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BMPRII-driven treatment response in heritable and idiopathic pulmonary arterial hypertension

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List of abbreviations

ALK: activin-like kinase

BM: basement membrane

BMP: bone morphogenetic protein

BMPRII: bone morphogenetic protein receptor type II

CCB: calcium channel blocker

CTEPH: chronic thromboembolic pulmonary hypertension

CHD: congenital heart disease

CTD: connective tissue disease

ERK: extracellular signal-regulated kinase

ICAM: intercellular adhesion molecule

Id1: inhibitor of DNA binding

HLMVEC: human lung microvascular endothelial cell

MAPK: mitogen-activated protein kinase

MCT: monocrotaline

mPAP: mean pulmonary arterial pressure

PH: pulmonary hypertension

PAH: pulmonary arterial hypertension

PAEC: pulmonary arterial endothelial cell

PASMC: pulmonary arterial smooth muscle cell

IPAH: idiopathic pulmonary arterial hypertension

HPAH: heritable pulmonary arterial hypertension

NO: nitric oxide

PGI_2: prostacyclin

PVR: pulmonary vascular resistance

p38 MAPK: p38-mitogen activated protein kinase

RHC: right heart catheterization

RV: right ventricle

Smad: small mothers against decapentoplegic

TGFβ: transforming growth factor beta

TGFβRII: transforming growth factor beta receptor type II

VEGF: vascular endothelial growth factor

VEGFR: vascular endothelial growth factor receptor

Chapter 1: General introduction

1.1. Introduction

Pulmonary hypertension (PH) is a condition characterized by an increased blood pressure in the pulmonary arteries, veins or capillaries. Different pathological features and therapeutic approaches segregate five categories of PH including: 1) pulmonary arterial hypertension (PAH), 2) PH due to left heart disease, 3) PH due to lung disease and/or hypoxia, 4) chronic thromboembolic PH (CTEPH) and 5) PH with unclear multifactorial mechanisms. PAH is further classified as depicted in table 1.1 (1).

Table 1.1: Updated subclassification of pulmonary arterial hypertension (6th World Symposium on Pulmonary Hypertension, Nice, 2018)(1).

1. Pulmonary arterial hypertension
1.1 Idiopathic PAH
1.2 Heritable PAH
1.3 Drug- and toxin-induced PAH
1.4 PAH associated with:
1.4.1 Connective tissue disease
1.4.2 HIV infection
1.4.3 Portal hypertension
1.4.4 Congenital heart disease
1.4.5 Schistosomiasis
1.5 PAH long-term responders to calcium channel blockers
1.6 PAH with overt features of venous/capillaries (PVOD/PCH) involvement
1.7 Persistent PH of the newborn syndrome

PAH is a life threatening disease characterized by occlusion of the pre-capillary pulmonary arteries, which results in increased pulmonary vascular resistance (PVR), right ventricular (RV) hypertrophy, right heart failure and ultimately death if left untreated (2). Despite constant improvements in PAH specific therapy there is no curative treatment available, leaving lung transplantation as the only therapeutic solution for a selection of patients (3).

PAH is a rare but severe condition with a median life expectancy of 2.8 years when left untreated (4). Although treatment options and patient care improved considerably in the last decades, we are still unable to cure the disease. Moreover, many aspects of the pathophysiology of PAH need to be further elucidated (5) to improve outcome, quality of life, and to reduce morbidity and mortality. In the following paragraphs, the current knowledge concerning the epidemiology, clinical and genetic aspects, pathophysiology and pathobiology of PAH is reviewed. Next, we focus on the important role of endothelial dysfunction as a hallmark of PAH, and the impact of *BMPR2* mutations. Finally, the link between loss of *BMPR2* and the BMPRII protein expression and endothelial dysfunction is highlighted.

1.2. PAH: a rare and severe disease

1.2.1. Definition and classification of PAH

PAH is defined by a mean pulmonary arterial pressure (mPAP) greater or equal to 20 mmHg , a normal capillary wedge pressure less than 15 mmHg and a pulmonary vascular resistance (PVR) above 3 wood units (1). PAH is a rare but severe, progressive and incurable disorder affecting the pulmonary vasculature, as well as the RV. The disease is characterized by a progressive obstruction of the pre-capillary pulmonary arteries resulting in increased PVR and elevated mPAP leading to RV hypertrophy, right heart failure and death if left untreated (2). Idiopathic PAH (IPAH) corresponds to sporadic cases without any familial history or identified risk factors (6). When PAH occurs in a hereditary context, germline mutations in the *bone morphogenetic protein receptor type 2* (*BMPR2*) gene, a member of the transforming growth factor-β (TGFβ) superfamily, are detected in 70% of cases (7). Interestingly, *BMPR2* gene mutations are also detected in 11-40% of apparently sporadic cases without any familial history (8–10). Moreover, PAH can be associated with drug/toxin intake such as anorexigens, dasatinib or interferon, or connective tissue disease (CTD), congenital heart disease (CHD), portal hypertension, schistosomiasis and human immunodeficiency virus (HIV) (6). According to the updated classification, PAH also includes pulmonary veno-occlusive disease or pulmonary capillary hemangiomatosis and persistent PH of the newborn (1).

1.2.2. Epidemiology and prognosis

PAH is considered a rare disease with an incidence of 2.5 – 7.1 cases per million and a prevalence ranging from 15 to 50 subject/million inhabitants, according to the French (11) and Scottish registries (12). IPAH is the most commonly diagnosed subtype of PAH, accounting for 40% of cases and occurs 15 times more frequently than HPAH. In the subgroup of associated PAH, the most common subtypes are PAH associated with CTD or CHD (13,14). According to the original National Institutes of Health IPAH cohort (1980-1985) the mean age of patients with PAH was initially diagnosed around 36 years-old (15). However, since the REVEAL (2006-2007) and COMPERA (2007) PAH registries, PAH is diagnosed in elderly patients with a median age at diagnosis ranging from 50 to 65 years-old (16,17). A female predominance (60 – 76%) is observed in patients diagnosed with IPAH (14). Patients with PAH generally display unspecific symptoms, including fatigue, shortness of breath, syncope or dyspnoea and despite a significant increase in the awareness of the disease, the diagnostic delay is still unacceptable. The mean duration from symptom onset to diagnosis is 2.8 years, resulting in frequent diagnosis at advanced stages of the disease (11).

In the absence of PAH-specific treatment, the median survival of patients with IPAH is 2.8 years with a 1-, 3- and 5- year survival of 68%, 48% and 34%, respectively. Notably, advances in patient

management and treatment options contribute to increased survival rates to a median around 7 years with 1-, 3- and 5- year survival rates between 85-87%, 68-76% and 49-67% (18–21). Although younger patients often present with a more severe hemodynamic profile, female and younger patients have a better survival (22). To date, there is no curative treatment for PAH. Despite effective vasodilator therapies, lung transplantation remains the only option in patients with severe PAH refractory to currently available medical therapies.

1.2.3. Aetiology or causation

In line with the sub-classification of PAH, there are different causes associated with the development of PAH. PAH can result secondary to other diseases including CTD, CHD, PoPH, HIV or schistosomiasis (3). Other risk factors of developing PAH include exposure to drugs or toxins such as anorexigens, dasatinib, amphetamines and interferon therapy (3,38). At diagnosis, an acute vasoreactivity test using nitric oxide (NO) or prostacyclin (PGI_2) is performed during right heart catheterization (RHC) to identify responders, who will be orientated towards low-priced calcium channel blockers (CCB). Long-term CCB responders are considered as a specific entity with a different clinical course, management and pathophysiology. Current used PAH therapeutics and treatment algorithm are discussed in paragraph 1.2.5. In addition, pulmonary veno-occlusive disease is a rare form of PAH and the pathology includes a fibrotic vasculopathy affecting the smallest branches of the pulmonary venous system (39). Further subclassification depending on the aetiology of PAH includes persistent PH of the newborn which develops when infants fail to decrease PVR at birth, causing severe respiratory distress and hypoxemia (1).

In both HPAH and IPAH, several mutations are known to be responsible for an increased susceptibility to develop the disease. Although mutations in the *BMPR2* gene are the most frequent, using whole genome sequencing (WGS) and whole exome sequencing (WES) in large cohorts of IPAH patients revealed mutations in several different genes. These include mutations in the *activin receptor-like kinase 1* (*ALK1*), *endoglin* (*ENG*) (26), small mothers against decaptoplegic homolog 1 (*Smad1*), *Smad4* (27) and *Smad9* (28), which are genes interfering with the BMPRII pathway. In addition, mutations are found in caveolin-1 (*CAV1*) (29), *potassium channel subfamily K member 3* (*KCNK3*) (30), *T-box transcription factor 4* (*TBX4*) (31) and *eukaryotic transcription initiation factor 2 alpha kinase* 4 (*EIF2AK4*) (32). More recently, mutations in *aquaporin* (*AQP1*), *SRY-box 17* (*Sox17*), *cation transporting ATPase 13A3* (*ATP13A3*) (33), *ATP binding cassette subfamily C member 8* (*ABCC8*) (34), *growth differentiation factor 2* (*GDF2*) (35) and *cerebellin 2* (*CBLN2*) (36) as well as *kallikrein (KLK1)* and *gamma-glutamyl carboxylase (GGCX)* (37) have been found to be associated with PAH. Finally, preliminary evidence suggests a role for two putative loss-of-function mutations of *BMP10,* a BMPRII ligand, in disease susceptibility (35).

Among mutations mentioned above, mutations in the *BMPR2* gene are the most frequent genetic risk factor for development of HPAH. However, *BMPR2* mutations show a penetrance of only 20%, suggesting that a secondary insult is required to trigger the progression of the disease, including environmental and genetic modifying factors. *BMPR2* mutation carriers develop PAH 10 years earlier than non-carriers, with a more severe clinical and hemodynamic phenotype at diagnosis (23). Depending on the type of *BMPR2* mutation, penetrance and severity can vary between, or even within families. A comprehensive description of the pathobiology of BMPRII signalling is detailed in paragraph 1.5.

1.2.4. Pathophysiology

The pulmonary vascular system is a low pressure, high compliance and high-flow system. The pathogenesis of PAH includes obstruction of the precapillary pulmonary arteries, resulting in a progressive increase of PVR. As a result, the RV must cope with an increased afterload. To further maintain the systolic function and cardiac output, RV hypertrophy develops and an increase in mPAP gradually occurs. When, the RV can no longer compensate, the RV starts dilating which eventually results in right heart failure (41).

The pathophysiological process of PAH is an intricate process that involves vasoconstriction, vascular remodelling, inflammation and thrombosis, contributing to the obstruction of precapillary arteries (42). Pulmonary vascular remodelling is a complex process triggered by different stimuli like hypoxia, shear stress, inflammation, drugs/toxins and genetic susceptibility (39). Vascular remodelling involves thickening of intima, media and adventitia due to hypertrophy and hyperplasia of pulmonary arterial smooth muscle cells (PASMCs), endothelial cells (PAECs) and fibroblasts. In addition, distal extension of PASMCs resulting in muscularization of peripheral arteries, loss of precapillary arteries, neointima formation and ultimately formation of occlusive or plexiform lesions are observed in severe PAH (Figure 1.1) (40).

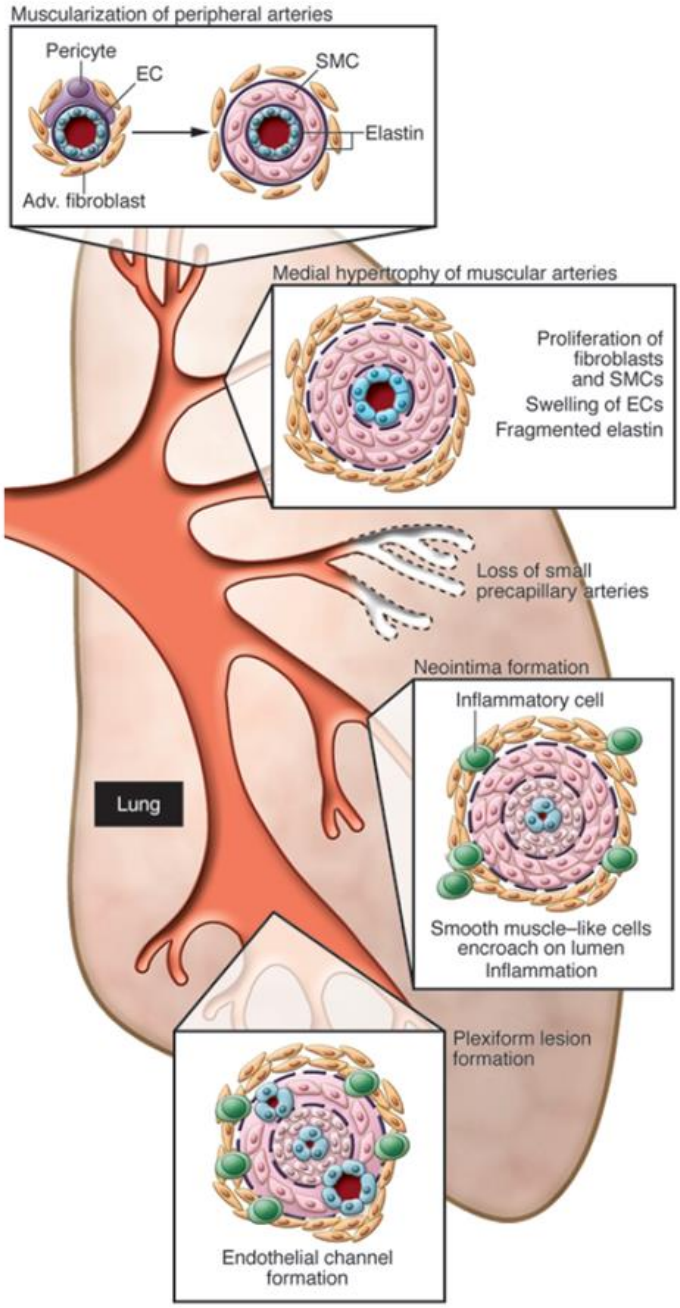

Figure 1.1: pathology of pulmonary hypertension. Schematic illustration of the different vascular abnormalities associated with PH. Figures based on (40) with permission.

These plexiform lesions result from disorganized proliferation of PAECs to become endothelial channels supported by stroma containing matrix proteins and fibroblasts, obliterating the vascular lumen (43). An intricate interaction exists among several signalling molecules within the pulmonary vasculature to maintain homeostasis. Deregulation of these signalling molecules may lead to endothelial dysfunction and results in multiple effects including endothelial dysfunction, impaired vascular dilatation, alterations in the expression of NO, ET1 and serotonin, increased expression of inflammatory cytokines and chemokines and disordered proteolysis of extracellular matrix contribute to the pathogenesis of PAH (44).

Therefore, endothelial dysfunction is considered a predominant hallmark in the pathophysiology of PAH. The endothelium has more functions than only acting as a mechanical barrier and regulates the underlying media and adventitia by excretion of regulating factors. Current PAH-specific therapeutics target endothelial dysfunction by restoring the imbalance in vasoreactivity. An overview on the different aspects of endothelial dysfunction is provided in Figure 1.2.

The presence of inflammatory cells such as T- and B-lymphocytes, macrophages and dendritic cells is frequently observed in plexiform lesions and adventitia of remodelled vessels, suggesting that an inflammatory process may contribute to the pathogenesis of PAH (45). In parallel with increased inflammation, endothelial dysfunction implies a loss of barrier function, which may lead to increased influx of circulating growth factors and cytokines and initiate an inflammatory cascade (90).

Altered angiogenesis is another important aspect and whether vascular endothelial growth factor (VEGF), one of the most potent angiogenic factor, plays a role in the development of PAH remains unclear. While VEGF and VEGF receptor (VEGFR) expression levels are elevated within plexiform lesions and serum from patients with PAH (46), the combination of hypoxia with the administration of the antiangiogenic VEGFR inhibitor, SU5416, causes obliterative PAH in rodents (47).

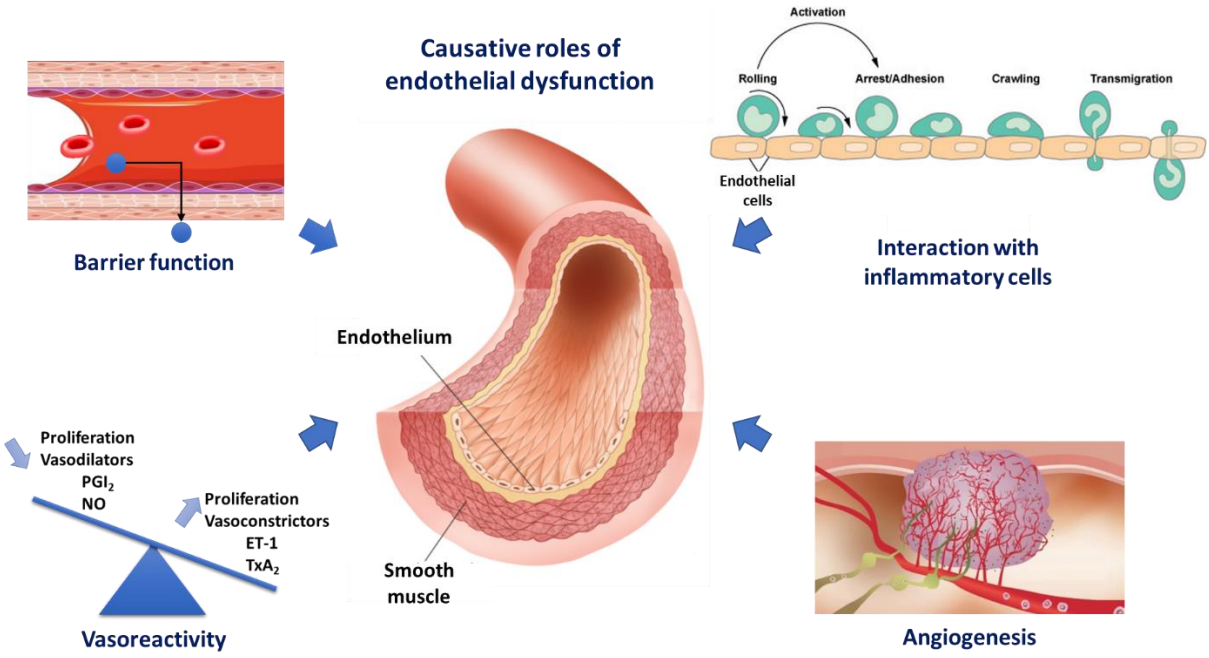

Figure 1.2: Schematic illustration of different physiological aspects in which the endothelium plays a role in the pathogenesis of PAH. Figure adapted from (48)

1.2.5. PAH therapies

Endothelial dysfunction is considered a predominant hallmark of the pathophysiology of PAH, and is defined by an imbalance between vasodilators including PGI_2 or NO, and vasoconstrictors such as endothelin-1 (ET-1), serotonin or thromboxane A_2, as well as potentially influencing contraction and relaxation of PASMCs, vascular remodelling and coagulation homeostasis (49).

The identification of molecules involved in the regulation of pulmonary vasoreactivity including PGI_2, NO and ET-1, has led to the development of different classes of approved PAH therapies, which target three major pathways (Figure 1.3). These drugs have a vasodilatory effect on the blood vessels, which will decrease the workload of the heart necessary to pump de-oxygenated blood through the lungs.

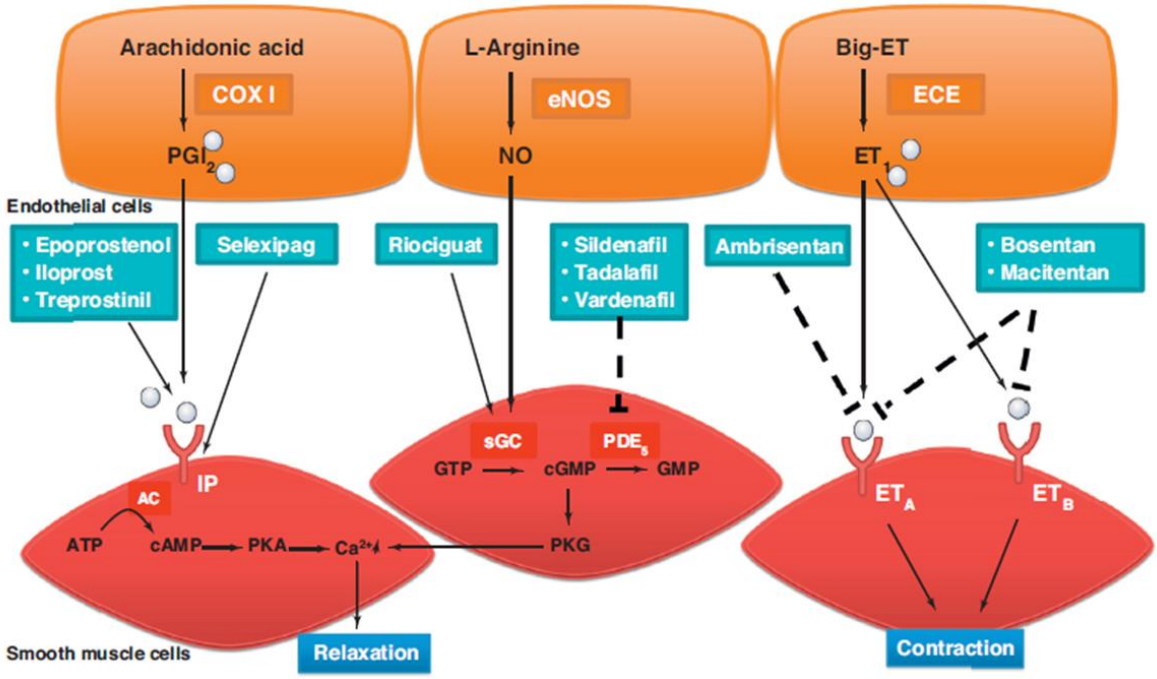

Figure 1.3: Schematic overview of current targeted pathways and available PAH-specific therapies (66). COX 1 – cyclooxygenase 1; PGI_2 – prostacyclin; IP – I-prostanoid receptor; AC – adenylate cyclase; ATP adenosine tri-phosphate; cAMP – cyclic adenosine monophosphate; PKA – protein kinase A; NO – nitric oxide; eNOS - endothelial NO synthase; PDE_5 – phosphodiesterase type 5; ET1 – endothelin-1; ET_A and ET_B – endothelin receptor A and B. Reproduced with permission from (66).

1.2.5.1. Targeting the prostacyclin pathway

1.2.5.1.1. Prostacyclin analogues

PGI_2 expression is reduced in the lungs of patients with severe PAH (50). PGI_2 is synthesized from arachidonic acid by cyclooxygenase-1 (51) and produced by ECs, in which it mainly inhibits platelet activation, thereby decreasing the risk of thrombosis. Additionally, PGI_2 is an effective vasodilator and reduces vascular remodelling as it works in homeostasis with thromboxane by counteracting its effect. Binding of PGI_2 to its receptor, the prostaglandin I2 receptor (IP receptor), activates downstream adenylate cyclase, resulting in production of cyclic adenosine monophosphate (cAMP) and

phosphorylation of protein kinase A (52). This leads to decreased calcium concentration in SMC, inducing vasodilation of the pulmonary artery and reducing vascular SMC proliferation (53). PGI_2 analogues include intravenous administration of epoprostenol, inhaled iloprost and intravenous or subcutaneous and oral (not approved in Europe) administration of treprostinil, which all require progressive up-titration and patient specific dosing adjustments to maximally improve exercise capacity, pulmonary haemodynamics and mortality (54).

1.2.5.1.2. Selective prostacyclin IP receptor agonist

The selective agonist of the IP receptor, selexipag, activates the IP receptor specifically. The main advantage of this first-in-class molecule is that it allows oral intake. Selexipag also requires dosing adjustments to the highest tolerable dose (55).

1.2.5.2. Targeting the nitric oxide pathway

1.2.5.2.1. Phosphodiesterase type 5 inhibitors

PAH is associated with NO deficiency and impaired NO-cGMP axis, probably due to reduced expression in endothelial nitric oxide synthase (eNOS). Endothelial NO activates the downstream production of cyclic guanosine monophosphate (cGMP) in PASMCs, resulting in vasodilation. Commonly, cGMP is degraded by phosphodiesterase type 5 (PDE_5) before it may activate protein kinase G (PKG). Inhibiting PDE_5 prevents the breakdown of cGMP, leading to increased PKG activation and resulting in decreased contractility. Therefore, an effective therapeutic approach exists in enhancing NO-cGMP signalling using PDE_5 inhibitors (PDE_5I) which include sildenafil, tadalafil and vardenafil (56,57). PDE_5I decrease PVR, improve 6-minute walking distance (6MWD) and haemodynamics (58,59).

1.2.5.2.2. Stimulator of soluble guanylate cyclase

Another approach targeting the NO pathway consists of increasing the production of cGMP by activating soluble guanylate cyclase (sGC). Soluble GC is an enzyme converting guanosine-5'-triphosphate (GTP) to cGMP. Riociguat, a stimulator of soluble sGC, directly activates sGC even in the absence of NO. It is an approved therapy for PAH, improving exercise capacity, functional class, 6MWD and time to clinical worsening (60).

1.2.5.3. Targeting the endothelin pathway

The endothelin (ET) pathway, which appears to be upregulated in lung tissue of patients with PAH, results in increased circulating levels of ET-1 leading to vascular SMC constriction. ET-1 is the most potent pulmonary vasoconstrictor and mitogen produced by ECs, and acts on two receptors in the cardiovascular system, ET-1 receptor A (ET_A) and B (ET_B) (61). ET_A and ET_B are both present on PASMCs and stimulate vasoconstriction by increasing intracellular calcium, whereas only ET_B is present on ECs, in which it stimulates activation and release of vasodilating agents such as NO and PGI_2 (62). In PAH,

ET_B receptors are upregulated on PASMCs and downregulated on PAECs. Therefore, inhibiting the ET cascade using ET-receptor blockers appears to be an effective therapy, with endothelin receptor antagonists (ERA) preventing vasoconstriction by inhibiting ET_A and ET_B receptors. This blockade can be selective for ET_A (ambrisentan), or dual against both ET_A and ET_B (bosentan and macitentan). Macitentan, a last generation ERA, shows increased efficacy *in vitro* by enhanced tissue penetration and prolonged receptor binding (63–65).

1.2.5.4. Treatment algorithm of PAH

Available approved vasodilatory therapies do not reverse pulmonary vascular remodelling in patients with PAH, and patients may respond differently to treatment. Currently only an acute vasoreactivity test using inhaled NO or prostacyclin is performed to orientate treatment towards low-priced calcium channel blockers (53,67).

The current treatment strategy is based on the severity of the newly diagnosed PAH as assessed by a multiparametric risk stratification approach (67). Clinical parameters, exercise capacity, right ventricular function and haemodynamic parameters are combined to define a low-, intermediate- or high-risk status. The current treatment algorithm provides the most appropriate initial strategy, including combination therapy and further escalation towards triple combination therapy (67). Treatment escalation is required when low-risk status is not achieved in planned follow-up assessments. Lung transplantation is currently considered as the only option in most advanced cases on maximal medical therapy (Figure 1.4).

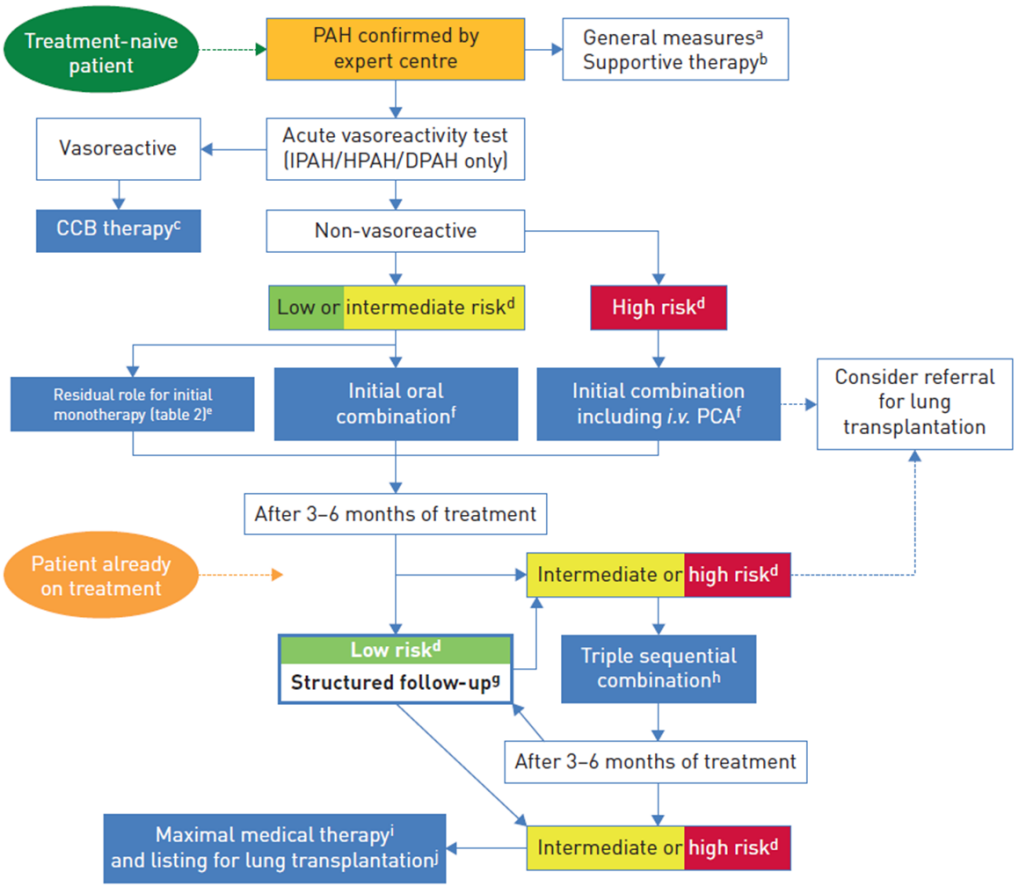

Figure 1.4: Treatment algorithm for pulmonary arterial hypertension (67). CCB: calcium channel blocker; PCA: prostacyclin analogue; a: 2015 ESC/ERS PH guidelines table 16; b: 2015 ESC/ERS PH guidelines table 17; c: 2015 ESC/ERS PH guidelines table 18; d: 2015 ESC/ERS PH guidelines table 13; e: 2015 ESC/ERS PH guidelines table 19; f: 2015 ESC/ERS PH guidelines table 20; g: 2015 ESC/ERS PH guidelines table 14; h: 2015 ESC/ERS PH guidelines table 21; i: maximal medical therapy is considered triple combination therapy including a s.c. or an i.vi PCA; j: 2015 ESC/ERS PH guidelines table 22 (6). Reproduced with permission.

Treatment escalation has been replaced by immediate upfront combination therapy since the results of the AMBITION clinical trial, which evidenced that initial combination therapy with ambrisentan and tadalafil resulted in a significantly lower risk of clinical-failure events compared to monotherapy (68). The TRITON study, a phase 3b trial, was designed to further confirm the efficacy of upfront combination therapy by comparing upfront triple therapy of macitentan, tadalafil, and selexipag versus a combination of macitentan and tadalafil (69).

1.2.6. Experimental models of PAH

In order to better understand the mechanisms and remodelling process involved in the pathogenesis of PAH, and to identify novel therapeutic agents, a variety of experimental *in vivo* and *in vitro* models has been developed and characterized. In the following paragraph the most often used experimental models are discussed.

1.2.6.1. In vivo models

Experimental *in vivo* models mimic several histological and molecular features of the human PAH pathophysiology, including endothelial dysfunction, muscularization of pre-capillary pulmonary arteries and increased medial thickness of muscularized arterioles, *in situ* thrombosis, local inflammation and plexiform lesions. Administration of monocrotaline (MCT), a pyrrolizidine alkaloid, (70) or chronic hypoxia combined with administration of a VEGF receptor-2 inhibitor, SU5416 (71), are the most widely used animal models. Several genetically modified animal models that mimic the pathophysiology of PAH are well-suited to study genetic factors contributing to the development of PAH. Homozygous *BMPR2*$^{(-/-)}$ knockout mice are not viable and die *in utero* (72), while heterozygous *BMPR2*$^{(+/-)}$ mice develop normally without harbouring any pathological phenotype, except if a conditional PASMC-specific knockout is applied (73,74). A heterozygous *BMPR2*$^{(+/-)}$ rat model is also available and develops age-dependent spontaneous PAH with a penetrance of about 20%, similar to that observed in humans (75). In parallel, a heterozygous *BMPR2*$^{(+/-)}$ rat model was developed, in which lung inflammation induced by 5-lipoxygenase results in severe PAH and intimal remodelling, indicating that in addition to a *BMPR2* mutation, a second hit is necessary to trigger PAH (76).

1.2.6.2. *In vitro* models

Isolation of patient material offers a great opportunity to study the pathophysiological process and can give more insight into patient specific differences. Besides histological analysis, isolation of cellular material including PAECs and PASMCs is feasible at the time of lung transplantation. Major drawbacks are that cells from patients are rare, at very end-stages of the disease and exposed to combined therapies for months or even years. Currently, there is no method available to isolate or study pulmonary microvascular cells from treatment-naïve patients at diagnosis. Therefore, alternative methods have been developed to overcome this limitation.

Silencing of *BMPR2* in commercially available PAECs resulted in decreased BMPRII expression which allows to investigate and focus on the isolated role of impaired BMPRII signalling and the effect in endothelial cells (24,77). Furthermore, Gu and colleagues highlighted the ability of induced pluripotent stem cells to differentiate into PAECs or PASMCs in order to investigate patient-specific differences

and collect cells from *BMPR2* mutation carriers, non-mutation carriers and unaffected mutation carriers. They emphasized the potential use of correcting the *BMPR2* mutation as a future therapeutic option (78). Alternatively, the use of blood outgrowth endothelial cells, only requiring a patient blood sample, has already shown its application potential (79). Although both abovementioned methods have somehow proven their potential, they are limited in use since the differentiated cells originate from a skin or a blood sample.

One group reported the feasibility of harvesting PAECs from the tip of Swan-Ganz catheters used during right heart catheterization in 2013 (80). Using direct flow cytometric-based measures of Bcl-2 activity, they were further able to differentiate PAH from heart failure with preserved ejection fraction without presenting additional risk to the patients (81). Most recently, another group has proven the applicability of cultured PAECs collected from pulmonary artery catheter balloon tips in pulmonary vascular diseases (82).

While these techniques have potential applications, more research is necessary to refine existing technologies and expand the use of patient specific materials in order to better understand the interindividual differences and the complexity of PAH.

1.3. Endothelial dysfunction in PAH

1.3.1. Endothelial barrier function in PAH

Since the endothelium primarily acts as a mechanical barrier, endothelial dysfunction implies a loss of the endothelial barrier function. Accordingly, increased endothelial cell permeability has been consistently reported in MCT-induced PAH in rats (83). Using animal models, most reports of endothelial dysfunction highlight increased lung wet to dry ratios, which implies an accumulation of interstitial oedema. This was confirmed in mice overexpressing a *BMPR2* mutant, which spontaneously develop mild PH and show impaired intrinsic, as well as LPS-stimulated endothelial barrier function (84,85). In addition, PAECs isolated from these mice display increased permeability (85). Burton and colleagues demonstrated that *BMPR2* silencing in PAECs impairs endothelial barrier function and favours leukocyte transmigration (86). This implies that loss of endothelial barrier function results in increased permeability and infiltration of circulating cytokines, growth factors and/or inflammatory cells within the pulmonary vascular wall.

