zhou L, Cao C and Fei G. Identifying Involvement of H19-miR-675-3p-IGF1R and H19-miR-200a-PDCD4 in Treating Pulmonary Hypertension with Melatonin. *Mol Ther Nucleic Acids*. 2018;13:44-54.
19. Galie N, Hoeper MM, Humbert M, Torbicki A, Vachiery JL, Barbera JA, Beghetti M, Corris P, Gaine S, Gibbs JS, Gomez-Sanchez MA, Jondeau G, Klepetko W, Opitz C, Peacock A, Rubin L, Zellweger M, Simonneau G and Guidelines ESCCfP. Guidelines for the diagnosis and treatment of pulmonary hypertension: the Task Force for the Diagnosis and Treatment of Pulmonary Hypertension of the European Society of Cardiology (ESC) and the European Respiratory Society (ERS), endorsed by the International Society of Heart and Lung Transplantation (ISHLT). *European heart journal*. 2009;30:2493-537.
20. Hoeper MM, Bogaard HJ, Condliffe R, Frantz R, Khanna D, Kurzyna M, Langleben D, Manes A, Satoh T, Torres F, Wilkins MR and Badesch DB. Definitions and diagnosis of pulmonary hypertension. *J Am Coll Cardiol*. 2013;62:D42-50.
21. Geenen LW, Baggen VJM, Koudstaal T, Boomars KA, Eindhoven JA, Boersma E, Roos-Hesselink JW and van den Bosch AE. The prognostic value of various biomarkers in adults with pulmonary hypertension; a multi-biomarker approach. *Am Heart J*. 2019;208:91-99.
22. Menting ME, McGhie JS, Koopman LP, Vletter WB, Helbing WA, van den Bosch AE and Roos-Hesselink JW. Normal myocardial strain values using 2D speckle tracking echocardiography in healthy adults aged 20 to 72 years. *Echocardiography*. 2016;33:1665-1675.
23. Galie N, Humbert M, Vachiery JL, Gibbs S, Lang I, Torbicki A, Simonneau G, Peacock A, Vonk Noordegraaf A, Beghetti M, Ghofrani A, Gomez Sanchez MA, Hansmann G, Klepetko W, Lancellotti P, Matucci M, McDonagh T, Pierard LA, Trindade PT, Zompatori M, Hoeper M and Group ESCSD. 2015 ESC/ERS Guidelines for the diagnosis and treatment of pulmonary hypertension: The Joint Task Force for the Diagnosis and Treatment of Pulmonary Hypertension of the European Society of Cardiology (ESC) and the European Respiratory Society (ERS): Endorsed by: Association for European Paediatric and Congenital Cardiology (AEPC), International Society for Heart and Lung Transplantation (ISHLT). *Eur Heart J*. 2016;37:67-119.
24. Tu L, Desroches-Castan A, Mallet C, Guyon L, Cumont A, Phan C, Robert F, Thuillet R, Bordenave J, Sekine A, Huertas A, Ritvos O, Savale L, Feige JJ, Humbert M, Bailly S and Guignabert C. Selective BMP-9 Inhibition Partially Protects Against Experimental Pulmonary Hypertension. *Circ Res*. 2019;124:846-855.
25. Hedlund L, Lischko MM, Rollag MD and Niswender GD. Melatonin: daily cycle in plasma and cerebrospinal fluid of calves. *Science*. 1977;195:686-7.
26. Sack RL, Lewy AJ, Erb DL, Vollmer WM and Singer CM. Human melatonin production decreases with age. *J Pineal Res*. 1986;3:379-88.
27. Zisapel N. New perspectives on the role of melatonin in human sleep, circadian rhythms and their regulation. *Br J Pharmacol*. 2018;175:3190-3199.

28. Cajochen C, Krauchi K and Wirz-Justice A. Role of melatonin in the regulation of human circadian rhythms and sleep. *J Neuroendocrinol*. 2003;15:432-7.
29. Wehr TA. Melatonin and seasonal rhythms. *J Biol Rhythms*. 1997;12:518-27.
30. Backlund PS, Urbanski HF, Doll MA, Hein DW, Bozinoski M, Mason CE, Coon SL and Klein DC. Daily Rhythm in Plasma N-acetyltryptamine. *J Biol Rhythms*. 2017;32:195-211.
31. Fukuda T, Akiyama N, Ikegami M, Takahashi H, Sasaki A, Oka H, Komori T, Tanaka Y, Nakazato Y, Akimoto J, Tanaka M, Okada Y and Saito S. Expression of hydroxyindole-O-methyltransferase enzyme in the human central nervous system and in pineal parenchymal cell tumors. *J Neuropathol Exp Neurol*. 2010;69:498-510.
32. Abrahao MV, Dos Santos NFT, Kuwabara WMT, do Amaral FG, do Carmo Buonfiglio D, Peres R, Vendrame RFA, Flavio da Silveira P, Cipolla-Neto J, Baltatu OC and Afeche SC. Identification of insulin-regulated aminopeptidase (IRAP) in the rat pineal gland and the modulation of melatonin synthesis by angiotensin IV. *Brain Res*. 2019;1704:40-46.
33. Qiu J, Zhang J, Zhou Y, Li X, Li H, Liu J, Gou K, Zhao J and Cui S. MicroRNA-7 inhibits melatonin synthesis by acting as a linking molecule between leptin and norepinephrine signaling pathways in pig pineal gland. *J Pineal Res*. 2019;66:e12552.
34. Drijfhout WJ, van der Linde AG, de Vries JB, Grol CJ and Westerink BH. Microdialysis reveals dynamics of coupling between noradrenaline release and melatonin secretion in conscious rats. *Neurosci Lett*. 1996;202:185-8.
35. Schomerus C and Korf HW. Mechanisms regulating melatonin synthesis in the mammalian pineal organ. *Annals of the New York Academy of Sciences*. 2005;1057:372-83.
36. Velez-Roa S, Ciarka A, Najem B, Vachiery JL, Naeije R and van de Borne P. Increased sympathetic nerve activity in pulmonary artery hypertension. *Circulation*. 2004;110:1308-12.
37. de Man FS, Tu L, Handoko ML, Rain S, Ruiter G, Francois C, Schalij I, Dorfmuller P, Simonneau G, Fadel E, Perros F, Boonstra A, Postmus PE, van der Velden J, Vonk-Noordegraaf A, Humbert M, Eddahibi S and Guignabert C. Dysregulated renin-angiotensin-aldosterone system contributes to pulmonary arterial hypertension. *Am J Respir Crit Care Med*. 2012;186:780-9.
38. Maron BA and Leopold JA. The role of the renin-angiotensin-aldosterone system in the pathobiology of pulmonary arterial hypertension (2013 Grover Conference series). *Pulm Circ*. 2014;4:200-10.
39. Sharma RK, Oliveira AC, Kim S, Rigatto K, Zubcevic J, Rathinasabapathy A, Kumar A, Lebowitz JJ, Khoshbouei H, Lobaton G, Aquino V, Richards EM, Katovich MJ, Shenoy V and Raizada MK. Involvement of Neuroinflammation in the Pathogenesis of Monocrotaline-Induced Pulmonary Hypertension. *Hypertension*. 2018;71:1156-1163.
40. de Man FS, Handoko ML, Guignabert C, Bogaard HJ and Vonk-Noordegraaf A. Neurohormonal axis in patients with pulmonary arterial hypertension: friend or foe? *Am J Respir Crit Care Med*. 2013;187:14-9.
41. MacLean MMR. The serotonin hypothesis in pulmonary hypertension revisited: targets for novel therapies (2017 Grover Conference Series). *Pulm Circ*. 2018;8:2045894018759125.
42. Jiki Z, Lecour S and Nduhirabandi F. Cardiovascular Benefits of Dietary Melatonin: A Myth or a Reality? *Front Physiol*. 2018;9:528.

43. Tan DX, Manchester LC, Esteban-Zubero E, Zhou Z and Reiter RJ. Melatonin as a Potent and Inducible Endogenous Antioxidant: Synthesis and Metabolism. *Molecules*. 2015;20:18886-906.
44. Gonzalez-Candia A, Veliz M, Carrasco-Pozo C, Castillo RL, Cardenas JC, Ebensperger G, Reyes RV, Llanos AJ and Herrera EA. Antenatal melatonin modulates an enhanced antioxidant/pro-oxidant ratio in pulmonary hypertensive newborn sheep. *Redox Biol*. 2019;22:101128.
45. Ciarka A, Doan V, Velez-Roa S, Naeije R and van de Borne P. Prognostic significance of sympathetic nervous system activation in pulmonary arterial hypertension. *Am J Respir Crit Care Med*. 2010;181:1269-75.
46. Forfia PR, Mathai SC, Fisher MR, Housten-Harris T, Hemnes AR, Champion HC, Girgis RE and Hassoun PM. Hyponatremia predicts right heart failure and poor survival in pulmonary arterial hypertension. *Am J Respir Crit Care Med*. 2008;177:1364-9.



