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Therapeutics in pulmonary arterial hypertension

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Therapeutics in pulmonary arterial hypertension

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Editorial: Therapeutics in pulmonary arterial hypertension

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Editorial on the Research Topic
Therapeutics in pulmonary arterial hypertension

Pulmonary arterial hypertension (PAH) is characterized by significant morbidity and mortality and despite advances, the prognosis remains poor, highlighting the critical need for new therapies and non-invasive methods to monitor disease progression. Although defined by strict hemodynamic criteria, PAH is a syndrome based on diverse etiologies and pathogenesis (1, 2). This Research Topic aimed to highlight the recent advances in PAH research and includes 9 papers, comprising 4 original research articles (basic and translational), 4 reviews and 1 opinion article.

In this issue, 2 outstanding basic research original studies were published. Recent investigations demonstrated that the thromboxane (TX) A2 receptor (TP) antagonist NTP42 attenuates experimental PAH across key hemodynamic parameters in the lungs and heart (3), and Mulvaney et al. aimed to validate the efficacy of *NTP42:KVA4*, a novel oral formulation of NTP42 recently validated in a Phase I clinical trial (4), in experimental PAH. Together, the findings from two independent preclinical models (monocrotaline and pulmonary artery banding) demonstrated that *NTP42:KVA4* not only alleviates pulmonary pathologies akin to those observed in clinical PAH, but also may act as a direct cardioprotective agent in settings of right ventricle (RV) pressure overload. This is relevant because RV function is widely viewed as the most important determinant of clinical outcome in PAH (5). This work points to a cardioprotective effect for *NTP42:KVA4* as a component of its potential to be a disease-modifying therapy in PAH and other cardiac conditions.

In the last two decades, more than 20 genes have been linked to a genetic predisposition to PAH, including those encoding ATP-sensitive K + channels (KATP) (6). The ABCC9 gene encodes two regulatory subunits of KATP channels: SUR2A and SUR2B. Le Ribeuz et al. showed that Kir6.1 and SUR2 are expressed in human and rat lungs and in pulmonary artery smooth muscle cells (PASMC) and PA endothelial cells (PAEC). SUR2A is upregulated in PASMCs from PAH patients but downregulated in

rat lungs with monocrotaline-induced PAH. The KATP channel opener pinacidil inhibited proliferation in healthy but not PAH endothelial cells and reduced the proliferation and migration of control and PAH-PASMCs. Pinacidil caused stronger relaxation in human control compared to PAH pulmonary arteries, a result not replicated in the rat model, and preventive or curative treatment with pinacidil in vivo reduced the severity of experimental PAH. These findings suggest complex changes in KATP channels in PAH and indicate that the monocrotaline model may not accurately reflect human PAH. However, SUR2 activators might effectively treat human PAH through vasodilator and antiproliferative effects on PASMC. Further studies are needed to confirm these interesting findings and explore therapeutic benefits.

In this issue, 4 comprehensive reviews were published. Mamazhakypov and Lother discuss the recent advances in mineralocorticoid receptor (MR) signaling in pulmonary vascular cells based on preclinical research and the potential in bringing MR antagonists (MRAs) into clinical application. During the past decade, a series of experimental studies investigated the role of aldosterone and its receptor, the MR, in pulmonary vascular remodeling and a potential benefit of MRAs for PH patients. Indeed, MR is an important and highly versatile transcription factor that regulates various key signaling pathways in the PH pathogenesis, inducing adverse cellular processes that lead to pulmonary vascular remodeling, such as endothelial cell apoptosis, smooth muscle cell proliferation, pulmonary vascular fibrosis, and inflammation (7). In other very interesting review, Santos-Gomes et al. revise potential circulating biomarkers for PAH, aiming to enhance diagnostic accuracy and therapeutic monitoring. This review underscores the evolving landscape of PAH management through innovative biomarker-driven approaches. In fact, RV catheterization is the current gold standard for diagnosis (1), but its invasiveness limits routine use, and, in this context, biomarkers show promise in predicting prognosis and therapy response if they are easy to detect and objective. Also, in this issue, Simmons Beck et al. review two transcription factors, SRY-box transcription factor 17 (SOX17) and one of its downstream targets, Runt-related transcription factor 1 (RUNX1), and the emerging data that implicate their roles in the pathogenesis of PAH, from their own work and other studies. Preclinical studies in endothelial cell SOX17 deficient mice or transgenic mice with mutations SOX17 resembling those present in some PAH patients have replicated many of the pathologic features of human PAH. Consistently, SOX17 downstream targets such as Notch signaling or BMPR2 have been widely involved in PAH. Also, RUNX1 may play a role in mediating the effect of impaired SOX17 expression in PAH and targeted deletion of RUNX1 in either myeloid or endothelial cells or pharmacological inhibition of RUNX1 reverse experimental PAH. Thus, they propose that RUNX1 inhibition may be an effective approach to treat PAH. Also, Körbelin et al. comprehensively review the pivotal role of dysregulated transcription factors (TFs) in PAH pathogenesis and underscore their potential as targets for vasculoregenerative or reverse remodelling therapies. Indeed, PAH arises from dysfunction in vessel wall cells and remodelling of the pulmonary vasculature, and TFs, pivotal in regulating gene expression, intricately modulate these responses through complex networks. Complex TF networks and chromatin remodeling add layers of complexity. While emerging TF-based therapies hold promise, addressing off-target effects, possibly through gene therapy (8), is crucial. Moreover, validation through clinical trials is imperative for advancing these innovative treatments effectively.

In the landscape of clinical hypertension research, 3 published articles have provided crucial insights into the management and prognosis these patients. Over the years, PAH-specific therapies have significantly enhanced patient survival. One such therapy is selexipag, an oral selective prostacyclin receptor agonist approved for PAH treatment (1). In the study from Ciu et al., use of selexipag in triple combination therapy has shown promise in improving outcomes for Chinese PAH patients. While selexipag has demonstrated efficacy in reducing clinical worsening events and improving right heart parameters, further research is needed to fully understand its benefits in specific patient populations like the Chinese. The evolving landscape of PAH treatment strategies underscores the importance of continued investigation into optimizing therapies to enhance patient outcomes.

In terms of assessment and management PAH represents a clinical challenge and despite advancements in risk stratification and specialized care, individual patients with PAH remain difficult to evaluate accurately (9). Thus, the use of linear models to evaluate pulmonary and systemic pressure in PAH patients as described by Doyle et al. offers a promising approach to address existing paradoxes in understanding these conditions. These models provide a comprehensive set of metrics that can aid in monitoring patients without the need for invasive procedures like right heart catheterization. By directly assessing pressure and cardiac status at a component level, these models have the potential to facilitate the translation of therapeutic benefits between different trials.

In recent years, the widespread use of mobile phones has raised concerns about their potential impact on health, particularly in relation to hypertension (10) and, in an opinion article, Bhattacharya et al. shed light on the association between mobile phone usage and the development of hypertension. The study highlighted that mobile phone users had a 7% increased risk of developing hypertension compared to non-users over a 12-year follow-up period, emphasizing the need to consider mobile phone guidelines in hypertension management protocols.

All these new findings and updated reviews collectively contribute to advancing our understanding of PAH pathophysiology and managing and treatment strategies, paving the way for improved care and outcomes for patients with this debilitating condition. However, ever more research is crucial to explore existing and innovative therapies that could further improve patient prognosis and survival.

Author contributions

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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An Overview of Circulating Pulmonary Arterial Hypertension Biomarkers

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Pulmonary arterial hypertension (PAH), also known as Group 1 Pulmonary Hypertension (PH), is a PH subset characterized by pulmonary vascular remodeling and pulmonary arterial obstruction. PAH has an estimated incidence of 15–50 people per million in the United States and Europe, and is associated with high mortality and morbidity, with patients' survival time after diagnosis being only 2.8 years. According to current guidelines, right heart catheterization is the gold standard for diagnostic and prognostic evaluation of PAH patients. However, this technique is highly invasive, so it is not used in routine clinical practice or patient follow-up. Thereby, it is essential to find new non-invasive strategies for evaluating disease progression. Biomarkers can be an effective solution for determining PAH patient prognosis and response to therapy, and aiding in diagnostic efforts, so long as their detection is non-invasive, easy, and objective. This review aims to clarify and describe some of the potential new candidates as circulating biomarkers of PAH.

Keywords: biomarkers, pulmonary arterial hypertension, pulmonary hypertension, prognosis, diagnosis, circulating levels

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INTRODUCTION

Pulmonary hypertension (PH) is classified into five groups based on etiology and pathogenesis (1). Patients in the first group are considered to have pulmonary arterial hypertension (PAH) (2, 3), a chronic and severe cardiopulmonary disease with a poor prognosis (4). According to the latest revised World Symposium on Pulmonary Hypertension, PAH can be subclassified into 7 distinct groups, as described in **Table 1** (5). Although PAH is considered a rare disease, it is estimated to have an incidence of 15–50 people per million in the United States and Europe. Most PAH cases (52.6%) are idiopathic, heritable, and/or anorectic-induced PAH (6). The incidence of PAH can, however, be associated with other morbidities (7).

PAH is characterized by excessive pulmonary vascular remodeling, which involves medial hypertrophy—an early event in PAH and even reversible, appearing in all PAH subgroups -, proliferative and fibrotic changes of the intima, adventitious thickening, and thrombosis *in situ* (1–3, 8–11). The mechanisms behind these transformations are not fully understood (12)—several pathophysiological processes are entailed, such as migration and proliferation of pulmonary arterial smooth muscle cells (PASMCs) and endothelial cells (ECs) (1, 2), endothelial dysfunction, endothelial-to-mesenchymal transition (13), enhanced adventitial pulmonary artery fibroblast

proliferation, migration, and differentiation (14), inflammation (15, 16) and oxidative stress (2, 3, 8, 9). Particularly, ECs may be involved in synthesizing growth factors that stimulate non-cellular matrix deposition and smooth muscle hypertrophy, contributing to the formation of plexiform lesions (1, 4, 10, 17). Moreover, necrotic and fibrotic tissue, as well as inflammatory cells, can also accumulate in the arterial wall, resulting in arteritis (1, 2, 4). Finally, there are changes in the production of vasoactive molecules, namely nitric oxide (NO), prostacyclins and endothelin-1 (ET-1) (2, 18), contributing to endothelial dysfunction and vasoconstriction.

All these alterations culminate in pulmonary arterial obstruction, with an increase in pulmonary vascular resistance (PVR), which will lead to right ventricular overload and, eventually, right ventricle (RV) failure (1, 9, 19)—the major cause of PAH mortality and morbidity (8). Early studies, before the introduction of PAH-specific therapies, suggested that patients' survival after diagnosis was only 2.8 years (20, 21).

Patients present with symptoms which reflect RV dysfunction and are non-specific (2, 3, 18). Because of this, the diagnosis is often delayed for ≥2 years, with many patients being diagnosed only in advanced stages (22). To characterize PAH, current guidelines require patients to undergo a right heart catheterization, with measurements of mean pulmonary artery pressure (mPAP) > 20 mmHg, pulmonary artery wedge pressure ≤ 15 mmHg and a PVR ≥ 3 Wood Units at rest, in the absence of other causes (5).

Upon establishing a diagnosis of PAH, adequate treatment must be started. Therapy for PAH patients has evolved considerably in the past decades, in parallel with improvements in patient survival and quality of life (21). A multidimensional approach is recommended, involving general measures (supervised physical rehabilitation, infection prevention and psychosocial support), supportive therapy (such as diuretics and supplementary oxygen) and PAH targeted drug therapy (17). Specific PAH therapies are targeted at the three main molecular pathways altered in dysfunctional pulmonary endothelium. Endothelin receptor antagonists (ERAs) modulate the endothelin (ET) pathway; phosphodiesterase type 5 (PDE-5) inhibitors and soluble guanylate cyclase stimulators act *via* the NO pathway; and prostacyclin analogs and prostacyclin receptor agonists act

TABLE 1 | Updated clinical classification of pulmonary hypertension.

1. Pulmonary arterial hypertension
1.1 Idiopathic PAH
1.2 Heritable PAH
1.3 Drug- and toxin-induced PAH
1.4 PAH associated with other conditions
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
2. PH due to left heart disease
2.1 PH due to heart failure with preserved LVEF
2.2 PH due to heart failure with reduced LVEF
2.3 Valvular heart disease
2.4 Congenital/acquired cardiovascular conditions leading to post-capillary PH
3. PH due to lung diseases and/or hypoxia
3.1 Obstructive lung disease
3.2 Restrictive lung disease
3.3 Other lung disease with mixed restrictive/obstructive pattern
3.4 Hypoxia without lung disease
3.5 Developmental lung disorders
4. PH due to pulmonary artery obstructions
4.1 Chronic thromboembolic PH
4.2 Other pulmonary artery obstructions
5. PH with unclear and/or multifactorial mechanisms
5.1 Hematological disorders
5.2 Systemic and metabolic disorders
5.3 Others
5.4 Complex congenital heart disease

PH, pulmonary hypertension; PAH, pulmonary arterial hypertension; HIV, Human immunodeficiency virus; PVOD, pulmonary veno-occlusive disease; PCH, pulmonary capillary hemangiomatosis; LVEF, left ventricle ejection fraction.

Abbreviations: 15-F2t-IsoP, 15-F2-isoprostanes; 5-HT, serotonin; 5-HTT, serotonin transporter; 6MWD, 6-minute walk distance; aa, amino acids; ADM, adrenomedullin; ADMA, asymmetric dimethylarginine; Ang-1, angiopoietin-1; Ang-2, angiopoietin-2; ANP, atrial natriuretic peptide; AVP, arginine vasopressin; BMP9, bone morphogenic protein 9; BNP, brain natriuretic peptide; CD40L, CD40 ligand; cGMP, cyclic guanosine monophosphate; CHD, congenital heart disease; COPD, chronic obstructive pulmonary disease; CRP, C-reactive protein; CTD, connective tissue diseases; CTEPH, chronic thromboembolic pulmonary hypertension; cTn, cardiac troponins; cTnI, cardiac troponin I; cTnT, cardiac troponin T; CysC, cystatin C; ECs, endothelial cells; Eng, endoglin; eNOS, endothelial nitric oxide synthase; ERAs, endothelin receptor antagonists; Es, endostatin; ET, Endothelins; ET-1, endothelin-1; ETA, endothelin type A receptor; ETB, endothelin type B receptor; F2-isoP, F2-isoprostanes; Gal-3, galectin 3; GDF-15, growth differentiation factor-15; HETEs, hydroxyeicosatetraenoic acids; HF, heart failure; HODEs, hydroxyoctadecadienoic acids; hsTnT, high-sensitive cTnT; IDO, indoleamine 2, 3-dioxygenase; IL, interleukin; IL-1R1, interleukin-1 receptor-1; IPAH, idiopathic PAH; MCP-1, monocyte chemoattracting protein-1; mPAP, mean pulmonary artery pressure; MPs, microparticles; mRAP, mean right atrial pressure; MyD88, molecular adapter 88; NF-κB, nuclear factor kappa B; NFAT, nuclear factor of activated T cells; NYHA, New York Heart Association; NO, nitric oxide; NOS, nitric oxide synthase; NT-proBNP, N-terminal prohormone; OPN, osteopontin; PAH, pulmonary arterial hypertension; PAP, pulmonary arterial pressure; PASMCs, pulmonary arterial smooth muscle cells; PDE, phosphodiesterase; PH, pulmonary hypertension; PIM-1, Moloney Murine Leukemia Provirus Integration Site; PVR, pulmonary vascular resistance; PoPH, portopulmonary hypertension; RAP, right atrial pressure; RDW, red blood cell distribution width; RV, right ventricle; sCD40L, soluble CD40 ligand; sEng, soluble endoglin; SMCs, smooth muscle cells; SSc, systemic sclerosis; STAT3, signal transducers and activators of transcription-3; sVEGFR-1, soluble vascular endothelial growth factor receptor type 1; SvO2, mixed venous saturation of oxygen; TMs, tryptophan metabolites; TNF- α, tumor necrosis factor- α; UA, uric acid; VEGF, vascular endothelial growth factor; VEGFR-1, vascular endothelial growth factor receptor type 1; VEGFR-2, vascular endothelial growth factor receptor type 2; VEGF-A, vascular endothelial growth factor A; VEGF-B, vascular endothelial growth factor B; VEGFR, vascular endothelial growth factor receptors; VSMCs, vascular smooth muscle cells; vWF, von Willebrand factor; WHO, World Health Organization.

in the prostanoid pathway. These agents act mainly by inducing pulmonary vasodilation. Initial treatment usually entails dual combination therapy, and patients are regularly evaluated for disease control and adjustment of treatment accordingly (17).

Right heart catheterization is the current gold standard for establishing both disease diagnosis and prognosis, particularly when considering the adjustment of treatment. However, its invasiveness prevents it from being used in routine clinical practice or patient follow-up, being replaced by measurements of systolic pulmonary artery pressure (PAP) and transthoracic echocardiography (23).

Thus, it is essential to find non-invasive techniques for disease monitoring. Biomarkers can be an effective solution for establishing diagnosis and prognosis, and evaluating response to therapy, so long as their detection is non-invasive, easy, and objective. This review aims to clarify and describe some of the potential candidates as a biomarker for the diagnosis and prognosis of PAH.

WHAT IS A BIOMARKER?

By definition, a biomarker is "a characteristic that is measured objectively and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention" (12, 24). More succinctly, we can define a biomarker as a molecular change in tissues and/or body fluids as result of a disease process (12). Ideally, a biomarker should represent clinical outcomes, that is, reflect how the patient feels and what stage of disease he is in; it should also serve as tool for diagnosis and prognosis, and as a therapeutic marker, providing information on the patient's response to a specific treatment (12, 25). Moreover, it must display a series of properties that make it ideal, namely, high sensitivity and specificity, easiness to obtain/collect and measure, total availability, non-invasive, and it must also be a sign of disease activity (risk stratification, responsiveness to treatment, anticipation of clinical worsening) or a treatment target (12, 25). The search for ideal biomarkers is constant and, in situations where an ideal candidate is not yet known, the one(s) considered to be more accessible, cheaper, and easier to measure is (are) used. However, as these can be less sensitive and less specific, they should not be used alone as a clinical decision tool, and a set of factors must be used to make a decision (25).

Although a PAH biomarker that is detected by a single and simple test is not yet known, there are already several well-known and well-defined biomarkers that could prove to be potent diagnostic and prognostic indicators in the future (23, 26). These biomarkers can be categorized according to the (patho)physiological mechanism they are associated with, which reflects the complexity of this syndrome: endothelial function, inflammation, oxidative stress, cardiac function, myocardial injury, metabolism, and gene expression (**Figure 1**).

BIOMARKERS OF CARDIAC FUNCTION/DAMAGE

Natriuretic Peptides

Natriuretic peptides are a family of genetically distinct hormones that share a similar molecular structure (12). This family includes atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), C-type natriuretic peptide (3, 27) and dendroaspis natriuretic peptide, a D-type natriuretic peptide, each having specific functions (27). They are fundamental in cardiac homeostasis (28), as they are involved in the regulation of blood volume and blood pressure through their diuretic, natriuretic, vasodilatory (3, 29) and kaliuretic (27) activities, in addition to inhibiting the renin-angiotensin-aldosterone system (27, 29) and regulating the proliferation of ECs (27). These hormones are secreted essentially by the heart, kidneys, and brain (3).

ANP and BNP represent the main hormones of the natriuretic peptide system (12, 30), both are released from cardiac myocytes, ANP is released essentially from atrial tissue, while BNP is released from ventricular tissue. Both are secreted in response to increased heart pressure and volume overload (31). BNP, being secreted by the ventricular tissue, is more sensitive to ventricular diseases when compared to ANP (12).

BNP is a transcription product of the *NPPB gene*, first forming a precursor of 134 amino acids (aa), preproBNP, which is enzymatically cleaved, forming proBNP (3, 30). ProBNP is cleaved into two distinct fragments: an active peptide, mature 32 aa BNP and an inactive N-terminal fragment, the N-terminal prohormone (NT-proBNP) (3, 27, 30). BNP acts by binding to receptor A, which is mainly expressed in the kidneys, adrenal, lung, terminal ileum, aorta, and adipose tissue (3). The activation of this receptor induces an increase in the levels of cyclic guanosine monophosphate (cGMP), which triggers a vasodilator, natriuretic response and inhibits aldosterone (3). On the other hand, although the function of NT-proBNP is not yet clear (3), it is eliminated by the kidneys and, therefore, its plasma levels increase significantly when there are changes in renal function; the same does not happen with BNP (30). Mature BNP has a short plasma half-life (about 22 min), while NT-proBNP has a 2-h half-life (30) and therefore has greater stability (3, 12) and it becomes easier to measure (12). The levels of cardiac and circulating BNP increase significantly in response to hypertrophy and/or ventricular overload, demonstrating that BNP is an excellent marker of ventricular dysfunction (28).

In PH pathophysiology: BNP is elevated in several forms of PAH, including idiopathic PAH (IPAH) (32), PAH associated with connective tissue diseases (CTD-PAH) (33), congenital systemic-pulmonary shunts (34), PH associated with lung diseases (35), PH with chronic obstructive pulmonary disease (COPD) (28), chronic thromboembolic PH (CTEPH) (29) and PH associated with acute pulmonary embolism (36). BNP levels, in several studies, are closely correlated with New York Heart Association (NYHA) functional class, 6-min walk distance (6MWD) test, and hemodynamic parameters (3, 29, 32, 37). Nagaya et al. it also demonstrated that BNP levels correlate positively with mPAP and inversely with cardiac output, showing a strong correlation with total pulmonary resistance (38).

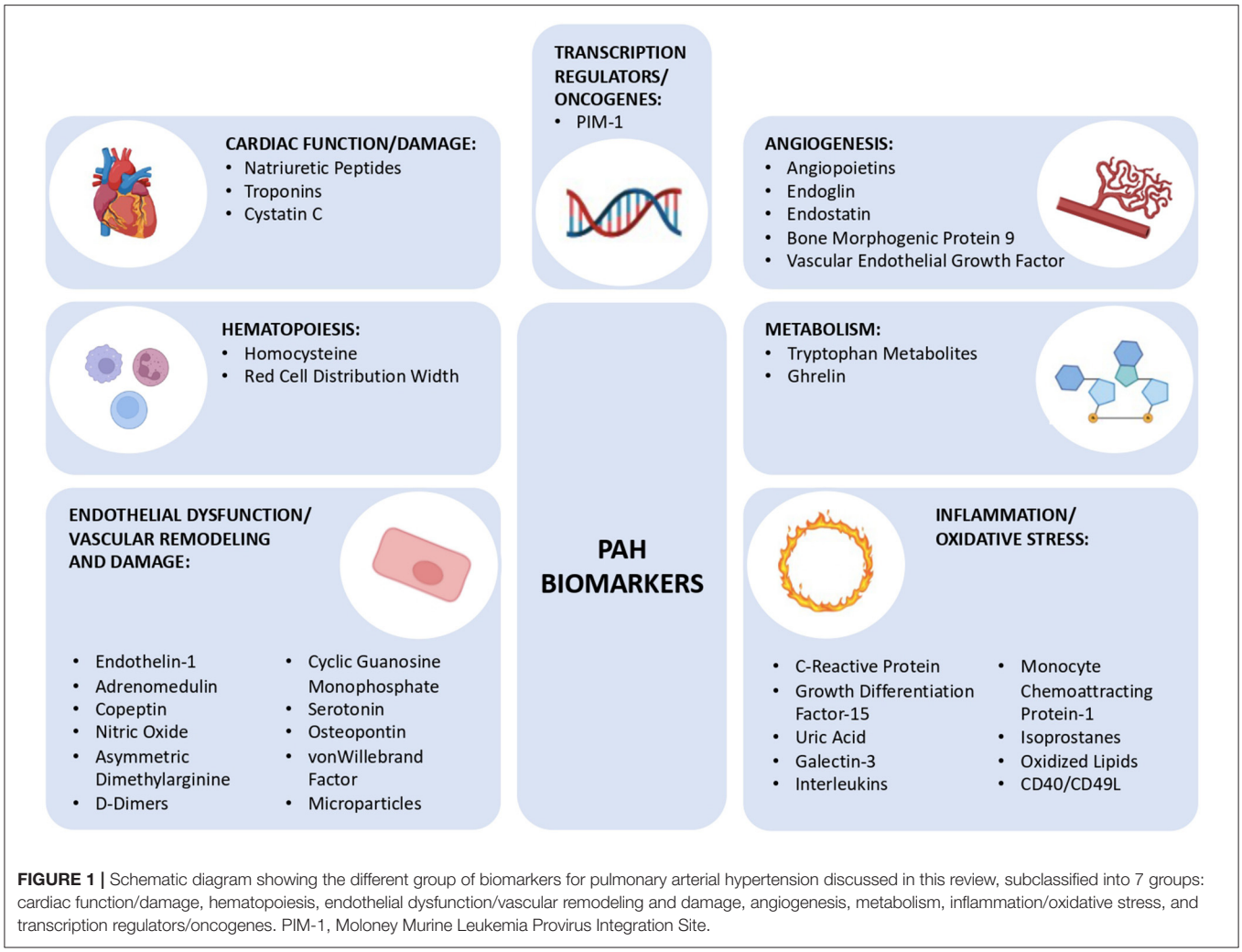

FIGURE 1 | Schematic diagram showing the different group of biomarkers for pulmonary arterial hypertension discussed in this review, subclassified into 7 groups: cardiac function/damage, hematopoiesis, endothelial dysfunction/vascular remodeling and damage, angiogenesis, metabolism, inflammation/oxidative stress, and transcription regulators/oncogenes. PIM-1, Moloney Murine Leukemia Provirus Integration Site.

NT-proBNP is also increased in different forms of PAH such as IPAH (28) and systemic sclerosis-associated PAH (SSc-PAH) (39). In the latter case, NT-proBNP correlates with mPAP, PVR, mean right atrial pressure (mRAP) and cardiac index (3), and it can effectively work as a predictor of survival in PAH (23). ANP levels are also increased in IPAH (40) and CTEPH. Like BNP, ANP levels are positively correlated with the mPAP and inversely with cardiac output (38). However, ANP has a short half-life in humans subjects—about 2 min (41, 42)—while BNP has a much longer half-life (43), making BNP a better candidate as biomarker (3).

Thus, BNP and NT-proBNP are sensitive and specific biomarkers for risk stratification in PAH (they are, so far, the only biomarker included in current guidelines) (23). They are also sensitive markers of RV dysfunction and of treatment efficacy (23, 26, 28). In addition, BNP, as a pulmonary vasodilator and antihypertrophic agent, has a therapeutic potential to alleviate pulmonary vascular remodeling (28). However, there are always factors that must be considered, namely the patients' gender and age, the presence of left heart disease, renal dysfunction, and obesity (12, 26). In any situation of left heart disease, BNP levels should not be used as predictors of diagnosis or prognosis for PAH (30).

Troponin

Cardiac troponins (cTn) are a set of 3 proteins—troponin C, troponin I (cTnI) and troponin T (cTnT) -, whose main function is to regulate the thin actin filaments of the heart muscle. The level of troponins is closely related to damage to the myocardium since the rupture of the cardiomyocyte membranes causes its release into the peripheral blood. Therefore, it is possible to detect it by highly sensitive assays in plasma (12, 26). The measurement of cTnT and cTnI is essential in the diagnosis and prognosis of patients with acute coronary syndrome, as well as of all pathologies associated with myocardial lesions which present increased levels of cardiac troponins, as is the case of myocarditis (12).

In PH pathophysiology: several studies linked cTnT to the poor prognosis of PH. Torbicki et al. demonstrated that in patients with PAH and CTEPH, cTnT levels are increased, which is probably explained by the damage to the RV myocardium. However, in this study, only 14% of patients (8 out of 56

patients) had an elevation of cTnT levels—since the test used to measure cTnT levels only allows detection of levels >0.01 ng /ml. Nonetheless, when comparing cTnT(+) *vs.* cTnT(-) patients, it was found that they had similar pulmonary hemodynamics, but cTnT(+) patients had a higher heart rate, lower mixed venous saturation of oxygen (SvO_2), higher serum NT-proBNP and less resistance to exercise (measured by 6MWD) (44). In addition, in patients undergoing treatment for PH, it was found that cTnT levels decrease, becoming even undetectable; in contrast, levels increase with the progression of the disease (44).

More recent studies present tests with greater sensitivity to cTn, with significantly lower detection levels. Heresi et al. demonstrated, using an immunoanalyzer with a detection limit of <0.008 ng/mL, that cTnI elevation was detected in 25% of patients with PAH. cTnI(+) patients, when compared to cTnI(-) patients, had a higher functional class, larger right atrial area, lower 6MWD, and higher levels of BNP and C-reactive protein (CRP). Furthermore, the survival of cTnI(+) patients was significantly lower than that of cTnI(-) patients (44% *vs.* 85%) (45). Filusch et al. evaluated cTnT levels in patients with PAH, comparing the conventional assay with the high-sensitive cTnT (hsTnT) assay, with a detection limit lower than 2 pg/ml. In 90.9% of patients, cTnT was detectable using the hsTnT assay *vs.* 30.9% using the conventional assay. In addition, the hsTnT assay measurements were significantly associated to systolic RV dysfunction and an impaired 6MWD. Furthermore, hsTnT predicted a World Health Organization (WHO) functional class of II or higher better than NT-proBNP, and predicted death as effectively as NT-proBNP (46). In another study, using the new hsTnI assay, cTnI levels were detectable in 95% of patients with PH, including PAH patients. Higher cTnI levels are associated with higher BNP levels, lower 6MWD, more severe hemodynamic abnormalities, and cardiac magnetic resonance imaging abnormalities (47).

Although cTn are also related to some markers on the left side of the heart (47), which can be a confounding factor, studies indicated that they can be used as indicators of disease severity. Furthermore, use of the new hsTn assays provides new prognostic information and these assays have the potential to detect more patients, at higher risk, and therefore facilitate risk stratification. Nevertheless, there are confounding factors that must be considered, namely, the presence of concomitant left heart disease or renal failure (12, 26).

Cystatin C

Cystatin C (CysC) is a 13 kDa non-glycosylated protein (48, 49), is produced at a constant rate by all investigated nucleated cells (50), and it is a member of the cystatin superfamily that comprises inhibitors of cysteine proteinases (51). In recent years, the importance of CysC has increased due to its free filtration in the glomerulus, complete reabsorption and catabolism in the proximal tubule and lack of tubular secretion. It is believed that the plasma concentration of CysC depends almost completely on the glomerular filtration rate, thus making it an ideal marker of renal function (48, 49). In addition, it is more sensitive than serum creatine since it detects smaller reductions in glomerular filtration rate (48, 49). Several studies have shown that CysC can also function as a cardiovascular risk marker since it predicts left heart failure (HF) and cardiovascular mortality in general (48, 49).

In PH pathophysiology: Fenster et al. showed that patients with PAH have abnormally high serum CysC levels and that these levels correlate with RV function (52). These studies show that RV systolic pressure is highly elevated in PAH patients and that it correlates positively with CysC serum levels. Furthermore, RV end-diastolic volume, RV end-systolic volume, mass index, strain and strain rate are positively correlated with CysC levels, and RV ejection fraction is negatively correlated (52). Thus, CysC can be a sensitive biomarker for assessing PAH and it also has additional advantages over standard biomarkers, BNP and NT-proBNT, of being independent of muscle mass, age, and gender (50).

BIOMARKERS OF HEMATOPOIESIS

Homocysteine

Homocysteine is a sulfur-containing intermediate product of the normal metabolism of methionine, an essential amino acid, obtained essentially from animal protein (53). Homocysteine must be recycled by a route that requires folic acid and vitamins B_6 and B_{12}. Alterations in this pathway, such as deficits in vitamin B_6 and B_{12} levels, genetic defects, or polymorphisms in the main enzymes of this pathway, can promote an increase in homocysteine levels, which can be harmful (53). Homocysteine is an inhibitor of dimethylarginine dimethylaminohydrolase, an enzyme that metabolizes asymmetric dimethylarginine (ADMA); ADMA is an endogenous inhibitor of the NO synthase (NOS) pathway. Thus, an increase in homocysteine levels, known as hyperhomocysteinemia, results in a decrease in NO bioavailability. It is believed that this decrease in NO happens because homocysteine inhibits the activity of dimethylarginine dimethylaminohydrolase, promoting the accumulation of ADMA and, consequently, a decrease in the production of NO by ECs (54). Furthermore, it is also believed that homocysteine is involved in the oxidative degradation of NO (54, 55). Since homocysteine essentially affects ECs, coagulation, and platelet function, it is possible to understand why the endothelial vasodilator function is impaired in individuals with hyperhomocysteinemia, as well as associated with platelet dysfunction, facilitating coagulation; thus, increasing the risk of cardiovascular diseases and an acceleration in vascular diseases (54, 55).

In PH pathophysiology: studies show that total plasma homocysteine levels—includes homocysteine and its oxidized derivatives—mixed homocysteine-cysteine disulphide and protein-bound homocysteine—are higher in patients with PAH compared to healthy controls (53). Furthermore, Sanli et al. showed that homocysteine levels are higher in patients with PAH associated with congenital heart disease (CHD-PAH), as are ADMA levels, but they did not correlate with hemodynamic factors (56). Thus, both studies suggest that homocysteine may be an important factor in the development of PAH, which can be well-justified by the endothelial damage caused by hyperhomocysteinemia. However, more studies should be done, since the sample size in both existing studies is relatively low (56).

Red Cell Distribution Width

Red blood cell distribution width (RDW) is a parameter that reflects the variation in the size of circulating red blood cells; it is routinely measured in a complete blood count, and it is essential in the differential diagnosis of anemia (57–59). Several studies showed that RDW values can be useful in predicting malignant tumors (60, 61). RDW is associated with several pathophysiological mechanisms, such as inflammation, iron metabolism, renal dysfunction, nutritional status, and oxidative stress which result in a decrease of erythropoietic output (62). Increased levels of RDW are closely associated with impaired erythropoiesis or erythrocyte degradation (63). RDW has proven to be a promising predictor of the clinical outcome of renal diseases, cardiovascular diseases, pulmonary diseases (57–59), such as HF, PH of various etiologies (58), acute myocardial infarction, community-acquired pneumonia, pulmonary embolism, and COPD; it has also been shown as a predictor of mortality in patients with COPD and PAH (64).

In PH pathophysiology: Ulrich et al. demonstrated that RDW is a factor that correlates with survival in PAH; and that iron deficiency is usually seen in patients with PAH (65, 66). RDW levels increase with decreasing iron levels, since the available body iron levels do not respond to the demand for iron from red blood cell synthesis, resulting in varied size of red blood cells (67). Rhodes et al. showed that RDW levels correlate with WHO functional class and 6MWD (68); they also showed that RDW can function as an independent predictor of survival, even when measured in combination with 6MWD, NT-proBNP and other clinical indices (68).

In conclusion, RDW is closely related to the severity of the disease and can be used to predict the survival of patients with IPAH (67, 68). Thus, the use of RDW as a PAH biomarker should be considered for new approaches with multiple biomarkers for PAH stratification; since the levels of RDW in combination with the levels of NT-proBNP showed better detection of high-risk cases, than just using NT-proBNP (68).

BIOMARKERS OF ENDOTHELIAL DYSFUNCTION OR/AND VASCULAR REMODELING AND DAMAGE

Endothelin-1

The ET system consists of three ET isopeptides (ET-1, ET-2, and ET-3), activating peptidases with different isoforms and two G protein-coupled receptors—ET type A receptor (ETA) and ET type B receptor (ETB) (69). ETs are isopeptides of 21 aa, presenting a high homology and similarity to each other. They are expressed essentially in ECs; however, they are also expressed in cardiac myocytes, pulmonary epithelium, glomerular renal cells, mesangial cells, smooth muscle cells (SMCs), leukocytes, macrophages (7, 70) and fibroblasts (12). ETs are recognized as being the most potent endogenous vasoconstrictors (69) but in addition, they are multifunctional peptides with cytokine or hormone-like activity (71).

ET-1 is the most expressed form in the cardiovascular system (7) and in the pulmonary vasculature (12); it binds to both receptors. ETA and ETB are distributed in various tissues and cells, but their expression is variable; ETA is primarily expressed on vascular smooth muscle cells (VSMCs) (71) and myocytes (7), while ETB is primarily located on ECs (ETB1 receptors), and on VSMCs (ETB2 receptors) (7). Activation of ETA primarily mediates vasoconstriction as well as cell proliferation (69). In contrast, ETB activation in ECs promotes an indirect vasodilator action, anti-proliferation of myocardial and vascular tissues, and renal blood pressure regulation. On the other hand, ETB2 receptors have some vasoconstrictor role, however, their most important action is the ET-1 clearance (69). Vasoconstriction is mediated by activation of phospholipase C, increase in inositol triphosphate and diacylglycerol, with a subsequent increase in intracellular calcium, promoting cell contraction. On the other hand, vasodilation mediated by the activation of endothelial receptors ETB1 stimulates the release of NO and prostacyclins, inducing relaxation of the vascular wall (7). ET-1 clearance occurs by internalization of the receptor to which ET-1 has bound; the lungs clear about 50% of circulating ET-1 (7).

ET-1 induces intense and prolonged vasoconstriction of the pulmonary arteries and veins, even when present in low concentration, and it is also capable of stimulating the proliferation of pulmonary fibroblasts. At the cardiac level, ET-1 is involved in increasing myocardial contractility and heart rate (positive inotropic and chronotropic effect, respectively), in addition to stimulating the production of cytokines, growth factors, and matrix proteins in other tissues (7).

In PH pathophysiology: most patients have increased ET-1 levels (72); and it has been implicated as a mediator of increased vascular tone and vascular remodeling (73). In PAH, there is an evident increase in the expression of ET-1 in the pulmonary vasculature (73) including in the plexiform lesions (7), characteristic of the disease. Also, ET-1 plasma levels are increased and are closely correlated with RAP and pulmonary artery oxygen saturation (5, 74), PVR, and 6MWD (75). Endothelial damage, characteristic of PAH, potentiates the constrictive action of ET-1, causing dysregulation in the ET system (7), and reduces the endothelium's capacity to release vasodilators (72). Thus, this dysregulation and overexpression of ET-1 promote an increase in PVR (7) in part due to the lack of vasodilators (72), and to an abnormal pulmonary vascular remodeling (19). High levels of ET-1 are also associated with an inflammatory response and increased fibrosis (1). The increase in ET-1 plasma levels in PAH may result from an increase in ET-1 release or a reduction in ET-1 clearance by the pulmonary vasculature or even a combination of both factors (1, 72).

Furthermore, inhibition of ET receptors by the action of ERAs is effective in the treatment of PAH, reducing PAP and inhibiting vascular remodeling. Bosentan demonstrated improvements in hemodynamic parameters, in 6MWD, and in WHO functional class in patients with PAH (76); Sitaxsentan showed improvements in 6MWD, WHO functional class, PVR, and cardiac index (77). Ambrisentan improves exercise capacity, WHO functional class, hemodynamics parameters and death in PAH patients; also, it is associated with a low risk of aminotransferase abnormalities (78, 79). In addition, the AMBITION trial showed that dual combination therapy with

Ambrisentan and Tadalafil, a PDE-5 inhibitor, reduced the risk of PAH-related hospitalization by 63% compared with just monotherapy (80). Macitentan improves mPAP, RAP, PVR, cardiac index, and the levels of NT-proBNP (81).

Therefore, the ET-1 system plays a fundamental role in the pathology of PAH and can even be used as a prognostic marker of the disease. However, the use of ET-1 as a marker has some limitations that must be considered, since it essentially spreads through vascular structures, its plasma levels do not accurately represent the concentration of ET-1 in the tissue (12, 72). Moreover, demographic characteristics such as ethnicity, sex, and age should always be considered since plasma ET-1 levels are higher in African ethnicity, males, and older age, thus representing potential confounding factors (82).

Adrenomedullin

Adrenomedullin (ADM) is 52 aa peptide hormone associated with long-lasting pulmonary vasodilator effect (83, 84). ADM was first isolated in 1993 from human phaeochromocytoma (83, 84) and was subsequently found as a circulating hormone (12). ADM is produced by ECs and VSMCs and diffuses between blood and interstitium (83, 85). Despite having receptors and binding sites throughout the body, the density of receptors is higher in the cardiovascular and pulmonary tissues, thus its functioning essentially in these two systems (83, 85). The ADM, in addition to its vasodilating effect, has diuretic and natriuretic effects, inhibits the renin-angiotensin-aldosterone system, and it is involved in angiogenesis and regulation of inflammation (12). Given its vasodilating action, and natriuretic and diuretic effect, it is understandable that ADM is involved in the regulation of body fluid and, therefore, in cardiac homeostasis (85). Also, it has the capability to act as a regulator of pulmonary vascular tone and vascular remodeling (83). Previous studies demonstrated that plasma levels of ADM are increased in patients with hypertension and HF.

In PH pathophysiology: plasma levels of ADM are increased in patients with PAH and increase in proportion to the severity of PH (83, 85). Moreover, ADM levels correlate with clinical parameters including mRAP, 6MWD, and NT-proBNP levels, with ESC/ERS and REVEAL risk scores, and may reflect overall patient survival (86). Studies also showed the importance of ADM as a therapeutic target for PAH—-administration of exogenous ADM resulted in significant hemodynamic improvements (increase in cardiac index and a decrease in PVR) in patients with PAH (83); therefore, acting as a disease-regulating hormone in PAH, in addition to functioning as an alternative prognosis and severity marker (86).

Copeptin

Copeptin is a 39 aa glycopeptide and it is especially known as arginine vasopressin (AVP)-associated glycopeptide (87). Copeptin, together with AVP and neurophysin II, is derived from a precursor, the pre-pro-vasopressin (87, 88). These three peptides are stoichiometrically secreted by the pituitary gland, and it is possible to use copeptin levels as reporters of AVP levels (87, 88). AVP is produced in the hypothalamus and secreted by the pituitary in response to hemodynamic and/or osmotic stimuli (87). AVP binds to two types of receptors: vasopressin1, which mediate arteriolar vasoconstriction (87, 88), and vasopressin2, which has antidiuretic effects (87), promoting water reabsorption through of the induction of aquaporins in the kidney collecting ducts (88). However, AVP has a short plasma half-life (89) and, since it is unstable, the circulating AVP is mostly linked to platelets (88) and therefore it is impossible to measure. Copeptin is a prognostic marker for several cardiovascular pathologies (12, 87, 88).

In PH pathophysiology: levels of circulating copeptin are increased in PAH patients and are positively correlate with NYHA class and negatively with 6MWD (88). In addition, it was found that the increase in plasma volume and low plasma sodium concentrations are closely related to mortality in patients with PAH (88). Thus, copeptin can function as a predictor of death, transplantation, or hospitalization (90) and provide information about response to treatment in patients with PAH (88).

Nitric Oxide

NO is a potent vasodilator produced in ECs by the action of the NOS enzyme in the conversion of L-arginine into L-citrulline and NO (26, 91). In addition to the endothelial NOS (eNOS), there are two other isoforms, the neuronal isoform, and an inducible isoform, which promotes the high diversity of NO biological functions (4). NO can function as a signaling molecule, a toxin, a pro-oxidant, and a potential antioxidant (92). It is involved in the regulation of vascular tone and neurotransmission (93), platelet aggregation, inhibition of VSMCs proliferation (12, 91), destruction of pathogens, and it is a precursor of oxidizing and nitrating species (93). Several stimuli mediate the release of NO, the main stimulus being shear stress (4). NO induces its vasodilatory activity when it is released from the pulmonary vascular endothelium, diffuses through adjacent VSMCs, and stimulates the production of cGMP which, consequently, leads to the activation of cGMP-dependent kinases (91), which in turn increases the myosin light chain phosphatase activity (4), inducing relaxation in VSMCs (4, 12, 91). Given the vasodilator effect of NO and the endothelial dysfunction associated with PAH, it would be expected that patients with PAH would express low levels of NO (30). However, since NO is too unstable to be measured in its gaseous form in the blood, there are several complementary techniques to assess endogenous NO, namely via exhaled NO, nasal NO and nitrate metabolite levels (NOx) in plasma and urine (94). Considering that there are several methodologies to quantify NO levels, we must compare results obtained respecting the same methodology in order to achieve a consistent analysis with the least possible discrepancy.

In PH pathophysiology: several studies showed that exhaled NO levels are reduced in patients with IPAH (94–96). Nevertheless, some studies refute these results, showing that exhaled NO levels may remain similar to healthy patients, or even increase (97–99). Still, there are confounding factors that must always be considered, namely, age, sex, atopy, infections, and medications (100), in addition to methodological differences that may explain the diversity of results (94). The levels of nitrates, nitrites and S-nitrosothiol proteins—products of the NO biochemical reaction -, are in agreement with the levels of exhaled

NO, and are also decreased in patients with PAH. Furthermore, NO and oxidative reactions in the lung are correlated with the increase of PAP in PAH (95). Several studies demonstrate that exhaled NO levels increase in PAH patients after treatments with Bosentan (94), and with Epoprostenol—a prostacyclin analog (96, 98). In addition, the use of phosphodiesterase (PDE) inhibitors such as Zaprinast was shown to increases the vasodilator responsiveness to inhaled NO in the lungs of rats challenged with endotoxin (lipopolysaccharide) (101).

Nevertheless, it should be noticed that NO levels are also conditioned by eNOS expression (96). Studies show that there is very little eNOS expression in the vascular endothelium of pulmonary arteries in patients with PH and that the decrease in eNOS expression is inversely correlated with the increase in vascular resistance (102). However, more studies in this sense should be done, since the results are not in agreement; several works assume that there is no expression of eNOS in plexiform lesions (102), while others suggest that there is (103). Hence, since this phenomenon is still not well-understood, the fact of whether there is an increase in the expression of an enzyme does not imply that the enzyme is active (103). Therefore, although there are alterations in the expression of NOS in the pulmonary endothelium, it is not evident that there is a greater sensitivity to the action of NO, as well as a greater activity of this enzyme (4).

Thus, although NO is not functional as a biomarker for PAH, in association with some additional data, it may be useful to understand patients' responses to therapy with prostacyclins (96) and Bosentan (94).

Asymmetric Dimethylarginine

ADMA is a natural amino acid, derived from the catabolism of proteins containing methylated arginine residues (23, 26). Interest in ADMA has grown over time because it is an endogenous inhibitor of the NOS pathway (104). ADMA was first recognized as a NOS inhibitor in patients with renal failure. In these patients, it was found that ADMA levels increased with the decrease in renal clearance, effects that were reversed with dialysis and restored endothelial function (104). Thus, ADMA is associated with vascular diseases and several risk factors (104) and its plasma levels can perfectly function as a marker of endothelial dysfunction.

In PH pathophysiology: several studies have shown that ADMA plasma levels are increased in patients with PH of different categories including IPAH (11, 105), CHD-PAH (56, 106, 107) and CTEPH (11, 108) and correlate with some hemodynamic parameters. In patients with IPAH, ADMA levels correlated with mPAP, PVR index, SvO_2, RAP, cardiac index, and survival (11, 105); in patients with CHD-PAH, ADMA levels correlated positively with RAP and negatively with SvO_2, cardiac output, cardiac index and survival rate (56); and in patients with CTEPH, ADMA levels correlated with mPAP, mRAP, cardiac output, cardiac index, PVR and SvO_2 (11, 108).

Therefore, circulating levels of ADMA can function as markers of endothelial dysfunction in PAH since the increase in ADMA induces NO synthesis inhibition and consequently will affect the NO/cGMP pathway that is responsible for regulating pulmonary vascular tone, increasing vascular resistance. Some studies also suggested that ADMA can promote pulmonary endothelial dysfunction due to changes in connexin 43 expression and activity, however, the mechanism is not yet known (11). Thus, the fact that ADMA is related to some hemodynamic parameters can be useful to study and monitor the severity of the disease, the effectiveness of the therapies implemented, and assist in risk stratification in patients with PAH or other types of PH (11).

Cyclic Guanosine Monophosphate

cGMP is the predominant intracellular second messenger of NO. NO activates guanylate cyclase, increasing intracellular cGMP concentrations, thereby inducing vascular smooth muscle relaxation (109). In this way, plasma cGMP would be an alternative marker of eNOS activity (30) since NO inhalation has been shown to induce a significant increase in plasma cGMP levels. On other hand, as cGMP is produced by the activation of the enzyme guanylate cyclase, in addition to being an indirect marker of NO, it will also be an indirect marker of natriuretic peptides, as these are also involved in the activation of guanylate cyclase (110, 111). Furthermore, cGMP has a short life span due to rapid degradation by cyclic nucleotide PDE, with PDE-5 being the most active isoform in lung tissue (112).

In PH pathophysiology: Ghofrani et al. demonstrated that cGMP and ANP levels are high in PAH patients, and that they are closely related to each other and to disease severity (111). In addition to plasma, cGMP can also be measured in urine (30). Bogdan et al. demonstrated that urine cGMP levels are increased in PAH patients and that these levels were higher in patients with severe hemodynamic impairment and may therefore reflect the hemodynamic status of patients with PAH (113). However, besides being related to hemodynamic measures, cGMP is also a relevant therapeutic target, as PDE-5 inhibitor drugs have been increasingly recognized for their action as inhibitors of cGMP degradation. Moreover, the high levels of PDE-5 in the lung VSMCs provide a strong molecular basis for PDE5 inhibitor treatment for PH (114): in patients with PAH, Tadalafil was well-tolerated and improved exercise capacity and quality of life measures and reduced clinical worsening (115); Sildenafil, in an animal model of hypoxia-induced PH, has been shown to reduce PAP, pulmonary vascular muscularization and prevent induced PH (116). Furthermore, Sildenafil proved to be a more effective and selective vasodilator than inhaled NO as it decreases mPAP and PVR and increases the cardiac index, without increasing wedge pressure (117).

Thus, cGMP is a notorious treatment target in PAH, and its plasma and urinary levels can also function as markers of disease severity and hemodynamic impairment.

D-Dimers

D-dimers are fibrin degradation products, that result from the degradation of the blood clot by plasmin. Each molecule is constituted by two D-domains, linked by disulfide bonds. The detection of D-dimers in the serum is a marker for thrombolytic activity, and this measure is fundamental in the diagnosis of venous thromboembolism—such as pulmonary embolism—and

disseminated intravascular coagulation (118, 119). However, D-dimers may also be elevated in other contexts in which there is an activation of the coagulation cascade, such as inflammation, cancer, or pregnancy; furthermore, levels increase with age. Nonetheless, there is a growing interest in the use of D-dimers for diagnostic and prognostic purposes in different clinical settings, like PH.

In PH pathophysiology: PAH pathogenesis involves endothelial dysfunction and *in situ* thrombosis. Moreover, Tournier et al. have described PAH patients with a hypercoagulable phenotype (120). Therefore, considering that D-dimers are products of these phenomenon, they may be useful as diagnostic, prognostic, and therapeutic biomarkers in the context of disease. Shitrit et al. demonstrated that D-dimer levels were elevated in IPAH patients. They also showed that D-dimer levels were correlated with IPAH disease severity, as shown by higher NYHA functional class and mPAP, and lower values of oxygen saturation and 6MWD. Additionally, higher values seemed to predict lower 1-year survival (121, 122). Tournier et al. established that elevated D-dimers in IPAH patients were independent of systemic inflammation (120). However, both studies were conducted with a small number of patients, which limits data interpretation. As for other PAH subtypes, Remková et al. found that elevation of D-dimers in patients with Eisenmenger syndrome (a subtype of CHD-PAH) was non-significant (123). In SSc patients, although D-dimers have been shown to be significantly elevated, no correlation was found with the development of PAH (124), or its severity, as measured by RV systolic pressure (125)—this may be due to a lesser role of microthrombosis in this PAH subtype, or to the low number of patients under investigation.

The main limitation for using D-dimers as PAH biomarkers is that they can be elevated due to inflammatory or pro-coagulatory states of various etiologies, therefore they have very low specificity for diagnostic purposes (119). However, they may be useful for evaluating disease severity.

Serotonin

Serotonin or 5-hydroxytryptamine (5-HT) is a vasoactive molecule that acts both as a systemic vasodilator and a pulmonary vasoconstrictor. It is produced by enterochromaffin cells, in the gastrointestinal tract, as well as pulmonary neuroepithelial bodies, and then stored in platelets. This storage is dependent on the action of the 5-HT transporter (5-HTT) and results in low plasma levels of 5-HT (10, 19).

In the 1960's, it was suggested that there was an association between the use of appetite-suppressant drugs and PAH, and it was later shown that it was due to interactions with 5-HTT, for which these pharmacological agents act as substrates (126). Since then, new investigations have established that 5-HT promotes pulmonary VSMCs hypertrophy and hyperplasia and pulmonary vasoconstriction, via 5-HTT and serotonin 1B receptor, respectively (1, 10). It can also induce local microthrombosis (127). Moreover, other medications that implicate the serotoninergic system have been shown to increase the risk of developing PAH, such as selective serotonin reuptake inhibitors (17, 128, 129). Regarding 5-HTT, PAH patients have higher transporter expression on vessels and platelets (130), and polymorphisms may implicate disease severity (131–133). Additionally, a study using cultured pulmonary artery cells obtained from PAH patients found that selective 5-HTT inhibitors appeared to have a role in preventing the development of hypoxic PAH, supposedly due to inhibition of the mitogenic response this transporter mediates (130).

In PH pathophysiology: evidence regarding the use of 5-HT as a biomarker for PAH is contradicting. Hervé et al. and Kéreuver et al. both demonstrated that IPAH patients had an increase in plasma serotonin concentrations (and a decrease in platelet levels of 5-HT) (134, 135), that was sustained after heart-lung transplantation, suggesting that higher levels are not secondary to PAH (134). However, 5-HT plasma levels were not predictive of disease severity (135). Later, Zeinali et al. and Lederer et al. failed to find significant different serum measurements of 5-HT between controls and IPAH patients and could not establish a relation between these values and disease severity and 6MWD (136, 137). These discrepancies may be due to a small sample size (the largest study enrolled only 16 patients and 16 controls), different quantification techniques, or even the fact that various PAH subtypes were included in the same samples. 5-HT levels have also been shown to be elevated in PAH associated with ventricular septal defect (138). Recently, Manaud et al. using a rat model of pulmonary veno-occlusive disease induced by mitomycin exposure, demonstrated that pulmonary serotonin level was increased at the very end of pulmonary veno-occlusive disease development, when PH and pulmonary vascular remodeling were already established, indicating that serotonin plays a role late in pathogenesis and/or serves as a marker of PH severity (139).

In conclusion, these findings highlight the potential use of 5-HT as a diagnostic, prognostic, and therapeutic biomarker.

Osteopontin

Osteopontin (OPN) is a 32-kDa glycoprotein, first described as secreted by malignant epithelial cells (140, 141). It is now recognized to be expressed and secreted by various other cell types, such as cardiomyocytes and fibroblasts, in the context of inflammatory or neoplastic processes (12). It can exist both as a component of the extracellular matrix and a soluble cytokine, acting by increasing cell proliferation, migration, remodeling, and fibrosis (12, 142). Saker et al. found that OPN was one of the most highly expressed matricellular proteins in PASMCs undergoing replicative senescence, and that its release stimulated the migration and proliferation of these cells (143). Later, Mura et al. demonstrated that OPN is among the top five overexpressed genes in transplanted lungs of PAH patients, involved in VSMCs angiogenesis, death, and proliferation pathways; furthermore, its expression was correlated with disease severity (144). Overall, OPN seems to be an important agent in pulmonary vascular remodeling, and, therefore, to the pathophysiology of PAH (145).

In PH pathophysiology: OPN plasma levels were elevated in group I PH, when compared to healthy controls, and predicted all-cause mortality (146, 147). In addition, OPN increased with mRAP, age, 6MWD and NYHA class (146). Rosenberg et al. also found that higher plasma OPN was related to RV

remodeling and dysfunction (148). Finally, in the context of IPAH, OPN correlated with NT-proBNP at baseline and during follow-up, providing independent and incremental prognostic information—while NT-proBNP is specific to hemodynamic alterations, OPN may describe the general condition of the patient (148). More recently, OPN plasma levels have been shown to be elevated in other subgroups of PAH, such CTD-PAH (specifically, SSc-PAH) (140) and CHD-PAH (149). In SSc-PAH, OPN was also correlated to patient age (140).

Contrary to all the findings above, a study analyzing group I PH patients irrespective of their disease subtype found that OPN serum measurements conducted at the time of pulmonary artery catheterization did not differ between those and group II PH patients and had no correlation with mPAP (144). As with other biomarkers, the main limitation of these studies is the sample size, that hinders the extrapolation of the findings to other types of PAH. Moreover, the elevation of OPN in both CTEPH (150) as well as PAH (144) leads to the conclusion that this biomarker is most likely related to the development of PH irrespective of its etiology. And, as mentioned earlier, OPN can be produced in the context of other pathologies, such as mesothelioma, breast cancer, or systemic inflammatory disorders, which limits its specificity as a biomarker (140, 148).

VonWillebrand Factor

Von Willebrand factor (vWF) is a glycoprotein that is produced by the endothelium and acts as a carrier protein for factor VIII. It mediates platelet aggregation and adhesion in response to endothelial activation (12, 26).

In PH pathophysiology: both vWF and its antigen activity (vWF:Ag) have been postulated as group 1 PAH biomarkers. vWF (120, 151–153) and vWF:Ag (154–157) are significantly elevated in primary PAH. It has been shown that elevated baseline vWF significantly predicts short- and long-term survival (151, 155, 158). Functional class and 6MWD were also associated with higher vWF activity; however, in this same study, Al-Naamani et al. determined that lower vWF activity predicts higher risk of death and lung transplant, which is not in concordance with earlier studies (159). Finally, Veyradier et al. and Friedman et al. found lowering in vWF values, parallel with hemodynamic improvements, in PAH patients exposed to prostacyclin treatment (152, 154). In respect to specific PAH etiologies, starting with CHD-PAH, this patient subgroup also showed elevated levels of vWF:Ag (123, 160, 161), and these levels predicted mortality (160, 161). Furthermore, vWF:Ag level may have a role in predicting PAH in SSc, however investigations have not been consensual (162–164). Finally, PAH associated with congenital systemic-pulmonary shunts had significantly raised vWF, that correlated with raises in NT-proBNP (165).

Microparticles

Microparticles (MPs) are small vesicles formed from membrane blebs (12). They are shed from eukaryotic cells that have been activated or damaged or during apoptosis and, therefore, constitute hallmarks of cell damage. There can be several types, depending on the cell that they derive from: ECs (EMPs), platelets (PMPs) or leukocytes (LMPs) (166) and many authors have postulated their role as biomarkers in cardiovascular diseases (167). Tual-Chalot et al. established a potential role of MPs in PAH pathogenesis (particularly, platelet- and erythrocyte-derived MPs), by determining that these particles acted in reducing eNOS activity and NO bioavailability and increasing production of reactive oxygen species, contributing to dysfunction of pulmonary endothelium (168).

In PH pathophysiology: Bakouboula et al. found that pro-coagulant CD105 or tissue factor positives EMPs were elevated in PAH, and the later subtype correlated with disease severity (6MWD and NYHA class of 3 or more) (169). Similarly, Amabile et al. found heightened levels of various MPs (EMPs - $PECAM^+$, $VE\text{-}cadherin^+$, $E\text{-}Selectin^+$ - and LMPs) in PAH patients, and significantly correlated $PECAM^+$ and $VE\text{-}cadherin^+$ MPs with hemodynamic severity (170). The investigators then prospectively followed the cohort and observed a significantly higher incidence of negative outcomes (death or decompensated right HF) associated with $E\text{-}Selectin^+$ MPs, with this biomarker classified as a potential independent predictor of prognosis (171). EMPs can also be found in urine samples, and this marker was elevated in PAH and correlated with tricuspid annular plane systolic excursion, therefore acting as a biomarker for RV function that can be easily measured in a non-invasive manner (172).

Finally, concerning specific PAH subgroups, IPAH was correlated with elevated levels of LMPs—specifically derived from T-cells (173), and PMPs (CD42a and $CD42b^+$) (174). Raised EMPs ($CD31^+/CD42b^-$) have been found in both IPAH and SSc-PAH, and variation in measurements taken before and after treatment initiation did not reflect clinical improvement, suggesting this as a marker of endothelial injury (175). Regarding SSc-PAH in particular, elevated $VE\text{-}cadherin^+$ MPs have also been reported, which measurements appear to independently predict PAH in SSc patients without PH (176). This marker, in addition to $CD146^+$ MPs, was also elevated in PAH patients with Eisenmenger Syndrome (177).

Despite significant advancements in this field of research in recent years, one of the main challenges we are now facing regards quantification and phenotyping of MPs. Flow cytometry surpasses previous techniques, such as annexin-V labeling (167), being able to both detect and determine cellular origin of MPs. Moreover, it can be more easily scaled up, allowing for the study of large patient cohorts (172). However, even the most sophisticated equipment available is limited in the particle size range it can detect, with most MPs being under the minimum size limit (178). This hinders the reliability of new findings. Still, MPs arise as a promising biomarker for disease severity and prognosis in PAH.