The basement membrane (BM) which is in tight contact with endothelial cells may also play a fundamental role in normal barrier function (87). Endothelial dysfunction may result in changes in BM composition which can influence cell junction formation and result in decreased barrier function and increased adhesion capacities, thus contributing to vascular remodelling (88). In patients with HPAH carrying a *BMPR2* mutation, the BM composition shows upregulation of fibrillin and integrin expression compared to controls, which acts as an initiating mechanism for endothelial to mesenchymal transition (89).

1.3.2. Inflammation in PAH

Immune dysregulation is one of the key effectors of endothelial dysfunction and is therefore tightly associated with the pathogenesis of PAH, in which it plays an important role. As such, PAH is associated with inflammatory diseases like HIV or schistosomiasis, and autoimmune diseases such as lupus and systemic sclerosis. Increased circulating levels of inflammatory mediators, including CRP, cytokines and chemokines such as interleukins (IL)-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18 and TNFα have been reported in PAH patients and may consequently contribute to the pathogenesis of PAH (90–93). Tuder and colleagues highlighted the presence of several inflammatory cells including B-cells, T-cells, macrophages and dendritic cells in plexiform lesions in patients with PAH (94), which was further confirmed by Dorfmuller and colleagues (95).

Although it is unclear whether inflammation is a cause or a consequence, various reports favour a second-hit scenario. These reports demonstrate that a co-incidence of *BMPR2* heterozygosity in combination with an inflammatory insult may trigger the progression of PAH, in line with the low

penetrance of *BMPR2* mutations in PAH (24). Accordingly, *BMPR2*$^{+/-}$ knockout mice overexpressing 5-lipoxygenase are more susceptible to develop PAH suggesting that *BMPR2* deficiency-induced endothelial dysfunction combined with an inflammatory insult contribute to the development of PAH and that *BMPR2* mutations could enhance the vulnerability to an inflammatory second hit (96).

1.3.3. Angiogenesis in PAH

Angiogenesis plays an important role in the progression of PAH, but the exact role of angiogenesis remains a matter of debate. Angiogenesis is the physiological process through which new blood vessels develop from pre-existing vessels and is a vital process in growth and development, as well as wound healing. During early angiogenesis, activated endothelial cells will form a tip cell from which a new blood vessel will develop. As such, endothelial dysfunction plays a significant role in the initiation of both sprouting and intussusceptive angiogenesis (97). However, whether enhanced angiogenesis should be considered beneficial or detrimental in the pathophysiology of PAH remains unclear and is discussed below.

1.3.3.1. Angioproliferative hypothesis

Angiogenesis is a fundamental process in tumour formation, and it is thought that increased angiogenesis stimulates further progression of PAH.

PAH is initially described as a proliferative disease triggered by endothelial cell injury resulting in apoptotic resistant EC, and is therefore considered as a cancer-like disorder by Voelkel and Tuder (94). This hypothesis is based on the observation of elevated serum levels of vascular endothelial growth factor (VEGF), a potent angiogenic factor, in patients with severe PAH and highly expressed VEGF and its receptor within plexiform lesions (98,99). Moreover, upregulation of other remodelling-associated genes, such as tumour growth factor β-1 (TGFβ_1), hypoxia induced factor 1a (HIF1a), thrombospondin, angiopoietin-1 and its receptor Tie-2, as well as sprouting-associated markers, including NOTCH and matrix metalloproteinase, were found in human plexiform lesions (100). Voelkel and Tuder based their concept on the quasi-malignant and monoclonal nature of PAECs in plexiform lesions arising from only a few cells able to switch towards an apoptotic resistant and hyperproliferative phenotype (98). However, rats exposed to hypoxia and pre-treated with a single injection of SU5416, an inhibitor of angiogenesis, develop severe PAH with occlusive neointimal lesions and plexiform lesions. This paradox was confirmed by the French registry, which reported the development of PAH in nine patients receiving an anti-cancer treatment with dasatinib (101). On the other hand, imatinib, another tyrosine kinase inhibitor which acts as a platelet-derived growth factor (PDGF) inhibitor, was investigated as an add-on therapy in the IMPRES trial and resulted in beneficial hemodynamic effects, but severe adverse events and discontinuation of 94% of patients (100).

1.3.3.2. Degenerative hypothesis

Lately the hypothesis of PAH being a proangiogenic disease has started to erode with arising alternative hypotheses. Recently, microvascular rarefaction could be attributed to a degenerative process suggesting that enhancing angiogenesis could be beneficial in patients with PAH. Chaudhary and colleagues postulated that increased PAEC apoptosis in patients with PAH can result in EC dropout, resulting in precapillary regression and microvascular rarefaction (102). This hypothesis implies that excessive cell proliferation is rather a consequence than a cause due to increased pressures and shear stress. As such, treatment with a broad-spectrum caspase inhibitor resulted in increased EC cell survival and prevented the development of severe PAH in both MCT and Sugen-hypoxia rat models with a reduction in caspase activity, correlated with improved hemodynamic parameters (103). In addition, Jurazs and colleagues hypothesized that the fragile precapillary arterioles, consisting of endothelial tubes, may be extremely vulnerable to apoptosis due to the lack of underlying SMCs or supportive pericytes (103). Moreover, there is clinical evidence showing that the number of occlusive lesions poorly correlates with compromised haemodynamics in patients with PAH, suggesting that other mechanisms than proliferative PAECs and PASMCs are involved.

Although angioproliferative and degenerative concepts may both contribute to the pathogenesis of PAH, the role of angiogenesis in PAH is yet to be elucidated. There are direct and indirect mechanisms by which PAEC apoptosis could lead to vascular pruning. Increased apoptosis of PAECs can lead directly to a degenerative loss of microvasculature, since pre-capillary arteries only consist in a single layer of endothelial cells (104). On the other hand, PAEC apoptosis can indirectly lead to emergence of apoptotic-resistant cells, which may result in angioproliferative obstructive lesions (98). Although the angioproliferative hypothesis has been first postulated and consequently received plenty of scientific attention, recent data from experimental models and human studies supports the degenerative hypothesis as a driving mechanism in the early stages of the disease (102,104).

1.4. BMP/BMPRII classification and signalling pathway

As abovementioned, mutations in *BMPR2* are associated with 70% of HPAH and BMPRII signalling pathway is impaired in IPAH. BMPRII, encoded by the *BMPR2* gene, is a serine/threonine receptor kinase involved in different cellular processes including osteogenesis, cell growth and cell differentiation in skeletal, kidney and lung development. BMPRII is present in all tissues but most abundantly expressed in pulmonary tissue, more specifically in PAECs and PASMCs (105).

1.4.1. *BMPR2* mutations

In 1997, a gene responsible for heritable forms of PAH was found on chromosome 2q33-34 (106). This gene, later defined as *BMPR2,* was shown to be associated with apparently sporadic cases of PAH (107). The *BMPR2* gene consists of 13 exons encoding four functional domains including an N-terminal ligand-binding domain, a single transmembrane region, a serine/threonine kinase domain and a cytoplasmic domain (Figure 1.5) (108,109). In a study investigating more than 800 patients with PAH, 486 distinct variants were detected in the *BMPR2* gene and considered likely pathogenic (110). Mutations in *BMPR2* are spread across the whole gene, with an exception of exon 13, and exhibit all types of mutations including missense mutations, nonsense mutations, splice site deletions, duplications or frameshift rearrangements (111). The majority of *BMPR2* mutations are frameshift (23%) and nonsense mutations (27%), triggering the nonsense-mediated decay pathway resulting in degradation of the protein and leading to haploinsufficiency (112). Specifically, 25% of PAH-specific variations in *BMPR2* are missense mutations that result in substitutions of highly conserved amino acids producing a stable mRNA construct, but mutant protein. HPAH patients carrying these mutations harbour more severe forms of PAH with lower age at diagnosis and reduced time to lung transplantation (113), suggesting a dominant-negative effect on the BMPRII function. These so-called nonsense-mediated mRNA decay (NMD)-negative mutations can affect the ligand-binding domain, the kinase domain or the cytoplasmic tail (114).

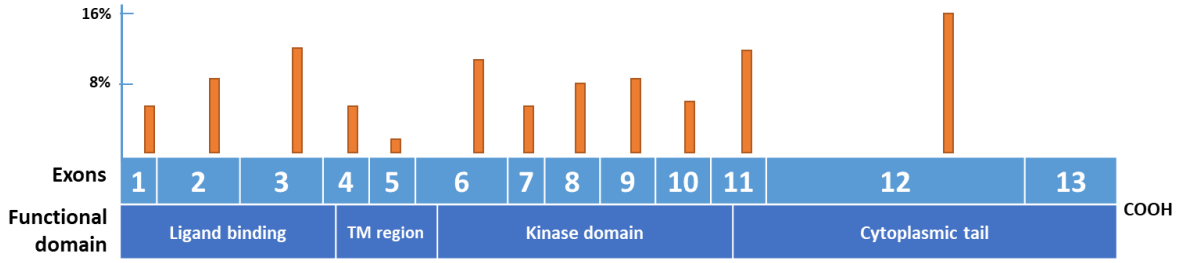

Figure 1.5: BMPR2 mutations associated with PAH. Orange bars represent relative occurrence (in %) of mutations in each of the different exons. Figure reproduced with permission (66).

Most mutations are located in the kinase domain, which consequently fails to activate by phosphorylation downstream transcription factors. These non-functional receptors translocate properly to the cell membrane where they fail to activate BMP signalling and have a dominant-negative

effect, and result in a non-functional oligomer (115). Cysteine substitutions in the ligand binding, or mutations in the kinase domain result in impaired intracellular membrane trafficking (115). This results in retention of dysfunctional mutant receptors within the endoplasmic reticulum or Golgi compartments (116,117), without reduction in the global expression of BMPRII. Missense mutations located in the cytoplasmic tail result in disrupted interaction with signalling partners of Smad-independent pathways (112), without impairing the capacity of the protein to be localised to the cell surface or its ability to activate BMP/Smad signalling (114). A *BMPR2* mutant containing a very small cytoplasmic tail showed inability to bind and inhibit LIM kinase-1 (LIMK-1). This is a key regulator of actin dynamics through inactivation by phosphorylation of cofilin and is important in the reorganization of actin filaments and to stimulate migration (118). Further analysis showed interaction between LIMK-1 and BMPRII preventing LIMK-1 activation, which could be alleviated by BMP4 ligand binding. Interestingly, the spectrum of genetic variants appears to be similar both in IPAH and HPAH (110).

1.4.2. BMP superfamily and BMPRII receptor signalling

Bone morphogenetic proteins (BMPs) are ligands which can bind the BMPRII receptor. These ligands are part of the TGFβ superfamily, which is a large family of structurally similar polypeptide growth factors and related receptors, including more than 30 different ligands such as TGFβs, activins, inhibin's, Nodal, Left-right determination factors (Lefty), bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs) and anti-Mullerian hormone (AMH) (Figure 1.6) (119,120).

In addition, these ligands can transduce signalling through a variety of transmembrane receptors. The signalling starts by formation of a heterocomplex of two groups of serine/threonine kinase receptors. The signalling cascade proceeds by formation of a heterotetrametric complex composed of two type I and two type II receptors and may potentially form an oligomeric complex when bound with a co-receptor. The different type I receptors are named activin-like kinase 1 to 7 (ALK1-7) and the different type II receptors are known as activin type II receptor a and b (ActRIIa and ActRIIb), bone morphogenetic protein receptor type II (BMPRII), transforming growth factor β receptor type II (TGFβRII) and Müllerian hormone receptor type II (AMHRII) (121). Co-receptors of this complex of two type I and two type II receptors include endoglin and betaglycan, and are involved in specific ligand-receptor binding and regulation of receptor signalling pathways (122). Further regulation of this receptor complex is possible by endogenous antagonists including gremlin-1, follistatin, noggin or chordin (Figure 1.6) (123,124).

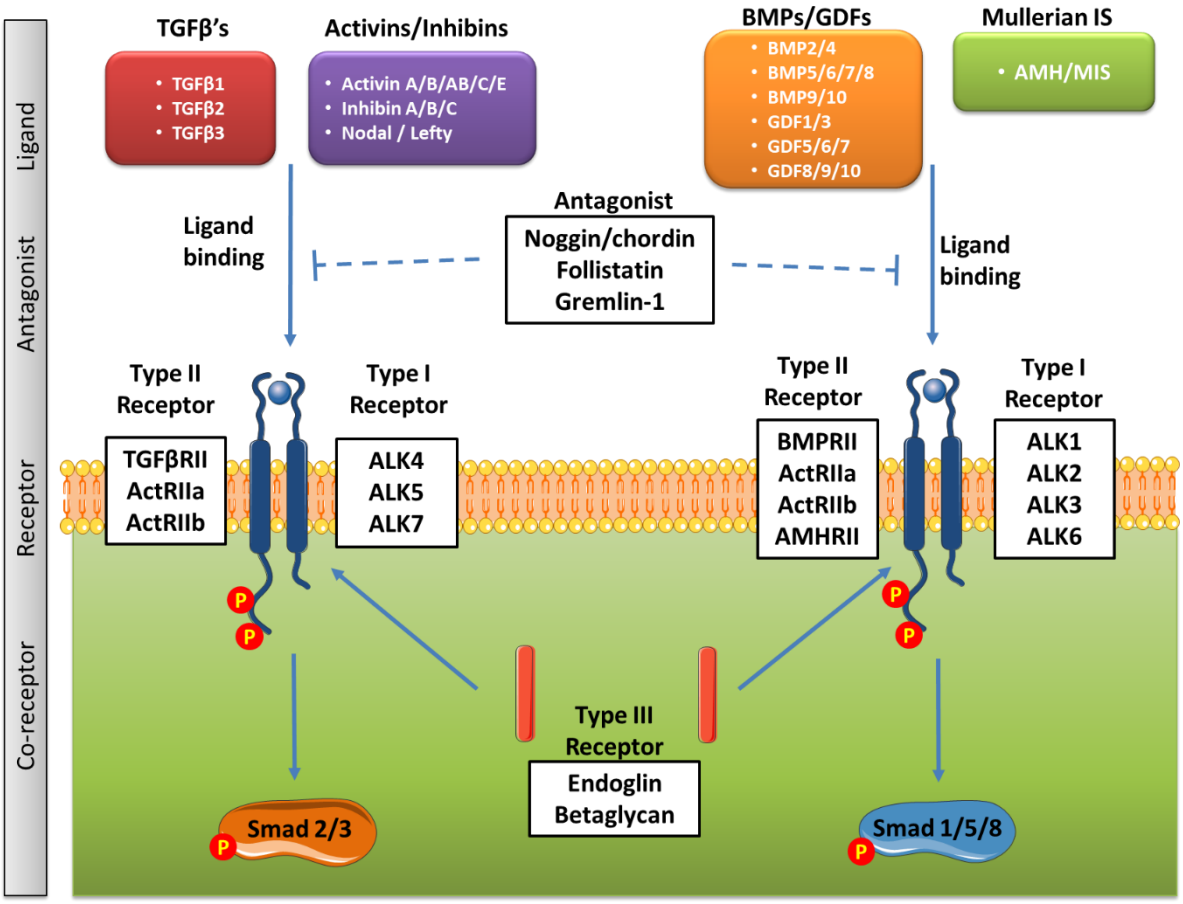

*Figure 1.6: Schematic diagram of TGFβ superfamily. Depending on ligand and receptor complex, different signalling pathways can be activated which are strictly regulated by ligand binding antagonists and co-receptors. Active forms of TGFβ bind to its receptors and induces Smad2/3 phosphorylation whereas BMPs induce Smad1/5/8 phosphorylation. Abbreviations: ActRII, activin type 2 receptor; ALK, activin-like kinase; AMH, anti-Müllerian hormone; AMR, anti- M*üllerian hormone receptor type II; BMP, bone morphogenetic protein; BMPRII, bone morphogenetic protein receptor type II; GDF, growth differentiation factor; MIS, Müllerian inhibiting substance; Smad, small mothers against decapentoplegic; TGFβ, transforming growth factor beta; TGFβRII, transforming growth factor beta receptor type II. Figure reproduced with permission *(66).*

The main signal transducers for the receptors of the TGFβ superfamily are transcription factors named Small mothers against decapentaplegic (Smad). There are 3 types of Smad proteins including receptor-Smad (Smad4), receptor-mediated Smads (Smad1, Smad2, Smad3, Smad5 and Smad8) and inhibitory Smads (Smad6 and Smad7). A transfer complex formation of a receptor regulated Smad and the co-Smad, Smad4, can enter the nucleus to regulate gene expression (125,126). It is generally assumed that downstream TGFβ receptor II signalling activates Smad2/3 by phosphorylation, while BMPs and BMPRII further regulate gene expression by activation of Smad1/5/8 (Figure 1.6) (121).

However, the situation in human is much more complex and numerous combinations of receptor pairs may occur between type I and type II receptors. In addition, different ligand-receptor binding affinities may induce different downstream Smad signalling (127). Smad activation can vary depending on the type I receptor, specific ligand binding or co-receptor. In endothelial cells, TGFβRII can induce Smad1/5/8 phosphorylation through formation of a heterotetrametric complex with ALK1 which is

able to antagonize TGFβRII/ALK5 signalling (127,128). Therefore, different cell types express specific type I or type II receptors as well as co-receptors to regulate cell specific signalling. In smooth muscle cells, BMPRII primarily signals with ALK3 or ALK6 in response to BMP2 or BMP4, whereas BMP6 and BMP7 have a higher binding affinity to a complex of ALK2 and BMPRII (129). A comprehensive overview on the complexity of the different ligand-receptor binding possibilities with downstream effectors is shown in table 1.2.

	ligand	type I receptor	type II receptor	R-Smad activation
BMPs/GDFs	BMP2/4	BMPRIa BMPRIb	BMPRII ActRIIA ActRIIB	Smad1/5/8
	BMP5/6/7/8	Alk2 BMPRIa BMPRIb	ActRIIA ActRIIB BMPRII	Smad1/5/8
	GDF5/6/7	BMPRIa BMPRIb	BMPRII ActRIIA ActRIIB	Smad1/5/8
	BMP9/10	Alk1	BMPRII ActRIIB ActRIIA	Smad1/5/8
	GDF1/3	ActRIb	ActRIIA ActRIIB	Smad2/3
	BMP3/GDF10	ActRIb	ActRIIA ActRIIB	Smad2/3
	GDF8(myostatin)/ GDF11	ActRIb TβRI	ActRIIA ActRIIB	Smad2/3
	BMP15/ GDF9	BMPRIb ActRIb TβRI	BMPRII BMPRII	Smad1/5/8 Smad2/3
TGFβs	TGFβ1/2/3	TβRI Alk1	TβRII	Smad2/3 Smad1/5/8
activins*	activin A	ActRIb	ActRIIA ActRIIB	Smad2/3
	activin B	ActRIb Alk7	ActRIIA ActRIIB	Smad2/3
	nodal	ActRIb Alk7	ActRIIB	Smad2/3
outsider	AMH	BMPRIa BMPRIb Alk2	AMHRII	Smad1/5/8
	GDF15	?	?	?

Table 1.2: *Schematic overview of potential combinations of ligand – receptor binding resulting in differential downstream signalling. Depending on the heterotetrametric receptor-formation, downstream canonical Smad signalling may differ. Table reproduced with permission* (130)

1.4.3. BMPRII signalling cascade

Upon BMP ligand binding to BMPRII, activation by phosphorylation of a type I receptor can initiate downstream signalling through canonical (Smad-dependent) or non-canonical (Smad-independent) pathways. Activation of the downstream non-canonical pathway induces a variety of intracellular

proteins including phosphatidyl-inositol 3-kinase/protein kinase B, extracellular signal-regulated kinase (ERK), p38 mitogen activated protein kinase (p38 MAPK), c-Jun N-terminal kinases, TGF-activated kinase-1 and protein kinase C (131). Regulation of these signalling cascades involve multiple partners and cross communication with other signalling pathways including LIM kinase, Rho/Rho-kinase, Wnt or Notch signalling, phosphoinositide 3-kinase PI3 kinase (131,132).

Receptor dimerization plays a major role in the distinction between downstream canonical or non-canonical signalling. According to Morrell and colleagues, preformed BMP receptor complexes, encompassing homodimers of type I and II receptors, would preferentially induce canonical Smad-dependent signalling pathways, whereas ligand-induced oligomerisation of type I and type II receptors would predominantly activate noncanonical non-Smad signalling pathways (133). Therefore, the ligand will first bind a type I receptor complex and further recruit a type II receptor for phosphorylation and downstream signalling (Figure 1.7).

This partially explains the increase in non-canonical signalling in *BMPR2* mutation carriers, with decreased BMPRII expression, that may favour heterodimer type II receptor-complex formation. This is an important phenomenon explaining a gain in non-canonical signalling. This highlights the multiple signalling possibilities within the TGFβ superfamily and the complex regulation of the BMPRII signalling cascade.

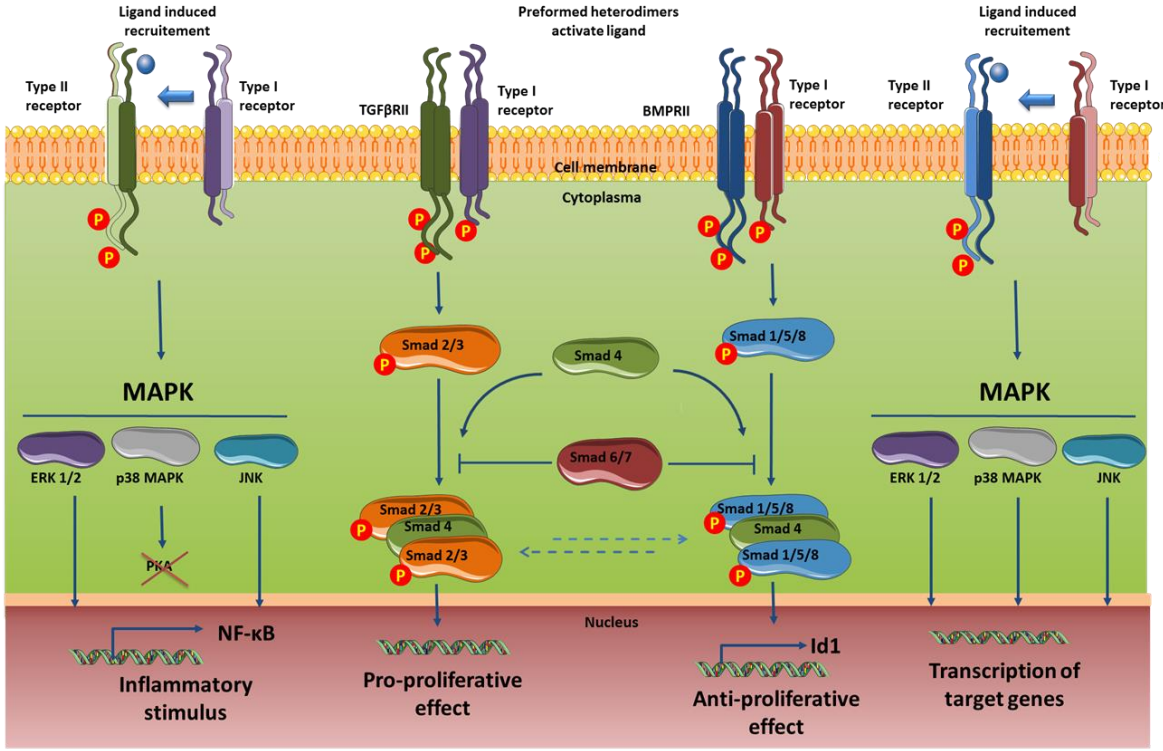

Figure 1.7: Schematic overview of different TGFβ and BMPRII signalling pathways. Abbreviations: ERK, extracellular signal-regulated kinase; Id1, inhibitor of DNA binding; JNK, c-Jun N-terminal kinases; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells. p38 MAPK, p38-mitogen activated kinase. Figure reproduced with permission (66).

1.5. Role of *BMPR2* in PAH

As described above, endothelial dysfunction is a major hallmark of the pathophysiology of PAH. Additionally, *BMPR2* is an important genetic factor linked with the development of PAH. Hence, the link between BMPRII and endothelial dysfunction will be further discussed.

1.5.1. BMPRII expression in the pulmonary vasculature

Atkinson and colleagues stated already in 2002 that the expression of BMPRII was decreased in pulmonary tissue of PAH patients with or without a *BMPR2* mutation (105). Although our research group/we showed the same trend in the present research, we did not observe a significant difference in BMPRII protein expression in lung tissue from *BMPR2* mutation carriers, non-carriers and healthy controls due to large variations between patients (24). This highlights the high variety between patients and that BMPRII protein expression can depend on type of mutation or patient-specific characteristics.

Reduced expression of BMPRII levels and abnormal signalling were observed in pulmonary tissue from experimental animal models of PAH, such as MCT- and hypoxia induced PH in rats (134,135). Restoring decreased BMPRII expression by performing targeted gene delivery was shown to prevent (136) and attenuate disease progression in both animal models (137).

1.5.2. Role of BMPRII in the development of PAH

Since BMPRII is mainly expressed within the endothelium of pulmonary arteries, it is involved in the progression of plexiform lesions (138). Accordingly, loss of functional BMPRII is associated with increased PAEC apoptosis and emergence of apoptosis-resistant vascular cells resulting in obliterated arterial remodelling (52). *BMPR2* mutation carriers develop PAH earlier (10 years) and display a more severe haemodynamic profile than non *BMPR2* mutation carriers (140).

Rats expressing human *BMPR2* mutations develop mild PH after 1 year, with increased pulmonary arterial pressures and reduced cardiac output, muscularization of pulmonary vessels and activation of non-canonical pathways with increased phosphorylation of p38 MAPK and ERK1/2 protein in lung tissue (141).

Focussing on the molecular mechanisms, the current hypothesis is that BMPRII and TGFβRII work in a strict equilibrium, in which BMPRII buffers the effects of TGFβRII, by competing for the available type I receptors and co-receptors to form receptor complexes and further downstream for the availability of receptor-Smad4. When an imbalance occurs, either by decreased levels of BMPRII or by increased TGFβRII levels, the predominant effect shifts to TGFβ signalling resulting in a proliferative phenotype. TGFβ is involved in inflammation and immune cell function, angiogenesis, differentiation, extracellular matrix synthesis and exerts effects on PASMC proliferation (142). Dysfunctional BMPRII/TGFβRII signal

transduction results in altered expression of transcription factors, including Smads and Id (inhibitor of DNA binding). Additionally, ALK5 inhibition prevents disease progression in MCT rats, suggesting a shift from BMPRII/Smad1/5/8 signalling to TGFβ/Smad2/3 signalling as a potential mechanism for PAH development (127).

1.5.3. BMPRII and endothelial dysfunction

As discussed above, endothelial dysfunction is an important hallmark in the development of PAH and current PAH-specific therapies intent to restore the imbalance between vasodilators and vasoconstrictors. Nevertheless, *BMPR2* and the BMPRII signalling are indisputably associated with increased susceptibility to develop PAH. Currently, little is known about overlapping pathways between the BMPRII signalling and other signalling cascades involved in vasoreactivity, which are the targets of current PAH-specific therapies. There is clinical evidence showing that BMPRII dysfunction impairs pulmonary vasoreactivity. As such, a large majority of PAH patients with *BMPR2* mutations had poorer outcome (143). Interestingly, *BMPR2* mutations result in impaired eNOS activation in human PAECs and, HLMVECs with different *BMPR2* mutants showed decreased NO production. In addition, PAECs from patients with PAH carrying *BMPR2* mutations or transient silencing of *BMPR2* in HLMVECs produce significantly higher levels of ET-1 (24,144). Accordingly, adenoviral-mediated BMPRII overexpression restores the imbalance between ET-1 and eNOS by increasing eNOS phosphorylation and limiting TGFβ1-triggered ET-1 secretion (145).

1.5.4. BMPRII and inflammation

Inflammation plays an important role and may promote vascular cell dysfunction in PAH. Since BMPRII signalling maintains normal function of PAECs and PASMCs, a defective BMPRII function could result in increased susceptibility to an inflammatory insult. *BMPR2*-silencing in human PAECs results in higher response to inflammatory stimuli leading to increased permeability and increased adhesiveness to monocytes (86,146). Heterozygous null *BMPR2* mutant mice do not develop spontaneous PH unless challenged by an inflammatory stimulus (147). This was recently confirmed in *BMPR2* monoallelic mutant rats, in which overexpression of an inflammatory mediator, 5-lipoxygenase, resulted in lung inflammation, development of severe PAH accompanied by extensive vascular remodelling with neointima formation and increased mortality rate (148). In addition, microvasculopathy and PAH development was reversed by a TGFβ antagonist in these *BMPR2* mutant rats, suggesting that the balance between BMPRII/TGFβRII signalling is crucial in neointimal formation (89,149).

Downregulation of BMPRII expression in HLMVECs is also accompanied by the upregulation of inflammatory genes including IL-6 and CXCL12 (150). In addition, PAECs isolated from PAH patients with a *BMPR2* mutation produced increased levels of IL-6 (24). This was confirmed in human PASMCs knocked down for *BMPR2*, resulting in increased IL-6 expression. In addition, it was demonstrated that

this occurred by alternative pathways including the non-canonical p38 MAPK pathway (151).

1.5.5. BMPRII and angiogenesis

As mentioned above, both proliferative and degenerative concepts contribute to the pathogenesis of PAH. While a loss of BMPRII expression results in hyperproliferation of PASMCs, it may promote PAEC apoptosis (152). Therefore, one can hypothesize that impaired BMPRII signalling and PAEC apoptosis would contribute to disordered angiogenesis in PAH. Accordingly, progenitor stem cells from *BMPR2* mutation carriers further differentiated into PAECs were found to display reduced angiogenic capacities *in vitro* compared to progenitor stem cells from *BMPR2* mutation non-carriers. In addition, in the presence of BMP4, cell migration and angiogenesis were enhanced in patient-specific induced pluripotent stem cell-derived endothelial cells (78). Whereas the density of pulmonary microvessels increased over time in wild type rats, it remained stable in rats expressing a *BMPR2* mutant (141). Tacrolimus or FK506, known to activate BMPRII signalling, is able to induce angiogenesis *in vitro* in the same extend as the angiogenic factor VEGF (153). The exact role of BMPRII and its function in angiogenesis is often contradictory and rather unexplored. By contrast, Long *and colleagues* state that BMP9 inhibits angiogenesis and prevents endothelial apoptosis (85).

Similarly, although BMPRII signalling promotes angiogenesis, TGFβRII displays anti-angiogenic properties, strengthening the hypothesis that a fair balance between TGFβRII and BMPRII signalling is required to maintain intact endothelium function. Interestingly, an ALK5 kinase inhibitor counteract the anti-angiogenic effect of TGFβ by enhancing EC growth and integrity in mouse embryonic stem cell-derived EC (154). However, contradicting results in later studies indicate that TGFβ ligands may stimulate angiogenic factors by lateral TGFβRII signalling through activation of type I receptor, ALK1. The relative expression levels of TGFβ seem to partially explain these discrepancies (155,156).

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Chapter 2: Rationale and objectives

Rationale

Endothelial dysfunction, a hallmark of PAH pathophysiology, affects pulmonary vasoreactivity, but also inflammatory response, endothelial barrier function and angiogenic capacity of pulmonary endothelial cells. However, current available PAH-specific therapies only target the consequences of the endothelial dysfunction through vasodilatory effects towards smooth muscle.

BMPR2 mutations represent a major risk factor for heritable PAH. BMPRII signalling is involved in inflammation, angiogenesis and endothelial barrier function. A current comprehensive view of i) the potential effects of BMPRII on the different aspects of endothelial dysfunction, and ii) the interaction between BMPRII signalling and PGI_2, NO and ET-1 therapeutic pathways is crucially missing.

PAH is a multifactorial disease and above all, each PAH patient responds differently to the available drugs. However, access to pulmonary tissue and pulmonary vascular cells from patients with PAH is rather limited, making the implementation of precision medicine approaches highly challenging. The current use of patient cells collected after lung transplantation provides limited translational applicability and mainly remains a tool for mechanistic studies.

We consequently hypothesize that impaired BMPRII signalling may affect different aspects of endothelial dysfunction and that vasodilatory PAH-specific drugs targeting PGI_2, NO and ET-1 pathways may interfere with angiogenic capacities and endothelial barrier function by interacting with BMPRII signalling. In addition, investigating the effects of PAH-specific drugs on PAECs collected at diagnosis could further orientate optimal treatment in individual patients.

General objectives

The general objective was to investigate the effects of PAH specific drugs *in vitro* on endothelium barrier function and angiogenic capacities, in a context of impaired BMPRII signalling. Alternatively, the aim was to overcome the limited access to pulmonary vascular cells from PAH patients by establishing a stable silencing of *BMPR2* in pulmonary microvascular endothelial cells to reproduce some pathophysiological aspects and by optimizing an innovative technique, which allows collecting PAECs at diagnosis in treatment-naïve patients and at follow-up.

We consequently aim to address the following objectives:

I. Develop and optimize alternatives to the limited access of primary pulmonary endothelial cells from patients with PAH (Chapter 3 & Chapter 5)
II. Investigate the effect of *BMPR2* silencing and a concomitant inflammatory insult on BMPRII signalling and on endothelial barrier function in pulmonary microvascular endothelial cells (Chapter 3);

III. Evaluate the effect of *BMPR2* silencing on PAEC angiogenic capacity of pulmonary microvascular endothelial cells (Chapter 4);

IV. Explore the effect of current PAH-specific drugs on BMPRII signalling, endothelial barrier function and angiogenic capacities in pulmonary microvascular endothelial cells (Chapter 4).

Cytokines trigger disruption of endothelium barrier function and p38 MAP kinase activation in *BMPR2*-silenced human lung microvascular endothelial cells

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Abstract

Background

The bone morphogenetic protein receptor II (BMPRII) signalling pathway is impaired in pulmonary arterial hypertension and mutations in the *BMPR2* gene have been observed in both heritable and idiopathic pulmonary arterial hypertension. However, all *BMPR2* mutation carriers do not develop pulmonary arterial hypertension, and inflammation could trigger the development of the disease in *BMPR2* mutation carriers. Circulating levels and/or lung tissue expression of cytokines such as tumor necrosis factor-a (TNFα) or interleukin-18 (IL-18) are elevated in patients with pulmonary arterial hypertension and could be involved in the pathogenesis of pulmonary arterial hypertension.

Hypothesis

We consequently hypothesized that cytokines could trigger endothelial dysfunction in addition to impaired BMPRII signalling. Our aim was to determine whether impairment of BMPRII signalling might affect endothelium barrier function and adhesiveness to monocytes, in response to cytokines.

Methods

BMPR2 was silenced in human lung microvascular endothelial cells (HLMVECs) using lentiviral vectors encoding microRNA-based hairpins. Effects of tumor necrosis factor-a and IL-18 on HLMVEC adhesiveness to the human monocyte cell line THP-1, adhesion molecule expression, endothelial barrier function and activation of p38 MAPK were investigated *in vitro*.

Results

Stable *BMPR2* silencing in HLMVECs resulted in impaired endothelial barrier function and constitutive activation of p38 MAPK. Adhesiveness of *BMPR2*-silenced HLMVECs to THP-1 cells was enhanced by tumor necrosis factor-a and IL-18 through ICAM-1 adhesion molecule. Interestingly, tumor necrosis factor-a induced activation of p38 MAPK and disrupted endothelial barrier function in *BMPR2*-silenced HLMVECs.