Chapter 6

General discussion

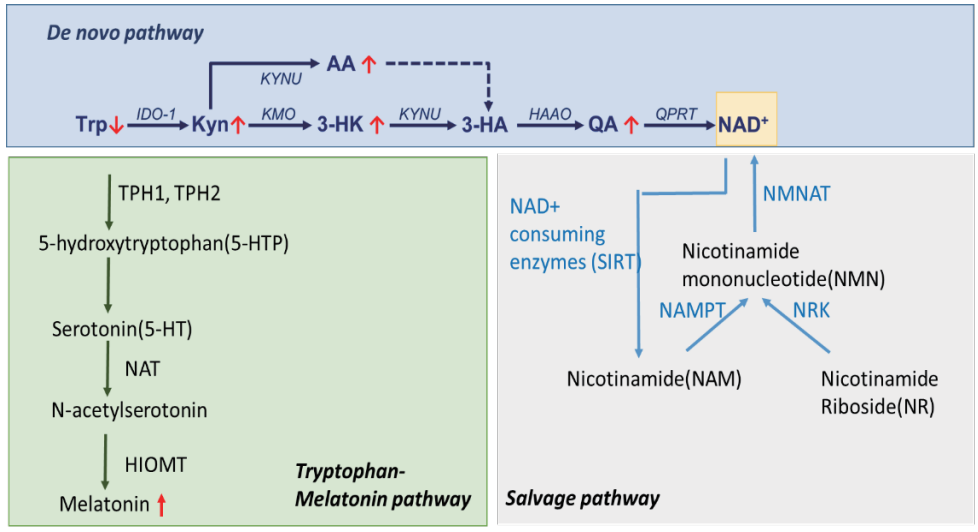


Figure 3. De novo pathway and salvage pathway of NAD$^+$ and link to tryptophan melatonin pathway. NAD$^+$ is synthesized through the de novo pathway and the salvage pathway in mammalian cells. Tryptophan is metabolized through the serotonin and melatonin. NAMPT: nicotinamide phosphoribosyl-transferase. NRK: nicotinamide riboside kinases. NMNAT: nicotinamide mononucleotide adenylyltransferase. TPH: tryptophan hydroxylase. (Adjusted from Chapter 4).

There is emerging evidence showing that IL6 signaling plays a primary role in the inflammatory of PAH. Elevated circulating levels of the pro-inflammatory cytokines IL6, have been observed in PAH patients in many studies.[48, 49] Furthermore, it was found that the membrane bound IL6R was upregulated in the smooth muscle layer of remodeled vessels in human IPAH and experimental PAH animal models, which suggests a key role for IL6R in pulmonary arterial smooth muscle cell activation in PAH.[50, 51] In **Chapter 4**, we stimulated the main three types of human lung cells (microvascular endothelial cells, smooth muscle cells, fibroblast) with the IL6/IL6Rα, which mimicked the KP metabolites profile observed in the PAH patients, suggesting that activated the IL6/IL6Rα signaling contributes to KP activation in PAH patients. Conversely, stimulation with IL6 alone failed to induce such a similar KP profile, and the other cytokines (TNF-α and TGFβ) and hypoxia also failed to induce the KP profile like the PAH patients.

Despite this indication that IL6/IL6Rα signaling is involved in pathogenesis of PAH, a phase 2 study using tocilizumab, an IL6R antagonist in PAH (TRANSFORM-UK), altering IL-6 signaling, demonstrated no overall change in pulmonary vascular resistance (PVR) through 6 months of therapy.[52, 53] Unfortunately, RV function was not assessed in TRANSFORM-UK. It is possible that the IL6R antagonist alone is insufficient to normalize the KP metabolites and hence does not change the PVR and that inhibition of IL6Rα is also necessary. Further studies into the role of KP metabolites as biomarkers for PH and into inhibition of IL6/IL6Rα are required to establish whether this pathway may provide a new therapeutic target in future studies.

Melatonin and pulmonary hypertension

Tryptophan is metabolized not only along with the kynurenine pathway but also via the serotonin pathway (Figure 3), resulting in serotonin and melatonin. Balancing both pathways has been proposed to be critical for maintaining the healthy homeostasis.[54] Melatonin is the end product of tryptophan metabolism of the serotonin pathway. Melatonin is a neurohormone with mutiple effects on vascular function and antioxidant functions.[55] Melatonin treatment reduces inflammatory factors, including TNFα, IL1β and IL6[56, 57] and prevents hypoxia induced PH in rat models by inhibiting inflammation and proliferation in pulmonary arterial smooth muscle cells.[58, 59] In 2015, Maarman et al treated rats with monocrotaline (MCT) induced PAH with melatonin showed a reduction in RV hypertrophy, improved RV function and reduced plasma oxidative stress.[60] In 2020, Zhang et al verified that melatonin treatment attenuated inflammation-associated vascular disorders by directly improving endothelial leakage and decreasing the formation of inflammatory in macrophages in PAH mice models.[61]

In the same study, they used a small cohort (15 PAH patients and 8 healthy control) to show that melatonin was lower in the serum of PAH patients.[61] Contrary to this previous study, in **Chapter 5,** we demonstrated that the plasma melatonin levels in our cohort were higher in treatment-naïve PH patients compared with healthy controls, which was supported by the findings in two experimental PH animal models. One potential explanation may be that we used a more sensitive method of determination, liquid chromatography-tandem mass spectrometry (LC-MS/MS), compared with Dr. Zhang's study which used enzyme-immunoassay.[58] Another important reason is that blood sampling in our study was conducted during a longer period (between 9.00 and 18.00), while Dr. Zhang's study took blood samples between 9.00 and 10.00. As melatonin shows a diurnal rhythm, being low in the morning, this may explain why our measurements resulted in higher melatonin levels. Despite the overall higher melatonin levels in PAH patients, in **Chapter 5**, we demonstrated that the lower levels of melatonin were predictive of worse long-term survival within our PAH cohort (43 PAH patients and 21 CTEPH patients *VS* 111 healthy controls). We therefore concluded that the higher levels of melatonin observed in treatment-naïve PAH patients which were shown in **Chapter 5**, and were also observed in some types of hypertension,[62] may be a counter-regulatory adaptive mechanism against-sympathetic overstimulation and/or endothelial dysfunction. Taken together, melatonin is certainly of interest in PAH studies but further clarification about its therapeutic potential is needed.

Future studies

Future studies, to fully understand the melatonin function in PAH, and to examine the differences in male and female PAH patients, are required as there are sex differences in the circadian rhythm regulation and up to four-fold more women than men develop PAH.[58, 63] Since melatonin has a strong diurnal rhythm, a full

24hours profile of melatonin in PAH patients and healthy controls should be measured.

Given the complex multifactorial nature of PH, no single animal model can per se completely mimic the human condition.[64] In Chapter 4, we indeed found that the KP metabolites in 2 PH animal models were different from PAH patients. For translational studies, animal models that exhibit a KP metabolite profile similar to human PAH patients should be identified and used. In such a model a preclinical trial could be performed to evaluate the effect of inhibition of the de novo NAD^+ synthesis through the KP.

3. Future perspectives

For the treatment of PH, the options are still limited to the pulmonary vascular vasodilator therapies but these do not reverse the vascular remodeling. Changes in pulmonary vascular stiffness are now recognized as early drivers of pulmonary vascular remodeling, which occurs both in the proximal and distal pulmonary arteries. Mechanotransduction pathways have been demonstrated to play crucial roles in vascular pathogenesis.[65] The mechanosensors and mechanotransduction responsible for this vascular stiffening process have become increasingly well-recognized.[66] In the future, more efforts should be directed towards further investigate the regulatory mechanisms of this vascular stiffening early process, and corresponding research and discovery of novel targets on mechanotransduction signaling as potential therapeutics is warranted.

Animal models play a critical role in studying the mechanotransduction pathways as well as investigating new therapeutic targets in PH. In PAH, pulmonary vascular remodeling is typically found at branching points where disturbed flow or altered shear stress occurs.[67] The altered shear stress leading to vascular remodeling may

also show in the animal models. However, the classic PH animal models like chronic hypoxia models and monocrotaline (MCT)-induced PH rodent models, are associated with a less severe response in the worsening of hemodynamics and are lack of typical complex vascular neointimal lesions.[67, 68] The large animal models seem to have a special interest since they more closely resemble humans and allow the identification of biomarkers (e.g., circulating biomarkers in the blood, hemodynamic biomarkers or ECG traits) to facilitate early detection of disease and/or disease progression.[69-71] Besides, large animal models enable clinically applicable interventions, including catheter-based approaches, which give us more insight into the mechanotransduction pathways involved in vascular remodeling.[69] Given the complex multifactorial nature of PH, no single animal model can completely mimic the human condition.[64] Taken together, due to inherent limitations of currently available animal models, it is recommended to develop large animal models of PH and to test interventions in different animal models, which may further help us to translate the new therapies into the clinical setting.

Biomarkers are useful tools in PH, providing crucial information regarding risk assessment, response to therapy, and prognosis.[72] The ideal biomarkers should have the following characteristics: high sensitivity and specificity, reproducibility, and easy to measure and capable of monitoring the course of PAH and its response to therapy,[73, 74] A variety of biomarkers have been explored in PAH, but only the brain natriuretic peptide (BNP) and the N-terminal fragment (NT) of pro-BNP (NT-proBNP) biomarkers are recommended by current guidelines for risk stratification and assessing response to treatment.[75, 76] PAH is a highly complex multifactorial disease, which involves many different pathogenic mechanisms.[77] Nevertheless, most studies only focused on a single biomarker, even though there is increasing evidence that a multiple biomarker approach could be more appropriate to obtain as much information as possible on the stage of disease and prognosis.[77, 78] Thus,

using a multi-biomarker approach can contribute to better risk stratification in PH and should be further investigated in larger studies with the use of continuous levels in a multivariable analysis.[79]

References:

1. Shimoda LA and Laurie SS. Vascular remodeling in pulmonary hypertension. *J Mol Med (Berl)*. 2013;91:297-309.
2. Simonneau G, Montani D, Celermajer DS, Denton CP, Gatzoulis MA, Krowka M, Williams PG and Souza R. Haemodynamic definitions and updated clinical classification of pulmonary hypertension. *Eur Respir J*. 2019;53.
3. Tuder RM. Pulmonary vascular remodeling in pulmonary hypertension. *Cell Tissue Res*. 2017;367:643-649.
4. Sommer N, Ghofrani HA, Pak O, Bonnet S, Provencher S, Sitbon O, Rosenkranz S, Hoeper MM and Kiely DG. Current and future treatments of pulmonary arterial hypertension. *Br J Pharmacol*. 2021;178:6-30.
5. Humbert M, Guignabert C, Bonnet S, Dorfmuller P, Klinger JR, Nicolls MR, Olschewski AJ, Pullamsetti SS, Schermuly RT, Stenmark KR and Rabinovitch M. Pathology and pathobiology of pulmonary hypertension: state of the art and research perspectives. *Eur Respir J*. 2019;53.
6. Roux E, Bougaran P, Dufourcq P and Couffinhal T. Fluid Shear Stress Sensing by the Endothelial Layer. *Front Physiol*. 2020;11:861.
7. Celestin C, Guillot M, Ross-Ascuitto N and Ascuitto R. Computational fluid dynamics characterization of blood flow in central aorta to pulmonary artery connections: importance of shunt angulation as a determinant of shear stress-induced thrombosis. *Pediatr Cardiol*. 2015;36:600-15.
8. Dummer A, Rol N, Szulcek R, Kurakula K, Pan X, Visser BI, Bogaard HJ, DeRuiter MC, Goumans MJ and Hierck BP. Endothelial dysfunction in pulmonary arterial hypertension: loss of cilia length regulation upon cytokine stimulation. *Pulm Circ*. 2018;8:2045894018764629.
9. Li J, Hou B, Tumova S, Muraki K, Bruns A, Ludlow MJ, Sedo A, Hyman AJ, McKeown L, Young RS, Yuldasheva NY, Majeed Y, Wilson LA, Rode B, Bailey MA, Kim HR, Fu Z, Carter DA, Bilton J, Imrie H, Ajuh P, Dear TN, Cubbon RM, Kearney MT, Prasad RK, Evans PC, Ainscough JF and Beech DJ. Piezo1 integration of vascular architecture with physiological force. *Nature*. 2014;515:279-282.
10. Wang S, Chennupati R, Kaur H, Iring A, Wettschureck N and Offermanns S. Endothelial cation channel PIEZO1 controls blood pressure by mediating flow-induced ATP release. *The Journal of clinical investigation*. 2016;126:4527-4536.
11. Albarran-Juarez J, Iring A, Wang S, Joseph S, Grimm M, Strilic B, Wettschureck N, Althoff TF and Offermanns S. Piezo1 and Gq/G11 promote endothelial inflammation depending on flow pattern and integrin activation. *J Exp Med*. 2018;215:2655-2672.
12. Liao J, Lu W, Chen Y, Duan X, Zhang C, Luo X, Lin Z, Chen J, Liu S, Yan H, Chen Y, Feng H, Zhou D, Chen X, Zhang Z, Yang Q, Liu X, Tang H, Li J, Makino A, Yuan JX, Zhong N, Yang K and Wang J. Upregulation of Piezo1 (Piezo Type Mechanosensitive Ion Channel Component 1) Enhances the Intracellular Free Calcium in Pulmonary Arterial Smooth Muscle Cells From Idiopathic Pulmonary Arterial Hypertension Patients. *Hypertension*. 2021;77:1974-1989.
13. Stenmark KR, Frid MG, Graham BB and Tuder RM. Dynamic and diverse changes in the functional properties of vascular smooth muscle cells in pulmonary hypertension. *Cardiovasc Res*. 2018;114:551-564.

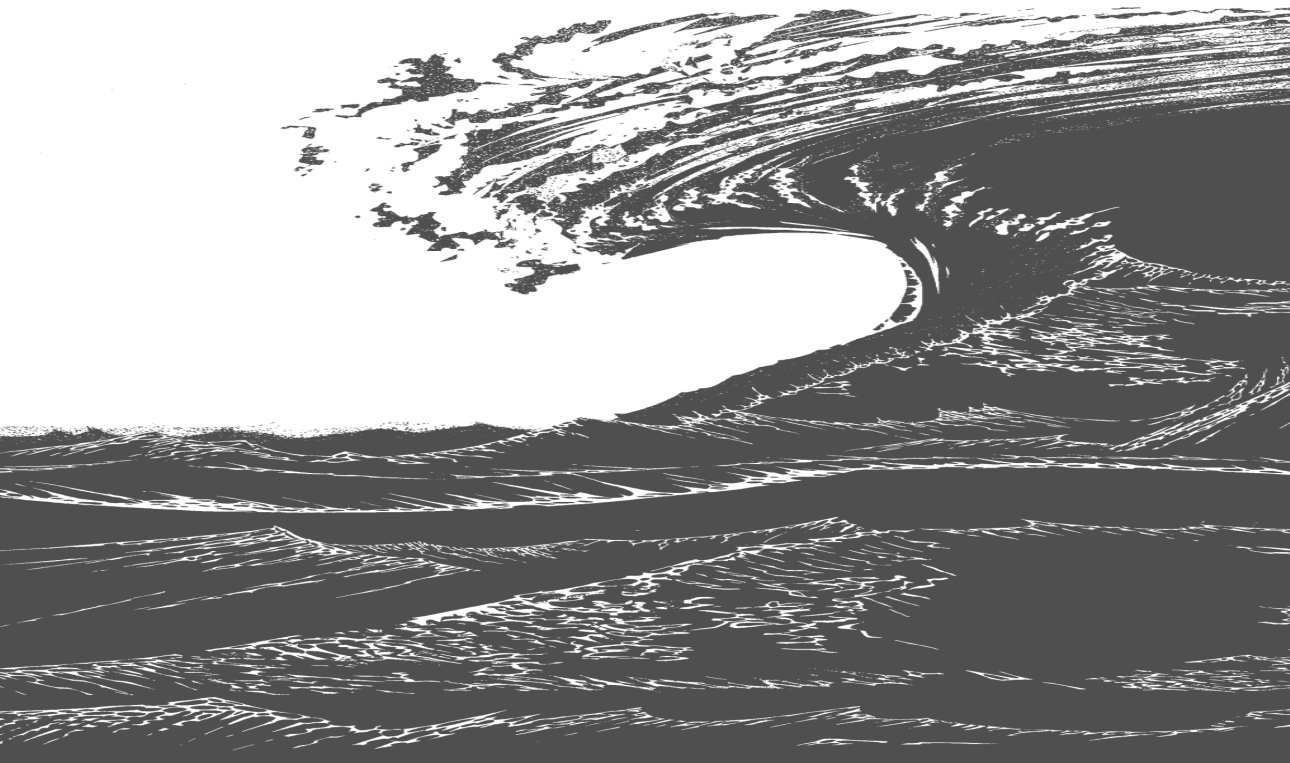
14. Lambert M, Capuano V, Olschewski A, Sabourin J, Nagaraj C, Girerd B, Weatherald J, Humbert M and Antigny F. Ion Channels in Pulmonary Hypertension: A Therapeutic Interest? *Int J Mol Sci*. 2018;19.
15. Hong J, Arneson D, Umar S, Ruffenach G, Cunningham CM, Ahn IS, Diamante G, Bhetraratana M, Park JF, Said E, Huynh C, Le T, Medzikovic L, Humbert M, Soubrier F, Montani D, Girerd B, Tregouet DA, Channick R, Saggar R, Eghbali M and Yang X. Single-Cell Study of Two Rat Models of Pulmonary Arterial Hypertension Reveals Connections to Human Pathobiology and Drug Repositioning. *American journal of respiratory and critical care medicine*. 2021;203:1006-1022.
16. Pardo-Pastor C, Rubio-Moscardo F, Vogel-Gonzalez M, Serra SA, Afthinos A, Mrkonjic S, Destaing O, Abenza JF, Fernandez-Fernandez JM, Trepat X, Albiges-Rizo C, Konstantopoulos K and Valverde MA. Piezo2 channel regulates RhoA and actin cytoskeleton to promote cell mechanobiological responses. *Proc Natl Acad Sci U S A*. 2018;115:1925-1930.
17. Yang W, Dong M, Rabinovitch M, Chan FP, Marsden AL and Feinstein JA. Evolution of hemodynamic forces in the pulmonary tree with progressively worsening pulmonary arterial hypertension in pediatric patients. *Biomech Model Mechanobiol*. 2019;18:779-796.
18. Gaikwad AV, Eapen MS, McAlinden KD, Chia C, Larby J, Myers S, Dey S, Haug G, Markos J, Glanville AR and Sohal SS. Endothelial to mesenchymal transition (EndMT) and vascular remodeling in pulmonary hypertension and idiopathic pulmonary fibrosis. *Expert Rev Respir Med*. 2020;14:1027-1043.
19. Ranchoux B, Antigny F, Rucker-Martin C, Hautefort A, Pechoux C, Bogaard HJ, Dorfmuller P, Remy S, Lecerf F, Plante S, Chat S, Fadel E, Houssaini A, Anegon I, Adnot S, Simonneau G, Humbert M, Cohen-Kaminsky S and Perros F. Endothelial-to-mesenchymal transition in pulmonary hypertension. *Circulation*. 2015;131:1006-18.
20. Gorelova A, Berman M and Al Ghouleh I. Endothelial-to-Mesenchymal Transition in Pulmonary Arterial Hypertension. *Antioxidants & redox signaling*. 2021;34:891-914.
21. Frid MG, Kale VA and Stenmark KR. Mature vascular endothelium can give rise to smooth muscle cells via endothelial-mesenchymal transdifferentiation: in vitro analysis. *Circulation research*. 2002;90:1189-96.
22. Ghigna MR, Guignabert C, Montani D, Girerd B, Jais X, Savale L, Herve P, Thomas de Montpreville V, Mercier O, Sitbon O, Soubrier F, Fadel E, Simonneau G, Humbert M and Dorfmuller P. BMPR2 mutation status influences bronchial vascular changes in pulmonary arterial hypertension. *Eur Respir J*. 2016;48:1668-1681.
23. Szulcek R, Happe CM, Rol N, Fontijn RD, Dickhoff C, Hartemink KJ, Grunberg K, Tu L, Timens W, Nossent GD, Paul MA, Leyen TA, Horrevoets AJ, de Man FS, Guignabert C, Yu PB, Vonk-Noordegraaf A, van Nieuw Amerongen GP and Bogaard HJ. Delayed Microvascular Shear Adaptation in Pulmonary Arterial Hypertension. Role of Platelet Endothelial Cell Adhesion Molecule-1 Cleavage. *Am J Respir Crit Care Med*. 2016;193:1410-20.
24. van Uden D, Koudstaal T, van Hulst JAC, Bergen IM, Gootjes C, Morrell NW, van Loo G, von der Thusen JH, van den Bosch TPP, Ghigna MR, Perros F, Montani D, Kool M, Boomars KA and Hendriks RW. Central Role of Dendritic Cells in Pulmonary Arterial Hypertension in Human and Mice. *Int J Mol Sci*. 2021;22.
25. Long L, Ormiston ML, Yang X, Southwood M, Graf S, Machado RD, Mueller M, Kinzel B, Yung LM, Wilkinson JM, Moore SD, Drake KM, Aldred MA, Yu PB, Upton PD and