BIOMARKERS OF ANGIOGENESIS

Angiopoietins

Angiopoietins, in particular angiopoietin-1 and 2 (Ang-1 and Ang 2, respectively), are angiogenic factors, produced by SMCs and precursor pericytes to regulate the development of lung vasculature (12, 19). Their actions are mediated via Tie-2 receptor, with Ang-2 acting as an antagonist to Ang-1 (12). It

has been difficult to establish the role of angiopoietins in PAH, but it is now widely recognized that the Ang-Tie2 signaling pathway plays a major role in PAH pathogenesis, through the regulation of vascular hyperplasia (12). However, tonic pathway activation appears to also play a protective role. On one hand, Tie-2 stimulation leads to inhibition of *BMPR2* signaling—the gene that is altered in hereditary cases of PAH—and increase in production of serotonin by pulmonary arteriolar ECs. These changes concur to promote PAH. On the other hand, the Tie-2 receptor pathway can downregulate endothelial activation and remodeling, therefore mitigating its deleterious effect (19, 179). Moreover, Ang-2 expression has been found to be up-regulated in plexiform lesions from lung tissue samples (180).

In PH pathophysiology: Kumpers et al. determined that, while venous plasma levels of Ang-1 and Ang-2 were elevated in IPAH patients, only Ang-2 correlated with mRAP, PVR, NYHA functional class, cardiac index and SvO_2. Ang-2 also significantly predicted mortality, and values after initiating treatment varied inversely to changes in mRAP, PVR, and SvO_2 (180). Another study showed that Ang-2 was elevated in IPAH patients, and its decrease reflected improvements in 6MWD during Treprostinil monotherapy (181). Recently, Ang-2 was also shown to be elevated in SSc patients that develop PAH, but studies regarding correlation with disease severity have yet to be conducted (182). As for Ang-1, significantly different levels between PAH patients and healthy individuals have been inconsistently found; and there was no association with clinical or laboratory parameters (180, 183).

Therefore, Ang-2 independently predicts disease survival, severity, and response to treatment, and appears to outperform NT-proBNP in doing so, and it is equally easy to determine (180).

Vascular Endothelial Growth Factor

Vascular endothelial growth factor (VEGF) is an angiogenic factor. It has several isoforms which act as ligands to specific receptors. Regarding PAH, the isoforms VEGF-A and -B and the receptors VEGFR-1 and−2 have been most extensively researched and linked to PH pathogenesis (19). They have been traditionally regarded as promoters of angiogenesis deregulation (184, 185), but recent investigations postulate a protective role against these pathologic alterations (186).

In PH Pathophysiology: VEGFR expression is increased in IPAH, with VEGFR-2 being found mostly overexpressed in plexiform lesions (184, 185). VEGFR-2 is lower in PAH (187), whereas soluble VEGFR-1 (sVEGFR-1) is elevated (188–190). sVEGFR-1 is the most well-researched marker, and it has been shown to predict functional class, disease severity (188, 190), patient survival (188), and adverse events (191). Particularly, Kylhammar et al. studied sVEGFR-1 in SSc-PAH and IPAH and found that both patient groups had significantly elevated values of this marker; additionally, sVEGFR-1 was able to predict treatment response, and was higher is SSc patients that would later develop PAH (189). This later finding is supported by other studies (192). Finally, it has been shown that sVEGFR-1 correlates to RV systolic pressure and the capacity of diffusing carbon monoxide in SSc-PAH (192); therefore, sVEGFR-1 arises as a potential non-invasive screening tool for SSc-PAH (190).

Regarding the VEGFR ligands, they have been documented as significantly increased in various forms of PAH, namely IPAH (180, 187, 189, 193) and SSc-PAH (162, 189, 194). Contrary to this, a 2014 systematic review found that, although serum VEGF levels were not increased and did not correlate with hemodynamic alterations, VEGF expression on arterial vascular ECs was significantly higher in CHD-PAH and IPAH patients when compared with healthy controls; additionally, higher expression predicted worse outcomes post-surgical treatment of the patients with CHD (106).

Endoglin

Endoglin (Eng), also known as CD105, is an anti-angiogenic agent that exists in two forms, soluble (sEng) and membrane bound. It is expressed in proliferating ECs and has an important role in vascular development and pathogenesis of vascular diseases, such as pre-eclampsia and tumor angiogenesis. Eng acts as an auxiliary receptor for transforming growth factor-β (TGF-β), modulating several signaling pathways, and its overexpression is associated with dysregulated angiogenesis (195)—in PAH, enhanced Eng expression has been found in plexiform lesions, which is in favor of Eng as an indicator of vascular proliferation and remodeling (188).

In PH pathophysiology: sEng was elevated in PAH patients and had higher sensitivity for the presence of PAH than NT-proBNP. Likewise, sEng was predictive of NYHA functional class and performed significantly better than NT-proBNP, being sensitive even among mildly symptomatic patients (NYHA Class I-II); moreover, an equally weighted combination of sEng and NT-proBNP was not significantly better than sEng alone. Finally, sEng independently predicted patient survival (188). Another study detected significantly raised levels of sEng on SSc-PAH patients *vs.* healthy controls (196).

Overall, sEng arises as a biomarker that appears to be more sensitive for risk stratification than NT-proBNP. Moreover, it is not influenced by RV dysfunction, making it more specific (188). Finally, it could detect PAH even in patients with minimal symptoms. Therefore, Eng is a promising diagnostic and prognostic biomarker and deserves further studies to validate these findings.

Bone Morphogenic Protein 9

Bone morphogenic protein 9 (BMP9), also known as Growth Differentiation Factor 2, is a liver derived protein present in peripheral circulation, belonging to the TGF-β superfamily (197, 198). It can act as a hematopoietic (199), hepatogenic (198), osteogenic (200), or chondrogenic factor (201), and several BMP9 genetic variants have been associated with different pathological states (197).

In PH pathophysiology: BMP9 is implicated in the BMP9/*BMPR2*/endoglin/ALK1 signaling axis - it is a physiological ligand to the *BMPR2*/ALK1 receptor complex, and to coreceptor endoglin—acting as a circulating factor for maintenance of vascular quiescence, by inhibiting EC migration and growth in the pulmonary vasculature (202–204). Mutations of the *BMP9* gene and its receptor and coreceptor are among the most common mutations present in hereditary cases of PAH

(205–208) and several pathogenic *BMP9* genetic variants have also been found IPAH patients (209–211). Moreover, in IPAH patients, median plasma levels of BMP9 have been found to be significantly lower than those of control patients (209, 210, 212), with even lower plasmatic levels and plasma activity in patients carrying certain BMP9 mutations (209, 211).

In recent years, research efforts have been focused on the implications of BMP9 levels and genetic variants in patients with PAH associated with severe liver disease—portopulmonary hypertension (PoPH). Nikolic et al. studied BMP9 plasma levels in patients with PAH of groups 1, 2 and 3, focusing particularly PoPH, and found markedly lower BMP9 levels in PoPH patients *vs.* healthy controls, *vs.* PAH of other etiologies, and *vs.* group 2 and 3 PH (213). Moreover, BMP9 was able to distinguish PoPH from liver disease without PAH in humans and rat models, suggesting a potential diagnostic application; and was predictive of transplant-free survival in all patients with group 1 PAH, therefore also implicating disease prognosis. No correlation with measures of RV function and disease severity was found. The same research group studied rat models of PAH with portal hypertension and cirrhosis and described an exacerbation of PH phenotype and pulmonary vascular remodeling upon administration of a BMP9 ligand trap, highlighting an apparent protective effect of endogenous BMP9 (213). These findings are supported by those of Long et al., who found that administration of exogenous BMP9 ameliorated PH induced by toxin exposure or *BMPR2* mutation, as shown by improvements in vascular remodeling and RV hypertrophy in several animal models (214). Contrary to these findings, a recent investigation by Tu et al. associated the loss of BMP9, either by deletion or inhibition, with protection against hypoxia and monocrotaline induced PAH; additionally, they described lower mRNA levels of ET-1, and higher levels of ADM, translating a de-regulation of endogenous vasoactive agents (215).

These opposing findings give insight into the complexity of BMP9 signaling and its potential implications in several mechanisms underlying PAH pathophysiology. In addition, they highlight the many potential clinical uses of BMP9, either as a diagnostic and/or prognostic biomarker, or as a novel therapeutic target. Regarding PoPH and considering the high fatality rate associated with this PAH subtype (216), BMP9 may constitute an important tool for clinical screening of high-risk populations; nonetheless, more studies evaluating biomarker performance are still needed to validate these new concepts.

Endostatin

Endostatin (Es) is an angiostatic peptide that results from the cleavage of collagen XVIII, which exists on the extracellular matrix, predominantly that of the vasculature (217). It is known as an inhibitor of endothelial proliferation, angiogenesis, and tumor growth (218). The action mechanism via which it intervenes in PAH has yet to be determined; however, recent findings showed that Es inhibits pulmonary artery ECs proliferation and migration and promotes ECs apoptosis, in line with PAH pathogenesis (219).

In PH pathophysiology: Es has been found to be elevated in PAH, particularly in IPAH (220, 221), CTD-PAH (221) and CHD-PAH (222). In IPAH, elevated Es levels correlated with unfavorable hemodynamic alterations (higher mPAP and PVR, lower cardiac index and cardiac output), worse functional class, and reduced exercise tolerance (220, 223). Moreover, Es strongly predicted disease mortality in this disease group (223). Similarly, regarding IPAH and CTD-PAH, Simpson et al. determined that the elevation of Es on patient blood samples independently predicted disease severity (measured by mRAP, mPAP, PVR, 6MWD, pulmonary artery compliance and stroke volume) and mortality—this association was particularly strong in IPAH (221). Considering the case of SSc, Es appeared to predict the development of PAH in these patients (224). Thirdly, Daly et al. investigated Es in CHD-PAH and found that, besides predicting worse hemodynamics and functional capacity, Es elevation was directly correlated to several echocardiographic alterations, predicting RV dysfunction; also, its levels lowered as patients showed clinical and hemodynamic improvement (222). Lastly, Es has been tested has a tool to predict clinical outcomes in IPAH, and CHD and CTD-PAH, in addition to tools that are currently validated [REVEAL, ESC/ERS (221), and NT-proBNP (222)], and it was able to improve their performance in risk discrimination and mortality stratification.

Es arises as a robust prognostic biomarker and a potential candidate for the update and refinement of current risk assessment strategies. Additionally, investigation into the pathobiology of Es in PAH is also needed—should it prove to be an agent in PAH pathophysiology, that would wield it a degree of specificity surpassing that of NT-proBNP.

BIOMARKERS OF INFLAMMATION/OXIDATIVE STRESS

C-Reactive Protein

CRP belongs to a family of highly conserved proteins, the pentraxins (225). CRP is predominantly synthesized in hepatocytes in response to cytokines such as interleukin (IL)-6 and IL-1, functioning as a representative of the acute state (225, 226) and as a sensitive marker of underlying systemic inflammation (227). However, during inflammation, CRP can be produced in different forms (225). In addition to being prevalent in inflammation, CRP also plays a key role in endothelial dysfunction (227), atherosclerosis and cardiovascular diseases, having been recognized as a risk predictor of cardiovascular diseases (225, 226, 228) and a risk predictor of pulmonary arterial diseases (229).

In PH pathophysiology: studies showed that CRP levels are increased in PAH patients (227, 228). There is evidence that CRP induces the production of IL-6 and monocyte chemoattracting protein-1 (MCP-1), known systemic inflammatory markers, both associated with the development of PAH in animal models (229). This was corroborated by Li et al., which showed that these pro-inflammatory agents are also increased in cultured VSMCs from PAH patients (227). Li et al. suggest that CRP regulates the expression of IL-6 and MCP-1 in VSMCs by nuclear factor kappa B (NF-κB) pathway (227). NF-kB is a crucial transcription factor in the inflammatory response,

involved in the transcription of several cytokines, chemokines, and adhesion molecules. Furthermore, it appears to be involved in the development of PAH: in an animal model of PAH, the administration of a NF-kB inhibitor improves PH manifestations (230). Moreover, it was found that treatment with atorvastatin has an anti-inflammatory action associated with the NF-kB pathway, decreasing the levels of MCP-1 and IL-6 induced by CRP in a dose-dependent manner (227). Quarck et al. showed that in addition to CRP levels being higher in patients with PAH, these correlate with RAP, NYHA functional class, 6MWD and survival, and predict outcome and response to therapy (228). Furthermore, Quarck et al. demonstrated that in PAH patients whose treatments are effective and stabilize plasma CRP levels, the survival rate is significantly higher, accompanied by a decrease in NYHA functional class and an increase in cardiac index (228). In CTEPH, plasma CRP levels decrease significantly after surgery (pulmonary endarterectomy) (228).

Therefore, this evidence shows that CRP can function as a biomarker of PAH, suggesting the inflammatory status of these patients and guiding the level of therapeutic options.

Growth Differentiation Factor-15

Growth differentiation factor-15 (GDF-15) or macrophage inhibiting cytokine is a stress-responsive member of TGF-β cytokine superfamily (231). GDF-15 is closely involved in tissue differentiation, remodeling, and repair (232), and it is strongly expressed in activated macrophages and epithelial cells (233). Under normal conditions, GDF-15 is poorly expressed in tissues, however, in pathological conditions—acute injury, tissue hypoxia, inflammation or oxidative stress—its expression is significantly increased (234). At the heart level, in normal situations, the myocardium does not express GDF-15, which increases dramatically after pressure overload or myocardial ischemia (235). Studies showed that circulating levels of GDF-15 are increased in patients with cardiovascular diseases, namely with chronic HF (236) and acute coronary syndrome (237), proving GDF-15 levels provide prognostic information and function as a biomarker of risk of death in these patients (236, 237). Lankeit et al. also showed that GDF-15 levels are increased in patients with pulmonary embolism and that these are related to the increased risk of death and major complications in the first 30 days after diagnosis. Furthermore, the prognostic information of GDF-15 is complementary to that of the biomarkers NT-proBNP, cTnT and the echocardiographic findings of RV dysfunction (235).

In PH pathophysiology: Nickel et al. showed that GDF-15 levels are increased in patients with IPAH, being closely related to more severe disease and poor prognosis in these patients (234). In addition, it was found that GDF-15 levels do not correlate with hemodynamic parameters and cannot be used as a diagnostic marker for PH, but, on the other hand, they do correlate with baseline NT-proBNP levels (234), which is in line with what was observed in patients with chronic HF (236) and acute pulmonary embolism (235). Moreover, measuring GDF-15 levels in combination with NT-proBNP achieved an improvement in the detection of high-risk cases (234). Nickel et al. also demonstrated that the expression of GDF-15 is increased in the lungs of patients with PAH and that GDF-15 is predominantly located in vascular ECs and the center of plexiform lesions (238). Therefore, GDF-15 arises as a potential marker for disease severity that correlates with NT-proBNP measures and can improve the prognostic performance of this validated biomarker.

Uric Acid

Serum uric acid (UA) is the end-product of adenine oxidation and guanine purine metabolism (239, 240). When this oxidative metabolism is impaired, there is an increase in UA levels due to the decrease/depletion of ATP levels, promoting the catabolism of adenine nucleotides into inosine, hypoxanthine, xanthine and UA, by increasing the expression of the xanthine enzyme oxidase (239, 240). In situations of hypoxia, ischemia, and some pathologies, such as chronic HF, cyanotic congenital heart disease, and COPD, UA levels are increased as a reflection of compromised oxidative metabolism (240, 241). Hyperuricemia is closely correlated with symptom severity and high mortality in patients with chronic HF, and serum UA may function as an independent marker of impaired prognosis in these patients (241, 242).

In PH pathophysiology: Nagaya et al. demonstrated that PAH patients have increased levels of UA compared to controls. Furthermore, serum UA levels positively correlate with more severe NYHA functional class, total pulmonary resistance, and mortality, and negatively correlate with cardiac output (240). Also, an approach with vasodilator therapy promotes a decrease in UA levels, associated with a reduction in total pulmonary resistance (240). Additionally, other studies corroborate these results and effectively show that serum UA levels are elevated in patients with PAH and correlate positively with NYHA functional class and mortality and negatively with the 6MWD (243). Recent studies showed that high serum UA levels are associated with a poor prognosis at first follow-up. Furthermore, the increased levels of UA promote a slight increase in the growth of PASMCs in IPAH patients when compared to controls (244). Thus, UA levels are elevated and correlated with disease severity in PAH (239, 240, 243), can be used as a non-invasive clinical prognostic indicator during follow-up (244), and may even function as a therapeutic marker.

The use of serum UA in combination with other biomarkers greatly increases the possibilities of diagnosis, so its use should be combined with more markers (245). However, it should always be considered that serum UA levels are affected by renal activity since approximately two-thirds of UA is excreted through the kidneys and one third through the gastrointestinal tract. Therefore, patients with renal failure or patients recommended for diuretic therapy should not be considered as their serum UA levels may be misleading (239). In addition, some hormonal factors and/or circulating substances (such as catecholamines, angiotensin II, ET, thromboxane, ANP) may influence impaired renal UA removal (239) and therefore interpretation may be difficult in some patients.

Monocyte Chemoattracting Protein-1

MCP-1 is one of the main pro-inflammatory chemokines, which are a family of chemo-attracting cytokines, subdivided

into four families (246). MCP-1 has a strong chemoattractant activity for monocytes and macrophages, being responsible for the regulation/activation and infiltration of monocytes and macrophages (246), leading to the induction of cytokine secretion and expression of adhesion molecules (247). MCP-1 is essentially produced in response to inflammation, and it is synthesized by several cells, including monocytes/macrophages, vascular ECs, VSMCs and fibroblasts (227, 247). In addition, MCP-1 is also released by pulmonary artery ECs, having a direct role in the infiltration of monocytes in the injured vessel wall, as well as in the proliferation of PASMCs (227).

In PH pathophysiology: Sanchez et al. demonstrated that MCP-1 overproduction may be a feature of the abnormal pulmonary ECs phenotype in IPAH, contributing to the inflammatory process and to pulmonary vascular remodeling (248). Previous studies showed that plasma MCP-1 levels are elevated in patients with CTD-PAH (247). Hashimoto et al. showed that serum MCP-1 levels are elevated in patients with IPAH and that there is a response to Epoprostenol therapy (249). Itoh et al. also showed that MCP-1 plasma levels are elevated in patients with IPAH, and this elevation was particularly marked in the early stage of disease. However, they do not correlate with the duration of the disease, nor do they correlate significantly with the hemodynamic variables (247), which was verified by Hashimoto et al. (249). Moreover, studies in rats with monocrotaline induced PH show that inhibition of MCP-1 signaling inhibits the increase of MCP-1 levels as well as pulmonary vascular remodeling, improving disease prognosis (250). Thus, although MCP-1 does not correlate with hemodynamic variables, it should be noticed that it contributes to the development of PH. Thus, the assessment of MCP-1 levels can become a useful tool in the early diagnosis of PH (247).

Galectin-3

Galectin 3 (Gal-3) is a member of the lectin family and of the β-galactoside-binding protein family (251, 252). It is expressed in the nucleus, cell surface and extracellular space and it is also expressed in inflammatory cells, fibroblasts, and myocardium (251, 252). Gal-3 binds to several substrates, which include signaling molecules, transcriptional regulators, ribonucleoproteins, cell surface receptors and matrix proteins and, for this reason, plays a fundamental role in several biological functions, namely, in proliferation, migration, adhesion, differentiation, angiogenesis, inflammation, apoptosis and fibrosis (251). That is why it is involved in remodeling and vascular stiffness (253). Gal-3 has pathogenic activity at the level of cancer, inflammatory and fibroproliferative diseases, such as pulmonary, cardiac, and hepatic fibrosis, and therefore, depending on the disease, Gal-3 is increased in different types of cells, which include macrophages, fibroblasts, and carcinogenic cells (251). Gal-3 is a well-known biomarker of fibrosis and of chronic left ventricular HF (254, 255). When expressed by activated macrophages and ECs, Gal-3 activates an inflammatory response and extracellular matrix remodeling (254, 255), inducing fibroblast proliferation and collagen synthesis, which contribute to cardiac remodeling, a determining factor in the development and progression of HF (251).

In PH pathophysiology: several studies show that Gal-3 serum levels are increased in patients with PAH (256), which may explain the development of vascular and RV fibrosis (254, 255). A systematic analysis of blood cells mRNA profiles from pre-transplant patients of the lung showed that the gene that most contributes to PAH, according to the Prediction Analysis of Microarrays, was Gal-3 (*LGALS3*). Gal-3 seems to be involved in RV HF, one of the most common causes of death in PAH patients. Moreover, this over-expression was validated in an independent PAH group by qPCR (257). Fenster et al. argue that, possibly, Gal-3 is released by the RV myocardial macrophages in response to changes in pulmonary artery pressure and volume. In addition, the levels of tissue inhibitor of metalloproteinase-1, a marker of myocardial matrix renewal, were increased in serum of PAH patients and were positively correlated with Gal-3; they may reflect the metabolism status of the RV myocardial extracellular matrix (256). On the other hand, Mazurek et al. also showed that Gal-3 levels are high in patients with PAH but that there is no correlation between Gal-3 levels and the structural and functional parameters of RV. They further argue that increasing Gal-3 levels are strongly predictive of mortality in any etiology of PH, including PAH. It should be noticed that all these studies are limited by the small sample size (256, 258). Furthermore, Scelsi et al. showed that plasma levels of Gal-3 increase linearly in the five risk strata on the REVEAL 2.0 risk scale. Furthermore, plasma levels of Gal-3 are also increased in patients who have a greater impairment of RV performance (259).

We can conclude that plasma levels of Gal-3 are associated with the various risk profiles of PAH (259). However, although Gal-3 has the potential to be a PAH biomarker, there are some confounding factors such as age, sex, diabetes, systemic hypertension, body mass index, BNP and NT-ProBNP (260). In addition, Gal-3 can be used as a biomarker of renal failure and it is present in pulmonary and hepatic fibrosis, making it useful as a specific biomarker of PAH only when these comorbidities are not present (256).

Interleukins

Inflammation is a characteristic of PAH, with high levels of circulating cytokines in patients with PAH (261). Several studies show the role of inflammatory cytokines in the development of IPAH; in addition, some animal studies also support the role of inflammatory cytokines in the initiation and progression of PAH (261). IL-1β, IL-6 and tumor necrosis factor- α (TNF- α) are pro-inflammatory cytokines, produced by monocytes, macrophages, and ECs (252). Additionally, they can induce the proliferation of fibroblasts and SMCs and promote thrombosis. Thus, they have a preponderant role in the initiation and progression of the growth of SMCs, fibroblasts and ECs and the occurrence of microthrombotic lesions commonly associated with severe PH (252).

In PH pathophysiology: serum levels of IL-1β, IL-6 and TNF-α are increased in patients with PAH (252, 262). Humbert et al. showed that IL-6 levels had an impact on the survival of patients with PAH; however, they did not correlate with hemodynamic parameters (252). Consistently with these results, Soon et al. showed that other cytokines are increased in circulation, namely,

IL-2, IL-4, IL-8, IL-10, and IL-12p70. Thus, it can be concluded that there is a deregulation of the circulating cytokines in PAH patients. Additionally, it also showed that the levels of IL-2, IL-6, IL-8, IL-10, and IL-12p70 are predictors of survival in these patients (261). Therefore, serum levels of cytokines are more correlated with patient survival than with the indexes of RV function; there was no correlation between cytokines and hemodynamic parameters, which may mean that cytokines are associated with the pathogenesis of PAH and not only with RV function (261).

The levels of IL-1β and IL-6 are clearly increased after exposure to hypoxia, and this increase is closely related to the activation of the immune system, responsible for inflammatory activity (263). However, the molecular mechanism involved is still not well-established and, therefore, several animal studies have been carried out in this direction. Savale et al. demonstrated that IL-6 appears to have a preponderant role in the modulation of inflammation, vascular remodeling, and the development of PH, presenting an exaggerated response to hypoxia. In studies with IL-6-deficient mice (IL-$6^{-/-}$), there was a clear attenuation of PH and RV hypertrophy; even when IL-$6^{-/-}$ mice were exposed to hypoxia, they presented lower recruitment of inflammatory cells in the lungs than wild-type mice (264). On the other hand, Hashimoto-Kataoka et al. identified IL-21 as a target downstream of IL-6 signaling in PAH, suggesting that the IL-6/IL-21 signaling axis is involved in the pathogenesis of PAH, together with an accumulation of M2 macrophages in the lungs. In addition, they also found an increase in IL-21 expression and M2 macrophage markers in lung samples from patients with IPAH (265). Regarding IL-1β, Parpaleix et al. suggest that binding of IL-1β to its receptor, IL-1 receptor1 (IL-1R1), promotes recruitment to the primary adapter myeloid differentiation protein of molecular adapter 88 (MyD88) and induces the synthesis of IL-1, IL-6, and TNF-α through NF-κB activation. This pathway appears to play a major role in the pathogenesis of PH, affecting the proliferation of VSMCs and the recruitment of macrophages. In addition, patients with IPAH and mice with hypoxia-induced PH have an increased expression of IL-1R1 and MyD88 in the pulmonary vessels. Moreover, MyD88-deficient mice (MyD$88^{-/-}$) showed attenuation in PH, which suggests effects mediated by IL-1β in PASMCs and macrophages. Therefore, the IL-1R1/MyD88 signaling axis is closely involved in the remodeling and inflammation of the pulmonary vessels (263).

Thus, we can conclude that, although cytokines do not stand out for the role of PAH biomarkers, it is possible to establish a relationship between some cytokines and the survival of patients with PAH. Additionally, Duncan et al. showed that there is an increase in levels of circulating cytokines and growth factors and their correlation with outcome in pediatric PAH (191). Some pathways could be potential targets of study in the treatment of PAH, namely, the pathways IL-6/IL-21 (265) and IL-1β/IL-1R1 (263).

Isoprostanes

Isoprostanes are formed when reactive oxygen species such as peroxide, superoxide and peroxynitrite react with unsaturated membrane lipids, such as arachidonic acid (92, 266) (molecules with a cyclopentane ring and two cis alkyl chains between them), isomers of prostanoids (with two trans chains between them) (267). Isoprostanes are essentially biomarkers of oxidative stress, however, they have several biological effects (267). F2-isoprostanes (F2-isoP) are one of the most stable biomarkers of lipid peroxidation when measured in urine (267, 268). 15-F2t-isoprostane (15-F2t-IsoP), or 8-iso-prostaglandin F2, in one of the most abundant isoprostane *in vivo* (268) and can be measured in urine and plasma (267). 15-F2t-IsoP has potent vasoconstrictor activity—it is involved in vasoconstriction of pulmonary arteries and resistance microvessels—and mitogenic activity in VSMCs (267). In addition, 15-F2t-IsoP stimulates ECs proliferation and ET-1 synthesis in pulmonary arteries (269).

In PH pathophysiology: studies suggest that F2-isoP may function as a marker in the early stages of PAH (270) as F2-isoP is increased in both urine (271, 272) and plasma (272) in different subgroups of PAH (including IPAH, hereditary PAH and asymptomatic patients predisposed to PAH because of *BMPR2* loss-of-function mutations) (273). Increased plasma levels of 15-F2t-IsoP in patients with IPAH correlate with WHO functional class severity, lower 6MWD and SvO_2, and higher mRAP and BNP levels (272). Additionally, studies demonstrated that increased levels of urinary F2-isoP are inversely correlated with pulmonary vasoreactivity (274) and that urine 15-F2-isoP levels, when quantified during initial diagnosis, were independently associated with an increased hazard of death in a cohort of patients with PAH (268). These results suggest that urinary F2-isoP may represent a biomarker with prognostic potential in PAH.

Oxidized Lipids

The role of oxidized fatty acids and oxidized phospholipids in atherosclerosis and other inflammatory diseases is well-established (275, 276). Biological metabolites of arachidonic and linoleic acid, hydroxyeicosatetraenoic acids (HETEs), and hydroxyoctadecadienoic acids (HODEs), respectively, play a critical role in the pathogenesis of atherosclerosis (275, 276).

In PH pathophysiology: concentrations of oxidized fatty acids- 5-, 12-, and 15-HETE, and 9- and 13-HODE - are increased in the plasma and in lung tissues of patients with PAH (277–279) and in several animal models of PH (280, 281). Increased 15-HETE levels in the context of PH have been shown to induce VSMCs pro-proliferative/anti apoptotic phenotype (282), inflammation (277), and fibrosis (283). Recently, Ruffenach et al. (284) demonstrated that dietary 15-HETE is sufficient to induce PH in mice. In this study, unbiased large-scale transcriptomics identified key pathways that are dysregulated by dietary 15-HETE and further confirmed the dysregulation of similar pathways in patients with PAH. They established that increased ECs apoptosis by 15-HETE via a T cell–dependent mechanism is one of the mechanisms triggering PH in mice. They also demonstrated that the apolipoprotein A-I mimetic peptide Tg6F (transgenic 6F), which has previously been shown to reduce plasma oxidized lipids and atherosclerosis (285, 286), can prevent and rescue PH development in mice.

CD40/CD49L

CD40 is a type I transmembrane receptor belonging to the TNF superfamily of receptors. Its ligand (CD40L) and its soluble form (sCD40L) have immunomodulating activity and likewise belong to the TNF superfamily (287). CD40 is expressed primarily on B cells, but also on other cells of the immune system (macrophages, basophils, T cells), on epithelial cells, fibroblasts, ECs, VSMCs, platelets and dendritic cells (287). Thus, the interaction of CD40 with CD40L, in its soluble and/or transmembrane form, induces the activation of inflammatory and coagulatory pathways of the vascular endothelium and promotes the activation of immune and non-immune cells (288), triggering an inflammatory response, degradation of the matrix and formation of thrombi (289).

In PH pathophysiology: Damås et al. demonstrated that patients with PAH have increased levels of sCD40L. They also suggested that the CD40-CD40L interaction contributes to the increased expression of chemokines in patients with PAH: recombinant sCD40L induced the production of IL-8 and MCP-1 in ECs, and plasma levels of these chemokines increased in PAH patients, significantly correlating with sCD40L and hemodynamic parameters. Furthermore, platelets from patients with PAH showed greater release of sCD40L compared to the control group (289). Another study suggests that endothelial progenitor cell transplantation, a novel and experimental therapeutic option for PAH, could yield higher efficacy when turning off the CD40 pathway is turned off in the transplanted cells may have advantages in therapies used to treat PAH, namely endothelial progenitor cell transplantation (288).

METABOLIC BIOMARKERS

Tryptophan Metabolites

Tryptophane is an essential amino acid that can be metabolized through two different enzymatic pathways. The tryptophane hydroxylase pathway results in the conversion of tryptophane to serotonin, whereas the indoleamine 2, 3-dioxygenase (IDO) pathway can produce various tryptophan metabolites (TMs), such as kynurenine, kynurenate, anthranilate and quinolinate (290, 291).

In PH pathophysiology: a recent study conducted on rats with monocrotaline induced PAH found that TMs pathways, among others, are dysregulated in PAH, due to probable metabolic reprograming events in disease pathogenesis (292). IDO-TMs, and not tryptophane hydroxylase metabolites, were elevated in PAH and strongly correlated with RV-pulmonary vasculature dysfunction (resting RAP, PAP, PVR, exercise PVR and change in cardiac output during exercise) in a cohort of patients with PAH (291). Moreover, the predominant IDO-TM, kynurenine, has been more thoroughly investigated and, aside from being significantly higher in PAH patients (293–295), it correlated with mPAP and PVR (293) and could predict negative patient outcomes (294).

Ghrelin

Ghrelin is traditionally viewed as a "hunger hormone," produced in the gastrointestinal tract. It acts as an endogenous ligand for growth hormone secretagogue receptors and can regulate energy metabolism, glucose metabolism, gastrointestinal motility, and food intake. However, it is also involved in modulating cardiovascular function, playing a role in some cardiovascular diseases (296) and has been shown to be a physiological antagonist of ET-1 mediated vasoconstriction *in vitro* (297). In the serum, ghrelin can be found as acyl-ghrelin or des-acyl ghrelin, with the latter constituting the larger but inactive fraction (298).

In PH pathophysiology: in IPAH, total ghrelin plasma levels were elevated and predicted RV hemodynamics (RV diameter and pulmonary artery systolic pressure) (299). As for CHD-PAH, acyl-ghrelin was raised, and correlated, additionally, with pulmonary artery diastolic pressure, RV systolic pressure, mPAP, and pulmonary artery trunk diameter (300). Lastly, ghrelin levels correlated with N-BNP (299), ET-1 and NO measures (299, 300). In atrial septal defect patients with PAH, ghrelin is increased and correlates negatively with mPAP, which can suggest that ghrelin levels can predict the severity of PH in patients with atrial septal defect and PAH (301).

Furthermore, studies in animal models of PH showed that ghrelin can modulate PH: in chronically hypoxic rats, exogenous administration of ghrelin attenuated the development of PH, pulmonary vascular remodeling, RV hypertrophy, and overexpression of eNOs and ET-1 (302); also, in rats with PH induced by monocrotaline, exogenous administration of ghrelin attenuated PH, RV hypertrophy, wall thickening of peripheral pulmonary arteries, and RV diastolic disturbances and ameliorated left ventricle dysfunction, without affecting its endogenous production (303). This highlights a potential role for ghrelin a new therapeutic weapon against PAH.

TRANSCRIPTIONAL REGULATORS AND ONCOGENES EXPRESSION

PIM-1

PIM-1 (Moloney Murine Leukemia Provirus Integration Site) is a proto-oncogene that encodes a serine/threonine kinase (304, 305). Its expression is induced by a variety of cytokines, growth factors, and mitogens (304). PIM-1 is closely related to cell cycle regulation, being involved in cell proliferation and survival (304), and overexpression of PIM-1 is related to the development and progression of various cancers, by increasing cell proliferation and resistance to apoptosis (306).

In PH pathophysiology: PIM-1 has been associated with the development of PAH, becoming a potential PAH biomarker. Katakami et al. demonstrated that PIM-1 is involved in VSMCs proliferation and neointima formation when associated with arterial wall lesions; that is, lesions in the arterial wall promote the release of mitogens and the induction of proto-oncogenes, which, in turn, will promote the proliferation of VSMCs and neointimal formation. Furthermore, they suggest that PIM-1 expression in cultured VSMCs is markedly induced by oxidative stress (304). Although the mechanism of action of PIM-1 in VSMCs proliferation is not totally understood, it is known that PIM-1 phosphorylates and activates Cdc25A, a phosphatase that

promotes cell cycle progression, and c-Myb, a transcription factor that is essential for VSMCs replication (304). In addition, it cooperates with c-Myc, which plays an important role in VSMCs proliferation (304, 305). Moreover, studies demonstrated that PIM-1 contributes to the activation of the nuclear factor of activated T cells (NFAT)/signal transducers and activators of transcription-3 (STAT3) signaling pathway (306), which is a pathway highly involved in pro-proliferative and anti-apoptotic phenotype of VSMCs in PAH (307, 308). Paulin et al. showed that PIM-1 is increased in PAH, both in experimental studies and in patients with PAH, and that it is dependent on STAT3 activation. In addition, PIM-1 expression parallels NFAT activation and pulmonary artery remodeling and pressure and correlates with disease severity, suggesting that NFAT activation via PIM-1 is specific to pulmonary vascular remodeling (306). STAT3 and NFAT are expressed in several tissues, including the pulmonary artery, contrary to PIM-1, which has a lower expression in healthy tissues, and it is essentially expressed in PAH-PASMCs (306). Renard et al. studied the levels of PIM-1 in patients with PAH (IPAH, CTD-PAH, vasoreactive-IPAH and CHD-PAH) and verified that the levels of PIM-1 in plasma are increased in patients with IPAH and CTD-PAH, patients essentially characterized by active pulmonary vascular remodeling. On the other hand, patients with vasoreactive-IPAH and CHD-PAH had normal plasma levels of PIM-1, suggesting that vascular remodeling via the STAT-3/NFAT/PIM-1 pathway is limited in some PAH phenotypes. In addition, it was found that among PAH patients, PIM-1 levels correlated with traditional markers of disease severity and predicted mortality (309), which makes PIM-1 a potential biomarker for PAH, essentially as a representative biomarker of pulmonary vascular remodeling.

DISCUSSION

In recent years, there have been clear advances in understanding the pathophysiology of PAH, with more and more therapeutic options available to combat this disease. However, in the absence of an exact cure, the combination of medication to obtain the best possible result is still complex and constantly evolving. Most patients with PAH are of advanced age and, therefore, have different associated comorbidities (8). Thus, interest arises in studying and discovering non-invasive biomarkers to use as a means of screening and diagnosing, to understand the severity and prognosis of the disease, and to monitor the response to therapies (12). Although there are already many known biomarkers, the truth is that an ideal one has not yet been found. An ideal biomarker must be fast, inexpensive, non-invasive, easy to measure, reproducible, and should apply to all classes of disease. Furthermore, it should not have confounding factors, that is, it should not vary with factors such as comorbidities, age, gender, race, etc. Thus, it is difficult to find an ideal biomarker, however, it is possible that a specific set of biomarkers can respond and provide all relevant information about the patient/disease.

The biomarkers addressed in this review article are non-invasive and easy to detect and therefore are of low cost and easy access. All biomarkers investigated have been found to be significantly altered in PAH patients of various subtypes when compared with healthy controls (**Supplementary Table 1**)—NO and VEGFR-2 appear lower in patient blood when compared to healthy controls, while all other markers are elevated. Moreover, almost all were found to be good indicators of disease severity and/or prognosis (excluding homocysteine, serotonin, MCP-1, oxidized lipids and CD40/CD49L). However, six biomarkers stand out as more informative and potentially more useful, as they were able to show similar predictive value to tools that are currently used in clinical practice, or, in some cases, were able to outperform these tools—these were RDW, osteopontin, angiopoietin-2, endoglin, endostatin and GDF-15 (findings listed in **Table 2**). Therefore, we consider these specific biomarkers to be the current best candidates for introducing into clinical practice in the near future.

TABLE 2 | Major findings regarding novel biomarker performance against validated clinical tools.

Findings	Biomarkers
Incremental predictive value to REVEAL risk scale	Endostatin (221)
Incremental predictive value to ESC/ERS criteria	Endostatin (221)
Incremental predictive value to NT-proBNP	Red cell distribution width (68), Osteopontin (148), Endoglin (222), Growth differentiation factor-15 (234)
Outperform NT-proBNP	Osteopontin (148), Angiopoietin-2 (180)

However, when interpreting new scientifically evidence, one must be cautious. Most evidence is still new and limited due to the difficulty in forming large patient cohorts, which limits the statistical power of new findings. Moreover, as previously mentioned, PAH pathophysiology is complex, and the role of each biomarker in disease development is yet to be determined. Finally, there is a clear lack of comparative evidence between the novel markers and measures that have been previously validated for patient evaluation, therefore these markers cannot be safely recommended alongside or in replacement of those validated tools; and we were not able to find any research work regarding a multibiomarker approach.

CONCLUSION

Although no biomarker has yet been found to be ideal, there are undoubtedly some that stand out for being more informative and that could be part of the set of standard biomarkers to be studied in patients with PAH. Research into PAH biomarkers is an emerging and highly promising scientific field with the potential to revolutionize PAH patient medical care.

AUTHOR CONTRIBUTIONS

CB-S, JS-G, and IG: conceptualization. JS-G and IG: writing—original draft preparation and visualization. JS-G, IG, RA, FP, and CB-S: writing—review and editing. CB-S and RA: supervision.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2022.924873/full#supplementary-material

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Selexipag-based triple combination therapy improves prognosis in Chinese pulmonary arterial hypertension patients

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Aim: Selexipag is an oral selective prostacyclin receptor agonist approved for treatment of patients with pulmonary arterial hypertension (PAH). In the present study, we aim to assess the safety and efficacy of selexipag in triple combination therapy with endothelial receptor antagonists (ERAs) and PDE5is for Chinese PAH patients.

Methods and results: A single center retrospective study was performed on group 1 PAH patients (n = 68) initiating triple combination therapy with selexipag from 1 February 2020 to 31 August 2021 in Qilu Hospital of Shandong University (Shandong, China). Adolescents, children, and PAH patients with unrepaired congenital heart disease were excluded. The French pulmonary hypertension network (FPHN) non-invasive risk assessment, echocardiogram parameters, and clinical data, including tolerability, safety, and death/hospitalization events associated with PAH, were collected. Of the 68 patients, 31 (45.6%) patients had tolerable side effects while only a single patient discontinued selexipag due to severe diarrhea. In the analysis of the efficacy set of 62 patients, the median selexipag treatment time from selexipag initiation to last risk assessment was 27 (21, 33) weeks. Compared to baseline parameters, the percentage of WHO FC III/IV decreased from 77.4% (48) to 24.2% (15) (p = 0.000), median 6-min walk distance (6MWD) increased 82 m [from 398 (318, 450) to 480 (420, 506) m; p = 0.000], and NT-proBNP levels decreased from 1,216 (329, 2,159) to 455 (134, 1,678) pg/mL (p = 0.007). Patients who improved to three low-risk criteria increased from 9.7 to 38.7%. Right ventricular diameter (RV) diameter also decreased and was accompanied by an improved tricuspid annular plane systolic excursion (TAPSE). Patients transitioning from subcutaneous treprostinil to selexipag continued to show improvements in WHO FC, 6MWD (404 ± 94 vs. 383 ± 127 m) and NT-proBNP levels (2,319 ± 2,448 vs. 2,987 ± 3,770 pg/mL). Finally, the 1-year event free survival rate was 96.7% for patients initiating the triple combination therapy within 3 years of PAH diagnosis.

Conclusion: Triple combination therapy with selexipag was safe and effective in Chinese PAH patients, which was confirmed by acceptable tolerability, and improved exercise capacity, right heart function, risk assessment, and prognosis.

KEYWORDS

pulmonary arterial hypertension, triple combination, selexipag, tolerability, risk assessment

Introduction

Pulmonary arterial hypertension (PAH) is a devastating disease characterized by progressive pulmonary artery remodeling and right heart failure with high mortality (1). In the past decades, PAH-specific therapy has greatly improved the survival rate of patients with this disease (2, 3). The combination of drugs targeting endothelin-1, nitric oxide, and prostacyclin (IP) have further improved patient response (4, 5). To date, the rationale for PAH treatment strategy is based on disease severity assessed with multi-parametric risk stratification approaches to help PAH patients achieve and maintain a low-risk status (6, 7).

Selexipag, an orally available selective IP receptor agonist, is an approved therapy for PAH (5). In the GRIPHON trial, patients receiving selexipag either as monotherapy or in addition to endothelial receptor antagonists (ERAs) and/or phosphodiesterase type 5 inhibitors (PDE5is) showed a 40% risk reduction in clinical worsening events (5). As a randomized controlled trial, the GRIPHON trial established the efficacy and safety of selexipag, but real-world evidence has also been important in providing complementary data for routine clinical practice. For instance, in GRIPHON trial, 14.3% patients discontinued selexipag treatment due to adverse event, most frequently reported was headache. However, in the SPHERE Registry from the United States the most reported side effect was gastrointestinal disorders, the discontinuation rate due to adverse event related to selexipag was 7.2% and the titration of selexipag is more complicated and proceeds more slowly in general practice (8). Another real-world study carried out in Germany intriguingly indicated that patients with side effects during titration responded better to selexipag treatment (9).

To date, data are limited for the treatment of Asian PAH patients with selexipag. Two hundred and twenty-three Asian patients were included in the GRIPHON trial with half of them treated with selexipag and the results did not show any benefit for this subgroup (5). Although the JAPIC trial carried out in Japan demonstrated that selexipag is effective in Japanese PAH patients (10), efficacy in Chinese PAH patients has not yet been rigorously evaluated.

Here, we performed a retrospective study in Chinese PAH patients treated with selexipag to investigate the efficacy and safety of the drug in general practice.

Materials and methods

Study design and patient enrollment

This single center, retrospective uncontrolled study was carried out in Qilu Hospital of Shandong University in northern China. Group 1 PAH patients diagnosed by right heart catheterization, including idiopathic PAH (IPAH), heritable PAH (HPAH), post-operative PAH associated with congenital heart disease (post-operative CHD-PAH), PAH associated with connective tissue diseases (CTD-PAH), and PAH associated with HIV infection were screened. Those who has Eisenmenger syndrome, met all three low-risk criteria with stable target therapy treatment or unwilling to take selexipag treatment were excluded from selexipag initiation. Those who began triple treatment which included selexipag, ERAs and PDE5is from 1 February 2020 to 31 August 2021, were recruited for the study. For sequential addition of selexipag to ERA and PDE5i, only patients who had already received at least 3 months stable treatment with ERA and PDE5i were included. Adolescents, children and patients with PAH related to uncorrected congenital heart disease (having treat-to-repair therapy) who took selexipag were excluded from the current analysis. The flow chart of enrollment was shown in **Figure 1**. All patients were recommended to undergo clinical evaluation every 3 months. The cutoff date for follow-up data collection was 31 December 2021. For the analysis, the safety set included all patients (n = 68) taking at least one dose of selexipag, while the efficacy set included patients (n = 62) taking continuous selexipag treatment over 12 weeks and undergoing at least one risk assessment during follow-up. The study was approved by the Institutional Human Ethics Committee of Qilu Hospital (reference number: 2021072) and written informed consent was exempted.

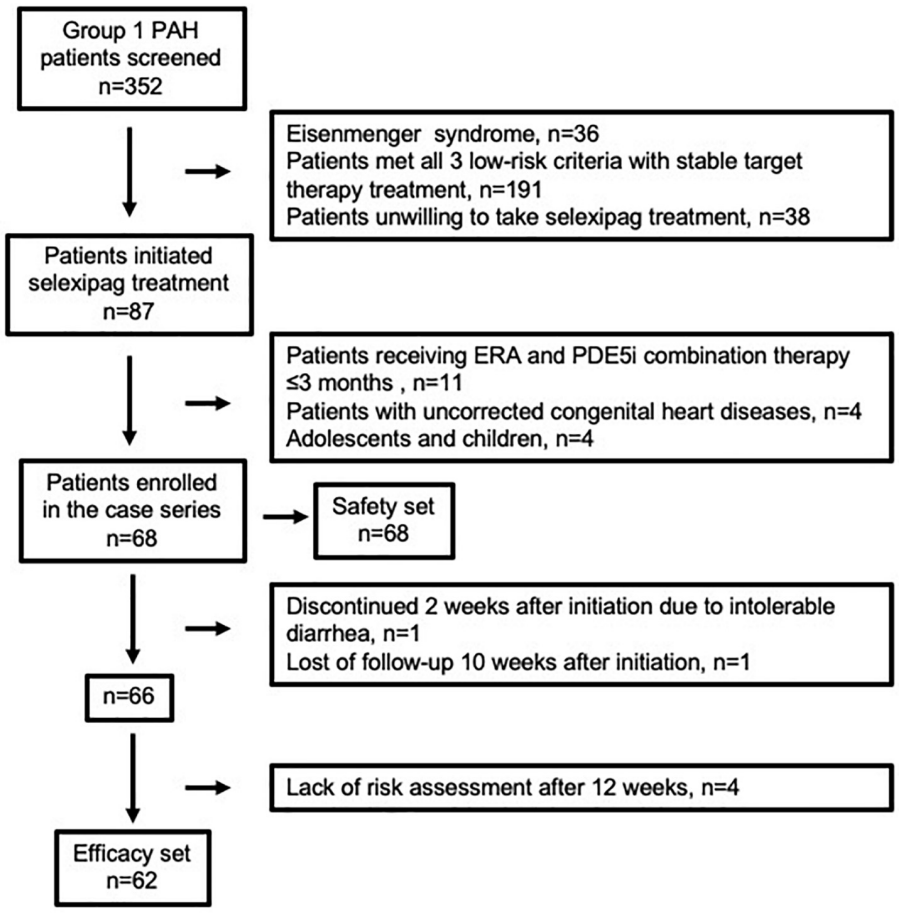

FIGURE 1
Enrollment flow chart for safety and efficacy set in the present case series.

Data collection

Non-invasive parameters collected at baseline and follow-up included clinical characteristics, disease characteristics, concurrent/previous PAH therapy within the last 3 months, selexipag titration, transition between selexipag and parenteral prostacyclin, WHO functional class (FC), 6-min walk distance (6MWD), right atrial area (RAA), right ventricular diameter (RV), tricuspid annular plane systolic excursion (TAPSE), hemoglobulin (HGB), and total bilirubin (TB). The clinical data obtained at the last clinical visit on or before the initiation of selexipag treatment were defined as baseline. The WHO FC was determined by an experienced physician, and the same technician performed transthoracic echocardiography during follow-up visits.

Risk assessment and outcome measures

The French Pulmonary Hypertension Network (FPHN) non-invasive risk assessment strategy using three low-risk criteria is more convenient to use in clinical practice compared to other risk stratification strategies (11–13), and has shown accurate identification of patients with excellent long-term survival (14). In the present study, non-invasive risk assessment was performed using three parameters, including WHO FC, NT-proBNP levels, and 6MWD, as recently suggested (13). Low-risk criteria were defined as follows: (1) WHO FC I or II, (2) NT-proBNP <300 pg/mL, and (3) 6MWD >440 m. The number of low-risk criteria present at baseline and each clinical visit were recorded. For patients with multiple risk assessments before the cutoff date, only the last event was noted for efficacy analysis. Events including hospitalization, death and initiation of parenteral PGIs associated with PAH progression were observed until the cutoff date or until the withdrawal date for patients who discontinued selexipag. For patients with multiple events, only the first event was noted.

Selexipag dosage titration

Selexipag was initiated in patients at 200 μg twice daily and further up-titration was suggested when side effects subsided.

A weekly increase of 200 μg twice daily was recommended, but the 200 μg increment, daily or at longer intervals, was also allowed depending on the tolerance of patients to side effects. The maximum dose allowed was 1,600 μg twice daily. The maintenance dose was defined as the twice daily dose received for the longest duration. Dose levels were defined as follows: low-dose, 200–400 μg twice daily; medium-dose, 600–1,000 μg twice daily; and high-dose, 1,200–1,600 μ g twice daily.

Transition from subcutaneous treprostinil to oral selexipag

The treprostinil dose was down-titrated every 3 days by 2.5 ng/kg/min, and a standard weekly increase in selexipag of 200 μg twice daily was initiated on the same day of the week.

Selexipag-based initial triple combination

Macitentan 10 mg or ambrisentan 10 mg once daily, and tadalafil 20 mg once daily or sildenafil 25 mg three times daily were initiated on day 1. Selexipag dose titration began on day 8–15.

Statistical analysis

Analysis was performed with SPSS software, version 13.0 (SPSS Inc., Chicago, IL, USA). Categorical data are presented as counts or percentages. Normal distribution was evaluated using the Kolmogorov–Smirnov test. Continuous variables are presented as the mean with the standard deviation when distributed normally, or otherwise as the median with the interquartile range (IQR). Paired *t*-test, paired rank sum test or the chi-square test were used to compare the differences between baseline and follow-up values where appropriate. Significant differences were defined as $p < 0.05$ (two-tailed test).

Results

Baseline characteristics

Sixty-eight patients, 57 (83.8%) females and 11 males (16.2%), were included in the study. The mean age was 31.9 ± 9.5 years. The mean time from PAH diagnosis to initiation of treatment with selexipag was 4.8 ± 4.9 years. All patients had WHO group 1 pulmonary arterial hypertension. The most common type was IPAH (61.8%), followed by post-operative CHD-PAH (25.0%) and CTD-PAH (10.3%). Two patients were also diagnosed as HPAH (2.9%). Fifty patients (73.5%) were already receiving ERA and PDE5i combination treatment (for ≥3 months), 11 patients (16.2%) were on triple therapy including subcutaneous treprostinil, and seven patients (10.3%) were newly diagnosed and given triple therapy, including ERA, PDE5i, and selexipag, at diagnosis. The WHO FCs at baseline were mainly class III (60.3%) and class II (23.5%).

Efficacy

Sixty-two patients were included in the efficacy analysis. The baseline characteristics of the patients are shown in Table 1. The average follow-up visits per patient was 2.6 ± 1.6 times. The median treatment time from initiation to the last risk assessment was 27 (21, 33) weeks. Compared to baseline, the percentage of WHO FC III/IV decreased from 77.4% (48) to 24.2% (15) ($p = 0.000$; Figure 2A). The median 6MWD increased 82 m [from 398 (318, 450) to 480 (420, 506) m; $p = 0.000$], and NT-proBNP levels decreased from 1,216 (329, 2,159) pg/mL to 455 (134, 1,678) pg/mL ($p = 0.007$) (Table 2). Sixty-one patients underwent echocardiographic assessment at baseline and follow-up to assess the effects of selexipag on right heart size and function (Table 2). The RV diameter decreased in treated patients [38 (31, 47) vs. 35 (29, 45) mm; $p = 0.001$], and the RA area showed a decreasing trend from 25 (19, 34) to 21 (15, 33) cm^2 although this difference was not statistically significant ($p = 0.113$). TAPSE increased from 16 (14, 18) to 18 (15, 20) mm ($p = 0.002$). Finally, total serum bilirubin decreased with treatment ($p = 0.020$).

TABLE 1 Baseline characteristics of the case series.

	Total population *n* = 68	Efficacy set *n* = 62
	57 (83.8)	53 (85.5)
Age, mean (SD), years	31.9 (9.5)	31.8 (8.4)
Time from PAH diagnosis to selexipag initiation		
Mean (SD), years	4.8 (4.9)	4.8 (5.0)
PAH etiology, *n* (%)		
IPAH	42 (61.8)	40 (64.5)
Post-operative CHD-PAH	17 (25.0)	15 (24.2)
CTD-PAH	7 (10.3)	6 (9.7)
HPAH	2 (2.9)	1 (1.6)
Combination of selexipag, *n* (%)		
Third add-on to ERA and PDE5i combination	50 (73.5)	46 (74.2)
Transition from subcutaneous treprostinil to selexipag	11 (16.2)	10 (16.1)
Upfront triple combination in newly diagnosed PAH	7 (10.3)	6 (9.7)

Continuous data are expressed as the "mean (SD)." IPAH, idiopathic pulmonary arterial hypertension; CHD-PAH, PAH associated with congenital heart disease; CTD-PAH, PAH associated with connective tissue diseases; HPAH, heritable PAH.

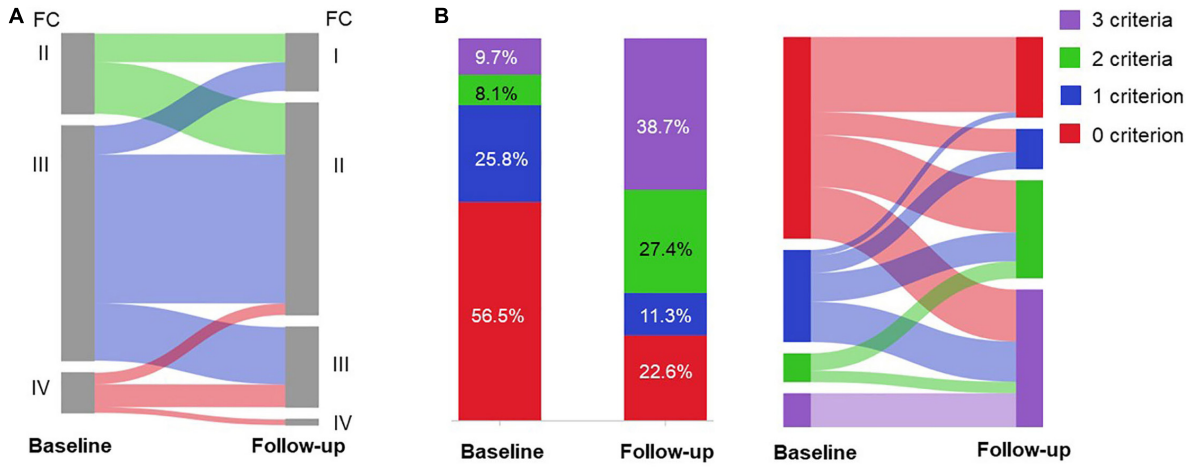

FIGURE 2
Risk assessment of the patients (n = 62) in the efficacy set at baseline and follow-up. Change in **(A)** WHO functional class (FC); and **(B)** the number of low risk criteria after selexipag treatment.

TABLE 2 Risk assessment parameters of the efficacy set at baseline and follow-up.

	Baseline	Follow-up	*P*
WHO FC, *n* (%)			<0.0001
I	0 (0.0)	10 (16.1)	
II	14 (22.6)	37 (59.7)	
III	41 (66.1)	14 (22.6)	
IV	7 (11.3)	1 (1.6)	
6MWD, median (IQR), m	398 (318, 450)	480 (420, 506)	<0.0001
NT-proBNP, median (IQR), pg/mL	1,216 (329, 2,159)	455 (134, 1,678)	0.007
RAA, median (IQR), $cm^{2\dagger}$	25 (19, 34)	21 (15, 33)	0.113
RV, median (IQR), mm†	38 (31, 47)	35 (29, 45)	0.001
TAPSE, median (IQR), mm†	16 (14, 18)	18 (15, 20)	0.002
TBIL, median (IQR), μmol/L†	14.1 (10.2, 24.2)	9.6 (11.6, 19.3)	0.020

Continuous data are expressed as the mean (SD) or if not normally distributed as the median (IQR) and compared using paired-*t* test or Wilcoxon matched-pairs signed rank test. Categorical data are compared using Fisher's exact test. *$P < 0.05$ between two groups. †Data missing for some subjects. WHO-FC, World Health Organization functional class; 6MWD, six-min walking distance; NT-proBNP, *N*-terminal pro B-type natriuretic peptide; RAA, right atrial area; RV, right ventricle diameter; TAPSE, tricuspid annular plane systolic excursion; TBIL, total bilirubin.

Based on FPHN non-invasive risk assessment, the percentage of patients with all three low-risk criteria increased from 6 (9.7%) to 24 (38.7%), while the proportion of patients with no low-risk criteria decreased from 35 (56.5%) to 14 (22.6%) ($p < 0.001$; Figure 2B). For those 35 patients with no low-risk criteria at baseline, 22 (62.9%) of patients reached at least one low-risk criterion and 25.7% improved to all three low-risk criteria.

Six patients deteriorated due to their PAH during the period from selexipag initiation to the cutoff date (a maximum of 92 weeks). The incidence rate of clinical worsening events was 15.9%/person-year. A single patient died due to PAH progression, while five patients were hospitalized due to declining right heart function related to PAH. One of the hospitalized patients continued selexipag treatment but switched to riociguat from tadalafil, two began to receive subcutaneous injection of treprostinil instead of selexipag, and two patients maintained their course of treatment. Time to clinical worsening was 39 ± 16 weeks and overall 1-year event-free survival was 90.2% (Supplementary Figure 1). Because the disease course correlates with severity, we further compared event-free survival in patients with PAH disease course of >3 years to ≤3 years. One-year event-free survival was 78.7 vs. 96.7%, respectively. Statistical analysis was not carried out due to the small sample size.

Tolerability, patient disposition, and safety

All 68 patients were included in the safety set. The maintenance dose for most patients (n = 40; 58.8%) was 600–1,000 μg selexipag twice daily. Seventeen (25.0%) patients received high dose selexipag, while 11 (16.2%) patients received low dose selexipag (Figure 3A). The median time for dose titration was 8 (5, 15) weeks. The side effects reported during the titration phase included headache, nausea/vomiting/diarrhea, myalgia, jaw pain, arthralgia, and extremity pain. All patients experienced at least one side effect during this period. Tolerable side effects occurred in 31 (45.6%) patients during the maintenance phase. The

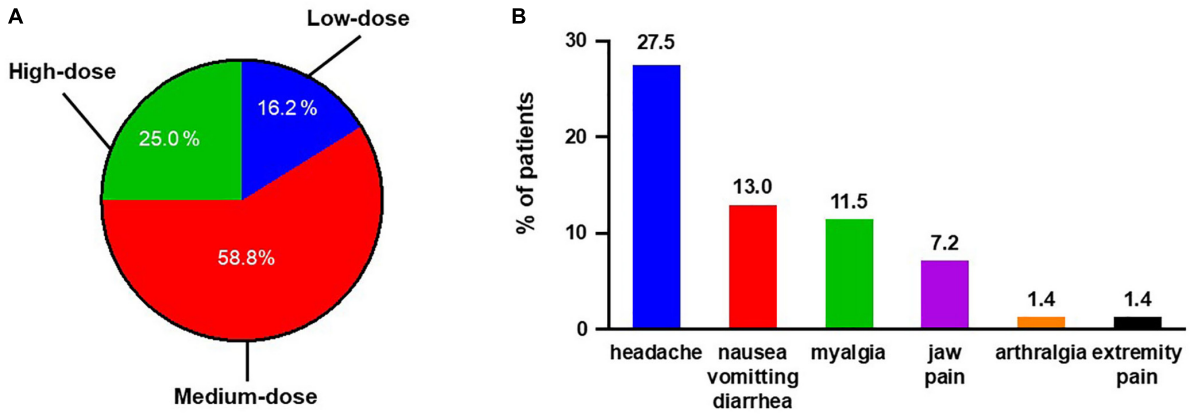

FIGURE 3
Summary of selexipag usage for all 68 Chinese PAH patients. **(A)** Maintenance dosage of selexipag in Chinese PAH patients: low-dose, 200–400 μg twice daily; medium-dose, 600–1,000 μg twice daily; and high-dose, 1,200–1,600 μg twice daily. **(B)** Side effects reported during maintenance period.

most commonly reported side effects were headache (27.9%), followed by nausea/vomiting/diarrhea (13.2%) and myalgia (11.8%) (Figure 3B). Hepatotoxicity or other unknown side effects were not reported.