Conclusion

Altogether, our findings showed that stable *BMPR2* silencing resulted in impaired endothelial barrier function and activation of p38 MAPK in HLMVECs. In *BMPR2*-silenced HLMVECs, cytokines enhanced adhesiveness capacities, activation of p38 MAPK and impaired endothelial barrier function suggesting that cytokines could trigger the development of pulmonary arterial hypertension in a context of impaired BMPRII signalling pathway.

Introduction

Pulmonary arterial hypertension (PAH) is a severe and life-threatening disease resulting in right heart failure and need for lung transplantation in many patients. Despite the recent development of several specific therapies for PAH, there is still no cure and the prognosis of the disease remains poor (1). Current PAH-specific therapies mainly substitute pulmonary arterial endothelium-derived vasodilatory mediators, but do not reverse pulmonary vascular remodelling characterized by endothelium dysfunction, loss of pre-capillary pulmonary arteries and proliferation of pulmonary vascular cells resulting in obstruction of the vessel lumen.

Among patients harbouring a familial form of PAH, 70% carry an autosomal dominant mutation resulting in haploinsufficiency or loss-of-function of *bone morphogenetic protein receptor type 2* (*BMPR2*). *BMPR2* gene mutations are also present in 20% of sporadic cases of idiopathic PAH (2); mutations in other receptors of the transforming growth factor (TGFβ) family such as *activin receptor-like kinase-type 1* (3) and *endoglin* (4), and affecting BMP signalling including *Smad9* (5), *caveolin-1* (6) and *potassium channel sub-family K member 3* (KCNK3) (7) have been also identified in patients with PAH.

However, *BMPR2* mutation displays a low penetrance since only 20% of the *BMPR2* mutation carriers develop clinical symptoms of PAH. In addition, this low penetrance suggests that *BMPR2* mutation carriers may harbour enhanced susceptibility to an inflammatory insult, for instance (8). Endothelium dysfunction plays a major role in the initiation and the progression of PAH and is associated with impaired BMP signalling as illustrated by decreased pulmonary arterial endothelial cells (PAECs) survival in response to injury (9), impaired adhesion and migration (10,11), enhanced adhesiveness for monocytes in response to inflammatory mediators (12) and disordered angiogenesis (13). Reduced expression of BMPRII results in impaired canonical BMP signalling including Smad1/5/8 and Id proteins as well as in the implementation of compensatory alternative pathways including p38 MAPK pathway (14–17). Accordingly, inflammatory mediators may contribute to p38 MAPK activation in a context of impaired BMPRII function (18). Considering the potential role of inflammatory cytokines and chemokines including IL-1 (19), IL-6 (20), IL-8 (21), CCL2 (22), CXCL10 (23), CCL5 (24,25) and fractalkine (26) in PAH and the recent demonstration of IL-6 production by PAECs from *BMPR2* mutation carriers in response to inflammatory mediators (12), we hypothesized that cytokines could negatively affect the endothelial function in a context of impaired BMPRII signalling pathway. Interestingly, interleukin-18 (IL-18), a pro-inflammatory cytokine, implicated in the pathogenesis of atherosclerotic disease (27), may display adverse effects on endothelium function by inducing apoptosis and increasing permeability of pulmonary microvascular endothelial cells (ECs) (28), increasing the expression of

adhesion molecules in human dermal microvascular ECs (29) or inducing the expression of cytokines by ECs (30). In addition, plasma levels of IL-18 and its downstream chemokine, CXCL10, are increased in PAH patients, and medial pulmonary smooth muscle cells (SMCs) are a source of IL-18 and its receptor, IL-18R, in PAH patients (31). Moreover, IL-18 disruption has been recently shown to suppress hypoxia-induced PAH in mice (32). Eventually, tumor necrosis factor-a (TNFα), another pro-inflammatory cytokine, is involved in the pathogenesis of PAH (33), note-worthy by potentially contributing to endothelial dysfunction through p38 MAPK (34).

Due to the limited access to native PAECs from PAH patients, we knocked down *BMPR2* in human lung micro-vascular endothelial cells (HLMVECs) to obtain a stable *BMPR2*-silenced HLMVEC lineage, in which we investigated the effects of cytokines, including IL-18 and TNFα, on endothelium function and on activation of the p38 MAPK pathway.

Methods

Tissue collection

Lung parenchyma was collected at the time of lung transplantation from patients with idiopathic PAH (n=8), from control subjects at the time of lobectomy or pneumonectomy for suspected localized lung tumour (n=8) and from an unused donor lung (n=1). After collection, lung tissue was immediately snap-frozen and stored at 80°C until use. The study protocol was approved by the Institutional Ethics Committee of the University of Leuven and participants gave written informed consent. Demographic characteristics of patients and control subjects are briefly described in Table 3.1.

Table 3.1: Characteristic of control subjects and IPAH patients

	CONTROLS (n=9)	**IPAH (n=8)**
Age (years)	66 ± 12	46 ± 17
Gender (M/F)	5/4	4/4

Generation of lentiviral transfer plasmids and lentiviral vector production

Short interfering RNA (siRNA) sequence targeting human *BMPR2* mRNA at positions 1920, 4277 and 7048 were developed using siSearch software (http://www.dharmacon.com/DesignCenter/DesignCenterPage.aspx). The number refers to the respective positions in the reference human *BMPR2* mRNA (NM_001204.6) that are recognized by the siRNA. Based on this sequence, simian immunodeficiency virus (SIV)-based lentiviral vectors, gift of D. Nègre (ENS Lyon, France) and encoding microRNA 30 (miR30)-based knockdown hairpins derived from the aforementioned siRNA, were generated to allow stable knockdown as previously described (35) (referred to as LV_miR_*BMPR2*_1920, LV_miR_*BMPR2*_4277 or LV_miR_*BMPR2*_7048, respectively), in addition to the control hairpins directed against the firefly luciferase (fLuc; LV_miR_fLuc).

The transfer plasmid constructs, pGAE SIV SFFV-eGFP-P2Azeo-miRNA-Hs*BMPR2*-WPRE and pGAE-SFFV-eGFPP2A-zeo-miRNA-fLuc-WPRE contain a zeocin resistance cassette (zeo) driven from an SFFV (spleen focus forming virus) long terminal repeat promoter, followed by the respective miRs and the WPRE (woodchuck hepatitis virus posttranscriptional regulatory element). In addition, the constructs contain the cDNA for enhanced green fluorescent protein (eGFP) cassette as a reporter gene that allows monitoring of the transduced cells by flow cytometry. The eGFP reporter cDNA and the ZeoR cDNA are connected by a peptide2A sequence, which allows equimolar expression of both proteins from the same mRNA transcript (36). All cloning steps were sequence verified. All lentiviral vector plasmids were designed and cloned at the Leuven Viral Vector Core and vector production was performed as previously described (36). Briefly, vesicular stomatitis virus glycoprotein (VSV-G) pseudo

typed lentiviral vector particles were produced by triple transient polyethylenimine transfection in HEK293T cells using pMDG.2, which encodes the vesicular stomatitis virus glycoprotein envelope, pAD_SIV3+, packaging plasmid and the transfer plasmid pGAE-SFFV-eGFP-P2A-zeo-miRNA-Hs*BMPR2*-WPRE, to generate LV_miR_*BMPR2*_1920; _4277; _7048. In parallel, a control vector was produced with miR-based hairpins which target the mRNA of fLuc, resulting in LV_miR_fLuc. The latter vector will be referred to as ‘‘control’’ throughout the text. Quality control tests for lentiviral vector production were conducted: transduction units/mL were assessed on 293T cells and p24 concentrations in pg/mL was determined by p24/p27 ELISA (Innotest HIV Ag mAb 480T, Innogenetics-Fujirebio).

Lentiviral vector transduction efficiency in HLMVEC

Stable *BMPR2* knockdown and controls in HLMVEC were generated following lentiviral transduction. Briefly, HLMVECs were seeded in a T75 flask at a density of 200,000 cells. When HLMVECs were 40% confluent, cells were transduced with a serial dilution series of lentiviral vectors LV_miR_*BMPR2*_1920, LV_miR_*BMPR2*_4277, LV_miR_*BMPR2*_7048 and LV_miR_fLuc, as control. After 48 h, the medium was replaced with growth medium containing 200 mg/mL zeocin to select transduced cells. Transduction efficiency was evaluated by flow cytometry for eGFP expression. Transduction efficiencies in HLMVECs (% eGFP positive cells in the population) were similar for the different lentiviral vectors, LV_miR_*BMPR2*_7048, LV_miR_*BMPR2*_4277, LV_miR_*BMPR2*_1920, for the respective dilutions (Figure S3.1A). Expression of eGFP was slightly lower in HLMVECs transduced with the control vector (1/20: 90.7%; 1/40: 71.7%; 1/80: 62.2%). Consequently, we opted for a 1/40 dilution of the lentiviral vectors and determined the relative *BMPR2* mRNA expression at this dilution. Relative *BMPR2* mRNA expression was significantly lower (ANOVA, $p<0.01$) in HLMVECs transduced with LV_miR_*BMPR2*_4277 ($7.5 \times 10^{-4} \pm 2.0 \times 10^{-4}$) or LV_miR_*BMPR2*_1920 ($2.5 \times 10^{-5} \pm 7.0 \times 10^{-5}$) compared with LV_miR_*BMPR2*_7048 (2.2 x 10-3 ± 6.0 x 10-4) or control vector (2.5 x 10-3 ± 7.0 x 10-5) (Figure S3.1B). The viability of HLMVECs transduced with the control vector was 95.3 ± 1.6% and with LV_miR_*BMPR2*_1920 was 97.0 ± 0.64%. The percentage of HLMVECs expressing eGFP were 78.8 ± 10.4% for the control vector and 89.2 ± 9.4% for LV_miR_*BMPR2*_1920 (Figure S3.1C). Consequently, LV_miR_*BMPR2*_1920 was chosen and named as *BMPR2*-KD.

Phenotyping of HLMVECs

Lentiviral vector-transduced HLMVECs were phenotyped by labeling cells with Dil-Ac-LDL and by immunofluorescence using antibodies against human CD31, VE-cadherin and von Willebrand factor (vWF), as previously described (37). To quantify immunofluorescence staining, five images from non-overlapping fields on each slide were captured at 40x magnification. After having separated the

different channels, red staining was measured using the ImageJ software and expressed as arbitrary units (AU). In addition, CD31 positive, viable HLMVECs were quantified by flow cytometry (FacsCanto II, Becton Dickinson) as described elsewhere.12

Cell adhesiveness assay

HLMVECs were seeded at a density of 12,500 cells/cm^2 on gelatin-coated 12 well-plates. Subconfluent HLMVECs were starved in starving medium for 24 h. The cells were stimulated with human recombinant TNFα (10 ng/mL) or IL-18 (10 ng/mL) for 3 h. Human monocytic THP-1 cells were radio-labeled with 1 mCi [3H]-thymidine per 10^6 cells for 48 h and added (5 x 10^5 per well) to the endothelial cell monolayer for 3 h at 37°C. Non-adherent cells were washed out. Radioactivity incorporated into monocytes in suspension and attached to the EC monolayer was quantified as previously described (38). Data are expressed as percentage of adhering cells over cells initially added.

Assessment of HLMVEC permeability

HLMVECs were seeded onto 24-well Transwell inserts (6.5mm diameter, 0.4 mm pore size, Corning) at full confluence (100,000 cells/insert) to ensure formation of endothelial monolayer. After 24 h, HLMVECs were starved for 18 h. The HLMVEC monolayer was then stimulated with TNFα (10 ng/mL) or IL-18 (10 ng/mL) for 4 h, washed twice with starvation medium, and 200 mL fluorescein isothiocyanate (FITC)-labeled bovine serum albumin (BSA; 0.1 mg/mL) in starvation medium was added to the upper chamber and 1mL of starving medium was added to the lower chamber. Leakage of FITC-labeled BSA into the lower chamber was assessed by collecting 50 mL of sample from the lower chamber at baseline, after 30 min, 1, 2 and 4 h. Absorbance was measured at 450nm using a FLUOstar Omega microplate reader (BMG Labtech). Concentrations of BSA were calculated and plotted vs. time; area under the curve (AUC) was calculated for each condition.

Adhesion molecule expression

Subconfluent HLMVECs seeded onto fibronectin-coated chamber slides were starved for 18 h and further stimulated with 10 mg/mL TNFα or IL-18 for 3 h at 37°C. HLMVECs were not permeabilized in order to detect adhesion molecules expressed only at the cell surface. ICAM-1 and VCAM-1 expression was detected by immunofluorescence using antibodies against ICAM-1 and VCAM-1 as previously described (12). Quantification of immunofluorescence staining was performed as mentioned above.

BMPR2, IL-18 and IL-18R mRNA expression

Total RNA was extracted from pulmonary tissue and HLMVECs; mRNA expression levels of *BMPR2*, IL-18, IL-18 receptor (IL-18R) and the housekeeping gene β-actin were determined by qrtPCR as previously described (12).

Western blotting

HLMVECs were grown to 90% confluence in six well plates and starved in starving medium for 24 h and further stimulated with 10 mg/mL TNFα or IL-18 for 1 h at 37°C. HLMVECs were washed twice in ice-cold phosphate-buffered saline, pH 7.4 and lysed for 30 min at 4°C in ice-cold RIPA buffer. The samples were centrifuged at 12,000 g for 15 min and protein concentrations were determined using the Bradford method. Proteins were separated by SDS-PAGE and transferred to polyvinylidene fluoride filters by electroblotting. Filters were blocked in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-Tween) and either 3% BSA or 5% non-fat dry milk for 1 h at room temperature (RT). Filters were incubated with primary antibodies overnight at 4°C in TBS-Tween containing either 3% BSA or 5% non-fat dry milk. Horseradish peroxidase-conjugated secondary antibodies were incubated in TBS-Tween for 1 h at RT and peroxidase staining was revealed by chemiluminescence, imaged and analysed.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 7.03 (GraphPad Software Inc., La Jolla, California). Differences between groups were analysed using Student's t-test, one-way or two-way ANOVA followed by a post-hoc Tukey test. All p-values are for two-sided tests. A value of $p<0.05$ was considered statistically significant. Continuous and normally distributed values are expressed as mean ± SD. A detailed method section is available in the online supplemental data.

Results

Phenotyping of lentiviral vector-transduced HLMVECs

HLMVECs transduced with the control vector and *BMPR2*-silenced HLMVECs both expressed CD31 (Figure 3.1a and b) and VE-cadherin (Figure 3.1c and d) at their surface, contained vWF in Weibel-Palade bodies (Figure 3.1e and f) and demonstrated Ac-LDL uptake (Figure 3.1g and h). Immunofluorescence staining was further quantified and did not show any significant difference in the expression of specific endothelial markers, e.g. CD31, VE-cadherin, vWF and Ac-LDL uptake, between HLMVECs transduced with the control vector and *BMPR2*-silenced HLMVECs (Figure S3.2).

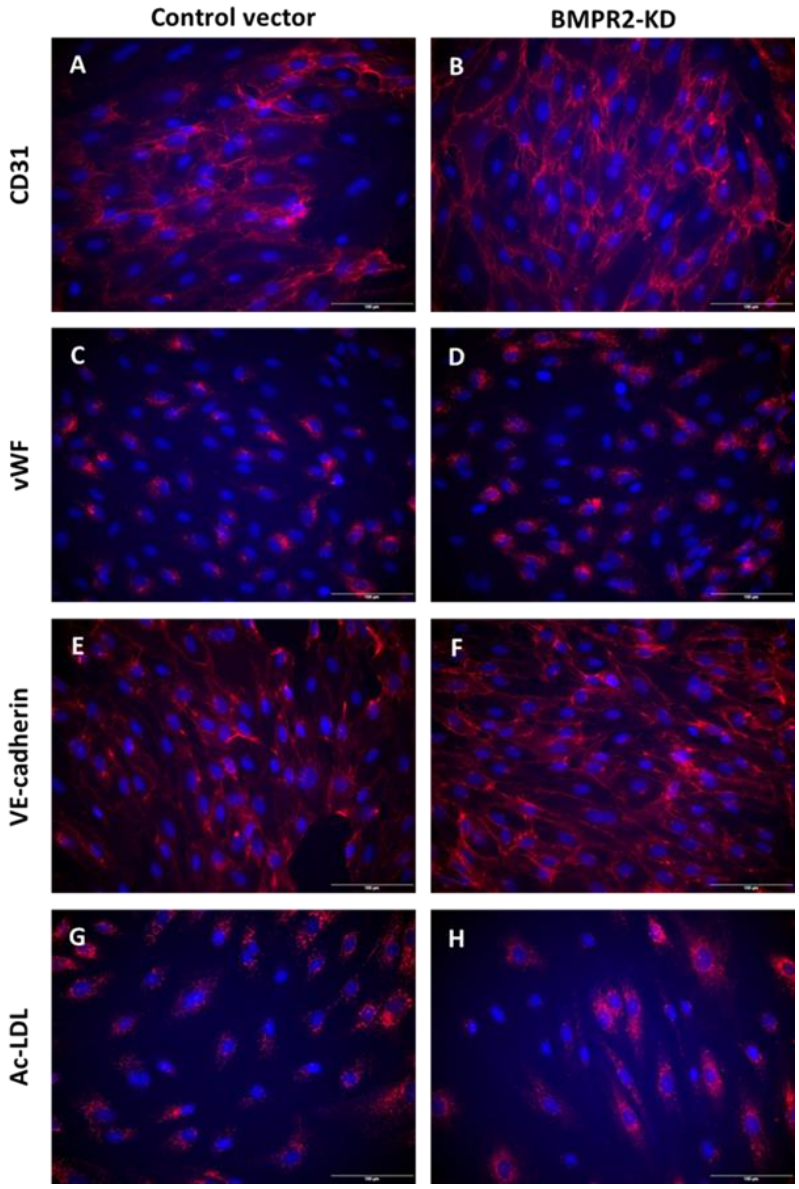

Figure 3.1: Phenotyping of HLMVECs knocked-down for *BMPR2*. HLMVECs transduced with control or *BMPR2*-KD lentiviral vectors were immuno-labelled with antibodies raised against human CD31 (A, B), vWF (C, D), VE-cadherin (E, F) and stained with DiI-Ac-LDL (G, H). Nuclei were counterstained using DAPI (blue). Experiments were performed in HLMVECs at passage 5. Scale: 100 µm.

BMPR2 silencing affects BMP signalling

Lentiviral vector transduction of HLMVECs with *BMPR2*-KD vector resulted in an 89% decrease in *BMPR2* mRNA expression (p=0.0001; Figure 3.2a) and an 87% decrease in BMPRII protein expression (p=0.0001; Figure 3.2b and c), compared to cell transduced with control vector. We further observed that in resting HLMVECs knocked down for *BMPR2*, Smad1/5/8 phosphorylation (0.42 ± 0.13) was significantly lower (p=0.015) compared with resting control HLMVECs (0.81 ± 0.13; Figure 3.3a and b). In addition, we found that silencing of *BMPR2* in resting HLMVECs resulted in significantly higher phosphorylation levels of p38 MAPK compared with resting control HLMVECs (0.78 ± 0.11 vs. 0.52 ± 0.07; p=0.003; Figure 3.3c and d).

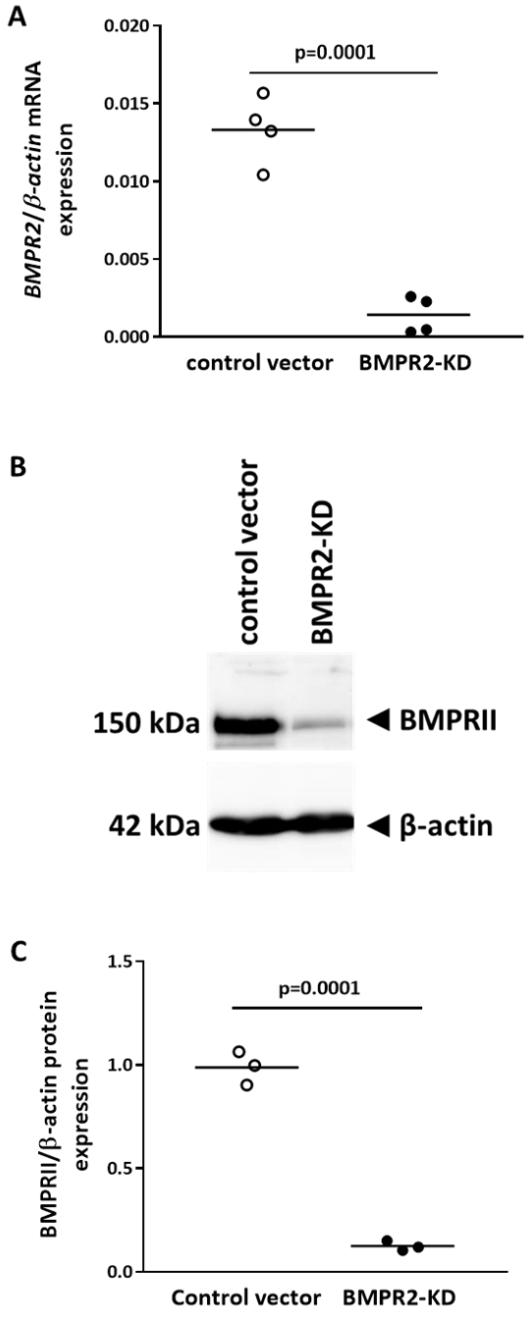

Figure 3.2: BMPRII expression in HLMVECs transduced with control or *BMPR2*-KD lentiviral vectors. (A) Relative *BMPR2* mRNA expression. Independent experiments were performed in quadruplicate for HLMVECs between passages 4 and 7. (B) Representative Western blot of BMPRII and β-actin protein expression. (C) Quantitative expression of BMPRII protein. Independent experiments were performed in triplicate in HLMVECs between passages 4 and 7.

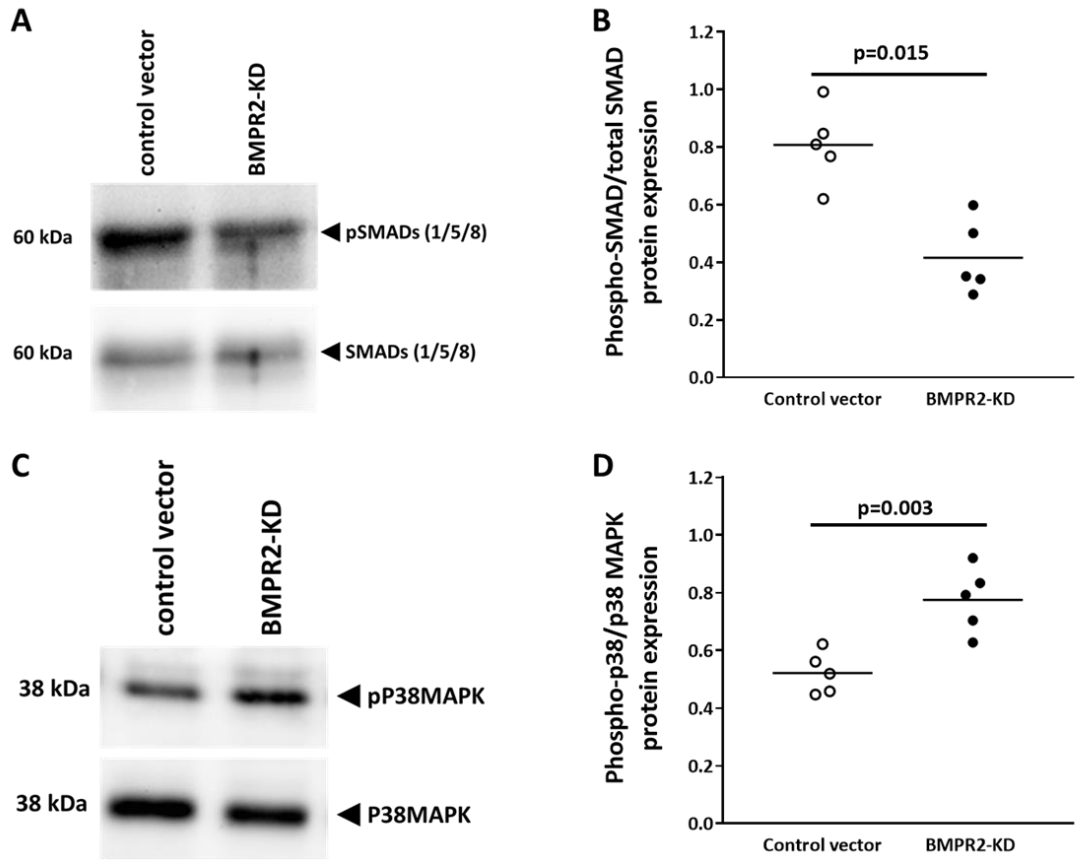

Figure 3.3: Activation of Smad1/5/8 and p38 MAPK proteins in HLMVECs transduced with control or *BMPR2*-KD lentiviral vectors. (A) Representative Western blot of phosphorylated and total Smad1/5/8 proteins. (B) Quantitative expression of phosphorylated versus total Smad1/5/8 proteins. Results are presented as relative protein expression ratio of phospho-Smad and total-Smad. Independent experiments were performed in quintuplicate for HLMVECs between passages 4 and 7. (C) Representative Western blot of phosphorylated and total p38 MAPK proteins. (D) Quantitative expression of phosphorylated versus total p38 MAPK proteins. Results are presented as relative protein expression ratio of phospho-p38 MAPK and total-p38 MAPK. Independent experiments were performed in quintuplicate in HLMVECs between passages 4 and 7.

BMPR2 silencing negatively affects endothelium function

Next, we determined the effect of *BMPR2* silencing on HLMVEC barrier function. We assessed the leakage of FITC-labeled BSA through the monolayer of HLMVECs transduced with either control or *BMPR2*-KD vectors over time. The increase in FITC-labeled BSA leakage through the monolayer of resting *BMPR2*-silenced HLMVECs was significantly higher compared to resting control HLMVECs over time (Figure 3.4a). After a 4-h period, BSA-FITC leakage was significantly higher (p=0.002) through *BMPR2*-silenced HLMVECs compared to control HLMVECs (2.07 ± 0.10 vs. 1.58 ± 0.16; Figure 3.4b). Considering that activated endothelium may influence the attraction and infiltration of inflammatory cells, we investigated whether impaired BMPRII signalling may affect the adhesiveness capacities of HLMVECs to monocytes. We did not observe any difference in the amount of adhering human monocytic cells (THP-1) between resting *BMPR2*-silenced HLMVECs and control HLMVECs (28.9 ± 3.4% vs. 28.4 ± 3.3%; Figure 3.4c). Accordingly, we did not find any differential expression in adhesion

molecules ICAM-1 and VCAM-1 at the surface of resting *BMPR2*-silenced HLMVECs and control HLMVECs (Figure 3.5a, d, g, j; Figure 3.7c–f).

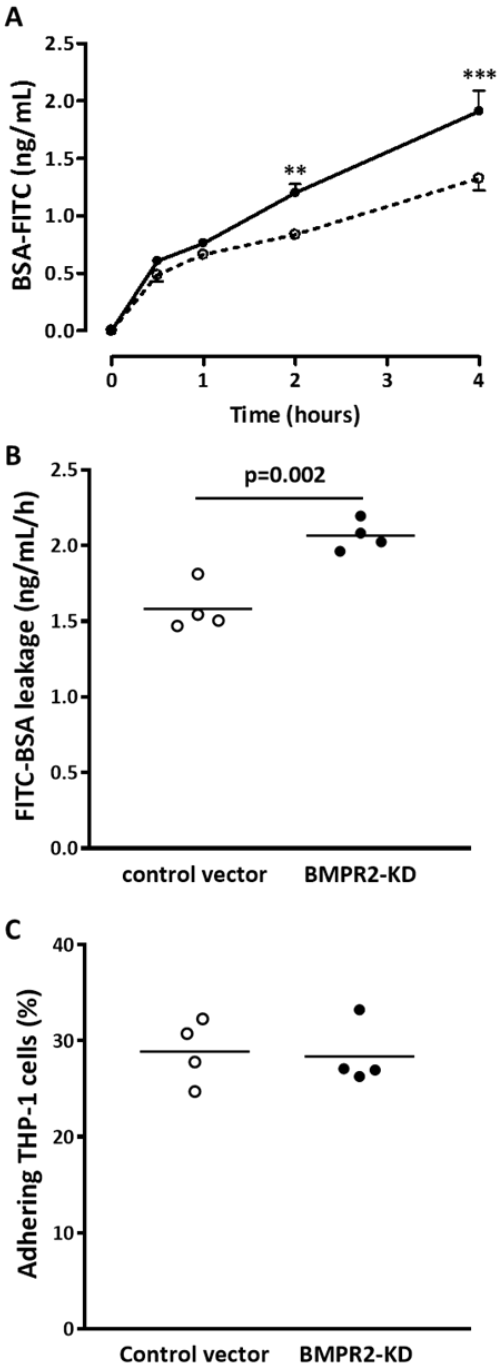

Figure 3.4: *Barrier function and adhesiveness to monocytes of HLMVECs. Leakage of FITC-labelled bovine serum albumin (BSA) through HLMVEC monolayers seeded onto Transwell® inserts, transduced with control or BMPR2-KD lentiviral vectors, was assessed after 1, 2, 3 and 4h in resting HLMVECs. (A) BSA-FITC leakage over time in one independent experiment performed in triplicate; (dotted line, control vector; full line, BMPR2-KD); **p<0.001, ***p<0.0001 vs. control vector. (B) BSA-FITC leakage after 4 hours in three independent experiments performed in triplicate or quadruplicate in HLMVECs between passages 4 and 7. Results are expressed as BSA-FITC leakage (ng/mL/hour). (C) Adhesiveness of resting HLMVECs, transduced with control or BMPR2-KD lentiviral vectors, to the monocytic cell line THP-1 in four independent experiments performed in triplicate in HLMVECs between passages 4 and 7.*

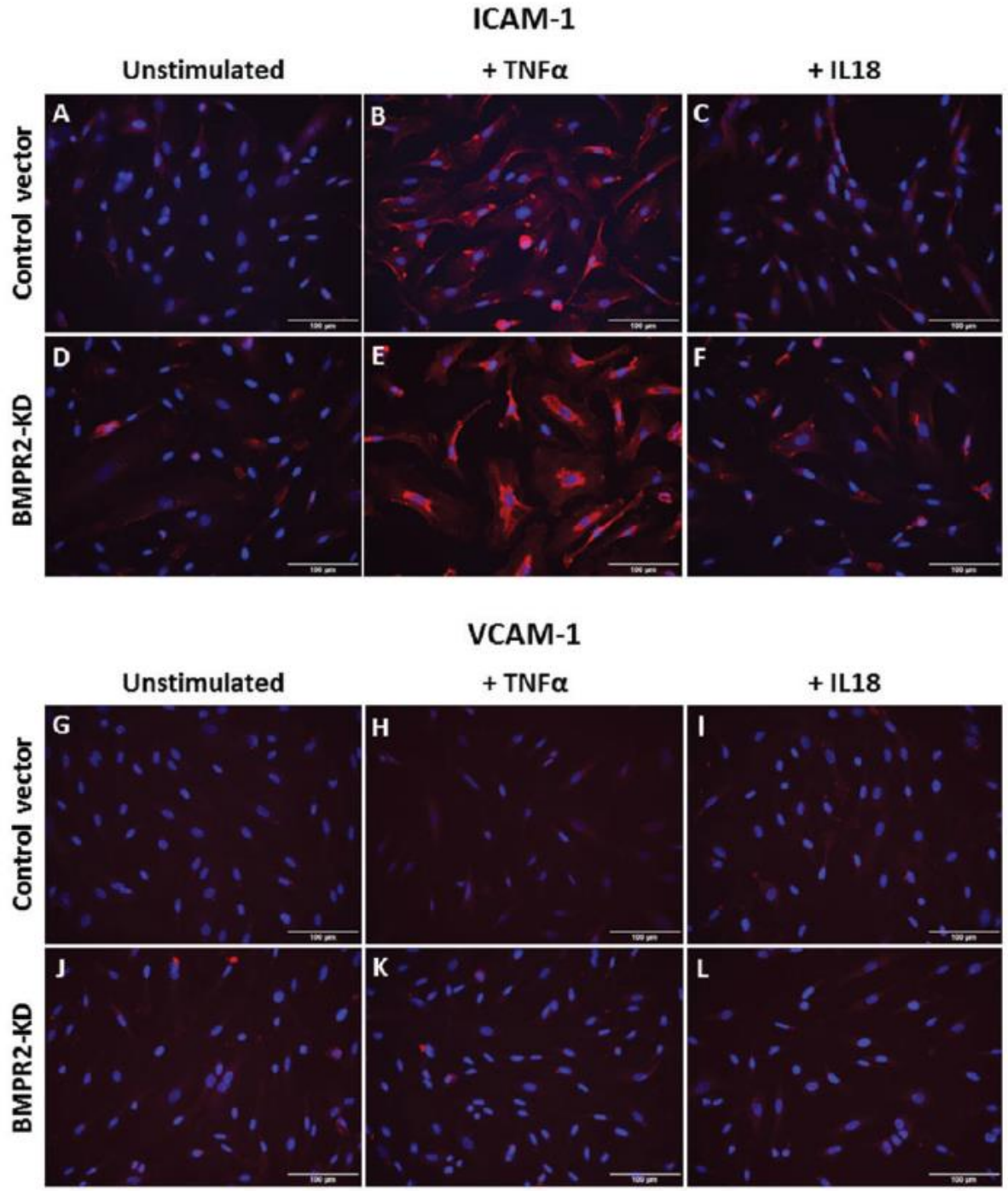

Figure 3.5: *Effect of TNFα and IL-18 on adhesion molecule ICAM-1 and VCAM-1 expression in HLMVECs. Expression of ICAM-1 (a-f) and VCAM-1 (g-i) in HLMVECs transduced with control (a-c; g-l) or BMPR2-KD lentiviral vectors (d-f; j-l), at rest (a, d, g, j), or in response to TNFα (b, e, h, k) or IL-18 (c, f, I, l). Experiments were performed in HLMVECs between passages 4 and 7. Scale: 100 µm.*

TNFα enhances p38 MAPK activation in BMPR2-silenced HLMVECs

We found that expression of IL-18 mRNA was 1.6-fold higher in pulmonary tissue from patients with IPAH compared with controls (p=0.02; Table 3.2). By contrast, expression of IL-18 receptor mRNA was similar in pulmonary tissue from patients with IPAH compared with controls (Table 3.2).

Table 3.2: Expression of IL-18 and IL-18R mRNAs in human pulmonary tissue samples

	Controls	IPAH	p-Value
IL18	$7.5 \times 10^{-3} \pm 2.8 \times 10^{-3}$	$12.4 \times 10^{-3} \pm 4.7 \times 10^{-3*}$	***0.02***
IL18R	$4.3 \times 10^{-3} \pm 2.8 \times 10^{-3}$	$8.1 \times 10^{-3} \pm 5.9 \times 10^{-3}$	0.10

Moreover, considering that the non-canonical p38 MAPK pathway is involved in the inflammatory process mediated by cytokines such as TNFα (33), we investigated whether IL-18 and TNFα could further activate p38 MAPK in *BMPR2*-silenced HLMVECs. We observed that IL-18 did not induce any significant increase in p38 MAPK activation both in control (0.52 ± 0.07 vs. 0.58 ± 0.15; p=0.39) and *BMPR2*-silenced HLMVECs (0.77 ± 011 vs. 0.85 ± 0.11; p=0.30; Figure 3.6b and d). By contrast, TNFα induced a significant increase in p38 MAPK phosphorylation in *BMPR2*-silenced HLMVECs (0.77 ± 0.11 vs. 1.01 ± 0.17; p=0.03), but not in control HLMVECs (0.52 ± 0.07 vs. 0.69 ± 0.23; p=0.10; Figure 3.6a and c).