Morrell NW. Selective enhancement of endothelial BMPR-II with BMP9 reverses pulmonary arterial hypertension. *Nat Med*. 2015;21:777-85.
26. Zhang B, Niu W, Dong HY, Liu ML, Luo Y and Li ZC. Hypoxia induces endothelialmesenchymal transition in pulmonary vascular remodeling. *Int J Mol Med*. 2018;42:270-278.
27. Huang J, Pu Y, Zhang H, Xie L, He L, Zhang CL, Cheng CK, Huo Y, Wan S, Chen S, Huang Y, Lau CW, Wang L, Xia Y, Huang Y and Luo JY. KLF2 Mediates the Suppressive Effect of Laminar Flow on Vascular Calcification by Inhibiting Endothelial BMP/SMAD1/5 Signaling. *Circ Res*. 2021;129:e87-e100.
28. Luo JY, Zhang Y, Wang L and Huang Y. Regulators and effectors of bone morphogenetic protein signalling in the cardiovascular system. *J Physiol*. 2015;593:2995-3011.
29. Dyer LA, Pi X and Patterson C. The role of BMPs in endothelial cell function and dysfunction. *Trends in endocrinology and metabolism: TEM*. 2014;25:472-80.
30. Yao Y, Jumabay M, Wang A and Bostrom KI. Matrix Gla protein deficiency causes arteriovenous malformations in mice. *The Journal of clinical investigation*. 2011;121:2993-3004.
31. Leibovich A, Steinbeisser H and Fainsod A. Expression of the ALK1 family of type I BMP/ADMP receptors during gastrula stages in Xenopus embryos. *Int J Dev Biol*. 2017;61:465-470.
32. Yao Y, Yao J, Radparvar M, Blazquez-Medela AM, Guihard PJ, Jumabay M and Bostrom KI. Reducing Jagged 1 and 2 levels prevents cerebral arteriovenous malformations in matrix Gla protein deficiency. *Proc Natl Acad Sci U S A*. 2013;110:19071-6.
33. Girerd B, Montani D, Coulet F, Sztrymf B, Yaici A, Jais X, Tregouet D, Reis A, Drouin-Garraud V, Fraisse A, Sitbon O, O'Callaghan DS, Simonneau G, Soubrier F and Humbert M. Clinical outcomes of pulmonary arterial hypertension in patients carrying an ACVRL1 (ALK1) mutation. *American journal of respiratory and critical care medicine*. 2010;181:851-61.
34. Urness LD, Sorensen LK and Li DY. Arteriovenous malformations in mice lacking activin receptor-like kinase-1. *Nat Genet*. 2000;26:328-31.
35. Bostrom KI, Guihard P, Blazquez Medela AM, Yao J, Moon JH, Penton A and Yao Y. Matrix Gla protein limits pulmonary arteriovenous malformations in ALK1 deficiency. *Eur Respir J*. 2015;45:849-52.
36. Roman BL and Cuttica MJ. Classifying Pulmonary Hypertension in Hereditary Hemorrhagic Telangiectasia. Hemodynamics Matter. *American journal of respiratory and critical care medicine*. 2017;196:1244-1246.
37. Yao Y, Nowak S, Yochelis A, Garfinkel A and Bostrom KI. Matrix GLA protein, an inhibitory morphogen in pulmonary vascular development. *J Biol Chem*. 2007;282:30131-42.
38. Andueza A, Kumar S, Kim J, Kang DW, Mumme HL, Perez JI, Villa-Roel N and Jo H. Endothelial Reprogramming by Disturbed Flow Revealed by Single-Cell RNA and Chromatin Accessibility Study. *Cell Rep*. 2020;33:108491.
39. Delle Vedove A, Storbeck M, Heller R, Holker I, Hebbar M, Shukla A, Magnusson O, Cirak S, Girisha KM, O'Driscoll M, Loeys B and Wirth B. Biallelic Loss of Proprioception-Related PIEZO2 Causes Muscular Atrophy with Perinatal Respiratory Distress, Arthrogryposis, and Scoliosis. *Am J Hum Genet*. 2016;99:1406-1408.

40. Nonomura K, Woo SH, Chang RB, Gillich A, Qiu Z, Francisco AG, Ranade SS, Liberles SD and Patapoutian A. Piezo2 senses airway stretch and mediates lung inflation-induced apnoea. *Nature*. 2017;541:176-181.
41. Chesler AT, Szczot M, Bharucha-Goebel D, Ceko M, Donkervoort S, Laubacher C, Hayes LH, Alter K, Zampieri C, Stanley C, Innes AM, Mah JK, Grosmann CM, Bradley N, Nguyen D, Foley AR, Le Pichon CE and Bonnemann CG. The Role of PIEZO2 in Human Mechanosensation. *N Engl J Med*. 2016;375:1355-1364.
42. Dubin AE, Schmidt M, Mathur J, Petrus MJ, Xiao B, Coste B and Patapoutian A. Inflammatory signals enhance piezo2-mediated mechanosensitive currents. *Cell Rep*. 2012;2:511-7.
43. Guihard PJ, Guo Y, Wu X, Zhang L, Yao J, Jumabay M, Yao Y, Garfinkel A and Bostrom KI. Shaping Waves of Bone Morphogenetic Protein Inhibition During Vascular Growth. *Circ Res*. 2020;127:1288-1305.
44. Jasiewicz M, Moniuszko M, Pawlak D, Knapp M, Rusak M, Kazimierczyk R, Musial WJ, Dabrowska M and Kaminski KA. Activity of the kynurenine pathway and its interplay with immunity in patients with pulmonary arterial hypertension. *Heart*. 2016;102:230-7.
45. Pawlak K, Domaniewski T, Mysliwiec M and Pawlak D. The kynurenines are associated with oxidative stress, inflammation and the prevalence of cardiovascular disease in patients with end-stage renal disease. *Atherosclerosis*. 2009;204:309-14.
46. Castro-Portuguez R and Sutphin GL. Kynurenine pathway, NAD(+) synthesis, and mitochondrial function: Targeting tryptophan metabolism to promote longevity and healthspan. *Exp Gerontol*. 2020;132:110841.
47. Heng B, Lim CK, Lovejoy DB, Bessede A, Gluch L and Guillemin GJ. Understanding the role of the kynurenine pathway in human breast cancer immunobiology. *Oncotarget*. 2016;7:6506-20.
48. Humbert M, Monti G, Brenot F, Sitbon O, Portier A, Grangeot-Keros L, Duroux P, Galanaud P, Simonneau G and Emilie D. Increased interleukin-1 and interleukin-6 serum concentrations in severe primary pulmonary hypertension. *American journal of respiratory and critical care medicine*. 1995;151:1628-31.
49. Cracowski JL, Chabot F, Labarere J, Faure P, Degano B, Schwebel C, Chaouat A, Reynaud-Gaubert M, Cracowski C, Sitbon O, Yaici A, Simonneau G and Humbert M. Proinflammatory cytokine levels are linked to death in pulmonary arterial hypertension. *The European respiratory journal*. 2014;43:915-7.
50. Tamura Y, Phan C, Tu L, Le Hiress M, Thuillet R, Jutant EM, Fadel E, Savale L, Huertas A, Humbert M and Guignabert C. Ectopic upregulation of membrane-bound IL6R drives vascular remodeling in pulmonary arterial hypertension. *J Clin Invest*. 2018;128:1956-1970.
51. Pullamsetti SS, Seeger W and Savai R. Classical IL-6 signaling: a promising therapeutic target for pulmonary arterial hypertension. *The Journal of clinical investigation*. 2018;128:1720-1723.
52. Toshner M and Rothman A. IL-6 in pulmonary hypertension: why novel is not always best. *The European respiratory journal*. 2020;55.
53. Hernandez-Sanchez J, Harlow L, Church C, Gaine S, Knightbridge E, Bunclark K, Gor D, Bedding A, Morrell N, Corris P and Toshner M. Clinical trial protocol for TRANSFORM-UK: A therapeutic open-label study of tocilizumab in the treatment of pulmonary arterial hypertension. *Pulm Circ*. 2018;8:2045893217735820.