Overall, eight patients (11.8%) discontinued selexipag before the cutoff date, and the median time for selexipag treatment was 43 (20, 50) weeks. Two patients transitioned to subcutaneous treprostinil due to unchanged risk status, one patient died due to right heart failure, and four patients were unwilling to continue treatment. Treatment in only one patient was terminated 2 weeks after initiation due to intolerable diarrhea and vomiting with 200 μg twice daily selexipag.

Subgroups of special interest

We also separately evaluated patients who transitioned from subcutaneous treprostinil to oral selexipag (n = 10). Treatment with treprostinil was terminated in two patients due to intolerable side effects, and in eight patients who were unwilling to continue due to economic burden. The average time to the last risk assessment was 29 ± 15 weeks. Six-minutes walking distance increased from 383 ± 127 to 404 ± 94 m (Figure 4A), and NT-proBNP levels decreased from 2,987 ± 3,770 to 2,319 ± 2,448 pg/mL (Figure 4B). At baseline, only one patient was diagnosed as WHO FC II, while the other nine patients were diagnosed as WHO FC III/IV. All patients showed improvement in the WHO functional class (Figure 4C). At baseline, six patients exhibited zero low-risk criteria, and four patients exhibited one low-risk criterion. After selexipag treatment, two patients reached all three low-risk criteria, two patients improved to meet more low-risk criteria, and four patients remained stable without PAH-associated hospitalization (Figure 4D). Two patients continued to deteriorate and were hospitalized due to right heart failure 9–39 weeks after transition, respectively.

We next focused on the characteristics of patients considered to respond to selexipag treatment. Patients with 0–1 low-risk criteria at baseline and improved in at least two low-risk criteria at follow-up were defined as selexipag responders. All other patients were considered as non-responders, except for patients with all three low-risk criteria at baseline, who were excluded from this analysis. Baseline characteristics between responders and non-responders are summarized in Table 3. Only descriptive analysis was carried out due to the limited sample size. Sex, age, baseline WHO FC, maintenance dosage of selexipag, and total treatment duration at follow-up were comparable between the two groups. Responders included more IPAH patients (81.5 vs. 51.7%), shorter PAH history, lower NT-proBNP levels [947 (334, 1,736) vs. 1,840 (957, 2,588) pg/mL] and smaller RAA [24 (19, 33) vs. 28 (22, 37) cm^2].

Discussion

The GRIPHON trial demonstrated that selexipag targeting the PGI pathway is an effective treatment for PAH. However, the results of the subgroup analysis based on geographic region showed no benefit of the drug for Asian patients (5). Only limited numbers of Chinese patients were included in the GRIPHON trial. Therefore, the efficacy and safety for selexipag in Chinese PAH patients remains unclear. In the present study, we demonstrated that selexipag effectively improves WHO FC, 6MWD, and NT-proBNP, and was accompanied with better risk assessment without unreported side effects. The results indicate that triple combination treatment including selexipag is effective and safe for Chinese PAH patients.

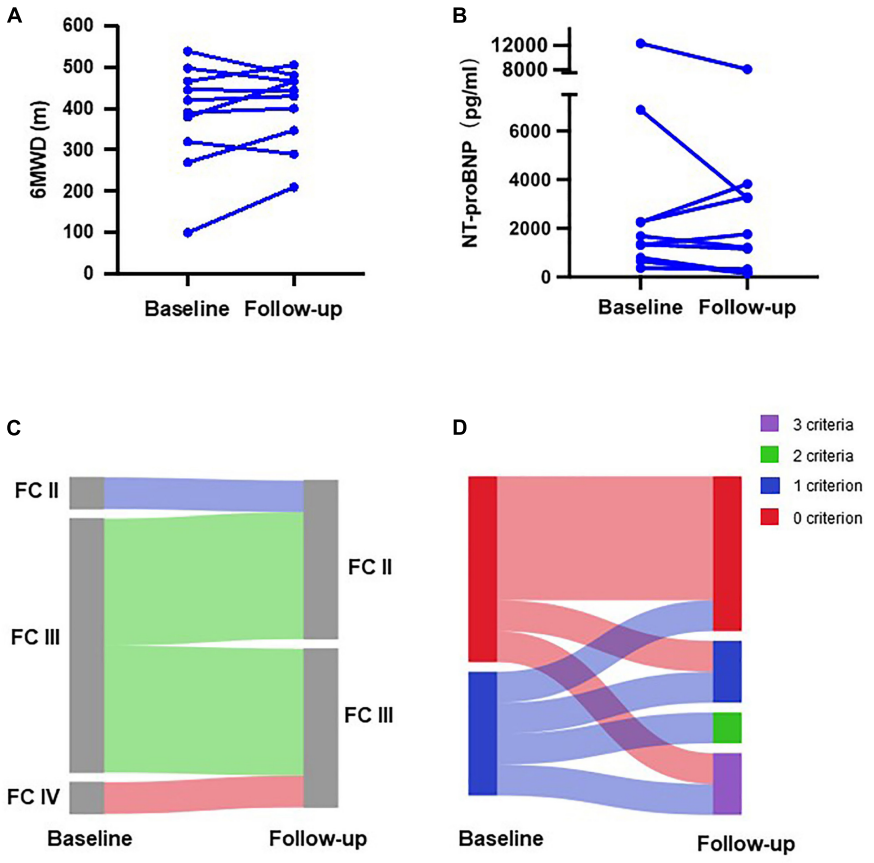

FIGURE 4
Changes in clinical parameters of patients transitioning from subcutaneous treprostinil to selexipag. Changes in **(A)** 6-min walking distance (6MWD); **(B)** *N*-terminal pro B-type natriuretic peptide (NT-proBNP) level; **(C)** WHO functional class (FC); and **(D)** number of low-risk criteria.

Nowadays, dozens of drugs specific for PAH treatment are commercially available. The treatment strategy for PAH patients, dual combination therapy containing ERA and PDE5i, which evolved in the past decade, is now widely accepted (15, 16). However, approximately 50% of patients treated initially with the combination therapy of ambrisentan and tadalafil for 2 years remain in a medium/high risk status. These results indicate that triple upfront combination therapy with drugs targeting the prostacyclin pathway may be necessary to achieve a more substantially improved prognosis in PAH patients (17). The GRIPHON trial demonstrated that the addition of selexipag in patients treated with ERA and PDE-5i further improved long-term outcomes (18). In the present study, 72.5% patients were already receiving a stable dose of ERAs and PDE5i, but we also noticed that sequential combination with selexipag improved the risk status. This finding is consistent with the results of the GRIPHON study.

Follow-up risk assessment following treatment has been shown to be more reliable in predicting patient survival than the initial risk assessment (6, 13). In the present study, 36 patients still met no low-risk criteria at baseline and 63.86% patients reached at least one low-risk criterion at follow-up. This rate is much higher than that in the COMPARA cohort (48%) and comparable to the French registry (72%) (14). Therefore, selexipag is effective for management of high-risk Chinese PAH patients.

Although parenteral prostacyclin analogs (PGIs) are the suggested treatment for high-risk PAH, there are several limitations for long-term utilization of PGIs, including not only the economic burden, but also the inconvenience of medication and systemic adverse effects (19).

Treprostinil was previously the only commercially available PGI in China, but the drug is not covered by insurance. In this case, patients are often unwilling to continue treatment with treprostinil, predominantly due to economic burden, even though they remain in a high-risk status. In the present study, most patients who transitioned from treprostinil to selexipag continued to improve or at least remained stable without further deterioration in their disease status. Only one patient continued to decline, which treatment with treprostinil did not prevent. Therefore, for high-risk patients who are unable to afford or unwilling to continue parenteral PGIs,

TABLE 3 Baseline characteristics of selexipag responder and non-responder.

	Non-responder $n = 29$	Responder $n = 27$
Female, *n* (%)	24 (82.8)	24 (88.9)
Age, mean (SD), years	32.5 (8.2)	31.3 (9.3)
PAH diagnosis to selexipag initiation ≤6 months, n (%)	3 (10.3)	7 (25.9)
PAH etiology, *n* (%)		
IPAH	15 (51.7)	22 (81.5)
CHD-PAH	11 (37.9)	1 (3.7)
CTD-PAH	2 (6.9)	4 (14.8)
HPAH	1 (3.4)	0 (0.0)
Selexipag treatment		
Medium/high-dose	24 (82.8)	22 (81.5)
Duration, median (IQR), weeks	27 (20.5, 34.5)	25 (20, 32)
Upfront triple combination	0 (0)	6 (22.2)
WHO FC III/IV, *n* (%)	24 (82.7)	24 (88.9)
NT-proBNP, median (IQR), pg/mL	1,840 (957, 2,588)	947 (334, 1,736)
RAA, median (IQR), cm^2	28 (22, 37)†	24 (19, 33)
RV, mean (SD), mm	41 (9)†	41 (9)
TAPSE, mean (SD), mm	16 (2.7)†	17 (3.2)

Continuous data are presented as the "mean (SD) or median (IQR)." †Data missing for one patient. WHO-FC, World Health Organization functional class; NT-proBNP, *N*-terminal pro B-type natriuretic peptide; RAA, right atrial area; RV, right ventricle diameter; TAPSE, tricuspid annular plane systolic excursion.

selexipag could be a substitute therapy. However, although several case series demonstrated successful transition from parenteral prostacyclins to selexipag after achieving a low-risk status in PAH patients, some cases exhibited a trend of decline in hemodynamic parameters with relatively stable clinical evaluation, especially in those who respond well to parenteral prostacyclins (20–23). As such, we have to pay close attention to this special transition group during routine clinical visit and hemodynamic monitoring is necessary.

In the present study, we also noticed that improvement in risk assessment and right heart remodeling do not occur in parallel. Although right ventricular diameter and TAPSE improved significantly, the trend in decreasing RA area did not reach statistical significance. Several other studies have also demonstrated that changes in right heart structure and function lagged behind NT-proBNP levels and exercise ability (24, 25). A recently published meta-analysis indicates that the improvement in RV systolic function appeared as long as 6 months and in the right atrial area, 12 months, after initiation of targeted therapy in PAH patients (26).

Post-hoc subgroup analysis of data from the GRIPHON trial showed that patients with WHO FC II or III symptoms at baseline similarly benefit from sequential combination of selexipag to background combination therapy with an ERA and PDE-5i (27). Although we only performed descriptive analysis, we also noticed that the WHO FC status between selexipag responders and non-responders was comparable. Recently published retrospective data from French Pulmonary Hypertension Registry illustrate that initiating triple-combination therapy at diagnosis seems to be associated with a higher survival rate in PAH (28). Exploratory analysis of the TRITON study also revealed a trend toward long-term outcome improvement in the initial triple combination treatment group (29). In the present study, patients with shorter PAH history, initial triple combination treatment and relatively minor disease severity, characterized by lower NT-proBNP levels and smaller RAA (30), tended to respond better to selexipag. It is also intriguing that IPAH is the predominant etiology in the responder group. We therefore propose that IPAH patients tend to have a better outcome than post-operative CHD-PAH patients with the same treatment in China.

There are several limitations to our study. First, this is an observational study with limited subjects from a single center, and the follow-up duration was not long enough. Second, invasive homodynamic parameters were not included for analysis. Although hemodynamic risk assessment criteria are independent predictors of transplant-free survival in PAH patients, repetitive RHC procedure is invasive and results in extra economic burden since it is only partially covered by insurance in China. In case that non-invasive risk assessment was already proved useful in identifying patients at low risk (13) and all patients have had at least one RHC before enrollment, only non-invasive parameters were used in the current case series.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by the Institutional Human Ethics Committee of Qilu Hospital. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

XC and QJ conceived and drafted the manuscript. WL, DZ, XL, and HuL performed the data collection and interpretation. LQ and HaL validated the data and formal analysis. QJ approved the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2022.991586/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Event-free survival in selexipag-treated Chinese PAH patients. Kaplan–Meier curve for time from selexipag initiation to first hospitalization or death event associated with PAH up to the cutoff date of 31 December 2021. Event-free survival of patients in the efficacy set. Patients with PAH disease course >3 years and disease course ≤3 years were calculated separately.

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The thromboxane receptor antagonist *NTP42* promotes beneficial adaptation and preserves cardiac function in experimental models of right heart overload

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Background: Pulmonary arterial hypertension (PAH) is a progressive disease characterized by increased pulmonary artery pressure leading to right ventricular (RV) failure. While current PAH therapies improve patient outlook, they show limited benefit in attenuating RV dysfunction. Recent investigations demonstrated that the thromboxane (TX) A_2 receptor (TP) antagonist *NTP42* attenuates experimental PAH across key hemodynamic parameters in the lungs and heart. This study aimed to validate the efficacy of *NTP42:KVA4*, a novel oral formulation of *NTP42* in clinical development, in preclinical models of PAH while also, critically, investigating its direct effects on RV dysfunction.

Methods: The effects of *NTP42:KVA4* were evaluated in the monocrotaline (MCT) and pulmonary artery banding (PAB) models of PAH and RV dysfunction, respectively, and when compared with leading standard-of-care (SOC) PAH drugs. In addition, the expression of the TP, the target for *NTP42*, was investigated in cardiac tissue from several other related disease models, and from subjects with PAH and dilated cardiomyopathy (DCM).

Results: In the MCT-PAH model, *NTP42:KVA4* alleviated disease-induced changes in cardiopulmonary hemodynamics, pulmonary vascular remodeling, inflammation, and fibrosis, to a similar or greater extent than the PAH SOCs tested. In the PAB model, *NTP42:KVA4* improved RV geometries and contractility, normalized RV stiffness, and significantly increased RV ejection fraction. In both models, *NTP42:KVA4* promoted beneficial RV adaptation, decreasing cellular hypertrophy, and increasing vascularization. Notably, elevated expression of the TP target was observed both in RV tissue from these and related disease models, and in clinical RV specimens of PAH and DCM.

Conclusion: This study shows that, through antagonism of TP signaling, *NTP42:KVA4* attenuates experimental PAH pathophysiology, not only alleviating pulmonary pathologies but also reducing RV remodeling, promoting beneficial hypertrophy, and improving cardiac function. The findings suggest a direct cardioprotective effect for *NTP42:KVA4*, and its potential to be a disease-modifying therapy in PAH and other cardiac conditions.

KEYWORDS

pulmonary arterial hypertension (PAH), thromboxane receptor, NTP42, right ventricle (RV), heart failure

Introduction

Pulmonary arterial hypertension (PAH) is a rare yet devastating disease with progressively debilitating symptoms and high mortality. The underlying etiology of PAH is characterized by excessive vasoconstriction and remodeling of the pulmonary vasculature leading to increased pulmonary vascular resistance (PVR). However, the ultimate determinant of survival in PAH patients is the response of the right ventricle (RV) (1, 2).

Upon elevated afterload due to increased PVR, the RV in PAH patients initially responds through compensatory mechanisms termed adaptive hypertrophy. These physiological responses aim to preserve systolic and diastolic right heart function and are characterized by an increased RV wall thickness facilitated by hypertrophic remodeling, increased angiogenesis, altered sarcomere organization and increased intrinsic cardiomyocyte contractility (1, 3, 4). However, in most PAH patients, these adaptive mechanisms are either insufficient or become exhausted, and RV hypertrophy ultimately transitions to pathological maladaptive mechanisms. Maladaptive remodeling of the RV is characterized by a transition to a more eccentric pattern of hypertrophy and a progressive RV dilation resulting in a leftward septal shift impacting left ventricular (LV) function (5, 6). In addition, the consequent elevation in RV wall tension results in an increased metabolic demand and a simultaneous decrease in myocardial perfusion capacity, leading to decreased RV contractility despite progressive increases in afterload (7). Furthermore, RV diastolic function is compromised through increased stiffness primarily due to cardiomyocyte hypertrophy and fibrosis. In PAH patients, all these factors contribute to progressive right heart dysfunction, ultimately resulting in heart failure.

While considerable advances have been made in the clinical management of PAH, patient mortality remains high. Current PAH standard-of-care (SOC) therapies include the phosphodiesterase type-5 inhibitors (PDE5is), endothelin receptor antagonists (ERAs), prostacyclin analogs (PCAs) or prostacyclin receptor agonists (PRAs) and soluble guanylate cyclase (sGC) stimulators, with various other pipeline compounds in clinical development. The key focus of the SOCs and those in clinical development is to reduce PVR,

either by alleviating excessive pulmonary vasoconstriction or enhancing pulmonary vasodilation, and/or reduce pulmonary vascular remodeling. However, while it is recognized that RV function is the main determinant of prognosis in PAH, current SOC therapies have limited cardiac-specific effects (8, 9). Furthermore, in PAH there is a paradox where RV function continues to deteriorate and have poorer survival outcomes despite reductions in PVR observed using current PAH SOC therapies (10). Consequently, there has been a shift in the clinical thinking from solely considering the effects of PAH therapies on PVR to instead investigating their potential for directly addressing the effects on the RV.

The thromboxane (TX) A_2 receptor, or TP, primarily mediates signaling of the prostanoid TXA_2 and of the free-radical derived isoprostane 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$), as well as other endogenous ligands, regulating processes including platelet aggregation, and constriction and proliferation of vascular and pulmonary smooth muscle. TP-mediated signaling also mediates potent *pro*-inflammatory, *pro*-mitogenic, and *pro*-fibrotic effects, and levels of TXA_2, 8-iso-$PGF_{2\alpha}$, and TP expression are elevated in many cardiovascular and pulmonary diseases, inflammatory disorders and in certain cancers (11, 12). Multiple studies have shown the importance of TP signaling in the development and progression of PAH (13–19). TXA_2 mimetics induce ventricular arrhythmia, and TP signaling contributes to cardiac hypertrophy and fibrosis in multiple animal models (19–26). TP expression has been demonstrated to be specifically elevated in certain pathological cardiac conditions, and both TP receptor occupancy and expression is elevated in the RV of PAH patients compared to non-diseased subjects (18, 19). In addition, the TXA_2/TP signaling axis contributes to cardiac hypertrophy in multiple animal models of systemic hypertension (24, 25). While activation of the TP is profibrotic in multiple systems, including within the heart, TP antagonism with CPI211 (Ifetroban) decreased RV fibrosis and improved cardiac function in a pulmonary artery banding (PAB) model of RV pressure overload (19). Furthermore, TP antagonism improved cardiac output, increased ejection fraction while decreasing cardiac fibrosis and transforming growth factor (TGF)−β signaling in mouse models of Duchenne muscular dystrophy (DMD) (26). Taken together, these studies suggest a broader pattern of deleterious consequences of TP activation on the heart.

The TP antagonist *NTP42* is currently in clinical development for PAH and other cardiopulmonary indications. Previous efficacy evaluations demonstrated that *NTP42* attenuates preclinical PAH in both the monocrotaline (MCT)- and Sugen/Hypoxia (SuHx)-induced animal models of PAH (27, 28). As a drug specifically developed as an oral formulation for clinical use, *NTP42:KVA4* was recently evaluated in a randomized, placebo-controlled first-in-human Phase I clinical trial (NCT04919863) in 79 healthy male volunteers where it was confirmed as safe, well-tolerated, with good pharmacokinetic and pharmacodynamic profiles following single and repeat oral dosing. To specifically assess its potential to impart direct cardioprotective effects on the RV, the aim of this study was to validate the efficacy of *NTP42*, delivered as the orally formulated *NTP42:KVA4*, in the MCT-PAH model while also exploring its effect in the pulmonary artery banding (PAB) preclinical model of RV dysfunction and pressure overload. Furthermore, in this study, we also examined expression levels of the TP, the target receptor for *NTP42*, in RV tissues from several highly relevant disease models as well as in clinical specimens from subjects with PAH and dilated cardiomyopathy (DCM), where the data further supports the hypothesis that the TP is a *bona fide* target for treatment of PAH and certain other cardiac dysfunctions.

Materials and methods

Animal models

All experiments were carried out in accordance with US NIH guidelines. Male Sprague-Dawley rats (Charles River Laboratories) were used in all models. MCT-PAH was induced using a single injection of 60 mg/kg MCT, where twice-daily oral treatment with placebo, *NTP42:KVA4* (1 mg/kg), the PDE5i Sildenafil (50 mg/kg), the ERA Macitentan (30 mg/kg), the PRA Selexipag (1 mg/kg), or the sGC stimulator Riociguat (5 mg/kg) was commenced on Day 7 post-MCT and continued to Day 28 (**Supplementary material**). The PAB model used surgical banding of the pulmonary artery, where twice-daily oral treatment with placebo, *NTP42:KVA4* (1 mg/kg), or the sGC stimulator Riociguat (5 mg/kg) was started on Day 2 post-PAB and continued to Day 27 (**Supplementary material**).

Human tissues

Human tissues, following autopsy, were obtained from the Institute of Cardiometabolism and Nutrition BioCollection (Paris, France), detailed in **Supplementary Table 2**. Protocols to obtain human biospecimens conformed with the recommendations of the Declaration of Helsinki.

A detailed description of the Materials and methods is presented in **Supplementary material**. Detailed descriptions of all materials and methods used in this study, including chemicals, animals and surgical procedures, tissue harvesting, preparation and histological staining and analysis, isolated cardiomyocyte force transduction experiments, quantitative real-time PCR, Western blotting, and statistical analyses are presented in **Supplementary material**. Statistical methods are also summarized in each figure legend, where values are expressed as mean ± standard error of the mean (SEM) and number of replicates (n). Throughout, P-values < 0.05 were considered to indicate significant differences.

Results

NTP42:KVA4 improves right ventricular adaptation in the monocrotaline-pulmonary arterial hypertension model

We previously reported that *NTP42* alleviates pulmonary pathologies and cardiopulmonary hemodynamics in both the MCT- and SuHx-induced PAH animal models (27, 28). In those studies, *NTP42* was administered orally as the active pharmaceutical ingredient (API) following its dissolution in an organic-based drug vehicle unsuited and not approved for use in man. Hence, for use in the clinical setting, *NTP42* has since been uniquely formulated with the widely used pharmaceutical polymer Kollidon® VA 64 yielding the investigational medicinal product (IMP) referred to as *NTP42:KVA4*. Herein, it was first necessary to confirm or validate the oral efficacy of *NTP42* administered as *NTP42:KVA4* in preclinical PAH. Thereafter, the study specifically aimed to investigate the preclinical efficacy of *NTP42:KVA4* in the PAB model of RV pressure overload to assess the potential of *NTP42* to impart direct cardiac benefits.

Hence, efficacy of *NTP42:KVA4* was first evaluated in a delayed interventional MCT-PAH model in rodents, where disease was allowed to develop for 7 days prior to initiating treatment, where efficacy was also compared with drugs from each of the four clinical SOC PAH therapies.

MCT led to increased RV systolic pressure (RVSP) and RV hypertrophy, as measured by Fulton's Index (**Figures 1A,B**). Quantification of cardiomyocyte size, measured as cross-sectional area at the cellular level, and vascularization demonstrated that this RV hypertrophy was typical of maladaptive processes. Specifically, RV cardiomyocytes from MCT-treated animals were significantly enlarged, while RV capillary density was significantly reduced which, combined, resulted in a decrease in the RV Adaptation Index, the ratio of vascularization to cardiomyocyte size (**Figures 1C–E,G**). Consistent with the myocardial disorganization that occurs in abnormal hypertrophic processes, Masson's trichrome staining also revealed pronounced fibrosis in the RV of MCT-treated animals (**Figures 1F,H**). Treatment with *NTP42:KVA4* significantly alleviated the MCT-induced increases in RVSP and Fulton's Index parameters and alleviated the MCT-induced increase in cardiomyocyte size (**Figures 1A–C**). While *NTP42:KVA4* did not significantly affect RV vascularization in this PAH model *per se*, it did result in an improvement in the RV Adaptation Index (**Figures 1D–E,G**). Moreover, *NTP42:KVA4* significantly attenuated MCT-induced RV fibrosis (**Figures 1F,H**). In line with previous investigations,(27) treatment with *NTP42:KVA4* also significantly alleviated the MCT-induced increase in mean pulmonary arterial pressure (mPAP), and significantly attenuated pulmonary pathologies, including vessel occlusion and muscularization, $CD68^+$ macrophage infiltration, perivascular fibrosis, and edema (**Supplementary Figure 1**).

Treatment with the PAH SOCs Sildenafil, Macitentan and Riociguat, but not with Selexipag, also resulted in decreased mPAP, RVSP and Fulton's Index (**Supplementary Table 4**). In contrast to *NTP42:KVA4*, none of these SOCs significantly reduced cardiomyocyte size (**Supplementary Table 4**). Sildenafil alone resulted in increased RV vascularization while Sildenafil, Macitentan, and Riociguat, but not Selexipag, significantly increased the RV Adaptation Index and decreased the levels of RV fibrosis (**Supplementary Table 4**). While the PAH SOCs decreased vessel occlusion and muscularization as well as $CD68^+$ macrophage infiltration (**Supplementary Table 4**), they did not significantly reduce perivascular fibrosis or edema (**Supplementary Table 4**). Notably, while neither *NTP42:KVA4*, Sildenafil or Macitentan affected mean systemic arterial pressure (mAP) or heart rate (HR) *per se*, animals treated with Riociguat or Selexipag displayed either increased mAP or decreased HR, respectively (**Supplementary Table 4**).

NTP42:KVA4 attenuates right ventricular structural changes and dysfunction in the pulmonary artery banding model

Following validation of the efficacy of the formulated *NTP42:KVA4* in the MCT-PAH model, the potential for a direct benefit of TP antagonism on cardiac adaptation and function was investigated using the PAB model of RV pressure overload. Notably, while RV hypertrophy and dysfunction are features of the MCT-PAH model, and findings of cardiac benefits for therapeutic agents in this model are indeed valuable, the MCT-PAH model has two important limitations in this regard (29). Firstly, as the RV and the pulmonary vasculature are functionally coupled, direct cardiac-specific effects for an interventional therapy cannot be readily distinguished from afterload reductions due any benefits of the therapy on the pulmonary vasculature, i.e., its pulmonary-specific effects. In addition, the toxic MCT alkaloid itself may have direct effects on the RV, inducing confounding pathologies including myocarditis and arrythmias (30). The use of the PAB model herein to induce a chronic pressure load on the RV aimed to circumvent these limitations. Treatment with *NTP42:KVA4* (1 mg/kg PO BID) or Riociguat (5 mg/kg PO, BID) was initiated 2 days after PAB surgery, where Riociguat was chosen as an appropriate comparator compound as, in previous preclinical investigations, it has been shown to prevent deterioration of RV function induced by PAB (31). In addition, Riociguat is the only PAH SOC which has demonstrated potential clinical uses in heart failure settings, (32) where other SOC compounds have demonstrated conflicting findings, often with adverse

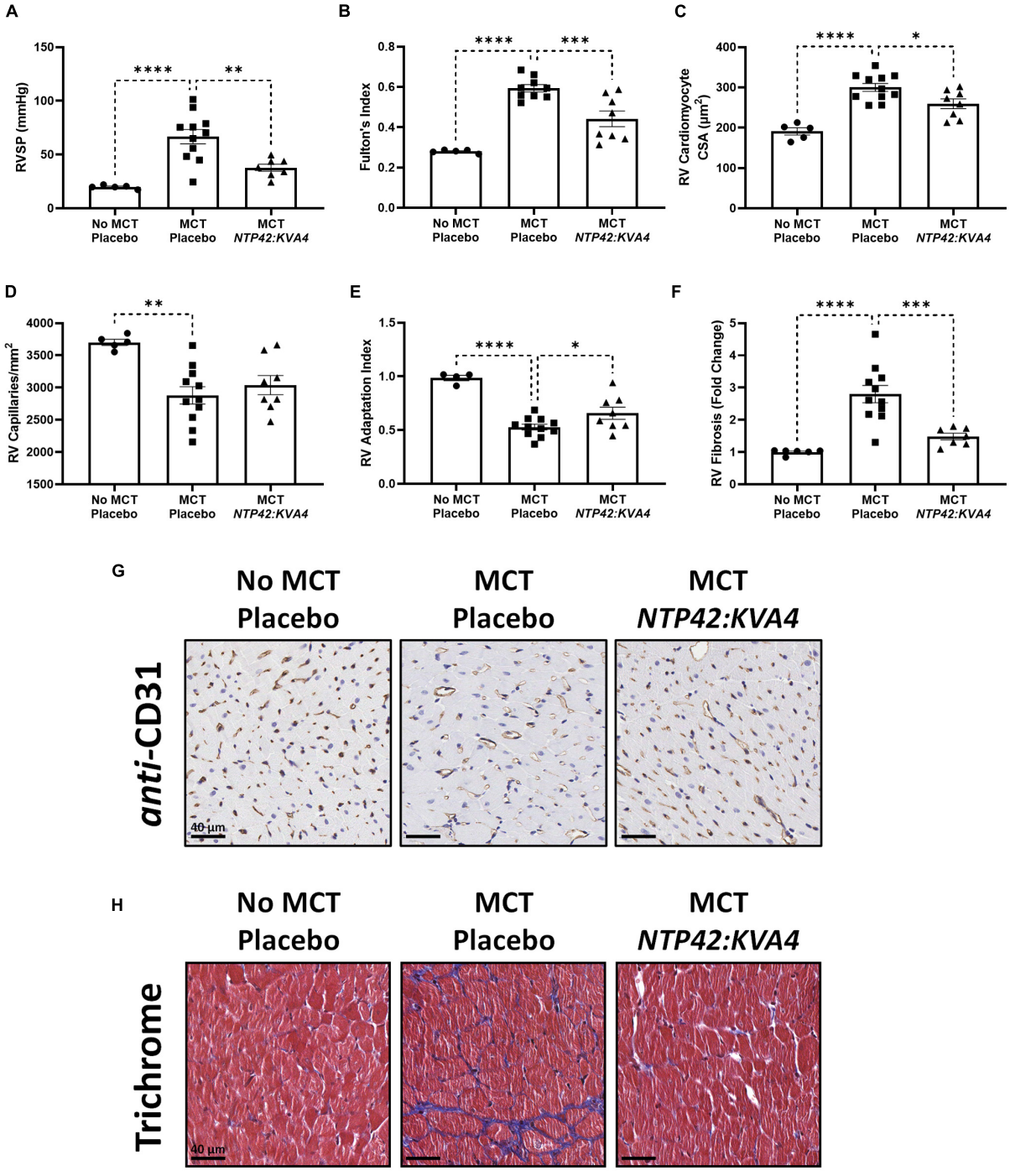

FIGURE 1
Effect of *NTP42:KVA4* treatment on RV hypertrophy and pathology in the MCT-PAH model. Normal control rats ("No MCT—Placebo") and rats injected with MCT (60 mg/kg) were treated twice daily orally (PO BID) with either drug placebo ("MCT—Placebo") or *NTP42:KVA4* (1 mg/kg PO BID), starting from Day 7 following administration of MCT. **(A–F)** show: **(A)** RVSP in the "No MCT—Placebo," "MCT—Placebo," and *NTP42:KVA4* groups [n = 5, 11, 7], **(B)** Fulton's Index [n = 5, 9, and 8, respectively]; **(C)** RV cardiomyocyte size [n = 5, 11, 8]; **(D)** RV vascularization [n = 5, 11, 8]; **(E)** RV adaptation index [n = 6, 11, 8], and **(F)** RV fibrosis [n = 5, 11, 7]. Data presented are the mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ vs. "MCT—Placebo," according to one-way analysis of variance (ANOVA) with Holm-Šídák correction applied for multiple comparisons. **(G,H)** Show representative photomicrographs, selected from a random animal from each treatment group, of: **(G)** *Anti*-CD31-stained RV tissue captured at 200 × magnification (scale bars represent 40 μm), and **(H)** Masson's trichrome-stained RV tissue captured at 200 × magnification (scale bars, 40 μm). Note that while **Supplementary Table 1** provides details on numbers of animals enrolled into the studies reported herein and those that survived through to terminal surgery, the numbers (n) given in the square brackets in all figure legends refer to the number of input data used for the individual experimental parameter following removal of any justifiable outliers identified using the method of Interquartile Range with Tukey fences.

outcomes (33, 34). Pre-treatment echocardiogram (ECHO) showed robust and comparable pulmonary arterial (PA) pressure gradients across the randomized PAB animal groups (**Supplementary Figures 2A,B**). At study termination, animals subjected to PAB showed comparable bodyweight and no differences in bodyweight gain over the course of the study was observed (**Supplementary Figures 2C,D**).

In ECHO assessments, PAB resulted in increased RV free wall thickness (RVFWT) and RV dilation as evidenced by increased RV end-diastolic dimension (RVEDD) and RV end-diastolic area (RVEDA) (**Figures 2A–C**). In addition, the right atrial area (RAA) was enlarged (**Figure 2D**). Treatment with *NTP42:KVA4* improved RV geometries and attenuated RV dilation, where both RVEDD and RVEDA were reduced (**Figures 2B,C**). Notably, the attenuated RV dilation observed following *NTP42:KVA4* treatment was not paralleled by compromised RV hypertrophy, where RVFWT was unchanged (**Figure 2A**). In addition, *NTP42:KVA4* alleviated PAB-induced RAA enlargement (**Figure 2D**). Comparable benefits on right heart geometry were not observed following treatment with Riociguat (**Supplementary Table 5**).

Detailed pressure-volume (PV) loop analyses showed that PAB animals demonstrated profound RV overload with marked signs of RV dysfunction (**Figure 3**). In PAB animals, HR was decreased with unchanged mAP (**Figures 3A,B**), and RV end-systolic pressure (ESP) was fourfold higher than in Sham animals (**Figure 3C**). RV filling pressure (end diastolic pressure, EDP) was also increased in PAB animals (**Figure 3D**), and RV dilation was observed when considering both end-systolic and end-diastolic volumes (ESV and EDV; **Figures 3E,F**). While cardiac output (CO) was reduced upon PAB (–18%, P = 0.1998, **Figure 3G**), the RV ejection fraction (RV EF) was significantly compromised relative to the Sham control (**Figure 3H**). End-systolic elastance (Ees) was increased in PAB animals (**Figure 3I**), and significant diastolic dysfunction was evident in this group, as demonstrated by increased end-diastolic elastance (Eed) (**Figure 3J**).

In this model, treatment with *NTP42:KVA4* significantly improved RV function. In line with ECHO data (**Figure 2**), *NTP42:KVA4* markedly reduced RV dilation, where both ESV and EDV were reduced relative to the PAB control (**Figures 3E,F**). Most notably, *NTP42:KVA4* significantly improved RV EF relative to PAB control (**Figure 3H**), resulting in near-normalized values compared with Sham animals. In addition, *NTP42:KVA4* trended toward an improvement (27%, P = 0.0641) in diastolic function, as measured by Eed (**Figure 3J**).

While treatment with Riociguat led to improvements in measures of RV dilation, and increased RV EF, albeit to a lesser extent than *NTP42:KVA4*, Riociguat treatment trended toward a further worsening in Eed (24%, P = 0.2908; **Supplementary Table 5**).

NTP42:KVA4 promotes an adaptive pattern of right ventricular hypertrophy and reduces expression of genes associated with cardiac dysfunction in the pulmonary artery banding model

While *NTP42:KVA4* treatment did not lead to reductions in the gross RV wall enlargement induced by PAB (Fulton's Index, **Figure 4A**), it significantly decreased cardiomyocyte size relative to PAB controls (**Figures 4B,I**), consistent with findings from the MCT-PAH model (**Figure 1C**). In addition, in this PAB model, *NTP42:KVA4* treatment significantly increased RV vascularization (**Figures 4C,I**). Considering both factors, *NTP42:KVA4* treatment resulted in a trend toward improvement in the RV Adaptation Index (P = 0.0797; **Figure 4D**). Furthermore, expression analysis of genes associated with RV hypertrophy showed that levels of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) were significantly increased upon PAB, relative to Sham levels (**Figures 4E,F**). Treatment with *NTP42:KVA4* significantly reduced ANP levels (**Figure 4E**) and trended toward reduced BNP levels (**Figure 4F**).

Notably, RV fibrosis, while increased upon PAB (**Figures 4G,J**), was less pronounced than that observed in the MCT-PAH model (**Figure 1G**), and reductions in this gross level of fibrosis were not observed with *NTP42:KVA4* in the PAB model. Notably, fibrosis-mediated myocardial stiffness is influenced by the predominant collagen isoform, where an increased ratio of the stiff type I isoform relative to the elastic type III isoform is linked with more severe RV dysfunction (35). Herein, analysis of collagen isoform expression levels demonstrated a marked increase in the collagen I/III ratio upon PAB (**Figure 4H**). Treatment with *NTP42:KVA4* significantly reduced the collagen I/III ratio (**Figure 4H**), indicative of predominant expression of the more flexible collagen III isoform.

In this model, total heart weight was significantly increased in all PAB groups (**Supplementary Figure 3A**). Consistent with ECHO assessments showing RA chamber enlargement (**Figure 2D**), measurements of the RA wall weight demonstrated that substantial RA remodeling occurred in response to PAB, and where *NTP42:KVA4* treatment led to significant reductions in this index (**Supplementary Figure 3B**). Notably, while PAB did not induce changes in left ventricular (LV) cardiomyocyte size or vascularization, an increased level of LV fibrosis was appreciable which was somewhat reduced by *NTP42:KVA4* treatment (–28%, P = 0.1386, **Supplementary Figure 3C–G**).

In these analyses, the PAH SOC Riociguat did not lead to significant improvements in Fulton's Index, cardiomyocyte

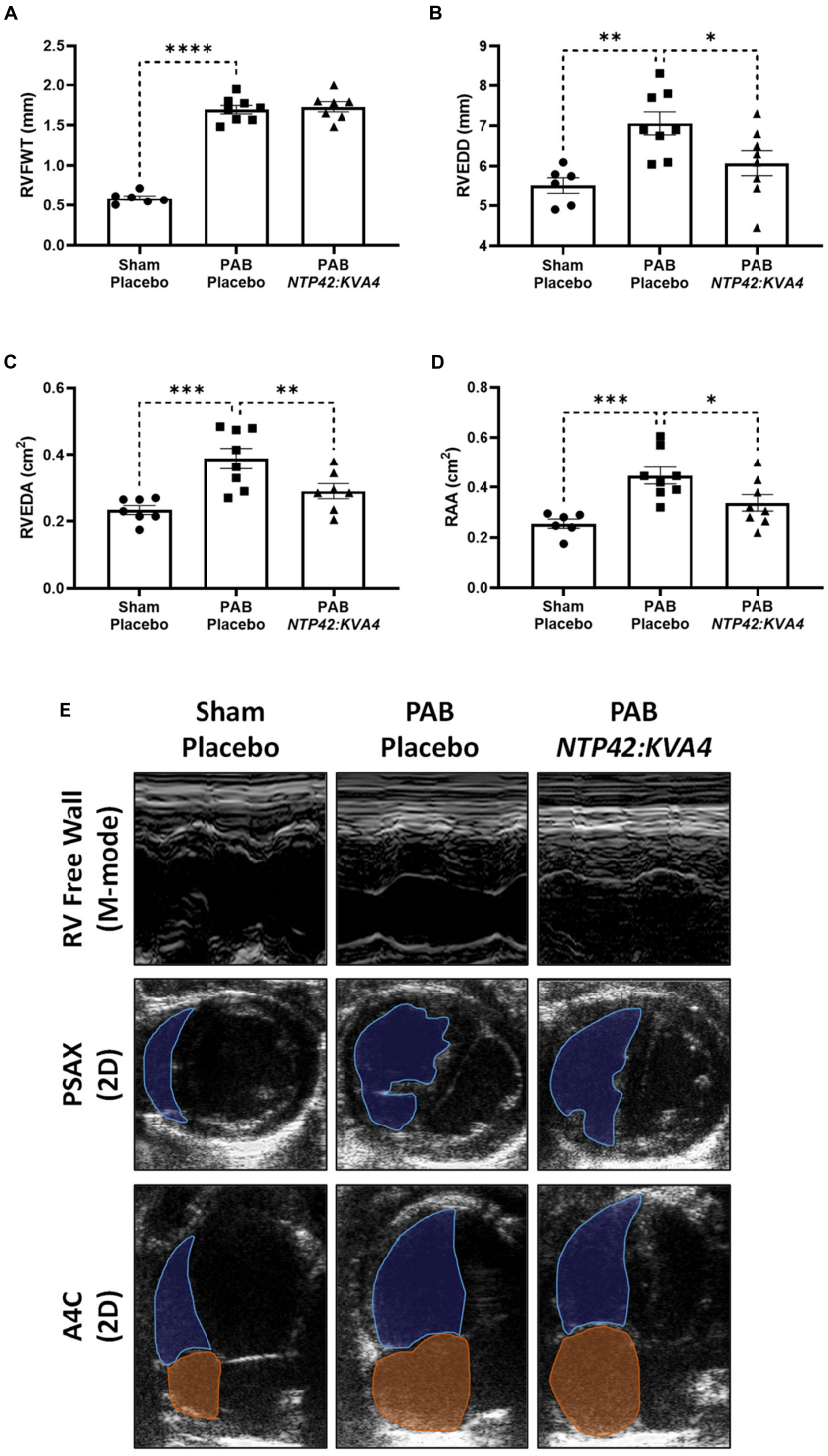

FIGURE 2
Effect of *NTP42:KVA4* treatment on right heart dimensions and geometry in the PAB model. Normal control rats ("Sham—Placebo") and rats subjected to pulmonary arterial banding (PAB) were treated with either drug placebo ("PAB—Placebo") or *NTP42:KVA4* (1 mg/kg PO BID), starting from Day 2 following PAB. **(A–D)** Show ECHO-derived measurements of: **(A)** RVFWT in the "Sham—Placebo," "PAB—Placebo," and *NTP42:KVA4* groups [n = 6, 8, and 7, respectively]; **(B)** RVEDD [n = 6, 8, 8]; **(C)** RVEDA [n = 7, 8, 7], and **(D)** RA area (RAA) [n = 6, 8, 7]. Data presented are the mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ vs. "PAB—Placebo," according to one-way ANOVA with Holm-Šídák correction. **(E)** Shows representative ECHO images from M-mode recordings and from 2D parasternal short-axis (PSAX) and apical four-chamber (A4C) views selected from a random animal from each treatment group and where blue and orange shading and lines delineate the RV and RA, respectively.

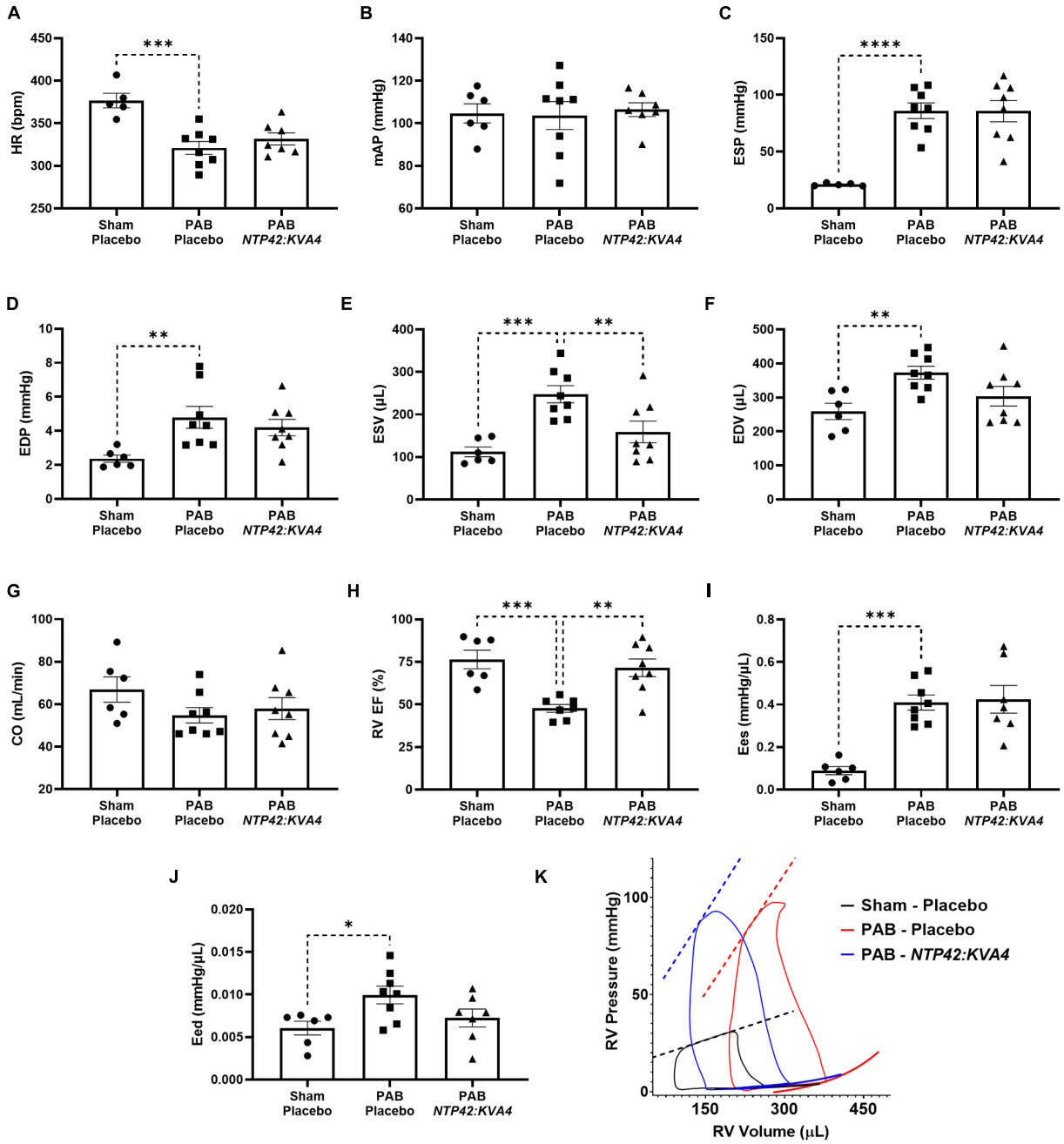

FIGURE 3
Effect of *NTP42:KVA4* treatment on RV pressure, volume and function in the PAB model. Hemodynamic measurements of: **(A)** HR in the "Sham—Placebo," "PAB—Placebo," and *NTP42:KVA4* groups [n = 5, 8, and 7, respectively]; **(B)** mAP [n = 6, 8, 7]; **(C)** RV ESP [n = 5, 8, 8]; **(D)** RV EDP [n = 6, 8, 8]; **(E)** RV ESV [n = 6, 8, 8]; **(F)** RV EDV [n = 6, 8, 8]; **(G)** CO [n = 6, 8, 8]; **(H)** RV EF [n = 6, 7, 8]; **(I)** RV Ees [n = 6, 8, 7], and **(J)** RV Eed [n = 6, 8, 7]. Data presented are the mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 vs. "PAB—Placebo," according to one-way ANOVA with Holm-Šídák correction. **(K)** shows representative RV PV loops from PAB study animals. The linear end-systolic and exponential end-diastolic PV relationships within each group are displayed as thick dashed or solid lines, respectively. Maximum/minimum PV points on the displayed representative loops, and the PV relationships plotted thereon, were adjusted to correspond approximately with the average values determined within treatment group.

size, RV vascularization, ANP mRNA expression levels, or RV fibrosis, and an improved pattern of adaptive hypertrophy was not observed upon Riociguat treatment (**Supplementary Table 5**). However, like *NTP42:KVA4*, Riociguat led to a reduction in the collagen I/III ratio (**Supplementary Table 5**).

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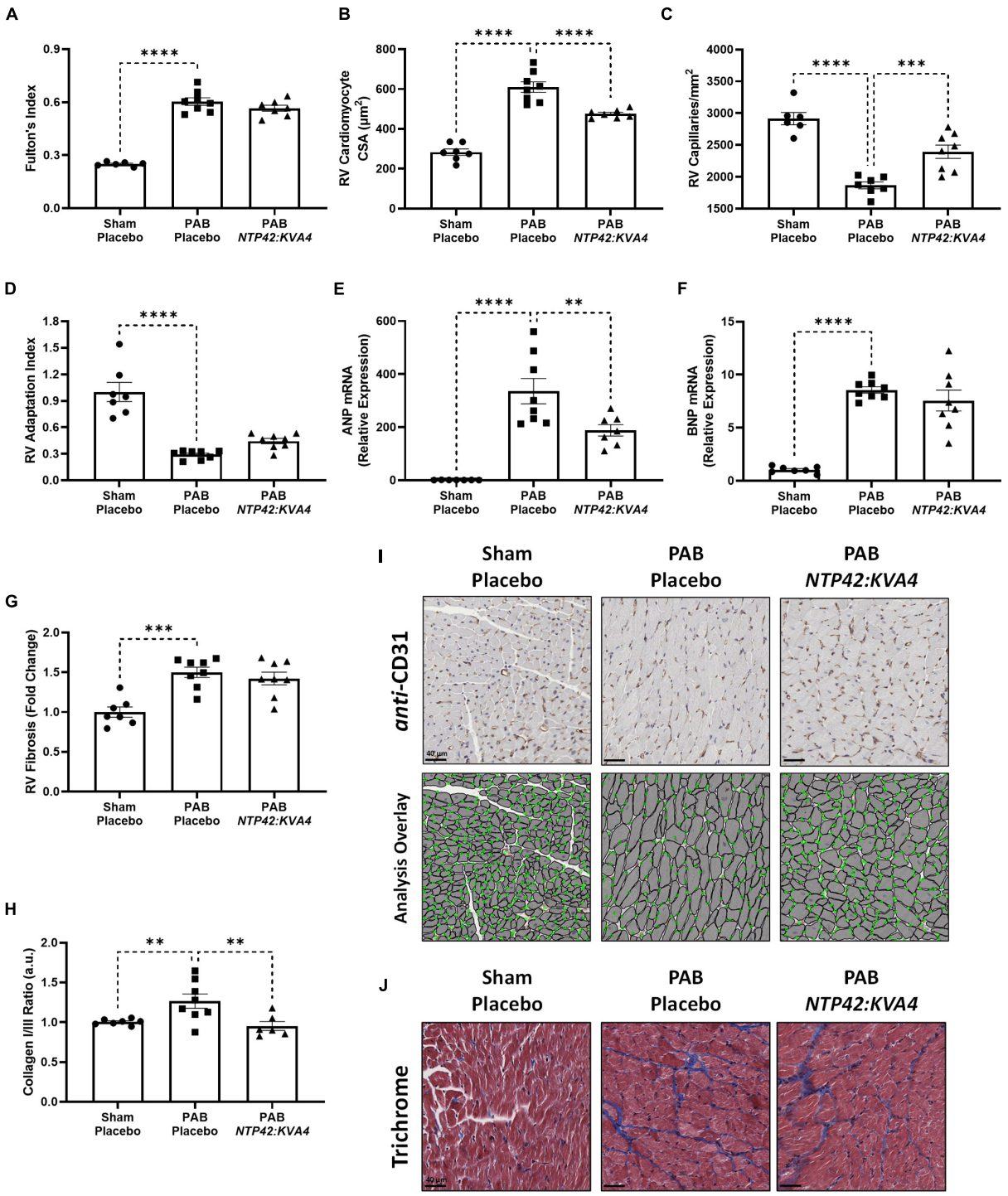

FIGURE 4

Effect of *NTP42:KVA4* treatment on RV hypertrophy and pathology in the PAB model. **(A–H)** show: **(A)** Fulton's Index in the "Sham—Placebo," "PAB—Placebo," and *NTP42:KVA4* groups [n = 6, 8, and 7, respectively]; **(B)** RV cardiomyocyte size [n = 7, 8, 7]; **(C)** RV vascularization [n = 6, 7, 8]; **(D)** RV adaptation index [n = 7, 8, 8], **(E)** RV ANP mRNA expression [n = 7, 8, 7], **(F)** RV BNP mRNA expression [n = 7, 8, 8], **(G)** RV fibrosis [n = 7, 8, 8], and **(H)** RV collagen type I/III ratio, calculated from the RV collagen type I and III mRNA expression for each animal, and expressing the ratio in arbitrary units (a.u.) [n = 7, 8, 6]. Data presented are the mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ vs. "PAB—Placebo," according to one-way ANOVA with Holm-Šídák correction. **(I,J)** show representative photomicrographs, selected from a random animal from each treatment group, of: **(I)** (Upper panel): *Anti*-CD31-stained RV tissue captured at 400 × magnification (scale bars represent 40 μm), (Lower panel): Image analysis overlay showing annotated cardiomyocyte cross-sectional area (gray outline) and $CD31^{+}$ vessels (green dots), and **(J)** Masson's trichrome-stained RV tissue captured at 400 × magnification (scale bars, 40 μm).

NTP42:KVA4 results in normalized cardiomyocyte passive tension and reduces proteolytic degradation of calcium-handling proteins in the pulmonary artery banding model

While PAB-induced right heart pressure overload resulted in significant structural remodeling and systolic and diastolic dysfunction, intrinsic changes in cardiomyocyte tension development were also observed in this model. Passive tension (PT) was significantly increased across all sarcomere lengths in PAB animals, indicative of increased cardiomyocyte stiffness (**Figure 5A**). Consistent with the improvement in diastolic function observed for *NTP42:KVA4* (**Figure 3J**), PT was significantly attenuated in cardiomyocytes isolated from *NTP42:KVA4*-treated animals (**Figure 5A**) and was indistinguishable from the profile of Sham control animals. Regarding systolic function, cardiomyocytes isolated from PAB control animals showed somewhat increased maximum active tension (AT) compared with Sham animals (**Figure 5B**), while relative AT profiles were similar between Sham, PAB and *NTP42:KVA4* groups (**Figure 5C**). Similar benefits on intrinsic cardiomyocyte function were not observed following Riociguat treatment in this model and both PT and AT were increased in comparison with the PAB control (**Supplementary Table 5**).

Notably, despite the altered profiles of contraction, no changes in calcium sensitivity were observed between the groups (**Figure 5D**). Besides altered calcium sensitivity, a further mechanism contributing to cardiomyocyte dysfunction involves decreased capacity for diastolic calcium clearance (36). For efficient cardiomyocyte relaxation, cytosolic calcium levels must promptly drop following contraction, where this is facilitated either by its efflux from the cell by Na^+/Ca^{2+} exchanger 1 (NCX1) or sequestration into internal cellular stores by sarco/endoplasmic reticulum Ca^{2+}-ATPase 2a (SERCA2a) (37). Alterations in the expression of these calcium-handling proteins contribute to cardiomyocyte dysfunction during pressure-induced hypertrophy and cardiac failure (38–40), and decreases in NCX1 and SERCA2a have been observed in the RV from PAH patients (36). Furthermore, degradation and inactivation of NCX1 and SERCA2a, mediated by the calcium-activated protease calpain, occurs in multiple animal models of heart failure (41–44). Herein, while expression of intact full-length NCX1 was unchanged, a significant decrease in SERCA2a protein was observed in PAB animals (**Figures 5E–H**). Moreover, elevated degradation of both NCX1 and SERCA2a were observed following PAB (**Figures 5E,F,I,J**). Specifically, increased expression of a single degradation fragment of NCX-1 and up to three SERCA2a degradation fragments were observed (**Figures 5E,F**), where these inactive fragments have been previously described (41, 44). Treatment with *NTP42:KVA4*, but not Riociguat, attenuated calcium-handling protein degradation, with significantly reduced NCX1 and SERCA2a degradation fragments evident (**Figures 5E,F,I,J**).

Thromboxane receptor expression is elevated in the right ventricle in experimental models and in human pulmonary arterial hypertension and other right ventricular conditions

While signaling through the TP is implicated in pathological cardiac conditions, few studies have examined TP expression levels in RVs of subjects with PAH. Thus, as a rationale for the therapeutic potential and utility of TP antagonists *per se*, expression of the TP was examined in RV tissues from experimental PAH and cardiac disease models, as well as in clinical specimens of PAH and dilated cardiomyopathy (DCM).

While low levels of TP expression were noted in the RV myocardium in No MCT and Sham animals, increased TP expression occurred in all diseased groups (**Figures 6A–D**). In addition, genomic analysis confirmed elevated TP expression levels in the PAB model (**Figure 6E**). Administration of *NTP42:KVA4* in both MCT-PAH and PAB models led to a non-significant trend toward reduction in TP expression ($P = 0.2091$ and $P = 0.0920$, respectively; **Figures 6A–D**). As no significant effects on TP expression were observed following treatment with PAH SOCs in either model (**Supplementary Tables 4**, **5**), a potential effect on TP expression following specific receptor engagement with *NTP42:KVA4* is notable and may indicate a mechanism whereby TP antagonism may lead to beneficial RV effects.

Further evidence for increased TP expression in the RV in PAH and, potentially in other cardiac dysfunctions, was found in RV specimens from both the Sugen5416/Hypoxia (SuHx)-induced PAH model (**Figures 6F,I**), and notably from rat strains harboring a mutation in the BMPR2 gene, the primary genetic cause of heritable PAH in humans (**Figures 6G,J**). Furthermore, TP expression was also increased in RVs from obese ZSF1 rats, a recognized model of heart failure with preserved ejection fraction (HFpEF) which also manifests RV dysfunction (**Figures 6H,K**).

In clinical specimens from healthy human donors (**Figure 7A**), TP expression was observed at a low level throughout the myocardium, with stronger expression evident in perinuclear regions, consistent with previous reports (19). In diseased tissue, TP expression was augmented in the enlarged cardiomyocytes of RV samples from PAH patients (**Figure 7B**), with increased expression also observed in RV samples from DCM (**Figure 7C**), a primary cardiomyopathy which results in ventricular dilation and functional impairment. Quantitative analysis confirmed these elevated TP expression levels in PAH & DCM cases, relative to healthy donors (**Figure 7D**). Notably, while increased TP expression occurred in RVs in experimental models and in clinical PAH samples, no significant changes were observed in the matching LV tissues (**Supplementary Figure 4**).

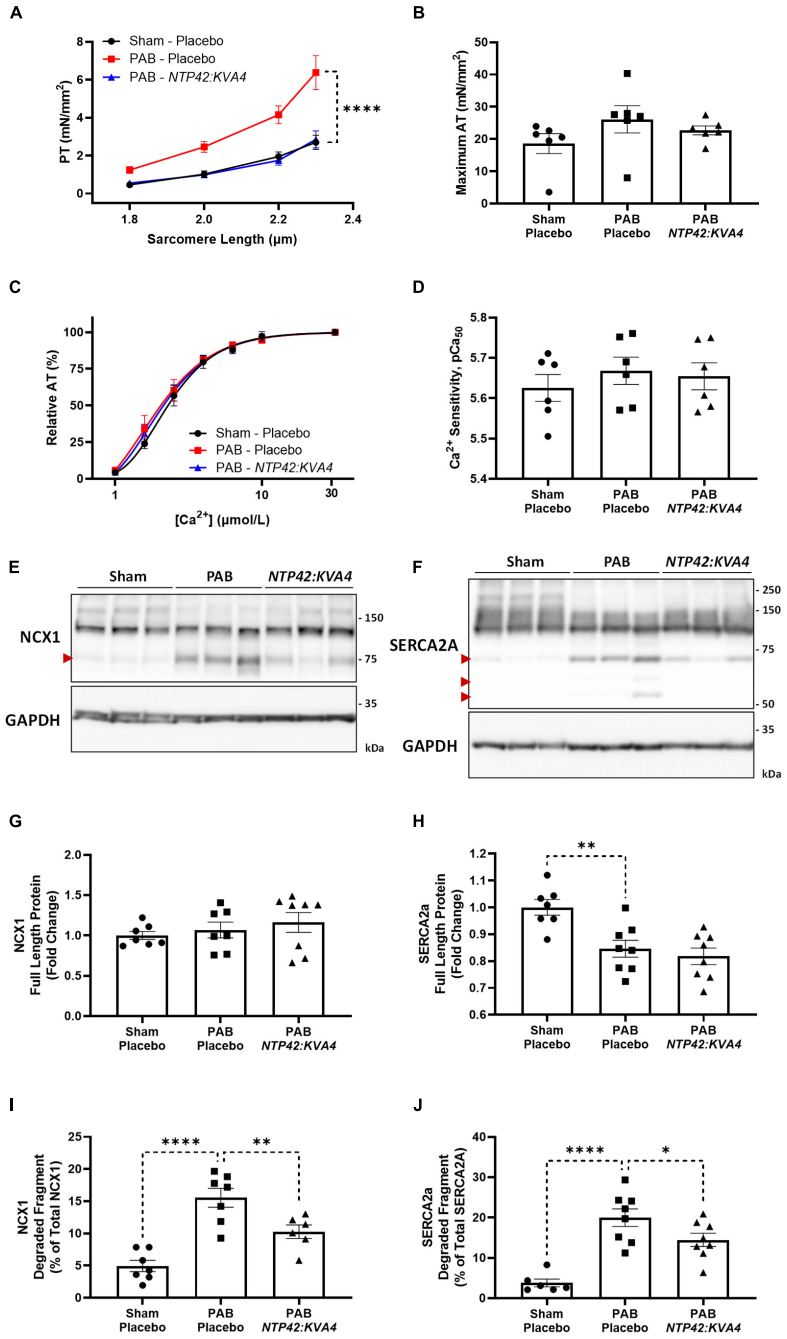

FIGURE 5
Effect of *NTP42:KVA4* treatment on isolated cardiomyocyte force transduction and calcium-handling protein expression in the PAB model. **(A–D)** show: **(A)** Steady-state PT measured at increasing sarcomere lengths (1.8–2.3 μm) from cardiomyocytes isolated from animals in the "Sham—Placebo," "PAB—Placebo," and *NTP42:KVA4* groups [*n* = 6, 6, and 6, respectively]; **(B)** Maximum AT development at [Ca^{2+}] 31.6 μmol/L [*n* = 6, 6, 6]; **(C)** Relative AT development in response to increasing submaximal free Ca^{2+} concentration ([Ca^{2+}], 1–31.6 μmol/L) [*n* = 6, 6, 6], where calcium response curves were fitted using non-linear regression, and **(D)** Calcium sensitivity (pCa_{50}) determined from individual regression analyses [*n* = 6, 6, 6]. **(E,F)** Show western blot protein expression of: **(E)** NCX1 and **(F)** SERCA2a, where representative RV lysates are displayed from 3 random animals from each treatment group and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression levels were used as loading control. The relative positions of the molecular size markers (kDa) are indicated to the right of the panels and the positions of the observed degraded fragments of NCX1 and SERCA2a are marked with red arrowheads to the left of the panels. **(G–J)** Show mean relative RV expression levels of **(G)** NCX1 full-length protein (120 kDa) [*n* = 7, 7, 8]; **(H)** SERCA2a full-length protein (110 kDa) [*n* = 7, 8, 8]; **(I)** NCX1 75 kDa degraded fragment, expressed as a percentage of total NCX1 [*n* = 7, 7, 6], and **(J)** SERCA2a 70 kDa degraded fragment, expressed as a percentage of total SERCA2a [*n* = 6, 8, 8]. Data presented are the mean ± SEM, and where in **(A–D)**, results are presented from 6 animals per group, with an average of 5 independent cardiomyocytes (technical replicates) analyzed per animal. *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$ vs. "PAB—Placebo," according to two-way ANOVA with Holm-Šídák correction **(A,C)** or one-way ANOVA with Holm-Šídák correction **(B,D,G–J)**.