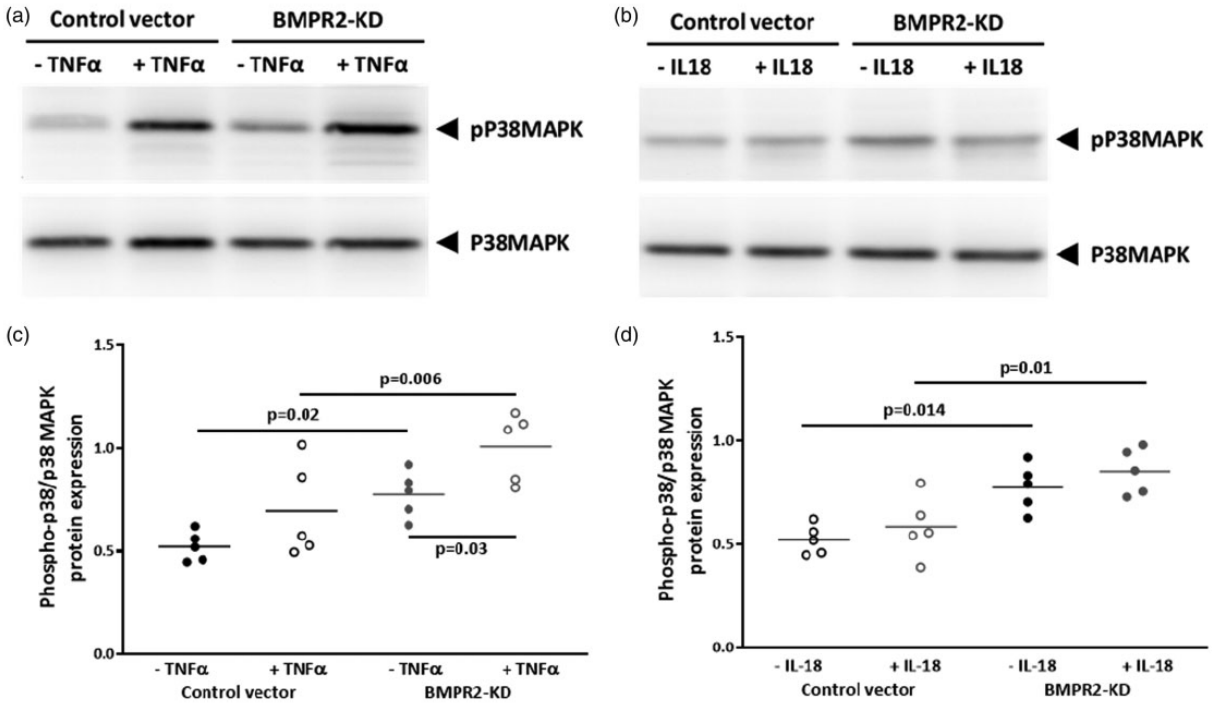

Figure 3.6: Effect of TNFα and IL-18 on activation of p38 MAPK proteins in HLMVECs. Representative Western blot of phosphorylated and total p38 MAPK proteins in HLMVECs transduced with control or *BMPR2*-KD lentiviral vectors stimulated with TNFα (a) or IL-18 (b). Quantitative expression of phosphorylated vs. total p38 MAPK proteins in HLMVECs stimulated with TNFα (c) or IL-18 (d). Results are presented as relative protein expression ratio of phosphor-p38 MAPK and total-p38 MAPK. Independent experiments were performed in quintuplicate for HLMVECs between passage 4 and 7.

TNFα and IL-18 enhance adhesiveness of BMPR2-silenced HLMVECs

Adhesiveness of *BMPR2*-silenced HLMVECs to THP-1cells was significantly higher compared to that of control HLMVECs in the presence of IL-18, (fold-increase: 1.18 ± 0.35 vs. 0.83 ± 0.06, p=0.03; Figure 3.7b) as well as in the presence of TNFα (fold-increase: 2.31 ± 0.75 vs. 1.35 ± 0.36, p=0.03; Figure 3.7a). In resting control HLMVECs or *BMPR2*-silenced HLMVECs, both ICAM-1 and VCAM-1 adhesion molecules were poorly expressed at the cell surface (Figure 3.5a, d, g, j). TNFα significantly induced the expression of ICAM-1 at the surface of *BMPR2*-silenced HLMVECs (Figure. 3.5d, e and Figure 3.7c), but not at the surface of control HLMVECs (Figure 3.5a, b and Figure 3.7c). IL-18 did not induce the expression of ICAM-1 at the surface of both control and *BMPR2*-silenced HLMVECs (Figure. 3.5a–f and Figure 3.7d). By contrast, neither IL-18 nor TNFα induced VCAM-1 expression at the surface of control and *BMPR2*-silenced HLMVECs (Figure 3.5g–l and Figure 3.7e, f). These results suggest that TNFα-induced adhesiveness of *BMPR2*-silenced HLMVECs could be attributed to enhanced ICAM-1 expression at their surface.

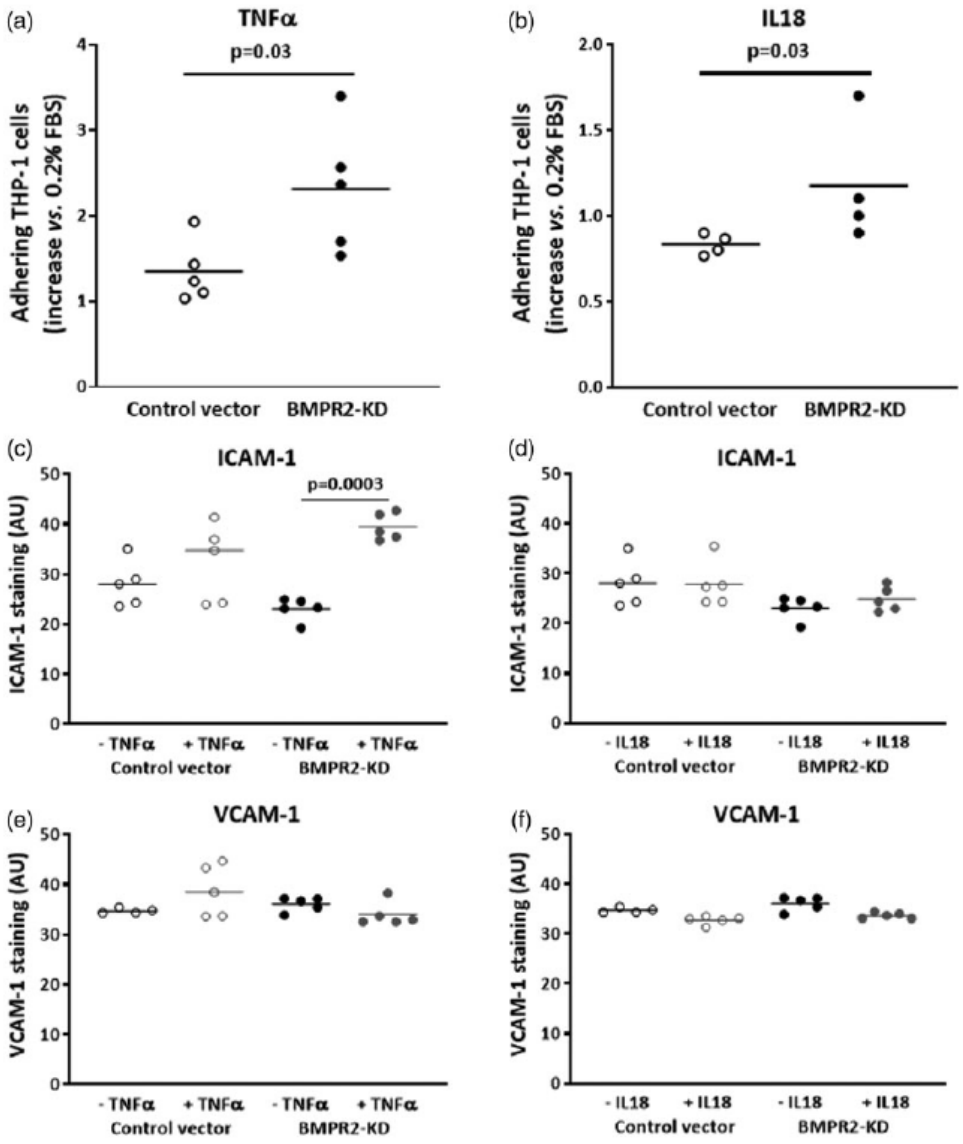

Figure 3.7: Effect of TNFα and IL-18 on adhesiveness to monocytes and quantitative expression of adhesion molecules ICAM-1 and VCAM-1 in HLMVECs. Adhesiveness of HLMVECs, transduced with control or *BMPR2*-KD lentiviral vectors, to the monocytic cell line THP-1 in response to TNFα (a) or IL-18 (b). Results were expressed as fold-increase vs. resting HLMVECs (0.2% FBS); four to five independent experiments were performed in triplicate in HLMVECs between passage 4 and 7.

TNFα impairs endothelial barrier function in BMPR2-silenced HLMVECs

TNFα significantly reduced endothelium barrier function in both control and *BMPR2*-silenced HLMVECs (AUC: 2.29 ± 0.16 vs. 2.96 ± 0.20 ng/ml/h, p=0.049 and 3.29 ± 0.13 vs. 4.02 ± 0.13 ng/ml/h, p=0.031; Figure 3.8a and c). The TNFα induced loss of endothelium barrier function was similar in *BMPR2*-silenced and control HLMVECs (31 ± 9% vs. 23 ± 3% increase, p=0.42). IL-18 did not display any effect on endothelium barrier function both in control HLMVECs or *BMPR2*-silenced HLMVECs (AUC: 2.29 ± 0.16 vs. 2.24 ± 0.21 and 3.29 ± 0.13 vs. 3.48 ± 0.213 ng/ml/h; Figure 3.8b and d).

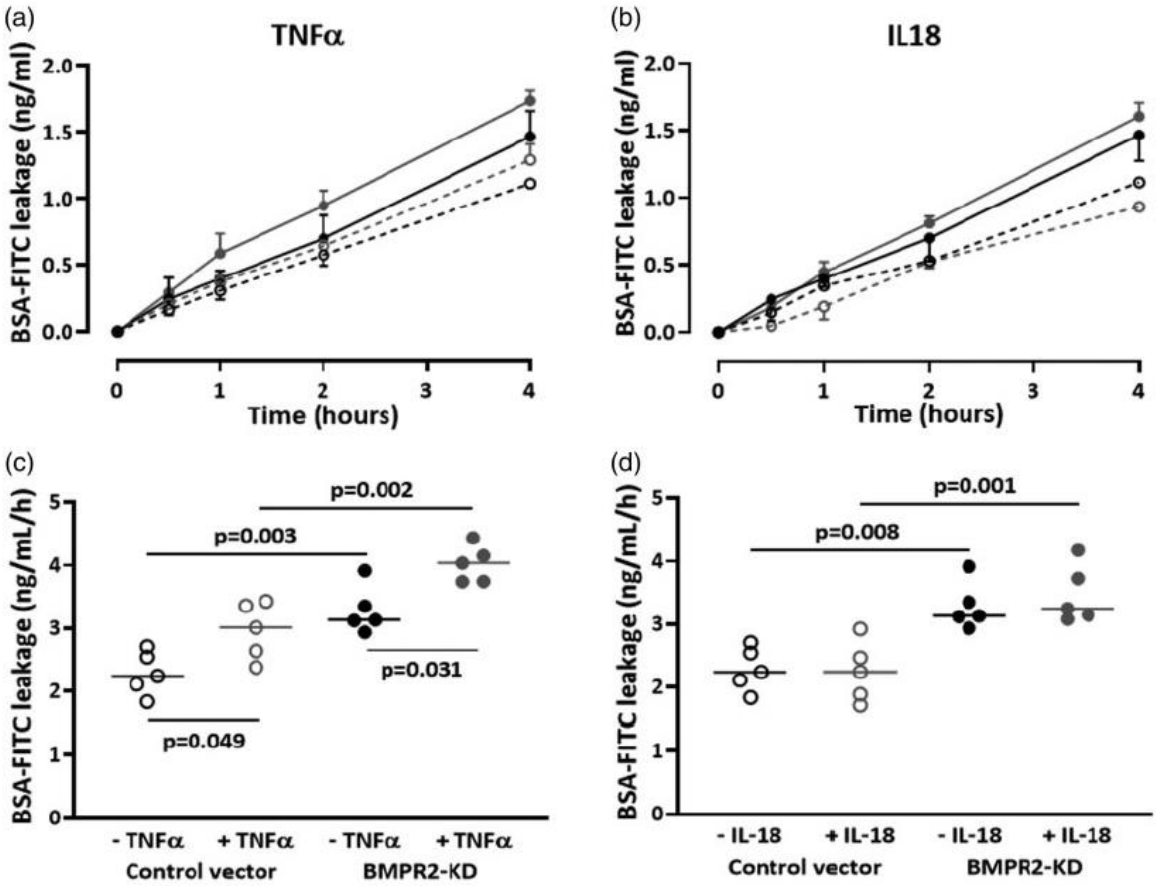

Figure 3.8: Effect of TNFα and IL-18 on barrier function of HLMVECs. Leakage of BSA-FITC through HLMVEC monolayers seeded onto Transwell® inserts, transduced with control or *BMPR2*-KD lentiviral vectors, was assessed after 30 min, 1, 2 and 4h. BSA-FITC leakage over time in one independent experiment perfomed in triplicate, in response to TNFα (a) or IL-18 (b). BSA-FITC leakage after 4h in five independent experiments performed in triplicate or quadruplicate in HLMVECs between passage 4 and 7, in response to TNFα (c) and IL-18 (d). Results are expressed as BSA-FITC leakage (ng/m:/h).

Discussion

In the present study, stable silencing of *BMPR2* in HLMVECs resulted in reduced expression of BMPRII protein, decreased phosphorylation of Smad1/5/8 proteins, loss of endothelium barrier function and activation of p38 MAPK. Additionally, cytokines such as IL-18 and TNFα induced adhesiveness of *BMPR2*-silenced HLMVECs to THP-1 monocytic cells, presumably through the adhesion molecule ICAM-1. Finally, TNFα-induced loss of endothelium barrier function in *BMPR2*-silenced HLMVECs was accompanied by p38 MAPK activation. Altogether, these results suggest that in the absence of functional BMPRII signalling, cytokines such as IL-18 and TNFα impair endothelial function, and concomitantly activate p38 MAPK.

Effects of reduced BMPRII expression on pulmonary endothelial function

We found that stable silencing of *BMPR2* in HLMVECs resulted in significantly decreased activation of the canonical downstream effectors of BMPRII, namely Smad1/5/8 proteins, similar to the decrease in Smad protein activation observed in PASMCs from IPAH patients (39), whereas transient *BMPR2* silencing in HLMVECs or PASMCs did not alter Smad1/5/8 protein phosphorylation (14,40). Interestingly, we observed that impairment of BMPRII signalling resulted in a loss of the endothelium barrier function. Accordingly, transient *BMPR2* silencing resulted in increased permeability of human PAEC monolayer (41); in addition, an increase in pulmonary vascular reactivity has been observed in vivo in heterozygous *BMPR2*-deficient mice (21,42). These findings, together with the demonstration that SRC-dependent caveolar dysfunction may contribute to endothelial barrier dysfunction of PAECs from heterozygous null *Bmpr2*$^{-/-}$ mutant mice (42), suggest that BMPRII plays a role in maintaining pulmonary endothelial barrier function. This is also consistent with a previous study whereby (i) BMP9 prevented LPS-induced loss of endothelial barrier function of blood outgrowth EC monolayers from PAH patients with *BMPR2* mutations and (ii) silencing of Smad1/5/8 eliminated the capacity of BMP9 to enhance BMPRII expression in PAECs (43). In addition, our current findings showing a concomitant loss of endothelial barrier function and decreased in Smad protein activation in *BMPR2*-silenced HLMVECs confirms that canonical Smad signalling is important in maintaining PAEC barrier function. Surprisingly, *BMPR2* silencing did not affect either HLMVEC adhesiveness properties to monocytic cells, or the expression of the adhesion molecules ICAM-1 and VCAM-1, whereas we previously found increased adhesiveness to monocytic cells concomitant with increased expression of ICAM-1 mRNA in HLMVECs isolated from *BMPR2* mutation carriers PAH patients (12). Discrepancies between HLMVECs from PAH patients with a *BMPR2* mutation and *BMPR2*-silenced HLMVECs could be attributed to the use of siRNA to knockdown *BMPR2*. Whereas siRNA targets cellular mRNAs, mutations in patients are permanent alterations in the structure of the *BMPR2* gene, which can be responsible for alterations in the trafficking of BMPRII to the cell surface (44). In this respect, cysteine substitution in the ligand-

binding or kinase domains results in reduced trafficking of mutant BMPRII to the cell surface, whereas variants carrying non-cysteine mutations in the kinase domain reaches the cell surface, but fail in activating Smad signalling pathway (16).

In parallel to a decrease in canonical Smad-dependent BMPRII signalling pathway, we also observed that *BMPR2* silencing resulted in increased activation of noncanonical Smad-independent p38 MAPK. Accordingly, increased phosphorylation of p38 MAPK has been observed in hypoxia- and monocrotaline-induced PH in rats (45,46) as well as in heterozygous *BMPR2* deficient and mutant mice (47). In addition, a loss of BMPRII expression in PASMCs results in activation of p38 MAPK (48). These results suggest that impaired BMPRII function may result in abnormal activation of alternative pathways such as the p38 MAPK pathway.

BMPR2 silencing and cytokine-induced endothelial dysfunction

Considering that effects of the cytokines IL-18 or TNFα on the endothelium are potentially mediated by activation of p38 MAPK (49,50), we consequently aimed to investigate whether IL-18 and TNFα would (i) worsen endothelial dysfunction in addition to silencing of *BMPR2* and (ii) activate the non-canonical p38 MAPK pathway. Despite higher levels of IL-18 mRNA observed in lung parenchyma from PAH patients, IL-18 failed to further increase *BMPR2* silencing-induced p38 MAPK activation in HLMVECs. By contrast, a synergic effect of TNFα and *BMPR2* silencing on p38 MAPK activation was observed in HLMVECs. These results indicate that TNFα, but not IL-18, is able to potentiate the non-canonical Smad-independent p38 MAPK pathway, in a context of a loss of BMPRII in HLMVECs. Accordingly, loss of BMPRII induced prolonged phosphorylation of p38 MAPK in response to TNFα in human PAECs. In parallel, we observed that TNFα was able to potentiate the effects of a loss of BMPRII expression on the pulmonary endothelium barrier function. Endothelial barrier compromise is accompanied by an activation of the p38 MAPK cascade in human PAECs (51); in rats with hypoxia-induced PH, whereby BMPRII expression is reduced in the lungs, p38 MAPK inhibitor reversed impaired endothelium dependent relaxation in isolated pulmonary artery rings (52). In addition, TNFα has been previously shown to induce endothelium permeability, concomitantly with an activation of p38 MAPK (49); treatment with TNFα resulted in increased permeability of *BMPR2* knockdown human PAEC monolayers (41). Although IL-18 has been previously shown to induce vascular SMC proliferation and migration (53,54), and increase permeability of rat PMVEC monolayers (28), we did not observe any additional effects of IL-18 on endothelial barrier function in HLMVECs with a loss of BMPRII. Whereas silencing of *BMPR2* had no effect on adhesiveness capacities of HLMVECs to monocytic cells, we observed that both IL-18 and TNFα induced adhesion of *BMPR2*-silenced HLMVECs to THP-1. Accordingly, we previously found that CRP and TNFα increased adhesion of U937 monocytic cells to PMVECs isolated from PAH patients with a *BMPR2* mutation (12). Interestingly, lentiviral vector-

mediated overexpression of *BMPR2* in HLMVECs has been recently shown to suppress LPS-induced neutrophil-endothelial adhesion (55). TNFα-induced adhesiveness was mainly attributable to induced expression of ICAM-1. In addition, we have also observed that PMVECs isolated from PAH with a *BMPR2* mutation displayed increased mRNA or protein expression of ICAM-1 in response to CRP and TNFα (12). Inversely, when *BMPR2* is overexpressed in HLMVECs, LPS-induced ICAM-1 expression was dramatically reduced (55). Eventually, our results are in line with the hypothesis that coincidence of *BMPR2* heterozygosity with an inflammatory insult is required to develop PAH (56).

Limitations

Findings of the present study are mostly based on *in vitro* investigation, using ECs in which *BMPR2* has been depleted. Silencing does not exactly reproduce the pathological situation of *BMPR2* mutations and *in vitro* cellular assays only allow to investigate some mechanistic aspects of the consequences of a loss of BMPRII. However, considering that access to microvascular pulmonary ECs from PAH patients is extremely limited and only possible at the time of lung transplantation, silencing of *BMPR2* in HLMVECs is a rather fair alternative to overcome this challenge and further investigate the consequences of a loss of BMPRII on the pathobiology of pulmonary vascular cells.

Conclusion

In the present study, we observed that a loss of BMPRII in HLMVECs resulted in increased permeability and activation of p38 MAP kinase. In addition, we observed that TNFα enhanced the effects of a loss of BMPRII expression on the pulmonary endothelium barrier function, likely through the activation of p38 MAPK. Finally, TNFα- and IL-18-induced adhesion capacities of *BMPR2* silenced HLMVECs were partially attributable to enhanced expression of ICAM-1 at the cell surface. Altogether, our results suggest that cytokines such as TNFα or IL-18 could trigger the development of PAH, in addition to a loss of BMPRII.

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Supplemental data

MATERIALS AND METHODS

Materials

Cell culture medium RPMI, fetal bovine serum (FBS), penicillin, streptomycin, fungizone, dispase, phosphate buffered saline (PBS), 4',6-diamidino-2-phenylindole (DAPI), TRIzol, SuperScript™ III First-Strand Synthesis System, primers and RT2 SYBR Green/ROX qPCR Master Mix were purchased from Life Technologies. TNFα was purchased from R&D Systems. IL-18 was from Medical and Biological Laboratories. Gelatin, bovine serum albumin (BSA) and fluorescein isothiocyanate (FITC)-labelled albumin were purchased from Sigma-Aldrich. [3H]-thymidine (specific activity: 74 GBq.mmol-1) was from Perkin Elmer. RNeasy Mini Kit and RNase-Free DNase set were purchased from Qiagen. Anti-human CD31 antibody coupled to allophycocyanin fluorochrome (CD31-APC) was from Miltenyi Biotec. Lab-Tek chamber slides from Nunc. THP-1 cells were a gift from the Lung Toxicology Research Group (KU Leuven). Monoclonal antibodies raised in mouse against human CD31 (clone JC70A, #M0823) and human von Willebrand factor (vWF; clone F8/86, #M0616) were purchased from Dako. Monoclonal antibodies raised in mouse against human ICAM-1 (#BBA3) and VCAM-1 (#BBA5) were from R&D Systems. Rabbit anti-phospho-p38 MAPK (clone D3F9, #4511), anti-p38 MAPK (#9212), anti-phospho Smad1/5/8 (#13820), anti-Smad1/5/8 (#6944) and VE-cadherin (clone D87F2, #2500) monoclonal antibodies were from Cell Signalling. Polyclonal antibodies raised in rabbit against peptide comprising amino-acids 950 to C-terminus of human BMPRII (ab115239) and mouse monoclonal antibody against human β-actin (clone AC-15, #ab6276) were from Abcam. Secondary antibodies conjugated with horseradish peroxidase were from Jackson Immuno Research and secondary antibodies Alexa Fluor™ 488 goat anti-mouse and anti-rabbit were from ThermoFisher. Acetylated low-density lipoprotein (LDL) coupled to a fluorescent carbocyanine dye, 1,1\'-dioctadecyl – 3,3,3\',3\'-tetramethyl-indocarbocyanine perchlorate (DiI-Ac-LDL) was purchased from Tebu-Bio.

Human lung microvascular endothelial cells (HLMVECs) were purchased from Cell Applications. HLMVECs were cultured in microvascular endothelial cell growth medium (Cell Applications) containing 6% growth supplement, 100 U/mL penicillin, 100 µg/mL streptomycin and 1.25 µg/mL fungizone and starved in microvascular endothelial cell basal medium (Cell Applications) supplemented with 0.2% growth supplement.

BMPR2, IL-18 and IL-18R mRNA expression

Primers used were as follows: *BMPR2*, forward 5'-TGCAGGTTCTCGTGTCTAGG-3' and reverse 5'-GGTCCCAACAGTCTTCGATT-3'; IL-18, forward 5'-GGGAAGAGGAAAGGAACCTC-3' and reverse 5'-CCATCTTTATTCCTGCGACA-3'; IL-18R, forward 5'-TGGTCAACAGCACATCATTG-3' and reverse 5'-

ACCCCTGATCTTCAAACTCG-3'; β-actin, forward 5'-GGACATCCGCAAAGACCTGT-3' and reverse 5'-CTCAGGAGGAGCAATGATCTTGAT-3'.

Western blotting

HLMVECs were grown to 90% confluence in 6 well-plates and starved in starving medium for 24h and further stimulated with 10 µg/mL TNFα or IL-18 for 1 h at 37°C. HLMVECs were washed twice in ice-cold phosphate-buffered saline, pH 7.4 and lysed for 30 min at 4°C in ice-cold RIPA buffer (50 mM Tris [pH 7.4], 150 mM sodium chloride, 1% [vol/vol] NP 40, 0.5% [wt/vol] sodium deoxycholate, 1 mM ethyleneglycoltetraacetic acid [EGTA], 10 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride [PMSF], leupeptin [10 µg/mL] and antipain [10 µg/mL]. Samples were centrifuged at 12,000 × g for 15 min and protein concentrations were determined by the Bradford method. Proteins were further separated on a 10% acrylamide gel by SDS-PAGE and transferred to polyvinylidenefluoride filters by electroblotting for 3 h in a transfer buffer containing 25mM Tris, pH 8.1-8.5, 192 mM glycine and 20% methanol. Filters were blocked in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-Tween) and 3% BSA (β-actin antibody) or 5% nonfat dry milk (phospho-Smad1/5/8, phospho-p38 MAPK, total Smad1/5/8, total p38 MAPK and BMPRII antibodies) for 1h at RT. Filters were incubated with primary antibodies overnight at 4°C in TBS-Tween and 3% BSA (anti-β-actin) or 5% nonfat dry milk (anti-phospho-p38 MAPK, anti-p38 MAPK, anti-phospho Smad1/5/8, anti-Smad1/5/8, BMPRII). The following horseradish peroxidase-conjugated secondary antibodies: donkey anti-rabbit immunoglobulin (Ig)G (anti-BMPRII, anti-phospho-p38 MAPK, anti-p38 MAPK, anti-phospho Smad1/5/8, anti-Smad1/5/8) and donkey-anti-mouse (Ig)G (anti-β-actin) antibodies were incubated in TBS-Tween for 1h at room temperature. Peroxidase staining was revealed by chemiluminescence and imaged with the Proxima 2850T imaging system (Isogen life technologies, NL) and analyzed with Totallab 1D (Isogen life technologies, NL).

FIGURES

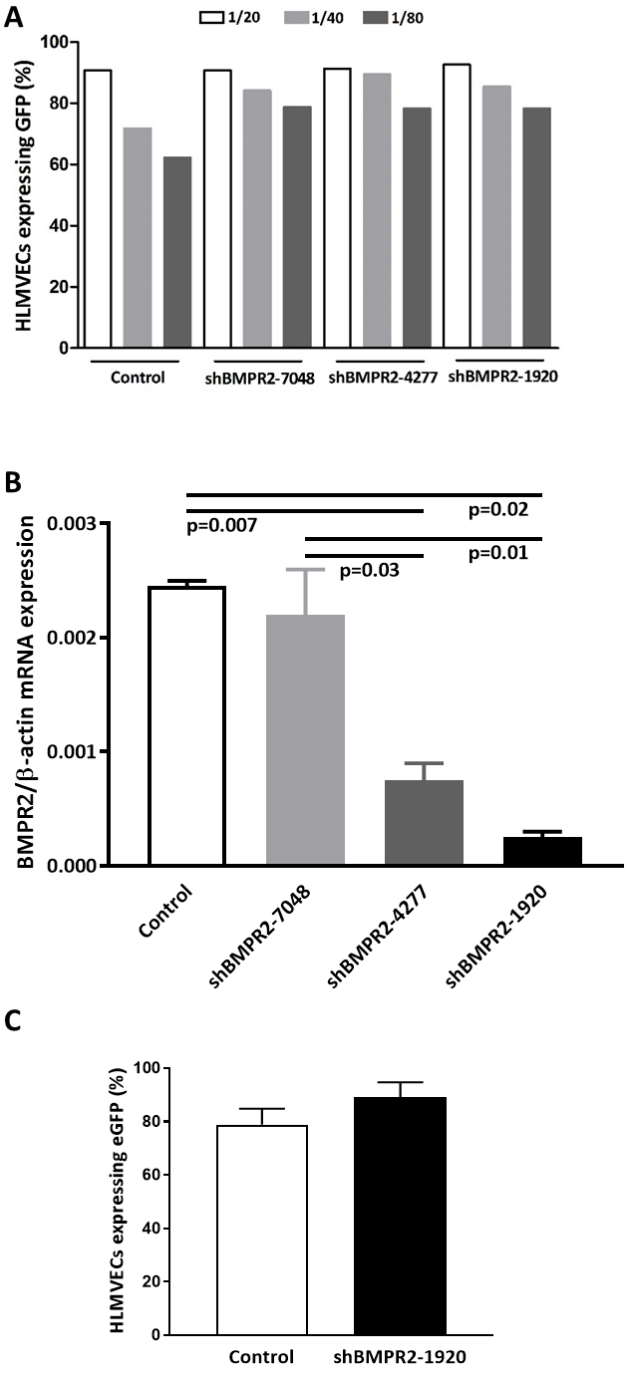

Figure S3.1: *Lentiviral vector transduction efficiency in HLMVECs. Percentage of HLMVECs expressing eGFP (A) and BMPR2 mRNA expression in HLMVECs (B) transduced with control and shBMPR2-7048, 4277 and 1920. ANOVA, p<0.005. (C) Viability of HLMVECs transduced with control and LV_miR_BMPR2_1920 was assessed by FACS. Experiments were performed in triplicate in HLMVECs at passage 4. Panel (A) shows the results of one independent experiment.*

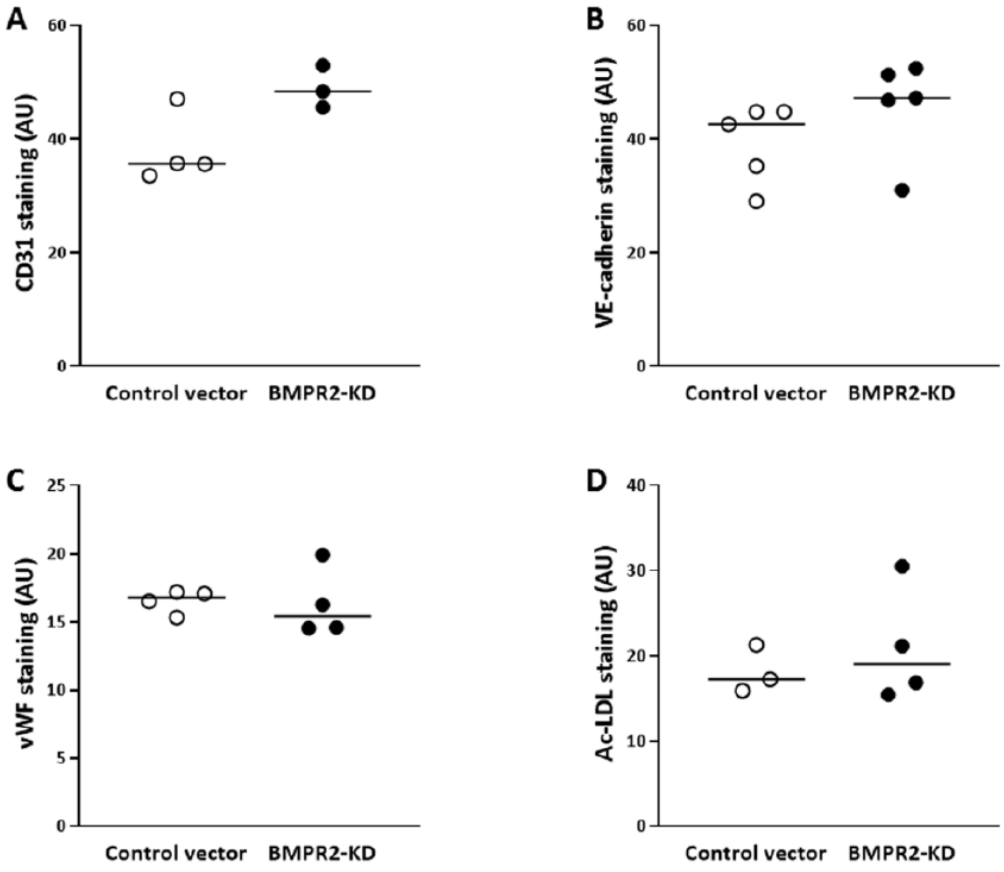

Figure S3.2: *quantification of the expression of specific endothelial markers in HLMVECs. Expression of CD31 (A), VE-Cadherin (B), vWF (C) and Ac-LDL uptake (D) were quantified both in control and BMPR2-KD HLMVECs.*

Effect of macitentan, selexipag and tadalafil on angiogenesis and barrier function in *BMPR2*-silenced human lung microvascular endothelial cells

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Abstract

Background

The BMPRII signalling pathway is impaired in PAH and mutations in the *BMPR2* gene have been observed in heritable and idiopathic PAH. Endothelial dysfunction, an important hallmark of PAH pathogenesis, is currently treated by vasodilatory therapies targeting the endothelin (ET-1), nitric oxide (NO) and prostacyclin (PGI_2) pathways. Endothelial dysfunction implies endothelial cell apoptosis, loss of barrier function and dysregulated angiogenesis. Current experimental and clinical evidence is inconclusive about the role of angiogenesis in PAH and potential effects of therapies.

Hypothesis

We consequently hypothesized that i) BMPRII loss of function may impair pulmonary microvascular endothelial cell angiogenesis and that PAH therapies could restore angiogenic capacities and pulmonary endothelial barrier function. The aim of this study was to investigate whether *BMPR2* silencing can impair angiogenic capacities and the effects of the most recently launched vasodilatory therapies targeting the *in vitro* angiogenic capacity and endothelial barrier function.

Methods

Using stable *BMPR2* silencing in human lung microvascular endothelial cells (HLMVECs) we investigated angiogenesis using a 2D migration and a 3D tube formation cell assay. The effects of macitentan, tadalafil and selexipag targeting ET-1, NO and PGI_2 pathways, respectively, on the activation of BMPRII downstream effectors, Smad1/5/8 and p38 MAPK, and of VE-Cadherin, migration, tube formation and endothelial barrier function were investigated *in vitro*.