54. Li Y, Hu N, Yang D, Oxenkrug G and Yang Q. Regulating the balance between the kynurenine and serotonin pathways of tryptophan metabolism. *FEBS J*. 2017;284:948-966.
55. Astorga CR, Gonzalez-Candia A, Candia AA, Figueroa EG, Canas D, Ebensperger G, Reyes RV, Llanos AJ and Herrera EA. Melatonin Decreases Pulmonary Vascular Remodeling and Oxygen Sensitivity in Pulmonary Hypertensive Newborn Lambs. *Front Physiol*. 2018;9:185.
56. Paredes SD, Marchena AM, Bejarano I, Espino J, Barriga C, Rial RV, Reiter RJ and Rodriguez AB. Melatonin and tryptophan affect the activity-rest rhythm, core and peripheral temperatures, and interleukin levels in the ringdove: changes with age. *J Gerontol A Biol Sci Med Sci*. 2009;64:340-50.
57. Rodriguez MI, Escames G, Lopez LC, Lopez A, Garcia JA, Ortiz F and Acuna-Castroviejo D. Chronic melatonin treatment reduces the age-dependent inflammatory process in senescence-accelerated mice. *J Pineal Res*. 2007;42:272-9.
58. MacLean MR. Melatonin: shining some light on pulmonary hypertension. *Cardiovascular research*. 2020;116:2036-2037.
59. Jin H, Wang Y, Zhou L, Liu L, Zhang P, Deng W and Yuan Y. Melatonin attenuates hypoxic pulmonary hypertension by inhibiting the inflammation and the proliferation of pulmonary arterial smooth muscle cells. *J Pineal Res*. 2014;57:442-50.
60. Maarman G, Blackhurst D, Thienemann F, Blauwet L, Butrous G, Davies N, Sliwa K and Lecour S. Melatonin as a preventive and curative therapy against pulmonary hypertension. *J Pineal Res*. 2015;59:343-53.
61. Zhang J, Lu X, Liu M, Fan H, Zheng H, Zhang S, Rahman N, Wolczynski S, Kretowski A and Li X. Melatonin inhibits inflammasome-associated activation of endothelium and macrophages attenuating pulmonary arterial hypertension. *Cardiovasc Res*. 2020;116:2156-2169.
62. Pechanova O, Paulis L and Simko F. Peripheral and central effects of melatonin on blood pressure regulation. *Int J Mol Sci*. 2014;15:17920-37.
63. Santhi N, Lazar AS, McCabe PJ, Lo JC, Groeger JA and Dijk DJ. Sex differences in the circadian regulation of sleep and waking cognition in humans. *Proc Natl Acad Sci U S A*. 2016;113:E2730-9.
64. Boucherat O, Agrawal V, Lawrie A and Bonnet S. The Latest in Animal Models of Pulmonary Hypertension and Right Ventricular Failure. *Circ Res*. 2022;130:1466-1486.
65. Lin C, Zheng X, Lin S, Zhang Y, Wu J and Li Y. Mechanotransduction Regulates the Interplays Between Alveolar Epithelial and Vascular Endothelial Cells in Lung. *Front Physiol*. 2022;13:818394.
66. Dieffenbach PB, Maracle M, Tschumperlin DJ and Fredenburgh LE. Mechanobiological Feedback in Pulmonary Vascular Disease. *Front Physiol*. 2018;9:951.
67. Dickinson MG, Bartelds B, Borgdorff MA and Berger RM. The role of disturbed blood flow in the development of pulmonary arterial hypertension: lessons from preclinical animal models. *Am J Physiol Lung Cell Mol Physiol*. 2013;305:L1-14.
68. Sztuka K and Jasinska-Stroschein M. Animal models of pulmonary arterial hypertension: A systematic review and meta-analysis of data from 6126 animals. *Pharmacol Res*. 2017;125:201-214.
69. Stam K, Clauss S, Taverne Y and Merkus D. Chronic Thromboembolic Pulmonary Hypertension - What Have We Learned From Large Animal Models. *Front Cardiovasc Med*. 2021;8:574360.

70. Cai Z, Klein T, Geenen LW, Tu L, Tian S, van den Bosch AE, de Rijke YB, Reiss IKM, Boersma E, Duncker DJ, Boomars KA, Guignabert C and Merkus D. Lower Plasma Melatonin Levels Predict Worse Long-Term Survival in Pulmonary Arterial Hypertension. *J Clin Med*. 2020;9.
71. Stam K, van Duin RWB, Uitterdijk A, Cai Z, Duncker DJ and Merkus D. Exercise facilitates early recognition of cardiac and vascular remodeling in chronic thromboembolic pulmonary hypertension in swine. *Am J Physiol Heart Circ Physiol*. 2018;314:H627-H642.
72. Marra AM, Bossone E, Salzano A, D'Assante R, Monaco F, Ferrara F, Arcopinto M, Vriz O, Suzuki T and Cittadini A. Biomarkers in Pulmonary Hypertension. *Heart Fail Clin*. 2018;14:393-402.
73. Warwick G, Thomas PS and Yates DH. Biomarkers in pulmonary hypertension. *Eur Respir J*. 2008;32:503-12.
74. Rosenthal JL and Jacob MS. Biomarkers in pulmonary arterial hypertension. *Curr Heart Fail Rep*. 2014;11:477-84.
75. Galie N, Humbert M, Vachiery JL, Gibbs S, Lang I, Torbicki A, Simonneau G, Peacock A, Vonk Noordegraaf A, Beghetti M, Ghofrani A, Gomez Sanchez MA, Hansmann G, Klepetko W, Lancellotti P, Matucci M, McDonagh T, Pierard LA, Trindade PT, Zompatori M and Hoeper M. 2015 ESC/ERS Guidelines for the diagnosis and treatment of pulmonary hypertension: The Joint Task Force for the Diagnosis and Treatment of Pulmonary Hypertension of the European Society of Cardiology (ESC) and the European Respiratory Society (ERS): Endorsed by: Association for European Paediatric and Congenital Cardiology (AEPC), International Society for Heart and Lung Transplantation (ISHLT). *Eur Respir J*. 2015;46:903-75.
76. Swaminathan AC, Zhu H, Tapson V, Lokhnygina Y, Poms A, Kelleher Z, Gaspard E, Kennedy K, Fee BE, Fortin T, Mason SN, Parikh K and McMahon TJ. Treatment-related biomarkers in pulmonary hypertension patients on oral therapies. *Respir Res*. 2020;21:304.
77. Pezzuto B, Badagliacca R, Poscia R, Ghio S, D'Alto M, Vitulo P, Mule M, Albera C, Volterrani M, Fedele F and Vizza CD. Circulating biomarkers in pulmonary arterial hypertension: update and future direction. *J Heart Lung Transplant*. 2015;34:282-305.
78. Cracowski JL and Leuchte HH. The potential of biomarkers in pulmonary arterial hypertension. *Am J Cardiol*. 2012;110:32S-38S.
79. Geenen LW, Baggen VJM, Koudstaal T, Boomars KA, Eindhoven JA, Boersma E, Roos-Hesselink JW and van den Bosch AE. The prognostic value of various biomarkers in adults with pulmonary hypertension; a multi-biomarker approach. *Am Heart J*. 2019;208:91-99.



Chapter 7

Summary
Samenvatting

Summary

Pulmonary hypertension (PH) is a disease of the pulmonary vascular system. PH is clinically defined as a pulmonary arterial pressure above 20 mmHg at rest. Greater pulmonary vascular resistance causes tremendous increase in afterload of the right ventricle, ultimately leading to failure of the right ventricle and death. PH is classified into 5 groups based on the pathology 1) pulmonary arterial hypertension (PAH), including idiopathic, heritable and drug/toxin-induced PH; 2) PH due to left heart disease; 3) PH due to interstitial lung diseases and/or hypoxia, including chronic obstructive pulmonary disease (COPD); 4) chronic thromboembolic PH (CTEPH); and 5) PH with unclear and/or multifactorial origin. The current PH treatment options principally focus on reducing pulmonary vascular resistance by inducing pulmonary vasodilation and a general inhibition of cell proliferation/mitogenic properties, but do not specifically target the altered pulmonary vessel.

Several factors may contribute to pulmonary vascular remodeling including altered shear stress, dysregulation in mechanotransduction signaling and a different metabolite profile. Yet, given the lack of targeted treatment of pulmonary vascular remodeling, there is an urgent need to better identify pathological mechanisms of pulmonary vascular remodeling to provide novel targets for the treatment of PH. Allowing reversal of pulmonary arterial lumen narrowing, perivascular inflammation and metabolite dysfunction. Consequently, this thesis aimed to elucidate the mechanism of pulmonary vascular remodeling and to identify possible targets as a fundament for novel treatment of PH patients.

In Part 1 **(Chapter 2-3)**, summarized the role of mechanotransduction in development and progression of PH and specifically investigated the

mechanotransduction channel Piezo2 and the involvement in PH. In Part 2 **(Chapter 4-5)**, a cohort of PH patients with long-term follow-up was analyzed to investigate metabolites of tryptophan through the kynurenine pathway (KP) and determine the possible prognostic value.

In **Chapter 2**, we summarized current knowledge about altered shear stress in the pulmonary circulation in PH. Abnormally low shear stress is found in the main pulmonary artery and proximal arteries in PAH patients. We also summarized multiple pathways activated by unidirectional as well as altered shear stress on the endothelial cells. Furthermore, the altered crosstalk between mechanical force and endothelial cell dysfunction, impacting the pulmonary vascular smooth muscle cells and thereby inducing media thickening, was also reviewed in Chapter 2.

Subsequently, in **Chapter 3** we went on to investigate mechanosensitive channels in our animal models of pulmonary hypertension. We found altered expression of Piezo2 in the lung. Whether the altered shear stress on mechanosensitive Piezo2 channels contribute to plays a role in physiological regulation of blood flow modulating the morphology of the pulmonary vasculature in favour of PH is investigated in Chapter 3. It is likely that reduced Piezo2, either through impaired NO production in the MVECs, with subsequent reduction in cGMP-mediated inhibition of smooth muscle cell proliferation or through direct activation of EndMT, plays a central role in this pulmonary vascular remodeling. However, future studies identifying the exact molecular mechanisms by which Piezo2 induces vascular remodeling are required, to further substantiate the importance and therapeutic potential of targeting Piezo2 in PH.

In **Chapter 4** We demonstrated that several tryptophan metabolizing pathways are altered in PAH and tryptophan metabolites may potentially be used as biomarkers

for disease progression. We also found that the KP metabolites reflect disease severity in PAH patients. We then went on to investigate which (inflammatory) triggers may be responsible for altered KP metabolites in PAH, in vitro. In Chapter 4, we stimulated the main three types present in human lung tissue(microvascular endothelial cells, smooth muscle cells, fibroblast) with the IL6/IL6Rα. This mimicked the KP metabolites profile observed in the PAH patients, suggesting that activated the IL6/IL6Rα signaling contributes to KP metabolism activation in PAH patients.