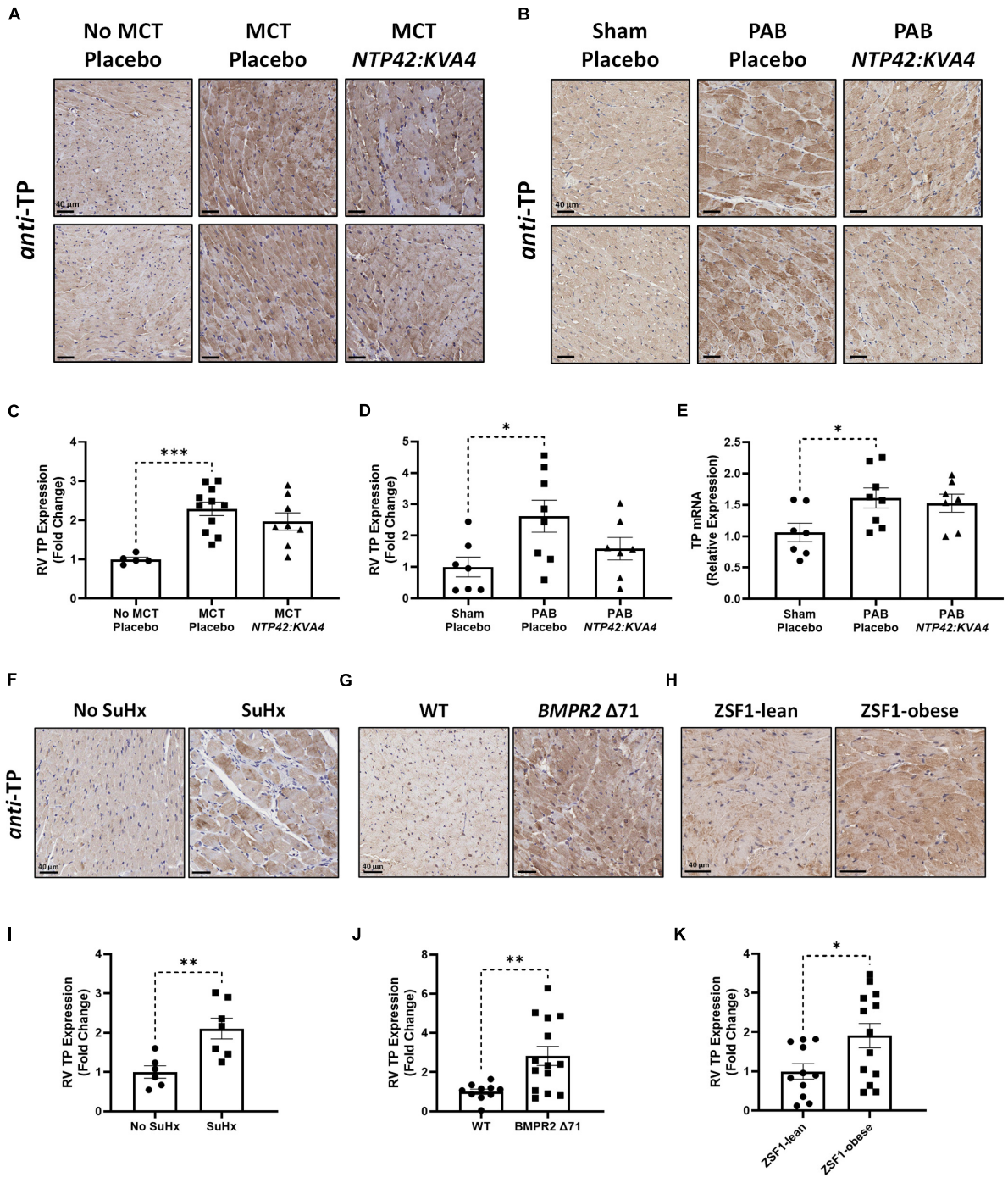

FIGURE 6
RV expression of the TP in preclinical models of PAH and RV dysfunction. **(A,B)** Show two representative photomicrographs, selected from a random animal from each treatment group, of *anti*-TP-stained RV tissue from the: **(A)** MCT-PAH model, and **(B)** PAB model, where images were captured at 400 × magnification (scale bars represent 40 µm). **(C)** Shows relative immunohistochemical (IHC) expression of the TP in the MCT-PAH model in RV tissue from animals of the "No MCT—Placebo," "MCT—Placebo," and *NTP42:KVA4* groups [*n* = 5, 11, and 8, respectively]. **(D–E)** Show: **(D)** relative TP IHC expression in the PAB model in RV tissue from animals of the "Sham—Placebo," "PAB—Placebo," and *NTP42:KVA4* groups [*n* = 7, 8, and 7, respectively], and **(E)** RV TP mRNA expression [*n* = 7, 8, 7]. **(F–H)** Show representative photomicrographs, selected from a random animal from each treatment group, of *anti*-TP-stained RV tissue from: **(F)** Control groups of a previously described SuHx-PAH model (28), namely "No SuHx" and "SuHx," where normal control rats ("No SuHx") and rats treated with SuHx ("SuHx") were treated PO BID with vehicle upon removal from hypoxia and continuing in normoxia until Day 49 (i.e., 4 weeks duration); **(G)** *BMPR2* Δ 71 rats or their WT counterparts, and **(H)** ZSF1-obese rats or their lean counterparts (ZSF1-lean). **(I–K)** show relative expression levels of the TP in RV tissue from: **(I)** "No SuHx" and "SuHx" animals [*n* = 6 and 7, respectively]; **(J)** WT or *BMPR2* Δ71 animals [*n* = 14 and 11, respectively], and **(K)** ZSF1-lean or ZSF1-obese animals [*n* = 13 and 11, respectively]. Data presented are the mean ± SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. the respective disease control group in each case according to one-way ANOVA with Holm-Šídák correction **(A–C)** or unpaired Student's *t*-tests **(I–K)**.

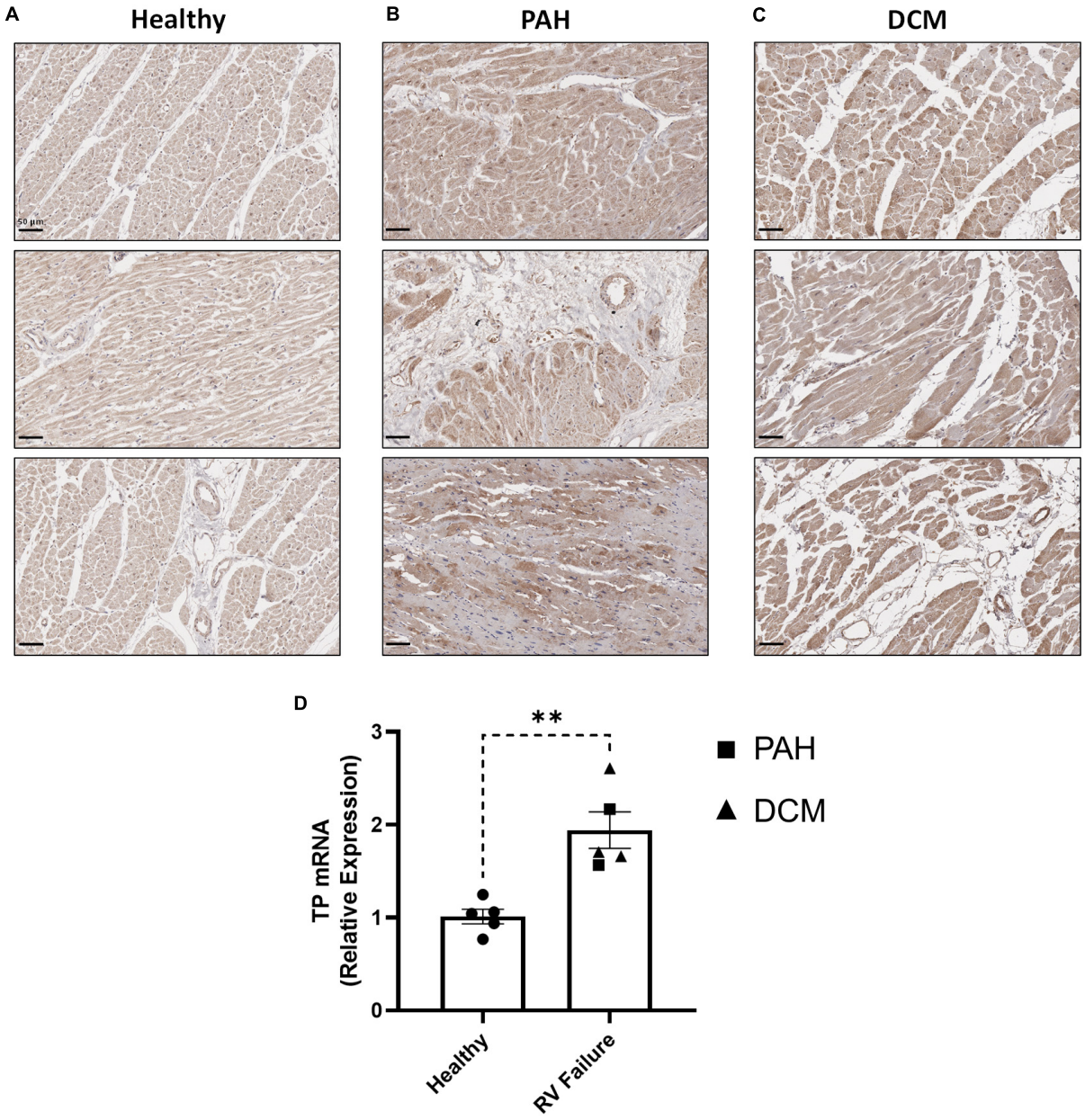

FIGURE 7
RV expression of the TP in human PAH and other RV pathologies. **(A–C)** show three representative photomicrographs of *anti*-TP-stained human RV tissue obtained from: **(A)** Healthy donors; **(B)** PAH patients, and **(C)** DCM patients, where all images were captured at 150 × magnification (scale bars represent 50 μm). **(D)** Shows relative RV TP mRNA expression levels in Healthy (n = 5) or RV Failure patients (n = 5, data for PAH and DCM combined). Data presented are the mean ± SEM. $^{**}P < 0.01$ according to unpaired Student's t-test.

Discussion

Right heart function is widely viewed as the most important determinant of clinical outcome in PAH and indeed in various other forms of pulmonary hypertension (PH) (45). Insufficient or aberrant RV adaptation, the development of RV dysfunction, and the progression to right heart failure in PAH involves complex pathological mechanisms and a precise understanding of the causes underlying these mechanisms remains to be fully elucidated (46). Unfortunately, no currently available PAH SOC therapy directly targets right heart adaptation and function.

While PAH SOCs demonstrate robust pulmonary vasodilatory enhancing effects and lead to meaningful reductions in PVR, they show limited evidence of directly targeting right heart adaptation and function. In PAH, there is an unmet need to not only alleviate pulmonary pathology

and PVR, but to also directly target mechanisms underlying RV dysfunction to enhance patient quality-of-life and improve survival. Current PAH SOCs have variable effects on RV function in both experimental and clinical settings (47). In preclinical models, the prostacyclin analog Iloprost improved RV contractility, and a recent trial in PAH patients demonstrated that Iloprost increases contractility and RV-PA coupling (48). However, large-scale clinical trials of Epoprostenol in heart failure patients demonstrated an association with increased mortality (49, 50). In preclinical models, endothelin receptor blockade worsens cardiomyocyte contractility (51). Clinical trials of endothelin receptor antagonists in patients with heart failure have never fully reported, making it impossible to assess efficacy or, at worst, suggestive of unfavorable effects (52–54). In PAH patients, while acute treatment with the phosphodiesterase (PDE)5 inhibitor Sildenafil improved RV diastolic function (55), recent trials in non-PAH heart failure patients with Sildenafil or the soluble guanylate cyclase (sGC) stimulator Riociguat failed to meet their primary clinical endpoints (56, 57).

Early studies targeting the TXA_2/TP pathway in preclinical PAH demonstrated conflicting results (58, 59). However, through recent evaluations in both the MCT- and SuHx-induced PAH models, we have demonstrated that the TP antagonist *NTP42* attenuates multiple features of experimental PAH (27, 28). In this study, the efficacy of the *NTP42:KVA4*, a novel oral formulation of *NTP42* specifically developed for clinical use and recently validated in a Phase I clinical trial (NCT04919863) in alleviating pulmonary pathologies in the MCT-PAH model was confirmed to be in line with previous findings using the *NTP42* API. Moreover, this MCT-PAH study demonstrated that *NTP42:KVA4* attenuates RV hypertrophy and promotes a more beneficial pattern of RV adaptation. Thereafter, we aimed to investigate the potential for *NTP42:KVA4* to directly target the compromised RV using the PAB model of right heart pressure overload. With an absence of confounding pulmonary pathologies, the PAB model allows for a direct assessment of therapeutic intervention on RV structure and function (60). In this PAB model, *NTP42:KVA4* reduced RV dilation as observed from ECHO and PV loop indices and also alleviated RA enlargement. While not affecting gross RV hypertrophy *per se* as evidenced by unchanged wall thickness and Fulton's Index, *NTP42:KVA4* treatment also resulted in a more adaptive pattern of hypertrophy, with significantly decreased cardiomyocyte size and a significant increase in capillary density in RV tissue. In individually isolated cardiomyocytes, the PAB-induced increase in passive tension development, a key indicator of cell stiffness, was attenuated by *NTP42:KVA4* being indistinguishable from that of healthy control animals, and decreased degradation of the Ca^{2+}-handling proteins NCX1 and SERCA2a. Moreover, due to the overall improved RV geometry, decreased RV dilation, and attenuated profiles of intrinsic diastolic and systolic cardiomyocyte tension development, treatment with *NTP42:KVA4* resulted in significantly improved RV function in PAB animals. Most notably, *NTP42:KVA4* markedly improved RV EF, near normalizing this key parameter relative to control Sham animals.

Together, the findings from these two independent preclinical models demonstrate that *NTP42:KVA4* not only alleviates pulmonary pathologies akin to those observed in clinical PAH, but also may act as a direct cardioprotective agent in settings of right heart pressure overload. Throughout these preclinical models, the efficacy seen with *NTP42:KVA4* was similar or indeed greater than the PAH SOCs used herein (**Supplementary Tables 4**, **5**). Furthermore, in translating the preclinical efficacy findings generated in these rodent PAH models to that predicted to clinically occur in man, it is important to also note that the API *NTP42* was rationally designed and selected using the human and not the rodent TP drug target (61, 62). Thus, due to key evolutionary differences in the TP in primates vs. lower species (63), *NTP42* is a highly potent antagonist of both TPα and TPβ isoforms of the human TP (61, 62), inhibiting TXA_2 mimetic U46619-induced calcium mobilization in cell lines stably over-expressing the human TP, and U46619-induced aggregation of human platelets, with IC_{50} values of 8.86 and 10.6 nM, respectively (27). However, in similar studies in the rat, *NTP42* is substantially less potent inhibiting U46619-induced calcium mobilization by the rat TP and aggregation of rat platelets *ex vivo* with IC_{50} values of 1.91 and 3.2 μM, respectively (**Supplementary Figure 5**). Thus, based on its relative IC_{50} for the TP in rats and humans, *NTP42* will be substantially more efficacious (250–300-fold) in man than in rat. Consistent with this proposition, in the recent Phase I clinical trial of the IMP *NTP42:KVA4* in healthy subjects (NCT04919863), the IC_{50} of *NTP42* for inhibition of U46619-induced aggregation of human platelets *ex vivo* was confirmed to be 9.9 nM. Extending this translation of preclinical to predicted clinical data for the PAH SOCs, based on their relative IC_{50} in rats and humans, both Sildenafil (IC_{50} in rat and man, approx. 3 nM) (64, 65) and Macitentan (IC_{50} in rat and man, 1 nM) (66, 67) are expected to be equally efficacious in both species. In contrast to this, based on its EC_{50} of 170 and 4 nM in rat and man (67, 68), the prostacyclin receptor agonist Selexipag is predicted to be 42.5-fold more efficacious in man than in rat and, therefore, is likely to elicit a better outcome clinically than observed in this or other preclinical studies. Indeed, this may account for the poor efficacy observed for Selexipag on key parameters such as mPAP, RVSP and Fulton's Index (**Supplementary Table 4**). With regard to Riociguat, based on its relative potency (EC_{50} in rat and man, 30 and 80 nM, respectively) (69, 70), it is predicted to be 2.6-fold more efficacious in rat than in man, likely generating a poorer clinical outcome than it does in the preclinical studies carried out in the rat. In addition to such key species-dependent differences in target specificity, it was also notable there were fewer systemic effects apparent for *NTP42:KVA4* relative to the PAH SOCs. Specifically, while Selexipag and Riociguat led to changes in HR

and mAP, respectively, and treatment with all the PAH SOCs tested led to increased liver weight indices, similar indicators of potential off-target or toxicological effects were not observed with *NTP42:KVA4* (**Supplementary Tables 4**, **5**). In addition, while demonstrating effects in the compromised RV, PV loop analysis showed that *NTP42:KVA4* did not affect LV parameters (data not shown). As an important regulatory-compliant safety parameter required by both the European and US EMA and FDA agencies before proceeding to FIH Phase I clinical trials, the *in vivo* effect of *NTP42:KVA4* on the cardiovascular system was also investigated in conscious telemetered dogs, where no inotropic or chronotropic effects were observed at doses up to 450 mg/kg *NTP42:KVA4* PO (**Supplementary Figure 6**).

In previous studies investigating the role of the TXA_2/TP pathway on RV dysfunction, TP antagonism was protective against mild RV pressure overload in a mouse PAB model, where pressures and cardiac output were improved (19). TP antagonism also attenuated PAB-induced increases in end-diastolic calcium levels and improved cardiac repolarization and reduced ECG abnormalities through restoration of the gap junction protein connexin 43 (71–73). Targeting of TXA_2/TP pathway has been previously investigated in PAH clinical therapy. Terbogrel, a dual TP antagonist and TXA_2 synthase (TXAS) inhibitor was evaluated in a Phase II clinical trial, but this study was prematurely terminated during enrolment due to the development of acute leg pain in trial participants (74). As subsequently reported, this leg pain occurred due to Terbogrel's inhibition of TXAS which, while blocking TXA_2 generation, resulted in a shift toward synthesis of prostacyclin, a potent pain inducer. In contrast to Terbogrel, *NTP42* is a highly selective TP antagonist, which does not inhibit TXAS and therefore, as also confirmed in the recent Phase I clinical trial even at high doses, will not induce leg pain (27). In addition, the specificity of *NTP42* for the TP has been previously reported, with no agonist or antagonist activity at the 7 other prostanoid receptors, namely the prostaglandin (PG) D_2 (DP_1), PGE_2 (EP_1, EP_2, EP_3, EP_4), $PGF_{2\alpha}$ (FP) and PGI_2/prostacyclin (IP) receptors, with no agonist activity at the TP itself (27).

As depicted in the model in **Figure 8**, there are many putative mechanisms by which signaling via the TXA_2/TP pathway may elicit detrimental effects within the myocardium, where the findings from this study provide important mechanistic insights into this and how *NTP42* alleviates this dysfunction. Consistent with a large body of data, including from this laboratory (63), signaling through the TP, TXA_2 induces profound increases in intracellular calcium (Ca^{2+}) in many cell types. Specifically, in cardiomyocytes, basal and peak Ca^{2+} concentrations as well as width of Ca^{2+} transients are increased following treatment with the TXA_2 mimetic U46619, and prolonged stimulation results in irregular Ca^{2+} oscillations and a marked increase in cytosolic-free Ca^{2+} concentrations (20). Direct injections of U46619 also induce ventricular arrhythmia in rabbits, where this effect occurs through a mechanism independent of reductions in coronary blood flow or activation of the autonomic nervous system (22). As a G protein-coupled receptor, the TP primarily couples to Gq, resulting in phospholipase (PL)Cβ activation and liberation of inositol trisphosphate (IP_3) and diacylglycerol (DAG) from phosphatidylinositol 4,5-bisphosphate (PIP_2) cellular stores (63). IP_3 release leads to rapid mobilization of intracellular Ca^{2+} from the sarco/endoplasmic reticulum (SR) via activation of ligand-gated IP_3 receptors (IP_3Rs). In this regard, treatment of isolated cardiomyocytes with U46619 increased intracellular Ca^{2+} in a dose-dependent manner, where these increases were blocked by the TP antagonist SQ29548 or inhibitors of the IP_3 pathway (23). IP_3-mobilized Ca^{2+}, along with the depolarization phase of the action potential and extracellular Ca^{2+} influx via L-type Ca^{2+} channels (LTCCs), triggers a larger Ca^{2+} release from the SR via ryanodine receptors (RyRs), leading to cardiac contraction. A further mechanism through which the TP may lead to increased intracellular Ca^{2+} is following protein kinase (PK) C activation due to second-messenger DAG liberation from PIP_2. It has been shown by multiple groups that TP stimulation in vascular smooth muscle cells leads to dysregulation of resting membrane potential and subsequent activation of LTCCs (75–78). Mechanistically, Cogolludo et al. demonstrated that U46619 treatment of pulmonary artery smooth muscle cells directly inhibits voltage-gated K^+ channels via a PKCζ-mediated mechanism, leading to subsequent depolarization, LTCC-mediated increase in intracellular Ca^{2+}, and vasoconstriction (76). Chronically elevated or dysregulated intracellular Ca^{2+} cycling plays a central role in hypertrophic signaling in cardiomyocytes (79). In particular, the distinct Ca^{2+} duty cycle produced following IP_3-mediated Ca^{2+} release has been shown to activate pro-hypertrophic pathways, including those involving nuclear factor of activated T cells (NFAT) transcriptional mechanisms (80–82). Notably in the context of TP-mediated signaling, Ca^{2+} overload, aberrant Ca^{2+} cycling, or PKC-mediated phosphorylation, activates the cysteine protease calpain (83, 84). In turn, activated calpain proteolytically degrades myofibrillar proteins including myosin and titin, as well as Ca^{2+}-handling proteins including NCX1 and SERCA2a (85–87). Herein, this study shows decreased degradation of both NCX1 and SERCA2a following *NTP42:KVA4* treatment in the PAB model. We propose that this is one possible mechanism where *NTP42* antagonism of TP signaling may be involved in preventing progression to cardiac dysfunction and heart failure. Besides this, there are other mechanisms which may drive cardiac dysfunction and account for the observed benefits of *NTP42*, including potentially involving free-radical mechanisms and/or contractile machinery modifications (35, 36). Levels of 8-iso-$PGF_{2\alpha}$, a non- enzymatic-, free-radical- derived product of arachidonic acid, are increased in line with heart failure severity and associated with increased ventricular dilation

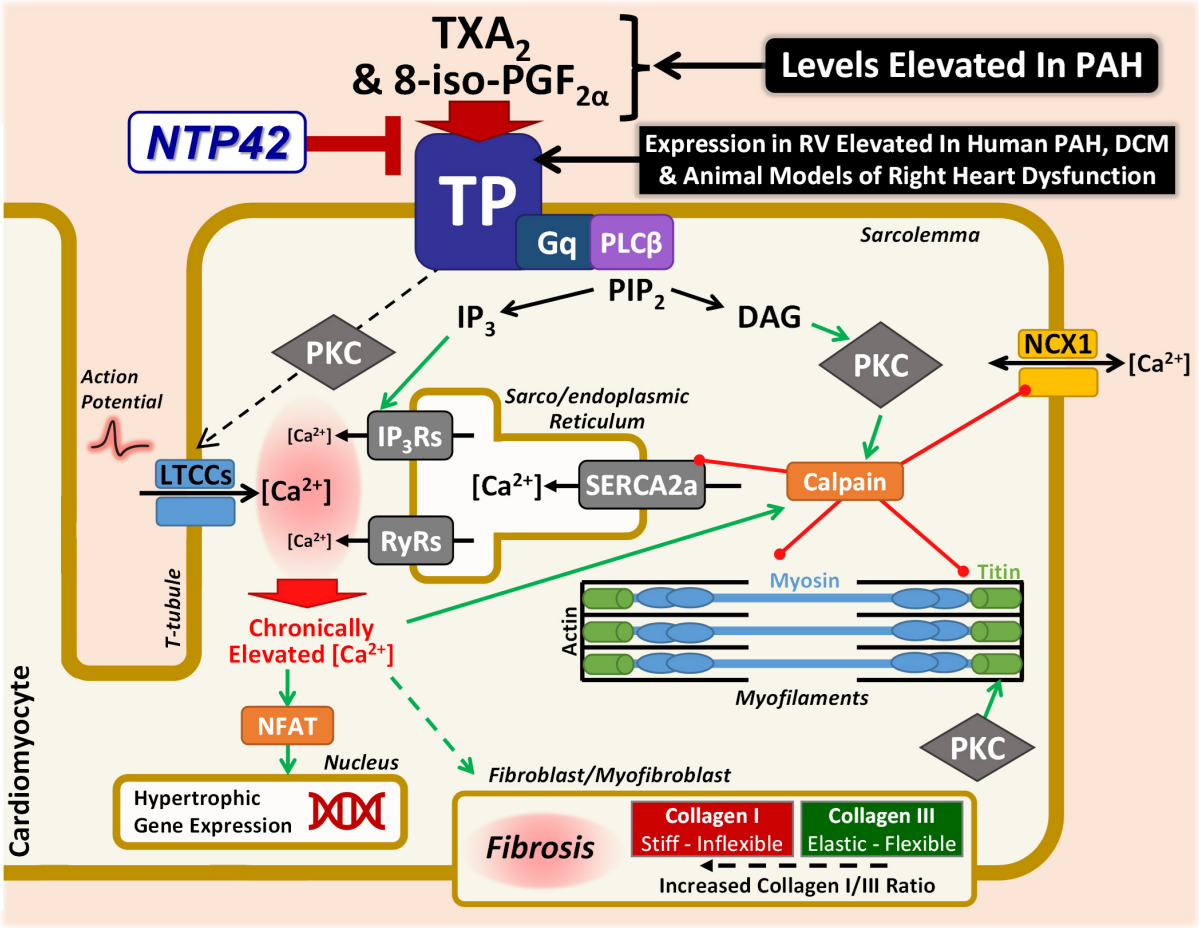

FIGURE 8
Specific antagonism of TP signaling may alleviate key mechanisms involved in cardiac dysfunction in PAH and other cardiac conditions. Schematic representation focusing on the potential mechanisms through which the TXA_2/TP signaling axis may lead to cardiac dysfunction in PAH and other cardiac conditions. Signaling through the Gq-coupled TP leads to PLCβ activation, liberating IP_3 and DAG from PIP_2. IP_3 release mobilizes Ca^{2+} from the sarco/endoplasmic reticulum via IP_3R activation. IP_3-mobilized Ca^{2+}, along with extracellular Ca^{2+} influx via LTCCs, triggers a larger Ca^{2+} release via RyRs. LTCC-mediated Ca^{2+} influx may also occur via a TP-mediated PKC mechanism. Chronically elevated Ca^{2+} leads to hypertrophic signaling via NFAT. Ca^{2+} overload also activates the protease calpain, which degrades myofibrillar proteins including myosin and titin, as well as Ca^{2+}-handling proteins including NCX1 and SERCA2a. This study has demonstrated decreased degradation of both NCX1 and SERCA2a following *NTP42:KVA4* treatment. Furthermore, PKC-mediated phosphorylation of titin leads to increased stiffness, and it is hypothesized that the decreased cardiomyocyte stiffness observed herein following TP antagonism may be as a result of reduced PKC-mediated titin phosphorylation. In addition, TP antagonism may also act on the cardiac fibroblast/myofibroblast, decreasing cardiomyocyte stiffness by resulting in a decreased Collagen I/III ratio and/or net RV fibrosis levels, as demonstrated in this study. Finally, not only are levels of the TP ligands TXA_2 and 8-iso-$PGF_{2\alpha}$ increased in PAH and in other cardiopulmonary conditions, but this study has demonstrated elevated expression of the TP in the myocardium in clinical PAH and dilated cardiomyopathy (DCM), as well as in multiple independent experimental models of PAH and/or cardiac dysfunction. Both TXA_2 and 8-iso-$PGF_{2\alpha}$ would be predicted to further compound the contribution of the TXA_2/TP pathway to detrimental effects within the diseased myocardium.

(88). Notably, the TP also mediates the actions of 8-iso-$PGF_{2\alpha}$, where uniquely TP antagonism is predicted to have the additional benefit of blocking this important pathological mediator of oxidative injury. Furthermore, in the context of the myofibril machinery, as a key determinant of myocardial passive stiffness, the distensibility of titin is heavily regulated by phosphorylation (89). In contrast to PKA/PKG-mediated effects, PKC phosphorylation of titin is widely known to increase cardiomyocyte stiffness (90–92). Mechanistically, as a Gq-coupled receptor directly linked to PKC activation (63), a plausible working hypothesis of how TP antagonism by *NTP42* may lead to decreased stiffness is by reducing PKC-mediated titin phosphorylation. While beyond the scope of this study, further mechanistic investigations are warranted to explore these proposed TXA_2/TP-mediated mechanisms.

Several limitations with the current study are acknowledged. While anesthesia may lead to depressed cardiac function and systemic hemodynamic effects, the anesthetics isoflurane or

sevoflurane used in our studies are known to have only mild effects on cardiac function in rodents (46, 93). Furthermore, as anesthetic regimens were used identically in all animal groups, we would not contend that this affected the study findings. Regarding the pathologies seen in the MCT-PAH and PAB models, it is accepted that these develop in a short time frame and that these preclinical studies may not completely recapitulate the changes that develop progressively over many years in the human condition. However, it is important to acknowledge that our data shows that increased expression of the TP occurred in RVs of several independent and relevant preclinical models, including in the MCT- and SuHx-PAH models, in the PAB model of RV overload, in the ZSF-1 model of heart failure and spontaneously in the *BMPR* Δ71 rodents without intervention. Importantly, increased expression of the TP was also found in clinical RV specimens from subjects with PAH as well as from DCM subjects. While historically most clinical attention in the study of DCM has been LV function and morphology, recent advances in cardiac imaging show that RV involvement is common in DCM, and the presence of RV dysfunction is in fact a major negative prognostic determinant in DCM morbidity and mortality (94). The observations reported herein for the potential involvement of the TP and the TXA_2 pathway in DCM pathogenesis or progression warrant further investigation in relevant preclinical models. The studies reported herein are in line with the current recommendations that, where possible, the effects of an intervention be tested in multiple animal models (27–29). With regard to the choice of preclinical models, and the specific timing of intervention used herein, treatment with *NTP42:KVA4* or PAH SOC was commenced in as delayed a schedule as possible while still permitting sufficient animal survival for analyses through complementary modalities, including invasive assessments of pressure-volume relationships. While the findings from the early interventional MCT-PAH approach reported herein add substantially to previous reports from a preventative MCT-PAH model (27) it is acknowledged that the therapeutic effects of *NTP42:KVA4* could be further investigated using conditions viewed as more reminiscent of a reversal approach, such as by further delaying treatment in an MCT-PAH or PAB model. Finally, regarding the findings from the PAB model, while *NTP42:KVA4* treatment resulted in normalization of isolated cardiomyocyte passive tension, statistical significance for the functional consequence of this improvement in diastolic stiffness, such as in Eed, was not achieved ($P = 0.091$, **Figure 3J**). While trending toward benefit, a possible explanation for this disparity is that while Eed measurement corresponds with the stiffness of the heart *in vivo*, tension measurements are taken *ex vivo* from individual isolated cardiomyocytes. While this isolation procedure preserves the structural and functional properties of the myofibrillar apparatus, these cells present with sarcolemmal damage and loss of intracellular organelles (95). These isolated cardiomyocytes also lack supporting extracellular matrix, fibrotic deposition, or cell-cell interactions that may contribute to diastolic stiffness *in vivo* (95). In addition, while assessments of NCX-1 expression showed unchanged levels of intact functional protein following PAB, the finding of increased degraded protein likely indicates that net expression of NCX-1 may be increased upon PAB, presumably as a compensatory or adaptative mechanism to cope with elevated Ca^{2+} levels and aberrant Ca^{2+} cycling in the compromised cardiomyocyte. Increased NCX-1 degradation in this setting would hence be predicted to have a negative effect on this adaptive response. However, and as demonstrated in this study, treatment with *NTP42:KVA4* led to significantly decreased levels of NCX-1 degradation and this benefit on NCX-1 turnover is hypothesized to be a component of the mechanism of TP antagonism in this model.

In conclusion, these preclinical studies provide evidence that, through antagonism of TP signaling, *NTP42*, administered orally as the clinical formulation *NTP42:KVA4*, may attenuate PAH pathophysiology by not only alleviating pulmonary pathologies but also by reducing RV remodeling and promoting beneficial hypertrophy, resulting in improved cardiac function. These findings in experimental models point to a cardioprotective effect for *NTP42:KVA4* as a component of its therapeutic potential not only in PAH, but possibly in other RV dysfunctions. Finally, expanding on the growing evidence for the role for the TP in PAH and the potential for antagonism of the TP as a therapeutic strategy in its clinical management, such as with *NTP42:KVA4*, this current study also demonstrated elevated expression of the TP in RV tissue from human PAH and other cardiomyopathy patients, validating the potential of this largely ignored target ripe for pharmaceutical intervention.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Protocols for the collection of human biomaterials used in this study were reviewed and approved by the Institute of Cardiometabolism and Nutrition BioCollection (Paris, France). All patients/participants, or their surrogates, provided written informed consent for the use of these biomaterials for research purposes. Animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of IPS Therapeutique (Sherbrooke, QC, Canada), or by the Ethical

Committee of the University of Porto (Porto, Portugal), certified by the Portuguese National Authority for Animal Health.

Author contributions

EM, FR, RA, ED, LB, VS, HR, GC, JG, AL-V, J-BM, CB-S, CL, LH, DM, MH, AV, FP, PM-F, and BK: substantial contributions to the conception or experimental design of the work or the acquisition, analysis, and interpretation of data for the work. EM, LH, DM, MH, AV, FP, PM-F, and BK: drafting the manuscript or revising it critically for important intellectual content. All authors have read and approved the final manuscript.

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Conflict of interest

EM, FR, LB, VS, HR, and BK were employees of ATXA Therapeutics Limited at the time of conducting the work reported in this manuscript. BK was also the founder and a member of the board of directors of ATXA Therapeutics Limited. Studies performed at the University of Porto or at IPS Therapeutique Inc./ToxiPharm Laboratories Inc. were sponsored by ATXA Therapeutics Ltd. LH reported grants from Janssen, honoraria for speaking from Janssen and MSD, supported for attending congresses from Janssen, and participates on the advisory board or steering committee for Janssen, Gossamer Bio, MSD, and Acceleron. DM reported grants from Acceleron, Janssen, and Merck, consulting fees from Acceleron and speakers' honoraria from Bayer, Janssen, and Merck. MH reported grants from Acceleron, AOP Orphan, Janssen, Merck, and Shou Ti, consulting fees from Acceleron, Aerovate, Altavant, AOP Orphan, Bayer, Chiesi, Ferrer, Janssen, Merck, MorphogenIX, and United Therapeutics, speakers' honoraria from Janssen and Merck, and participates on the advisory board for Janssen, Altavant, Merck, United Therapeutics, and Acceleron.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2022.1063967/full#supplementary-material

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Transcription factors in the pathogenesis of pulmonary arterial hypertension—Current knowledge and therapeutic potential

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Pulmonary arterial hypertension (PAH) is a disease characterized by elevated pulmonary vascular resistance and pulmonary artery pressure. Mortality remains high in severe cases despite significant advances in management and pharmacotherapy. Since currently approved PAH therapies are unable to significantly reverse pathological vessel remodeling, novel disease-modifying, targeted therapeutics are needed. Pathogenetically, PAH is characterized by vessel wall cell dysfunction with consecutive remodeling of the pulmonary vasculature and the right heart. Transcription factors (TFs) regulate the process of transcribing DNA into RNA and, in the pulmonary circulation, control the response of pulmonary vascular cells to macro- and microenvironmental stimuli. Often, TFs form complex protein interaction networks with other TFs or co-factors to allow for fine-tuning of gene expression. Therefore, identification of the underlying molecular mechanisms of TF (dys-)function is essential to develop tailored modulation strategies in PAH. This current review provides a compendium-style overview of TFs and TF complexes associated with PAH pathogenesis and highlights their potential as targets for vasculoregenerative or reverse remodeling therapies.

KEYWORDS

epigenetics, epigenetics (chromatin remodeling), transcriptomics, targeted therapy, reverse remodeling, pathogenesis, pulmonary hypertension (PAH)

Introduction

Pulmonary arterial (PA) hypertension (PAH), whether idiopathic (IPAH), hereditary (HPAH), or associated with other conditions (APAH), is a rare, serious and progressive pulmonary vascular disease. Despite improvements in the management of PAH, overall 5-year mortality remains around 30% (1).

PAH is characterized by elevated resistance and pressure in precapillary pulmonary vessels leading to right heart failure, if untreated (2, 3). Pathophysiologically, PAH is characterized by an initial loss of small pulmonary microvessels via endothelial cell (EC) apoptosis in combination with neointima formation through the uncontrolled growth of smooth muscle cell (SMC)-like cells, adventitial fibroblasts (AF), pericytes and mesenchymally transdifferentiated endothelial cells (endothelial-mesenchymal transition, EndMT) (4–6). Although the origin of hyperproliferative neointimal cells in PAH is still not fully understood, recent lineage-tracing studies suggest that the neointima mainly consists of propagating SMC, while EndMT can be detected in a smaller fraction of pathologically remodeled lung vessels (7). Upon persistent vascular inflammation, PAECs also undergo a phenotypic switch from initially increased propensity to apoptosis toward a more apoptosis-resistant and hyperproliferative state thereby further contributing to intraluminal PA obstruction (4, 8).

Currently available pharmacological options in PAH comprise vasodilatory drugs with selectivity for the pulmonary vasculature that attenuate disease progression; namely endothelin receptor antagonists (ERA: bosentan, ambrisentan, and macitentan), phosphodiesterase 5 inhibitors (PDE5i: sildenafil, tadalafil), or soluble guanylate cyclase (sGC: riociguat) stimulator in addition to prostanoids/prostacyclin receptor agonist (epoprostenol, iloprost, treprostinil, selexipag) (9, 10). However, all of the currently available drugs fail to meaningfully reverse PAH-associated structural remodeling of pulmonary blood vessels and lung transplantation remains the only cure. Therefore, novel therapeutic approaches are needed to attenuate PAH progression but also reverse prevalent structural remodeling of the pulmonary vasculature.

In this light, disease-modifying drugs have been an important research focus in PAH over the last few years. Bone morphogenic protein receptor type II (BMPR2) has evolved as a promising molecular target (11, 12). BMPR2 is a transmembrane serine/threonine receptor kinase and a member of the transforming growth factor (TGF)-β superfamily and is a pivotal player in differentiation, inflammation, apoptosis, and proliferation pathways of the pulmonary vasculature (4, 13–15). Pathogenic variants in the *BMPR2* gene account for approx. 75% of HPAH cases and for ∼20% of IPAH cases (16, 17). In addition to germline mutations, BMPR2 expression and BMPR2 signal transduction is universally impaired in all PAH forms, including APAH (16, 18–20) and other precapillary PH forms such as chronic thromboembolic PH and interstitial lung disease associated PH (21, 22) by a plethora of pathological mechanisms [reviewed in (23)]. Pharmacological strategies to re-activate or re-balance BMPR2 signaling in the pulmonary vasculature have been able to restore PA endothelial function, suppress PASMC proliferation and successfully treat PH in experimental models (24–28) and early clinical trials (29–31).

Downstream of BMPR2, non-canonical transcription factors (TFs) can be pharmacologically harnessed to reverse experimental PH (28, 32–35) and repair prevalent DNA damage in PAEC from PAH patients harboring BMPR2 mutations (28) uncovering an additional BMPR2-dependent disease-modifying approach.

This review, therefore, summarizes the current knowledge regarding the role of TFs in PAH pathogenesis and explores their therapeutic potential as disease modifiers in PAH.

Transcription factors: Molecular basics

TFs are key cellular components that—as molecular switches—control gene expression: TFs are DNA-binding proteins that relay external and internal cellular stimuli to a molecular function enabling gene transcription (36). These processes require modification in chromatin structure by chemical modification of DNA and histones as well as other ribonucleoproteins. Therefore, TFs are part of a finely tuned interaction network with chromatin remodeling or histone-modifying proteins to regulate gene transcription (37). TFs bind to highly specific regulatory DNA elements, so called "motifs," within promoter or enhancer regions of their target genes to either activate or repress transcription (38, 39). TFs can regulate transcription either by recruiting chromatin remodeling proteins to induce conformational changes of chromatin to provide DNA accessibility or by directly binding to promotors and enhancers to facilitate the recruitment of additional components of the transcriptional machinery for transcription initiation (37). In this regard, TFs have much higher (> 1,000-fold) affinity to their specific DNA-binding sites within a target gene (= TF-binding site, TFBS) than to surrounding, non-specific DNA sequences (40). These TFBS (or "motifs") are usually found as DNA repeats in cis-regulatory and non-coding DNA elements (see above) (41). As TFs are pivotal to integrating a plethora of cellular processes, TF dysfunction, e.g., through mutations, or (epigenetic) inactivation, contributes to the pathogenesis of numerous diseases (41–45).

Transcription factors: Key regulators in PAH pathogenesis

In the pulmonary vasculature, TFs regulate crucial cellular functions such as proliferation, differentiation, inflammation, cell death, repair, and regenerative programs (39, 45). In PAH, TFs are responsible for altered expression of multiple disease-related genes thereby contributing to defective cellular homeostasis and vascular remodeling (46, 47). Members from eight out of ten TF superclasses (48) are crucially involved in PAH pathophysiology. In this section, we provide a short compendium of the most relevant TFs of each superclass with relevance to PAH (please also see **Table 1** and **Figures 1A,B**).

TF superclass 1

TFs belonging to the basic domains group superclass (S1) bind DNA through a basic region which becomes folded in an alpha-helically manner if added to DNA (48). *At least six members of this superclass contribute to PAH pathogenesis.*

CREB

Cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) functions as an anti-proliferative TF in healthy PASMC. In PAH and associated oxidative stress with excessive production of reactive oxygen species (ROS) like H_2O_2, CREB is downregulated leading to enhanced proliferation of PASMC (49).

HES5

The *hes family bHLH transcription factor 5* (HES5) binds to the Notch-receptor and promotes proliferative signals (50). In PASMCs, HES5 inactivation reverses the proliferative effect of NOTCH3 and induces a shift in gene expression toward a more differentiated phenotype (51).

MYC

MicroRNAs (miR/miRNA) regulate numerous disease pathways in the pulmonary vasculature and have been linked with PAH development (52–54). In this context, Zhang et al. showed that miR-449a-5p, which is downregulated in PAH, represses the activity of the TF *MYC proto-oncogene* (MYC). Lack of MYC repression in PAH PASMC is associated with mitochondrial and metabolic dysfunction as well as phenotype transformation (55).

TWIST1

Expression of *Twist-related protein 1* (TWIST1) is increased in the lungs of PAH patients and TWIST1 has been shown to mediate EndMT thereby contributing to pathological vascular remodeling in PAECs (56, 57).

TF superclass 2

The TF superclass 2 contains TFs with Zinc-coordination DNA-binding domains. Such zinc fingers, consisting of a repetitive pattern of cysteine and histidine residue, represent the most frequent DNA-binding motifs found in eukaryotic TFs (48). *The frequency of zinc fingers among DNA-binding motifs is also represented by the many members of the TF superclass 2 that play a role in PAH.*

PPARG

Peroxisome proliferator-activated receptor gamma (PPARγ) is a member of the nuclear hormone receptor superfamily of ligand-activated TFs. It is pivotal for the regulation of mulitple central processes in pulmonary vascular cells (47, 58–61). PPARγ, which is ubiquitously expressed, represents probably the best-studied TF in pulmonary hypertension. Norbert Voelkel and his group were first to demonstrate that PPARγ is downregulated in lungs from PAH patients and in PAH-associated vascular lesions (62). PPARγ dysfunction in PAEC or PASMC facilitates the hyperproliferative vascular phenotype typical for PAH (47, 63).

In PASMC, a downregulation of PPARγ by short interfering RNA leads to increased proliferation, decreased mitochondrial mass and increased mitochondrial ROS generation (47, 63), which is in part mediated by decreased levels of TFAM, GRP75, and MFN2 (47) and by NF-kB dependent NOX4 upregulation (64). In contrast, pharmacological PPARγ activation is sufficient to reverse experimental PH (58, 65).

In this regard, Hansmann et al. showed that PPARγ-mediated anti-proliferative BMP-2 signaling in PASMC and that loss of PPARγ function in PASMC was associated with the spontaneous onset of experimental PH. PPARγ agonists were able to restore anti-proliferative signaling in wildtype and BMPR2-mutant PASMC, suggesting early on that activation of PPARγ signaling may reverse PAH (66). Mechanistically, in PASMC this is mediated by BMP2-dependent upregulation of a protective autocrine PPARγ—Apolipoprotein E (ApoE)—Low density lipoprotein receptor-related protein 1 (LRP1) axis (66) and inhibition of TGF1-mediated SMAD3/4 and STAT3-FOXO1 signaling (see below) (67). In these studies, Chakraborty et al. used Cre-constructs driven by the Tagln/Sm22-promoter to delete PPARγ in SMC instead of more SMC-specific promoters such as Myh11 (68). The Tagln/Sm22 promoter has been shown to also be active in cardiomyocytes and non-muscle tissues such as myeloid cells and platelets [reviewed in: (68)]. Therefore, future studies need to evaluate to what extent PPARγ's protective function to reverse experimental PH relates to rehabilitation of SMC-specific signaling or also includes effects on additional cell types such as cardiomyocytes as suggested by a recent study of the same group (32).

In PAECs, Vattulainen-Collanus et al. suspected that a lack of PPARγ could result in increased expression of E2F1,

TABLE 1 Transcription factors in PAH.

TF superclass (S1–6)	TF name	Cell type	Expression and function in PAH	References
S1: Basic domains group	CREB	PASMC	Expression: ↓ Function: proliferation↓, migration↓, hypertrophy↓, dedifferentiation↓ and ECM production↓	(49)
	TWIST1	PAEC	Expression: ↑ Function: EndMT↑, vascular remodeling↑	(56)
	MYC	PASMC	Expression: ↑ Function: regulates mitochondrial and metabolic function (in PAH: under hypoxia-induced phenotype transformation proliferation↑ and hypoxia-induced mitochondrial dysfunction↑)	(55)
	HIF1A	PAEC, PASMC	Expression: ↑ Function: metabolic shift↑ (anaerobic glycolysis), angiogenesis↑, proliferation↑, inflammation↑, apoptosis↓	(150, 155, 156, 167–169)
	HIF2A	PAEC, LVEC	Expression: ↑ Function: EndMT↑ via SNAI1/2↑, vascular remodeling↑, occlusive lesions↑, influences vascular resistance	(172)
	HES5	PASMC	Expression: ↑ Function: proliferation effect of NOTCH3↑, gene expression shift into undifferentiated phenotype↑	(51)
	AP1	PASMC	Expression: c-fos↑, c-jun ↑ Function: involved in proliferative response via ET1	(147)
S2: Zinc-coordination DNA-binding domains	PPARγ	PAEC	Expression: ↓ Function: cell cycle progression↑, cell survival↑, apoptosis↓	(32, 46, 47, 60, 63, 66, 70)
		PASMC	Expression: ↓ Function: vessel remodeling↓, proliferation↓, mitochondrial integrity↑, apoptosis↑	
	SNAI2	PAEC	Expression: ↑ Function: EndMT↑ via HMGA1 after BMPR2↓	(57)
	EGR1	PASMC, PAAF	Expression: ↑ Function: vessel remodeling↑, medial hypertrophy↑	(73, 75, 233)
	ZNF740	PAEC	Expression: ↑ Function: proliferation↑, angiogenesis↑	(76)
	KLF2	PAEC	Expression: ↓ Function: proliferation↓, apoptosis↓, inflammation↓, vasodilation↑	(80–82)
	KLF4	PAEC	Expression: ↓ Function: vessel protection↑, regulation of vasodilation, inflammation↓, coagulation↓, and oxidative stress, chromatin accessibility for vasculoprotective genes↑	(83, 84, 234)
	KLF5	PASMC	Expression: ↑ Function: proliferation↑, apoptosis↓	(85)
	PPARGC1A (PGC1A)	PBMC	Expression: ↑ under hypoxia Function: Regulates total antioxidant status via CYTC and SOD, inflammation by activating CYTC	(20, 87, 88)
		PASMC	Expression: ↓ Function: mitochondrial integrity ↑, maintains proliferation-apoptosis rheostat	(88)
		PAEC	Expression: Under BMPR2-loss and normoxia↑, hypoxia↓ hypoxia-reoxygenation↓↓ Function: Promotes mitochondrial health and integrity upon oxidative stress via NRF2-TFAM cascade	(20)
	GATA6	PAEC	Expression: ↓ Function: transcription regulator of genes controlling vascular tone, inflammation and vascular remodeling	(89)
S3: Helix-turn-helix domains	FOXO1	PASMC	Expression: ↓ Function: proliferation↓	(93)
	FOXM1	PASMC	Expression: ↑ Function: proliferation↑, DNA-repair↑, resistance to apoptosis↑	(90, 92, 235)

(Continued)

TABLE 1 (Continued)

TF superclass (S1–6)	TF name	Cell type	Expression and function in PAH	References
	ELK1	PAEC	Expression: ↑ Function: proliferation↑	(95)
	MSX1	Lymphocytes	Expression: ↑ (under BMPR2-loss) Function: capillary regression↑	(96)
	OCT4	PASMC	Expression: ↑ (PSG1 + 5: ↓) Function: proliferation↑ under hypoxia	(98)
S4: Other all-alpha-helical DNA binding domains	SOX17	PAEC	Expression: ↓ Function: Regulates Notch-signaling in pulmonary EC development, PA remodeling↓, SNPs in SOX17 enhancer associated with impaired survival in PAH?	(101, 102, 105)
	TFAM	PAEC	Expression: under BMPR2-loss: normoxia↑, reoxygenation↓ Function: modulates inflammatory response, mtDNA integrity↑, EC survival↑	(20)
	NFY	PASMC	Expression: ↑ Function: regulates genes for proliferation, glycolysis, apoptosis-resistant phenotype↑	(106)
S5: alpha-Helices exposed by beta-structures	MEF-2	PAEC	Activity: ↓ Function: regulates expression of transcriptional targets involved in vessel homeostasis	(107)
S6: Immunoglobulin fold	NFAT	PASMC	Expression: ↑ Function: proliferation↑, migration↑, apoptosis-resistant phenotype↑, Warburg-phenotype↑	(58, 109, 110, 236)
	RUNX1	EPC	Expression: ↓ Function: EHT↑	(111)
	RUNX2	PASMC	Expression: ↑ Function: proliferation↑, vascular remodeling↑, calcification in PA lesions↑, resistance to apoptosis↑, transdifferentiation into osteoblast-like cells↑	(116)
	p53	PASMC	Expression: under hypoxia↓ Function: aerobic glycolysis↓, mitochondrial respiration↑, proliferation↓	(20, 128)
		PAEC	Expression: under BMPR2-loss and normoxia↑, hypoxia↓, hypoxia-reoxygenation↓ Function: p53↓: mtDNA deletion↑, apoptosis↑ p53↑: mitochondrial membrane potential↑, ATP production↑, glycolysis↑, production of cytokines↑	
	NF-KB	PAEC, PASMC	Expression: ↑ Function: inflammation by activation of macrophages, lymphocytes and endothelial cells↑, vascular remodeling↑, EndMT↑	(131)
	TBX4	–	TBX4 mutation associated with childhood-PAH and PAH with lung parenchymal maldevelopment	(136)
	STAT3	PASMC	Expression: ↑ Function: proliferation↑, resistance to apoptosis↑	(137, 184)
	STAT1	PAEC	Expression: ↑ Function: proliferation↑, migration↑, inflammation↑	(138)
S7: beta-Hairpin exposed by an alpha/beta-scaffold	SMAD3	PASMC	Expression: ↓ Function: proliferation↓, migration↓, vascular remodeling↓	(139)
		PAEC	Expression: ↓ (↑ under HERV-K dUTPase stimulation) Function: proliferation↓, migration↓, EndMT↑	(132)
S8: beta-Sheet binding to DNA	HMGA1	PAEC	Expression: ↑ Function: EndMT into SM-like phenotype↑ (with SNAI2)	(57, 139)

TFs by superclass as determined by comparison with the Human Transcription Factor Database (Animal TFDB 3.0; 232) and TFclass (48).

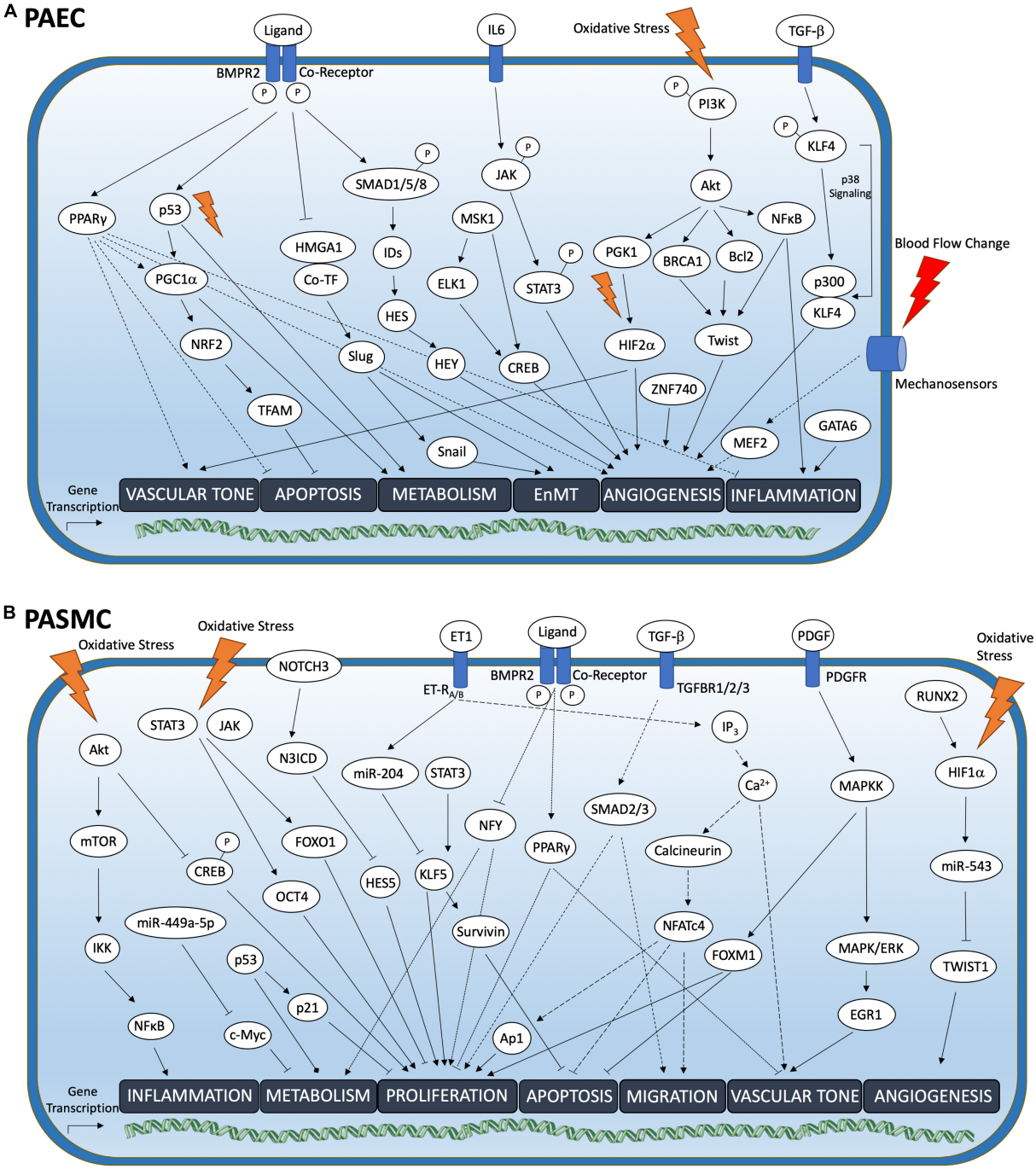

FIGURE 1
Transcription factor pathways in PAEC and PASMC associated with PAH. Overview of pathogenetically relevant transcription factor (TF) pathways in **(A)** pulmonary arterial endothelial cells (PAEC) and **(B)** pulmonary arterial smooth muscle cells (PASMC) upon activation by cell membrane anchored receptor signaling or cellular stress events. Depending on the pulmonary vascular cell type TF activation mediated gene transcription elicits cell-type specific downstream responses. Blue box represents cell membrane. Ca^{2+}, Calcium; IP_3, Inositol 1,4,5-trisphosphate; $ET\text{-}R_{A/B}$, Endothelin Receptor Type A and B; TGFBR1/2/3, TGF-ß receptor 1/2/3; –p, Phosphorylation.

which is associated with a dysregulated Wnt pathway and disturbed angiogenesis and migration (69). PPARγ may also play a role in PAEC's response to DNA damage (70). In cellular studies, depletion of PPARγ was sufficient to promote the development of a PH phenotype by upregulation of cell cycle- and angiogenesis-related genes (71). In an EC-specific

PPARγ knockout mouse model (using the Tie2 promoter), experimental PAH developed spontaneously (63). Additional information on the beneficial effects of PPARγ on the pulmonary vasculature can be found further down in the section on PPARγ TF complexes.

SNAI2

Snail family transcriptional repressor 2 (SNAI2), also known as Slug, a highly conserved zinc finger TF, has been implicated in epithelial-mesenchymal transition (EMT) and EndMT (72). In PAEC, loss of BMPR2 leads to increased expression of High-mobility group protein 1 (HMGA1) and Slug, which is associated with upregulation of SMC markers and EndMT (57).

EGR1

Expression of *early growth response protein 1* (EGR1) is increased in plexiform lesions of PAH (73, 74), is triggered by tissue damage and is associated with pathological remodeling of the lung vessel wall (75). Interestingly, EGR-1 is negatively regulated by PPARγ agonists (75).

ZNF740

Zinc finger proteins (ZNFs) bind classically to DNA, RNA, proteins, and other small molecules and are highly conserved in their binding specificity of a particular protein. Yu et al. identified a novel signaling pathway involved in proliferation and angiogenesis of PAECs and in vascular remodeling *in vitro*. This new signaling axis consists of ZNF740, GDF11, TGF-β-receptor I, and SMAD, which is also involved in the imbalance of pulmonary vascular homeostasis in PAH (76).