Results

We observed that stable *BMPR2* silencing resulted in loss of migration capacity and impaired tube forming ability. Inhibition of ET-1 pathway by macitentan and activation of NO and PGI_2 pathways by tadalafil and selexipag, respectively did not affect canonical Smad1/5/8 and non-canonical p38 MAPK downstream BMPRII effectors. Whereas macitentan was able to partially restore tube formation in *BMPR2*-silenced HLMVECs, tadalafil and selexipag did not. None of the drugs were able to restore endothelial barrier function in *BMPR2*-silenced HLMVECs. Importantly, we did not find any deleterious effect in response to the inhibition of ET-1 or the activation of NO and PGI_2 pathways on the *in vitro* angiogenic capacities, endothelial barrier function in *BMPR2*-silenced HLMVECs.

Conclusion

Altogether, our findings highlight the role of BMPRII in pulmonary microvascular endothelial cell angiogenesis in PAH. In addition, most recently launched vasodilatory drugs used to treat PAH display limited, but no deleterious effects on endothelial function when BMPRII is impaired. Eventually, our

results suggest that innovative therapeutic strategies targeting BMPRII signalling are needed to better restore endothelial function in PAH.

Introduction

Pulmonary arterial hypertension (PAH) is a severe and progressive disease characterized by a distal pre-capillary arteriopathy resulting in increased pulmonary vascular resistance, right ventricular hypertrophy, progressive right heart failure and ultimately death (1). PAH is a complex multifactorial disease involving abnormal vascular tone, endothelial dysfunction, inflammation, dysregulated angiogenesis, and enhanced thrombosis (2). In addition, plexiform lesions, a hallmark of severe PAH, are characteristically located at an arterial branch point or at the origin of a supernumerary artery; they are typically defined as a dynamic network of vascular channels, with the presence of phenotypically distinct endothelial cells, e.g. a quiescent phenotype lining the channels and a proliferating, apoptosis-resistant phenotype at the core of the lesion (3,4) as well as inflammatory cells (2).

More than 70% of patients with familial PAH and 20% of idiopathic PAH harbour heterozygous mutations in the *bone morphogenetic protein type 2 receptor* (*BMPR2*) gene (5–7). *BMPR2* transcodes for a transmembrane serine/threonine kinase receptor of the bone morphogenetic protein (BMP) pathway, which is essential for embryogenesis, development, and adult tissue homeostasis (8). Upon ligand binding, a hetero-dimer complex, formed by a combination of two type I receptors and two type II receptors, propagates the signal into a canonical pathway through phosphorylation of the Smad1/5/8 transcription factors or a non-canonical pathway by phosphorylation of p38 MAPK (9). To date more than 380 distinct *BMPR2* variants have been identified and most of them are pathogenic (10). In addition, mutations in other genes including the *activin receptor-like kinase 1* (*ALK1*), *endoglin* (*ENG*) (11), small mothers against decaptoplegic homolog 1 (*Smad1*), *Smad4* (12) and *Smad9* (13) have been found being associated to PAH, highlighting the crucial role of the BMP pathway in the pathogenesis of PAH (14).

Among the multifactorial processes associated with PAH, endothelial dysfunction and defective angiogenesis play a key role in initiating structural changes in the pulmonary vasculature. The pharmacological therapies currently used to treat PAH mainly display vasodilatory effects by targeting 3 pathways: endothelin-1 (ET-1), nitric oxide (NO) and prostacyclin (PGI_2) pathways (15).

Expression of vascular endothelial growth factor (VEGF) and its receptors is upregulated in pulmonary tissue from PAH patients, potentially resulting in the formation of plexiform lesions (16,17). By contrast, tyrosine kinase inhibitors (TKIs), used as chemotherapeutic and anti-angiogenic drugs, such as imatinib, have proven efficacious in patients with severe PAH, but serious side effects (intracranial bleeding) have prevented their further use (18). On the other hand, 9 patients with chronic myelogenous leukemia and treated with another TKI, dasatinib, developed severe PAH (19). Additionally, rats exposed to hypoxia and who received a single injection addition of SU5416, an

inhibitor of the VEGF receptor with anti-angiogenic properties developed severe PAH and plexiform-like lesions (20). Induced pluripotent stem cells (iPSCs) of PAH patients carrying a *BMPR2* mutation display reduced migration and tube forming capacity *in vitro* compared to iPSCs from control subjects or healthy *BMPR2* mutation carriers (21). Eventually, we recently observed that stable *BMPR2* silencing impair endothelium barrier function (22).

Consequently, we do believe that deficient endothelial barrier function and impaired angiogenesis, in addition to impaired BMPRII signalling contribute to PAH progression (23,24). Furthermore, we hypothesize that PAH-specific drugs may interfere with the BMPRII signalling pathway and could partially restore angiogenic deficiency and impaired endothelium barrier function. Therefore, we aim to investigate whether *BMPR2* silencing would worsen angiogenic capacities of pulmonary microvascular endothelial cells and further determine whether PAH-specific drugs would be able to restore endothelium barrier function and angiogenic capacities in a context of impaired BMPRII signalling.

Material & methods

Materials

Fetal bovine serum (FBS), penicillin, streptomycin, fungizone, phosphate buffered saline (PBS), 4',6-diamidino-2-phenylindole (DAPI), were purchased from Life Technologies. Gelatin and bovine serum albumin (BSA) were purchased from Sigma-Aldrich. Anti-human CD31 antibody coupled to allophycocyanin fluorochrome (CD31-APC) was from Miltenyi Biotec. Lab-Tek chamber slides were purchased from Nunc. Rabbit anti-phospho-p38 MAPK, anti-p38 MAPK, anti-phospho Smad1, anti-Smad1 and vascular endothelial (VE)-cadherin monoclonal antibodies were from Cell Signaling. Mouse monoclonal antibody against β-actin (clone AC-74) was from Sigma. Secondary antibodies conjugated with horseradish peroxidase were from Dako and Jackson Immuno Research.

Cell culture

BMPR2 was silenced in HLMVECs as previously described. HLMVECs were cultured in microvascular endothelial cell growth medium - EGM (Cell applications) containing 100 U/mL penicillin, 100 µg/mL streptomycin, and 1.25 µg/mL fungizone and starved in microvascular endothelial cell basal medium - EBM (Cell applications) supplemented with 0.2 % growth supplement.

Drug selection

From each of the different pathways, *i.e.* ET-1, NO and PGI_2, targeted by vasodilatory pharmacological therapies, we have chosen to select the most recently available drugs. Regarding ET-1 pathway, the dual endothelin receptor agonist (ERA), macitentan, was preferred because of its more tissue-specific effect, its longer lasting effect and its effect on morbidity/mortality outcome (25). To target the NO pathway, phosphodiesterase 5 inhibitor (PDE_5I), tadalafil, was elected because of its effects as upfront therapy together with macitentan (26). Finally, as concerns the PGI_2 pathway, prostacyclin receptor agonist, selexipag, was chosen because it is the first oral drug targeting this pathway, since so the most effective therapies activating PGI2 production were continuous parenteral intravenous delivery of epoprostenol (27).

Cell viability

Drug solutions were prepared with serial dilutions from a 10 mM solution in dimethylsulfoxide. The required concentrations were determined according to the manufacturer's datasheet and the maximum serum concentrations (Cmax) measured in the blood from patients with PAH. The Cmax was estimated as 1.35 µM, 0.06 µM and 1.32 µM for macitentan (28), selexipag (29) and tadalafil (30), respectively. Subsequently, a range of concentrations around the Cmax was used to establish a dose response curve and perform viability assay.

Potential drug toxicity was evaluated using the cell viability 2-(2-methoxy-4-nitrophenyl)-3-(4-

nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-1) method. HLMVECs (10^4 cells/well) were incubated in a 96-well plate in the presence of increasing drug concentrations (10^{-9} to 10^{-5} M) for 2, 4 and 6 hours and compared with non-exposed cells. Absorbance was measured at 550 nm and results were expressed as a percentage of values to non-exposed cells (Figure 4.1). If Cmax did not show any toxic effect this concentration was further considered.

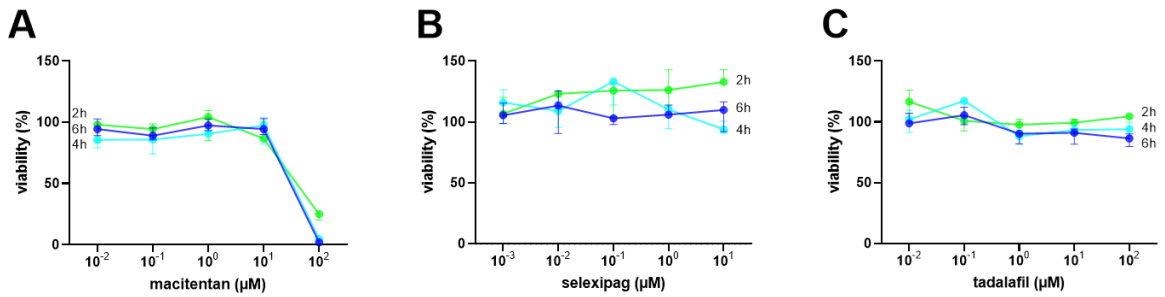

Figure 4.1: Viability of HLMVECs in response to increasing concentrations of macitentan (A), selexipag (B) and tadalafil (C) after 2, 4 and 6 hours, using a WST-1 assay. Results were expressed as percentage relative to non-stimulated cells.

Western blotting

Confluent HLMVEC layers were starved overnight and homogenized in fresh, ice-cold RIPA lysis buffer containing 50 mM Tris, pH 7.4, 1% octylphenoxypolyethoxyethanol (IGEPAL), 0.5% sodium-deoxycholate, 0.1% sodium dodecyl sulfate, 150mM NaCl, 1mM ethylene glycol tetra-acetic acid EGTA), 1% protease inhibitor cocktail 100x (Halt™), 1% phosphatase inhibitor cocktail 100x (Halt™). Homogenates were centrifuged at 12,000 g for 15 minutes at 4°C to remove insoluble material and supernatants were collected. Proteins were separated on a 10 % acrylamide gel by SDS-PAGE and further visualized as previously described (22). Briefly, non-specific binding sites were blocked and membranes were incubated with primary antibodies against p38 MAPK, phospho-p38 MAPK, Smad1/5/8 and phospho-Smad1/5/8 overnight at 4°C. Appropriate secondary antibodies were incubated for 1 hour at RT and revealed with a chemiluminescence kit (GE healthcare) and imaged using the Proxima 2850T imaging system.

A drug-induced time-response of activation of Smad1/5/8 and p38 MAPK was performed by Western blotting. Activation of Smad1/5/8 and p38 MAPK was optimal after 30 minutes (Figure 4.2), consequently, activation of BMPRII effectors was further evaluated upon 30 min in the presence of the different drugs.

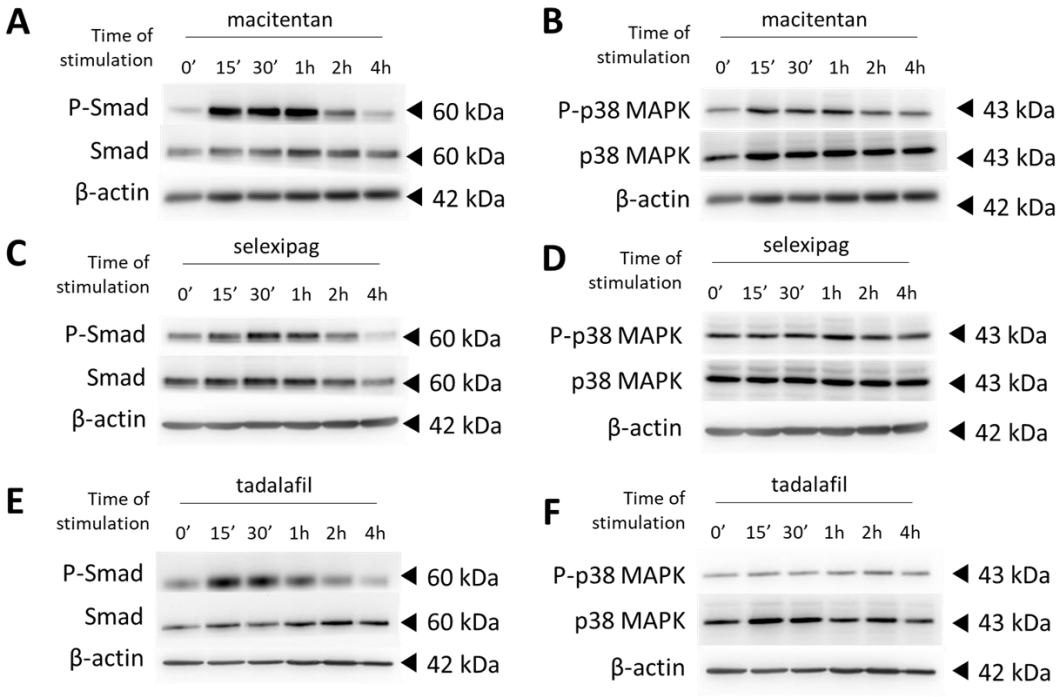

Figure 4.2: Smad and p38 MAPK activation in response to macitentan (A, B), selexipag (C, D) and tadalafil (D, E).

Migration scratch assay

HLVMECs were seeded in 2-well culture inserts (Ibidi, 81176; Figure 4.3A). Subconfluent cells were starved overnight. Inserts were removed, washed twice with growth medium and incubated in growth medium containing or not macitentan, selexipag or tadalafil. Pictures were taken every other hour using a Leica DMi1 inverted microscope. The distance of the gap was measured at 0, 2, 4, 6, 8, 10- and 12-hours using ImageJ. At each time point, the gap-distance was measured and subtracted from the initial gap-distance, further plotted over time and the area under the curve (AUC) representing migration velocity in µm/hour.

Tube formation assay

Angiogenesis µ-slide (Idibi, 81506) were filled with matrigel (BD Bioscience) and incubated for 30 min at 37°C. HLMVECs were starved overnight and trypsinized, resuspended in growth medium in the absence or presence of macitentan, selexipag or tadalafil and added on top of the matrigel. After 4 hours, images were taken using an Olympus IX71 inverted microscope, analysed by ImageJ. Number of tubes, nodes and meshes (Figure 4.3B) were quantified using the software 'Angiogenesis Analyzer' developed by Gilles Carpentier (31) .

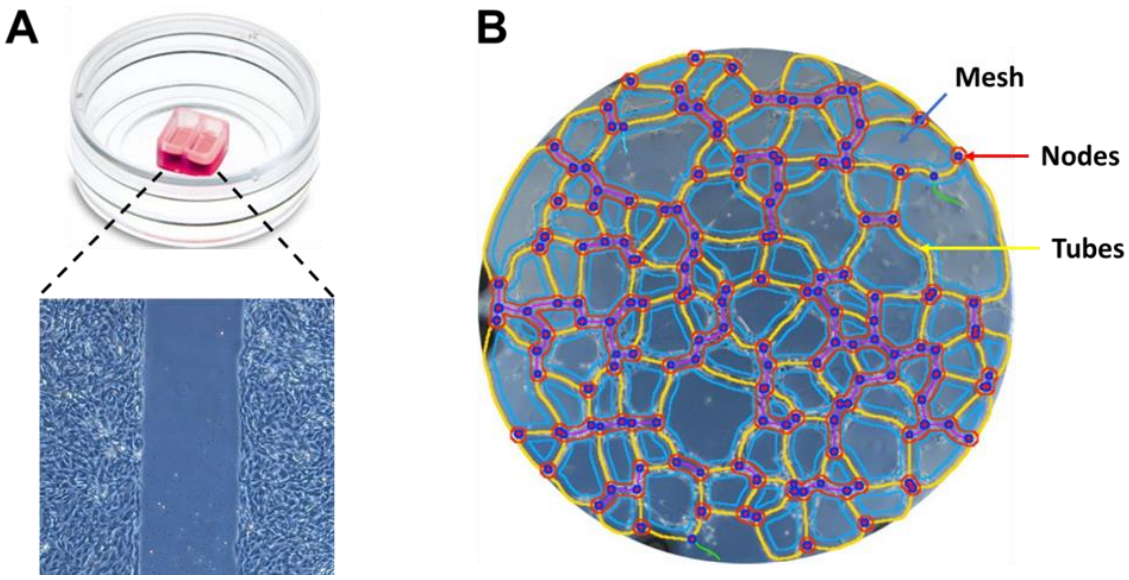

Figure 4.3: Representative figures of the migration assay set-up (A) and a tubular network formed 4 hours after seeding with corresponding analysis of tubes (yellow), nodes (red dots) and mesh (blue).

Assessment of HLMVEC permeability

Briefly, cells were seeded onto 24-well Transwell inserts (100,000 cells/insert) to form a monolayer and starved overnight. Cells were first stimulated with macitentan, selexipag or tadalafil for one hour, then fluorescently labelled bovine serum albumin (BSA; 0.1 mg/mL) was added to the upper chamber. Leakage of labeled BSA into the lower chamber was assessed as previously described.

Immunofluorescence

HLMVECs seeded onto fibronectin-coated chamber slides. A confluent cell layer was stained by immunofluorescence using antibodies against VE-cadherin and phospho-VE-cadherin (VE-cadherin 1:800, phospho-VE-cadherin 1:50), as described previously. Quantification of immunofluorescent images was performed using Image J software by measuring staining intensity, corresponding to VE-cadherin and phospho-VE-cadherin expression. The number of DAPI-stained nuclei was calculated. The staining intensity of each image was related to the number of cells and expressed as arbitrary unit (AU).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 8.1.2 (GraphPad Software Inc., La Jolla, California). Differences between groups were analyzed using Student t-test or paired t-test. All p values are for 2-sided tests. A value of $p<0.05$ was considered statistically significant. Continuous and normally distributed values are expressed as mean ± SD.

Results

Effect of *BMPR2* silencing on HLMVEC angiogenic capacities

Angiogenic capacities were assessed *in vitro* by investigating migration capacities and tube formation of control and *BMPR2*-silenced HLMVECs. These cells were transduced as previously described (22), see chapter 3. Migration velocity of *BMPR2*-silenced HLMVECs was significantly lower compared to control HLMVECs (25.2 ± 2. 7 vs 21.8 ± 3.2 µm/h; p<0.0001; Figure 4.4). The number of nodes (984 ± 38 vs 828 ± 36, p= 0.006; Figure 4.5A), tubes (325 ± 14 vs 274 ± 16, p= 0.02; Figure 4.5B) and mesh; 67 ± 5 vs 53 ± 5, p= 0.04; Figure 4.5C) were significantly lower in *BMPR2*-silenced HLMVECs compared to control HLMVECs.

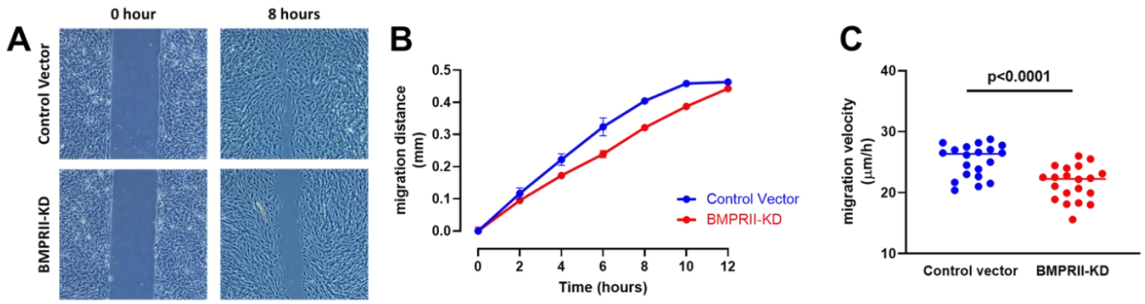

Figure 4.4: Effect of *BMPR2* silencing on migration capacity of HLMVECs. Migration capacity was evaluated by measuring the distance gap within control and *BMPR2*-silenced HLMVECs over time. (A) Representative pictures at 0 and 8 hours of control and *BMPR2*-silenced HLMVECs. (B) Representative time-curve migration of control (blue) and *BMPR2*-silenced (red) HLMVECs. (C) Migration velocity of control and *BMPR2*-silenced HLMVECs expressed as migration velocity (µm/h). Experiments were performed in 5 independent experiments ranging from 2 to 7 replicates between passages 5 and 8.

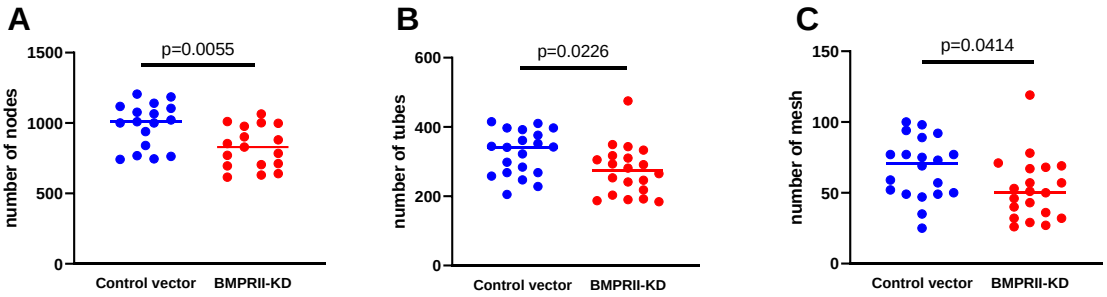

Figure 4.5: Effect of *BMPR2* silencing on tube forming capacity of HLMVECs. The number of nodes (A), tubes (B) and mesh (C) was measured in control and *BMPR2*-silenced HLMVECs. Experiments were performed in 5 independent experiments ranging from 2 to 7 replicates between passages 5 and 8.

Effects of macitentan, selexipag and tadalafil on canonical and non-canonical BMPRII signalling

We previously observed that silencing of *BMPR2* results in decreased canonical Smad1/5/8 and increased non-canonical p38 MAPK activation (22).

Here, we stimulated both control and *BMPR2*-silenced HLMVECs with macitentan, selexipag or tadalafil. After stimulation cells were lysed and analysed using western blotting. We did not observe

any effect of macitentan, selexipag or tadalafil on the canonical Smad1/5/8 signalling (Figure 4.6A and 4.6C) in *BMPR2*-silenced HLMVECs. In addition, macitentan, selexipag or tadalafil did not activate p38 MAPK protein activation (Figure 4.6B and 4.6D). Similarly, macitentan, selexipag or tadalafil did not display any effect on the activation of the canonical Smad1/5/8 (Figure 4.7A and 4.7C) and the non-canonical p38 MAPK (Figure 4.7B and 4.7C) signalling pathways in control HLMVECs.

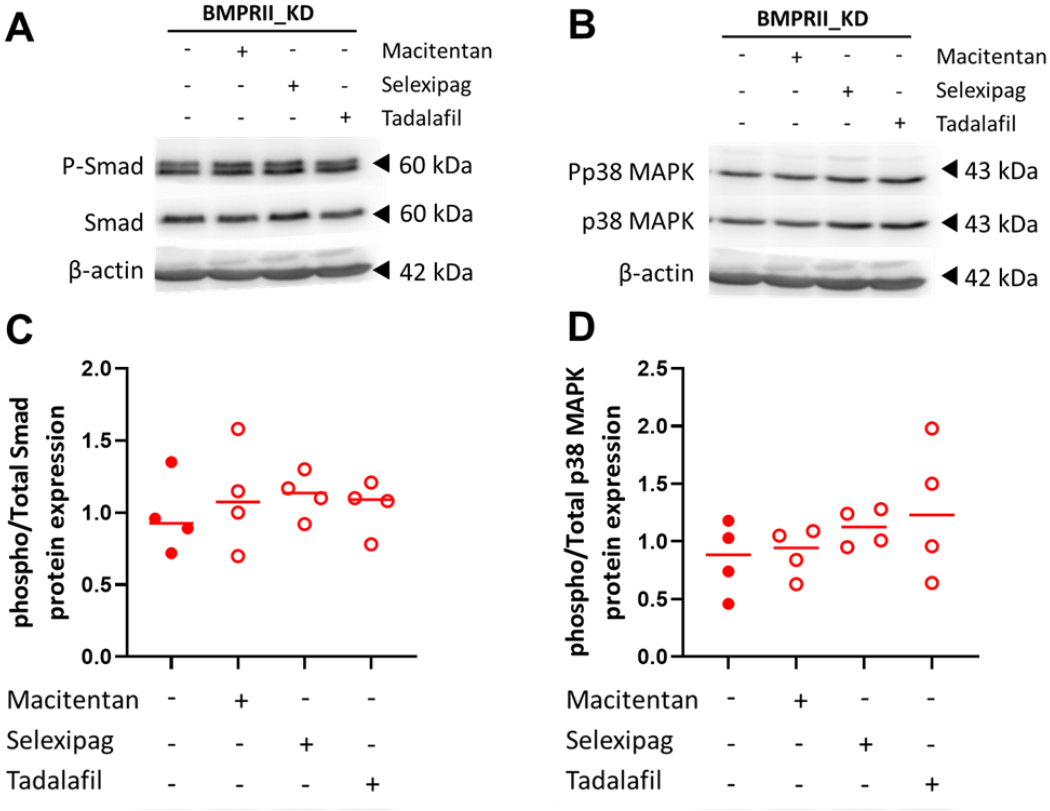

Figure 4.6: Effect of macitentan, selexipag and tadalafil on BMPRII canonical and non-canonical signalling pathways. Representative Western blot of phosphorylated and total Smad1/5/8 proteins (A) and p38 MAPK (B) in *BMPR2*-silenced HLMVECs in the absence and after incubation with macitentan, selexipag and tadalafil for 1 hour. Quantitative expression of phosphorylated vs. total Smad1/5/8 proteins (C) and p38 MAPK (D). Results are presented as relative protein expression ratio of phospho-Smad/p38 MAPK over total-Smad/p38 MAPK. Independent experiments were performed in 4 replicates for HLMVECs between passages 5 and 8.

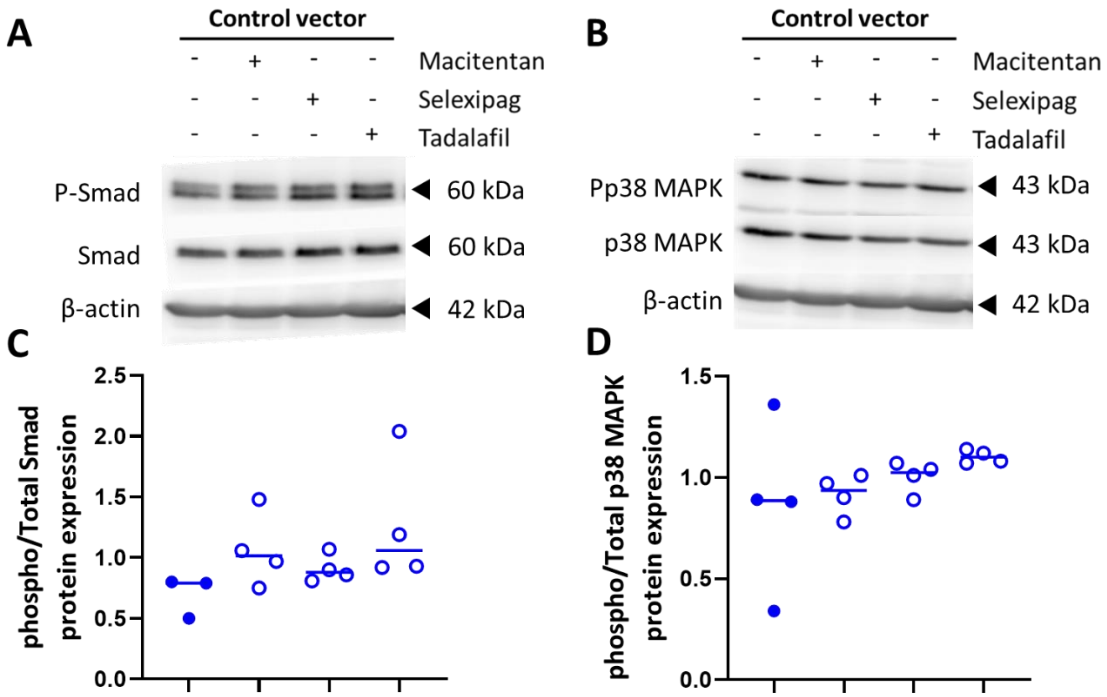

Figure 4.7: Effect of macitentan, selexipag and tadalafil on BMPRII canonical and non-canonical signalling pathways. Representative Western blot of phosphorylated and total Smad1/5/8 proteins (A) and p38 MAPK (B) in control HLMVECs in the absence and after incubation with macitentan, selexipag and tadalafil for 30 minutes. Quantitative expression of phosphorylated vs. total Smad1/5/8 proteins (C) and p38 MAPK (D). Results are presented as relative protein expression ratio of phospho-Smad/p38 MAPK over total-Smad/p3 8MAPK. Independent experiments were performed in 4 replicates for HLMVECs between passages 5 and 8

***In vitro* angiogenic capacities of HLMVECs in response to macitentan, selexipag and tadalafil**

Considering that *BMPR2* silencing resulted in reduced *in vitro* angiogenic capacities of HLMVECs, we next investigate whether drugs targeting ET-1, NO and PGI_2 pathways could restore them. Tadalafil significantly increased the migration velocity of *BMPR2*-silenced HLMVECs (21.4 ± 3.1 vs 23.3 ± 3.1 µm/h, p=0.001, Figure 4.8C), whereas macitentan (21.8 ± 3.2 vs 22.2 ± 2.3 µm/h, p=0.53; Figure 4.8A) and selexipag (21.8 ± 3.2 *vs.* 22.4 ± 3.1 µm/h, p= 0.21; Figure 4.8B) did not have any significant effect on the migration velocity. We did not observe any effects of macitentan on migration of control HLMVECs (25.1 ± 2.5 *vs.* 24.5 ± 2.7 µm/h, p= 0.32, Figure 4.9A). Surprisingly, selexipag (25.2 ± 2.4 *vs.* 24.3 ± 2.9 µm/h, p= 0.009; Figure 4.9B) and tadalafil (25.3 ± 2.5 vs 23.5 ± 2.9 µm/h, p= 0.002; Figure 4.9C) significantly decreased migration capacities of control HLMVECs (Figure 4.9).

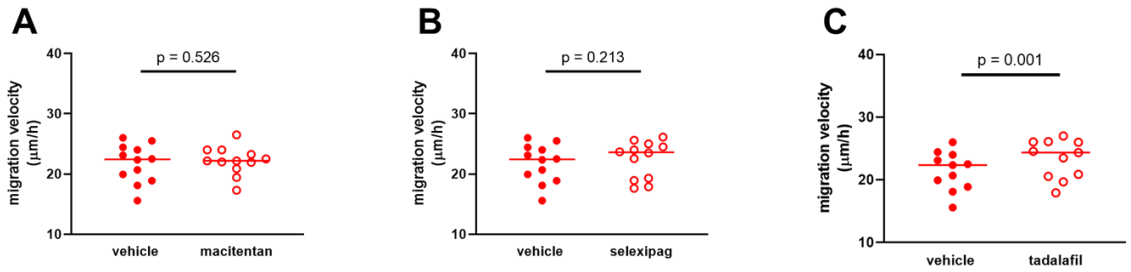

Figure 4.8: Migration capacity of *BMPR2*-silenced HLMVECs in response to macitentan (A), selexipag (B) and tadalafil (C). Results are expressed as migration velocity (µm/h). Experiments were performed in 4 independent experiments ranging from 2 to 5 replicates between passages 5 and 8.

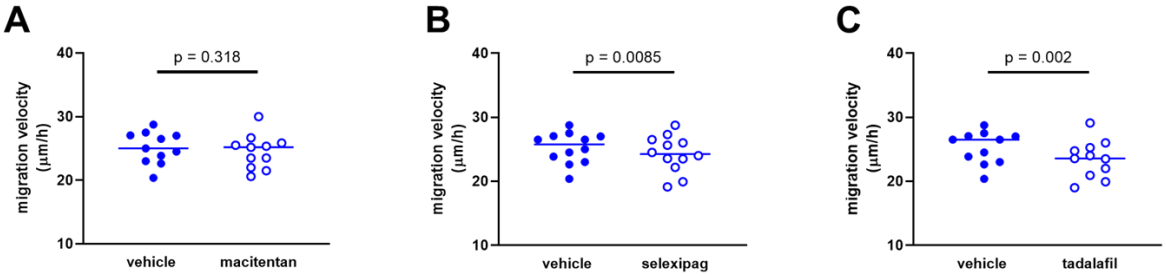

Figure 4.9: Migration capacity of control HLMVECs in response to macitentan (A), selexipag (B) and tadalafil (C). Results are expressed as migration velocity (µm/h). Experiments were performed in 4 independent experiments ranging from 2 to 5 replicates between passages 5 and 8.

Regarding *in vitro* tube formation, we found that only macitentan significantly enhanced tube formation capacities of *BMPR2*-silenced HLMVECs by increasing the number of nodes, tubes and meshes by 7% (p=0.015), 10% (P=0.007) and 21% (p=0.003), respectively (Figure 4.10A-C), whereas selexipag or tadalafil did not show any significant effects on tube formation (Figure 4.10D-F & Figure 4.10G-I). We did not observe any effects of macitentan or selexipag on *in vitro* tube formation of control HLMVECs (Figure 4.11A-C and 4.11D-F). Surprisingly, tadalafil significantly reduced *in vitro* tube formation of control HLMVECs in decreasing the number of nodes, tubes and meshes by 16% (p=0.020), 18% (p=0.027) and 23% (p=0.024), respectively (Figure 4.11G-I).

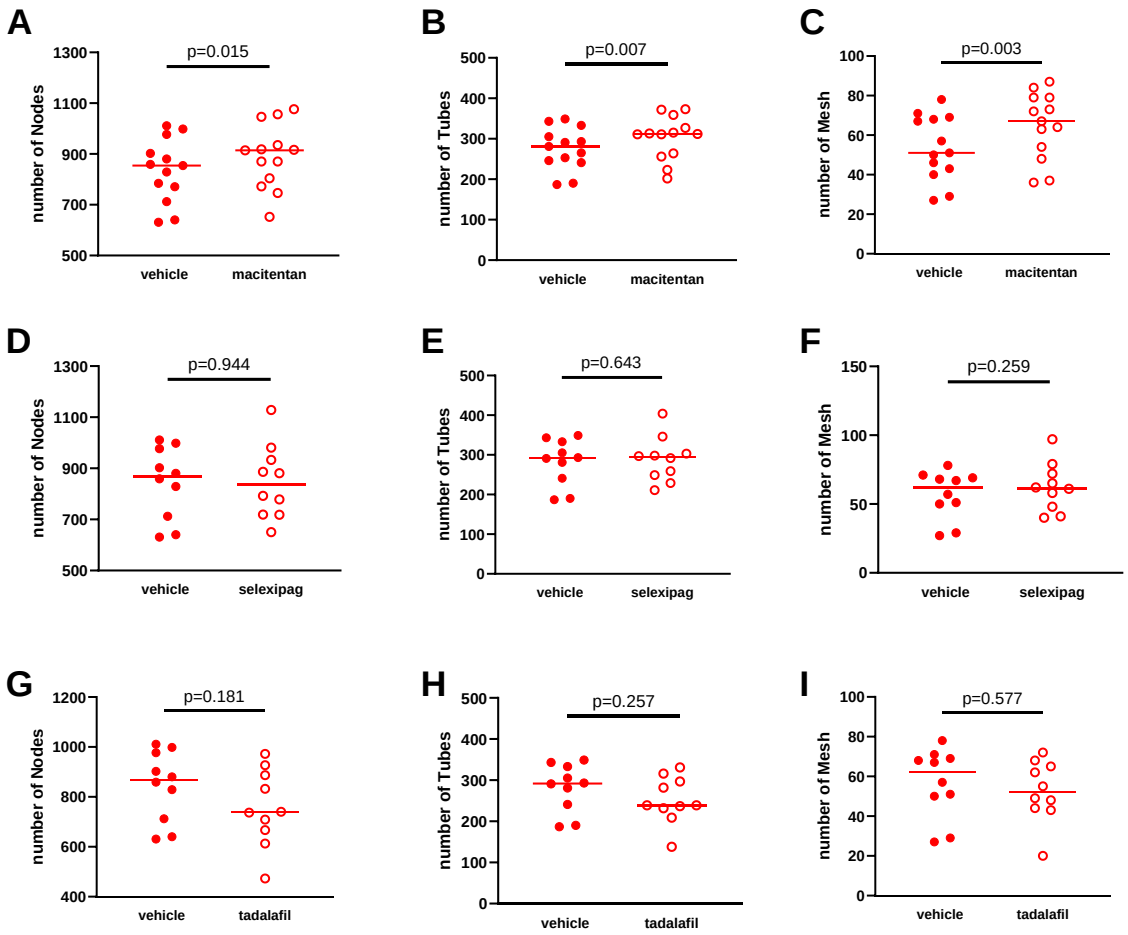

Figure 4.10: Tube formation capacity in *BMPR2*-silenced HLMVECs in response to macitentan (A, B, C), selexipag (D, E, F) and tadalafil (G, H, I). The number of nodes (A, D, G), tubes (B, E, H) and mesh (C, F, I) was measured. Experiments were performed in 4 independent experiments ranging from 2 to 7 replicates between passages 5 and 8.