Although animal studies suggest that exogenous melatonin might be beneficial for patients with PH, endogenous melatonin levels in animal models of PH, and in treatment-naïve patients with PH and their clinical significance are still unknown. In **Chapter 5**, we demonstrated that the lower levels of melatonin were predictive of worse long-term survival within our PAH cohort (43 PAH patients and 21 CTEPH patients VS 111 healthy controls), which was supported by the findings in two experimental PH animal models. Higher levels of melatonin were an independent risk factor of PAH in logistic regression analysis. However, lower levels of melatonin were predictive of worse long-term survival for PAH patients. Taken together, melatonin is certainly of interest in PAH studies but further clarification about its therapeutic potential is needed.

Future studies, to fully understand the melatonin function in PAH are needed and to since melatonin has a strong diurnal rhythm, a full 24hours profile of melatonin in PAH patients and healthy controls should be obtained. PAH is more prevalent in females compared to males and to highlight sex differences both sexes should be included in the research, in the circadian rhythm regulation and up to four-fold more women than men develop PAH. Since Besides, MGP as a potential downstream pathway of Piezo2 may also help us to better understand Piezo2's

function in pulmonary circulation and to contribute to identifying novel therapeutic strategies for the stimulation treatment of PH.

Nederlandse Samenvatting

Pulmonale hypertensie (PH) is een complexe ziekte die gekenmerkt wordt door een verhoogde pulmonale arteriële druk groter of gelijk aan 20 mmHg, gemeten in rust tijdens katheterisatie van het rechter hart. Deze verhoogde druk gaat vaak gepaard met structurele veranderingen in het longvaatbed (vasculaire remodelling), die zorgen voor een toename in de vaatweerstand van het pulmonale vaatbed. Deze continue hoge vaatweerstand doet de afterload van het rechter ventrikel (RV) stijgen en vraagt zo meer pompkracht om voldoende bloed de pulmonale circulatie in te pompen. Het RV gaat compenseren tegen deze verhoogde afterload door een toe te nemen in spiermassa (hypertrofie), dit zal geruime tijd onopgemerkt blijven tot het hart niet meer op kan tegen de hoge vaatweerstand in het longvaatbed en faalt. PH wordt op basis van de pathologie in 5 groepen ingedeeld 1) pulmonale arteriële hypertensie (PAH), waaronder idiopathische, erfelijke en door geneesmiddelen/toxinen veroorzaakte PH; 2) PH ten gevolge van linker hart aandoeningen; 3) PH ten gevolge van interstitiële longaandoeningen en/of hypoxie, waaronder chronisch obstructieve longaandoeningen (COPD); 4) chronische trombo-embolische PH (CTEPH); en 5) PH met onduidelijke en/of multifactoriële oorsprong.

De huidige PH-behandelingen richten zich voornamelijk op het verlagen van de pulmonale vaatweerstand door het induceren van pulmonale vasodilatatie. Daarnaast wordt er systemisch proliferatie/mitogene eigenschappen geremd, maar er wordt niet specifiek aangegrepen op de structurele veranderingen in het pulmonale vaatbed. Pulmonale vasculaire remodelling is gekenmerkt door proliferatie van pulmonale vasculaire cellen, verdikte tunica intima en/of media, toename van gladde spiercellen rondom het bloedvat en endotheel cellen met een gen-expressie profiel van gladde spiercellen. Verschillende factoren kunnen bijdragen aan het ontstaan van deze structurele veranderingen, waaronder

veranderde schuifspanning (shear stress), inflammatie, ontregeling in mechanotransductiesignalering en een afwijkend metabolisme. Om de prognose van patiënten te verbeteren en het ontwikkelen van rechter hartfalen te vertragen is er een beter begrip van de pathologische mechanismen nodig om zo nieuwe doelwitten te identificeren voor behandeling. Dit proefschrift is dan ook gericht op het onderzoeken van de pathofysiologie van PH en specifiek in te gaan op de vasculaire remodelling van het pulmonale vaatbed. Het ontrafelen van de beginselen die ten grondslag liggen aan het ontstaan en verloop van PH kunnen, kunnen mogelijkheden bieden om vasculaire remodelling tegen te gaan of zelfs om te keren.

In **Deel 1 (Hoofdstuk 2-3)** ligt de focus op de hemodynamische veranderingen in het longvaatbed van PH-patiënten en mechanotransductiekanalen. Endotheelcellen worden direct blootgesteld aan abnormale druk. Mechanotransductiekanalen regeren op deze veranderingen en spelen potentieel een rol in de ontwikkeling en progressie van PH, Piezo2 is uitgebreid onderzocht.
In **Deel 2 (Hoofdstuk 4-5)** is een cohort van PH-patiënten met langetermijnfollow-up geanalyseerd om de metabolieten die tryptofaan via de kynurenineroute (KP) omzetten in kaart te brengen en de mogelijke prognostische waarde te bepalen.

In **Hoofdstuk 2** is er uitgebreid onderzoek verricht naar shear stress in de longcirculatie bij PH-patiënten. Abnormaal lage shear stress wordt gevonden in de belangrijkste longslagader en proximale slagaders bij PAH-patiënten. Als reactie hierop worden verschillende cellulaire processen geactiveerd die mogelijk een rol spelen in de structurele vasculaire verandering van het pulmonale vaatbed. De interactie tussen mechanische krachten en endotheelcellen hebben ook hun uitwerking op de omliggende gladde spiercellen, die prolifereren en zo bijdragen aan een verdikte tunica media.

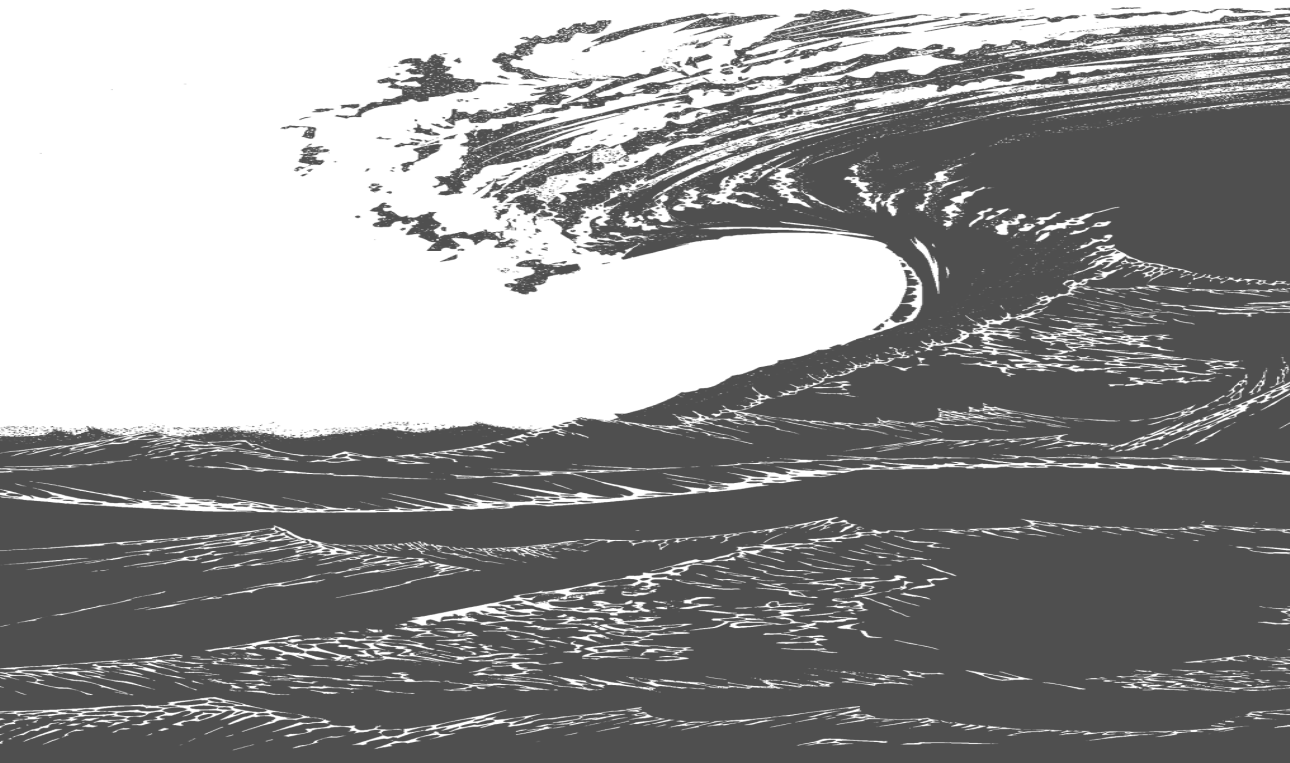
Vervolgens zoomde we in **Hoofdstuk 3** verder in op de rol van mechanosensitieve kanalen in diermodellen met pulmonale hypertensie. We vonden veranderde expressie van Piezo2 in long, deze mechanisch gevoelige ionenkanalen zijn permeabel voor kationen en worden geactiveerd door de shear stress op endotheelcellen. Het is al aangetoond dat Piezo1-activering als reactie op veranderingen in het stroompatroon resulteert in activering van het immuunsysteem en veranderde NO-productie in zowel systemische als pulmonale endotheelcellen. Wij vonden een verminderde Piezo2 expressie in de microvasculaire endotheelcellen in het distale longvaatbed. Mogelijk door een verminderde NO-productie, met daaropvolgende vermindering van cGMP-gemedieerde remming van gladde spiercelproliferatie, of door directe activering van een proces waarbij endotheelcellen transformeren naar mesenchymale cellen(EndMT). EndMT lijkt een centrale rol te spelen in pulmonale vasculaire remodellering. Toekomstige studies zullen de exacte moleculaire mechanismes moeten onderzoeken en zo de therapeutische mogelijkheden van Piezo2 te bestuderen.