KLF2

Krüppel-like Factor 2 (KLF2) is a vasculoprotective factor expressed in endothelial cells that is activated by laminar shear stress and is pivotal for normal lung vessel formation (77). Heterozygous germline missense mutations in KLF2 have recently been associated with HPAH (**Table 2**) (78–80) and KLF2 mRNA expression is strongly downregulated in lungs from rodents and humans with PAH (80, 81). Loss of KLF2 impairs NO synthesis and thereby contributes to the severity of hypoxia-induced PH in Apelin-deficient mice (82). In contrast, adenoviral transduction mediates anti-inflammatory, anti-apoptotic, and anti-proliferative effects in PAEC under nutrient stress (80). Additionally, miRNA isolated from exosomes derived from KLF2-overexpressing PAEC can be therapeutically harnessed to attenuate experimental PH in the Sugen/hypoxia mouse model (80).

KLF4

Krüppel-like Factor 4 (KLF4), a protective PAEC maintenance factor, is inactivated by posttranslational modification upon nitrosative stress, thereby disabling its protective function in the pulmonary vasculature (83). Recently, KLF4 was identified as an interaction partner of the SWI/SNF complex to increase accessibility of enhancer sites which regulate genes essential for endothelial homeostasis under laminar shear stress (84).

KLF5

Krüppel-like Factor 5 (KLF5) has been linked with an apoptosis-resistant and proliferative phenotype in PASMCs (85), as an upstream regulator of HIF1 in PASMC (86). In addition, KLF5 and HIF1 might form a TF complex with yet unknown function (86).

PGC1A

PPARγ coactivator-1α (PGC1A/PPARGC1A), which normally regulates oxidative metabolism and mitochondrial biogenesis, was found to regulate inflammation in blood cells of IPAH patients by activating cytochrome complex (CYTC) under hypoxia (87, 88). In PASMC, PGC1A regulates the expression of the Mitofusin-2 gene MFN2 to maintain mitochondrial integrity. PAH PASMC lacking PGC1A and MFN2 show heightened mitochondrial fragmentation associated with increased PASMC proliferation (88). In PAEC, PGC1A promotes EC survival and sustains mitochondrial membrane potential upon oxidative stress downstream of a non-canonical BMPR2-p53 axis (20).

GATA6

GATA sequence binding protein 6 (GATA6), a member of the ZNF TF family, is upregulated in inactive vasculature and downregulated during vascular injury (89). In PAECs, GATA6 directly regulates ET1 receptor type A (ETA), a gene for controlling vascular tone, as well as pro-inflammatory genes like 5-lipoxygenase-activating protein PAI-1, which is involved in vascular remodeling and increased vascular muscularization (89).

TF superclass 3

The helix-turn-helix superclass (S3) of TFs comprises a DNA-recognition helix that fits into the major DNA groove. Some important TFs regarding their relevance to PAH belong to this superclass.

FOXO1 and FOXM1

Forkhead box proteins O1 (FOXO1) and *M1* (FOXM1) have opposing roles in PAH pathogenesis. While FOXM1 is overexpressed in PASMC of PAH patients and promotes hypoxia-induced proliferation as well as resistance against apoptosis and DNA repair (90–92), FOXO1, which integrates multiple vasculoprotective pathways, shows reduced expression and/or is inactivated in PAH PASMC (93).

TABLE 2 Genetic variants in transcription factors associated with PAH pathogenesis.

Gene symbol	Identifier	Location	Mode of inheritance	Gene-disease validity assertion
SMAD9	HGNC:6774	Chr 13 (36844831.36920854)	Autosomal dominant	Definitive (ClinGen) high evidence (Genomics England) high evidence (BRIDGE consortium)
TBX4	HGNC:11603	Chr 17 (61452422.61485110)	Autosomal dominant	Definitive (ClinGen) high evidence (Genomics England) n/a (BRIDGE consortium)
SOX17	HGNC:18122	Chr 8 (54457935.54460892)	Autosomal dominant	In scope (ClinGen) high evidence (Genomics England) n/a (BRIDGE consortium)
SMAD1	HGNC:6767	Chr 4 (145480770.145559176)	(Pseudo-) autosomal dominant	In scope (ClinGen) low evidence (Genomics England) high evidence (BRIDGE consortium)
SMAD4	HGNC:6770	Chr 18 (51030213.51085042)	(Pseudo-) autosomal dominant	In scope (ClinGen) low evidence (Genomics England) high evidence (BRIDGE consortium)
KLF2	HGNC:6347	Chr 19 (16324826.16328685)	Autosomal dominant	In scope (ClinGen) n/a (Genomics England) n/a (BRIDGE consortium)

TFs are sorted by evidence level of variant-disease association as determined by three consortia (ClinGen Genomics England and BRIDGE consortium). Chr, Chromosome; HGNC, HUGO Gene Nomenclature Committee.

ELK1

ETS Like-1 protein (ELK1) is a member of the E-twenty-six (Ets) ternary complex family of TFs known to stimulate the expression of immediate early response genes involved in cellular proliferation and apoptosis (94). Phosphorylation of Elk-1 in concert with p38-mitogen-activated protein kinase (MAPK) induces PAEC proliferation (95).

MSX1

Msh homeobox 1 (MSX1) is upregulated in lymphocytes of IPAH patients and EC of BMPR2-deficicent mice. Lack of BMPR2-mediated suppression derepressed MSX1 expression which correlates with upregulation of MSX1 target genes in IPAH (96).

OCT4

The octamer-binding TF 4 (OCT4) is a marker for undifferentiated cells, highly expressed in human embryonic stem cells. Even though OCT4 is frequently silenced in differentiated somatic cells (97), Firth et al. detected weak expression of OCT4 isoforms A and/or B mRNA and strong expression of OCT4 pseudogene (PSG) 1 and 5 mRNA in PASMC from healthy controls. In PASMCs under hypoxia or isolated from IPAH patients, mRNA expression of OCT4A/B is upregulated, whereas OCT4 PSG 1 and 5 are downregulated (98). OCT4A/B upregulation in IPAH PASMC might be mediated by HIF2α, which has been shown to directly bind to the OCT4 promoter (99), and is a key regulator of the pro-proliferative response in PAAF (100). This is in line with a study by Bertero et al. showing that HIF2α-dependent OCT4 activation promotes early vascular stiffening as a central pathological event in PAH via induction of microRNA 130/301 (53). Therefore, hypoxia-associated OCT4 upregulation might also contribute to a hyperproliferative, dedifferentiated PASMC phenotype in IPAH.

TF superclass 4

TF superclass 4 comprises transcription factors with alpha-helical DNA-binding domains. At least three members of this superclass have important functions in PAH pathogenesis.

SOX17

SRY-related HMG-box (SOX) 17 is an endothelial-specific TF pivotal for cardiac and pulmonary development by integrating and regulating VEGF, WNT and NOTCH signaling [reviewed in (101)]. Activation of SOX17 represses PA remodeling in the monocrotaline PH model (102). Using genome-wide association studies in PAH, rare pathogenic variants within the coding region of SOX17 and SNPs in an enhancer region have been associated with PAH (103–105).

TFAM

Transcription Factor A, Mitochondrial (TFAM) is a crucial modulator of the inflammatory response to oxidative stress and maintains mitochondrial DNA integrity and cell survival in PAEC under oxidant stress downstream of the non-canonical BMPR2-p53 signaling axis (20).

NFY

Nuclear factor Y (NFY) is epigenetically activated in PASMC isolated from PAH patients to induce pro-proliferative and

glycolysis genes to facilitate the cancer-like hyperproliferative and glycolytic-switch phenotype of PAH PASMC (106).

TF superclass 5

Members of the alpha-helices exposed by beta-structures (S5), as the name suggests, possess alpha helices exposed by a scaffold of beta-strands (48). To our knowledge, there is a single TF of this group with a well-established role in PAH.

MEF2

Transcriptional activity of *myocyte enhancer factor 2* (MEF2) is inhibited in PAEC isolated from PAH patients by nuclear accumulation of histone deacetylases 4 and 5. Thereby, expression of vasculoprotective factors miR-424, miR-503, connexins 37 and 40 as well as KLF2 and 4 is impaired contributing to PAH pathogenesis (107).

TF superclass 6

The Immunoglobulin fold TF superclass (S6) comprises TFs that are characterized by a beta-core structure that induces a DNA contact. Many TFs of this group play a role in the context of PAH.

NFAT

Nuclear factor of activated T cells (NFAT), discovered approx. three decades ago (108), is increased in PAH and regulates PASMC calcium homeostasis in conjunction with calcineurin (CaN) as interaction partner (109). Increased CaN/NFAT promotes PASMC proliferation, survival and migration in chronic hypoxia and MCT-induced PAH (109). In addition, NFAT is upregulated by DNA-damage mediated PARP-1 activation facilitating pulmonary vascular remodeling which was reversible by PARP-1 inhibitors (110).

RUNX1

Liang et al. reported that bone-marrow derived endothelial progenitor cells (EPC) undergo endothelial-to-hematopoietic transition (EHT) to promote pulmonary arterial hypertension. Inhibition of the critical hematopoietic transcription factor *Runt-related transcription factor 1* (RUNX1), also known as acute myeloid leukemia 1 protein (AML1), blocked EHT *in vivo*, and attenuated progression of experimental PH by preventing bone-marrow egression of EPC (111). In addition, RUNX1 mediates expression of neutrophil elastase in PASMC contributing to ECM remodeling in the pulmonary vasculature (112).

RUNX2

RUNX family transcription factor 2 (RUNX2) promotes vascular remodeling and stiffening in vascular disease (113–115). RUNX2 activation promotes vascular calcification. Excessive proliferation of PASMCs in PAH is sustained over time by the loss of miR-204-mediated upregulation of RUNX2 contributing to the development of proliferative and calcified PA lesions (116).

TP53

Tumor protein p53 (p53), the *Guardian of the Genome* (117), is a crucial TF highly conserved in multicellular vertebrates, where it functions as a tumor suppressor by maintaining genome integrity and stability (118). In general, p53 controls many central cellular functions such as cell cycle, DNA repair, apoptosis as well as inflammatory and metabolic homeostasis via its numerous (direct) target genes (119, 120). In the vasculature, depending on the context, p53 exerts both, detrimental (121–123) and regenerative effects (124, 125). In the pulmonary vasculature, p53 fulfills protective functions: Mizuno et al. demonstrated that mice with global p53 knockout developed more severe PH upon chronic hypoxia (126). This is in concert with data showing that pharmacological inhibition of p53 transcriptional activity by Pifithrin-α was sufficient to spontaneously induce PH in rats and to aggravate MCT-induced PH (127). In addition, Wakasugi et al. found that reduced p53 expression in PASMC led to increased aerobic glycolysis and downregulation of mitochondrial respiration thereby contributing to the cancer-like hyper-proliferative "Warburg phenotype" found in PASMC isolated from PAH patients. PASMC-specific p53-knockout, however, did not aggravate hypoxia-induced PH (128). Activation of p53 in PASMC by Nutlin-3, on the other hand, prevented and reversed experimental PH mice (129). In PAEC, p53 is a non-canonical effector downstream of BMPR2 (20, 28). Under oxidative stress, BMPR2-defective PAEC are unable to stabilize and transcriptionally activate p53 and p53-dependent TFs PGC1A, nuclear factor erythroid 2-related factor 2 (NRF2), and mitochondrial transcription factor A (TFAM). Loss of BMPR2-p53 signaling destabilizes mitochondrial DNA integrity and biogenesis causing adenosine triphosphate (ATP)-crises-mediated PAEC apoptosis which is associated with an inability to recover from hypoxia-induced PH (20). While, strictly speaking, p53 itself is a TF complex by auto-multimerization, fine-tuning of cellular effects depends on additional context-specific interaction partners (120). In the pulmonary vasculature, in response to oxidative stress and other DNA-damaging agents, p53 forms a transcriptionally active, vasculoprotective complex with PPARγ in PAEC, PASMC, and PAAF which is BMPR2-dependent (28). This is discussed further in the section on TF complexes of this review.

NF-kB

Strictly speaking, *nuclear factor kappa-light-chain-enhancer of activated B cells* (NF-κB) resembles a TF

complex, best studied in cancer, that mediates transcription of proinflammatory cytokines and thus promotes unfavorable cell phenotypes (130). In advanced PAH, NF-κB is active in PAEC, PASMC and perivascular macrophages and lymphocytes of large and small pre-capillary vessels and is correlated with expression of pro-inflammatory cytokines (131). In PAEC, NF-κB contributes to leucocyte adhesion and inflammation facilitating EndMT (132). In contrast, genetic and pharmacological inhibition of NF-κB reversed and prevented experimental PAH in rodent models, respectively (133, 134). This indicates that targeting might be a reasonable therapeutic strategy.

TBX4

T-box TF 4 (TBX4) is necessary in embryonal development and a gene mutation leads into an autosomal-dominant disorder called small patella syndrome (135). Kerstjens-Frederikse et al. showed that genetically depleted TBX4 is associated with childhood-onset PAH, which, with 0.7 cases per million, is an even rarer disease than PAH (136).

STAT3

Several physiological processes, like cell growth and apoptosis, are affected by the pro-survival TF *signal transducers and activators of transcription*-3 (STAT3) and an inhibition always leads to dramatic changes in biological processes. In PASMCs, Paulin et al. demonstrated that STAT3 activation induces proliferation and resistance to apoptosis by activating NFAT (137).

STAT1

Gairhe et al. showed that *Signal Transducer and Activator of Transcription 1* (STAT1) is elevated in PAECs with caveolin1 loss-of-function. This results in a proliferative, hypermigratory phenotype (138). Also Otsuki et al. showed, that PAECs stimulated with human endogenous retrovirus K (HERV-K) dUTPase have a TLR4-STAT1-dependent inflammatory response (132).

TF superclass 7

This superclass features an alpha-/beta-scaffold in the DNA-binding domain (48).

SMAD3

SMADs, in particular phospho-SMAD1/5/8 are important downstream TF of BMPR2 signaling in the pulmonary vasculature (23). *SMAD family member 3* (SMAD3) is downregulated in lungs from PAH patients or animal models. Loss of SMAD3 is associated with a hyperproliferative and pro-migratory PASMC and PAEC phenotype in a myocardin-related transcription factor (MRTF)-dependent manner (139). In PAEC stimulated with HERV-K dUTPase, activation of SMAD3 can induce EndMT via SNAIL (132). More details about SMAD signaling can be found in the section on TF complexes of this review.

TF superclass 8

In this superclass, DNA binding occurs through β-sheets (48). *A single TF from this superclass has been associated with PAH pathogenesis.*

HMGA1

High Mobility Group AT-hook 1 (HMGA1) is upregulated in PAECs of PAH patients, which is associated with a loss of BMPR2. By inducing SLUG expression, HMGA1 promotes EndMT of PAECs into an SMC-like mesenchymal phenotype in the vasculature of BMPR2-mutant PAH patients (54).

Newly identified transcription factors with unknown impact

In addition to the above-listed TFs with well-established implications for PAH, there are other TFs that might contribute to the disease. A recent comprehensive analysis of chromatin remodeling in PAEC identified 18 novel TFs with differential activity in PAH compared with healthy control donors (more active in PAH: ATF1, ATF7, E4F1, CREB5, RFX3, RFX4, FOSL1, FOSL2, JUN, JUND, BATF; more active in controls: ARI3A, FOXG1, FOXJ3, FOXL1, TBX3, PITX2). These TFs are not discussed further in this review as the exact molecular mechanisms and associated pathophysiological ramifications remain to be elucidated (140).

Pathogenic genetic transcription factor variants in pulmonary arterial hypertension

Specific variants in at least 22 genes have been associated with PAH pathogenesis (78, 105, 141, 142). Of these, six genes code for TFs, namely KLF2, SMAD1, SMAD4, SMAD9, SOX17, and TBX4. Variants in two of these genes show definitive associations with PAH pathogenesis as classified by independent expert panel working groups (**Table 2**).

Transcription factor complexes: Basics

Finely tuned regulation of transcription requires sequence-specific DNA binding of TFs and co-factors. The combination

of multiple TFs is termed combinational control. Cooperation between multiple copies of the same TF, or between different TFs, can stimulate transcriptional synergy in which the regulatory effect of TFs working together is greater than the sum of the individual TFs (143). Cooperative TFs typically generate transcriptional output through such multi-protein complexes (144). A distinction must be made between *(1)* complexes that consist of several TFs, i.e., individual TFs that have a potentially transcriptional modulatory effect on their own, and *(2)* complexes in which TFs are influenced in their function by cofactors. Depending on the binding partner and cell type, a single TF can thus influence various signaling pathways through complex formation (145). Whether a TF complex consisting of at least two TFs is referred to as a TF complex, TF dimer, or TF multimer, or to what extent a distinction is made between TF-TF complex and TF-co-factor complexes has not yet been defined uniformly.

Transcription factor complexes in the pathogenesis of pulmonary arterial hypertension

Although a multitude of TF complexes are known to affect pulmonary cells, only a few of them have been identified to play a pivotal role in the pathogenesis of PAH or harbor therapeutic potential. A descriptive overview of the best-described TF complexes in PAEC and PASMC is given below and summarized in **Figure 2**.

AP1 complex

The activator protein 1 (AP1) TF complex, which is composed of c-JUN-c-FOS and c-JUN–c-JUN dimers, is regulated by many extracellular stimuli like peptide growth factors, pro-inflammatory cytokines, and other forms of cellular stress (146). In the vessel wall of lungs from IPAH patients, higher levels of total and phosphorylated c-FOS and c-JUN were detected, which results in an altered proliferative response in PASMCs mediated by the potent vasoconstrictor endothelin-1 (ET1) (147).

NOTCH1/RBP-Jκ TF-complex

The transmembrane protein *Neurogenic locus notch homolog protein 1* (NOTCH1), which can be activated by extracellular ligands or hypoxia, releases the *Notch intracellular domain* (NICD) through proteolytic cleavage, which then translocates to the nucleus. There, NICD binds to the *Recombinant Signal Sequence Binding Protein J kappa* (RBP-Jκ) to form a heterodimeric TF complex. This complex positively influences the proliferation of PAEC and exerts anti-apoptotic effects (148). In addition, PAEC-PASMC contact, mediated by BMPR2-activated NOTCH1, induces transcription of endothelial regeneration genes, and coordinates the link between PAEC metabolism and chromatin remodeling to activate vascular homeostasis and repair in response to endothelial injury (149).

Hypoxia-inducible factor complexes

Hypoxia-inducible factor (HIF) represents a TF complex that is highly responsive to subtle changes in the environmental oxygen content of the lung. The HIF complex is permanently formed and degraded under normoxic conditions (150) and HIF complex dynamics are finely tuned: With decreasing ambient oxygen content the complex is rapidly stabilized and is also degraded within minutes when reoxygenation occurs (150, 151). In the lung, HIF isoforms HIF1α and HIF2α, individually form a TF complex with HIF1β (also known as *aryl hydrocarbon receptor nuclear translocator*, ARNT) (152–154).

Under hypoxia, HIF1 (= HIF1α/HIF1β complex) recruits another co-factor, CREB/p300 to bind to the hypoxia-responsive element (HRE) in the promoter region of its target genes (151) to transcriptionally regulate angiogenesis, vascular tone and remodeling (150, 151, 155, 156).

HIF1 is the predominant hypoxia sensor in PASMC and promotes a hyperproliferative PASMC phenotype (157). HIF1α expression is increased in pulmonary arteries of PAH patients (158) and contributes to the hyperproliferation of PASMC by modulating the vascular tone through altered expression of membrane ion channels in PASMC (159, 160). HIF1 directly induces expression of angiogenetic genes like VEGF (156) via nitric oxide (NO) synthases 2 (NOS2) which synthesizes the most potent vasodilator NO (158, 161, 162). Under conditions of reduced NOS2 expression or impaired activity, the relaxing effect of NO on the PA vascular bed is attenuated thereby facilitating vascular remodeling and neointima formation via increased PASMC proliferation and resistance to apoptosis (163, 164). In addition to NO synthesis, Wang and Ying describe another mechanism influencing vascular tone: Loss of HIF1α induces expression of miRNA-543, which then downregulates Twist1 resulting in increased expression of the potent vasoconstrictor ET-1 in PASMC (44). Mitogenic effects of ET-1 have been shown to be associated with PA remodeling (165). Vascular remodeling in the lung is further facilitated by HIF1 via transcriptional repression of miRNA-223 in PASMCs leading to increased *PARP-1* expression (166), via a feedback loop with KLF5 (see above, 86) and by HIF1-dependent upregulation of plasminogen activator inhibitor-1 (PAI-1) (167) and Ras association domain-containing protein 1A (RASSF1A) to promote hypoxia-signaling to PASMC in PAH thereby likely conferring a cancer-like PASMC phenotype (168). Interestingly,

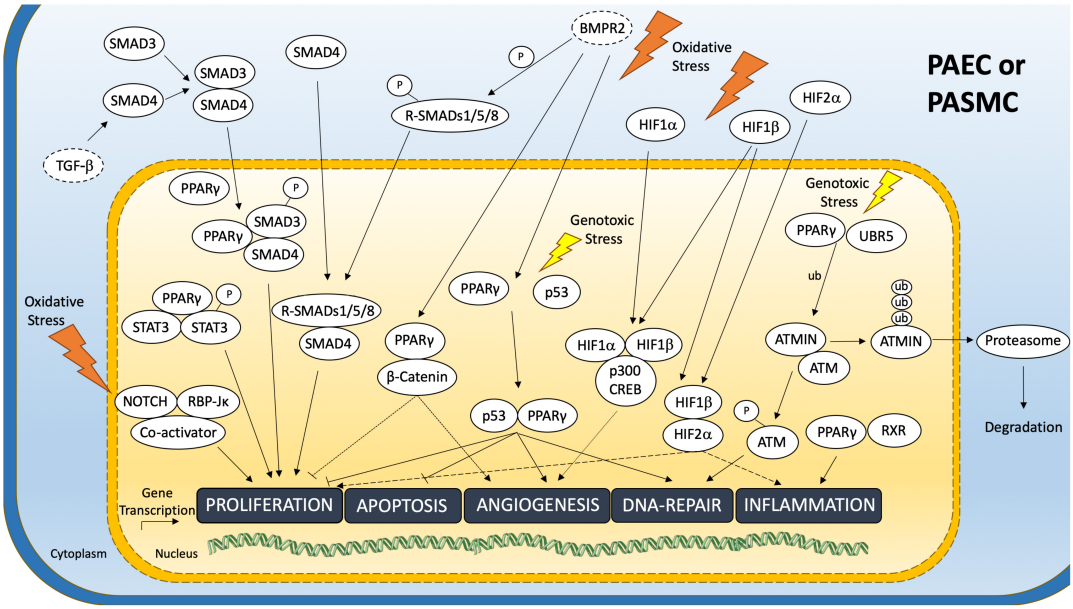

FIGURE 2
Transcription Factor Complexes in the Pulmonary Vasculature related to PAH. Overview of most relevant TF complex pathways in the pulmonary vasculature. TF complex formation upon complex inducing microenvironmental stimuli and downstream TF complex mediated gene transcription programs are shown. Yellow box represents nucleus. Blue box represents cell membrane. Dotted ovals indicate cell membrane anchored receptors. ub, Ubiquitin residue/Ubiquitination; −p, Phosphorylation.

PASMC proliferation caused by the transient HIF1 activation is attenuated by treatment with PPARγ activator rosiglitazone (169). HIF1α also mediates a metabolic shift to a cancer-like Warburg phenomenon in PAEC (158). Nevertheless, in PAEC HIF2α appears to be the predominant HIF isoform (147, 148).

HIF2α is increased in lung vascular ECs (LVECs) of IPAH patients which was associated with downregulation of HIF2α degrading enzyme prolyl hydroxylase domain protein 2 (PHD2). This resulted in induction of SNAI1/2 expression facilitating EndMT and formation of pulmonary vascular lesions (170). Endothelial-specific KO of PDH2 leads to experimental PH under normoxia which was dependent on HIF2α but not HIF1α (171). HIF2α also influences vascular resistance in the pulmonary vasculature. Mice with heterozygous global KO of HIF2α were protected from hypoxia-induced PH in an ET-1- and plasma catecholamine-dependent manner (172). Endothelial HIF2α disturbs EC NO homeostasis by upregulation of Arginase and mice with endothelial deletion of HIF2α were protected from hypoxia-induced PH (173). On the other hand, an activating mutation in the HIF2α gene is associated with erythrocytosis and pulmonary hypertension (174, 175), which, interestingly, seems to be mostly related to a phenotypic switch of PASMC but not PAEC (176).

It is likely that the HIF complex also interacts with additional TFs in the pulmonary vasculature to fine-tune hypoxia-associated gene expression. In this light, Palmer et al. suggest that HIF1α cooperates with activating Transcription Factor 1 (ATF-1) and/or CREB-1 either in the form of a complex or to functionally replace the two TFs in hypoxia (151). Additional interaction partners and their role in PA maintenance and remodeling remain to be elucidated.

SMAD complexes

Dysfunction of the BMPR2 signal transduction is found in all forms of PAH (24, 177, 178). Normally, BMPR2 activation triggers a canonical signaling pathway resulting in phosphorylation of *Receptor*-regulated *Small Mothers Against Decapentaplegic Homolog Family* members (R-SMADs, SMAD 1/5/8) (23). Activated R-SMADs form a heteromeric complex with common mediators (Co-SMADs, SMAD4). The R-SMAD-Co-SMAD complex translocates into the nucleus (179). SMAD proteins are crucial for cell development, the transcription of specific vasculoprotective target genes (180) and growth regulation by activating the TGF-β superfamily. While R/Co-SMADs activate the TGF-β pathway, I-SMADs disrupt the TGF-β pathway. Disturbed SMAD signaling leads to increased MSX1, which seems to be associated with IPAH and HPAH pathogenesis (96). The BMPR2-Smad axis is a promising therapeutic target as SMAD signaling can be pharmacologically reactivated on the BMPR2 level by tacrolimus (24, 181).

STAT3 homodimer

Signal transducer and activators of transcription-3 (STAT3) is a cytoplasmatic transcription factor, which is activated in response to cytokines (IL-6), growth factors (PDGF), and agonists (ET1) and mediates its function as a homodimer (182). It plays an important role in regulating the expression of multiple proteins and TFs associated with the pathogenesis of PAH such as HIF1α, Pim1, and NFAT. STAT3 signaling confers a cancer-like, hyperproliferative, anti-apoptotic phenotype to PAH PASMC (183). Functionally, STAT3 promotes pro-inflammatory processes by increasing the recruitment of inflammatory cells through induction of interleukin-6 (IL-6). In addition, STAT3 activation increases proliferation and migration of vascular SMCs in response to vascular injuries (184). STAT3 also regulates the miR-cluster17/92 and miR-204, which regulates BMPR2 translation. Via induction of KLF5, STAT3 augments transcription of the anti-apoptotic gene BIRC5 (survivin) to increase PASMC proliferation (184, 185). Another way, STAT3 promotes a pro-proliferative PASMC phenotype found in PAH patients is by increasing PIM1 gene expression and Nuclear Factor of Activated T Cells 2 (NFATC2) activity (183, 185).

YAP/TAZ/TEAD complex

Transcriptional co-regulators *Yes-associated protein 1* (YAP) and *Transcriptional Co-Activator with PDZ-Binding Motif* (TAZ, official gene symbol: WWTR1) form complexes with *TEA domain* (TEAD) transcription factors and function as mechanotransducers and -effectors of the Hippo signaling cascade (186). Altered mechanobiological properties are a well-established pathological feature of PAH and stiffening of the ECM initiates a vicious circle of vessel wall remodeling that is further promoting ECM rigidity [reviewed in: (187)]. In this context, Bertero et al. have shown that ECM remodeling activates YAP/TAZ, which then induces expression of miRNA-130/301 independent of TEAD (188). miRNA-130/301 then promoted PA collagen deposition, lysyl oxidase (LOX) activation with subsequent release of pro-fibrotic factors causing proliferation of PAEC, PASMC, and PAAF and subsequent vessel wall remodeling, ECM stiffening and thus further YAP/TAZ activation (188). In addition, pulmonary vascular stiffening-associated YAP/TAZ activation also promoted metabolic reprogramming of PAEC through direct transcriptional regulation of the key enzyme of glutaminolysis, GLS1 (189). YAP/TAZ activation also contributes to PAH severity by suppressing anti-inflammatory and vasodilatory cyclooxygenase-2 and prostaglandin $F_{1\alpha}$ in a TEAD-dependent fashion in PASMC (190).

PPARγ/RXRα complex

Peroxisome proliferator-activated receptors (PPARs) belong to a family of nuclear hormone receptors called nuclear factors. Different PPAR isoforms (α, β/δ, γ) are ubiquitously expressed, while PPARγ represents the main isoform in pulmonary vascular cells (66, 191). PPARs usually bind to a nuclear receptor response element (NRRE) in the promoter region of their target genes in complex with a co-repressor or co-activator and a histone deacetylase. Interaction with PPAR ligands forces co-repressor dissociation to activate the transcription machinery (192). Likewise, co-activators heavily influence the cellular response of this TF-complex by chromatin acetylation thereby making it accessible to RNA polymerase II (61). Typically, PPARs form heterodimers with their canonical interaction partner, *retinoid X receptor* (RXR), to control transcription of target genes that play a critical role in energy balance, including triglyceride and fatty acid metabolism and glucose homeostasis: processes that are dysregulated in obesity, diabetes, and atherosclerosis (58, 60, 61, 193, 194). It is highly likely, that most anti-inflammatory and vasculoprotective PPARγ effects in the pulmonary vasculature for which no exclusive PPARγ interaction partners have been identified are mediated by the PPARγ/RXRα complex (34, 59, 194).

PPARγ/MRN and PPARγ/UBR5 complexes

Although not a classical TF-TF complex, another DNA-associated PPARγ protein complex is of special interest for PAH pathogenesis. Upon genotoxic stress, PPARγ interacts with the DNA damage-sensing heterotrimer MRE11-RAD50-NBS1 (MRN) to facilitate DNA repair via the ATM pathway. This also requires the interaction of PPARγ with UBR5, an E3 ubiquitin-protein ligase, responsible for damage-associated degradation of ATM inhibitor (ATMIN) (70). Interestingly, the PPARγ-UBR5 interaction is disturbed in PAEC of PAH patients. This corresponds to an inability to activate the DNA damage response pathway upon genotoxic stress and to repair DNA damage (70).

PPARγ/β-catenin complex

The protein β-catenin is normally involved in cell adhesion and gene transcription. In PAECs, PPARγ forms a BMPR2-mediated TF complex with β-catenin (PPARγ/β-catenin complex). In PAH patients with a dysfunctional BMPR2-signaling, the expression of PPARγ//β-catenin inducible vasculoprotective genes such as Apelin is reduced. Apelin-deficient PAECs are prone to apoptosis and promote PASMC proliferation (46).

PPARγ/SMAD3 and PPARγ/STAT3 complexes

Two other interesting complexes are related to PASMC proliferation and metabolism: PPARγ/SMAD3 and PPARγ/STAT3. On the one hand, PPARγ inhibits the well-known canonical TGF-β1-pSMAD3/4 signaling pathway through interactions with SMAD3 and, on the other hand, the non-canonical TGF-β/STAT3-FoxO1 signaling, which is mostly unknown (33). Interestingly, the direct interaction between PPARγ-SMAD3 (cytoplasm) and PPARγ-STAT3 (nucleus) inhibits TGF-induced phosphorylation and shuttling of SMAD3/4 and STAT3/FoxO1 through pioglitazone which resulted in altered proliferation and metabolism (67).

PPARγ/p53 complex

Under conditions of genotoxic stress PPARγ and p53 form a TF complex in various cell types (28, 195). In the pulmonary vasculature, PPARγ and p53 interact physically in all cell types across the vessel wall, namely PAEC, PASMC, and PA adventitial fibroblasts, to activate a vasculo-regenerative gene transcription program, which in PAEC is BMPR2-dependent (20, 28). Of note, the PPARγ-p53 TF complex can be harnessed pharmacologically as Nutlin-3, a p53-stabilizing compound, induces complex formation even under conditions of dysfunctional or lacking BMPR2, thereby salvaging impaired transcription of vasculoprotective genes including but not limited to genes promoting EC metabolism, survival, angiogenesis, and DNA repair (28). In a genetic PAH model with endothelial cell-specific BMPR2 knockout Nutlin-3 induces formation of the PPARγ-p53 complex and upregulation of complex target genes in lung microvascular EC was associated with reversal of persistent pulmonary hypertension, PA remodeling, and regeneration of pulmonary microvessels (28).

Therapeutic potential of transcription factors in pulmonary arterial hypertension

Despite the many advances in recent decades, PAH remains a disease with a poor long-term prognosis. If left untreated, around 2–10% of patients die in the first year after diagnosis (196). Current therapies can only delay, but not prevent or reverse progression to right heart failure (11). Currently approved PAH-specific therapies target four different pathways: (1) the endothelin pathway promotes vasoconstriction and proliferation, therefore endothelin receptor blockers (ERA) are used, (2) prostacyclins or prostanoid receptor agonists directly promote vasodilatation and partially exert anti-proliferative effects, (3) activation of the NO-sGC-cGMP pathway has vasodilatory and anti-proliferative effects, and (4) in the subset of vasoresponsive PAH patients voltage-dependent calcium-channel blocker are used (196, 197). An early and upfront combination of these drugs is recommended to improve long-term outcomes (198). Thus, although long-term mortality has significantly improved, it remains high (199, 200).

Currently approved pharmacological options for PAH mainly influence the vascular tone. Therefore, current medication cannot significantly reverse the pathologically dysregulated signaling pathways that lead to vascular remodeling through inflammation, growth factor signaling, and metabolic dysfunction (10). New treatment options for PAH patients are therefore needed to further improve outcome.

In this light, TF-based therapies might pave way for reverse remodeling strategies. TFs are involved in numerous pathological conditions like cancer, diabetes, or cardiovascular diseases. However, TFs were long deemed "undruggable," yet targeting transcription factors for therapy has become reality (201). Strategies include the use of small molecule compounds to modulate TF activity, e.g., by inhibition of TF (-co-factor) complex formation or DNA binding or promotion of TF degradation [reviewed in (202)]. For some diseases, TF targeting therapies are clinically well established like TZD therapy in type 2 diabetes mellitus (203). For PAH, multiple novel compounds are currently in clinical trials with promising candidate TF pathways still in preclinical phases (204).

One such candidate is FOXO1 (205). Loss of FOXO1 function in PASMCs promoted a disease phenotype *in vitro* and *in vivo* and caused experimental PAH. On the other hand, pharmacological activation of FOXO1 was associated with reconstitution of a healthy PASMC phenotype and reversal of experimental PH (93). The multitude of routes and options for pharmacological FOXO1 activation (206) augurs well for FOXO1-based PAH treatment strategies (93, 206).

HIF has been a TF of interest as a therapeutic target for PAH for many years (150, 157). Early evidence indicated that pharmacological inhibition of HIF1 and HIF2α attenuated hypoxia-induced pulmonary hypertension, RV hypertrophy and PA remodeling by inhibiting intracellular Ca^{2+} release and pH changes upon hypoxia in PASMC (207). In general, at least 12 different pharmacological inhibitors of HIF1 and HIF2α were able to attenuate, prevent or reverse experimental pulmonary hypertension in rats or mice [reviewed in (157)] and multiple strategies appear feasible: Besides pharmacological inhibition of HIF signaling, destabilization of HIF via activation of HIF-degrading enzyme cascades or disruption of HIF complexes have all shown promising results as potential therapeutic strategies in experimental

PH (and, partly, in ECs isolated from PAH patients) (208–210). In addition, HIF augurs well for novel combination therapies since pharmacological inhibition of HIF2α with simultaneous activation of p53 was more effective in reversing experimental PH and vascular remodeling than either treatment alone (211). This is particularly interesting, as pharmacological activation of p53 has been shown to reverse experimental PH by PASMC- (129) and PAEC (28)-specific mechanisms (see below).

The YAP/TAZ/TEAD pathway can also be harnessed as a therapeutic target in PAH. Pharmacological blunting of YAP/TAZ activation by glutaminase inhibitors, LOX inhibitors, ApoE activators or gene therapy using adeno-associated viruses expressing shRNA against the newly identified YAP/TAZ upstream regulator HSP110 attenuated or reversed experimental PH in rodent models (188, 189, 212).

Despite recent evidence that emphasizes the beneficial role of HIF2α inhibition (in endothelial cells) as a therapeutic target for pulmonary hypertension (208–210), thorough selection of patients to test proof-of-concept of these results in humans will be necessary as endothelial HIF2α appears to be crucial for vascular survival and maintenance of a functional alveolar structure (213, 214). As an alternative, targeting HIF1 in PASMC might be a reasonable approach (157).

As mentioned before, PPARγ is pivotal for maintaining pulmonary vascular homeostasis via complex formation with various interaction partners. PPARγ activation through endogenous ligands or pharmacological compounds has been shown to convey a broad spectrum of beneficial functions in the pulmonary vasculature from facilitation of normal cell signaling to maintaining pulmonary vascular cell homeostasis and promoting reverse remodeling of pathological vascular changes associated with PAH (28, 32–34, 46, 53, 63, 66, 67, 69, 215–219). In PAH studies, pharmacological activation of PPARγ is achieved by using thiazolidinediones (TZD, including Rosiglitazone and Pioglitazone), a class of drugs that has been under scrutiny for some time due to unwanted and potentially harmful side effects (34).

Earlier studies mostly used Rosiglitazone showing beneficial effects in various animal models of PH (53, 66, 216, 219). The first evidence in PAH came from Hansmann et al. who showed a complete reversal of right ventricular and pulmonary arterial remodeling by inhibition of proliferation and promotion of insulin sensitization in PASMC (66, 216). This was further substantiated in additional PH animal models by Liu et al. (219) as well as Bertero et al. (53). In a PAEC-dependent mechanism, Rosiglitazone restored miR-98 expression to attenuate ET-1-mediated hypoxia-induced PH (220). Due to the more favorable side effect profile, recent PH studies have used Pioglitazone (34). Pioglitazone also reversed PA and RV remodeling through beneficial effects on PASMC and cardiomyocytes (32, 33, 67, 217) by inhibiting canonical and non-canonical TGFβ1 signaling (33, 67), restoring mitochondrial homeostasis and improving cellular energy production by optimization of β-oxidation and glucose utilization (32).

Recently, we have also shown that PPARγ signaling is essential for Nutlin-3-mediated vasculoregeneration by modulating PAEC-protective p53 signaling (28). The small molecule compound Nutlin, currently in clinical trials for various cancers (221–223), induces activation of the PPARγ/p53 complex and a vasculo-regenerative gene transcription program in PAEC, PASMC, and PAAF (28). This resulted in reversal of persistent pulmonary hypertension in mice with endothelial-specific loss of BMPR2 via restoration of endothelial function and regeneration of pulmonary microvessels (28). In PAECs harboring BMPR2 mutations that were isolated from patients with PAH, Nutlin-induced PPARγ/p53 target genes facilitated the repair of prevalent DNA damage (28). In PASMC-based PH models, Nutlin-3 was also successful in preventing and reversing experimental PH by inhibiting PASMC proliferation through induction of a quasi-senescent phenotype (129). Since the PPARγ/p53 TF complex is also formed in PASMC (28) it would be interesting to see to which extent the beneficial effects of Nutlin-3 on PASMC are mediated by PPARγ/p53 complex target genes.

In addition to the direct targeting of TFs, the therapeutic potential of modulating TF co-factors or epigenetic factors which alter TF DNA is currently being investigated [reviewed in (45)].

In light of the recent advances in molecular strategies to modulate TF function, it appears to be only a matter of time before TF-based therapies will become a clinical reality in PAH treatment regimens (35).

Conclusion

A growing body of evidence highlights the central role of TFs in the pathogenesis of PAH. Currently approved therapies mainly modulate vascular tone, but fail to significantly reverse pathological vascular changes in pulmonary arteries and microvessels or the right ventricle/heart. Dysregulation of TF function is closely associated with pathological remodeling of the pulmonary vasculature and the right heart. Multiple TFs have been identified that are related to either maintaining pulmonary vessel homeostasis or, if dysfunctional, contributing to vascular pathology. Even well-studied, pathways commonly dysbalanced in PAH such as BMPR2 and TGFβ signaling, often disembogue in a highly intertwined network of downstream TFs. These TFs often form complex protein-protein interaction networks (e.g., PPARγ) to elicit cell-specific, in part opposing functions, adding complexity.

However, critical knowledge gaps remain. Most importantly, available data suggest that TFs operate through elaborate networks comprising multiple TFs and Co-factors (28, 53, 70). Investigation of individual TFs may not fully reflect their biological function in the pulmonary vasculature and how they integrate a multitude of intracellular and extracellular signals. Therefore, additional systems biology approaches are needed to dissect the pathobiology of complex TF networks. An additional layer of complexity is added by chromatin remodeling phenomena in PAH, which directly affect TF activity (84, 140). In the pulmonary vasculature, it is thus necessary (a) to fully understand the underlying epigenetic mechanisms that facilitate three-dimensional chromatin conformation and accessibility and (b) how exactly epigenetic modifications affect TF networks. This is especially important in light of epigenetic modifiers emerging as druggable targets in PAH (224, 225).

While current data suggest that transcriptional dysfunction is an early event in PAH pathogenesis (4, 226, 227), the spatial resolution of TF dysfunction is less understood. Even though there is growing evidence that (microvascular) endothelial dysfunction precedes the pathological changes in PASMC and PAAF (228), it remains unclear what microniche-specific factors contribute to cell-type specific TF functions. In this regard, the application of single-cell epigenomics and multi-omics technologies will help uncover cell-type specific TF networks.

Therefore, the characterization of molecular TF functions, binding partners, and modes of action are essential for understanding PAH pathogenesis and identification of new therapeutic targets. Current experimental TF-based therapeutic strategies focus on modulating individual TF function, stability, or TF interaction partner network formation with very promising results.

Although specific targeting of dysregulated TF pathways in PAH is advantageous over the currently available broad and rather symptomatic therapeutic approaches, off-target effects need to be mitigated when using systemic drug strategies (229). Hence, utilizing gene therapy approaches with high selectivity (tropism) for specific pulmonary vascular cell types might be useful to overcome this (230, 231).

In summary, recent advances in our understanding of the underlying molecular mechanisms as well as tailored modulation of TF function pave the way for TF-based vasculo-regenerative or reverse remodeling therapies. The clinical usability of TF-based therapies needs to be validated in upcoming clinical trials.

Author contributions

CM, JR, and JKl reviewed the literature and collected the data. CM made the illustrations and drafted the manuscript with support of JKl, JKö, and JKH. LH and HK helped to revise the manuscript and contributed to important intellectual content. JKö and JKH were responsible for oversight, design, manuscript preparation, and revision. All authors have read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Involvement of SUR2/Kir6.1 channel in the physiopathology of pulmonary arterial hypertension

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Aims: We hypothesized that the ATP-sensitive K^+ channels (KATP) regulatory subunit (ABCC9) contributes to PAH pathogenesis. *ABCC9* gene encodes for two regulatory subunits of KATP channels: the SUR2A and SUR2B proteins. In the KATP channel, the SUR2 subunits are associated with the K^+ channel Kir6.1. We investigated how the SUR2/Kir6.1 channel contributes to PAH pathogenesis and its potential as a therapeutic target in PAH.

Methods and results: Using *in vitro*, *ex vivo*, and *in vivo* approaches, we analyzed the localization and expression of SUR2A, SUR2B, and Kir6.1 in the pulmonary vasculature of controls and patients with PAH as in experimental pulmonary hypertension (PH) rat models and its contribution to PAH physiopathology. Finally, we deciphered the consequences of *in vivo* activation of SUR2/Kir6.1 in the monocrotaline (MCT)-induced PH model. We found that SUR2A, SUR2B, and Kir6.1 were expressed in the lungs of controls and patients with PAH and MCT-induced PH rat models. Organ bath studies showed that SUR2 activation by pinacidil induced relaxation of pulmonary arterial in rats and humans. *In vitro* experiments on human pulmonary arterial smooth muscle cells and endothelial cells (hPASMCs and hPAECs) in controls and PAH patients showed decreased cell proliferation and migration after SUR2 activation. We demonstrated that SUR2 activation in rat right ventricular (RV) cardiomyocytes reduced RV action potential duration by patch-clamp. Chronic pinacidil administration in control rats increased heart rate without changes in hemodynamic parameters. Finally, *in vivo* pharmacological activation of SUR2 on MCT and Chronic-hypoxia (CH)-induced-PH rats showed improved PH.

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Conclusion: We showed that SUR2A, SUR2B, and Kir6.1 are presented in hPASMCs and hPAECs of controls and PAH patients. *In vivo* SUR2 activation reduced the MCT-induced and CH-induced PH phenotype, suggesting that SUR2 activation should be considered for treating PAH.

KEYWORDS

ATP, ABCC9, pulmonary arterial tone, migration, proliferation, metabolism

1. Introduction

Pulmonary arterial hypertension (PAH) is a severe and complex disease, defined as an elevation of >20 mmHg in the mean pulmonary artery (PA) pressure, pulmonary vascular resistance (PVR) of >2 Wood units at rest, and a PA wedge pressure of ≤15 mmHg (1). PAH is a complex and multifactorial disease that is associated with narrowing of the distal PA (diameter < 500 μm), leading to right ventricular (RV) hypertrophy and failure, and ultimately death (2). In the last two decades, more than 20 genes have been identified to be associated with a genetic predisposition to PAH, including two genes that encode for potassium channel proteins: *KCNK3* (Potassium Two Pore Domain Channel Subfamily K Member 3) and *ABCC8* (ATP-binding cassette subfamily C member 8) (3–5). In 2018, Bohnen et al. identified 12 loss of function (LOF) mutations in *ABCC8* (5). *ABCC8* encodes for the SUR1 protein, a regulatory subunit of ATP-sensitive-K^+ channels (KATP). *ABCC8* mutation carriers are younger at diagnosis than those with idiopathic PAH (median age at diagnosis 14 years vs. 42 years) (5).

Four Kir6.x constitutes KATP channels (Kir6.1 or Kir6.2) and four sulfonylurea receptor subunits (SUR1 or SUR2A or SUR2B, depending on the cell type) (6). KATP channels are activated by reducing cytosolic ATP concentrations or elevation of nucleotide-diphosphate concentrations (7). Classically, SUR1/Kir6.2 channel is described to constitute the pancreatic KATP. However, we have previously shown that SUR1/Kir6.2 are expressed in pulmonary circulation and that SUR1 activation may be considered a therapeutic target for PAH (8). SUR2, encoded by the *ABCC9* gene, can be spliced into two isoforms: SUR2A or SUR2B. SUR2A/Kir6.2 channel is generally considered the cardiac KATP, while SUR2B/Kir6.2 and SUR2A/Kir6.1 are usually vascular KATP channels (7).

The pharmacological SUR1 activators used in the previous study could also weakly activate SUR2, as the transcripts for SUR2B and Kir6.1 were previously observed in hPASMCs and rat pulmonary arteries (9). Additionally, the selective SUR2 activator, pinacidil, induced pulmonary artery relaxation (10), and the non-selective KATP channel opener iptakalim inhibited the proliferation of control hPASMCs (11). Moreover, previous work suggested that global KATP channel opener nicorandil or iptakalim reduced the severity of PH in the preventive approach (12–14). We hypothesized that SUR2 and Kir6.1 could be additional actors in the pulmonary circulation and PAH pathogenesis. We investigated the localization, expression, and function of SUR2A, SUR2B, and Kir6.1 in controls, PAH-human pulmonary arterial endothelial cells (hPAECs), and human pulmonary arterial smooth muscle cells (hPASMCs), and experimental models of pulmonary hypertension (PH). We evaluated the consequences of SUR2/Kir6.1 channel activation in the proliferation rate and migration of hPASMCs and hPAECs. We assessed the role of the SUR2/Kir6.1 channel in pulmonary arterial tone using myograph experiments on isolated PA from control rats and the monocrotaline (MCT)-induced PH rat model (MCT-PH). Additionally, we assessed the effect of pharmacological activation of SUR2 with pinacidil on healthy rats and pre-clinical PH rat models (MCT and chronic-hypoxia rats) in curative protocol at 1 mg/kg/day.

2. Materials and methods

2.1. Patients

Human lung tissues were obtained at lung transplantation in 12 patients with PAH and upon pneumonectomy or lobectomy for restricted lung cancer from 10 control subjects. PAs were isolated away from tumor areas in the lung specimens of control subjects. Transthoracic echocardiography was performed pre-operatively in the control subjects to rule out PH (15). The demographic information of PAH and control patients are presented in **Supplementary Table 1**.

The patients studied were part of the French Network on Pulmonary Hypertension, a program approved by our institutional Ethics Committee, and had signed informed consent forms (Protocol N8CO-08- 003, ID RCB: 2008-A00485-50, approved on June 18, 2008). All human tissues were obtained with signed informed consent from transplant recipients or families of organ donors in accordance with the Declaration of Helsinki.

2.2. Human PASMC and PAEC culture

Pulmonary artery were excised away from tumor areas. hPAECs and hPASMCs were cultured as described previously (16, 17) and were used in passages 4–5 for the study. Patients studied were part of a program approved by the institutional Ethics Committee and had given written informed consent (ID RCB: 2018-A01252-53, approved on June 18, 2006).

2.3. Pulmonary vascular cell proliferation measurement

Cell proliferation was evaluated by quantifying the incorporation of BrdU using a DELFIA cell proliferation kit (AD0200, PerkinElmer) accordingly to the kit recommendations. Different treatments were used to evaluate the cells' proliferation state: hPASMCs and hPAECs from control and PAH subjects were treated for 24 h with pinacidil (10 μmol/L) or the same amount of DMSO. Cell proliferation was stimulated by a medium containing 10 percent of FBS + EGF + insulin in the presence of 1 μmol/L BrdU for 24 h (Perkin Elmer). The fluorescence signal in the 96-well plate was read with an Envision 2103 plate reader (Perkin Elmer).

2.4. Animals and surgical procedures

The animal facility is licensed by the French Ministry of Agriculture (agreement N°C92-019-01). This study was approved by the Committee on the Ethics of Animal Experiments (CEEA26 CAP Sud). The animal experiments were performed in compliance with the guidelines from Directive 2010/63/EU on 22 September 2010 of the European Parliament on the protection of animals used for scientific purposes and complied with the French institution's animal care and handling guidelines.

In vivo experiments performed in the study were performed according to clinical trial standards: animals were randomly dispersed between groups, and we performed blinded analyses. All rats received a number known by only the experimenter who administered the treatment to rats. Before euthanasia (day 21), all rats underwent an evaluation with closed-chest right heart catheterization. This experiment was performed by a blinded experimenter who did not know the correspondence between ID and treatment. Hemodynamic parameters were analyzed blindly.

Male Wistar rats (4 weeks old) were used in different experimental procedures:

1. PH was induced by a single MCT injection (60 mg/kg, s.c.). MCT was dissolved in 1 N HCl and neutralized with 1 N NaOH. Control animals received the same volume of saline solution.

2. Pinacidil-exposed rats: Wistar rats were treated with pinacidil (1 mg/kg/day in DMSO, daily intraperitoneal injection) from day 0 to day 21. Pinacidil was dissolved in DMSO. Control animals received the same volume of DMSO.

3. MCT-exposed Wistar rats were treated with pinacidil by intraperitoneal injection (1 mg/kg/day dissolved in DMSO) from days 14 to 21 and from days 0 to day 21 with a chronic protocol. Pinacidil was dissolved in DMSO. Control animals received the same volume of DMSO.

2.5. Anesthesia and euthanasia

Rats were placed under general anesthesia (induction: isoflurane 5% at room air) and spontaneous breathing with an isoflurane Rodent Anesthesia System (Minerve Esternay, France) (maintenance: isoflurane 2% at room air). At the end of experimental procedures, animals were euthanized under general anesthesia (isoflurane 5%) by cervical dislocation.

2.6. Chemicals

MCT and pinacidil were obtained from Sigma. U46619 was obtained from R&D Systems.

2.7. Western blot analyses

Total protein from human or rat lungs or isolated PA tissue samples were prepared as described previously (8). The list of antibodies used is presented in **Supplementary Table 2**.

2.8. Reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was extracted using TRIzol. One μg of total RNA was reverse-transcribed using a QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA, USA; cat. no. 205311). Gene expression was quantified using qPCR following the standard protocol for ready-to-use TaqMan gene expression assays on a StepOne Plus Real-Time PCR System (Life Technologies). Pre-designed probe sets used for experiments are described in **Supplementary Table 3**.

2.9. Hemodynamic measurements and tissues collection

Under general anesthesia (isoflurane 2% at room air), hemodynamic measurements, such as right ventricular systolic pressure (RVSP; mmHg), cardiac output (CO; ml/min), and

mean carotid pressure (mCP; mmHg), were measured blindly in unventilated anesthetized rats using a closed chest technique. Hemodynamic parameters were blindly analyzed.

After catheterization, animals were euthanized under general anesthesia (isoflurane 5%) by cervical dislocation. Then tissues were collected, and Fulton's index (RV/LV + S) was calculated by weighing RV and LV plus septal (S).

2.10. Adult rat right ventricular myocytes isolation

Hearts from control rats were mounted on a Langendorff apparatus and perfused through the aorta with collagenase A (Roche, Meylan, France). The solution used to isolate myocytes was a Hanks-Hepes buffer solution containing (mM): NaCl, 117; KCl, 5.7; MgCl2, 1.7; KH2PO4, 1.5; NaHCO3, 4.4; HEPES, 21; glucose, 11.7; creatine, 10; taurine, 20; bubbled with 100% O2; pH 7-1. Digestion time varied between 40 and 50 min. After the enzymatic digestion, the right ventricles (RV) were excised, chopped finely, and agitated manually to dissociate individual myocytes.

2.11. Electrophysiological recordings

Borosilicate glass pipettes (Harvard Apparatus) were pulled with a Sutter puller, fired polished, and had a resistance between 1–2 MΩ. Series resistance was compensated up to 50% and was continually monitored during the experiment. The composition of the standard extracellular solution used to record APs was (mM): NaCl, 140; KCl, 4; CaCl2, 1.8; MgCl2, 1.1; HEPES, 10; glucose, 10; pH 7.4 (LiOH). When APs were recorded, the pipette solution contained (mM): KCl, 135; MgCl2, 4; Ethylene glycol-bis (2-aminoethyl ether)-N, N, N', N'-tetraacetic acid (EGTA), 10; Glucose 10; HEPES, 10; Na2-ATP 5; Na2-CP 3, pH 7.2 (LiOH). In a current-clamp configuration, action potential was measured in response to brief depolarizing current (1–2 ms) injections at 1 Hz as described (18).

2.12. Myograph experiments

Human PAs, rat PAs, and descending aorta were mounted in an emkaBATH4 modular tissue bath system (EMKA Technologies, Paris, France) coupled to IOX software (EMKA Technologies). Human PAs were set at optimal length by equilibration against a passive load of 0.6 g. Rat PAs were set at 0.250 g and the aorta at 1 g.

Vessels were bathed in Krebs solution containing (in mmol/L) 119 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.17 $MgSO_4$, 1.18 KH_2PO_4, 25 $NaHCO_3$, and 11 glucose at 37°C and continuously aerated with a mixture of CO_2/O_2 (5%/95%). Rat PAs were set at optimal length by equilibration against a passive load of 0.3 g. After adding Krebs solution, vessels were contracted with 100 mmol/L K^+-containing solutions (K100). Once a plateau was reached, the vessels were washed with Krebs solution for 30 min. Pinacidil (10 μmol/L) or the same volume of vehicle (DMSO) was used as pretreatments before the dose-response to KCl (10–90 mmol/L). After vessel contraction mediated by the thromboxane A2 analog U46619 (1 μmol/L), pinacidil was used to induce relaxation by increasing concentration (1 nmol/L to 100 μmol/L). Various KCl concentrations (K10 to K100) were prepared with an equimolar substitution of NaCl to maintain constant osmolarity and $[Cl^-]$ compared to standard Krebs solution. The contractile responses were normalized to the maximal response obtained with the K100 challenge. The relaxation responses were expressed as the percentage of the maximum contraction obtained with U46619.

2.13. Echocardiographic measurement

Trans-thoracic echocardiography (TTE) was performed with a digital ultrasound system (Vivid E9, GE Healthcare) using a high-frequency phased array transducer (12 S-D 4-12 MHz, GE Healthcare). The echocardiographic assessment was performed under general anesthesia and spontaneous breathing with an Isoflurane Rodent Anesthesia System (Minerve, Esternay, France) (maintenance isoflurane 2% at room air). Rats were shaved, and body temperature was controlled during experiments. Rats' experimental conditions were unknown by the operator during TTE examination and data interpretation. Measurement of pulmonary artery acceleration time (PAAT), heart rate (HR), and pulmonary artery velocity time integral (VTI) were performed as previously described (18). In the 4-cavity view performed, we measured RV and LV thickness, RV or LV end-diastolic diameter (RV EDd, LV EDd, respectively), and systolic diameter (RV EDs, LV EDs). RV and LV fractional shortening (FS) correspond to the percentage change in LV and RV cavity diameters. LV FS (%) = (LV EDd-LV EDs/LV EDd) * 100 or RV FS (%) = (RV EDd -RV EDs/RV EDd) * 100.

2.14. Immunofluorescence staining

Paraffin-embedded thick sections of lung samples (5 μm thickness) were mounted on SuperFrostPlus slides (Thermo Scientific, Villebon sur Yvette, France). Slices were saturated with human serum (10%) in PBS for 1 h at room temperature. We used primary antibodies against SUR2A (1/100), SUR2B (1/100), or Kir6.1 (1/100) against α-Smooth Muscle Actin (α-SMA, Sigma, F3777) (1/200). Primary Antibody was detected with the secondary antibodies goat anti-mouse and goat anti-rabbit (1/400). Slides were counterstained with 4′,6′-diamidino-2-phénylindole (DAPI).

Pulmonary vessel neovascularization was evaluated by immunostaining against alpha-SMA-FITC (SMC marker F3777 Sigma)/Von Willebrand Factor (endothelial marker, A0082 DAKO). Immunostaining was quantified under an LSM 700 microscope (Carl Zeiss, Le Pecq, France). Images were recorded and analyzed with ZEN software (Carl Zeiss).

2.15. Wound healing assay

After 48 h of starvation (medium without growth factors: FBS, EGF, and insulin), human PASMCs were plated in a culture insert (Cat. No. 90209; Ibidi) at a density of 1.2×10^4 cells per well in a fresh medium with cytosine arabinoside (10 μmol/L). After allowing cells to attach for 24 h, we removed the culture insert and washed the cells with phosphate-buffered saline to remove non-adherent cells. We added fresh medium with DMSO or pinacidil (at 10 μmol/L). We photographed the wound at time 0 and after 15 h for hPASMCs and between 0 and 8 h for hPAECs (corresponding to approximately 50% of wound recovery). Cell migration into the wound space was quantified using image J. Cell motility/invasion was assessed by the percentage of wound closure 15 h after initiation of wound healing {[(area T0–area T15)÷area T15] × 100}.

2.16. Pulmonary vessel and right ventricular remodeling analyses

The lungs were fixed in 4% paraformaldehyde, embedded in paraffin, and serially sectioned (5 μm). Pulmonary vascular remodeling was assessed in all the pulmonary vessels larger than 50 μm and less than 100 μm identified in 20 randomly selected microscopic fields per tissue section. The wall thickness was calculated according to the following equation: [External diameter–Internal diameter)/(External diameter)] × 100, as previously described (19).

Hearts were fixed in 4% paraformaldehyde, paraffin-embedded, and serially sectioned (5 μm). Heart sections were stained with Sirius red (0.1%) to assess heart fibrosis. As previously described, RV fibrosis was quantified using Image J software (20).

2.17. Statistical analyses

All statistical tests were performed using GraphPad Prism software (GraphPad, version 9.0 for Windows).

After checking with the Shapiro–Wilk, Kolmogorov-Smirnov, D'Agostino and Pearson test and Anderson-Darling Tests normality test whether the sample data followed a normal distribution, differences between the two were assessed using an unpaired *t*-test or Mann-Whitney test when conditions of parametric tests were not met. Kruskal-Wallis tests with *post-hoc* Dunn were used to compare three or more groups (all data with sample size $n < 6$/group and skewed data with sample size $n \geq 6$/group) or one-way ANOVA with Dunnett *post-hoc* test for normally distributed data with sample size $n \geq 6$/group. All values are reported as mean ± standard error of the mean. Representative images/figures were chosen to represent the mean of each quantification. For all experiments, *P*-value of <0.05 was considered statistically significant.

3. Results

3.1. The SUR2/Kir6.1 channel is expressed in the lungs of patients with PAH

To examine the localization and the expression of SUR2A, SUR2B, and Kir6.1 in controls and patients with PAH, paraffin-embedded lung sections of controls and patients with PAH were immunostained (**Figure 1A**). In both conditions, SUR2A, SUR2B, and Kir6.1 were expressed in hPAECs (blue arrow) and hPASMCs [yellow arrow, colocalization with smooth muscle actin isoform α (αSMA, in green)]. We subsequently quantified the relative amount of *ABCC9* and *KCNJ8* (coding for Kir6.1) mRNA levels in controls and patients with PAH, which showed that *ABCC9* and *KCNJ8* mRNA levels were unchanged in the lungs of PAH patients (**Figure 1B**). We performed western blot analysis using proteins from controls and PAH patients to determine the relative quantity of SUR2A, SUR2B, and Kir6.1 in the lung tissues. We found similar expression amounts of SUR2A, SUR2B, and Kir6.1 protein in the lung tissues of PAH patients and controls (**Figure 1C**).