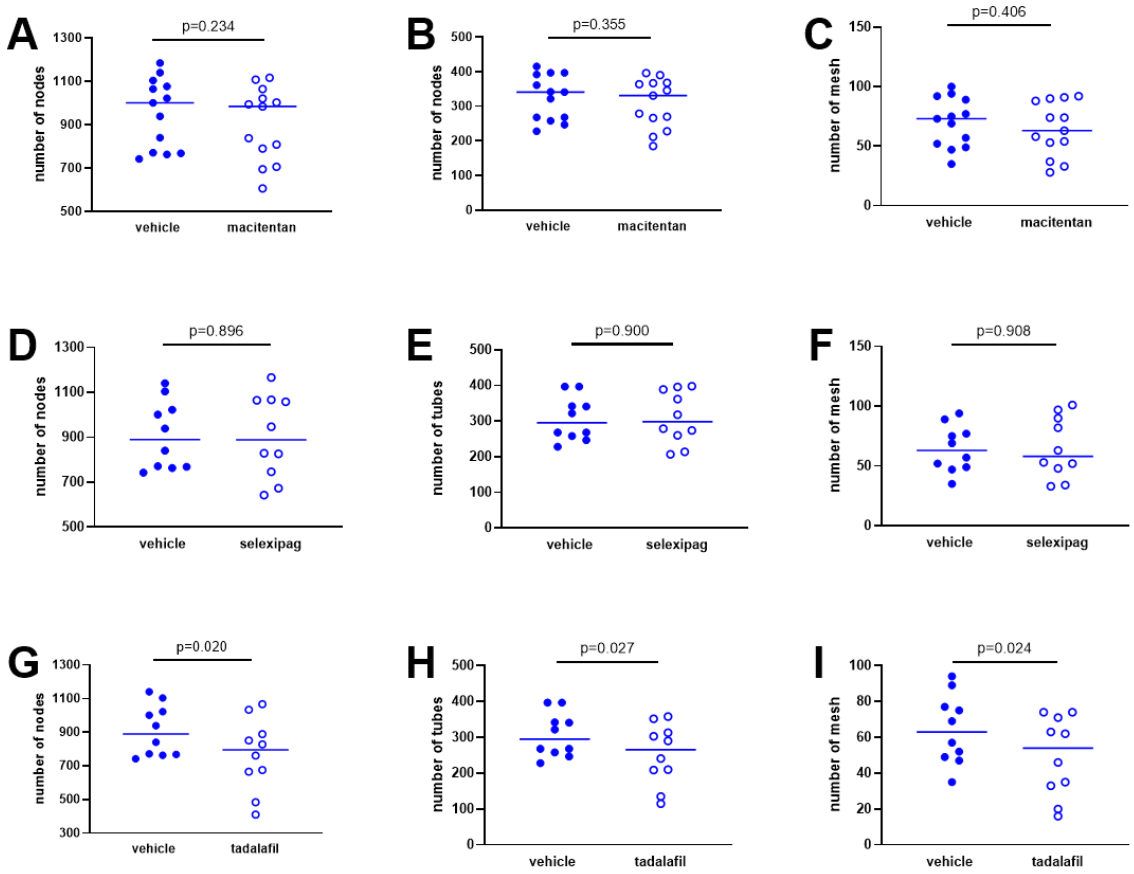

Figure 4.11: Tube formation capacity in control HLMVECs in response to macitentan (A, B, C), selexipag (D, E, F) and tadalafil (G, H, I). The number of nodes (A, D, G), tubes (B, E, H) and mesh (C, F, I) was measured. Experiments were performed in 4 independent experiments ranging from 2 to 7 replicates between passages 5 and 8.

Endothelial barrier function in response to macitentan, selexipag and tadalafil

We previously observed that *BMPR2* silencing resulted in impaired endothelial barrier function in HLMVECs, but without affecting VE-cadherin content (22). We therefore aim to investigate whether drugs targeting ET-1 (macitentan), NO (tadalafil) and PGI_2 (selexipag) pathways might restore the endothelial barrier function and/or affect VE-cadherin content and phosphorylation. We did not observe any significant effects of macitentan, selexipag or tadalafil in restoring endothelial barrier function in *BMPR2*-silenced (Figure 4.12) or control HLMVECs (Figure 4.13).

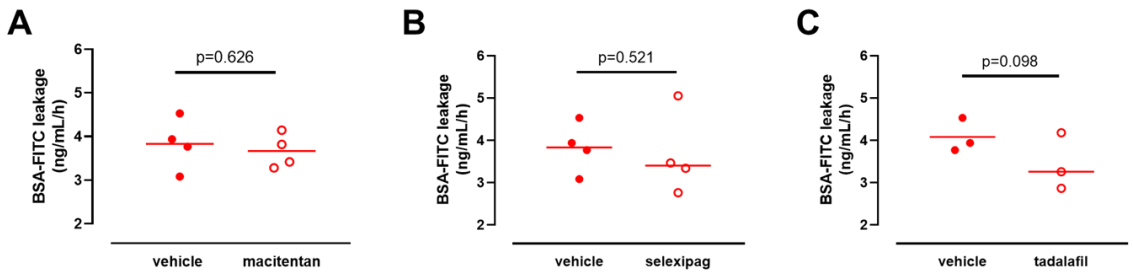

Figure 4.12: Endothelial barrier function of *BMPR2*-silenced HLMVECs in the presence of macitentan (A), selexipag (B) and tadalafil (C). Results are expressed as BSA-FITC leakage (ng/mL/h). BSA-FITC was measured in 4 independent experiments performed in triplicate in HLMVECs between passages 5 and 8.

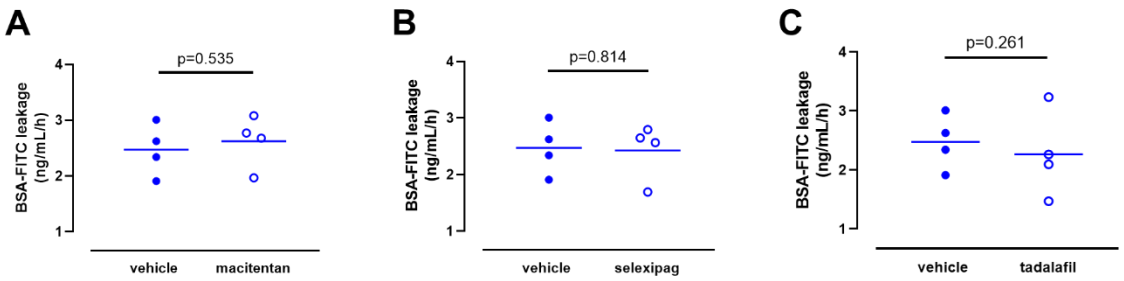

Figure 4.13: Endothelial barrier function of control HLMVECs in response to macitentan (A), selexipag (B) and tadalafil (C). Results are expressed as BSA-FITC leakage (ng/mL/h). BSA-FITC was measured 4 independent experiments performed in triplicate in HLMVECs between passages 5 and 8.

The impairment of endothelial barrier function previously observed in *BMPR2*-silenced HLMVECs (22), was not associated with a change in VE-cadherin expression or phosphorylation (Figure 4.14A-D). Immunofluorescent staining did not show any difference in VE-cadherin expression between *BMPR2*-silenced HLMVECs compared to control (Figure 4.14A and 4.8B). Interestingly, macitentan, selexipag and tadalafil tend to increase phosphorylation of VE-cadherin by 32%, 41% and 49% respectively in *BMPR2*-silenced HLMVECs (Figure 4.14C and 4.14D), whereas they had no effect on VE-cadherin phosphorylation in control HLMVECs (Figure 4.15A and 4.15B).

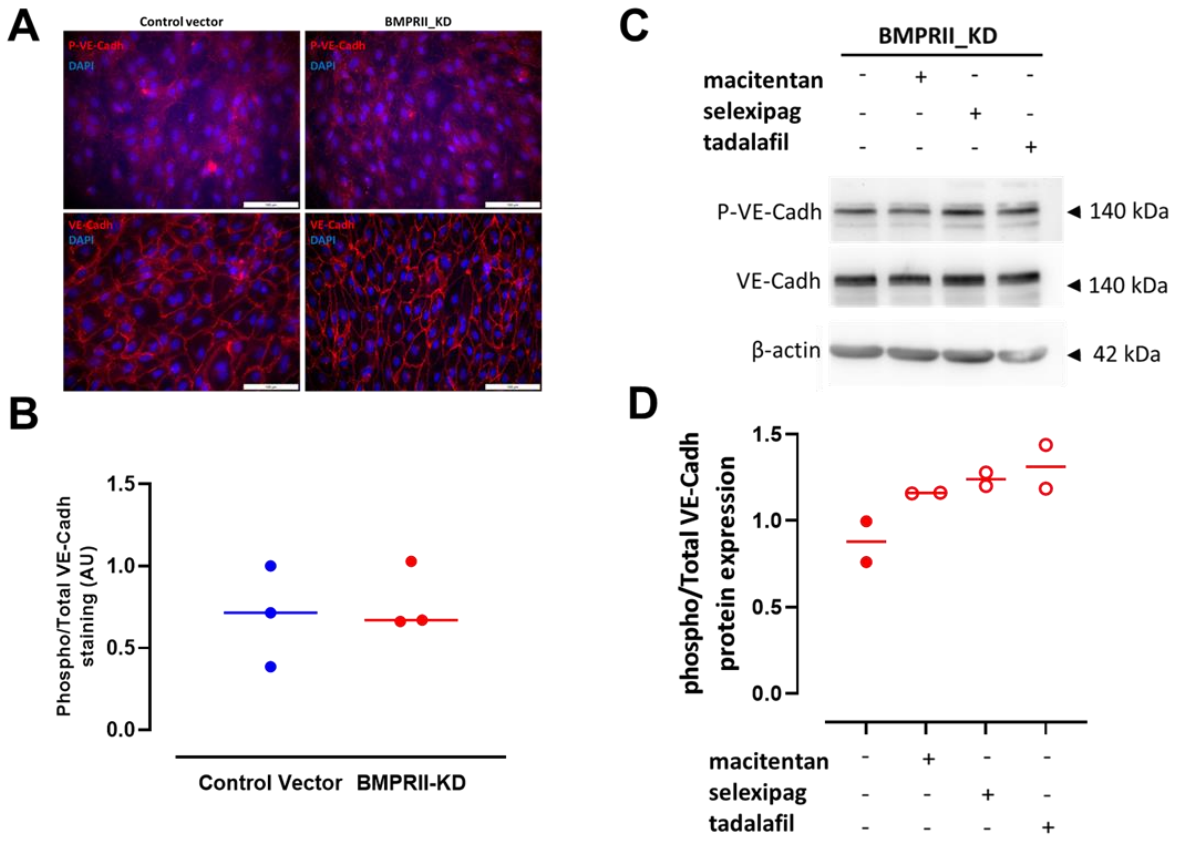

Figure 4.14: Effect of *BMPR2* silencing and PAH-specific drug on VE-cadherin activation. (A) Representative pictures of immunofluorescent staining with phospho-VE-cadherin and total VE-cadherin in HLMVECs transduced with control or BMPRII-KD lentiviral vectors. Nuclei were counterstained using DAPI. (B) Quantification of immunofluorescent staining with phospho-VE-cadherin and total VE-cadherin in 3 independent experiments. (C) Representative western blots of phospho VE-cadherin and VE-cadherin protein expression. (D) Quantification of activated VE-cadherin in HLMVECs transduced with BMPRII-KD lentiviral vectors and in response to macitentan, selexipag and tadalafil 1-hour stimulation. β-actin was used as loading control.

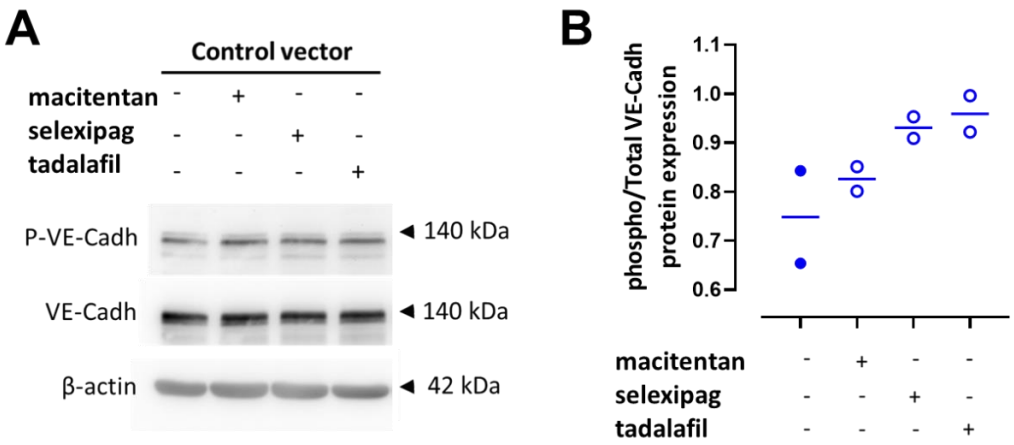

Figure 4.15: Effect of PAH-specific drug on VE-cadherin activation in control HLMVECs. (A) Representative western blots of phospho VE-cadherin and VE-cadherin protein expression. (B) Quantification of activated VE-cadherin in HLMVECs transduced with control lentiviral vectors and in response to macitentan, selexipag and tadalafil 1-hour stimulation. B-actin was used as loading control.

Discussion

In the present study, we observed that stable *BMPR2* silencing in HLMVECs resulted in impaired angiogenic capacity *in vitro*, as illustrated by decreased migration capacity and loss of tube forming capabilities. We evidenced that macitentan and tadalafil can improve angiogenic capacity. Unfortunately, we did not find any beneficial effects of PAH-specific drugs targeting the endothelin-1, nitric oxide or prostacyclin pathways on endothelial barrier function and tube forming capacity *in vitro*.

BMPR2 silencing & angiogenesis

We found that stable *BMPR2* silencing in HLMVECs resulted in impaired angiogenesis, as illustrated by decreased 2-dimension migration capacities and reduced 3-dimension tube formation. Accordingly, induced pluripotent stem cells (IPSCs) derived from *BMPR2* mutation carriers with PAH demonstrate decreased migration capacity and tube forming capacity compared to IPSCs from healthy individuals or unaffected *BMPR2* mutation carriers (21). Moreover, rats expressing a *BMPR2* mutant were shown to display a significantly decreased pulmonary network, in comparison to control rats at the age of 6 and 12 months (32), suggesting a loss of angiogenic capacities and pulmonary microvascular network maturation. In agreement, loss of downstream BMPRII effectors in Smad8$^{-/-}$ deficient mice resulted in impaired development of the pulmonary vasculature (33). Whereas angiogenesis has been previously considered deleterious in the early pathogenesis of PAH, recent findings have evidenced the potential beneficial effects of neovascularization in preventing PAH progression. Although the potent angiogenetic factor VEGF and its receptor VEGFR are robustly expressed in pulmonary vascular lesions from PAH patients (16,17), the angiogenic paradigm was initiated by Tuder and colleagues who showed that inhibition of angiogenesis by the antagonist of VEGFR-2 ,SU5416, resulted in severe PH in rats exposed to hypoxia (34). However, expression of VEGFR-3, which plays an important role in sprouting angiogenesis by binding VEGF C and VEGF D ligands (35), is significantly lower in pulmonary arterial endothelial cells (PAECs) from PAH patients, resulting in reduced angiogenesis (36). Interestingly, Hwangbo and colleagues identified a co-localization and interaction of VEGFR-3 and BMPRII at the surface of human umbilic vein endothelial cells (36). Consequently, a loss of BMPRII might affect normal VEGFR-3 activation. In addition, extracellular ligands and activators of BMPRII are also able to promote angiogenesis. Actually, BMP2-induced *in vivo* angiogenesis was observed in mice (37,38); BMP4-induced migration occurred in human microvascular ECs via activation of VEGF/VEGFR2 and Angiopoietin/Tie2 signalling (37,38); BMP6 induced sprouting angiogenesis both *in vivo* and *in vitro*, similarly to the most potent angiogenic factor VEGF (39). Ectopic expression of Id1, BMP/Smad target in ECs, mimicked BMP-induced effects by inducing EC migration and tube formation (40)**.** Interestingly, administration of BMP9 to mice bearing a human *BMPR2* mutation and in rat experimental models of PH (monocrotaline and Sugen-hypoxia) reversed PH by restoring tube formation and endothelial

barrier function (41). Downregulation of BMP signalling in ECs by the natural compound codonolactone impairs tumor angiogenesis (42). Altogether our current results and abovementioned studies highlight the beneficial role of BMPRII signalling-driven angiogenesis in the pathogenesis of PAH.

Effect of PAH specific drugs on BMPRII signalling

We previously showed that stable *BMPR2* silencing in HLMVECs resulted in decreased canonical Smad1/5/8 and increased non-canonical p38 MAPK activation (22). To further explore the drug mechanisms, we hypothesized that activation of the PGI_2 or NO or inhibition of the ET-1 pathways could, at least partially restore impaired canonical and non-canonical BMPRII signalling. Unfortunately, by stimulating PGI_2 or NO or inhibiting ET-1 pathways we did not observe any significant effect on Smad1/5/8 or p38 MAPK signalling in HLMVECs.

By contrast, in pulmonary arterial smooth muscle cells (PASMCs), bosentan, another dual antagonist of ET-1 receptors, is able to restore BMPRII expression and downstream signalling (43). Macitentan can inhibit the effects on TGFβ activity by preventing the formation of a ET-1/TGFβ receptor complex in fibroblasts (44). Sildenafil, a PDE_5 inhibitor, can also restore BMPRII signalling by enhancing canonical Smad signalling in PASMCs overexpressing a *BMPR2* mutant (45,46). *In vivo* administration of sildenafil results in increases expression of BMPRII in a monocrotaline rat model (47). Besides, iloprost is able to induce Id1 expression in PASMCs and both iloprost and treprostinil enhance Smad1/5 phosphorylation in response to BMP4 (48). Noteworthy, the abovementioned studies were all conducted in PASMCs or fibroblasts. In this study, we investigated the effects of PAH-specific drugs on microvascular pulmonary endothelial cells. In addition, maintaining endothelial cells in culture is known to be associated with the loss of the expression of cell markers and receptors (49,50). In order to reproduce the situation of treated patients, we have chosen to base the drug dose-response on their Cmax. In addition, the time of stimulation is also important and can varies between studies from 1 hour (43–45) to 24 hour (46) and may result in different effects. As such, we performed a time-response curve showing that one hour of stimulation displayed the highest activation response. Importantly, despite the absence of any effects of the drugs in restoring BMPRII signalling in HLMVECs, we did not experience any deleterious effect of PAH-specific drugs.

Effect of PAH specific drugs on endothelial function

Considering that dysregulated angiogenesis contributes to the pathophysiology of PAH, the potential effects of PAH-specific drugs on endothelial function, including barrier function and angiogenic capacities, remain unexplored. In the present study, we observed very limited effects of PAH-specific drugs on *BMPR2*-induced impaired angiogenesis and no effect on the endothelial barrier function.

We have previously reported that ET-1 secretion is significantly higher in pulmonary microvascular ECs from *BMPR2* mutation carriers compared to non-mutation carriers or controls (51) and activation of endothelial ET-1 receptors results in increased permeability (52). In addition, we have previously reported that stable *BMPR2* knock down in HLMVECs showed decreased barrier function (22). However, blocking ETA and ETB receptors by macitentan did not restore, but also did not further deteriorate barrier function in *BMPR2*-silenced HLMVECs. In addition, activation of the PGI_2 pathway by the IP receptor agonist, selexipag did not display any effect on the endothelial barrier function, whereas prostacyclin analogous previously shown to harbor endothelial barrier protective effects (53–55). Another intriguing aspect to perform comparison between the present and previously published studies is that in most of the abovementioned studies, endothelial barrier function was evaluated by measuring trans-endothelial electrical resistance, whereas we performed a permeability test using fluorescently labelled BSA. In addition, to target PGI2 pathway, we used selexipag, an agonist of the IP receptor, while in the other studies prostacyclin analogues were preferred; this may imply different downstream mechanisms (56).

Furthermore, *in vitro* studies have shown that activation of NO pathway via stimulation of eNOS or by PDE_5 inhibitors can improve the endothelial barrier function, potentially via activated sGC-driven production of cGMP and (57,58). However, tadalafil did not induce any improvement of the barrier function of *BMPR2*-silenced HLMVECs. This may be due to differences in stimulation time of maximum 30 minutes (57) up to 3 days (58) and the use of human brain endothelial cells (58).

Interestingly, we evidenced that macitentan and tadalafil can improve angiogenic capacity. Actually, macitentan did not display any effect on migration properties of *BMPR2*-silenced HLMVECs, but significantly increased their tube formation capacities, in line with the demonstration that macitentan significantly improves tube formation of PAECs (59). Whereas ET-1 has been shown to promote angiogenesis and induce neo-vascularization *in vivo* and *in vitro* (60–62), others evidenced that ET-1 impairs angiogenesis. Consequently, the beneficial role of ET-1 receptors antagonists on angiogenic capacities remains under debate.

Selexipag, a specific IP receptor agonist did not show any effect on angiogenic capacities of *BMPR2*-silenced HLMVECs. By contrast, altered prostanoid metabolism results in impaired angiogenesis (63) and iloprost showed pro-angiogenic capacities in a corneal model of angiogenesis, potentially by interacting with different receptors and alternative pathways (64). This may explain discrepancies

between our results and previous studies. Noteworthy, we found that selexipag reduced tube formation of control HLMVECs, suggesting a potential interaction between BMPRII and the IP receptor pathways and highlighting that loss of BMPRII expression could counteract deleterious effects of selexipag on angiogenic capacities. Tadalafil resulted in a significant improvement of migration capacity, but did not display any effect on tube formation in *BMPR2*-silenced HLMVECs. Surprisingly, tadalafil reduces tube formation by control HLMVECs expressing functional BMPRII, suggesting that PDE_5 inhibition could be detrimental in control endothelial cells whereas it has no impact on diseased endothelial cells. Sildenafil is more potent in increasing tube forming capacity, compared with iloprost (PGI_2 analogue) or bosentan (ERA), (65), in agreement with vardenafil- (66) and sildenafil (67) increased angiogenesis in a mouse hindlimb ischemia model.

To summarize, there are conflicting results regarding the effects of PAH-specific drugs on endothelial function potentially due to the variability of *in vivo* and *in vitro* used models.

Limitations

Although we generated a stable *BMPR2* silencing in HLMVECs to further investigate the effects of PAH-specific drugs targeting the 3 major therapeutic pathways, we are aware that this model does not cover the more than 300 identified *BMPR2* mutations/variants. However, stable *BMPR2* silencing in HLMVECs was developed to overcome the limited access to patient HLMVECs, so far only possible at lung transplantation of patients with a rare disease. In addition, *BMPR2* mutations result in different outcome regarding appropriate BMPRII trafficking and functionality (68,69). In the current study, we explore the effects the effect of each drug type separately. Considering the advent of combination therapy (26), we may consider in the future investigating the effects of 2 or even 3 combined drugs. Another limitation is the use of cell subcultures, which are known to be associated with the loss in the expression of surface markers and receptors, even if the phenotype appears to be maintained. By contrast to previously published *in vitro* studies, we have chosen to mimic the patient situation by using concentration of drugs close to their Cmax, which may have mitigated their *in vitro* effects, but which are more relevant of the physiopathology.

Conclusion

In the present study, we highlighted the key role of BMPRII in regulating angiogenesis of pulmonary microvascular endothelial cells in the context of the PAH pathogenesis. We showed that impaired BMPRII signalling results in reduced migration capacity and tube formation. However, we did not show any relevant effects of PAH-specific drugs on BMPRII signalling pathway-induced endothelial barrier function and very limited effects on angiogenic capacities. Interestingly, drugs targeting NO, PGI_2 and ET-1 pathways did not display any detrimental effects on BMPRII-mediated pulmonary endothelial function.

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In vitro primary culture of human pulmonary arterial endothelial cells harvested from Swan-Ganz catheters

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Abstract

Background

Pulmonary arterial hypertension (PAH) is a devastating condition affecting the wall and endothelium of pulmonary microvessels, resulting in their partial or total obstruction. Despite the availability of several vasodilatory therapies, PAH remains incurable. There is an urgent need of therapies targeting the remodelling process and of innovative methods allowing access to patient material in order to evaluate drug effects in individual patients. A technique, that allows harvesting pulmonary arterial endothelial cells (PAECs) from the balloon of the Swan-Ganz catheter used to perform right heart catheterization (RHC), was recently described.

Hypothesis

The aim of the present study was to optimize the initial technique and to phenotype PAECs along *in vitro* subcultures.

Methods

PAECs were harvested from Swan-Ganz catheters during routine diagnostic or follow-up right heart catheterization of patients with pulmonary hypertension. PAECs were phenotyped by immunofluorescence using antibodies against CD31, VE-cadherin von Willebrand factor (vWF) and α-smooth muscle actin, by DiI-Ac-LDL uptake through scavenger receptors and by flow cytometry.

Results

Within 4 years, we were able to harvest PAECs from 56 catheterization and to maintain them up to 7-12 subcultures for 23 catheterizations. In early subcultures (up to subculture 4), the endothelial phenotype remained stable. However, in advanced subcultures (starting subculture 6 and more), a progressive loss in endothelial phenotype, with decreased expression of CD31, VE-Cadherin and vWF, and reduction in DiI-Ac-LDL uptake, was observed. In addition, cell hypertrophy was observed in advanced subcultures.

Discussion

The present study highlights the unique opportunity to obtain homogenous subcultures of primary PAECs from patients at diagnosis and follow-up. In addition, it opens promising perspectives regarding tailored precision medicine for patients suffering from rare pulmonary vascular diseases.

Introduction

Pulmonary arterial hypertension (PAH) is a rare but devastating condition, characterized by a precapillary pulmonary arteriopathy resulting in partial or total obstruction of pulmonary microvessels, leading to increased pulmonary vascular resistance (PVR), elevated mean pulmonary arterial pressure (mPAP) and eventually right heart failure. PAH is also associated with mutations in the bone morphogenetic protein receptor type II (*BMPR2*) gene (1), impaired BMPRII signalling, pulmonary vascular remodelling and endothelial dysfunction (2). Despite effective vasodilator therapies, mortality rates remain high with a 5-year survival rate of 49–67% (3). Currently, there is no curative treatment available for PAH behalf lung transplantation being the only option in patients with severe PAH refractory to current medical therapies (4).

To better understand the onset and progression of PAH, various experimental animal models have been developed (5). Despite their obvious contribution to the insight of the pathogenesis, they all display limitations to efficiently translate their outcome to the bedside. *In vitro* alternatives based on patient material collected at lung transplantation imply major drawbacks including limited number of samples since lung transplantation occurs rarely, and the advanced stage of disease of the transplanted patients, who have been exposed to PAH-specific drugs for several years. Therefore, innovative methods to access patient material are necessary to better understand disease progression and heterogeneity.

To simulate the *in vivo* situation, transient and stable knock-down of *BMPR2* in human pulmonary vascular cells has been established to investigate the effect of impaired BMPRII signalling on disease progression including inflammation, thrombosis, proliferation and angiogenesis (6). The recent development of a technique to obtain endothelial-colony forming cells derived from peripheral blood of patients with PAH and healthy controls is a major step to collect endothelial cells and generate induced pluripotent stem cells (iPSC) from PAH patients at diagnosis and from healthy *BMPR2* mutation carriers (7). The use of iPSC was recently underscored to investigate patient specific characteristics and evidence different patient-specific mechanisms resulting in common EC dysfunction (8). This suggests that iPSC represent a precious tool to investigate disease heterogeneity and progression.

Interestingly, Pollet and colleagues reported an innovative technique that enables harvesting pulmonary arterial endothelial cells (PAECs) from the balloon of the Swan-Ganz catheter (9) commonly used to perform right heart catheterization, requested to establish any diagnosis of PAH (10). However, these authors discourage further *in vitro* cell culture experiments (11).

We therefore propose an optimization of this technique allowing to establish and maintain *in vitro* the phenotype of PAECs up to 8 subcultures.

Methods

Isolation of PAECs from Swan-Ganz catheters

Swan-Ganz catheters were collected from patients at the time of routine diagnostic or follow up right heart catheterization performed in patients with idiopathic PAH (IPAH), heritable PAH (HPAH), drug or toxin induced PAH, PAH associated with congenital heart or connective tissue diseases, porto-pulmonary hypertension, pulmonary veno-occlusive disease, chronic thromboembolic pulmonary hypertension and PH associated with lung or heart diseases, within the University Hospital of Leuven between 2015 and 2019. The study protocol was approved by the institutional ethical committee of the University of Leuven and all participants gave written informed consent. Patient characteristics were collected at the time of RHC.

To harvest PAECs from Swan-Ganz catheters, we have adapted and optimized the method initially described by Pollett and colleagues (9). The Swan-Ganz catheter balloon was withdrawn protected within the sheath and immediately placed in previously warmed (37°C) microvascular endothelial cell basal medium supplemented with 0.2% cell growth supplement (Cell Applications Inc.), antimycotics (1.25µg/mL amphotericin B; Thermo Fisher Scientific) and antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin; Thermo Fisher Scientific). The balloon was inflated and agitated thoroughly for 2 min. The tip of the Swan-Ganz catheter was cut, maintained in warm medium and transferred to the laboratory within 10 minutes for further processing (Figure 5.1).

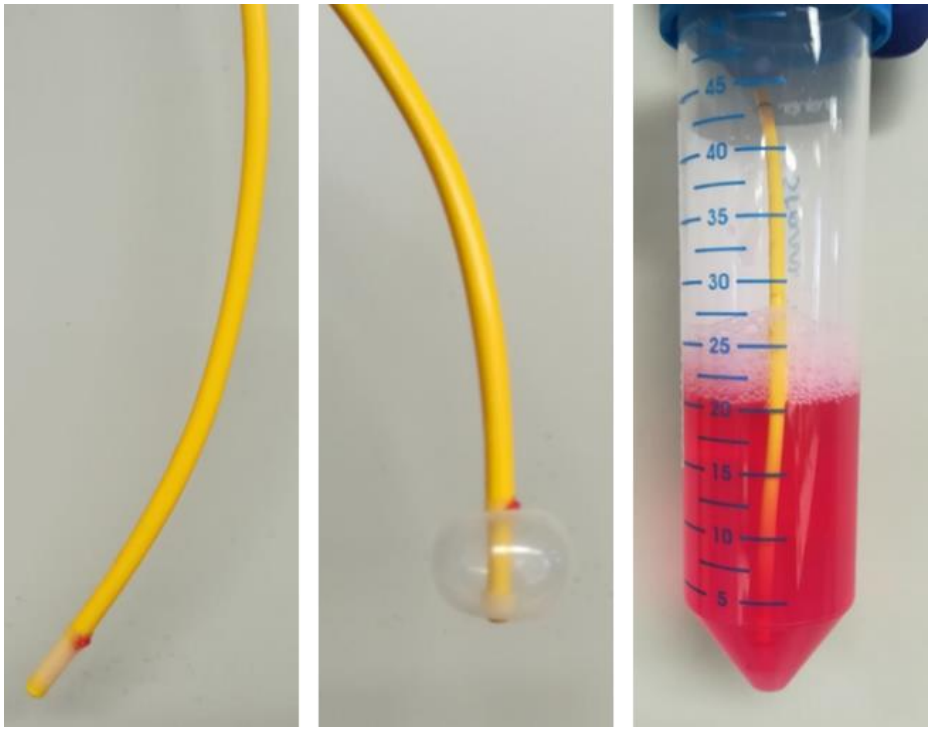

Figure 5.1: The Swan-Ganz catheter tip is presented when collected after right heart catheterization (left panel). Illustration of the inflated balloon at the tip of the Swan-Ganz catheter (middle panel). Collection and transportation tube in which the Swan-Ganz catheter is transported containing warm (37°C) basal medium with 0.2% cell growth supplement (right panel).

Under the laminar flow, the balloon was inflated with air using a 1-mL syringe and transferred in a 0.25% trypsin/0.91mM EDTA solution (Thermo Fisher Scientific). After 2 min, the catheter tip was washed briefly into fresh microvascular endothelial cell growth medium containing 6% cell growth supplement (Cell Applications Inc.), antimycotics and antibiotics, as abovementioned. This medium was mixed with the trypsin solution and centrifuged together with the collection tube at 500*g at room temperature for 10 min. To discard red blood cells, the resulting pellets were pooled and incubated in Ammonium-Chloride-Potassium lysis buffer (Thermo Fisher Scientific) for 5 min at room temperature, followed by a centrifugation at 500*g at room temperature for 10 min, with an excess of phosphate-buffered saline (PBS). The remaining pellet was resuspended in microvascular endothelial cell growth medium, seeded in 2 wells of a 12-well cell culture plate and maintained at 37°C under 5% CO_2. Cell culture medium was refreshed after 5 days and then every other day. Cells were maintained up to subculture 7 to 12, including trypsinization every 4 to 5 days, for 6 to 9 weeks.

In parallel, cells were expanded, trypsinized and transferred in a solution containing 90% fetal bovine serum and 10% dimethyl sulfoxide for further storage in liquid N_2 at -170°C. At least one vial containing 10^6 cells was stored.

Qualitative phenotyping of PAECs using immunofluorescence

Acetylated low-density lipoprotein (Ac-LDL) display the unique property of binding to scavenger receptor expressed by endothelial cells and is currently used to characterize endothelial cells as previously described (12,13). DiI (1,1'-dioctadecyl- 3,3,3',3'-tetramethylindocarbocyanine perchlorate) is a highly lipophilic molecule that can be noncovalently incorporated into lipoproteins. Once DiI-Ac-LDL is taken up by the cell, the lipoprotein molecule is acted upon by lysosomal enzymes and DiI accumulates in lysosomal membranes (14). Subconfluent PAECs were seeded onto fibronectin-coated (10µg/ml; R&D Systems) 4-chamber slides (Thermo Scientific) at a density of 20,000 cells/chamber and further labelled using 10 µg/mL Ac-LDL coupled with fluorescent DiI (Tebu Bio) for 4h at room temperature and fixed in PBS containing 4% paraformaldehyde.