In **Hoofdstuk 4** hebben wij aangetoond dat verschillende tryptofaan-metaboliserende routes veranderd zijn bij PAH-patiënten, de concentratie van tryptofaan-metabolieten kan gedetecteerd worden in serum en potentieel dienen als biomarker voor ziekteprogressie. In de thesis hebben we aangetoond dat de KP-metabolieten de ernst van de ziekte bij PAH-patiënten weerspiegelen. Vervolgens onderzochten we in vitro, welke (inflammatoire) triggers verantwoordelijk kunnen zijn voor veranderde KP-metabolieten profiel in PAH. De drie belangrijkste celtypen die aanwezig zijn in humaan longweefsel zijn; microvasculaire endotheelcellen, gladde spiercellen en fibroblasten, deze werden blootgesteld aan IL6/IL6Rα. Dit bootste het KP-metabolietenprofiel na dat werd waargenomen bij PAH-patiënten, wat suggereert dat geactiveerde IL6/IL6Rα-signalering bijdraagt aan KP-activering

bij PAH-patiënten. Omgekeerd kon stimulatie met IL6 alleen niet een dergelijk vergelijkbaar KP-profiel induceren, en de andere cytokinen (TNF-α en TGFβ) en hypoxie slaagden er ook niet in om het KP-profiel te induceren zoals de PAH-patiënten.

Tryptofaan wordt niet enkel door kynurenine gemetaboliseerd maar ook via serotonine, waarbij melatonine het eindproduct is. Hoewel dierstudies erop wijzen dat exogeen melatonine gunstig zou kunnen zijn voor patiënten met PH, zijn de endogene melatoninespiegels in diermodellen van PH en bij patiënten met PH die nog geen behandeling hebben gehad en hun klinische betekenis nog onbekend. Binnen ons PAH-cohort (43 PAH-patiënten en 21 CTEPH-patiënten versus 111 gezonde controles), hebben wij aangetoond dat de lagere niveaus van melatonine voorspellend waren voor een slechtere langetermijnoverleving. Dit werd ondersteund door de bevindingen in twee experimentele PH diermodellen In **Hoofdstuk 5**. Hogere niveaus van melatonine waren een onafhankelijke risicofactor van PAH, in logistische regressie analyse. Alles bij elkaar genomen is melatonine zeker van belang in PAH-onderzoeken, maar er is meer opheldering nodig over het therapeutisch potentieel ervan.

Toekomstige studies zijn nodig om de melatoninefunctie bij PAH volledig te begrijpen en aangezien melatonine een sterk circadiaans ritme heeft, moet een volledig 24-uurs profiel van melatonine bij PAH-patiënten en gezonde controles worden verkregen. De prevalentie van PAH is hoger onder vrouwen en daarom is het belangrijk dat beide sekse onderzocht worden. Bovendien kan MGP als een potentiële stroomafwaartse route van Piezo2 ons ook helpen om de functie van Piezo2 in de longcirculatie beter te begrijpen en bij te dragen aan het identificeren van nieuwe therapeutische strategieën voor de behandeling van PH.



Chapter 8

Appendix

PhD portfolio

List of publications

Acknowledgement

About the author

PhD training portfolio

PhD candidate Name: Siyu Tian

Department: Cardiology

Research School: Cardiovascular Research School (COEUR)

Promotor: Prof. dr. D. Merkus

Prof. dr. D.J. Duncker

Courses and Seminars	Year	ECTS
Advanced English spoken	2018	3.0
Science exchange day	2018	0.2
Create your career	2018	0.5
Animal training course: mouse handling	2018	2.0
Systemic review course	2018	1.0
Scientific integrity	2019	0.3
NHF course: Vascular Biology	2019	2.0
COEUR course – Pulmonary hypertension	2019	0.5
COEUR symposium – Biomechanics in cardiovascular disease	2019	0.1
COEUR PhD day	2019	0.3
Confocal Microscopy Introduction Course Leica SP5 part 1 and 2	2019	0.3
Confocal Microscopy Introduction Course Zeiss part 1 and 2	2019	0.3
Biomedical English writing	2020	2.0
The personal leadership &communication	2021	1.0
COEUR course – Pulmonary hypertension	2021	0.5
JMCC-ISHR Cardiovascular Webinar lectures	2021	0.5

Conferences	Year	ECTS
Dutch vascular biology meeting (poster)	2017	1.1
NHF course symposium (poster)	2019	2.0
Frontiers in Cardiovascular Biology (poster) postpone	2020	2.0
ESC congress online	2020	0.9
International cell culture under flow meeting (poster)	2020	1.1
International symposium on Biomechanics in Vascular Biology and Cardiovascular Disease (presentation)	2022	1.1
The 6th translational cardiovascular research meeting(poster)	2022	1.1
Monthly Cardiovascular Research Meeting	2020-2022	2.0
Weekly Cardiology journal club	2018-2022	2.0

Teaching activities	Year	ECTS
Supervising master internship (Rowan van Heiningen)	2021	1.1

Total ECTS: 31.2

List of publications

1. **Siyu Tian**; Zongye Cai; Payel Sen; Denise van Uden; Esther van de Kamp; Karin Boomars; Kim Van der Heiden; Maarten M. Brandt; Daphne Merkus. Loss of lung microvascular endothelial Piezo2 expression impairs NO synthesis, induces EndMT and is associated with development of pulmonary hypertension. Submit to AJP-heart and circulation.

2. Zongye Cai; **Siyu Tian**; Theo Klein; Ly Tu, et al… Christophe Guignabert; Daphne Merkus. Kynurenine Metabolites Predict Survival in Pulmonary Arterial Hypertension: A role for IL-6/IL-6Rα. Received by Scientific Reports.

3. **Siyu Tian**; Maarten M. Brandt; Daphne Merkus. Abnormal shear stress and BMP signaling in Pulmonary Hypertension. Ready to submit.

4. **Siyu Tian**; Jarno J. Steenhorst; Kim Van der Heiden; Daphne Merkus. Mechanosensing and Mechanotransduction in Pulmonary Hypertension. Chapter of book "Vascular Mechanobiology in Physiology and Disease" 2021.

5. Zongye Cai; Theo Klein; Laurie W Geenen; Ly Tu; **Siyu Tian** et al…Christophe Guignabert; Daphne Merkus. Lower Plasma Melatonin Levels Predict Worse Long-Term Survival in Pulmonary Arterial Hypertension. Journal of Clinical Medicine 2020 9(5):1248.

6. Yanming Tian; **Siyu Tian**; Dong Wang et al… Yi Zhang. Elevated expression of the leptin receptor ob-R may contribute to inflammation in patients with ulcerative colitis. Molecular Medicine Reports 2019.20(5).

7. Fang Yuan; Li Zhang; Yan-Qing Li; Xu Teng; **Si-Yu Tian**; Xiao-Ran Wang; Yi Zhang. Chronic Intermittent Hypobaric Hypoxia Improves Cardiac Function through Inhibition of Endoplasmic Reticulum Stress. Scientific Reports 2017. 7(1)

8. Hui-Jie Ma; **Si-Yu Tian**; Shuo Gu et al… Yi Zhang. Amelioration of Chronic Intermittent Hypobaric Hypoxia on Decreased L-type Ca2+ Currents by Ischemia/Reperfusion in Cardiomyocytes of Developing Rat. Adaptive Medicine, 2016.8(4):177-185.

9. MIAO Sui-bing; CHEN Hua; CUI Fang; **TIAN Si-Yu**; GU Shuo; GUAN Yue, Dexmendetomidine decreases spontaneous contraction of duodenal smooth muscle of rabbits in vitro. Chinese Pharmacological Bulletin 2016; 10.3969.

Acknowledgement

Time flies so fast. I still remember the first day I arrived in Erasmus MC. I feel really lucky to have this fantastic opportunity to do my PhD study in the Netherlands. During this learning process, some parts are really difficult and tough but some parts are full of happiness. It is definitely a wonderful and memorable time in my life. I would like to express thanks to my promoters, colleagues, my family, and friends, who supported me a lot. Please allow me to express my sincere gratitude to all of you!

To my promoter, dear **Prof. dr. Daphne Merkus**, thank you for providing me with this great opportunity to join your group! I still remember the first time we met where is in your office room. It was just like a casual interview. At that time, I feel extremely nervous but when we talked with each other, I could feel you are a kind and positive supervisor. You have always been supportive and helpful when I met some problems. When I saw you usually travel between Rotterdam and Munich, I admire your passion and energy in the research. I knew I still need to grow up in the research area and you still support and encourage me to continue. Thanks for your patience to revise my manuscripts, without your help, I won't be able to finish and defense my thesis. I feel very lucky that you as my supervisor!

To my co-promoter, dear **Prof. dr. Dirk-Jan Duncker**, I really admire your knowledge and determination in the research. I feel that you are a kind and generous professor. When I traveled back to China in 2020, the corona breakout at that time, I still remember that you care about my family and me and I feel really warm and sweet! Thanks for your help with the scientific writing, I have learned a lot with your suggestions and support!

I would like to acknowledge the members of my doctoral committee, **Dr. C. Cheng, Prof. dr. R.W. Hendriks, Prof. dr. M.J.T.H. Goumans, and Dr. ir. J.J. Wentzel, Prof. dr. I.K.M. Reiss**. Each one of you has been of tremendous help, I am so grateful for

your comments and effort! Dear Caroline, thanks a lot for your help with the RNA-seq data which help and support me a lot for the research direction. I enjoy talking with you about life and research and your passion and working style really impressed me. Hopefully, we still have chance to collaborate in future. I am really thankful for all your help!

I would like to express my sincere thanks to **Prof. Paul Evans**, thank you for offering me the fantastic opportunity to work with you and your group in UK. I am looking forward to joining your group and enjoying this research experience! Hopefully we could do some wonderful research work together in the future!

I would like to extend my gratitude to **Dr. Kim van der Heiden**, thank you for choosing me to present my data at Biomechanics in Vascular Biology symposium. It is a great chance for me to meet my future supervisor. Thanks for your insightful comments, which are extremely beneficial for the manuscript! **Dr. Karin Boomars**, many thanks for the collaborations. It is so precious for the manuscript! **Denise**, thanks for all your help and support for the mice's work! Best wishes for your training!