3.2. Pharmacological activation of the SUR2/Kir6.1 channel reduced the proliferation of control-hPAECs

Immunostaining revealed the expression of SUR2A, SUR2B, and Kir6.1 in the hPAECs. Using western blot analysis, we quantified their relative expression in the primary hPAECs. As shown in **Figure 2A**, SUR2A, SUR2B, and Kir6.1 protein were expressed in both control- and PAH-hPAECs. Compared to control-hPAECs, the expression of SUR2A and SUR2B proteins was unchanged, and Kir6.1 protein expression was increased in the PAH-hPAECs (**Figure 2B**). Therefore, we evaluated the role of SUR2 in the proliferation and migration of hPAECs following pharmacological activation of SUR2 with pinacidil (21, 22) (10 μmol/L) for 24 h. We chose to use pinacidil as an activator of SUR2 because contrary to other KATP channels, pinacidil activates SUR2B and SUR2A similarly

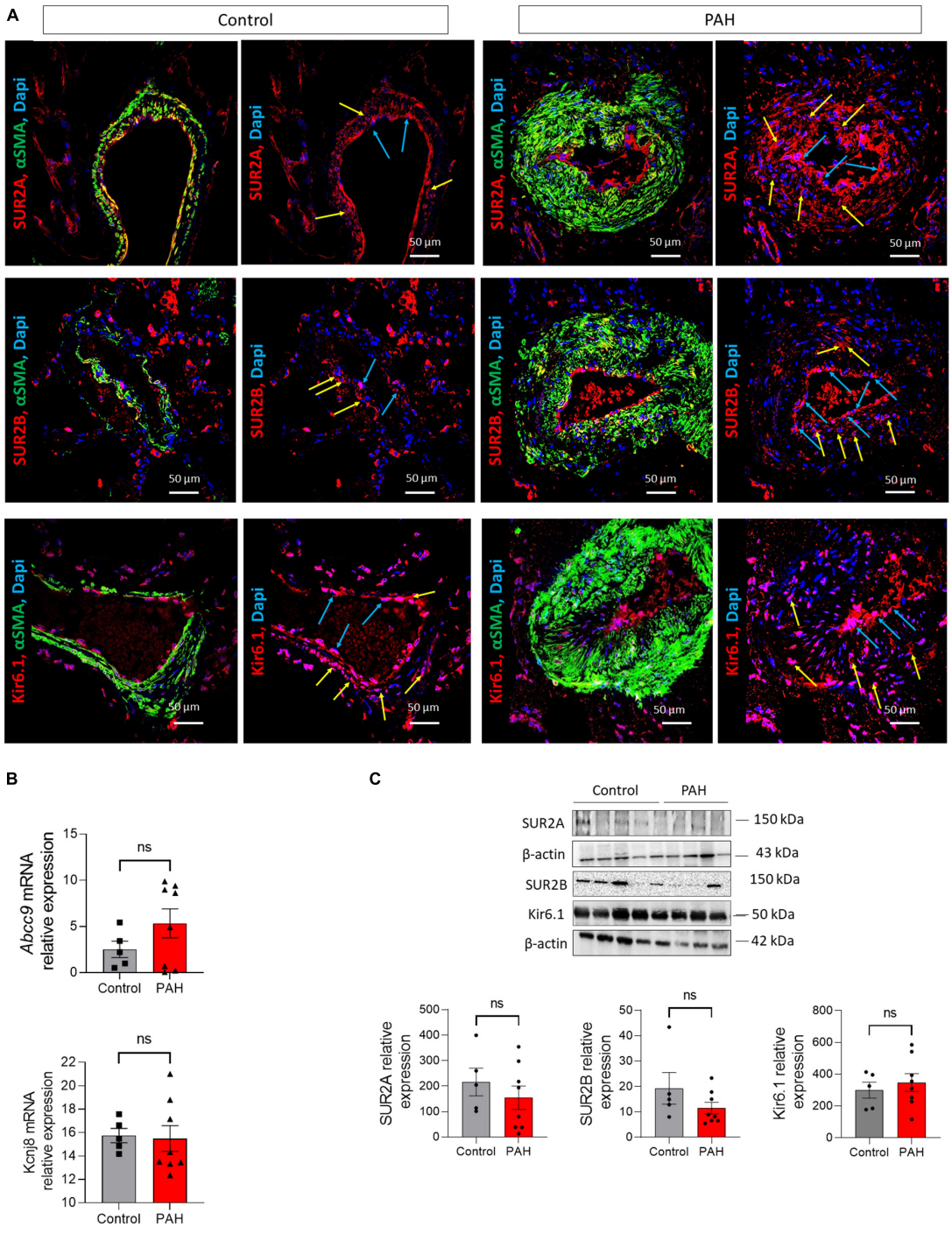

FIGURE 1
Expression of SUR2A, SUR2B, and Kir6.1 protein in lungs from control and patients with PAH. **(A)** SUR2A, SUR2B, or Kir6.1 immunostaining in paraffin-embedded lung sections from controls and patients with PAH; PAEC (blue arrow) and PASMC (yellow arrow) staining is visible. Scale Bar 50 µm. mRNA expression and representative western blots of *ABCC9* (coding for SUR2A and SUR2B) and *KCNJ8* (coding for Kir6.1) in the lungs of controls and patients with PAH [**(B,C)**, respectively] (n = 9–12). ns: non-significant vs. control. Two-tailed unpaired Student t-tests assessed the difference between the two groups.

(23) and has a very low affinity for SUR1 (24). Interestingly, pharmacological activation of SUR2 reduced the proliferation rate of control-hPAECs without altering the proliferation of PAH-hPAECs (**Figures 2C, D**). SUR2 activation by pinacidil had no consequence on the migration capacity of hPAECs in control- and PAH-hPAECs (**Figures 2E, F**).

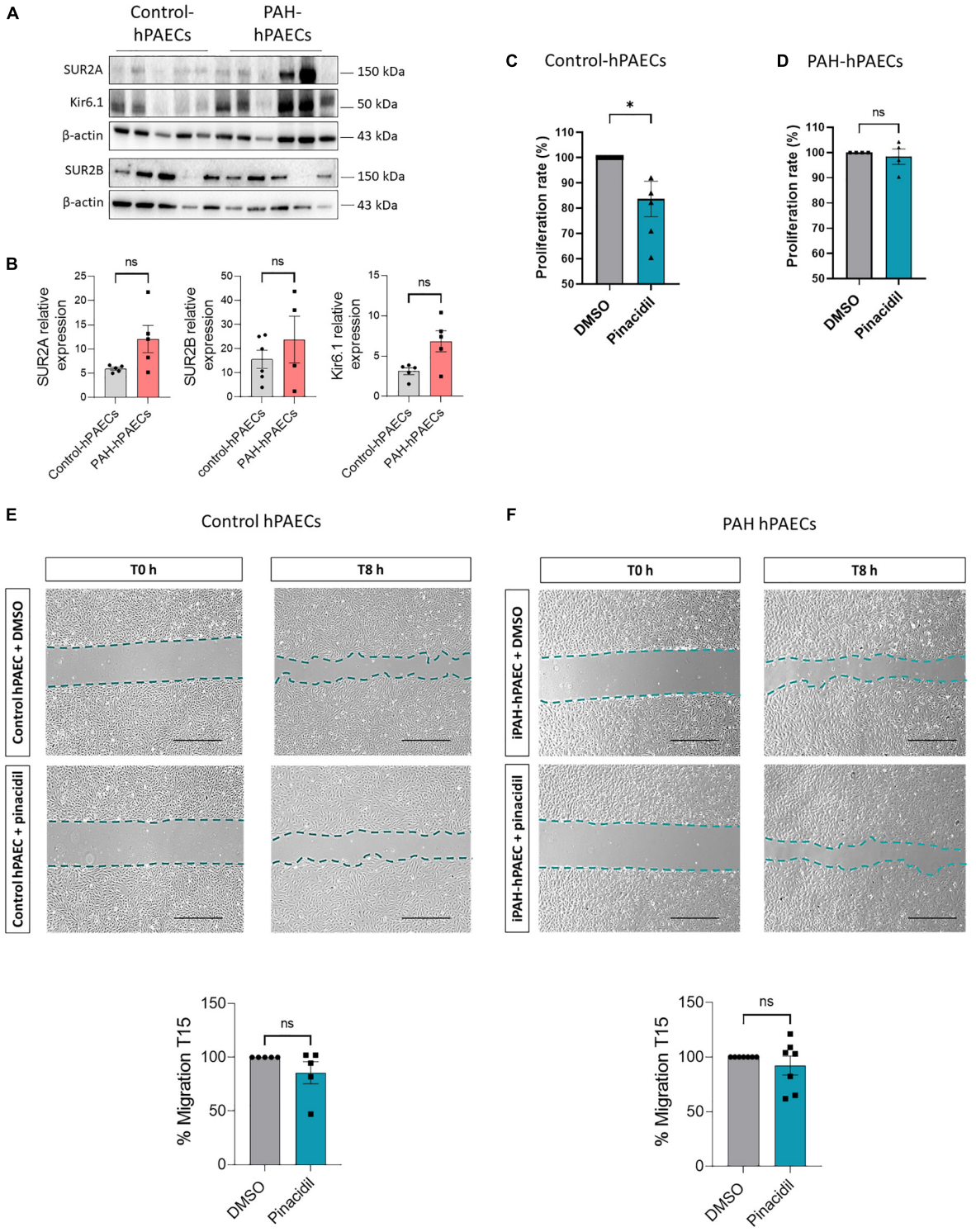

FIGURE 2
Pharmacological activation of SUR2/Kir6.1 channel reduced the proliferation in control hPAECs. **(A)** Representative western blots of SUR2A, SUR2B, and Kir6.1 in hPAECs in the lungs of controls and patients with PAH. **(B)** Quantification of SUR2A, SUR2B, and Kir6.1 expression in hPAECs from controls and patients with PAH (n = 4–6). **(C,D)** Analysis by Bromodeoxyuridine (BrdU) assay of the proliferation rate of hPAECs from controls and patients with PAH treated with pinacidil (10 μmol/L) or DMSO (n = 4–5 patients). **(E,F)** Analysis of the migratory capacity of hPAECs from controls and patients with PAH treated with pinacidil (10 μmol/L) or DMSO. Scale Bar = 500 μm (n = 5–7 patients). ns: non-significant. *$p < 0.05$. Two-tailed unpaired Student t-tests assessed the difference between the two groups.

3.3. Pharmacological activation of SUR2/Kir6.1 channel reduced the proliferation and migration of control- and PAH-hPASMCs and produced PA relaxation in PAH patients

We found that SUR2A, SUR2B, and Kir6.1 proteins were expressed in the control- and PAH-hPASMCs (**Figure 3A**). SUR2A protein was higher in the PAH-hPASMCs than in the controls. Further, SUR2B expression in the PAH-hPASMCs also tended to increase, though the increase was not significant (p = 0.053). Kir6.1 protein expression was unchanged in the PAH-hPASMCs (**Figures 3A, B**). Pharmacological activation of SUR2 reduced the proliferation rate of hPASMCs equally in both controls and PAH-hPASMCs (**Figures 3C, D**). SUR2 activation by pinacidil reduced the migration capacity of the control-hPASMCs but not the PAH-hPASMCs (**Figures 3E–H**).

We measured the consequence of SUR2 activation on the relaxation of PA in control and PAH patients (**Figure 3G**). We found pinacidil induced more potent PA relaxation in controls than in PAH conditions, suggesting that SUR2 contributes to the regulation of human PA tone, but SUR2-mediated PA relaxation was reduced in PAH patients.

3.4. SUR2 pharmacological activation produces the relaxation of PA in control and MCT-PH rats

We showed that the mRNA levels of *ABCC9* were reduced in the lungs of MCT-PH rats, while *KCNJ8* mRNA remained unchanged (**Figures 4A, B**). In addition, SUR2A expression was severely reduced, SUR2B expression was unchanged, and Kir6.1 expression increased in the MCT-PH rats' lungs (**Figures 4C, D**).

Next, we investigated the consequence of SUR2 activation in the contraction and relaxation of PA from the controls and MCT-PH rats. In the controls, the contractile response to increasing concentration of potassium chloride (KCl, 10 to 90 mmol/L) was shifted significantly to the right in the presence of pinacidil (**Figure 4E**), as indicated by increased EC50 values (**Figure 4E**). In PA isolated from the MCT-PH rats, we found that pinacidil application induced similar results in PA from control rats (**Figure 4F** and **Supplementary Figure 1**).

After pre-contraction of PA with 1 μmol/L U46619, we applied increasing concentrations of pinacidil or dimethyl sulfoxide (DMSO) (100 nmol/L to 100 μmol/L). As shown in **Figure 4G**, in the controls, the contraction of PA was progressively reduced by increasing the dose of pinacidil (until almost total PA relaxation) compared to increasing the dose of DMSO, indicating that SUR2 activation-mediated relaxation of PA was showed by the decrease in PA contraction (**Figure 4H**). Similar results were obtained in the PA isolated from MCT-PH rats (**Figure 4F** and **Supplementary Figure 1**).

SUR2 activation induced more potent relaxation of PA in the MCT-PH rats than in the controls, suggesting that pinacidil-mediated PA relaxation mainly depends on SUR2B expression in MCT-PH rats. In contrast, SUR2A expression was impaired in the MCT-PH rats.

As presented in **Supplementary Figure 2A**, in the absence of endothelium, the relaxation mediated by pinacidil was diminished by 40% (**Supplementary Figure 2A**), suggesting that the endothelial-SUR2 also contributes to the regulation of pulmonary arterial tone.

3.5. Expression of SUR2A, SUR2B, and Kir6.1 in the hearts of MCT-PH rats

Because SUR2 and Kir6.1 are expressed in the heart, we quantified the expression of SUR2 and Kir6.1 protein in the RV of control and MCT-PH rats by western blot analyses. In comparison with control rats, we found a reduced expression of SUR2A and an unchanged expression of Kir6.1 in the MCT-PH rats. SUR2B protein expression was not quantifiable in the RV of the control and MCT-PH rats (**Figure 5A**). At the same time, SUR2B appears to be expressed in the left ventricular (LV) (**Supplementary Figure 3B**). Moreover, SUR2A protein was decreased, and SUR2B and Kir6.1 expression were unchanged in the LV of the MCT-PH rats (**Supplementary Figure 3B**).

To study the contribution of SUR2 on RV action potential, we measured the effect of pinacidil on action potential duration (APD). The intake of pinacidil led to a shortening of APD without changes in resting membrane potential (**Figure 5B**, left panel). In the control RV cardiomyocytes, APD at 50% of AP repolarization was not affected by pharmacological activation of SUR2 (**Figure 5B**, middle panel). In contrast, APD at 90% of AP repolarization was significantly decreased (**Figure 7B**, right panel).

3.6. *In vivo* pharmacological activation of SUR2 increased the heart rate of control rats

To evaluate the consequence of *in vivo* pinacidil exposure on control rats, we exposed chronically healthy rats to 1 mg/kg/d pinacidil for 3 weeks (**Figure 5C**). *In vivo* chronic exposure to pinacidil had no pulmonary vascular consequences, as indicated by unchanged right ventricular systolic pressure (RVSP), cardiac output (CO), pulmonary vascular resistance (PVR) (evaluated by RVSP/CO ratio), RV hypertrophy (Fulton

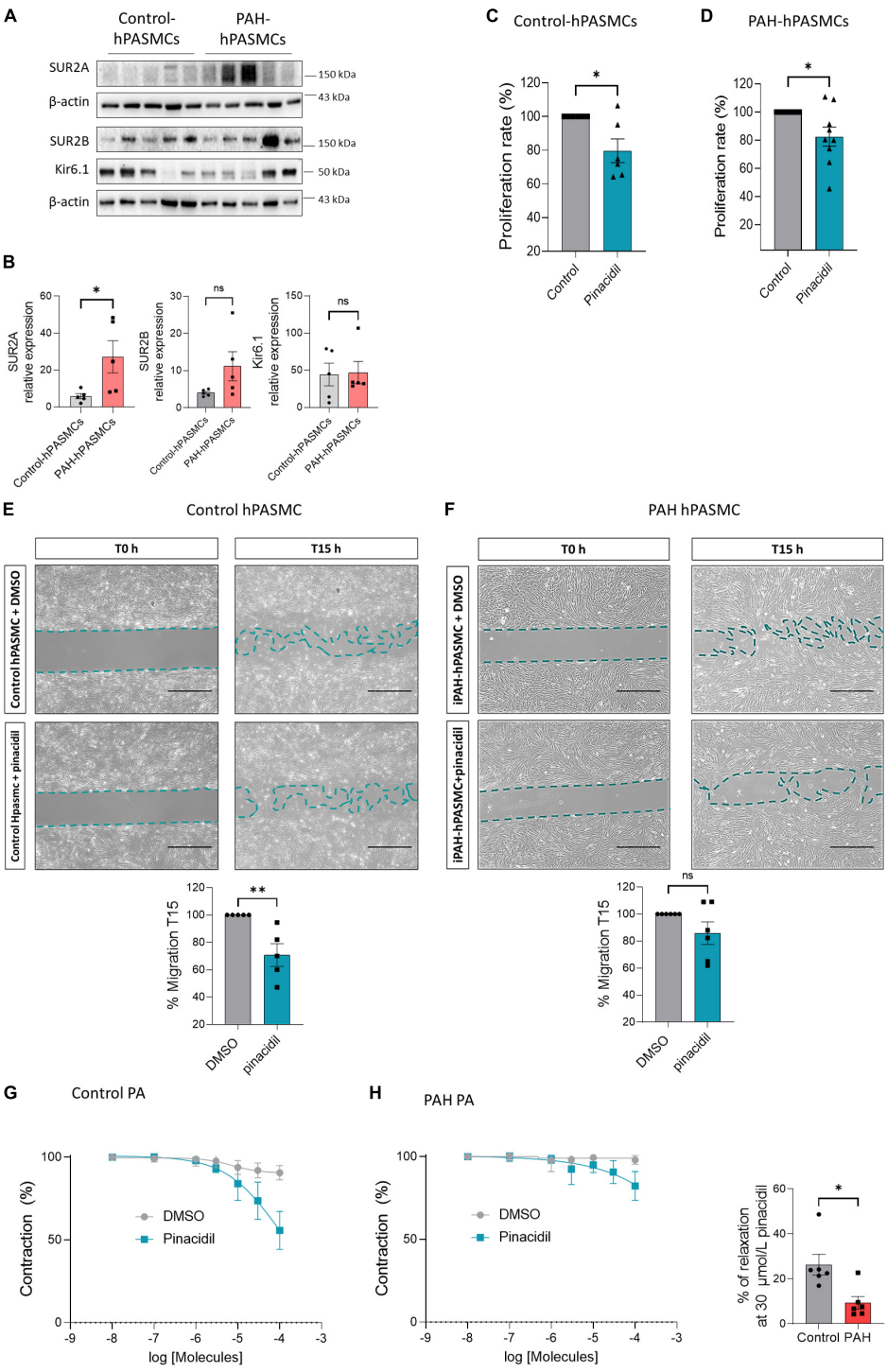

FIGURE 3
Pharmacological activation of SUR2/Kir6.1 channel reduced the proliferation and migration of control and PAH-hPASMCs. **(A)** Representative western blots **(A)** and quantification **(B)** of SUR2A, SUR2B, and Kir6.1 in hPASMCs from controls and patients with PAH (n = 5). Analysis by BrdU assay of the proliferation rate of hPASMCs from controls patients **(C)** and patients with PAH **(D)** treated with pinacidil (10 µmol/L) or DMSO (n = 6–7 patients). **(E,F)** Analysis of the migratory capacity of hPASMCs from controls **(E)** and patients with PAH **(F)** treated with pinacidil (10 µmol/L) or DMSO (n = 5–6 patients). Scale Bar = 500 µm. **(G)** Dose-response to pinacidil (100 nmol/L to 100 µmol/L) and DMSO on pre-contracted human PA control and iPAH **(H)** by 1 µmol/L of U46619. Graphics represent the relaxation percentage at 30 µmol/L of PA treated with pinacidil or DMSO (n = 6 patients). ns: non-significant. $^{*}p < 0.05$ $^{**}p < 0.001$.

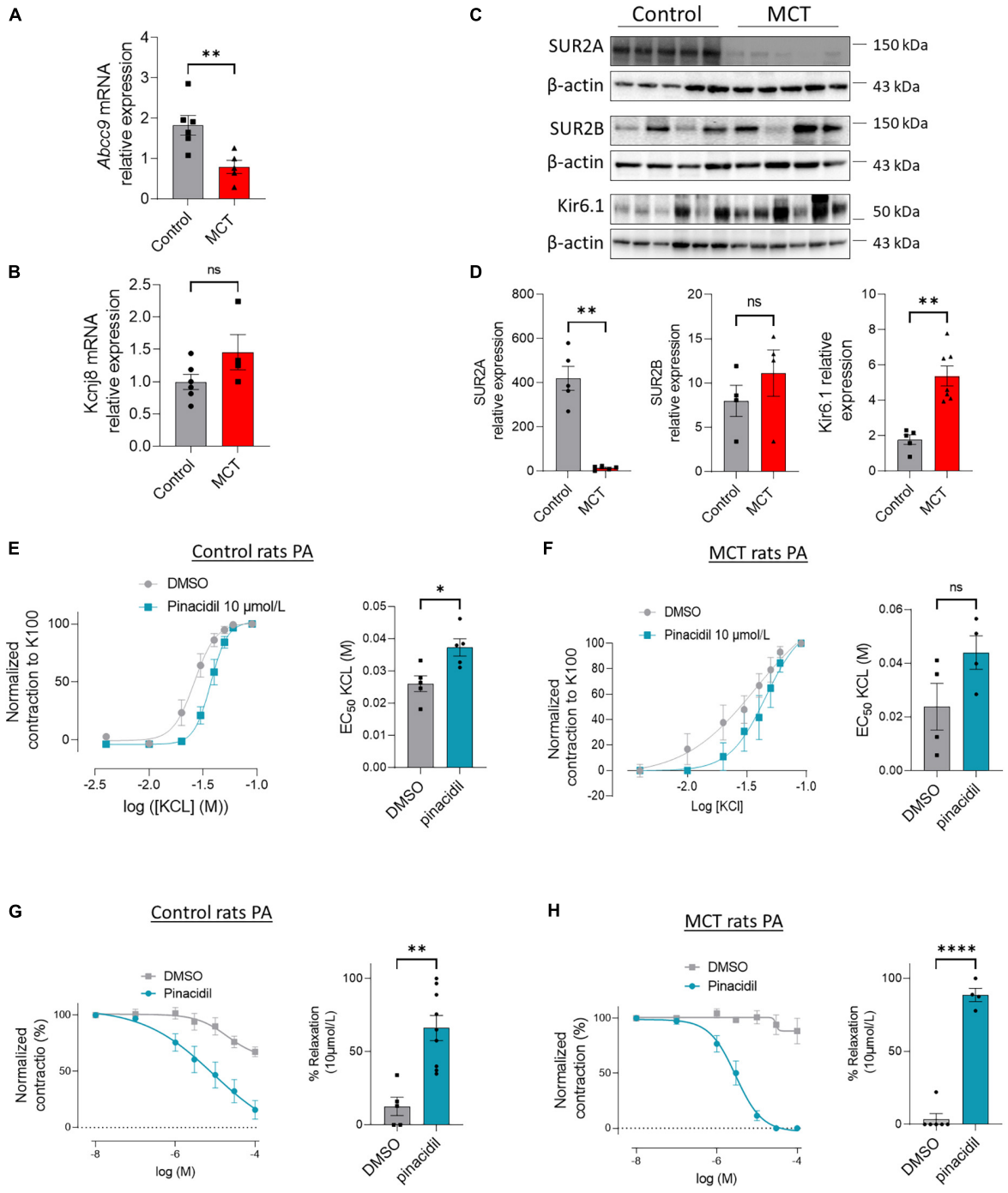

FIGURE 4
Pharmacological activation of SUR2 produces PA relaxation in control and MCT-PH rats. **(A,B)** *ABCC9* and *KCNJ8* mRNA expression was quantified in the lungs of control and MCT-PH rats (n = 4–6). **(C)** Representative western blots of SUR2A, SUR2B, and Kir6.1 in lungs from control and MCT-PH rats (3 weeks). **(D)** Quantification of SUR2A, SUR2B, and Kir6.1 expression in lungs of controls and MCT-PH rats (n = 4–6). **(E)** Dose-response curve (normalized to K100) was established by increasing concentrations of potassium chloride (KCl) to isolated PA from control rats in the presence of DMSO or pinacidil (SUR2 activator, at 10 µmol/L). Corresponding quantification EC50 values (n = 5 rats). **(F)** Dose-response curve (normalized to K100) was established by increasing concentrations of potassium chloride to isolated PA from control and MCT-PH rats in the presence of pinacidil at 10 µmol/L. Corresponding quantification EC50 values (n = 4 rats). **(G)** Dose-response to pinacidil (100 nmol/L to 100 µmol/L) and DMSO on precontracted control rat PA by 1 µmol/L of U46619. Graphics represent the contraction percentage at 10 µmol/L of PA treated with pinacidil or DMSO (n = 5–9 rats). **(H)** Dose-response to pinacidil (100 nmol/L to 100 µmol/L) and DMSO on pre-contracted MCT-PH rat PA by 1 µmol/L of U46619 (n = 4–5 rats). Graphics represent the contraction percentage at 10 µmol/L of PA treated with pinacidil or DMSO. ns: non-significant. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$.

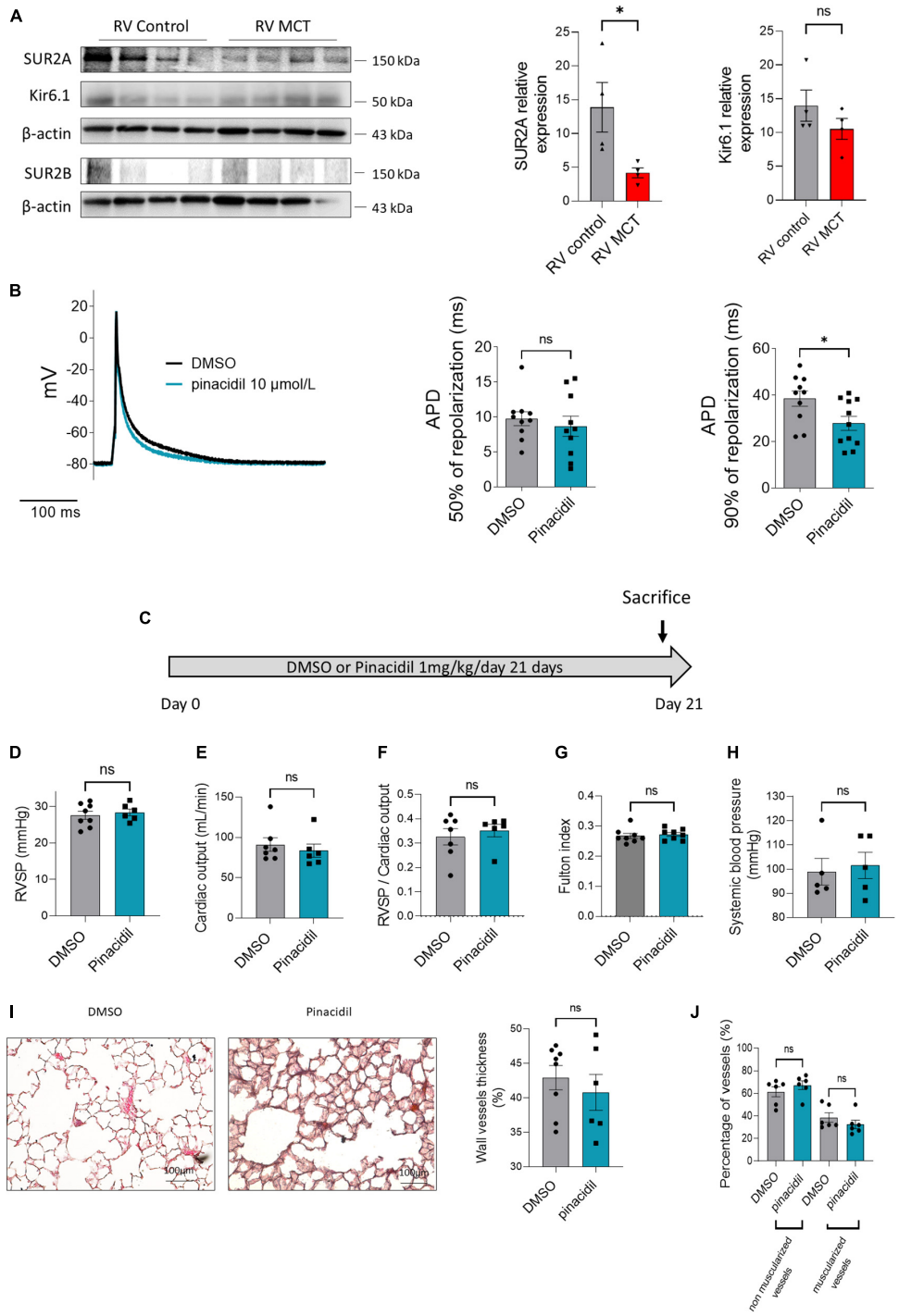

FIGURE 5
Consequences of SUR2 pharmacological activation in the RV compartment (*in vitro* and *in vivo*). **(A)** Representative Western blots of SUR2A, SUR2B1, and Kir6.1 in RV from control and MCT-PH rats (3 weeks), and quantification of SUR2A, SUR2B, and Kir6.1 expression in RV from control and MCT-PH rats (n = 5–8). **(B)** Representative action potential in RV cardiomyocytes isolated from control rats in DMSO or after perfusion of pinacidil. Analysis of action potential duration at 50 and 90% of AP depolarization in basal conditions and the presence of 10 µmol/L of pinacidil (12–14 cells from four different rats). **(C)** *In vivo* experimental design. Pinacidil (1 mg/kg/ day for 3 weeks) was administered long-term to healthy control rats by intraperitoneal injection. **(D)** RVSP (mmHg; n = 6–8 rats per condition). **(E)** CO (mL/min) (n = 6–8 rats per condition). **(F)** PVR (n = 5 rats per condition) **(G)** Fulton index (n = 6–8 rats per condition) **(H)** and carotid artery mean pressure (n = X–X rats). **(I)** Pulmonary vessel occlusion (%) was analyzed by hematoxylin-eosin Safran staining (HES) (n = 6–8 rats per condition). **(J)** Percentage of non-muscularised and muscularised vessels [100 vessels per rat, (n = 6–8 rats)] as measured by immunostaining against α-smooth muscle actin (αSMA) and Von Willebrand Factor (VWF). ns: non-significant. $^{*}p < 0.05$.

TABLE 1 Evaluation of right ventricular and left ventricular function by echocardiography measured in pinacidil or dimethyl sulfoxide-exposed rats.

	DMSO n = 8	Pinacidil n = 8
HR (bpm)	326.1 ± 15.42	29 ± 1.2**
Cylce length (ms)	159.4 ± 5.52	143.3 ± 3.19*
RVET (ms)	82.75 ± 2.38	71.63 ± 2.66**
PAAT (ms)	36.00 ± 1.36	29 ± 1.2**
PAAT/RVET	0.44 ± 0.016	0.40 ± 0.007
VTI Pulmonary Artery (ml)	5.30 ± 0.29	4.87 ± 0.16
RV EDd (mm)	6.24 ± 0.26	6.72 ± 0.26
RV EDs (mm)	1.47 ± 0.28	1.84 ± 0.13
RV tihickness (mm)	0.87 ± 0.06	0.93 ± 0.06
RV FS (%)	48.75 ± 4.98	43.95 ± 3.12
VTI Aorta (ml)	7.38 ± 0.28	7.09 ± 0.38
Stroke Volume Aorta (ml)	15.76 ± 1.25	15.17 ± 0.85
LV EDd (mm)	6.24 ± 0.26	6.72 ± 0.26
LV EDs (mm)	3.35 ± 0.22	3.47 ± 0.16
LV thickness (mm)	1.48 ± 0.14	1.50 ± 0.12
LV FS (%)	39.80 ± 4.76	47.70 ± 3.40

HR, Heart rate; RVET, right ventricular ejection time; PAAT, pulmonary artery acceleration time; VTI, velocity-time integral; RV EDd, right ventricular end-diastolic diameter; RV Eds, right ventricular end-systolic diameter; RV FS, right ventricular fractional shortening; LV EDd, left ventricular end-diastolic diameter; LV Eds, left ventricular end-systolic diameter; LV FS, left ventricular fractional shortening. ns, non-significant *$P < 0.05$, **$P < 0.01$.

index), systemic blood pressure (measured in the carotid artery) (**Figures 5D–H**). Pinacidil administration did not affect pulmonary vessel remodeling and neo-muscularization (**Figures 5I, J**).

We observed increased heart rate and decreased cycle length of the pinacidil-exposed rats (**Table 1**) without changes in heart morphometric parameters (**Supplementary Table 4**), which is in accordance with pinacidil-induced reduced APD in RV cardiomyocytes. PA acceleration time (PAAT) and RV ejection time (RVET) were reduced, while the PAAT/RVET ratio remained unchanged (**Table 1**). All the other measured parameters were unchanged, including RV and LV thickness and diameters (**Table 1**).

3.7. Preventive *in vivo* SUR2 pharmacological activation reduced the development of MCT-induced PH

In this study, SUR2 activation reduced the proliferation rate of PAH-hPASMCs and caused PA relaxation in the MCT-PH rats. Therefore, we assessed the consequences of *in vivo* pharmacological activation of the SUR2 channel in MCT-PH rats (**Figure 6A**). We administered pinacidil 1 mg/kg/day *in vivo* to MCT-PH rats from days 1 to 21 (**Figure 6A**). We measured a reduction in the RVSP (**Figure 6B**), an increase in CO (**Figure 6C**), a decrease in PVR (**Figure 6D**), and a reduction in RV hypertrophy in the MCT + pinacidil rats compared to the MCT + DMSO rats (**Figure 6E**). Further, Dp/dt min was decreased, and heart rate was increased in the MCT + pinacidil rats compared to the MCT + DMSO rats (**Supplementary Table 5**). At the same time, no difference was observed in systemic blood pressure (**Figure 6F**).

In vivo preventive treatment with pinacidil reduced the thickening of pulmonary wall vessels induced by MCT exposure (**Figure 6G**) and reduced the neo-muscularisation capacity of pulmonary vessels, as attested by the increased non-muscularised vessels and by the decreased of muscularised vessels (**Figure 6H**). Preventive pinacidil treatment also reduced RV fibrosis in the MCT + pinacidil rats compared to the MCT-DMSO rats (**Figure 6I**).

3.8. *In vivo* curative pharmacological activation of SUR2 reduced the severity of MCT and chronic-hypoxia (CH)-induced PH

In the curative strategy in the MCT-PH model (day 14 to day 21) (**Figure 7A**), pinacidil significantly reduced RVSP (**Figure 7B**), but not CO (**Figure 7C**), RVSP/CO ratio (**Figure 7D**), and Fulton index (**Figure 7E**) not systemic blood pressure (**Figure 7F**) and other morphometric parameters in the MCT-PH rats (**Supplementary Table 6**). Additionally, *in vivo* curative treatment with pinacidil reduced the pulmonary vascular remodeling induced by MCT exposure (**Figure 7G**) and pulmonary vessel neo-muscularisation (**Figure 7H**). Furthermore, curative pinacidil treatment did not significantly reduce RV fibrosis in the MCT-pinacidil rats compared to the MCT-DMSO rats (**Figure 7I**). These results suggest that pinacidil treatment may reduce the severity of PH in MCT-exposed rats.

Finally, we analyzed the protein expression of SUR2A, SUR2B, and Kir6.1 in in CH-PH rats. We found that SUR2A, SUR2B, and Kir6.1 lung protein expression were unchanged in CH-exposed rats compared with normoxia rats (**Supplementary Figure 4A**), like in human lung tissues. Then, we evaluated the consequences of *in vivo* pharmacological activation of the SUR2 by pinacidil in CH-PH rats (**Supplementary Figure 4B**). We treated with 1 mg/kg/day of pinacidil CH-PH rats from days 14 to 21. We measured an improvement in the RVSP (**Supplementary Figure 4C**), CO (**Supplementary Figure 4D**), and PVR (**Supplementary Figure 4E**), but not Fulton index, systemic

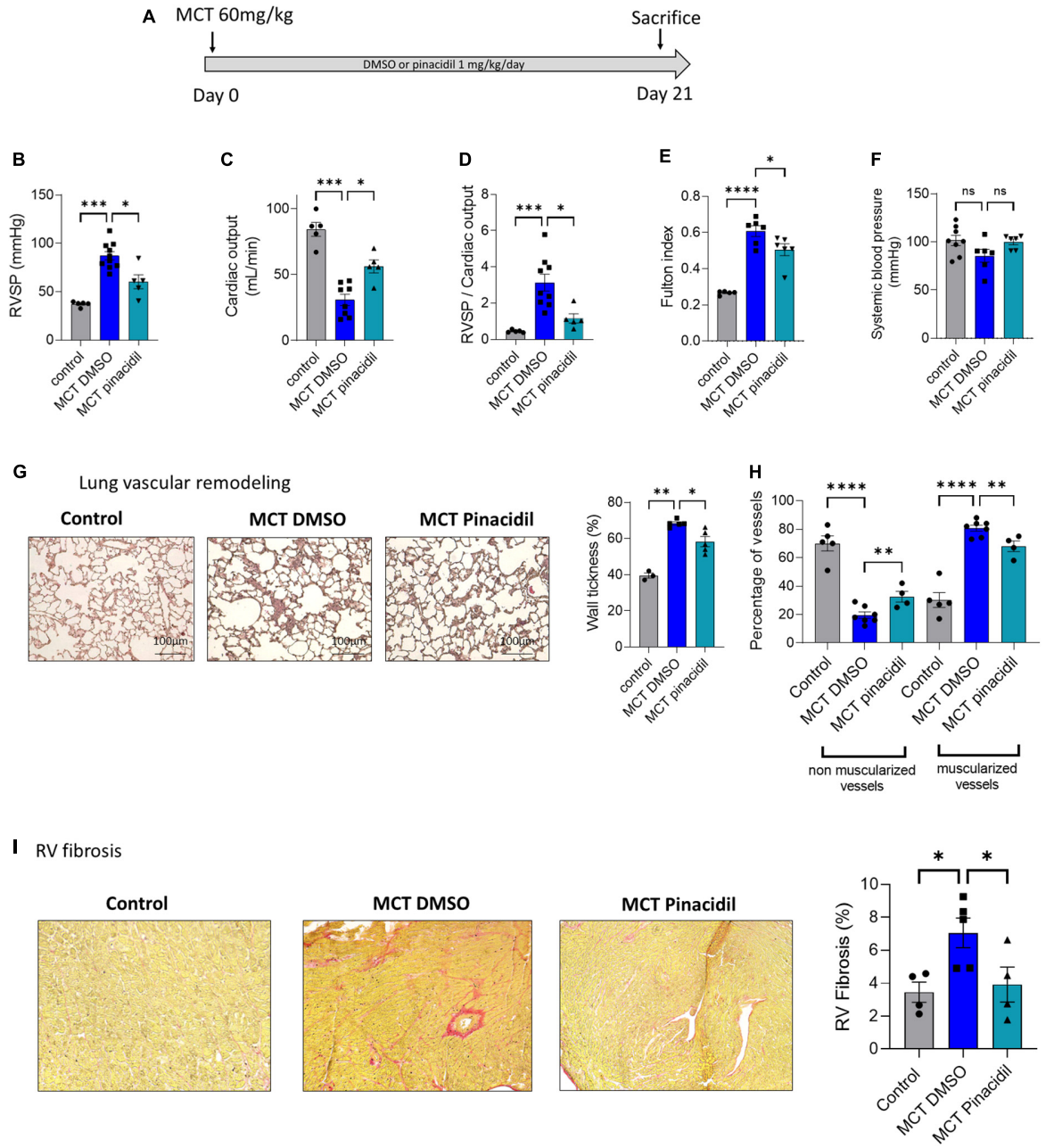

FIGURE 6
In vivo long-term pinacidil treatment interferes with MCT-PH development. **(A)** *In vivo* experimental design. Pinacidil (1 mg/kg/day for 3 weeks) was administered long-term during MCT exposure by intraperitoneal injection. **(B)** RVSP (mm Hg; n = 5–10 different rats per condition) **(C)**, Cardiac output (CO; ml/min) (n = 5–8 different rats per condition). **(D)** PVR (evaluated by the RVSP/CO ratio) (n = 5–8 different rats per condition). **(E)** Fulton index (RV/LV + septum) (n = 6 different rats per condition). **(F)** Pulmonary vessel occlusion (%) was analyzed by HES (n = 3–5 rats per condition). **(G)** Percentage of non-muscularised and muscularised vessels [100 vessels per rat, (n = 4–7 rats)] as measured by immunostaining against α-smooth muscle actin (αSMA) and Von Willebrand Factor (VWF). **(H)** Interstitial fibrosis was analyzed with Sirius red staining in the RV compartment from control, MCT + DMSO, and MCT + pinacidil rats. **(I)** Quantification of the percentage of fibrosis in RV tissue in each condition (n = 20 images per rat from 4–5 different rats). ns: non-significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

blood pressure, and Heart rate (Supplementary Figures 4F–H). The pulmonary vessel wall thickness and the pulmonary vessel neomuscularization were reduced by the pinacidil treatment (Supplementary Figures 4I, J).

4. Discussion

In this study, we report on several essential findings related to SUR2/Kir6.1 expression and function in the

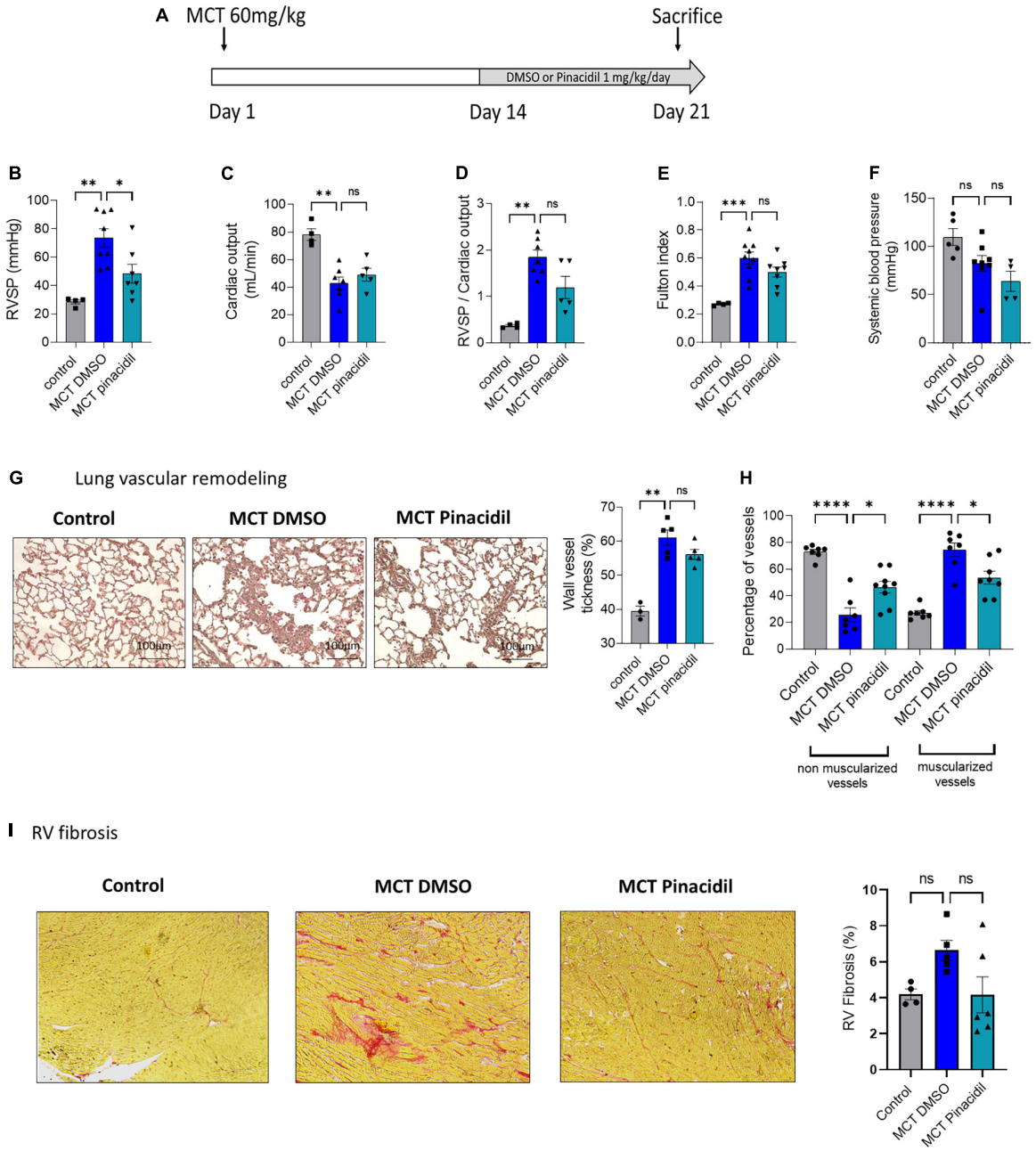

FIGURE 7
In vivo curative pinacidil treatment reduces the development of PH in MCT-PH rats. **(A)** *In vivo* experimental design. Pinacidil (1 mg/kg/day from day 14 to day 21) was administered short-term during MCT exposure by intraperitoneal injection. **(B)** RVSP (mm Hg; n = 4–8 different rats per condition) **(C)**, Cardiac output (CO; ml/min) (n = 4–7 different rats per condition). **(D)** PVR (evaluated by the RVSP/CO ratio) (n = 4–7 different rats per condition). **(E)** Fulton index (RV/LV + septum) (n = 4–8 different rats per condition). **(F)** Pulmonary vessel occlusion (%) was analyzed by HES (n = 3–5 rats per condition). **(G)** Percentage of non-muscularised and muscularised vessels [100 vessels per rat, (n = 6–8 rats)] as measured by immunostaining against αSMA and VWF. **(H)** Interstitial fibrosis was identified with Sirius red staining in the RV compartment from control, MCT + DMSO, and MCT + pinacidil rats. **(I)** Quantification of the percentage of fibrosis in RV tissue in each condition (n = 20 images per rat from 4–5 different rats). ns: non-significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

PAH pathogenesis. First, SUR2A, SUR2B, and Kir6.1 were expressed in hPASMCs and hPAECs. Second, SUR2 activation reduced the proliferation of control-hPASMCs, control-hPAECs, and PAH-hPASMCs and the migration of control-hPASMCsc. Third, pharmacological activation of SUR2 produced PA relaxation in both the control and PH rat models. Fourth, SUR2A and Kir6.1 were highly expressed at the RV level and contributed to potential

repolarization in the control rats. Fifth, chronic *in vivo* SUR2 activation with pinacidil increased heart rate without disturbing pulmonary circulation hemodynamics in the control rats. Finally, *in vivo* activation of SUR2 reduced the development of PH in two preclinical models of PH (MCT- and CH-PH rat models).

4.1. SUR2A/SUR2B localization expression

SUR2A shares 99% of homology with SUR2B (25). However, they have different localization and sensitivities to ATP. In the absence of pinacidil, when SUR2B is associated with Kir6.1, this channel is stimulated by ADP and ATP rather than inhibited by ATP (26). Moreover, the half-maximal inhibitory concentration of ATP for SUR2A and SUR2B are 100 and 300 μmol/L, respectively (27). Regarding their different localization, SUR2A is the primary isoform in the heart, skeletal muscle, and brain (28, 29), while SUR2B is more ubiquitous. The *in situ* hybridization technique indicated that SUR2B and Kir6.1 mRNA are found in many systemic conditions but not in PAs (30). Here, we demonstrated for the first time that SUR2A, SUR2B, and Kir6.1 are expressed at the protein level in human and rat lung tissues and isolated hPAECs and hPASMCs from controls and patients with PAH. SUR2A and SUR2B are predominantly associated with Kir6.1, but they can also be associated with Kir6.2, commonly associated with SUR1. We recently found that Kir6.2 is expressed in control and PAH hPAECs and hPASMCs and that Kir6.2 is increased in PAH-hPASMCs and lungs of MCT-PH rats (8). In the pulmonary vasculature, we could reasonably imagine that SUR2A or SUR2B can co-assemble with Kir6.1 and Kir6.2. Therefore the increase in Kir6.2 observed in the context of PAH may modify the properties of KATP (8).

Moreover, as suggested by Videbaek et al. (31) pinacidil is more potent in systemic and rat pulmonary arteries. In the present study, we confirmed that pinacidil application is more potent to relax the aorta artery than the pulmonary artery. KATP channels, including SUR2A and SUR2B, are expressed in the mitochondrial membrane (32) and may inhibit the mitochondrial radical oxygen species (ROS) production (33). As ROS are overproduced in PAH (34), we could hypothesize that SUR2 pharmacological activation reduces the severity of PH partly due to an inhibition of ROS production.

4.2. SUR2 function in the RV

SUR2, Kir6.1, and Kir6.2 are found in the heart (35). However, all current studies on the role of SUR2 in the heart involve the LV compartment. We found that RV cardiomyocytes also expressed SUR2A, Kir6.2, and Kir6.1 (8), while SUR2B expression appeared low in the RV compared to the LV. LV cardiomyocytes from guinea pigs administered with pinacidil showed significantly shortened APD (36). Inversely, LV cardiomyocytes isolated from *kir6.2*$^{-/-}$ mice displayed a prolonged APD in high glucose concentration compared with cardiomyocytes from Wild-Type mice (37).

Moreover, in the LV of control rats, SUR2A and SUR2B appear localized in the mitochondria, indicating that KATP could also exert cardio-protection based on their role as KATP in the mitochondria (32). The role of the SUR2A/Kir6.1 channel in APD and the mitochondrial function of cardiomyocytes may have cardioprotective consequences that could benefit from RV dysfunction occurring in MCT-PH models (18, 20). Indeed, Storey et al. demonstrated that KATP was crucial for regulating cardiomyocyte Ca^{2+} homeostasis under basal and pathological conditions. The reduction of KATP current increases Ca^{2+} overload by preventing mitochondrial membrane potential oscillations during oxidative stress (38). Here, we demonstrated that SUR2 activation leads to a reduction of APD in control RV cardiomyocytes. As we previously found, a pathological prolongation of APD in RV cardiomyocytes isolated from MCT-PH rats (39), we could hypothesize that the protective effect of SUR2 activation in the MCT-PH model could partly act by these mechanisms in RV cardiomyocytes. In LV cardiomyocytes, SUR2 activation by pinacidil was already demonstrated to modulate the action potential duration (40, 41). Additionally, we found that chronic pinacidil application in healthy rats enhances the heart rate of pinacidil-exposed rats, suggesting a potential direct action in the sinoatrial node, which is the dominant pacemaker in the mammalian heart. It was previously demonstrated that Kir6.1 containing KATP channels contributes to the sinoatrial node excitability and heart rate control (42).

4.3. *ABCC9* in Cantu syndrome

Recently, the gain-of-function mutations in *ABCC9* is the most important genetic cause of Cantu syndrome, characterized by congenital hypertrichosis, osteochondroplasia, cardiomegaly, dilated vasculature, and pericardial effusion. Interestingly, some patients with Cantu syndrome also develop pulmonary hypertension (43–46). Twelve different *ABCC9* mutations are gain-of-function (43) or LOF-of-function mutations (47). The pathophysiological mechanism involving the development of PH is complex, suggesting that the constitutive SUR2A/Kir6.1 opening leads to systemic vasorelaxation and hypotension, leading to compensatory cardiac hypertrophy and hypercontractility and PH (35). Moreover, some patients have also been diagnosed with PAH due to LV disease (48). This study found that daily pinacidil administration in control rats at

1 mg/kg/day for 3 weeks had no consequences on hemodynamic parameters and pulmonary vascular remodeling. Previous work suggested that long-term treatment (preventive protocol) with KATP openers treatment reduced the severity of PH (12–14). Our results demonstrated that curative SUR2 activation could be an interesting therapy for PAH.

5. Conclusion

We showed that pharmacological activation of SUR2 reduces the proliferation and migration capacity of PAH-hPASMCs and that SUR2 contributes to PA tone. Moreover, *in vivo* SUR2 curative activation effectively restores or ameliorates PH in MCT-PH and CH-PH rat models. Despite the SUR2 gain-of-function mutation being a cause of cardiovascular abnormalities observed in patients with Cantu syndrome, our results showed that pharmacological activation of SUR2 should be considered for treating PAH.

6. Limitations

Since SUR2 is expressed by other cell types such as fibroblasts or other tissues (liver, for example (28, 49), our results did not exclude that *in vivo* SUR2 activation reduces PH by acting in pulmonary and cardiac fibroblasts (50) or by improving liver function in MCT-PH model, which is characterized by liver dysfunction. The generation of cardiac or smooth muscle cell SUR2 overexpressing animals will constitute a powerful tool. As indicated in the **Supplementary Table 1**, our samples (lung, PAECs, PASMCs) used in the study were from control and PAH patients with different ages, so we did not exclude that these differences have any consequences on SUR2/Kir6.1 expression. However, using a human sample, there is no alternative strategy.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Protocol N8CO-08- 003, ID RCB: 2008-A00485-50, approved on June 18, 2008. The patients/participants provided their written informed consent to participate in this study. The animal facility is licensed by the French Ministry of Agriculture (agreement No. C92-019-01).

Author contributions

HL, VC, DM, and FA performed conception and design. HL, BM, MD, ABo, ABe, JS, VD, VC, and FA conducted the experiments and performed the data analysis. All authors drafted and brought the important intellectual content of the manuscript and reviewed and approved the final version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Therapeutic targeting of mineralocorticoid receptors in pulmonary hypertension: Insights from basic research

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Pulmonary hypertension (PH) is characterized by pulmonary vascular remodeling and associated with adverse outcomes. In patients with PH, plasma aldosterone levels are elevated, suggesting that aldosterone and its receptor, the mineralocorticoid receptor (MR), play an important role in the pathophysiology of PH. The MR plays a crucial role in adverse cardiac remodeling in left heart failure. A series of experimental studies from the past few years indicate that MR activation promotes adverse cellular processes that lead to pulmonary vascular remodeling, including endothelial cell apoptosis, smooth muscle cell (SMC) proliferation, pulmonary vascular fibrosis, and inflammation. Accordingly, *in vivo* studies have demonstrated that pharmacological inhibition or cell-specific deletion of the MR can prevent disease progression and partially reverse established PH phenotypes. In this review, we summarize recent advances in MR signaling in pulmonary vascular remodeling based on preclinical research and discuss the potential, but also the challenges, in bringing MR antagonists (MRAs) into clinical application.

KEYWORDS

pulmonary hypertension, aldosterone, finerenone, spironolactone, mineralocorticoid receptors, eplerenone, right heart failure

Introduction

Pulmonary hypertension (PH) is characterized by increased muscularization and thickening of the small pulmonary arteries (PAs), resulting in progressive elevation of pulmonary vascular resistance (PVR) and PA pressure (PAP) (1). With disease progression, increased right ventricular (RV) afterload leads to RV dysfunction and failure, resulting in markedly reduced functional capacity, quality of life, and life expectancy (2). PH is defined as a mean PAP (mPAP) of more than or equal to 20 mmHg and PVR $\geq$ 3 Wood Units (WU) for pre-capillary forms of PH, measured by right heart catheterization (3–5). PH combines heterogeneous pulmonary vascular conditions, classified into five groups as follows: Group 1—pulmonary arterial hypertension (PAH), including idiopathic, heritable, and drug/toxin-induced PH; Group 2—PH due to left heart disease; Group 3—PH due to lung disease and/or chronic hypoxia; Group 4—PH due to chronic thromboembolism; and Group 5—PH with unclear multi-factorial mechanisms.

The pathobiology of pulmonary vascular remodeling is characterized by PA endothelial cell (PAEC) dysfunction and apoptosis with the subsequent reactive proliferation of PA smooth muscle cells (PASMCs), increased extracellular matrix (ECM) deposition, and inflammatory/immune cell infiltration of the pulmonary vascular wall (1). Despite several decades of research in this field, disease development and progression mechanisms remain

incompletely defined (6). Alterations of several signaling pathways have been shown to participate in the pathogenesis of pulmonary vascular remodeling. This has led to the development and approval of therapies that primarily target calcium channels, prostaglandin receptors, endothelin receptors (ETs), phosphodiesterases, and soluble guanylate cyclase in PAH (5). However, these therapies provide only partial improvements in hemodynamics and outcome for PAH patients suggesting there are additional dysregulated signaling pathways contributing to the disease pathogenesis. In addition, available therapies are largely restricted to Group 1 PH patients (5) (except for riociguat in CTEPH) but not efficient or even detrimental in patients with other PH classes.

Therefore, a number of preclinical and early clinical trials have been conducted in order to test the ability of pharmacological agents targeting other signaling pathways to improve the pathophysiology of PH and patient outcomes (7). Among others, augmented activation of the mineralocorticoid receptor (MR), an aldosterone receptor belonging to the nuclear transcription receptor superfamily, has emerged as one of the underlying mechanisms driving disease development and progression in PH (8). Aldosterone is a critical effector hormone of the renin-angiotensin-aldosterone system (RAAS), which plays an important role in the regulation of normal cardiovascular homeostasis and the pathogenesis of diverse cardiovascular diseases. Activation of MR in cardiomyocytes, endothelial cells, vascular SMCs, or myeloid cells induces inflammation and adverse remodeling of the heart and the vascular system (9, 10). MR antagonists (MRAs) such as spironolactone and eplerenone are considered standard in left heart failure management and associated with significantly improved outcomes (10).

During the past decade, a series of experimental studies investigated the role of aldosterone and MR in pulmonary vascular remodeling and a potential benefit of MRAs for PH patients. In this review, we discuss mechanisms of MR signaling in pulmonary vascular cells with a focus on recent findings from genetically engineered animal models. We compare the impact of pharmacological MR blockade in different PH animal models and the association of aldosterone with PH patients' phenotypes and outcomes. Lastly, we discuss the translational potential of MRAs toward clinical application in PH, as well as new research initiatives that may lead to a better understanding of MR in PH and further developments.

Mechanisms of aldosterone and mineralocorticoid receptor signaling

The mineralocorticoid aldosterone is primarily synthesized in adrenocortical cells of the zona glomerulosa of the adrenal cortex and functions as one of the effector hormones in the RAAS. The classical role of aldosterone is to control salt and water balance *via* binding to the MR in kidney epithelial cells, however, MR is widely expressed in extrarenal tissues (11).

The MR belongs to the family of nuclear receptors of ligand-dependent transcription factors (12). Unbound MR is together with its chaperone proteins located in the cytosol. Ligand binding induces a conformational change of the MR, dissociation from the chaperone proteins, dimerization, and translocation into the nucleus to induce the expression of target genes (13). Besides aldosterone, cortisol acts as a ligand at the MR with similar degree of affinity as aldosterone (11). Given the high concentration of cortisol in tissues and circulation, aldosterone binding to the MR requires the expression of 11β-hydroxysteroid dehydrogenase 2 (HSD11B2), which inactivates cortisol into cortisone, which has low affinity for the MR (11, 14). In addition to regulating gene expression, MR also can promote non-genomic effects by modulating several other signaling pathways, including different membrane receptors (13).

Pharmacology of the mineralocorticoid receptor

Mineralocorticoid receptor antagonists comprise a group of pharmacological agents that antagonize the action of aldosterone at the MR. Several MRAs including spironolactone, eplerenone, and finerenone have been studied for their effects in the experimental models of PH (**Tables 1**, **2**). Despite the similar mechanisms of action of those agents, they may substantially differ between each other in term of their pharmacological properties, which may eventually account for the variability of the observed effects in humans and animal models. For example, spironolactone exhibits binding affinity not only to the MR but also to androgen and progesterone receptors, causing undesirable effects such as painful gynecomastia (15). This was improved with eplerenone, showing an improved selectivity for MR and less side effects due to unspecific binding to androgen and progesterone receptors compared to spironolactone (15). In addition, eplerenone exhibits lower plasma protein binding and has a shorter plasma half-life compared with spironolactone (16). Both, spironolactone and eplerenone are based on a steroidal backbone. More recently, non-steroidal MRAs such as finerenone or esaxerenone have been developed, which have specific pharmacological features that are distinct from steroidal MRAs. So far, finerenone has been tested in experimental PH. Finerenone is a dihydronaphthyridine-based compound with high selectivity for the MR over all other steroid hormone receptors and high binding affinity (17). Finerenone is excreted to a minor degree (<1%) by the kidney, has a short plasma half-life (2–3 h), also in patients with renal failure, and no active metabolites have been identified (18). These pharmacological features of finerenone may contribute to a significantly smaller increases in serum potassium levels and lower incidences of hyperkalemia compared to the steroidal MRA spironolactone (19). Collectively, the pharmacological characteristics of the MRAs outlined above should be carefully considered when interpreting and comparing the observed effects and side effects of these drugs. The pharmacological characteristics of the different MRAs outlined above should be taken into account when evaluating the potential utility of these compounds in preclinical and clinical studies of PH.