In addition, PAECs were phenotyped by immunofluorescence using antibodies against human CD31 (Clone JC70A, Dako), von Willebrand factor (vWF, clone F8/86; Dako) and Vascular Endothelial-Cadherin (VE-Cadherin, clone D87F2; Cell Signaling Tech.). Potential contamination with myofibroblasts at early subcultures or cell dedifferentiation in advanced late subcultures were investigated by immunostaining using antibodies raised against human alpha smooth muscle actin (α-SMA, clone 1A4; Dako). Cells were seeded onto fibronectin-coated 4-chamber slides (20,000 cells/chamber). Subconfluent PAECs were fixed in PBS containing 4% paraformaldehyde and permeabilized with 0.2% Triton-X100 (Sigma-Aldrich). Non-specific binding sites were saturated in PBS containing 3% bovine serum albumin (BSA; Sigma-Aldrich) for 1h at room temperature.

Immunolabelling of endothelial specific markers was performed using primary antibodies diluted in PBS containing 3% BSA for 2h at room temperature (CD31, 1:25 dilution; vWF, 1:50 dilution; VE-Cadherin, 1:600 dilution; α-SMA, 1:50 dilution). Fluorescent labelling was obtained using secondary antibodies Alexa594 (1:2000 dilution; Thermo Fisher Scientific) goat anti-mouse for CD31, vWF and α-SMA and goat anti-rabbit for VE-cadherin for 1h at room temperature. For negative control, primary antibodies were omitted.

Nuclei were visualized using 4',6-diamino-2-phenylindole (DAPI, Thermo Fisher Scientific). Slides mounted in FluoroSave (Calbiochem) medium were analysed under an inverted Olympus IX80 fluorescence microscope. To quantify immunofluorescence staining, images from non-overlapping fields on each slide were captured at 40X magnification. After having separated the different channels, red staining was measured using the ImageJ software and expressed as arbitrary units (AU). Quantification of cell hypertrophy was performed using immunofluorescent images of CD31 staining measuring the cell surface area of ten cells in three different fields using the ImageJ software.

Quantitative phenotyping by flow cytometry

Subconfluent PAECs were trypsinized, resuspended in microvascular endothelial cell growth medium and counted using the Countess™ automated cell counter (Invitrogen). One-hundred thousand cells were collected for negative control and for staining with allophycocyanin-conjugated anti-CD31 antibody (APC-conjugated CD31; Miltenyi Biotec), respectively. Cells were centrifuged at 300*g and 4°C for 10 min, resuspended in PBS containing 1% BSA (PBS-1%BSA) and incubated with APC-conjugated CD31 for 15 min in the dark at 4°C. Cells were further washed with PBS-1%BSA, fixed in PBS containing 4% paraformaldehyde during 15 min in the dark at 4°C and resuspended in PBS-1%BSA for further quantitative flow cytometry analysis (Canto HTS, BD bioscience). Analysis was standardized with fixed gates used to investigate CD31-APC positive cells as previously described (13). The forward scatter (FSC) and sideward scatter (SSC) of each individual cell is detected. These measures were used to evaluate cell size (FSC) and cell granularity (SSC). Evaluation of cell granularity is a rough estimation of cell aging and life span. A CD31-APC parameter histogram was used to identify CD31-APC positive cells.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 8.1.2 (GraphPad Software Inc., La Jolla, California). Differences between early and late subcultures were analyzed using paired t-test. Correlations between successful and unsuccessful cultures and patient characteristics were tested on normal distribution and analyzed using a parametric t-test or non-parametric Mann-Whitney U test accordingly. All p values are for 2-sided tests. A value of $p<0.05$ was considered statistically significant.

Values are expressed as mean ± SD.

Results

Starting August 2015 until 2019, we collected 132 Swan-Ganz catheters mainly from IPAH, HPAH, and chronic thromboembolic pulmonary hypertension (69%), but also from patients with PAH associated with congenital heart or connective tissue diseases, porto-pulmonary hypertension, pulmonary veno-occlusive disease or pulmonary hypertension secondary to lung or heart diseases, in order to optimize our methodology. From the 132 Swan-Ganz catheters collected at RHC, we were able to observe growing cells from 56 procedures, among them 33 were contaminated with bacterial or fungal infections or did not appropriately grow after a first subculture. Patient characteristics indicate that we did not observe any significant difference between successful and unsuccessful procedures (Table 5.1).

Table 5.1: Patient characteristics

	All (n=132)	Successful (n=56)	Unsuccessful (n=76)	p-value
Age, *years*	64 (23-89)	64 (27-89)	64 (23-83)	0.727
Gender, male (%)	33	37	30	
BMI kg/m^2	25 (17-47)	24 (18-47)	26 (17-41)	0.544
Aetiology				
IPAH	45	18	27	
HPAH	5	3	2	
Drug/toxin	8	4	4	
APAH	17	4	13	
PVOD	3	2	1	
PH sec to HD	7	1	6	
PAH sec to LD	3	2	1	
CTEPH	33	17	16	
No PH	11	5	6	
PAH therapy				
treatment-naive	65	25	40	
monotherapy	16	8	8	
bitherapy	28	11	17	
tritherapy	20	10	10	
CCB	3	2	1	
NYHA FC				0.433
I	12	5	7	
II	55	22	33	
III	55	23	32	
IV	5	3	2	
RAP (mmHg)	7 (1-25)	7 (2- 25)	7 (1-22)	0.456
mPAP (mmHg)	41 (11-106)	39.5 (11-70)	43 (13-106)	0.748
PAWP (mmHg)	10 (2-57)	10 (4-57)	10 (2-22)	0.700
PVR (dyn.s.sec^{-5})	530 (61-2187)	539 (61-2187)	527 (72-1706)	0.966
CI (L/min/m^2)	2.5 (1.1-4.7)	2.5 (1.1-3.9)	2.6 (1.3-4.7)	0.330
SvO_2 (%)	65.5 (37-95)	65 (39-80)	66 (37-95)	0.377
6-MWD (m)	360 (23–747)	386 (60-580)	349 (23-747)	0.661
CRP (mg/mL)	2.4 (0.3-64.4)	2.3 (0.3-14.6)	2.5 (0.3-64.4)	0.374
Vit D (ng/mL)	20.4 (2.3-60.7)	19.1 (2.3-60.7)	21.2 (4.5-60.4)	0.947
NT-proBNP (ng/mL)	807 (8-21834)	753 (53-21834)	807 (8-13395)	0.552

Results are expressed median (min-max). Successful cultures were considered when an expanding cell colony was observed, if not, these cultures were considered unsuccessful. BMI, body mass index; IPAH, idiopathic pulmonary arterial hypertension; HPAH, heritable PAH; APAH, associated PAH (including associations with connective tissue disease (CTD), congenital heart defects (CHD), porto-PH (PoPH) and HIV); PVOD, pulmonary veno-occlusive disease; PH sec to HD, pulmonary hypertension secondary to heart disease; PH sec to LD, PH secondary to lung disease; CCB, calcium channel blockers; RAP, right atrial pressure; mPAP, mean pulmonary arterial pressure; PAWP, pulmonary arterial wedge pressure; PVR, pulmonary vascular resistance; CI, cardiac index; SvO_2, mixed venous oxygen saturation; 6-MWD, six-minute walking distance; CRP, C-reactive protein; Vit D, vitamin D; NT-proBNP, N-Terminal pro brain natriuretic peptide.

Eventually, we were able to expand and store cells from 23 procedures. Despite several optimization attempts and personal learning curve, we were able to obtain a success rate of 35% of properly expanded and stored cells. (Table 5.2).

Table 5.2: Summary of total collected catheters and successfully stored PAECs.

	2015	**2016**	**2017**	**2018**	**2019**	**Total**
Total SG catheters collected	15	17	34	43	23	132
Stored PAECs (%)	4 (27%)	3 (18%)	2 (6%)	6 (14%)	8 (35%)	23 (17%)

SG, Swan-Ganz catheters; PAEC, pulmonary arterial endothelial cells.

PAECs freshly isolated from Swan-Ganz catheters display a typical cobblestoned morphology (Figure 5.2; subculture S2). Until subculture 6, we did not observe any major changes in the morphology of PAECs (Figure 5.2; S2-S6). By contrast, after 8 subcultures, cell hypertrophy and occurrence of myofibroblast-like cells could be observed, which was even enhanced after 10 subcultures (Figure 5.2, S8-S10). Concomitantly, the amount of CD31-positive PAECs measured by flow cytometry showed the presence of homogenous endothelial cell population over subcultures (Figure 5.2). However, the spreading of CD31-positive cells, shown by the enlargement of the base of the curve, indicates a higher variety of CD31 expression or physical properties of the cells (Figure 5.2).

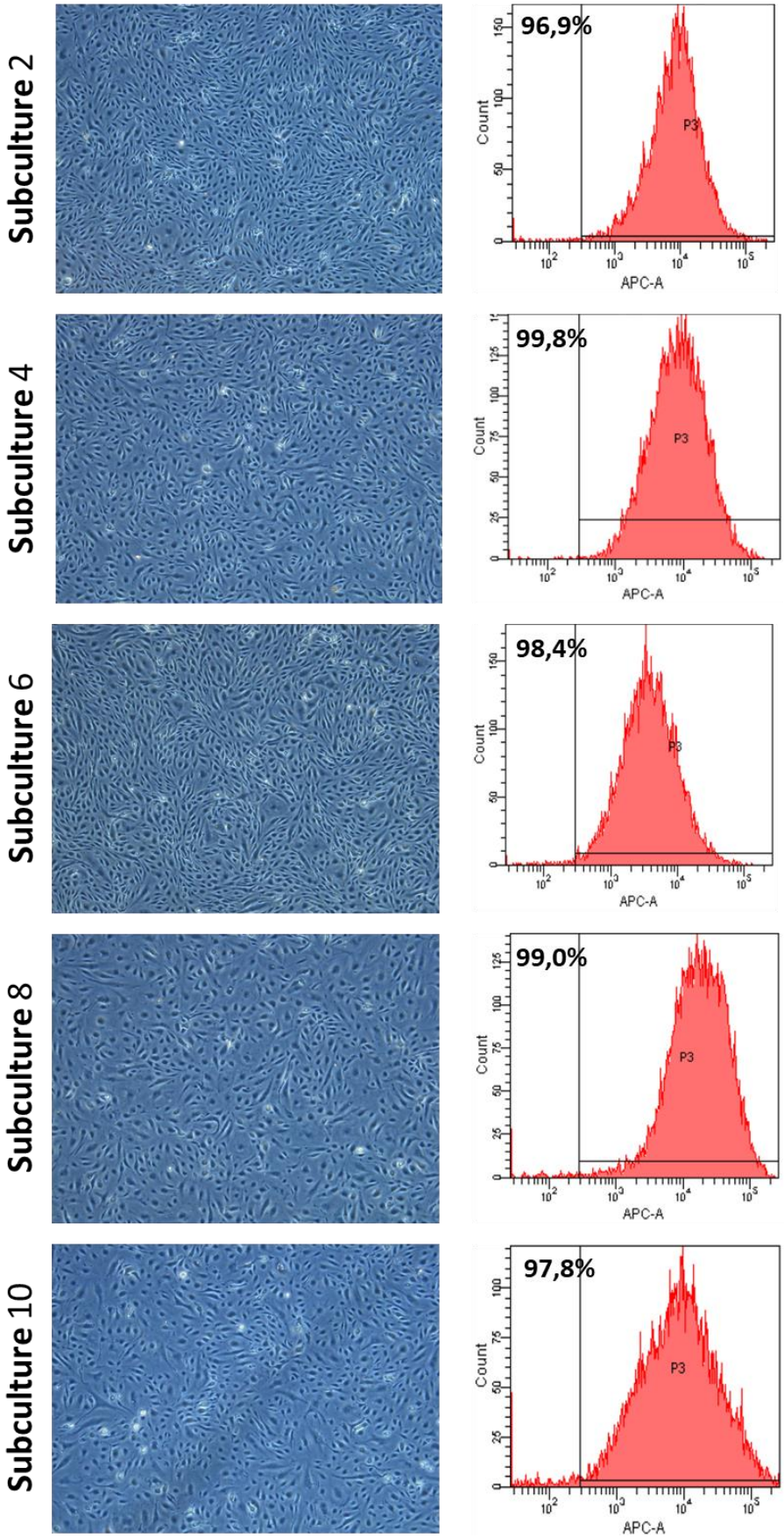

Figure 5.2: Representative pictures of PAECs showing the morphology along subcultures (left panel). Quantitative analysis of CD31-positive PAECs by flow cytometry along subcultures (right panel).

Accordingly, in early subcultures (S2-S3), a typical cobblestoned morphology of PAECs isolated from 4 different patients was observed. In advanced subcultures (S7-S10), elongated myofibroblast-like and hypertrophic cells occurred (Figure 5.3).

In early subcultures (S2-S3), a highly homogenous endothelial cell population was observed as shown by the percentage of 98.7 ± 1.1 % of CD31-positive cells detected by flow cytometry (Figure 5.4). Interestingly, in advanced subcultures (S8-S10), the percentage of CD31-positive cells remained unchanged 98.4 ± 0.6 %: p = 0.404, despite of an enlargement of the histogram width (Figure 5.4).

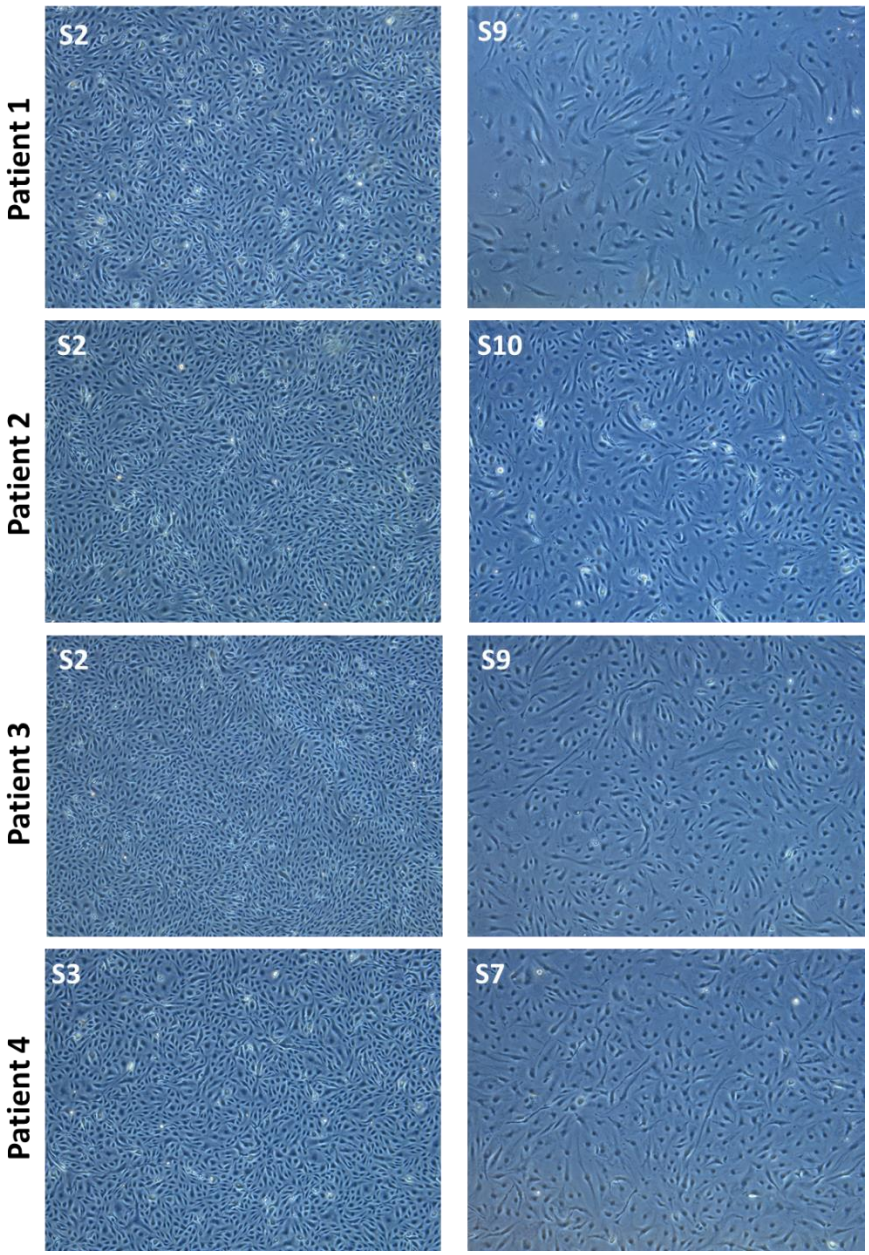

Figure 5.3: Representative pictures of PAECs in culture isolated from 4 different patients at right heart catheterization using Swan-Ganz catheters. left panel: early subcultures; right panel: advanced subcultures.

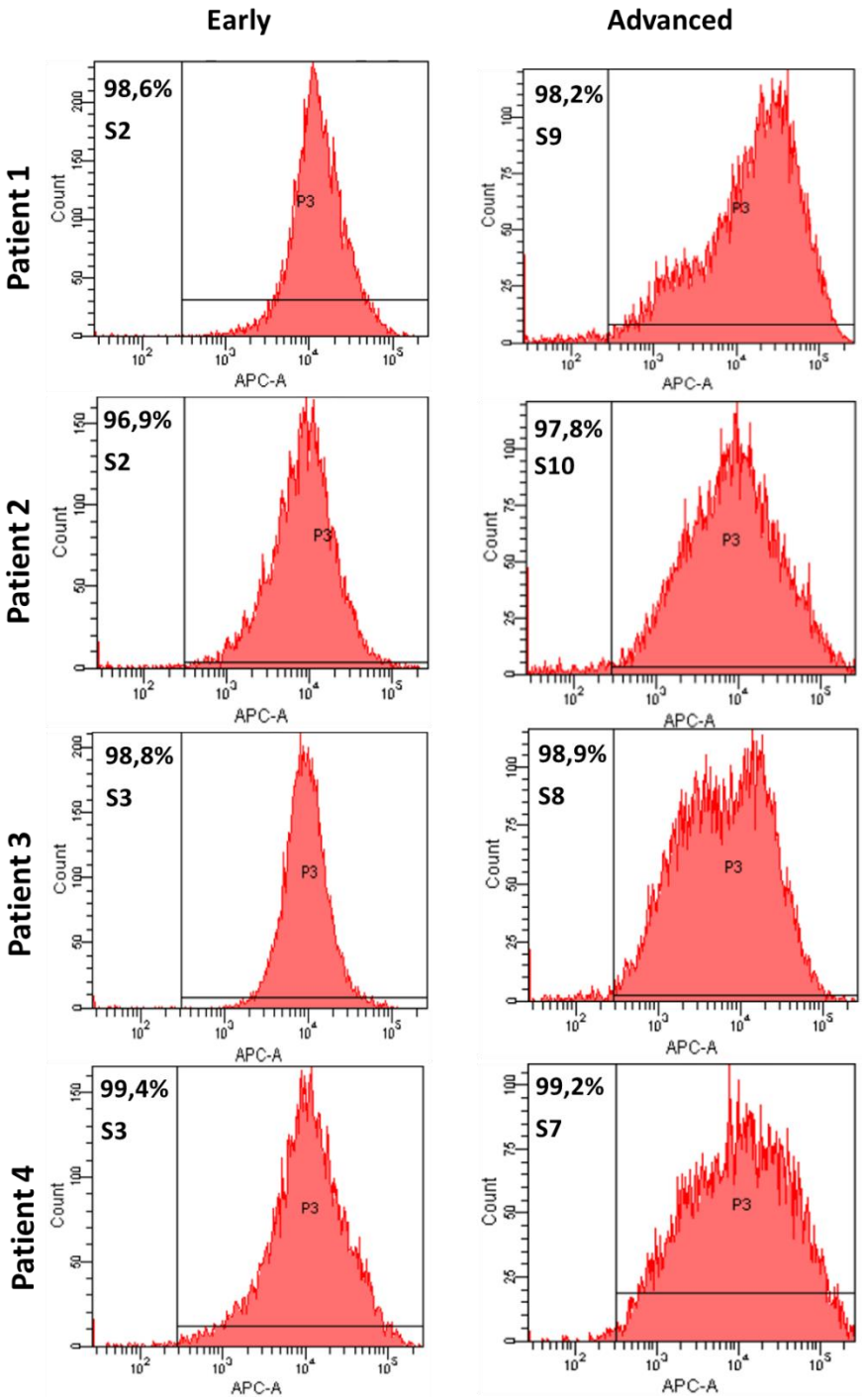

Figure 5.4: Quantitative phenotyping by flow cytometry of CD31-APC-labeled PAECs isolated from 4 different patients using Swan-Ganz catheter. Histograms at early (left panel) and advanced (right panel) subcultures.

In early subcultures (S2-S4), PAECs isolated from 3 different patients showed expression of the specific endothelial cell surface markers CD31 (Figure 5.5A-C) and VE-Cadherin (Figure 5.5G-I). In advanced subcultures (S7 to S10), expression of both CD31 (Figure 5.5D-F) and VE-Cadherin (Figure 5.5J-L) was mitigated, VE-cadherin staining was less homogenous with the presence of intracellular gaps (Figure 5.5J-L). Quantification of the immunofluorescence did not show any significant change in both CD31 and VE-cadherin staining between early and advanced subcultures (Table 5.3).

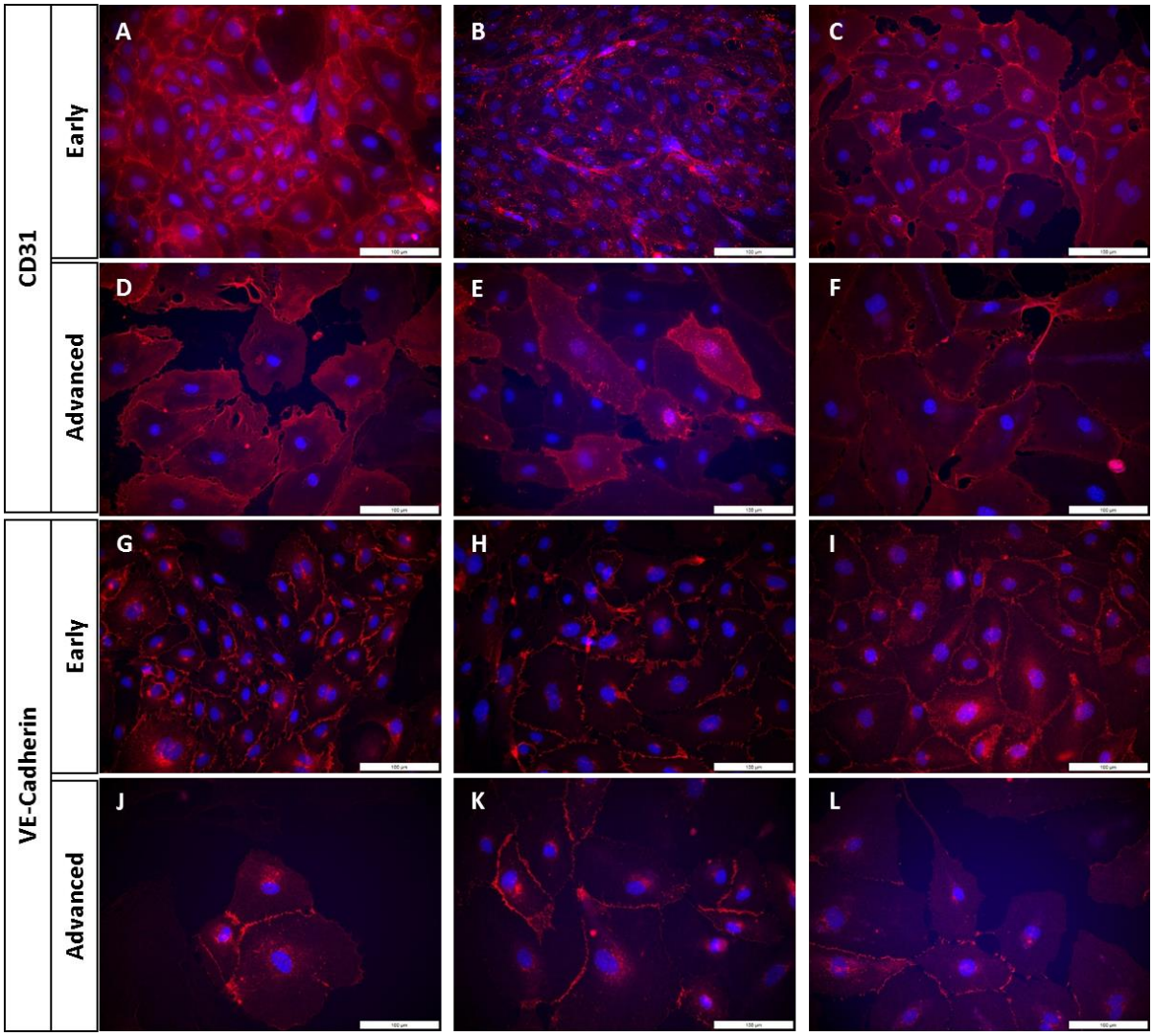

Figure 5.5: Qualitative phenotyping of PAECs using endothelial specific surface markers CD31 (A-F) and VE-Cadherin (G-L) by immunofluorescence performed in cells isolated from 3 different patients in early subculture (S2 or S3; A-C & G-I) and advanced subculture (S7-S10; D-F & J-L). Nuclei were counterstained using DAPI (blue). Scale = 100 µm.

Cell hypertrophy could be observed (Figure 5.5D-F & 5.5J-L), with a significant increase in cell surface area in advanced compared to early subcultures (Figure 5.8C). Intracellular expression of the specific endothelial marker vWF (Figure 5.6A-C) and the uptake of Ac-LDL by endothelial-specific scavenger receptors (Figure 5.6G-I) were profuse in early subcultures, but dramatically reduced in advanced subcultures (Figure 5.6D-F & 5.6J-L), with a significant decrease in Ac-LDL uptake in advanced compared to early subcultures (Table 5.3).

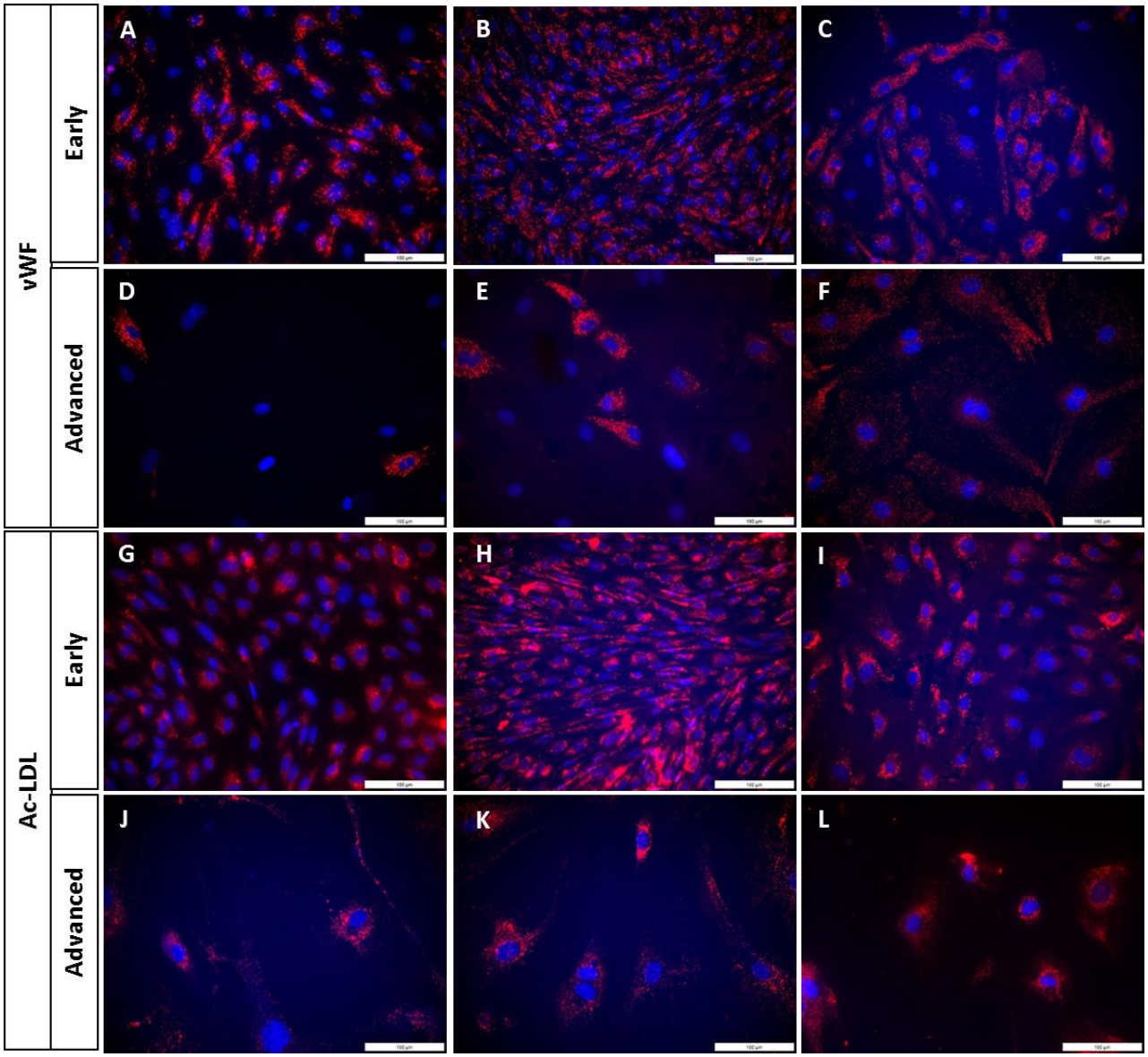

Figure 5.6: Qualitative phenotyping of PAECs using endothelial specific intracellular markers von Willebrand factor (A-F) and Ac-LDL (G-L) by immunofluorescence performed in cells isolated from 3 different patients in early subculture (S2 or S3; A-C & G-I) and advanced subculture (S8-S10; D-F & J-L). Nuclei were counterstained using DAPI (blue). Scale = 100 µm.

Table 5.3: Quantification of the expression of specific endothelial markers in PAECs

	Early subcultures	Advanced subcultures	p value
CD31	62.9 ± 24.0	43.3 ± 2.9	0.29
VE-cadherin	40.1 ± 7.8	30.0 ± 7.0	0.23
vWF	33.7 ± 15.8	19.5 ± 4.8	0.16
Ac-LDL uptake	39.1 ± 4.4	14.9 ± 4.1*	***0.0001***
α-SMA	21.7 ± 11.5	17.9 ± 1.6	0.63

Expression of CD31, vascular endothelium cadherin (VE-cadherin), von Willeband factor (vWF), and α-smooth muscle actin (α-SMA) and uptake of acetylated low-density lipoproteins (Ac-LDL) by PAECs were quantified in early subcultures and advanced subcultures and expressed as mean ± std. deviation.

Additionally, early subcultures of PAECs did not display any positive staining for α-SMA, whereas in advanced subcultures, α-SMA was expressed in the cytoplasm of hypertrophic cells (Figure 5.7). However, no significant change in α-SMA staining was observed between early and advanced subcultures (Table 5.3). Finally, the number of CD31-positive cells remained stable along subcultures (Figure 5.8A & 5.8C). Cell granularity, measured by SSC-A, remained unchanged until the fifth subculture, started to increase after the sixth subculture and is significantly 2-fold increased (p=0.03) after 9 subcultures (Figure 5.8B).

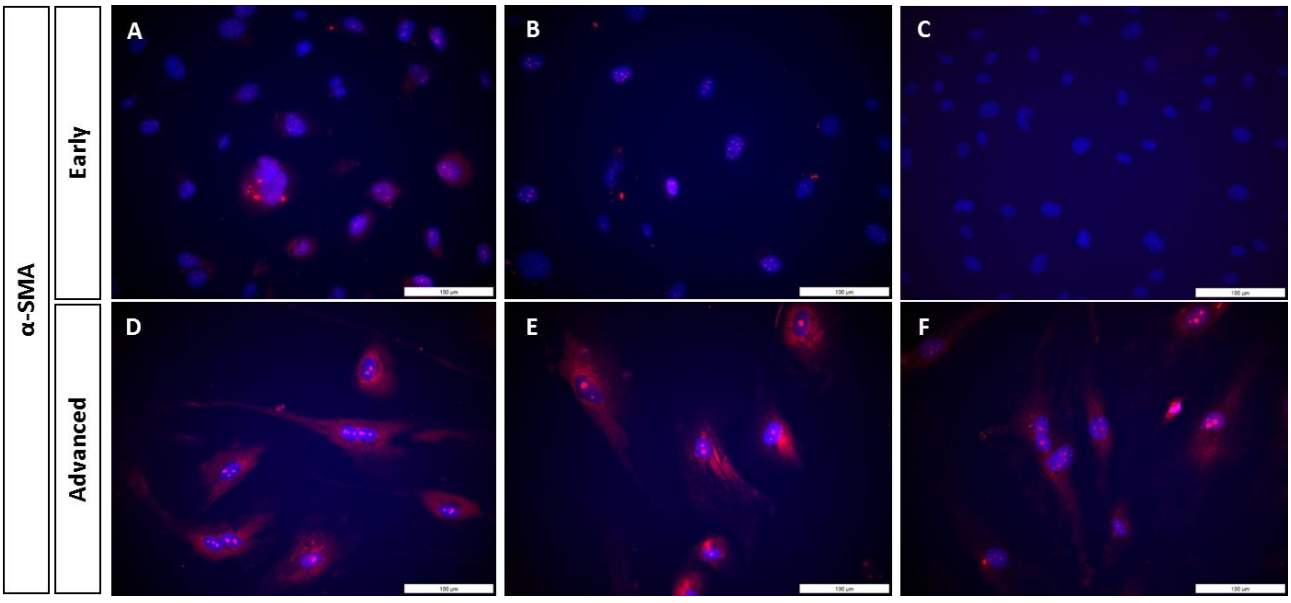

Figure 5.7: Immunostaining of cells isolated from 3 different patients in early subculture (S2 or S3; A-C) and advanced subculture (S8-S10; D-F) using an antibody against α-smooth muscle actin. Nuclei were counterstained using DAPI (blue). Scale = 100 µm.