I would like to thanks for my lovely paranymphs, Lotte and Ranga. Thank you for all your time to help me prepare the defense. **Lotte**, thanks for you revising my Dutch summary. Luckily, I could meet you in my last year of PhD. It is very coincidentally that both of us are living in Amsterdam. You may also continue with the Piezo2 project and RNA-seq data, and it is a very interesting project to continue and investigate. Best wishes for your projects and future! For **Ranga**, my friend! We talked a lot with each other, from research to life. Sometimes I feel very disappointed about the results of the experiments and you usually could understand my feeling and give me some positive points. You are also a lovely man, and I could see your different selfie photos in WhatsApp. All the best for your cool 3D chips paper!

I would like to thank my colleagues in Experimental of Cardiology. **Maarten**, thanks a lot for your help with the Piezo2 project. Since you held a meeting with Ranga, Jie and me, I feel that I find an organization. You provided me with a lot of insightful points, then I could do that project so confidently and independently in the meantime I learned a lot from you! Best wishes for your new career! For **Oana**, thanks a lot for your patience to listen to my story. When I looking for a job, I indeed met the situation of dilemma. You are willing to help and give me some useful suggestions which help me a lot. **Ruben**, thanks for sharing the DCVA video with us which is full of fun. You are always very friendly and helpful. Best wishes for your PhD projects! For **Ihsane,** you are a kind and humous man! When I started the PhD in 23rd floor, you prepared your defense. It is so interesting that we could meet again when I almost finish my PhD. Congratulations for your handsome baby! **Jens,** I admire you that you could finish and publish so many high qualities papers in 4 years. I am sure you could do even more brilliant in the clinical area! **Michelle**, I think you are a hard-working superwoman! Thank you for being a role model and inspire me a lot to investigate science. Looking forward to your new project about the Piezo1 and placenta!

Zongye(宗烨师兄), thanks a lot for guiding me to this wonderful group! Thank you very much for our collaborations in many projects! You are so hard-working and full of passion in research which inspires me a lot. Best wishes for your research and clinical training! **Jarno**, my previous office neighbor. Haha, I still remember that you always forgot something when you leave then you need to come back and force. You are also a wonderful and helpful friend! All the best for your clinical training! **Rahi**, my current office neighbor. You cook Asian food very well even though I have never tried your food before. Every time I smell your cuisine's flavor, I feel very hungry but enjoyable. **Lakshme**, it is so amazing that you could speak a little bit of Chinese and your Chinese pronunciation is very nice. Thanks for sharing your

fantastic experience and trips as internationals. **Martine**, we always see each other in the evening in the lab because we are night workers. Good luck for your projects!

Esther, we had together to Munich and enjoyed the typical German food. I really thank you for your continuous help and support in the lab. You are extremely friendly and are always very helpful! **Liesbeth**, thanks a lot for helping me to solve the computer problems! **Lau**, I think that you are really relaxing now. Enjoy your retirement time! **Monique**, thank you for organizing the management things for us! You always smile and help people in the department.

Many thanks and all the best to my previous and current colleagues in the 23rd floor! **Richard, Kelly, Annemarie, Marc, Maaike, Heleen, Aladdin, Dianne, Mathijs, Joaquim, Francesca, Marielle, Harold, Patricia, Bram, Kim, Rowan. Joop**, thanks a lot for lending some ibidi products for us. You are really kind and helpful!

To my Chinese friends

谢谢心内科中国的小伙伴！ **Jie**(高洁)师妹,谢谢你在 2021 年的年初来到这里， 顿时心内科有两个中国人了,感到非常亲切！ 你一直特别热情和充满活力,有时像个孩子一样天真可爱,还记得你给我的手写信,往事历历在目.希望我毕业走之后,你能快快长大,实验顺利,早日毕业！ **Heng**(刘恒)大哥,哈哈还是要叫你声大哥， 不得不说自从你来了以后， 我们 23 楼中国人才真正的融合到一起， 每每有你总是充满了欢声笑语,以后等大哥继续组局,我一定飞回来和大家相聚!

张爽， 感谢 EMC 遇到你!你一直是我的一个榜样， 你每次做实验的精神深深震撼着我,而且我们都在研究 chip,很多时候我们能够交流科研,彼此学习,我也向你学习了很多.在你的引领下,我也开始学习滑雪， 虽然见证了你的伤痛,但我也看到你滑雪坚持不懈的精神,让我也开始热爱这项运动.很舍不得你回国,不想让你离开荷兰,还想和你一起探索科研以及生活,但我知道做一名医生始终是你的梦想,希望你能回国后一切顺利， 顺利成为张主任!

----感谢 Amsterdam 的好邻居小伙伴!
马一鸣/尤艺虹， 谢谢邻居总是带我们尝试各种鸡尾酒， 带我们开启了新世界的大门,很开心能和你们一起瑞士之旅!也祝愿你们早日领证修成正果!
陈淼， 谢谢陈哥总是摆一桌好菜接待我们,你那里总是我们的开会聚集地,祝愿早日找到佳人!
徐龙福/黄意轩,羡慕你俩的厨艺,每次去你们那里吃饭,总能大呼好吃,以后一定还要一起去 hiking 露营,我们一定要约!
米雪/李德水,还记得我们一起去过意大利,巴黎,我俩还见证了你俩的爱情,祝愿你们在国内大展宏图,早日抱宝宝!
赵馨颖,谢谢小馨,在我刚来荷兰时我们相互陪伴相互倾诉,感谢那时我们一起游玩的日子,一起美好的回忆！ 祝愿赵老师国内一切顺利， 向赵主任进发!
孙晓晴师姐,谢谢师姐一路的指引,你是我来荷兰认识的第一个中国人而且居然又是老乡,在我遇到困难时,谢谢师姐的引荐与帮助,让我感受到了温暖,也祝愿你在荷兰科研界继续大放异彩,发 paper 拿 funding!

----感谢 EMC 的小伙伴!
23 楼的王宇辰,李泓辰,两位师弟加上刘总,可以凑一个群口相声,茶水间一起侃大山!谢谢王总高超摄影技术答应帮我答辩拍照,谢谢李总高超的艺术造诣总是语出金句,笑声连连记忆深刻!
依妮,师妹总是充满活力,很佩服你总是能安排的十分充实,愿师妹心想事成,实验一切顺利!张璐,中午我们总是能相伴一起吃饭,充满欢乐,让吃饭变成了欢乐的聚集地,祝愿师妹新婚快乐!

以及 23 楼的 Lucy，胡雨阳，韩艺创，谢谢你们带我去攀岩,带给我不一样的体验！
谢谢陈金鸾,葛周虹师姐,两位师姐都是学术道路上的楷模,从你们身上都能学到好多,受益匪浅！
谢谢这些朋友的相逢以及帮助：丽伟，孙源，谭仑波，林惠，雨婵，赵薇，施少军，燕宁,朱红振,吴亚宏/石雨薇,文秋,陈帅,利霞,向泓宪,郑佳豪,李彦霖,培培,王玲,忠丽师姐，德丽师姐，潘潘师姐，胡旭师姐.

To my master supervisors in China
特别感谢河北医科大学张翼教授，谢谢恩师指导，做您的学生真的很幸福，您的学术精神也一直感染着我，谢谢您当时对于我申请出国的大力支持,愿恩师能好好享受退休生活,身体健康,一切顺利！马会杰教授，感谢您以往的信任以及帮助，在我读博期间您还对我多多关注，钦佩您做科研的精神以及对学生的耐心和照顾，希望未来我们仍有合作的机会！也谢谢谷硕，陈金婷，王日，王亚萍，付丛蕊师姐在国内的鼎力帮助！

To my family
感谢我的家人的支持！谢谢爸爸妈妈，我知道有时我打电话频率很低，有时也真的忽略了你们,3 年了,也因为疫情回不去看你们，真的很愧疚！你们从来没有怪过我，包括我又要去英国漂，你们也没要求我回来，我真的很感谢你们一路以来的支持！我嘴上埋怨你们不关心我,不管我,其实内心知道你们真的很支持我的每一项选择!我爱你们,爸爸妈妈!谢谢姥姥,我真的很自责一直回不去看望您,真心希望我能明年就回去,马上飞奔到您面前！谢谢舅舅舅妈,小姨姨父,你们大老远还一直关心着我,你们一切都好，我就满足了！

To my lover
谢谢亲爱的佳贤，我们真的相识相伴已经快 5 年了,很幸运能在异国他乡认识你,让孤单的生活有了彼此！我们探索过欧洲最美丽的风景，吃过各个地方的美食，每一个地方都有你我的回忆.谢谢你在我最抑郁的时候的支持和帮助,无法想象那段日子如果没有你，我该如何度过.我真的慢慢的已经习惯了有你在的日子,你让我感受到异国他乡还有个家.我们未来的路还有很长,希望我们能够互相扶持,共同进步！爱你！

About the author

Siyu Tian was born on 3rd of May 1992 in Shijiazhuang, Hebei province, China.

In 2010, after her graduation from NO.17 high school in Shijiazhuang, she started the bachelor degree study of Nursing in Zunyi Medical University in Zhuhai, Guangdong province in China. In 2013, she started the nursing clinical training in the Fifth Affiliated Hospital Sun Yat-Sen University and got the Nursing Qualification License in China. In 2014, she finished the bachelor study and started her master study in Hebei Medical University, supervised by Prof. Yi Zhang at department of physiology. During her master, she mainly focused on the treatments for hypertension research. She developed a keen interest in cardiovascular research.

In 2017, after her master, she got a scholarship from China scholarship council (CSC) and decided to come to the Netherlands to continue with PhD study in cardiovascular research. She joined in Prof. dr. D. Merkus and Prof. dr. D.J. Duncker' s group at Department of Cardiology, Erasmus MC, and focused on the pulmonary hypertension research. Upon her PhD graduation, she will continue with her research in vascular disease and pursue the position as a post-doc researcher at University of Sheffield, in UK, under the supervision of Prof. Paul Evans.