Effects of aldosterone and MR activation on the course and severity of PH

Patients with PH with no evidence of left heart dysfunction display elevated aldosterone levels, which are associated with a wide range of clinical and hemodynamic indices (20, 21) (**Figure 1**). For example, in patients with PAH aldosterone levels

TABLE 1 Summary of studies evaluating the preventive application of mineralocorticoid receptor antagonists (MRAs) in rodent models of pulmonary hypertension (PH).

Agent	PH model	Agent details	Pulmonary hemodynamics		Vascular remodeling (histo)	RV remodeling (echo)	RV remodeling (ex-vivo, histo)	RV function (echo)	References
			Invasive	Non-invasive					
Spironolactone	Monocrotaline rats (3 weeks)	40 mg/kg/day (implanted pellet) (3 weeks)	↓ RVSP	NA	↓ PA muscularization	NA	↔ RV/(LV+S), ↓ RV myocyte, ↔ RV fibrosis	NA	(44)
Spironolactone	Monocrotaline rats (3 weeks)	25 mg/kg/day (drinking water) (3 weeks)	↓ PASP	NA	↓ PA muscularization, ↓ Vessel wall thickness	NA	↓ RV/(LV+S)	NA	(46)
Spironolactone	Monocrotaline rats (25 days)	25 mg/kg/day (drinking water) (25 days)	↓ PASP	↓ PAAT	↓ PA muscularization, ↓ PA fibrosis	↓ RVWT	NA	NA	(27)
Spironolactone	Hypoxia-sugen mice (4 weeks)	15 mg/kg/day (implanted pellet)	↓ RVSP	NA	↓ PA muscularization	NA	↔ RV/(LV+S), ↓ RV myocyte, ↓ RV fibrosis	NA	(47)
Spironolactone	Hypoxia mice (5 weeks)	15 mg/kg/day (implanted pellet) (5 weeks)	↓ RVSP	NA	↓ PA muscularization	NA	↔ RV/(LV+S), ↓ RV fibrosis, ↔ RV myocyte	NA	(44)
Eplerenone	Hypoxia mice (6 weeks)	200 mg/kg/d (chow) (5 weeks)	NA	↑ PAAT/PAET	↓ PA vessel thickness	↓ RVID/LVID	↓ RV myocyte	↑ TAPSE	(52)
Eplerenone	Hypoxia-sugen mice (3 weeks)	200 mg/kg/d (chow) (3 weeks)	↓ RVSP	NA	↓ PA muscularization, ↓ Vessel wall thickness	NA	↓ RV/BW	NA	(51)
Spironolactone	Monocrotaline rats (25 days)	25 mg/kg/day (drinking water) (25 days)	NA	NA	↓ PA wall thickness	NA	NA	NA	(54)

RVSP, right ventricular systolic pressure; mPAP, mean pulmonary artery pressure; PSAP, pulmonary artery systolic pressure; BW, body weight; RV, right ventricle; LV, left ventricle; S, septum; PA, pulmonary artery; TAPSE, tricuspid annular plane systolic excursion; RVWT, right ventricular wall thickness; RVID, right ventricular diameter at end-diastole; LVID, left ventricular internal diameter; NA, not applicable. ↔, Measured parameter in the treatment did not change significantly compared to placebo group; ↑, measured parameter in the treatment significantly increased compared to placebo group; ↓, measured parameter in the treatment significantly decreased compared to placebo group.

showed positive correlations with PVR, mPAP, transpulmonary pressure gradient, and WHO functional class (22, 23) but negative correlations with cardiac output (CO) (22). Furthermore, aldosterone level in PAH patients may differ depending on the underlying etiology. For example, in contrast to idiopathic PAH (IPAH) patients, circulating aldosterone levels are not increased in PAH patients associated with connective tissue diseases (PAH-CTD) (23). Taken together, these studies indicate that circulating aldosterone levels are increased in Group 1 PH and its increase may be associated with adverse functional and hemodynamic alterations. Available data suggest that both, adrenal and extra-adrenal sources may contribute to overall circulating levels of aldosterone in PAH. For example, in PH due to heart failure with preserved ejection fraction (HFpEF), transpulmonary aldosterone levels are increased indicating that pulmonary synthesis of aldosterone (24). In more advanced disease conditions, altered hemodynamics may contribute to an exaggerated increase of circulating aldosterone. For example, decompensated stage of PH with pronounced RV failure may lead to the release of renin secondary to chronically reduced kidney perfusion resulting in chronically activated RAAS (25).

Local and systemic aldosterone synthesis in pulmonary hypertension

The contribution of local aldosterone production in lung tissue during PH has been assessed in experimental models. In hypoxia-sugen rat (26) and monocrotaline rat (27) models, PH development is associated with not only increased circulating aldosterone but also lung tissue aldosterone content. Two rate-limiting enzymes catalyze the formation of aldosterone from cholesterol: steroidogenic acute regulatory protein (StAR), which transports cholesterol to the inner mitochondrial membrane, and aldosterone synthase (CYP11B2), which converts 11-deoxycorticosterone into aldosterone. In addition, aldosterone in PAECs may also be synthesized from other metabolic intermediates such as pregnenolone, rather than from cholesterol (28), which can explain the differences in the expression profiles of enzymes responsible for aldosterone synthesis in PAECs compared to cells of the adrenal cortex (28). *In vitro* studies have shown that stimulation of PAECs with ET-1 induces the binding of the steroidogenic transcription factors such as steroidogenic factor-1 (SF-1) and peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) to the CYP11B2 promoter, leading to CYP11B2 expression and aldosterone synthesis (27). Similarly, hypoxia also

TABLE 2 Summary of studies evaluating the therapeutic application of mineralocorticoid receptor antagonists (MRAs) in rodent models of pulmonary hypertension (PH).

Agent	PH model	Agent details	Pulmonary hemodynamics		Vascular remodeling (histo)	RV remodeling (echo)	RV remodeling (ex-vivo, histo)	RV function (echo)	References
			Invasive	Non-invasive					
Spironolactone	Monocrotaline rats (25 days)	25 mg/kg/day (drinking water) (10 days)	↓ RVSP	NA	NA	NA	NA	NA	(27)
Spironolactone	Monocrotaline rats (5 weeks)	40 mg/kg/day (implanted pellet) (2 weeks)	↓ RVSP	NA	↓ PA muscularization	NA	↔ RV/(LV+S), ↓ RV myocyte, ↔ RV fibrosis	NA	(44)
Spironolactone	Hypoxia-sugen rats (6 weeks)	25 mg/kg/day (drinking water) (4 weeks)	↓ PASP	NA	NA	NA	↓ RV/LV	NA	(26)
Spironolactone	Hypoxia-sugen rats (6 weeks)	25 mg/kg/day (drinking water) (3 weeks)	↓ PASP	NA	NA	NA	↓ RV/(LV+S)	NA	(46)
Spironolactone	Hypoxia-sugen rats (10 weeks)	40 mg/kg/day (chow) (5 weeks)	↔ RVSP	NA	↔ PA wall thickness	↔ LV ECI (MRI), ↔ RVEDV/LVEDV (MRI)	↔ RV fibrosis	↔ RV EF (MRI)	(53)
Eplerenone	Hypoxia-sugen rats (10 weeks)	100 mg/kg/day (chow) (5 weeks)	↔ RVSP	NA	↔ PA wall thickness	↓ LV ECI (MRI), ↓ RVEDV/LVEDV (MRI)	↔ RV fibrosis	↔ RV EF (MRI)	(53)
Eplerenone	Pulmonary artery banding mice (3 weeks)	200 mg/kg/d (chow) (3 weeks)	↔ RVSP	NA	NA	↔ RVID	↔ RV/BW, ↔ RV fibrosis, ↔ RV cardiomyocyte	↔ TAPSE	(51)
Finerenone	Monocrotaline rats (4 weeks)	1 mg/kg/day (per os) (2 weeks)	↓ mPAP	↑ PAAT/PAET	↓ PA muscularization, ↓ PA wall thickness	NA	↓ RV/(LV+S), ↓ RV fibrosis	NA	(43)
Finerenone	Hypoxia-sugen rats (8 weeks)	1 mg/kg/day (per os) (3 weeks)	↓ mPAP	↑ PAAT/PAET	↓ PA muscularization, ↓ PA wall thickness	NA	↓ RV/(LV+S), ↓ RV fibrosis	NA	(43)

RVSP, right ventricular systolic pressure; mPAP, mean pulmonary artery pressure; PSAP, pulmonary artery systolic pressure; BW, body weight; RV, right ventricle; LV, left ventricle; S, septum; PA, pulmonary artery; TAPSE, tricuspid annular plane systolic excursion; RVWT, right ventricular wall thickness; RVID, right ventricular diameter at end-diastole; LVID, left ventricular internal diameter; NA, not applicable. ↔, Measured parameter in the treatment did not change significantly compared to placebo group; ↑, measured parameter in the treatment significantly increased compared to placebo group; ↓, measured parameter in the treatment significantly decreased compared to placebo group.

promotes PAEC aldosterone synthesis by increasing StAR expression *via* hypoxia-induced c-Fos/c-Jun binding to the StAR promoter (26). Moreover, angiotensin-II (Ang-II) also has been shown to induce aldosterone synthesis in PAECs (27). Cumulatively, these studies indicate that various factors, including ET-1, Ang-II, and hypoxia, which have been known to modulate pulmonary vascular remodeling, may also directly stimulate *de novo* aldosterone synthesis in the pulmonary vasculature.

Aldosterone–MR signaling in pulmonary vascular cells

Mineralocorticoid receptor is expressed in PAECs and PASMCs and MR activation induces various cellular processes that contribute to pulmonary vascular remodeling in PH: in healthy PAECs, nitric oxide (NO) is produced by endothelial nitric oxide synthase (eNOS) and diffuses to the underlying PASMCs, where it promotes pulmonary vascular dilation (29). In addition, NO also acts locally on PAECs to prevent inflammation and thrombosis. In PH, this system becomes dysregulated and is considered a key driver of pulmonary vascular remodeling (30). MR activation by aldosterone as observed in PH disturbs NO signaling by promoting reactive oxygen species (ROS) production in pulmonary vascular cells. Aldosterone activates NADPH oxidase 4 in PAECs to generate excessive ROS (31, 32), which in turn leads to NEDD9 oxidation, disabling its association with SMAD3, and resulting in NEDD9 nuclear translocation (31). In the nucleus of PAECs, NEDD9 together with NKX2 activates the COL3A1 gene promoter (31). In addition, aldosterone-mediated expression of profibrotic factors including connective tissue growth factor (CTGF), collagen 1, matrix metalloprotease 2 (MMP2), and MMP9 in PAECs (26, 31) has been shown, which is considered

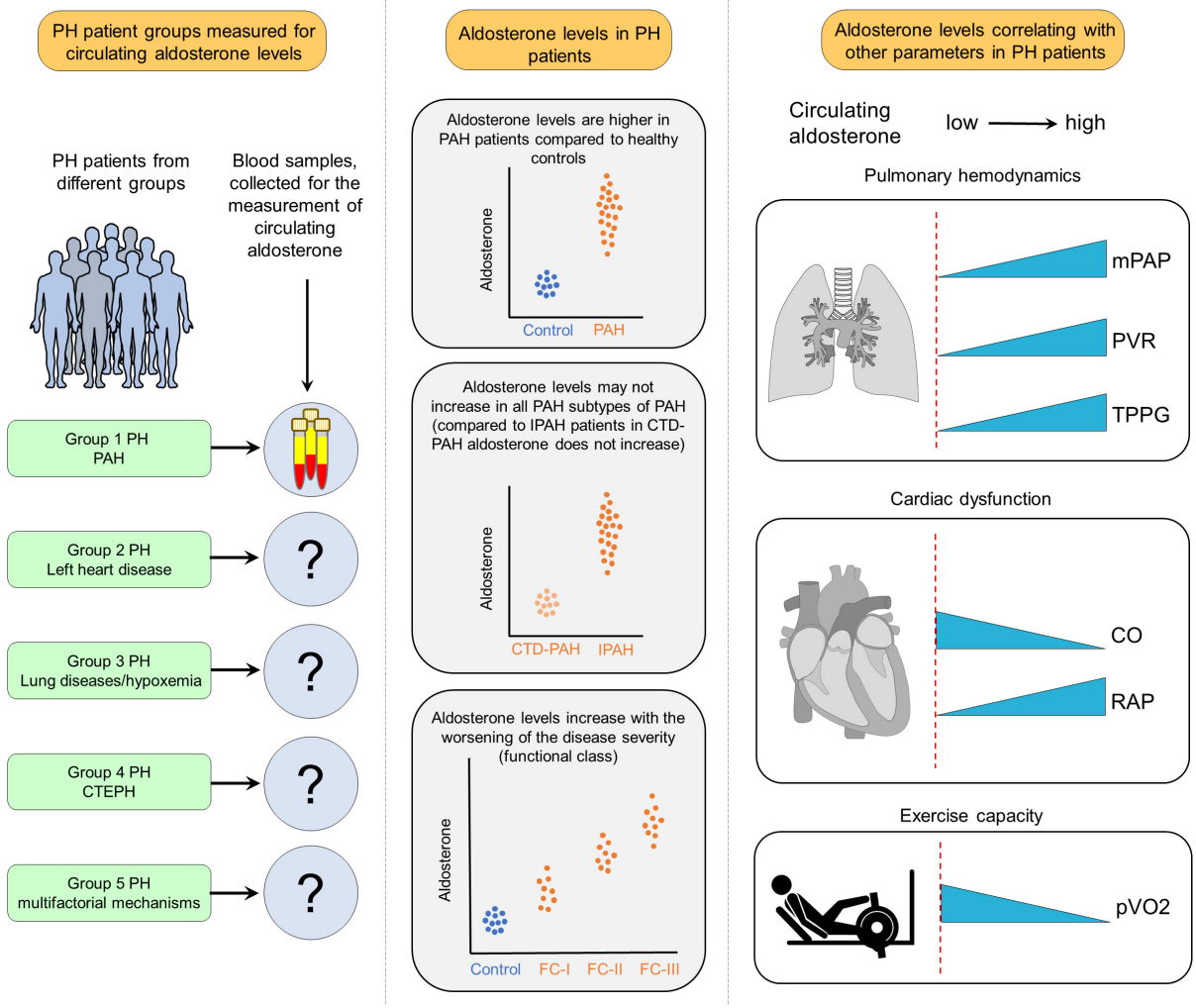

FIGURE 1
Clinical implications of increased aldosterone levels in pulmonary hypertension (PH). In available studies, aldosterone levels have been measured mostly in Group 1 PH also referred to as pulmonary arterial hypertension (PAH), while in other forms of PH, circulating levels of aldosterone remained neglected. The results of those studies revealed that aldosterone levels are increased in PAH patients compared to healthy controls. In addition, there are differences in PAH subtypes regarding aldosterone levels. For example, compared to idiopathic PAH (IPAH), circulating aldosterone levels are not increased in PAH patients due to connective tissue diseases (CTD-PAH). Moreover, in PAH patients, aldosterone levels are increased along with worsening the disease severity. Furthermore, aldosterone levels are correlated with several hemodynamic and functional parameters like mean PA pressure (mPAP), pulmonary vascular resistance (PVR), transpulmonary pressure gradient (TPPG), cardiac output (CO), right atrial pressure (RAP), and decreased maximal oxygen uptake (pVO2).

to ultimately result in excessive collagen deposition in the pulmonary vasculature.

Hydrogen peroxide (H_2O_2) generation in PAECs due to aldosterone-mediated NADPH oxidase-4–activity induces a sulfenic post-translational modification of the ET type-B (ET-B), resulting in the blockade of ET-B signaling pathway and impaired NO synthesis and bioavailability in the pulmonary vasculature (27). It is interesting to note that in the systemic vasculature, MR signaling also plays a role in promoting ROS formation and dampening NO signaling that is EC-specific. MR deletion in ECs prevents the aldosterone-induced increase in superoxide (O_2^-) production (33) and results in enhanced eNOS activity and improved endothelial function (34). Consequently, MR-mediated decrease in NO bioavailability within the pulmonary vasculature results in PASMC contractility, proliferation, and ECM synthesis (27), which are the main characteristics of the pathobiology of PH. Taken together, impaired NO signaling and excessive ROS formation in PH is considered one of the pathological mechanisms underlying PH development in response to excessive aldosterone.

Activated aldosterone-MR signaling has been shown to promote PAEC senescence and inhibit cell proliferation by down-regulating SIRT1 with consequent p53 and p21 up-regulation (35). Excessive EC apoptosis and pulmonary vascular remodeling due to secretin deficiency are at least partially mediated by aldosterone-mediated down-regulation of vascular endothelial growth factor (VEGF) (36). MR activation is a well-known driver of vascular inflammation (37, 38) and this seems to apply to the pulmonary vasculature as well. In PAECs, MR promotes leukocyte adhesion to ECs *via* up-regulating intercellular adhesion molecule-1 (ICAM-1) expression (39). Similarly, aldosterone-MR signaling also facilitates tumor necrosis factor alpha (TNFα)-induced proinflammatory gene expression in PAECs, which can be effectively prevented by the

application of spironolactone (40). In addition, aldosterone may promote pulmonary vascular remodeling by inducing endothelial-to-mesenchymal transition (EndoMT), as it has been demonstrated that MR activation promotes cardiac and renal fibrosis *via* activating EndoMT (41), which is also involved in the pathogenesis of PH (42). However, the direct effects of aldosterone mediated EndoMT in the pathogenesis of PH has not been studied and should be investigated in the future. Finally, MR may interfere with VEGF signaling in PAECs, however, this remains to be confirmed. Taken together, MR signaling activation in PAECs alters diverse cellular processes including NO signaling, ROS formation, cellular apoptosis, ECM synthesis, and inflammation which in crosstalk with PASMCs, fibroblasts, and immune cells promotes pulmonary vascular remodeling and PH development (**Figure 2**).

Beside indirect signaling *via* MR in PAECs, it has been shown that MR activation in PASMCs is involved in PH progression. A recent study has provided direct experimental evidence that MR is overexpressed in PASMCs of the remodeled PAs in patients with PAH as well as in monocrotaline and hypoxia-sugen rats with established PH (43). *In vitro*, aldosterone activates MR in PASMCs in a dose-dependent manner without affecting its expression level (44). The main effect of activated aldosterone-MR signaling in PASMCs is an augmented cell proliferation (44, 45), which can be prevented by MRAs (44, 45) or MR-directed siRNAs (43). Aldosterone has been shown to modulate various cellular signaling pathways in PASMCs to promote cell proliferation and survival (44). For example, in PASMC, aldosterone activates the Akt signaling pathway, which in turn induces mTOR signaling pathway activation resulting in cell survival and proliferation (46). In addition, aldosterone has been shown to promote PASMC proliferation *via* the ERK signaling pathway because of BMP2/7-mediated MR up-regulation (45). Similarly, aldosterone also promotes PASMC viability *via* up-regulating aquaporin and b-catenin (35). Moreover, aldosterone prevents oxidative stress-induced PASMC apoptosis (35). In addition to increased cell proliferation, aldosterone also promotes the profibrotic phenotype of PASMCs. For example, hypoxia-exposed PAECs promote CTGF up-regulation in PASMCs *via* aldosterone-mediated mechanisms (26). As outlined above, aldosterone-induced NEDD9 up-regulation in PAECs can cause exosome-mediated NEDD9 activation in PASMCs and collagen three up-regulation (31). MR activation in PASMC is also responsible for the perivascular inflammation in PH (47), likely through the production of a paracrine factor that enhances monocyte chemotaxis (48). Pharmacological MR blockade exerts protective effects against pulmonary vascular remodeling with decreased PASMC proliferation and reduced inflammatory cell infiltration (43). Likewise, a lower degree of perivascular lung inflammation in response to hypoxia-sugen was observed in mice with SMC-specific deletion of MR compared to wild type (47). In addition, aldosterone may exert adverse effects on PASMCs by promoting cell senescence as aldosterone has been shown as one the strong pro-senescent factors in the pathogenesis of cardiovascular diseases (49), which also play a crucial role in the pathobiology of PH (50). However, the evidence directly linking aldosterone signaling with cellular senescence in the development of PH is still lacking and should focus of the future investigation. Taken together, the pathologic effects of aldosterone-MR signaling in PASMCs are mainly driven due to their effects to promote cell proliferation, apoptosis resistance, ECM synthesis, and perivascular inflammation (**Figure 2**).

Pharmacological targeting of MR in PH animal models

Several studies have been conducted to evaluate the potential benefits of MRAs to prevent or reverse pulmonary vascular remodeling in animal models of PH. Those experimental studies have employed MRAs spironolactone, eplerenone, or finerenone in several models of PH (**Tables 1**, **2**). The overall finding of those studies is that MRAs can prevent and partially reverse pulmonary vascular remodeling and improve pulmonary hemodynamics in PH (43, 47, 51–53) (**Tables 1**, **2**). Preventive application MRAs attenuated the development of adverse pulmonary vascular remodeling as assesses by attenuated PA muscularization and wall thickening along with decreased PA pressure (27, 44, 46, 47, 51, 52, 54) (**Table 1**). Initiation of MRA therapy after the disease phenotype had established was able to decrease PA muscularization and wall thickening and improve pulmonary hemodynamics (26, 43, 44, 46, 53) (**Table 2**). This applied both to steroidal as well as the novel non-steroidal MRA finerenone (43). In most of these studies, both preventive and therapeutic applications of MRAs could improve RV dysfunction and remodeling induced by monocrotaline, hypoxia, or hypoxia-sugen (43). It has been questioned whether improved RV function was a direct effect of MRAs on cardiac cells or indirectly mediated by lowered RV afterload.

In one recent study, MRAs initiated after the onset of significant RV failure in the hypoxia-sugen rat PH model led to modest, but consistent beneficial effects on cardiac function and remodeling (53). Specifically, MRI imaging of the heart in hypoxia-sugen rats, revealed that MRAs improved cardiac index, the RVEDV/LVEDV ratio, and the degree of septal displacement although no significant reductions in either PA pressure or vessel remodeling were observed (53). The cardioprotective effect of MRAs in this study may be related at least in part to the attenuated pro-inflammatory gene expression in the RV (53), which is considered as a crucial mediator of adverse RV remodeling in response to pressure overload (55). In contrast to that, application of the MRA eplerenone after 1-week of PAB surgery in mice did not improve RV function and remodeling at 3 weeks (51). Similarly, in the rat PAB model of RV remodeling, preventive application of an Ang-II receptor blocker plus eplerenone for 11 weeks also did improve RV function (56).

Taken together, these findings suggest that MR blockade can exert beneficial effects on pulmonary vascular remodeling and subsequent RV failure when applied preventive or in established mild-to-moderate PH. In more advanced pulmonary vascular remodeling or in the setting of fixed RV pressure overload, their therapeutic potential seems to be limited.

Genetic manipulation of MR in PH animal models

During the past 10 years a series of experimental studies using transgenic mouse lines with cell type-specific targeting of the MR in the cardiovascular system have unraveled distinct roles for MR in SMCs, ECs, cardiomyocytes, and monocytes/macrophages. As a result of these studies, the specific contribution of in MR different cell types to hypertension, heart failure, or post-myocardial infarction remodeling could be defined (9, 10). A recent study has demonstrated that transgenic mice ubiquitously overexpressing

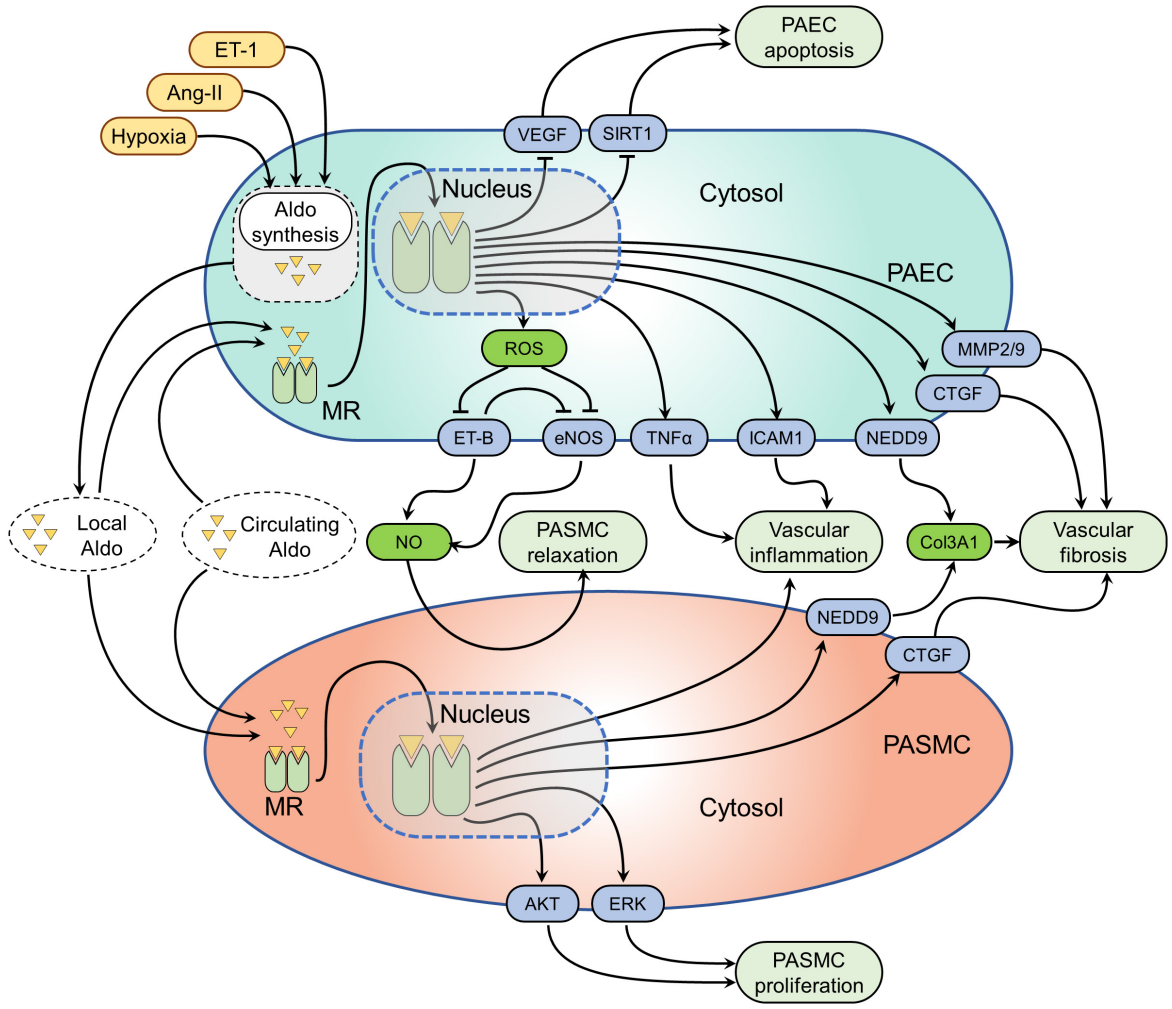

FIGURE 2
Mineralocorticoid receptor (MR)-mediated signaling pathways in pulmonary vascular cells. Several factors such as hypoxia, angiotensin-II (Ang-II), and endothelin-1 (ET-1) have been shown to induce aldosterone synthesis in pulmonary artery endothelial cells (PAECs). Both locally synthesized aldosterone and circulating aldosterone can activate MR in PAECs and PA smooth muscle cells (PASMCs). Activation of the MR alters several signaling pathways in pulmonary vascular cells. The effected pathways in PAECs are the following: (1) MR-induced reactive oxygen species (ROS) formation, which in turn inhibits endothelial nitric oxide synthesis (eNOS) and endothelin receptor B (ET-B) resulting in decreased nitric oxide (NO) formation. The resulting effect of decreased NO bioavailability is increased pulmonary vascular SMC (PASMC) contractility; (2) MR activation inhibits VEGF and sirtuin 1 (SIRT1) resulting in PAEC apoptosis; (3) MR leads to the overexpression neural precursor cell expressed developmentally down-regulated protein 9 (NEDD9), connective tissue growth factor (CTGF), matrix metalloproteinase-2 and 9 (MMP2/9) causing pulmonary vascular fibrosis; (4) MR also induces tumor necrosis factor alpha (TNFα) and intercellular adhesion molecule-1 (ICAM-1) overexpression, resulting in pulmonary vascular inflammation. While the MR effected pathways in PASMCs include: (1) MR activation leads to pulmonary vascular inflammation through unknown mechanisms; (2) MR induced neural precursor cell expressed developmentally down-regulated protein 9 (NEDD9) and CTGF activation cause pulmonary vascular fibrosis; (3) while MR-mediated activation of protein kinase B (AKT) and extracellular signal-regulated kinase (ERK) signaling pathways cause increased PASMC proliferation.

human MR (hMR$^+$ mice) spontaneously develop PH, characterized by increased RV systolic pressures, RV hypertrophy, and remodeling of small pulmonary vessels and a 2-fold increase in the percentage of proliferating PASMCs compared with their wall thickness (WT) littermates (43). This adds to an earlier study reporting that hMR$^+$ mice develop moderate dilated cardiomyopathy without cardiac fibrosis, with normal blood pressure, tachycardia, and a high occurrence of arrhythmia (57). Given the many parallels in the pathophysiology of cardiovascular disease, it seemed plausible that MR in these cells might also be involved in the development of PH (**Table 3**). Indeed, while mice with EC-specific MR deletion were protected from pulmonary vascular remodeling in response to hypoxia in a similar manner as seen with eplerenone treatment, transgenic mouse lines with specific MR deletion in SMCs, fibroblast, or myeloid cells displayed a similar degree of PH as observed in WT mice (52). These findings indicate that the beneficial effects of MRAs on PH may be mainly mediated through the blockade of MR in ECs with indirect effects on PASMCs. It is important to note that this finding could not be reproduced in another study using the hypoxia-sugen model (47). EC-specific MR deletion has been shown to exert benefits on the RV in the hypoxia-sugen mouse model by regulating RV E-selectin and collagen III expression and attenuating RV perivascular fibrosis but did not improve PH (47). This suggests that the protective effects of MR deletion in PAECs on the pulmonary vasculature may involve VEGF signaling (as this remains disturbed in the hypoxia-sugen model due to inhibition of VEGF receptor 2 by SU5416, independent of the MR). In line with this, we had reported before that MR activation is able to counterregulate VEGF

TABLE 3 Summary of studies evaluating the effects of genetic manipulation of mineralocorticoid receptor (MR) on the pulmonary vasculature and right ventricle in rodent models.

Cell type	PH model	Genetic model	Pulmonary hemodynamics		Vascular remodeling (histo)	RV remodeling (echo)	RV remodeling (ex-vivo, histo)	RV function (echo)	References
			Invasive	non-invasive					
SMC MR deletion	Hypoxia mice (6 weeks)	$Myh11^{MerCreMer}$–$MR^{fl/fl}$	NA	↔ PAAT/PAET	NA	NA	NA	↔ TAPSE	(52)
Macrophage MR deletion	Hypoxia mice (6 weeks)	$LysM^{Cre}$–$MR^{fl/fl}$	NA	↔ PAAT/PAET	NA	NA	NA	↔ TAPSE	(52)
FB MR deletion	Hypoxia mice (6 weeks)	$Tcf21^{CreERT}$–$MR^{fl/fl}$	NA	↔ PAAT/PAET	NA	NA	NA	↔ TAPSE	(52)
EC MR deletion	Hypoxia mice (6 weeks)	$Cdh5^{CreERT}$–$MR^{fl/fl}$	NA	↑ PAAT/PAET	↓ PA thickness	↓ RVID/LVID	↓ RV myocyte	↑ TAPSE	(52)
SMC MR deletion	Hypoxia-sugen mice (4 weeks)	$Acta2^{Cre}$–$MR^{fl/fl}$	↔ RVSP	NA	↔ PA muscularization	NA	↔ RV/(LV+S), ↔ RV myocyte, ↔ RV perivascular fibrosis, ↔ RV interstitial fibrosis	NA	(47)
EC MR deletion	Hypoxia-sugen mice (4 weeks)	$Cdh5^{CreERT}$–$MR^{fl/fl}$	↔ RVSP	NA	↔ PA muscularization	NA	↔ RV/(LV+S), ↔ RV myocyte, ↓ RV perivascular fibrosis, ↔ RV interstitial fibrosis	NA	(47)
Global MR overexpression	hMR expressing mice	P1 promoter into the B6D2F1 mouse strain	↑ RVSP	↔ PAAT/PAET	↑ PA muscularization, ↑ PA wall thickness	NA	↑ RV/(LV+S)	NA	(43)

EC, endothelial cells; FB, fibroblasts; SMCs, smooth muscle cells; RVSP, right ventricular systolic pressure; BW, body weight; RV, right ventricle; LV, left ventricle; S, septum; PA, pulmonary artery; TAPSE, tricuspid annular plane systolic excursion; RVID, right ventricular diameter at end-diastole; LVID, left ventricular internal diameter. NA, not applicable. Parameter ↑ increased, ↓ decreased, or remains ↔ unchanged compared to wild type control.

signaling in cultured endothelial cells (58). SMC-MR deletion did not improve PH and RV hypertrophy in both, hypoxia and hypoxia-sugen models of PH, compared to wild type mice (47, 52), although MR deficient mice displayed attenuated degree of lung perivascular inflammation (47). This finding suggests that PASMC-MR, activated *in vivo* in PH, contributes to the recruitment of inflammatory cells in the lung perivascular area. This most likely occurs through a yet to be defined paracrine factor that promotes chemotaxis of inflammatory cells. However, it is surprising that such anti-inflammatory benefits of SMC-MR deletion did not translate into the improved pulmonary hemodynamics and vascular remodeling. Taken together, these studies using transgenic mouse lines revealed that MR overexpression can cause the development of spontaneous PH while MR deletion can prevent the development of PH in response to hypoxia at least in part due to EC-specific actions.

Clinical application of MR antagonists in PH patients

Above discussed preclinical studies and the established utility of MRAs for the management of heart failure have led to several clinical studies evaluating the potential benefit of MRAs in patients with PH (59). For example, a retrospective analysis of spironolactone use in PAH patients in ARIES-1 and ARIES-2 trials showed a trend toward improved 6-min walking distance (6-MWD) and circulating B-type natriuretic peptide (BNP) levels with the combination of spironolactone and ambrisentan (ET-A antagonist) and compared to ambrisentan alone (22). Interestingly, spironolactone use was associated with more potent decrease in circulating inflammatory markers compared to PAH-specific therapies (40). Another retrospective study revealed that MRA use indicates disease severity in PH patients (60). This may be the result of prescribing MRAs only for those PH patients with more severe conditions. It was documented that a combination of spironolactone and hydrochlorothiazide almost completely reversed PH and RV dysfunction within 3 months of treatment initiation in a preterm infant with bronchopulmonary dysplasia (BPD) with associated severe PH and RV failure (61). Cumulatively, the results of these clinical studies suggest that MRAs may be beneficial for PAH patients, and their use is associated with a worse clinical condition, likely due to a delayed initiation of MRAs. In contrast, two recent studies indicated that MRA use was associated with increased mortality in patients with PH (60, 62). In one of these studies, the association of MRA use with decreased survival was not evident after adjustment for disease

severity, suggesting that MRAs were prescribed preferentially for those PH patients with more advanced disease condition (62). Another trial showed that spironolactone did not change tissue fibrosis biomarker levels as the primary end-point nor did it improve clinical outcomes, although spironolactone was well-tolerated and did not lead to significant adverse events in PAH patients (63). Of note, it was possible to analyse the effects of MRAs on PH patients in above discussed retrospective studies because MRAs are prescribed for PH patients with fluid overload. Whether MRAs improve outcomes of patients with PH due to left heart disease is unknown. The TOPCAT trial failed to demonstrate a benefit of spironolactone in patients with HFpEF, 36% of them showing PH (64, 65). However, the study had substantial methodological problems and must be interpreted with caution (66). Currently, there are two further prospective, randomized placebo-controlled trials are ongoing. The STAR-HF trial, assessing the ability of spironolactone to reduce RV ventricular wall stress (NCT03344159). Another study is evaluating the effect of spironolactone on exercise capacity, RV function, inflammatory markers, and potential side effects in PAH patients (NCT01712620). The results of these trials are expected to determine whether MR blockade is beneficial in managing PAH patients.

Summary

Mineralocorticoid receptor is an important and highly versatile transcription factor that regulates various key signaling pathways in the pathogenesis of PH. MR activation in PAECs promotes aberrant redox signaling through augmented expression of pro-oxidant enzymes, increased ROS production, and reduced NO bioavailability, resulting in PAEC senescence and apoptosis. In addition, MR activation drives pro-inflammatory and pro-fibrotic phenotypes of PAECs. In PASMCs, MR activation causes cell hyperproliferation and excessive ECM synthesis and perivascular inflammation. Cumulatively, these effects of MR activation in pulmonary vascular cells promote PH development and progression. Abundant evidence from preclinical studies demonstrates the therapeutic promise of MR blockade to prevent and reverse many pathobiological features underlying PH. Considering that MRAs are available as approved treatment for left heart failure, repurposing those agents for the treatment of PH patients appears a promising strategy. Indeed, based on the available evidence on the regulation of aldosterone in PH patients and the results of retrospective clinical studies, subpopulations of PAH patients can be identified that may benefit from MRA treatment. Experimental studies may help to identify suitable biomarkers to closer define patients that are responsive to MRA treatment. However, data from a prospective randomized trial with MRAs in addition to established PH therapy is warranted to make definitive conclusions about the efficacy of MR blockade in the management of PH.

Challenges and future directions

The use of animal models has proven to be a valuable tool in revealing the molecular mechanisms of PH and to test potential new therapeutic approaches. The available data from experimental studies have provided key insights into the role of MR signaling in the pathogenesis of PH and its potential as a therapeutic target. They indicate that increased MR activity leads to pulmonary vascular remodeling, ultimately resulting in the development of PH. MR activation modulates complex signaling pathways during PH pathogenesis, as a result of its cell-, tissue-, and organ-specific effects. In this regard, these multitude roles of MR signaling may also pose many challenges in the field of research because it makes it difficult to interpret and generalize the results obtained. A common problem encountered during preclinical research is the controversial results across available studies. For example, the effects of MRAs tested in rodent models of PH may differ substantially between studies. It is likely that the different methodological approaches employed in those studies account for the majority of controversial results and heterogeneous conclusions. Therefore, it is important to consider the following points in interpreting the results of already available studies or in designing new studies: (1) choosing the model that fits best with the PH class and severity of disease of the target patient collective (for example, hypoxia model leading to moderate PH, while hypoxia-sugen causes more severe PH phenotypes and interferes with specific signaling pathways); (2) whether studies include animals of both sexes (to take into account for the sex-specific features of MR signaling); (3) phenotyping the disease severity in a rodent model with state-of-the-art imaging methods such as echocardiography, MRI, and cardiac catheterization (for example MRI can help to detect subtle cardiac changes of MRAs more precisely, which may be missed by other imaging modality); (4) whether doses, durations, and routes of administration of MRAs are comparable across studies.

Majority of available studies evaluating the effects of MRAs in PH model, used only male animals. This makes it challenging to extrapolate the results obtained from male animals to their female counterparts due to several sex-specific differences in the cardiovascular physiology. For example, sex differences play an important role not only in the development of PH (67), but also in responses to MRAs (34, 68) and in vascular role of the MR (69, 70). A recent study demonstrated that increased aldosterone production in response to physiologic and pathophysiological stimuli, increased EC MR expression and increased susceptibility to aldosterone-induced EC dysfunction in females compared with males (71). In addition, there is evidence to suggest that therapeutic responses to MRAs may be greater in females compared to males (72, 73). Cumulatively, the results of these studies dictate that it is crucial to consider the sex-specific features in MR research in PH. Although, cell-specific roles of MR in PH models have been studied in major vascular cells including SMC, ECs, fibroblasts, and macrophages, the contribution of other cell type MRs in PH pathogenesis cannot be ruled out. For example, the MR in cardiomyocytes or T cells may also play a role in the pathogenesis of PH. In addition, differing dosages and routes of administration of MRAs may also account for some of the conflicting results obtained from the experimental studies. For example, MRAs were administered with chow (26, 27, 51, 52), from a subcutaneous continuously releasing pellet (44, 47), or with a drinking water (26, 27, 46, 54). Similarly, the dosages of MRAs differed by up to factor three between studies (51–53). Whether there is a dose-dependent effect of MRAs in PH has not been systematically assessed yet. In the end, well-designed prospective clinical trials will

be needed to properly assess a potential benefit of MRAs in PH. Considering the insights and challenges outlined above may help us to design future clinical studies that evaluate the effects of (1) different MRA compounds in (2) patients from different groups of PH at (3) different stages of disease.

Author contributions

AM and AL conceived, drafted, and revised manuscript and figures. Both authors contributed to the article and approved the submitted version.

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Conflict of interest

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Publisher's note

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The sine transform is the *sine qua non* of the pulmonary and systemic pressure relationship

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Assessment of therapeutic interventions in patients with pulmonary arterial hypertension (PAH) suffers from several commonly encountered limitations: (1) patient studies are often too small and short-term to provide definitive conclusions, (2) there is a lack of a universal set of metrics to adequately assess therapy and (3) while clinical treatments focus on management of symptoms, there remain many cases of early loss of life in a seemingly arbitrary distribution. Here we provide a unified approach to assess right and left pressure relationships in PAH and pulmonary hypertension (PH) patients by developing linear models informed by the observation of Suga and Sugawa that pressure generation in the ventricle (right or left) approximately follows a single lobe of a sinusoid. We sought to identify a set of cardiovascular variables that either linearly or via a sine transformation related to systolic pulmonary arterial pressure (PAPs) and systemic systolic blood pressure (SBP). Importantly, both right and left cardiovascular variables are included in each linear model. Using non-invasively obtained cardiovascular magnetic resonance (CMR) image metrics the approach was successfully applied to model PAPs in PAH patients with an r^2 of 0.89 ($p < 0.05$) and SBP with an r^2 of 0.74 ($p < 0.05$). Further, the approach clarified the relationships that exist between PAPs and SBP separately for PAH and PH patients, and these relationships were used to distinguish PAH vs. PH patients with good accuracy (68%, $p < 0.05$). An important feature of the linear models is that they demonstrate that right and left ventricular conditions interact to generate PAPs and SBP in PAH patients, even in the absence of left-sided disease. The models predicted a theoretical right ventricular pulsatile reserve that in PAH patients was shown to be predictive of the 6 min walk distance ($r^2 = 0.45$, $p < 0.05$). The linear models indicate a physically plausible mode of interaction between right and left ventricles and provides a means of assessing right and left cardiac status as they relate to PAPs and SBP. The linear models have potential to allow assessment of the detailed physiologic effects of therapy in PAH and PH patients and may thus permit cross-over of knowledge between PH and PAH clinical trials.

KEYWORDS

pulmonary hypertension, pulmonary arterial hypertension, mathematical model, physiology, ventricular interdependance

Abbreviations

4D, Four dimensional; 6MWD, Six minute walk distance; BP, Blood pressure; BSA, Body surface area; CI, Confidence interval; CMR, Cardiovascular magnetic resonance; CXA-10, 10-nitrooctadec-9(*E*)-enoic acid; EDV, End diastolic volume; EF, Ejection Fraction; HPm, Hydraulic pulmonary mean pressure; HPp, Hydraulic pulmonary pulsatile pressure; HSm, Hydraulic systemic mean pressure; HSp, Hydraulic systemic pulsatile pressure; Imp, Impedance; LV, Left ventricle; MA, Moving average; MPA, Main pulmonary artery; PAH, Pulmonary arterial hypertension; PAPs, Systolic pulmonary artery pressure; PH, Pulmonary hypertension; PR, P-wave to R-wave interval; QRS, Q-wave R-wave S-wave complex; QT, Q-wave T-wave interval; QTcF, Fridericia formulation of QT/RR ratio; RHC, Right heart catheterization; ROC, Receiver operating characteristics; RR, R-wave to next R-wave interval; RV, Right ventricle; SBP, Systolic blood pressure (systemic); SD, Standard deviation; SPSS, Statistical Processing for the Social Sciences; VIF, Variance inflation factor; WHO, World Health Organization.

1. Introduction

We sought to develop linear models of pulmonary and systemic pressure to provide a universal set of metrics to address key paradoxes that exist in the study of PAH and PH patients (1). Firstly, PAH, a rare, untreatable and progressive disease has dominated the interests of basic, translation and clinical scientist and the pharmaceutical industry. From the time of detection there is a 5–7 year half mortality burden (2). Conversely, PH is common, often secondary to left heart disease, and remains largely undetected until severe and irreversible damage is sustained, leading to a very poor prognosis once discovered (3–6). Secondly, the PAH population remains so small that traditional clinical trials often fail to find any statistically significant impact of treatment, despite the common observation that some patients demonstrate clinically relevant improvement (7). A recent meta-analysis of the use of time to clinical worsening as an end-point in PAH trials noted that while it may lead to shorter and smaller trials, time to clinical worsening cannot be considered as valid surrogates for mortality in PAH trials (8) and further, that there is no widely-agreed upon definition of this metric (9). Additionally, the distinction between PAH and PH can be clinically challenging, with each cohort presenting with similar symptoms (10). This difficulty is further confounded in patients with a combination of pre and post-capillary causes of PH (11). Many of the above difficulties in detection and characterization relate to a lack of access to key variables that adequately assess PA pressure and cardiac status (12–16). In part this is due to the wide variety of hemodynamic indices and concepts employed to characterize PAH and in part due to the difficulty in obtaining key measures of cardiac status such as cardiac outflow reserve. While right ventricular outflow reserve has been shown to be a good prognostic indicator, since it involves a time-consuming and potentially hazardous rest and stress examination, it is rarely performed and even more rarely performed in an ongoing manner during routine clinical assessment (17).

In developing a unified pair of linear models capable of characterizing and assessing both PAH and PH patients we consider the disease state commonalties as well as differences. The widely perceived major difference involves the role of the left ventricle (LV). While PH is predominantly the result of left-sided disease, PAH is, by strict World Health Organization (WHO) classification, not related to left-sided disease (18). In PAH this often leads to neglect of left-sided conditions and considerations (19). However, ventricular interdependence is inevitable due to (A) the non-compliant pericardial sac constraining the total volume of the combined left and right ventricles, (B) the left and right ventricles being connected in series and thus constrained to generate the same stroke volume, and (C) that both ventricles share a common septum which directly transmits pressure from one to the other (20). These conditions inform us of the mechanisms by which the ventricles interact and indicate that that both ventricles contribute to right and left pressure generation. However, they do not provide any quantitative means of assessing what the pressure conditions are and how in detail they relate. To develop quantitative linear models of pressure we hypothesized that various key cardiac variables adequately define the system's "state variables". State variables are a set of physical conditions such as ejection fraction (EF) and end systolic volume (ESV) that describe the state of the dynamic cardiovascular system (21). In this representation of the cardiovascular system, the heart generates pulmonary and systemic blood pressures via time-evolution of its state variables. Suga and Sugawa observed that during isovolumic contraction and relaxation, pressure generation can be modeled by a sinusoidal waveform (22, 23). Thus, we sought to identify the cardiac state variables by applying a sinusoidal transform to candidate variables to model blood pressure. The linear models presented here were successfully applied to PAH patients, clearly showing the mode and magnitude of linkage between right and left-ventricular pressure generation. Further the linear models identified key differences in the manner of pressure generation between PAH and PH patients despite a similar range of PAPs. Finally, the models naturally suggested the concept of RV contractile reserve which was shown to correlate with the 6 min walk distance (6MWD). The ultimate value of the linear models is that they provide a set of non-invasively obtainable quantitative metrics that can be used to assess both pulmonary and systemic pressure conditions. In particular, in the context of clinical trials that target structural remodeling directly, there is a shortage of suitable approaches to measure markers of biology that drive cardiovascular change (24). Even positive PAH trials suffer from a low rate of reproducibility (as low as 21%) in part due to the adequacy of the markers of benefit (25). Consider that improvement in the 6MWD only poorly correlates with survival benefit (26) and more recently, dependence on time to clinical worsening requires larger trials of longer duration (27). While 4D flow CMR can assess pulmonary arterial pressure via direct interrogation of the flow field, it lacks an assessment of cardiac metrics that correspond to pressure conditions (28). Further, echocardiography is most commonly used to non-invasively assess pulmonary artery pressure but primarily relies on assessment of leakage of the tricuspid valve, which may not be present in each case (29). Thus, our intent in developing linear models of pulmonary and systemic pressure was to identify the anatomic cardiac variables that could be directly measured to assess response to therapy.

2. Materials and methods

2.1. Study populations

2.1.1. Exclusive PAH cohort (complexa trial patients)

Data were collected from patients with clinically diagnosed pulmonary arterial hypertension ($n = 51$) who were enrolled in the Complexa clinical trial (30). In brief, this trial was a multicenter double-blinded, placebo-controlled study to evaluate

the safety, efficacy, and pharmacokinetics of the study drug, CXA-10 [10-nitrooctadec-9(*E*)-enoic acid] which is an endogenous compound. Subjects 18 to 80 years of age (target $n = 96$) with PAH were randomly assigned to 75 mg, 150 mg of CXA-10 or stable background therapy. This phase II trial was terminated early due to lack of efficacy (with no safety concerns). Only baseline data are reported here prior to administration of the study drug. The study was performed at multiple sites from August 1, 2018 to August 5, 2020 (see **Supplementary Material Table S1**). Approximately 4% of data was missing at random.

In the Complexa trial, all pulmonary hypertension patients with normal pulmonary capillary wedge pressure (<15 mm Hg) or normal left ventricular end diastolic pressure (<10 mm Hg) were diagnosed to have PAH. Upon enrollment, demographic data were collected along with 6MWD data, right heart catheterization (RHC) pressure data, and cardiovascular magnetic resonance (CMR) imaging assessment of the left and right heart. The CMR data was analyzed at our core lab. The key CMR image acquisitions were (1) a multi-stack short axis cine set of images covering the left and right ventricles and used to obtain cardiac chamber volumes, and (2) phase velocity quantitative flow scans positioned through the ascending aorta and main pulmonary artery. All baseline CMR, RHC and 6MWD measurements were performed within a thirty day period of each other. Assignment to WHO functional class was performed at each site using standard of care clinical assessments (functional class data were available in 49 patients, 94%). Baseline demographic and test measurements are provided in **Table 1**.

2.1.2. Suspected PH/PAH cohort

In addition to the Complexa trial patients we obtained data from a retrospective cohort presenting at our CMR facility. Other than selecting patients with sufficient CMR metrics to model pressure, no exclusion criteria were applied. The key CMR acquisition protocol was identical to that used for the Complexa trial patients. Data were collected from 49 consecutive patients referred to our CMR laboratory from 2011 to 2015 who had a suspicion of PH (34, 70%) or PAH (15, 30%). While SBP was measured in all patients, contemporaneous measures of PAPs were not available, while estimates of PAPs were available in 17 (34%) with 10/17 (59%) obtained by echocardiographic criteria involving measurement of the tricuspid regurgitation jet (33). Due to the sparsity and the low quality of the PAPs estimates these pressure data were not used to generate the linear model of PAPs. The purpose of including this patient cohort was to (1) extend the number of patients contributing to the SBP model and (2) to apply the linear models to identify key differences in pressure distribution between PAH and PH patients. Demographic and key test measures of this cohort are provided in **Table 2**.

2.2. CMR variables

Our hypothesis is that a limited number of key cardiac measures define the state variables of the pulmonary and systemic pressure systems. We sought to identify cardiac metrics obtained non-invasively via CMR imaging. The multi-slice short axis data sets, time resolved through the cardiac cycle and covering the left and right ventricles from base to apex, allowed standard ventricular metrics to be obtained separately for the left and right ventricles, including: EF, ESV and ventricular mass. In addition to the standard CMR functional examination (32), separate phase velocity mapping scans were obtained to quantify blood flow velocity through the ascending aorta just above the sinus and through the main pulmonary artery. This data, obtained at the interface of each ventricle to the vasculature, was used to calculate the ventricular-vascular impedance match (36). Here we focused on the impedance match between the ventricle and vasculature using a formulation previously introduce by us (35), with the general formula for the left impedance matching index given by:

$$\text{Left impedance matching index} = \frac{\text{cardiac time} \times \text{average aortic blood velocity}}{\text{aortic diameter}} \quad (1)$$

Originally, the "cardiac time" variable was the end-systolic duration, but subsequent work (not shown) has demonstrated that the cardiac cycle duration is more appropriate. Using parallel logic we define the right impedance index as;

$$\text{Right impedance matching index} = \frac{\text{cardiac time} \times \text{average main pulmonary artery blood velocity}}{\text{main pulmonary artery diameter}} \quad (2)$$

2.3. Linear model generation and optimization

2.3.1. PAPs and SBP models

Candidate variables for the PAPs and SBP linear models were selected from CMR and baseline demographic data. PAPs data were only available for the Complexa PAH cohort, while for the SBP model, data were available from the combined patient cohort, thus more data were available to fit SBP than PAPs. Each candidate variable was correlated separately with the PAPs data and with the SBP data. Additionally, a sine transform of the candidate variable was correlated with PAPs and SBP;

$$\text{Sine transform of variable} = \text{Sine}(\text{Frequency} \times \text{Variable} + \text{Phase}) \quad (3)$$

Where the Frequency and Phase parameters were optimized by a generalized reduced gradient nonlinear approach implemented in MS Excel to maximize the Pearson correlation r value (37). Variables with a correlation r value greater than 0.2 were entered into the candidate multivariable models. The two multivariable linear models were separately optimized by the systemic search algorithm to maximize each model's r^2 value by systematically changing each variable's sine transform Frequency and Phase parameters. In the final linear models, variable entry was allowed

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TABLE 1 Baseline demographics and measurement of exclusively PAH patients.

Variable	Full population (52)	WHO functional class II (49)	WHO functional class III (17)	*p* range
General demographics				
Age (years)	50.37 (SD 12.19)	49.95 (SD 12.84)	50.39 (SD 9.99)	0.91
Height (cm)	162.14 (SD 6.97)	162.4 (SD 7)	161.12 (SD 6.79)	0.55
Weight (kg)	94.6 (SD 29.71)	91.95 (SD 29.84)	100.82 (SD 29.92)	0.33
Male	6 (12.24%)	4 (12.9%)	2 (14.29%)	0.9
Ethnicity				
White	36 (73.47%)	24 (77.42%)	9 (64.29%)	0.36
Black	4 (8.16%)	1 (3.23%)	2 (14.29%)	0.17
Hispanic	8 (16.33%)	4 (12.9%)	3 (21.43%)	0.47
Other	9 (18.37%)	6 (19.35%)	3 (21.43%)	0.87
Substances				
Alcohol: current user	14 (29.17%)	10 (32.26%)	3 (21.43%)	0.46
Alcohol: former user	8 (16.67%)	7 (22.58%)	1 (7.14%)	0.21
Tobacco use	12 (25%)	9 (29.03%)	2 (14.29%)	0.29
Drug use				0.3
Respiratory				
Respiratory rate (breaths/min)	17.38 (SD 2.51)	16.81 (SD 2.09)	18.53 (SD 2.96)	<0.05
Forced expiratory volume in 1 s (%)	79.39 (SD 13.91)	80.1 (SD 12.26)	74.79 (SD 15.57)	0.22
Total lung capacity (%)	89.69 (SD 11.86)	90.4 (SD 11.22)	87.79 (SD 14.57)	0.52
PAH type				
Idiopathic	35 (71.43%)	24 (77.42%)	10 (71.43%)	0.67
Connective tissue	9 (18.37%)	3 (9.68%)	3 (21.43%)	0.28
Toxin/drug	2 (4.08%)	1 (3.23%)	1 (7.14%)	0.56
6 min walk test				
Oxygen saturation pre (%)	96 (94.25–98.75)	96 (94.5–98)	97 (94.25–98)	0.81
Oxygen saturation post (%)	93.5 (88–96.75)	92 (87–96)	94 (91.25–96.75)	0.78
Heart rate pre (beats/min)	81.28 (SD 11.9)	80.65 (SD 12.25)	81.86 (SD 12.66)	0.76
Heart rate post (beats/min)	112.96 (SD 35.75)	121.6 (SD 17.25)	109.36 (SD 19.37)	<0.05
Distance (m)	402.76 (SD 84.75)	425.13 (SD 74.31)	364.14 (SD 101.84)	<0.05
Electrocardiographic				
Mean heart rate (beats/min)	74.97 (SD 11.69)	73.73 (SD 11.72)	78.14 (SD 12.82)	0.26
PR interval (ms)	175.76 (SD 34.64)	169.9 (SD 29.77)	190.05 (SD 44.52)	0.08
QRS duration (ms)	90.53 (SD 11.5)	90.4 (SD 11.28)	93.45 (SD 11.46)	0.41
QT interval (ms)	410.86 (SD 30.25)	412.37 (SD 31.18)	403.52 (SD 30.7)	0.38
QTcF interval (ms)	440.8 (SD 22.64)	439.34 (SD 19.81)	438.95 (SD 29.32)	0.96
RR interval (ms)	799.27 (SD 144.38)	801.92 (SD 155.47)	793.56 (SD 143.24)	0.87
Right heart catheterization				
Cardiac output (L/min)	5 (4.15–5.61)	4.82 (4.03–5.39)	5.7 (4.9–6.3)	<0.05
Heart rate (beats/min)	76.31 (SD 10.46)	75.39 (SD 11.06)	78.75 (SD 8.84)	0.36
Mixed venous oxygen saturation (%)	68.12 (SD 13.71)	69.63 (SD 10.76)	70.13 (SD 8.37)	0.89
Systemic oxygen saturation by pulse oximeter (%)	92.07 (SD 14.07)	93.46 (SD 3.99)	94.17 (SD 3.33)	0.6
Mean right atrial pressure (mm Hg)	7.13 (SD 4.01)	6.98 (SD 3.44)	9.15 (SD 5.06)	0.11
Pulmonary artery diastolic pressure (mm Hg)	29 (SD 8.99)	29.9 (SD 9.97)	31 (SD 6.87)	0.72
Pulmonary artery systolic pressure (mm Hg)	76.29 (SD 18.42)	78.37 (SD 19.22)	79.54 (SD 17.27)	0.85
Pulmonary artery wedge pressure (mm Hg)	9.06 (SD 3.02)	9.21 (SD 3)	9.64 (SD 2.66)	0.69
Right ventricular end-diastolic pressure (mm Hg)	7.8 (SD 6.53)	7.17 (SD 5.93)	11.46 (SD 7.38)	0.05
Right ventricular systolic pressure (mm Hg)	73.65 (SD 22.99)	76.57 (SD 22.96)	79.15 (SD 17.11)	0.72
Systemic				
Systolic blood pressure (mm Hg)	114.98 (SD 14.86)	116 (SD 15.95)	112.5 (SD 14.24)	0.52
Diastolic blood pressure (mm Hg)	70.13 (SD 9.8)	70.63 (SD 11.4)	68.42 (SD 5.87)	0.53
Valvular disease				
Mitral	4 (8.33%)	2 (6.9%)	2 (13.33%)	0.48
Tricuspid	17 (35.42%)	11 (37.93%)	6 (40%)	0.89
Aortic	0 (0%)	0 (0%)	0 (0%)	
Pulmonary	6 (12.5%)	2 (6.9%)	4 (26.67%)	0.07
None	30 (62.5%)	18 (62.07%)	8 (53.33%)	0.58

(*Continued*)

TABLE 1 (Continued)

Variable	Full population (52)	WHO functional class II (49)	WHO functional class III (17)	*p* range
CMR variables				
Aortic flow (ml)	65.83 (SD 17.59)	62.91 (SD 14.13)	71.65 (SD 23.8)	0.13
MPA flow (ml)	63.34 (SD 19.04)	64.28 (SD 18.9)	62.97 (SD 22.06)	0.84
LV mass (g)	116.11 (SD 25.16)	109.24 (SD 23.73)	126.36 (SD 24.68)	<0.05
LV stroke volume (ml)	64.03 (SD 14.66)	61.56 (SD 12.86)	67.45 (SD 18.87)	0.23
LV end diastolic volume (ml)	96.67 (SD 20.34)	92.92 (SD 18.18)	101.73 (SD 23.39)	0.17
LVEF (%)	66.52 (SD 8.41)	66.44 (SD 6.8)	66.23 (SD 9.86)	0.94
LV end systolic volume (ml)	32.64 (SD 11.56)	31.36 (SD 9.38)	34.28 (SD 12.67)	0.39
RV cardiac output (L/min)	4.68 (SD 1.29)	4.55 (SD 1.22)	5.08 (SD 1.45)	0.21
RV mass (g)	105.84 (SD 34.07)	99.43 (SD 33.72)	115.44 (SD 30.04)	0.13
RV stroke volume (ml)	64.68 (SD 16.68)	63.2 (SD 16.74)	67.95 (SD 18.14)	0.39
RV end diastolic volume (ml)	147.58 (SD 38.27)	144.12 (SD 38.22)	151.51 (SD 34.58)	0.53
RVEF (%)	45.37 (SD 11.36)	45.26 (SD 10.64)	45.99 (SD 11.95)	0.84
RV end systolic volume (ml)	82.89 (SD 34.97)	80.92 (SD 34.71)	83.56 (SD 32.73)	0.81
BSA (m^2)	1.83 (SD 0.56)	1.91 (SD 0.56)	1.68 (SD 0.55)	0.21
LV mass index (g/m^2)	69.64 (SD 28.29)	62.68 (SD 24.66)	83.09 (SD 30.77)	<0.05
LV stroke volume index (ml/m^2)	38.23 (SD 14.65)	35.3 (SD 12.36)	43.89 (SD 17.36)	0.06
LV end diastolic volume index (ml/m^2)	57.6 (SD 20.84)	52.96 (SD 17.13)	66.58 (SD 24.83)	<0.05
LV end systolic volume index (ml/m^2)	19.37 (SD 8.56)	17.65 (SD 6.35)	22.7 (SD 11.24)	0.06
RV cardiac output index ($L/m/m^2$)	2.86 (SD 1.17)	2.59 (SD 0.92)	3.37 (SD 1.44)	<0.05
RV mass index (g/m^2)	62.38 (SD 26.07)	55.03 (SD 20.04)	76.59 (SD 30.92)	<0.01
RV stroke volume index (ml/m^2)	39.02 (SD 15.04)	36.31 (SD 13.58)	44.27 (SD 16.77)	0.1
RV end diastolic volume index (ml/m^2)	87.62 (SD 32.5)	80.64 (SD 25.21)	101.1 (SD 40.95)	<0.05
RV end systolic volume index (ml/m^2)	48.59 (SD 23.53)	44.33 (SD 17.34)	56.83 (SD 31.45)	0.1
LV EDV/LV mass index ($ml/g/m^2$)	0.85 (SD 0.18)	0.87 (SD 0.2)	0.82 (SD 0.17)	0.35
LV impedance match	8.51 (SD 3.59)	8.2 (SD 3.56)	9.15 (SD 3.71)	0.44
RV impedance match	3.75 (SD 1.94)	3.88 (SD 1.92)	3.48 (SD 2.02)	0.54

TABLE 2 Baseline demographics and measurement of suspected PH/PAH patients.