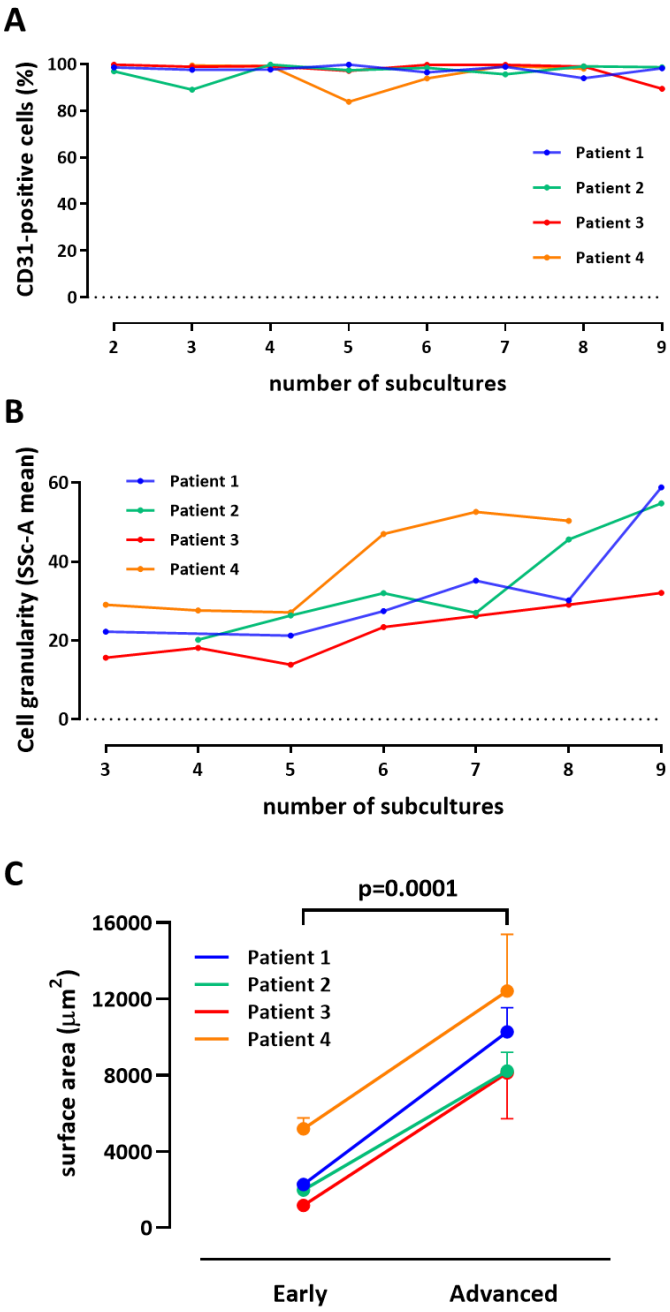

Figure 5.8: CD31-positive cells isolated from 4 individual patients along subcultures (A). Cell granularity as an indication of cell aging in PAECs isolated from 4 individual patients along subcultures (B). Quantitative analysis of endothelial cell hypertrophy from 4 individual patients at early and advanced subcultures (C).

Discussion

In the present study, we reported a detailed and optimized protocol allowing the isolation of PAECs from Swan-Ganz catheters, adapted from the initial description of the technique by Pollett and colleagues (15). More importantly, we demonstrated that PAECs harvested from Swan-Ganz catheters can be maintained *in vitro*, with an endothelial phenotype remaining stable for several weeks in culture.

A major finding of the present study is the possibility to maintain PAECs harvested from Swan-Ganz catheters in culture, with a rather stable phenotype along subcultures. This can be seen as an advance since Pollett and colleagues mentioned that one important limitation of their technique was the low number of viable isolated PAECs (11). As initially reported (15), we obtained homogenous culture of cobblestoned ECs, independently of disease aetiology. Consequently, we did not perform extensive characterization of the isolated cell populations by flow cytometry considering that potential contaminations by other cell types would be wiped out by the stringent EC culture conditions. In advanced subcultures, morphological changes including cell hypertrophy, dedifferentiation such as change towards myofibroblast phenotype, loss of endothelial markers, increased granularity illustrating some signs of cell aging could be noticed. This suggests that short-term subcultures would be more appropriate for potential translational applications, but matching previous findings highlighting the *in vitro* retention of several abnormal phenotypic features in cultured pulmonary endothelial cells from patients with PAH/CTEPH (16).

Compared to the technique developed by Pollett (15) and Passineau (11), we have consistently refined the initial protocol, *e.g.* immediate immersion of the catheter in warmed basal medium containing 0.2% cell growth supplement instead of iced collection, inflated balloon agitated in cell culture medium, omission of the micro-bead purification step, which might lower cell recovery yield (11), leaving the cells untouched for the first 5 days in culture instead of refreshing medium the day after isolation. In our hands, this refined protocol resulted in successful isolation of PAECs followed by subcultures from about 23 Swan-Ganz catheters within 4 years, indicating that this innovative technique is a unique and inestimable source of pulmonary arterial endothelial cells from patients at diagnosis and follow-up, without additional risk for the patient. Analysis of successful and unsuccessful collection did not show any correlation between patient's characteristics and successful collection of the culture. By contrast to Ventetuolo and colleagues, who found that successful culture was more likely in subjects with a lower cardiac index and higher pulmonary vascular resistance, we did not observe any significant statistics within the patient characteristics between successful versus not successful procedures (17).

A major asset of this technique is the assurance to exclusively harvest endothelial cells originated from the pulmonary vessel wall, excluding circulating endothelial cells, as previously argued by Pollett (15), in comparison with the use of BOECs (18) or IPSCs (8) derived from peripheral blood.

Consequently, this technique will undeniably open multiple translational and clinical applications, and offer promising perspectives. For instance, serial collection of patient material could be performed at diagnosis and follow-up, which is highly valuable to investigate disease progression, specific responses to medical, angioplasty or surgical treatment in rare forms of PH, and according to the genetic background. In addition, molecular signature would be possible as recently proposed for *in situ* expression of Bcl-2 in pulmonary artery endothelial cells of patients with pulmonary hypertension relative to heart failure with preserved ejection fraction (19). This is a major asset within the era of precision and personalized medicine, with for instance the implementation of alternative and innovative therapeutic approaches combining gene editing, cell therapy and oral medication, individually tailored for each patient (20).

Limitations

However, such a promising technique displays some limitations that need to be highlighted. Actually, by contrast to isolation of endothelial cells from peripheral blood, *e.g.* BOECs (18) or iPSCs (8), from any individual, the present technique does not offer the possibility to sample healthy controls or healthy *BMPR2* mutation carriers, since RHC is an invasive technique only performed in expert centres in case of PH suspicion. Nevertheless, synergy between these different methods will only increase the insights of endothelial cell biology during disease progression, disease heterogeneity and specific response to treatment. As previously noticed, the number of viable cells harvested is rather low and highly variable (15). This obviously conditioned the success rate of PAEC isolation and subcultures, which recently reached 35% in our hands, suggesting that further optimization is still necessary. Indeed, the presence of initial PAEC clones does not systematically ensure further sufficient expansion, which can depend on patient aetiology, technique of the physician, period during which the balloon is in contact with the vessel wall. As already highlighted by Pollett and colleagues, this location is generally the branch of a pulmonary artery, and not the pre-capillary/microvessels initially affected by the disease.

Conclusion

To conclude, the present study highlights the promising and unique opportunity to obtain homogenous and rather stable subcultures of primary PAECs from a larger cohort of patients, more frequently, at diagnosis and follow-up. Importantly and by contrast to a recent update of the technique discouraging cell culture experiments, we successfully overcame a step forward opening promising perspectives regarding tailored precision medicine for patients suffering from rare pulmonary vascular diseases.

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Chapter 6: General Discussion

Summary of main findings

1. Since access to patient material in PAH is extremely limited and mainly feasible following lung transplantation, we developed two alternatives. First, we created a stable *BMPR2* silencing in commercially available pulmonary human lung microvascular endothelial cells (HLMVECs) which partially reproduces the BMPRII loss of function, associated with PAH. Secondly, we optimized a unique technique and demonstrate feasibility to isolate and culture pulmonary arterial endothelial cells (PAECs) from the Swan-Ganz catheters used to perform right heart catheterization (RHC), the gold-standard method to establish any diagnosis of PH. It is an innovative and elegant approach, which allows collection of PAECs at diagnosis in treatment-naïve patients and at follow-up.
2. Using *BMPR2*-silenced HLMVECs, we demonstrated the effect of impaired BMPRII signalling on endothelial dysfunction by investigating endothelial barrier function and angiogenesis. Our major findings show that loss of BMPRII results in impaired barrier function and increased susceptibility to inflammatory mediators including TNFα and IL-18. These results highlight that impaired BMPRII signalling can generate an enhanced vulnerability to an inflammatory insult, in agreement with the second-hit hypothesis in PAH patients carriers of a *BMPR2* mutation (1).
3. Another key finding is the concomitant reduced angiogenesis and BMPRII loss of function. This is supporting the hypothesis of the microvascular rarefaction as a driving pathogenic process in PAH, by contrast to the pro-angiogenic hypothesis advocating the initial description of PAH as a cancer-like disorder as initially described (2). In addition, our results show that impaired BMPRII signalling leads to decreased angiogenesis indicating that PAH must be considered as an anti-angiogenic disease.
4. Finally, we found limited effects of recently launched vasodilatory PAH therapies on the endothelial barrier function and *in vitro* angiogenic capacities. Importantly, we did not observe any deleterious effects of these drugs in a background of BMPRII loss of function.

6.1. Alternatives to broaden access to pulmonary vascular cells from PAH patients

PAH is a rare disease implying that collection of patient pulmonary vascular cells is limited and exclusively possible through lung transplantation, at advanced stages of the disease and in patients exposed to drug combination for a long period. Therefore, there is a critical need for reliable approaches enabling access to pulmonary vascular cells at diagnosis of treatment-naïve patients. Considering this gap, we have implemented two different *in vitro* approaches.

1. *BMPR2* silencing in HLMVECs

Previous publications have described transient *BMPR2* silencing (3–5). To overcome this, we have established *BMPR2* silencing in HLMVECs, using lentiviral vectors, in tight collaboration with the Leuven Viral Vector Core (https://gbiomed.kuleuven.be/english/corefacilities/LVVC). Stable *BMPR2* silencing in HLMVECs results in reduced BMPRII expression as well as downstream impaired canonical Smad1/5/8 and non-canonical p38 MAPK signalling pathways, consequently reproducing some aspects observed in the human PAH pathophysiology. However, we are aware that this model is not fully representative of the human pathology, since we previously observed that not all PAH patients show down-regulation of BMPRII protein expression (6). In addition, *BMPR2* mutations have been identified to be associated with expression of a non-functional BMPRII protein or with dysfunctional intracellular trafficking resulting in retention of BMPRII in the endoplasmic reticulum or Golgi compartments. Both type of mutations do not show any change in the global BMPRII expression (7,8). Therefore, an alternative approach would be to perform lentiviral vector-mediated overexpression of BMPRII mutants in order to affect receptor dimerization, which plays a major role in BMPRII downstream signalling (9). As such, several preclinical studies have shown a potential benefit of therapeutics activating the BMPRII signalling pathway (10). For instance, correction of a *BMPR2* mutation in IPSCs derived from a HPAH patient resulted in normalisation of p38 MAPK signalling (11). In addition, upregulation of BMPRII expression by adenoviral gene delivery shows beneficial effects in animal models of PH (12,13). Current improvements in gene editing technology including TALEN or CRISPR methodology may be used in future research in order to develop more representative models of PAH. With these new techniques, we can easily and rapidly create cell lines which resemble all types of different mutations identified in individual patients (14).

2. Isolation of PAECs from Swan-Ganz catheters

As another alternative, we took advantage of an innovative technique initially described in 2013 by Pollett and colleagues (15), which allows the collection of PAECs at less advanced stages of the disease from PAH treatment-naïve patients, in comparison to cells collected from transplanted patients. Although the pioneers of this approach discouraged maintaining these cells in culture, we have optimized this innovative approach and succeeded to maintain stable subcultures of PAECs. Another potential application of such a technique is the implementation of cell signature analysis, which can be used to confirm a diagnosis as illustrated by Benza and colleagues who demonstrated Bcl-2 as an index to differentiate between PAH and PH due to HFpEF (16). Another advantage of this technique is the possibility of longitudinal exploration along the course of the disease and in response to treatment. This methodology can also counteract rather limited and unpredictable isolation of PAECs via lung transplantation.

Over the last 4 years, we collected 132 catheters tips after clinical routine right heart catheterization

of which we were able to expand 56 and properly store 23 PAECs from patients with HPAH, IPAH, APAH or CTEPH. These findings were supported by a recent report highlighting the feasibility of the methods at various stages of disease progression (17). Unaware of any progression, we developed a very similar methodological protocol and obtained likely success rates. The authors reported a success rate of 39%, while our reached 42% so far.

Concomitantly, other initiatives to overcome shortness of access to pulmonary vascular cells from PAH have emerged, such as induced pluripotent stem cells (iPSCs) (11) or blood outgrowth endothelial cells (BOECs) (18). As such, Gu and colleagues compared iPSC derived-ECs isolated from unaffected *BMPR2* mutation carriers with PAH affected *BMPR2* mutation carriers (11). BOECs generated from circulating endothelial progenitor cells in peripheral blood can be used as a surrogate to investigate patient-specific endothelial dysfunction (18). Ormiston and colleagues emphasized that BOECs-derived IPCs are suitable for disease modelling, drug screening and cell transplantation techniques (18). Although each of these techniques is able to acquire cells from patients in any stage of disease development, each method has advantages and drawbacks summarized in table 6.1.

Table 6.1: summary of advantages and drawbacks of different methods to isolate PAECs.

	PAECs after RHC	BOECs	iPSCs
Advantages	+ inexpensive + PAEC directly collected from pulmonary vasculature	+ Control and non-affected mutation carriers can be included + abundant number of cells + limited invasiveness	+ control and non-affected mutation carriers can be included + abundant number of cells + Long life span + limited invasiveness
Drawbacks	- Invasive - No controls - Limited amount of cells - Limited life span	- Time consuming - Blood derived EC	- Expensive - Time consuming - Cells are not derived from lung circulation

PAECs; pulmonary arterial endothelial cells, RHC; right heart catheterization, BOECs; blood outgrowth endothelial cells, iPSCs; induced pluripotent stem cells,

6.2. *BMPR2* silencing and PAH-associated physiopathology of pulmonary microvascular endothelial cells

We have consequently chosen to take advantage of the stable *BMPR2* silencing in HLMVECs to study effect of impaired BMPRII signalling, common to IPAH and to PAH with *BMPR2* mutations, on 2 different aspects of the physiopathology of pulmonary microvascular cells: barrier function and angiogenic capacities.

Barrier function

Maintaining a proper endothelial barrier function is crucial to hamper the infiltration of circulating growth factors, cytokines, chemokines and inflammatory cells into the subendothelial space and to

prevent excessive inflammatory responses. To illustrate this aspect, we showed that, in addition to reducing the barrier function, *BMPR2* silencing is also associated with an exacerbated response to inflammatory cytokines and activations of HLMVECs with an increased expression of adhesion molecules at their surface (see Chapter 3). Interestingly, we previously observed inflammatory stimuli significantly increased cytokine production by HLMVECs from PAH patients carriers of a *BMPR2* mutation compared to non-carriers and controls (6). Actually, increased expression of ICAM-1 results in enhanced infiltration of inflammatory cells into the vascular wall which may initiate pulmonary vascular remodelling (19). Considering the low penetrance (20%) of *BMPR2* mutation in PAH, our results highlight that inflammatory stimuli may trigger the progression of PAH within a background of genetic susceptibility (second hit hypothesis) (20). As such, patients with PAH are considered a very vulnerable group in this period of COVID-19 and may develop a more severe illness. Long-term effects of COVID-19 on incidence of PAH will appear in the near future.

In addition, *BMPR2* deficiency results in increased cytokine production and constitutive activation of bone marrow-derived macrophages leading to increase endothelial – immune communication (21,22). This emphasizes the major role of inflammation in triggering and perpetuating vascular remodelling. Interestingly, immunosuppressive agents can reverse experimental PH in rodents (23,24). Observational studies demonstrated that immunosuppressive treatment may be beneficial in a subset of patients (25). Therefore, precise characterization of patients is required and may lead to development of precision medicine strategies.

Angiogenesis

PAH has been initially considered as proliferative and pro-angiogenic disease (26) but since the publication of Chaudhary and colleagues suggesting that microvascular drop out can be one of the initiating pathogenic factors, the debate about PAH as a cancer-like versus microvascular rarefaction-driven disease remains a hot topic (27). Our own *in vitro* findings (Chapter 5) together with the abnormally low microvascular network in *BMPR2*$^{+/-}$ mutant rats (28) strongly support that microvascular rarefaction contribute to pulmonary vascular remodelling and to the pathogenesis of PAH (27) and additionally highlights the key role of the BMPRII pathway in driving beneficial angiogenesis to further orientate innovative therapeutic strategies preventing microvascular rarefaction and rescuing angiogenic capacities.

Our research outcome demonstrates that *BMPR2* and BMPRII signalling plays a significant role in endothelial dysfunction. However, depending on the type of *BMPR2* mutation, penetrance and severity can vary between families. Currently, more mutations are being related with the development of PAH. Several of these mutations have been identified in genes coding for proteins directly or indirectly interacting with the BMPRII signalling pathway and lung development. As such, *T-box*

transcription factor 4 (*TBX4*), the second most frequent mutation associated with PAH (29), is involved in embryonic development by regulating BMP signalling (30,31). Recently mutations in the gene coding for the kinase insert domain receptor (KDR), a VEGF receptor-2 able to physically interact with BMPRII at the cell surface, have been identified (32). Interestingly, such mutations may have consequences in vasculogenesis (32).

6.3. BMPRII signalling pathway and PAH-specific therapies

Since i) we observed an association between impaired BMPRII signalling and loss of pulmonary vascular endothelial function and ii) NO production is significantly decreased in PAECs from *BMPR2* mutation carriers (33,34), we hypothesized that PAH-specific drugs may restore endothelial function by driving BMPRII signalling pathway. Unfortunately, any of the investigated drugs (macitentan, tadalafil or selexipag), targeting the 3 vasodilatory therapeutic pathways did not improve BMPRII downstream signalling or BMPRII-driven impaired endothelial barrier function. Importantly, any of the abovementioned drugs displayed deleterious effects on BMPRII signalling or on endothelial barrier function. Interestingly, ERA (macitentan) and PDE5 inhibitor (tadalafil) did show some limited but significant effects on *in vitro* angiogenic capacities, whereas the IP receptor agonist, selexipag, did not. Although studies have elaborated on the potential beneficial effects of PAH-specific drugs on endothelial function (35–37), there is currently no established consensus. One can argue that vasodilatory PAH-specific drugs rather aim to target the pulmonary vascular smooth muscle layer than the endothelium. Endothelial dysfunction is defined by an imbalance between vasoconstrictors and vasodilators and current PAH-specific drugs rather target the consequences of endothelial dysfunction, but only delay the course of the disease without curing it (38). Consequently, new approaches targeting the endothelium, restoring damaged lung microcirculation and reversing the progression of the disease need to be explored. Noteworthy, an immunosuppressive drug, FK506 (Tacrolimus) able to activate BMPRII signalling, can rescue endothelial dysfunction and reverses experimental PAH by activation of the BMPRII receptor (39). A phase II clinical study showed the safety and efficacy of FK506 in a limited number of patients with severe PAH (40) and a randomised placebo-controlled safety trial (NCT01647945) evidenced that FK506 was well tolerated and increased BMPRII *expression* in a subset of PAH patients, opening perspectives for a future phase III clinical trial.

Although targeting the endothelium is a therapeutic option, we are aware that patients harbour different types of *BMPR2* mutations and phenotypes as abovementioned and may therefore respond differently on available therapies. This opens the hot topic of precision medicine and tailor-based therapy for PAH patients and consolidate the use of *BMPR2* silencing as a pre-clinical tool.

Limitations

Despite the novelty of our findings, we are aware of their limitations. The innovative technique allowing isolation of PAECs from patients at diagnosis and follow-up is not without drawbacks, since we were not able to isolate cells from each patient who undergoes a routine RHC. This could be attributable to technical limitations and according to Ventetuolo and colleagues to differences in hemodynamics (16), which we did not experienced. However, it will increase the frequency and decrease the degree of unpredictability in comparison with the so far experienced low occurrence of lung transplantation. We are nevertheless convinced that this initiative will push this technique forward by other groups worldwide to favour access to more patient material at early stages of the disease and open perspectives for more elaborated pre-clinical studies and precision medicine.

Primary PAECs cultures ineluctably precludes selection of most robust cell clusters and current *in vitro* studies on cells from patients lack the dynamic, interactive and evolving physiological microenvironment. In this project, we have chosen to dismantle the complex PAH phenotype by focussing specifically on BMPRII signalling. While the BMPRII pathway is a key player in the pathogenesis of PAH, the recent emergence of various mutations associated with this condition (42) compels to cautiously interpret *in vitro* findings and remain watchful regarding potential translational applications. Consequently, 3D technology involving crosstalk multi-culture systems, extracellular matrix, exposure to flow and shear stress such as organ on-a-chip methodology is an innovative alternative that should be considered in the future.

General conclusion

To eventually conclude, we showed that we were able to develop relevant alternatives to overcome the limited access to pulmonary vascular cells from patients suffering from a rare disease such as PAH. We further highlighted the key role of BMPRII signalling in endothelial dysfunction and emphasized the effect of loss of BMPRII on endothelial barrier function, angiogenesis and susceptibility to inflammatory insult. Finally, we were not able to evidence any beneficial, but importantly no detrimental effects of PAH-specific drugs regarding impaired BMPRII-driven endothelial dysfunction.

Future perspectives

In the present study we demonstrate a methodology to isolate and maintain patient PAECs following RHC. Further optimizations and continuous collections are necessary to increase the success rate and the amount of collected cells to better understand interindividual differences. Other methodologies such as the use of iPSC or BOEC and multicentric cooperative research programs should also contribute to implement individual tailored-based therapy, since our long-term objective is to create a tool to evaluate patient-specific drug responses. Using existing clinical studies to analyse treatment responses between the different PAH subtypes may be an initial step towards precision medicine. Individual analysis of phase 3 randomized controlled trials demonstrated that PAH treatment may be less effective in PAH associated with connective tissue disease compared to IPAH (43). Careful phenotyping and classification of the patient offers a great opportunity to restrict the selection of therapies for a specific subtype of patients.

Another possibility is to correct identified pathological mutations. There is evidence that bone marrow cells alone are sufficient to drive PAH (44), This suggests that autologous bone marrow cell transplantation can be used as a future therapeutic option. Collections of stem cells from PAH patients carrying a *BMPR2* mutation, correction of this mutation using the CRISPR-Cas9 method and re-injection of these genetically engineered stem cells may become a therapeutic approach (44). The use of endothelial progenitor cells (EPC) or blood-derived mononuclear cells to treat PAH appears very promising as illustrated by recent preclinical studies demonstrating reduced pulmonary arterial pressures and vascular remodelling in experimental models of PH (45,46). The Pulmonary Hypertension and Angiogenic Cell Therapy (PHACeT) trial (NCT00469027) was a first-in-human, phase I, dose escalation study examining the tolerability and potential efficacy of eNOS gene-enhanced progenitor cell therapy for PAH (47).

Gene therapy is an interesting approach in which direct insertion of human DNA in the human genome can be performed. Adeno-associated viruses can be used to administer and incorporate a corrected *BMPR2* gene into the cell and into the chromosome (48). Pre-clinical studies using adenoviral vector-mediated *BMPR2* gene delivery showed restoration of BMPRII expression, improve haemodynamics and attenuate vascular remodelling in monocrotaline-induced and chronic-hypoxia models of PH in rats (12,49). Direct upregulation of BMPRII protein expression using gene delivery resulted in significant improvements in right ventricular systolic pressure and Fulton index in a *BMPR2* mutant mouse model (13).

In addition, *in vivo* gene editing using CRISPR-Cas9 is a very promising technique to correct pathogenic mutations. However, these techniques are challenging, further progression in the field of gene editing is necessary to ensure the safety of the procedure and to ensure a long-term effect.

Besides gene therapy or cell therapy, the investigation towards new oral therapeutics needs to be further explored. The potential therapeutic effect of drugs targeting directly or indirectly the BMPRII signalling pathway (10) should be considered in the future. Potential therapeutic approaches directly affecting the BMPRII signalling include the use of tacrolimus (FK506), chloroquine, BMP9 or ataluren (40,50–52). Other therapeutic approaches targeting the TGFβRII signalling include a selective TGFβ ligand trap (sotatercept), an ALK5 inhibitor or schisandrin B (53–55). Besides drugs directly targeting the BMPRII signalling pathway, therapeutics which indirectly target BMPRII and are used as anti-cancer, anti-inflammatory or anti-fibrotic drugs show beneficial effects on the BMPRII signalling (56,57).

Consequently, we may dream that improvements in available oral therapies, advances in gene and cell therapy, may lead to reversal and eventually the cure of HPAH and IPAH (Figure 6.1) (58). Therefore, an extensive genetic screening of the patient may identify which genes are mutated and even identify the type of mutation. In combination with specific biomarkers and *in vitro* analysis, this will create an individual pathobiological perspective of each individual patient. This will allow the clinician to adapt the therapeutic strategies and progress towards precision medicine (Figure 6.1).

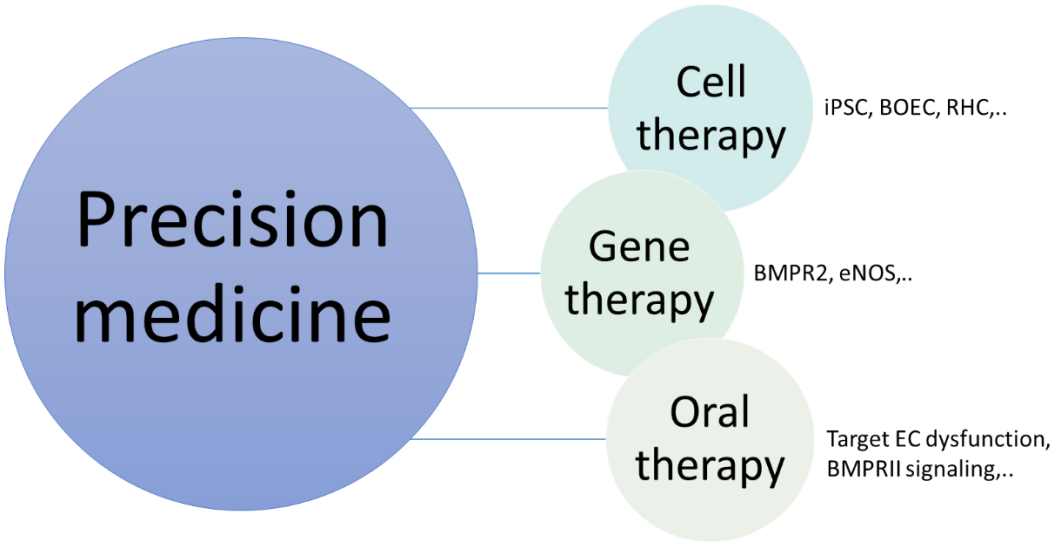

Figure 6.1: Future perspectives and potential focus for upcoming research to orientate treatment towards precision medicine.

Since upfront combination therapy is becoming general practise as emphasized by the AMBITION study (59) and recommended in the most recent guidelines (60). We planned to include combination testing to investigate the synergy between the different PAH-specific drugs that may show more convincing *in vitro* effects.

In addition, stable *BMPR2* silencing in HLMVECs is a unique and relevant tool to pre-clinically investigate and understand the impact of new therapeutic targets, which however deserves to be further elaborated. For instance, the potential therapeutic effects of drugs targeting or interfering directly and indirectly with the BMPRII signalling pathway (10) in promoting angiogenic capacity could

be evaluated.

Interestingly, *BMPR2*-silenced HLMVECs or patient PAECs could be used in *in vitro* organ on-a-chip dynamic models combining multi-culture systems, extracellular matrix exposed to flow and/or shear stress. In addition, the use of patient PAECs has great potential to generate patient specific cell signature using RNA sequencing, microarray analysis or single drug profiling to further orientate the therapeutic strategy. However, preclinical studies need to be implemented to carefully assess safety, feasibility and efficacy of new therapies.

Besides improvements in therapeutic options, there is still a diagnostic delay. As such, there is a need to identify biomarkers which allow a faster diagnosis of the disease and ideally may be used to distinguish between potential drug responders and non-responders and will further increase the implementation of a tailored-based therapy.

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Summary

Summary

Pulmonary arterial hypertension (PAH) is a severe condition characterized by remodelling of the pre-capillary pulmonary arteries. Vascular remodelling involves thickening of intima, media and adventitia leading to muscularization of peripheral arteries, loss of precapillary arteries and formation of plexiform lesions triggered by hypoxia, shear stress, inflammation, drug/toxin intake and genetic susceptibility. The pathophysiological process that leads to vascular remodelling is complex process which involves vasoconstriction, inflammation, thrombosis, vascular remodelling and impaired angiogenesis. As such, endothelial dysfunction is a major hallmark in the pathophysiology of PAH. When PAH occurs in a hereditary context, germline mutations in the *bone morphogenetic protein receptor type 2 (BMPR2)* are detected in 70-80% of cases. In this regard, we explored the role of *BMPR2* on endothelial function. We mimic the *in vivo* patient situation by creating a *BMPR2* knock down in human lung microvascular endothelial cells (HLMVEC).

We first evaluated if impaired BMPRII signalling is associated with endothelial barrier function. We found that *BMPR2*-silenced HLMVECs result in loss of barrier function and increased permeability of circulating cytokines and growth factors. In addition, we found that inflammatory mediators TNF and IL-18 enhanced adhesion molecule expression, THP1 monocyte adhesion and further decreased endothelial barrier function in *BMPR2* knockdown EC.

To further explore the effect of *BMPR2* on endothelial dysfunction we investigated the effect of impaired BMPRII signalling on angiogenesis and showed that loss of BMPRII expression results in impaired angiogenic capacity. This was investigated focusing on migration capacity and tube forming network.

Since current PAH-specific therapies target endothelial dysfunction focusing on vasodilation, we explored the effect of macitentan, selexipag and tadalafil on BMPRII signalling, endothelial barrier function and angiogenesis. We observed that PAH specific drugs did not affect BMPRII signalling neither restored normal barrier function. There was only limited beneficial effect on angiogenesis.

In parallel we developed an alternative approach to collect patient material. We were able to isolate and culture PAECs using the Swan-Ganz catheters following RHC. This includes a methodology which allow a more frequent collection and access of PAECs at the time of diagnosis and during follow-up.

We conclude that *BMPR2* plays an important role in endothelial dysfunction and the pathophysiology of PAH, but current available therapy lacks proper effect. The possibility of isolating patient specific PAECs has great potential in future research and progression towards precision medicine.

Samenvatting

Pulmonale arteriële hypertensie (PAH) is een ernstige aandoening die zich manifesteert in de capillaire pulmonale arteriën. Structurele veranderingen zoals verdikking van de vasculaire spierlaag, vermindering in pre-capillaire arteriën leiden tot vernauwing van de bloedvaten in de longen. De oorzaak waarom PAH zich ontwikkeld is complex en een combinatie van hypoxie, shear stress, inflammatie, gebruik van medicatie en genetische afwijkingen. Wanneer er een genetische oorzaak gevonden wordt is dit in 70-80% van de gevallen een mutatie in het *bone morphogenetic protein receptor type 2 (BMPR2)* gen. Pathofysiologisch bekeken ontstaan deze vernauwingen door een combinatie van vasoconstrictie, inflammatie, trombose en een verminderde angiogenese. Al deze factoren houden verband met een belangrijk kenmerk in PAH, namelijk endotheel dysfunctie. Daarom hebben we in dit project het belang van *BMPR2* in endotheel dysfunctie bestudeerd. Dit deden we door een *BMPR2*-knockdown model te creëren in commercieel verkrijgbare HLMVECs.

Allereerst hebben we het effect van BMPRII op endotheel permeabiliteit bestudeerd en zagen dat een verminderde BMPRII-expressie resulteert in een hogere permeabiliteit en dus een verhoogde mogelijkheid om cytokines en circulerende groeifactoren door te laten. Daarnaast bleek dat inflammatoire factoren zoals TNFα en IL-18 een groter effect hebben op de permeabiliteit en ICAM-1 expressie wanneer BMPRII-expressie verminderd is in vergelijking met controle. Een verminderde BMPRII-expressie resulteert ook in een daling van de angiogenese wat bleek wanneer we de migratie en netwerkvorming van de endotheelcellen vergeleken.

De huidige beschikbare geneesmiddelen tegen PAH zijn vooral gericht op het herstellen van endotheel functie en hebben hoofdzakelijk een vasodilaterend effect. Ons doel is om het effect van deze drug, namelijk macitentan, selexipag en tadalafil, op de BMPRII pathway en functie te bestuderen. Hierbij tonen we aan dat huidige gebruikte medicijnen geen effect hebben op het herstellen van de BMPRII pathway of op de permeabiliteit. Ze vertonen zeer variërende effecten op angiogenese.

Tegelijkertijd hebben we een nieuwe methode verfijnt die het mogelijk maakt om patiënt-eigen endotheelcellen te verzamelen op het punt van diagnose. Dit door middel van het verzamelen van de Swan-Ganz katheter die gebruikt wordt tijdens rechter hartkatheterisatie. Dit betekent dus dat, door deze methodologie, er een grotere toegankelijkheid is naar patiënt-specifieke cellen.

Uit ons onderzoek kunnen we concluderen dat *BMPR2* een belangrijke rol speelt in endotheel functie en de pathofysiologie van PAH, maar dat de huidige gebruikte medicatie slechts een minimaal effect vertoont wat erop wijst dat er nood is aan ontwikkeling van nieuwe therapie. Daarenboven hebben we een methode ontwikkeld die het toelaat om patiënt-eigen cellen in cultuur te brengen wat een belangrijke stap is richting het ontwikkelen van patiënt-eigen gepersonaliseerde geneesmiddelen.

Scientific acknowledgements, personal contributions and conflict of interest

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Personal contributions to the manuscript

Chapters 1,2 and 6: Birger Tielemans drafted these chapters

Chapter 3: Birger Tielemans has performed experiments including Western blotting, analysed data and performed revision of the paper before publication.

Chapter 4: Birger Tielemans has performed all experiments, processed and analysed the data, performed statistical analysis and drafted the manuscript.

Chapter 5: Birger Tielemans has performed all cell experiments, immunostaining and flow cytometry, analyzed the data, performed statistical analysis and drafted the manuscript. Extraction of patient characteristics from the patient database and analysis was performed by Dr. Rozenn Quarck.

Conflict of interest

This research was partially funded by the pulmonary hypertension vzw. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Short curriculum vitae

Birger Tielemans was born on April 26the 1991 in Lier, Belgium. After graduating from secondary school at Sint Gummarus college at Lier in 2009, he started his training in biochemistry and biotechnology at the University of Antwerp, Belgium, where he graduated in 2015. In August 2015, he started his PhD in the pulmonary circulation unit under supervision of Prof. Marion Delcroix, Prof Catharina Belge and Dr. Rozenn Quarck to investigate the effect of PAH-specific drug responses on endothelial dysfunction and pulmonary endothelial cells from patients with pulmonary arterial hypertension.

Publication list

Publications

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Communications

1. **Tielemans B,** Gijsbers R, Michiels A, Wagenaar A, Farré Marti R, Belge C, Delcroix M, Quarck R. BMPRII driven response of endothelial integrity and angiogenesis in human lung microvascular endothelial cells. GSK PAH Seminar - Dec 14-15, 2017 – Leuven, Belgium – Poster.
2. **Tielemans B,** Wagenaar A, Leys M, Belge C, Delcroix M, Quarck R. *Phenotyping of pulmonary arterial endothelial cells isolated from patients with idiopathic pulmonary arterial hypertension at diagnosis*. 6th World Symposium on Pulmonary Hypertension – Feb 27-28/March 1, 2018 – Nice, France – Poster.

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