Variable	Full population (34)	PAH (15)	PH (35)	*p* range
General demographics				
Age (years)	52.35 (SD 17.5)	60.93 (SD 14.08)	48.56 (SD 17.7)	<0.05
Height (cm)	167.28 (SD 12.13)	166.62 (SD 9.78)	167.57 (SD 13.15)	0.80
Weight (kg)	82.84 (SD 25.57)	93.11 (SD 25.37)	78.31 (SD 24.68)	0.06
Male	19 (38.78%)	4 (26.67%)	15 (44.12%)	0.25
Blood pressures				
Estimated pulmonary artery systolic pressure (mm Hg)	50.12 (SD 23.47)	55.86 (SD 28.71)	46.1 (SD 19.66)	0.42
Measured systemic systolic blood pressure (mm Hg)	133.84 (SD 16.41)	137.91 (SD 17.91)	132.05 (SD 15.65)	0.25
Key CMR variables				
LV mass (g)	99.45 (SD 36.78)	98.73 (SD 26.63)	99.76 (SD 40.82)	0.93
RV mass (g)	46.89 (SD 15.39)	49.37 (SD 13.81)	45.79 (SD 16.11)	0.46
RVEF (%)	54.06 (SD 9.94)	51.57 (SD 10.08)	55.15 (SD 9.83)	0.25
LVEF (%)	56.2 (SD 7.93)	58.11 (SD 5.4)	55.35 (SD 8.76)	0.26
LV impedance match	8.07 (SD 3.65)	7.53 (SD 3.49)	8.31 (SD 3.75)	0.49
RV impedance match	8.1 (SD 3.68)	6.87 (SD 3.9)	8.65 (SD 3.5)	0.12
LV end systolic volume (ml)	65.61 (SD 27.22)	66.67 (SD 18.71)	65.15 (SD 30.48)	0.86
RV end systolic volume (ml)	91.04 (SD 43.66)	103.53 (SD 43.16)	85.53 (SD 43.36)	0.19
BSA (m^2)	1.94 (SD 0.34)	2.06 (SD 0.32)	1.89 (SD 0.34)	0.12
LV mass index (g/m^2)	4.39 (SD 1.95)	4.33 (SD 1.67)	4.42 (SD 2.08)	0.88
RV mass index (g/m^2)	24.34 (SD 7.93)	24.3 (SD 7.21)	24.36 (SD 8.33)	0.98
LV end systolic volume index (ml/m^2)	16.59 (SD 7.15)	16.76 (SD 6.21)	16.51 (SD 7.61)	0.91
RV end systolic volume index (ml/m^2)	5.95 (SD 2.69)	6.43 (SD 2.36)	5.74 (SD 2.83)	0.41

at the $p < 0.05$ level. To ensure that highly correlated variables did not inflate the model's correlation r^2, the variance inflation factor (VIF) was calculated, and only variables with a VIF < 5 were retained. The linear model variables together with the sine transform variables are shown in Table 3 for both PAPs and SBP linear models.

TABLE 3 PAPs and SBP linear and sine transform components.

PAPs model	Sine frequency	Sine phase	Model coefficient (95% confidence interval)	*p* Value	VIF
Linear variables					
Intercept			74.76 (63.05–86.47)	<0.001	0
Age			−0.68 (−0.89 to 0.47)	<0.001	1.37
RV mass			0.39 (0.31–0.46)	<0.001	1.43
Sinusoidal transformed variables					
LVEF	0.33	0.36	−8.75 (−12.46 to 5.03)	<0.001	1.53
RVEF	1.25	2.79	−7.35 (−11.24 to 3.45)	<0.001	1.35
LV end systolic volume	0.90	−5.13	−6.75 (−10.3 to 3.19)	<0.001	1.27
RV end systolic volume	1.16	0.62	−11.74 (−15.28 to 8.21)	<0.001	1.16
LV impedance slow	0.98	1.91	13.65 (9.98–17.33)	<0.001	1.16
LV impedance rapid	2.75	−1.80	9.71 (6.1–13.33)	<0.001	1.33
RV impedance slow	1.91	−1.54	4.94 (0.98–8.89)	<0.05	1.21
SBP model					
Linear variables					
Intercept			103.62 (92.13–115.11)	<0.001	0
age X			0.45 (0.29–0.6)	<0.001	1.09
LV mass X			0.17 (0.09–0.25)	<0.001	1.29
RV mass X			−0.26 (−0.32 to 0.19)	<0.001	1.25
Sinusoidal transformed variables					
LVEF	0.70	−2.87	−8.87 (−12.42 to 5.31)	<0.001	1.2
RVEF	1.35	−5.99	5.57 (1.98–9.17)	<0.001	1.21
LV end systolic volume	0.98	−4.98	−9.55 (−13 to 6.09)	<0.001	1.1
RV end systolic volume	1.19	1.02	−7.63 (−11.23 to 4.03)	<0.001	1.17
LV impedance slow	1.14	0.34	7.78 (4.13–11.42)	<0.001	1.27
LV impedance rapid	2.57	−1.08	−8.88 (−12.57 to 5.18)	<0.001	1.15
RV impedance slow	2.09	−4.08	−19.31 (−25.25 to 13.38)	<0.001	3.73
RV impedance rapid	1.94	−0.51	−19.13 (−25.37 to 12.89)	<0.001	3.8

2.3.2. 6MWD model

To generate the multivariable model of the 6MWD for the Complexa PAH patients, candidate variables were selected based on a suitably high correlation r with the 6MWD either for the linear variables or the sinusoidally-transformed variables. Similar to the blood pressure models, the parameters of the sine transforms were selected by optimizing the correlation r^2 value by the automatic search algorithm.

2.3.3. Linear pressure relationship

To illustrate the nature of the relationship between SBP and PAPs we conducted a simulation using the linear models, whereby an artificially constructed set of parameters of the SBP model was generated to achieve a target SBP. Here two separate SBP targets were simulated: 120 and 140 mm Hg. Since multiple variables contribute to each pressure condition, multiple combinations of parameter values can achieve the same target SBP. In our case 20 combinations of variables were generated to achieve each target pressure. These variables were then entered into the PAPs linear model to generate the corresponding simulated PAPs data and the results were plotted using a box plot.

2.4. Mean and pulsatile blood pressure component representation

To aid in developing an intuitive understanding of the PAPs and SBP model components we combined pressure components to relate to the concept of hydraulic power. Conventionally, the steady hydraulic power is calculated as mean PAP × cardiac output while the remaining power component is associated with pulsatile blood flow (38). Hydraulic power is rarely measured since it requires simultaneous measures, time-resolved thought the cardiac cycle, of high-fidelity pressure and flow data (39). Here we assigned the sinusoidal components to the pulsatile hydraulic power component since the sinusoidal transform is integral to the time-varying aspect of ventricular-vascular interaction. The linear components were assigned to the mean hydraulic power since they contain only time-invariant components. Thus, four separate components of hydraulic pressure were generated:

$$\text{Hydraulic pulmonary pulsatile (HPp)} = \text{sum of pulmonary sinusoidal components} \quad (4)$$

$$\text{Hydraulic pulmonary mean (HPm)} = \text{sum of pulmonary linear components} \quad (5)$$

$$\text{Hydraulic systemic pulsatile (HSp)} = \text{sum of systemic sinusoidal components} \quad (6)$$

$$\text{Hydraulic systemic mean (HSm)} = \text{sum of systemic linear components} \quad (7)$$

2.5. Statistical analysis

Count statistics were represented as number and percentage, continuous data were analyzed as mean and standard deviation if normally or nearly normally distributed, and as median and interquartile range if non-normally distributed. Comparison between grouped variables was performed with *t*-testing or the non-normal equivalent, as appropriate. Analysis of grouped data were presented as box plots, with interquartile ranges and outliers identified by the "o" symbol. Linear regression modeling was performed using a stepwise forward inclusion approach. To estimate the variability of each linear regression model a bootstrapping approach was applied with 1,000 randomly resampled data sets. The mean and standard deviation of the bootstrapped model correlation r^2 was used to calculate the mean and 95% confidence intervals. For linear regression models that incorporated measured data the fitted regression line was generated by Deming regression that takes into account variation in dependent and independent variables (40). The linear model results were subjected to Bland-Altman analysis to yield the model bias and 95% confidence interval. The relationship between pulmonary and systemic hydraulic pressure components were plotted against increasing values of PAPs with a five-point moving average applied to allow data trends to be more easily visualized. Logistic regression modeling was used to distinguish PH from PAH patients. Discrimination capability of the model was measured by the area under curve (AUC) from a receiver operating characteristic (ROC) curve analysis. For an estimate of sensitivity and specificity a cut-off value was set to approximately equalize sensitivity and specificity. Statistical significance was regarded at $p < 0.05$. Statistical analyses were performed using SPSS 18.0 (SPSS Inc., Chicago, Illinois).

3. Results

For the 52 Complexa patients, the demographic and test results at baseline are shown in Table 1. Complete CMR and RHC data were available in 41 (79%) patients. The mean time between the RHC and the CMR examination was 9.5 days with a standard deviation of 11 days. For the 49 patients with a suspicion of pulmonary hypertension (PAH, $n = 34$, 70% or PH, $n = 15$, 30%) who were referred to receive a CMR examination, estimates of PH pressure were available in 17 (35%) with 15 (31%) having a suspicion of PAH. The demographic and test results are shown in Table 2 separately for the PAH and PH groups.

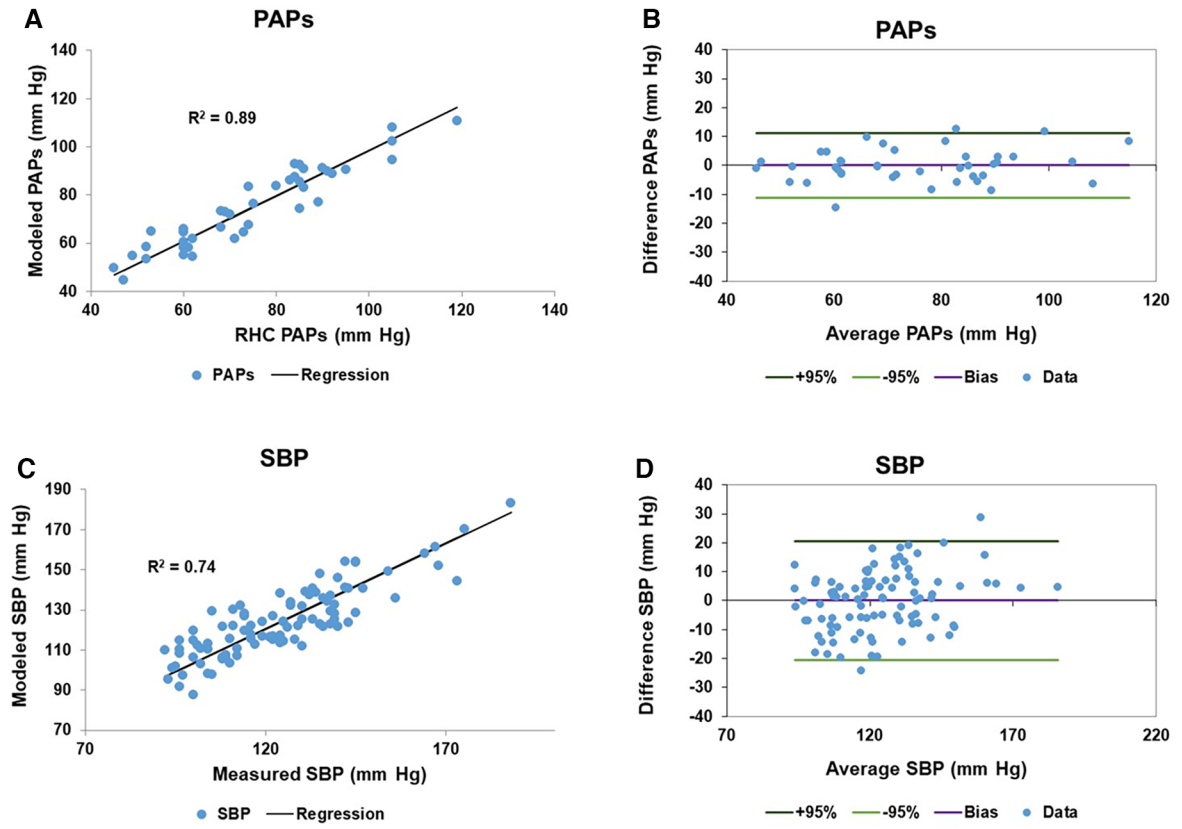

FIGURE 1
Model results of pulmonary artery systolic pressure (PAPs) and systemic systolic blood pressure (SBP): (**A**) the scatter plot of the right heart catheter (RHC) measured and fitted PAPs data has a regression r^2 of 0.89. (**B**) The corresponding Bland-Altman plot of the fitted and measured PAPs data has a bias is zero and the 95% confidence limits of agreement are ±11.2 mm Hg. (**C**,**D**) Are the corresponding scatter and Bland-Altman plots for the measured and fitted SBP data, respectively. The measured and fitted SBP data has a regression r^2 of 0.74, while the Bland-Altman bias is zero and the 95% confidence limits of agreement are ±20.5 mm Hg.

3.1. Pressure modelling

Only patients with RHC measures of PAPs (Complexa cohort) contributed to the linear model of PAPs. To generate the linear model of SBP, patients from both cohorts were entered in to the linear model. Each linear model included cardiac component variables from both right and left ventricles. In the case of left and right impedance match values we identified two sine transforms, one termed slow (lower frequency) and the other rapid (higher frequency) which were sufficiently independent for inclusion in the model since the VIF was <5. Table 3 itemizes the cardiac variables included in each linear model, including the sine

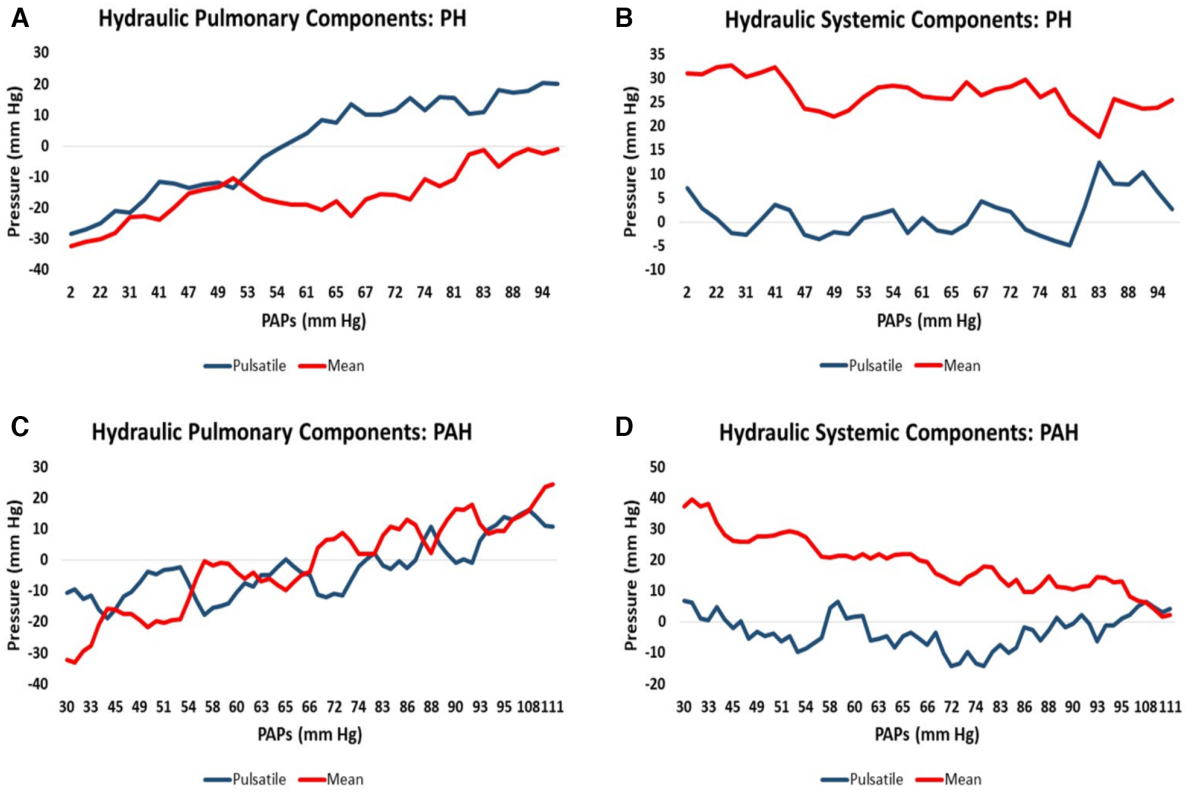

FIGURE 2
Moving average of hydraulic pressure components for pulmonary hypertension (PH) and pulmonary arterial hypertension (PAH) patients plotted against increasing pulmonary artery systolic pressure (PAPs): (A) the hydraulic pulmonary pulsatile (HPp) and hydraulic pulmonary mean (HPm) pressure for PH patients. (B) The corresponding hydraulic systemic pulsatile (HSp) and hydraulic systemic mean (HSm) pressure plots for PH patients. (C,D) Are the corresponding pulmonary and systemic data plots for PAH patients, respectively.

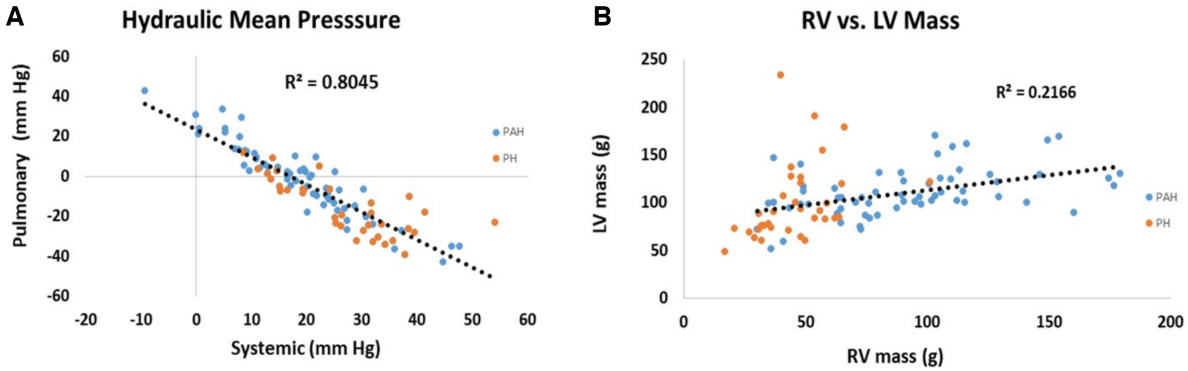

FIGURE 3
Scatter plots of (A) hydraulic pulmonary mean pressure (HPm) vs. hydraulic systemic mean pressure (HSm) and (B) scatter plots of right ventricular (RV) mass vs. left ventricular (LV) mass. Points are plotted separately for pulmonary arterial hypertension (PAH) patients (blue) and pulmonary hypertension (PH) patients (orange). Note, that mean pressure components are strongly inversely related ($r^2 = 0.80$) while ventricular masses are weakly positively correlated ($r^2 = 0.22$).

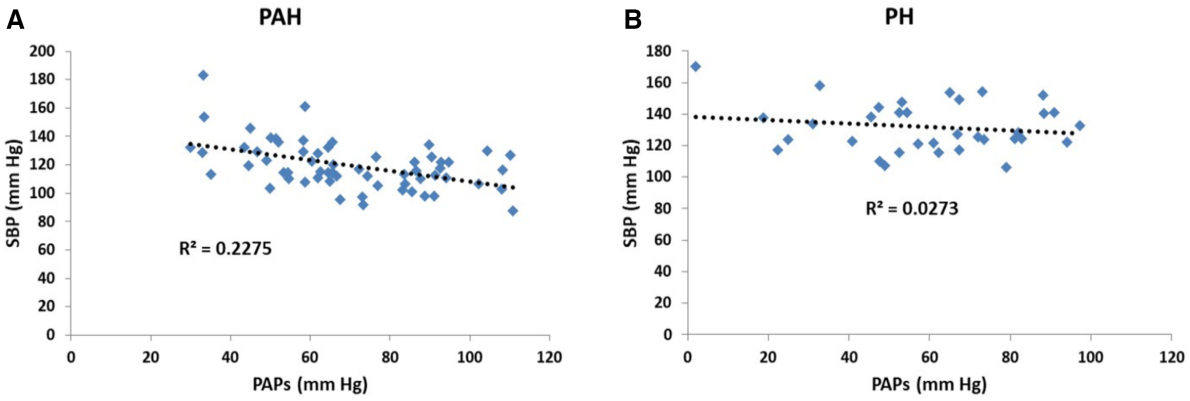

FIGURE 4
Scatter plots of (A) systolic blood pressure (SBP) vs. pulmonary arterial systolic pressure (PAPs) for patients with pulmonary arterial hypertension (PAH) and (B) corresponding scatter plots for patients with pulmonary hypertension (PH). In the PAH patients the pressures are weakly negatively related ($r^2 = 0.23$) while for PH patients there is no significant trend ($r^2 = 0.03$).

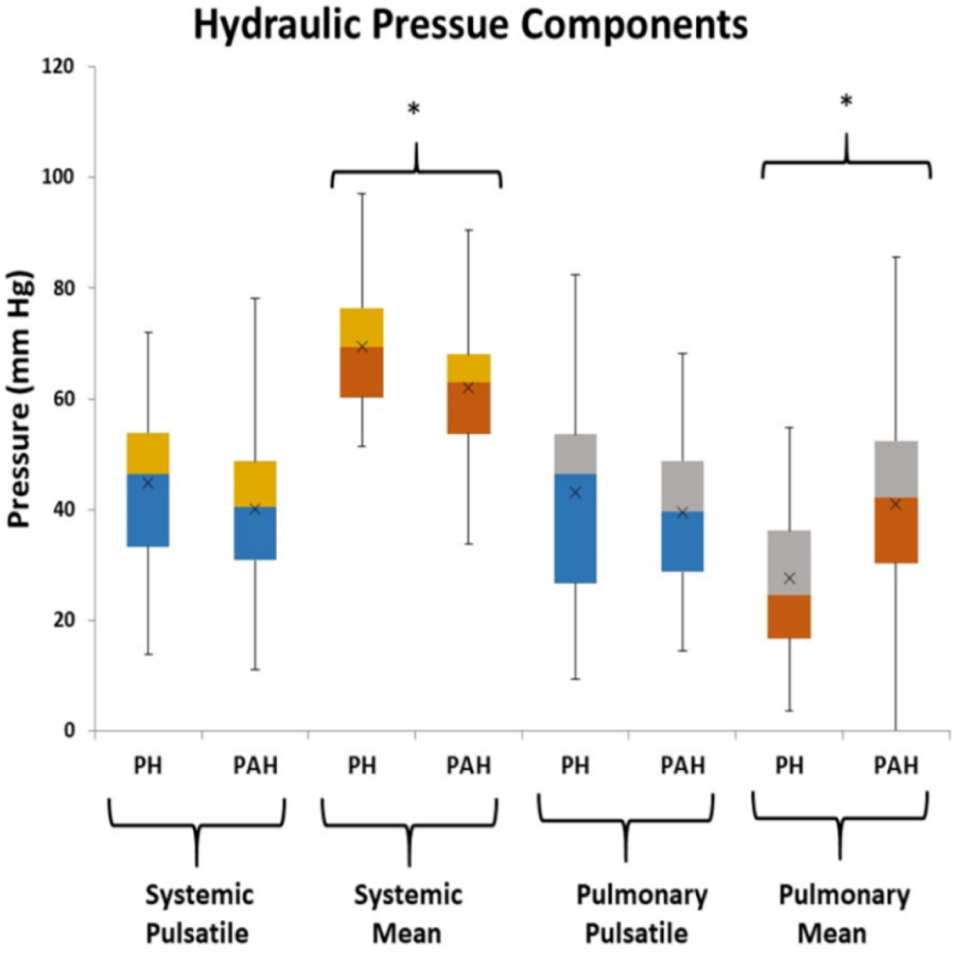

FIGURE 5
Box plots of the hydraulic pressure components for pulmonary hypertension (PH) and pulmonary arterial hypertension (PAH) patients (the asterisk indicates $p < 0.05$ between PH and PAH). The hydraulic systemic pulsatile pressure (HSp) and hydraulic pulmonary pulsatile pressure (HPp) are not significantly different between groups, while the hydraulic systemic mean pressure (HSm), and hydraulic pulmonary mean pressure (HPm) components are statistically different between groups.

transform Frequency and Phase parameters, the linear coefficient and 95% confidence interval, p value and VIF are noted. Age was the only non-CMR-determined variable. Importantly, the models did not contain any indication of which patients had PAH vs. PH. The fitted and modeled PAPs and SBP data were evaluated using linear regression and Bland-Altman analysis, Figure 1. The results of bootstrapping the models are: SBP model $r^2 = 0.77$ (95% CI: 0.64–0.91) and PAPs model $r^2 = 0.92$ (95% CI: 0.85–0.99).

3.2. Pressure relationships

Since the suspected PH/PAH patient group did not have RHC-measured PAPs contemporaneously with CMR we uniformly employed the modeled PAPs data to plot against each component of hydraulic pressure. Patients from this group were separated into PH ($n = 34$, 70%) and PAH ($n = 15$, 30%) to allow the moving average of the two pulmonary and two systemic hydraulic pressure components to be plotted separately for PH and PAH patients, Figure 2. The 34 PH patients were drawn exclusively from the suspected PH/PAH cohort, while the PAH patients comprised Complexa ($n = 52$) and an additional 15 patients (total $n = 67$) from the suspected PH/PAH cohort. Pressure data from linear models are plotted without regard to static offsets.

Scatter plots of the relationship between pulmonary and systemic hydraulic mean pressure components for PAH and PH patients are shown in Figure 3A. Note that for both PAH and PH patients there is a strong negative relationship between hydraulic pulmonary mean pressure and hydraulic systemic mean pressure, with the PAH data spread over a wider range compared to PH. In Figure 3B the scatter plot of right and left ventricular masses are plotted for PAH and PH patients, displaying a weak positive relationship. Scatter plots of the modeled PAPs and SBP for PAH and PH patients are shown in Figures 4A,B, respectively. Despite the strong negative correlation of hydraulic pulmonary mean and hydraulic systemic mean pressures there is only a weakly negative correlation between PAPs and SBP for PAH and PH patients.

3.3. Pressure components differences between PAH vs. PH Patients

From Figure 2 there are clearly discernible differences in the general pattern of pressure components between PAH and PH

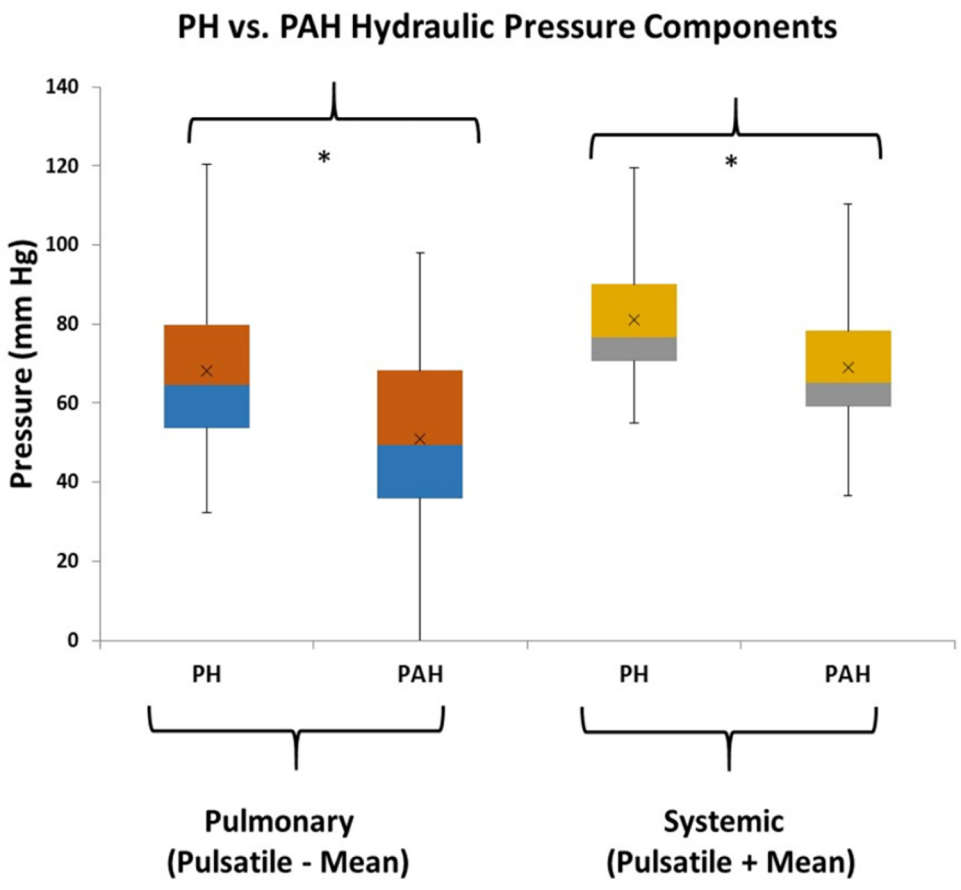

FIGURE 6
Box plots of combinations of hydraulic components that are different between pulmonary hypertension (PH) and pulmonary arterial hypertension (PAH) patients (the asterisk indicates $p < 0.05$). The pulmonary combination that is different between patient groups is the subtraction of pulmonary hydraulic mean (HPm) pressure from the pulmonary hydraulic pulsatile (HPp) pressure. The systemic combination that is different between patient groups is the summation of systemic hydraulic mean (HSm) pressure and the systemic hydraulic pulsatile (HSp) pressure.

patients. These differences are visible over a wide range of PAPs and also in the pressure component's mean and distribution, Figure 5. We explored what combination of hydraulic pressure components (without averaging) differentiated PAH vs. PH patients. In Figure 6 we show the box plot of two composite pressure components that are statistically different between PAH and PH patients: (A) the difference between hydraulic pulmonary pulsatile and hydraulic mean pressures and (B) the summation of the hydraulic systemic pulsatile and hydraulic mean pressures. These two composite variables were both significant $p < 0.05$) in a binary logistic regression model to distinguish between PAH and PH patients. The corresponding ROC plot had an area under the curve of 0.75, Figure 7. Setting the threshold to 0.66 resulted in a sensitivity of 67% with a specificity of 68%.

3.4. Right ventricular pulsatile components

The right ventricular hydraulic pulsatile component is formed from the summation of seven sine-transformed variables. The sine transform varies systematically from positive one to negative one and is further multiplied by a scaling factor for each variable. Thus, summation of the absolute magnitude of each sine-transformed variable identifies the maximum possible hydraulic pulmonary pulsatile value, i.e., corresponding to the condition when all sine contributions are maximally positive. In this case the maximum possible pulsatile pulmonary value is 56 mm Hg which represents the upper limit of the hydraulic pulmonary pulsatile component. Thus, the linear model of PAPs naturally produces the concept of RV contractile reserve, which we define as the difference between the current hydraulic pulmonary pulsatile value and the upper limit of 56 mm Hg. For PAH patients, the plots in Figure 8A show how the seven contributions add to produce the realized net and theoretical absolute maximum hydraulic pulmonary pulsatile component. Due to the mixture of positive and negative contributions (depending on the phase of each variable's sine wave) the realized net hydraulic pulmonary pulsatile pressure value (black line) ranges from negative through positive with increasing PAPs. However, by discarding the phase of each contribution (i.e., considering the absolute magnitude of each variable's contribution) the sum of each component of hydraulic pulmonary pulsatile component produces the theoretical absolute value (blue line). Note that the absolute summation of each of the seven variable's contributions do not systematically vary over the range of PAPs. The corresponding net and theoretically absolute maximal amplitudes of hydraulic pulmonary pulsatile pressure are plotted in Figure 8B for PH patients, respectively.

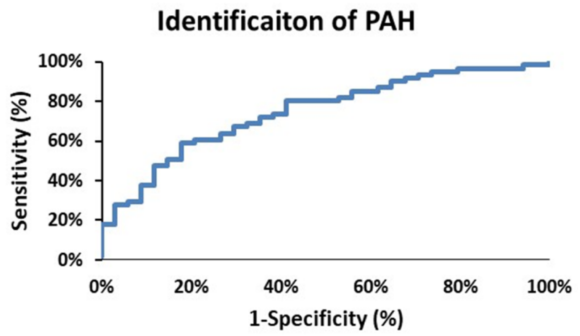

FIGURE 7
The receiver operator characteristic curve plot for identification of pulmonary arterial hypertension (PAH) patients. The area under the curve is 0.75, and selecting a threshold of 0.66 yields a sensitivity of 68% and a specificity of 68%.

The RV functional reserve is known to be a factor in determining the 6MWD along with biomechanical variables (41). We generated a linear model to predict the 6MWD in PAH patients which included the biomechanical variables of height, weight and age and the pulmonary pressure variables of hydraulic pulmonary pressure and the RV impedance match. Height, hydraulic pulmonary pressure and RV impedance match were related via sinusoidal transforms while weight and age were linearly related. Sine transform variables and model coefficients are given in Table 4. The linear model accounted for 45% of the variation in the 6MWD. The scatter plot of modeled and measured 6MWD is shown in Figure 9A with the corresponding Bland-Altman plot in Figure 9B. The bias term is zero and the 95% confidence limits are ±119 m.

3.5. Pressure simulation

The results of the pressure simulations for the two targeted SBP levels of 120 and 140 mm Hg are shown in the box plots of

TABLE 4 6 min walk distance linear and sine transform components.

6MWD components	Sine frequency	Sine phase	Model coefficient (95% confidence interval)	*p* Value	VIF
Linear variable					
Intercept			−36,110.66 (−65,395.92 to 6,825.4)	<0.05	0
Weight (kg)			−1.17 (−1.95 to 0.38)	<0.001	1.15
Age (years)			−2.41 (−4.24 to 0.57)	<0.05	1.08
Sinusoidal transformed variables					
Height (cm)	0.005	0.74	40,166.58 (11,031.79–69,301.38)	<0.05	1.05
Hydraulic pulmonary pulsatile (mm Hg)	0.005	1.51	−3,405.97 (−6,629.03 to 182.91)	<0.05	1.08
RV impedance match	1.16	1.83	−40.63 (−67.39 to 13.87)	<0.001	1.06

Figure 10. Here we see that for each target SBP (employing 20 sets of artificially generated parameters) the same set of parameters resulted in a wide range of PAPs values. Note that the lower SBP target of 120 corresponds to the higher PAPs data centered on 69 (SD 18) while the higher SBP target of 140 corresponds to the lower PAPs data centered on 56 (SD 21, $p < 0.05$). The lower and higher set of PAPs values were statistically different ($p < 0.05$) and clearly show that each target value of SBP produces a range of values of PAPs, i.e., a one to many relationship.

4. Discussion

We introduced two linear models that quantified the relationships that exists between the pulmonary and systemic pressure systems and which further show the nature of the interrelationships of the right and left ventricles. In particular, these interrelationships were demonstrated in a cohort of well documented PAH patients which met all WHO criteria, including the stipulation of hypertension not being secondary to left-sided disease. We interpreted the pressure component derived from linear variables as an index of mean hydraulic power and the pressure component derived from the sine-transformed variables as an index of pulsatile hydraulic power. For the combined patient cohort, which included PAH and PH patients, the linear models showed how the separate hydraulic pressure components varied with increasing PAPs and demonstrated that they exhibited distinct patterns and values that distinguished between PAH and PH patients with good sensitivity and specificity. The concept of right ventricular reserve naturally arose from the model with the reserve index significantly correlating with the 6MWD.

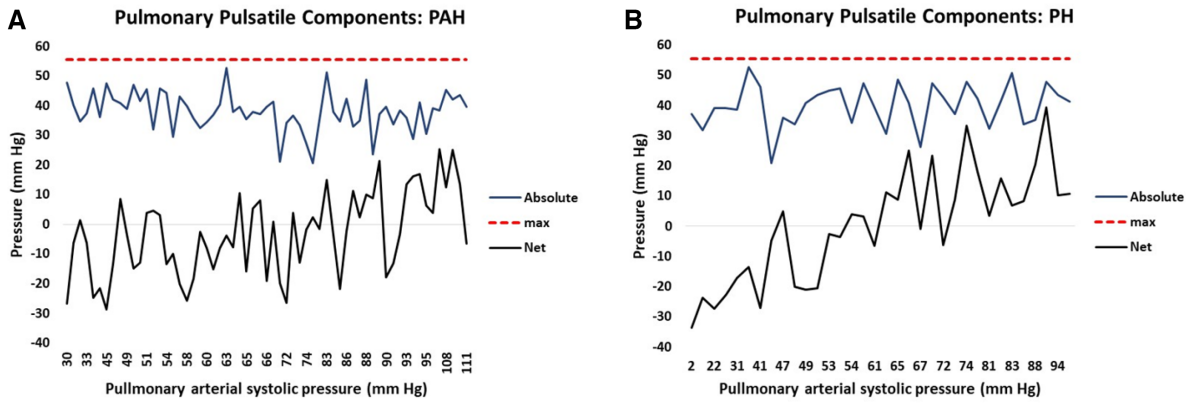

FIGURE 8
The attained net (black) and potential (blue) hydraulic pulmonary pulsatile pressure component plotted against increasing pulmonary arterial systolic pressure (PAPs) for (**A**) pulmonary arterial hypertension (PAH) patients and (**B**) pulmonary hypertension (PH) patients. The solid black line represents the net summation of each contributing variable of hydraulic pulmonary pulsatile pressure taking into account the phase (i.e. positive or negative contribution) of each variable. The solid blue line represents the net summation of each contributing variable of hydraulic pulmonary pulsatile pressure without taking into account the phase (i.e. each contribution is positive) of each variable. The punctuated red line represents the model-determined maximum value of hydraulic pulsatile pressure (56 mm Hg).

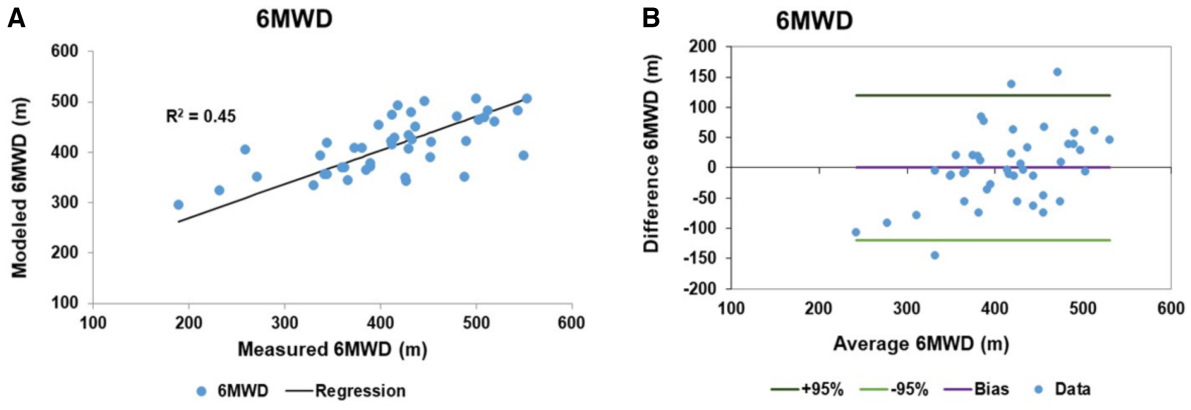

FIGURE 9
Linear model results of 6 min walk distance (6MWD): (**A**) the scatter plot of the 6MWD measured and fitted data with a regression r^2 of 0.45. (**B**) The corresponding Bland-Altman plot of the fitted and measured 6MWD data. The bias is zero and the 95% confidence limits of agreement are ±119 m.

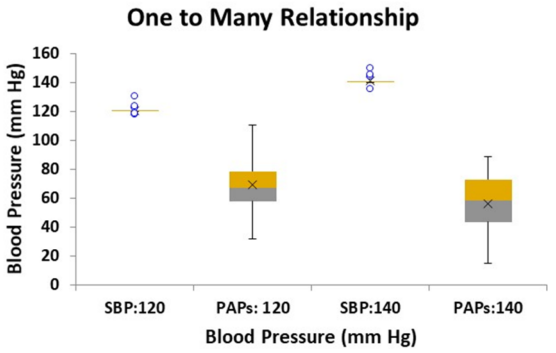

FIGURE 10
Box plot of simulated linear model results of conditions relating to two target systemic systolic blood pressure (SBP) levels: 120 and 140 mm Hg with the corresponding modeled systolic pulmonary artery pressure (PAPs) range of pressures. Twenty sets of conditions were modeled for each target SBP. The PAPs corresponding to SBP target of 120 mm Hg is centered on 69.1 mm Hg with a standard deviation of 4.0 mm Hg, while the PAPs range corresponding to SBP target 140 mm Hg is centered on 55.8 mm Hg with a standard deviation of 4.7 mm Hg, ($p < 0.05$).

There are several features of the models that are consequences of the construct adopted, namely that the heart conditions represent the state variables of the system. The concept of state variables is more natural to engineering than physiology, whereby the behavior of a dynamic system comprised of multiple parts (e.g., the breaking system of an automobile) can be characterized by a small number of state variables (e.g., the vehicle speed and road-tire coefficient of friction) (42). In the case of the heart, the state variables are weights (ventricular mass), volumes (ESV) and efficiency ratios including EF and the coupling efficiency at the interface to the vasculature (impedance match). The state space representation allows determination of the response of the system to a time-dependent action (e.g., in the case of the breaking system, the rate of application of the break). In the case of the heart the non-linear variables represent the state of the heart at end-systole and the sinusoidal transforms are related to the time course over the cardiac cycle of each variable. The sinusoidal time course was postulated from the observation of Suga and Sugawa concerning the time course of pressure generation in the ventricle. An important aspect of the state-space representation is that external conditions affect how the system behaves (e.g., in the case of the breaking system, icy road vs. dry road conditions). In the case of PAH vs. PH the external conditions are pre-capillary vs. post-capillary elevated resistance/impedance, respectively, and these conditions dramatically affect the response of the heart. The right and left ventricular masses are static over the time course of the cardiac cycle and were thus assigned as contributors to mean pressure. The only non-cardiac variable was age, **Table 3**. While it has been established that increasing age is associated with higher levels of PAPs in the general population (43) surprisingly the model coefficient of age was negative. However, while the positive association with age can be observed in the general population, here we considered a population with confirmed PAH or a high suspicion of PH/PAH and applied a model that adjusts for a number of cardiac variables which may in turn be age dependent. There are many potential applications of the model: (1) since all model variables are non-invasively obtained, clinical monitoring of PAH patients can be achieved without conducting a RHC, (2) since multiple variables contribute to each model the detailed effects of a therapy on each component can be assessed in an ongoing manner, (3) all model measurements are performed in the heart, and while the heart might not be the target of a therapy the effects of external conditions ultimately manifest in the heart, (4) the models provide estimates of pulmonary and systemic conditions, (5) since the PAH population is small, and does not easily lend itself to large randomized clinical trials (44), there is the possibility that trials might be designed to observe the effects of each therapy in PH patients to suggest how they might transfer to PAH patients and *vice versa* (45).

4.1. Model accuracy

From **Figures 1A,B** we see that the model accounted for 89% of the variation of PAPs. The Bland-Altman analysis showed that the 95% confidence limits were ±11 mm Hg, which is on the order of the reported spontaneous variation of PAPs of 20%–25% (46). We did not have reliable contemporaneous measures of PAPs for patients in the suspected PH/PAH cohort. However, the available estimated PAPs values were compared to the modeled values using a paired t-test which failed to reject the hypothesis that the measurements were different ($p = 0.40$). Others have shown that the use of CMR in studying PAH patients has excellent repeatability and registered a larger therapy effect size than either the 6MWD or N-terminal pro b-type natriuretic peptide (47).

The linear model of SBP accounted for 74% of the variation despite having considerably more patients contributing to the model. However, it has been noted that the typical clinical measurement of SBP is notoriously inaccurate (48, 49). Consider a hypothetical example where the standard deviation of measuring the SBP is on the order of 5 mm Hg. Under this scenario a person with a nominal SBP reading of 125 mm Hg (i.e., pre hypertensive) could well be hypertensive (upper 95% confidence level 135 mm Hg) or normotensive (lower 95% confidence level 115 mm Hg) (50). However, in routine clinical practice the standard deviation of SBP ranges from 14 to 26 mm Hg depending on measurement method and personnel (34, 51). The lower range of the clinical standard deviation is comparable to the standard deviation obtained here for the model SBP Bland-Altman analysis of 10.5 mm Hg. Thus, we believe that the lower level of agreement between modeled and measured SBP in part reflects the difficulty of obtaining a reliable measure of SBP.

4.2. Heart failure in PAH vs. PH

It is widely accepted that the mode of death in PAH and PH patients is predominantly due to RH-failure (31, 52). However,

heart failure remains a clinical syndrome with little indication of the details of how failure occurs (53). Examination of the relative pressure components may indicate differences in the mode of heart failure that are expected in PAH vs. PH. **Figure 2C** shows that in PAH patients both hydraulic pulmonary mean and pulsatile components increase in a broadly parallel manner with increasing PAPs. We speculate that this is due to the normally widely-distributed compliance of the pulmonary arterial tree becoming progressively concentrated in the pulmonary artery, such that the RV ejection pattern progressively resembles that of the LV, where 80% of the systemic compliance is localized in the aorta (54). Thus, as PAH progresses, the hydraulic pulmonary mean pressure increases, but since this is largely contributed to by RV mass (**Table 3**) at some point the heart may be physically limited in its ability to increase the hydraulic mean pressure, leading to RH failure. **Figure 2D** shows the that hydraulic systemic mean pressure steadily declines as disease progresses, consistent with the clinical observation that widespread end-organ damage is sustained due to low perfusion pressure (55). A careful study of the mode of death of PAH patients found that only 50% definitively suffered from RH-failure, while the remaining 50% died in the ICU (56), conceivably due to left-sided complications. Thus, interpretation of **Figures 2C,D** are consistent with observations concerning the changes in physiology, types of morbidity and modes of death observed in PAH patients.

In PH patients, as disease progresses the normally highly compliant pulmonary artery progressively loses compliance, which in turn requires the RV to increase pulse pressure (57). The pulmonary conditions for PH patients are shown in **Figure 2A** where there is an early elevation of both hydraulic pulmonary pulsatile and mean pressure components, but when PAPs exceeds about 50 mm Hg, the pulsatile component preferentially increases. This in turn causes the reflected pulse pressure wave to rapidly arrive back at the right ventricle prior to closing of the pulmonic valve, further opposing ventricular ejection during late systole (58). However, the model indicates that the increase in hydraulic pulmonary pulsatile pressure cannot proceed beyond an upper limit of 56 mm Hg. With reference to **Figure 8A** it can be appreciated that in advanced stages of PH the RV pulsatile component approaches the maximum value, i.e., approaching the condition of zero RV pulsatile reserve. This indicates that PH patients may be prone to RV failure due to an inability to increase the hydraulic pulmonary pulsatile component, which is indirectly supported by the association of higher mortality with increased pulse pressure (59). It has been noted that in patients with PH secondary to left-sided heart failure with preserved EF that the degree of loss of RV function greatly exceeds that of the LV (19, 60). In **Figure 2B** it is apparent that the systemic pressure adaptations are not as dramatic as those of the RV (**Figure 2A**). Thus, the model reveals several differences in pressure components between PAH and PH which may be explanatory of the different modes of heart failure and whether right or left ventricles are likely to fail or result in morbidity.

4.3. Interconnectedness of ventricular responses

The high-degree of interconnectedness of the pulmonary and systemic pressure systems that is demonstrated by the linear models may be a controversial aspect, especially in PAH patients where the role of the left ventricle may be generally underappreciated (61). While right ventricular changes gradually accrue, clinically relevant changes to the left ventricle tend to manifest at the end stage (62), leading to the concept put forth of the forgotten left ventricle in right ventricular pressure overload (63). In advanced PAH the left ventricle has been clinically noted to appear "small and underfilled" and hyperdynamic (20, 64, 65). This issue directly relates to the underlying determinants of ventricular responses to PAH (66). **Figure 3A** shows the close to perfect antisymmetric relationship between pulmonary and systemic mean hydraulic pressure components. If the determinant of ventricular response was due to circulating neurohumoral factors, a symmetric response of each ventricle would be expected. Conversely, if the ventricles were independent and responded to localized workloads they could be positively or negatively related, but the relationship would not be expected to be strong due to their independence. However, if the ventricles were interdependent then the response would be expected to be strongly negative, i.e., antisymmetric, which is what the model indicates. Note, that the strong anti-symmetric relationship between hydraulic pulmonary mean and hydraulic systemic mean pressure is not a feature forced by the linear models. Further, **Figure 3B** shows that RV mass exhibits a weak positive correlation with LV mass which, given the constrained total volume, indicates a reduction in LV chamber volume consistent with the small and underfilled LV. This reduction in LV afterload is expected to weaken the LV myocytes, an expectation that was confirmed in a recent study conducted in PAH patients undergoing cardiac transplantation where it was found that left ventricular myocytes were thinner and exhibited a reduced force generating capacity compared to those of donor hearts (67). The weaker LV myocytes indicate a reduced ability to sustain a high hydraulic systemic mean pressure, which is only partially compensated for by an increased mass. Thus, the exhibited ventricular interdependence of mean pressure is consistent with clinical and physiologic observations.

One may counter that for PAH patients, while there is evidence of left-sided dysfunction (68), the literature does not indicate that there is a clear inverse relationship between pulmonary and systemic pressure, with some observations even indicating a weak positive correlation (69, 70). **Figure 4A** indicates that the linear models established a weakly negative relationship between PAPs and SBP. Several aspects of the linear models explain why this is the case. Firstly, **Figure 10** shows that the linear models do not predict a one-to-one relationship between SBP and PAPs. In the simulated results of **Figure 10** several sets of parameter combinations were applied to generate a SBP close to 120 mm Hg which resulted in generating a wide range of PAPs values centered on 69 mm Hg. Conversely, the simulation for the higher SBP value of 140 mm Hg generated a range of wide PAPs values

centered on the lower value of 56 mm Hg. That is, there is a one-to-many relationship between SBP and PAPs while the overall trend is negative. Secondly, while there is a strong asymmetric relationship observed between the hydraulic systemic mean and hydraulic pulmonary mean pressures, we note that these are calculated components of the model and may not correspond to any easily-measured pressure component. Thirdly, the difficulty in obtaining accurate measures of SBP requires large numbers of patients to observe these trends. As has been noted, systemic pressure conditions in PAH are widely underappreciated and underreported in sufficient detail to allow determination of the relationships by performing Meta-analysis. Further, commonly applied therapeutic interventions in PAH patients may reduce left-sided pressure (55) and thus any relationship observed may be interpreted as being a side effect of medication.

4.4. Ventricular reserve

Right ventricular output reserve is the ability of the RV to increase output in response to acute exercise or pharmacologic stress. RV reserve can be accessed via multiple indices including cardiac output, pulmonary vascular resistance, pulmonary capacitance, tricuspid annular plane systolic excursion and pulmonary artery pulsatile pressure (71, 72). A common feature of all cardiac reserve assessments is the requirement for a comparison between rest and stress/exercise conditions. Typically, the relevant metric is obtained via non-invasive imaging (most commonly echocardiography) or via invasive RHC (73). An important aspect of the PAPs linear model is that the concept of ventricular reserve naturally emerges from the resting data. The hydraulic pulmonary pulsatile pressure component is generated by summation of sine-transformed variables and thus varies continuously from a net negative value at normal PAPs to a net positive value at high PAPs. Thus, for a continuous variable such as EF, the sine transform indicates that a high EF value is not necessarily "better" than a low EF value since the sine transform systematically cycles between positive and negative values several times over the expected EF range. **Figure 8** illustrates the result of combining several sine-transformed variables to generate the net hydraulic pulmonary pulsatile pressure that trends from negative through zero to positive values as PAPs increases. When all sine waves contribute at maximum positive value, the hydraulic pulmonary pulsatile pressure component cannot increase further. This is represented by the punctuated red line in **Figure 8**. In contrast, note the result of combining the absolute magnitude of each sine-transformed variable in **Figure 8** where it can be appreciated that the absolute magnitudes of each pulsatile pressure variables are not in general different between low and high PAPs levels. That is, the absolute magnitude of each contribution to pulsatile pressure is not the dominant determination of the experienced pressure, instead it is the phase (controlled by the sine transform) of each variable's contribution to pressure that primarily determines the experienced pressure. Here we define the right ventricular contractile reserve as the net difference between the instantaneous hydraulic pulmonary pulsatile value and the upper limit of 56 mm Hg. Thus, the hydraulic pulmonary pulsatile reserve can vary from a maximum of 112 when the phase of each variable component is wholly negative to 0 when the phase of each variable component is wholly positive. For our PAH cohort the highest hydraulic pulmonary pulsatile reserve was 84 and the lowest was 30. This range is similar to that observed for a study assessing PAPs increase between rest and exercise using echocardiographic estimation, where the 95% confidence limits of PAPs increase were 83 and 13 mm Hg (17).

Of course without a true assessment of cardiac reserve we cannot be certain that the assignment of RV contractile reserve was correct. However, the 6MWD measured in PAH patients is known to be related to impaired oxygen delivery as a result of decreased cardiac index and decreased RV contractile reserve (17, 41). A multi-variable linear model of 6MWD was constructed with the biomechanical elements of height and weight and the right-sided components of hydraulic pulmonary pulsatile pressure and RV impedance match index. **Table 4** shows that patient height related to the 6MWD through a sine transform as did the hydraulic pulmonary pulsatile pressure and RV impedance match index. The sine transform of height is likely related to the pendulum effect of each leg taking longer to "swing" for taller people, but accomplishing more distance per step (74). The full model r^2 was 0.45. An intriguing feature is that hydraulic pulmonary pulsatile pressure, which is the result of combining seven sine-transformed variables, was in turn predictive of 6MWD via the sine transform. This provides further evidence that the sine transform is fundamental to the relationship between cardiovascular variables and measures of outcome (such as between 6MWD and pressure components).

4.5. Individualized monitoring and management

The components of the linear model of pulmonary pressure were obtained from cardiac-measured variables. This is significant since it has been established that cardiac status, and in particular right ventricular output reserve, is a major determinant of long-term outcome (75). The survival half-life after initial PAH diagnosis is about 5–7 years (76) and assessment via the REVEAL score can further stratify risk (77). However, irrespective of their risk strata, some PAH patients may appear to being doing well and, without any predictors of impending decline, may rapidly deteriorate and die (7). In part this may be a consequence of stratifying patients by an outcomes-based risk factor which does not necessarily group them by common underlying physiology (78). Thus, despite appropriate risk stratification and management at specialized centers of excellence, individual PAH patients remain difficult to assess. The majority of monitoring approaches involve assessment of PA pressure, with the RHC being regarded as the gold standard (79) while some patients are assessed on a daily basis via indwelling pressure monitoring devices (80). However, improvement in pulmonary hemodynamics and quality of life rarely translate to a survival benefit (81). Consequently, use of hemodynamic markers

as surrogate end points for trials has declined over time while measures of functional status have increased (82, 83). More recently, trials that rely on time to clinical worsening have been shown to demonstrate survival benefit (84). However, the population size and duration of event-driven trials are dominated by the expected number of events (85) which typically leads to large trials of long duration. In an attempt to reduce trial size and duration it has been proposed that trial entry criteria could be skewed to predominantly include high-risk patients (27). However, this may present a further impediment to trial efficiency in that the therapy has to demonstrate an outcome benefit in patients with the most advanced disease. In traditional trial designs, which include a wide range of patients, it is possible that to a large extent the power of the trial is attributable to the clinical benefit realized by less sick patients. Thus, the ideal trial endpoint should reflect and quantify the degree of clinical benefit in addition to clinical worsening, irrespective of patient symptoms. By this means the difference between trial groups is essentially doubled compared to trials that measure only clinical worsening. The linear model presented here not only offers a means to non-invasively assess PA pressures via measurement of key cardiac variables, but importantly, offers the simultaneous ability to assess cardiac function. This is due to the key cardiac variables (state variables) being intimately involved in characterizing cardiac morphologic and functional status. Thus, the value of the linear model to future clinical trials is that the outcome of the trial could be assessed in terms of a vector of key variables that describe hemodynamic change, cardiac efficiency and RV contractile reserve, which can quantitatively express both beneficial and deleterious changes. The vector of end-points has the added power that even the small changes in several components may be beneficial and that this benefit can be captured and related to hemodynamic and cardiac functional status simultaneously.

Since the linear models provide the infrastructure to directly assess pressure and cardiac status at the component level, they may allow translation of therapeutic benefit between PH and PAH trials. Outside of a randomized clinical trial, assessment of the effects of therapy in PAH patients is hampered by the need to continuously adjust medications in response to evolving symptoms (86). Further, even within a randomized clinical trial, the rarity of PAH patients often renders the trial non-conclusive due to low numbers of trial participants and to the lack of suitable outcomes over the short term (26). Nevertheless, progress has been made in managing PAH patients, while advances in PH are rare. This has led to the unfortunate trend whereby PAH medications are indiscriminately offered off-label to PH patients, often with poor outcomes (87). Given the different etiologies of PH vs. PAH, translation of therapies between PH and PAH populations is fraught with difficulties. In part, these difficulties are the result of an insufficient framework with which to assess patients. The linear models presented here are an attempt to provide a uniform framework with which to assess PAH and PH patients based on hydraulic mean and hydraulic pulsatile pressure components. We note that these two components cannot typically be assessed with a routine RHC evaluation due to the requirement for high-fidelity pressure and flow measuring catheters (88). Further, a given therapy is likely to affect only a few of the variables influencing hydraulic pulmonary mean and pulsatile components, and potentially, while it may improve some components, it may adversely affect others, thus attenuating or even negating any benefit. This mixture of therapeutic benefit and detriment has potential to explain the common observation that some PAH patients improve while others decline, presumably due to differences in the combination of cardiac variables between seemingly similar patients in terms of cardiac status and pressure conditions. Since the linear model components are obtained from non-invasive CMR imaging at rest there is great potential to identify the detailed response of each therapy in a wide-ranging population. This has potential to address an often encountered complication of PAH, namely the deleterious influence on left-sided pressure (89). Since the models simultaneously identify variables that affect right and left pressure conditions there is the possibility that therapeutic interventions can be tailored to maximize the benefit-to-risk ratio on an individual basis.

It is unlikely that any one therapy can provide the required degree of benefit for effective PAH management, instead it may be more desirable to consider combining several therapies in a designer manner (90). While desirable, the feasibility of this approach has been hampered by several factors, including limited knowledge concerning therapeutic interaction and the perceived need to achieve large changes in order to make a clinical difference. Assessment of each trial patient with the linear models may address both of these obstacles. Firstly, the linear models have identified that the contributing variables are independent predictors and that they affect both pulmonary and systemic pressure, thus they each contribute non-overlapping data. Secondly, the linear models indicate that even small changes in key variable can be effective. For example, consider the contribution of EF, if pressure were linearly related to EF then clinically-significant benefit may only be achieved with a dramatic increase in EF. However, the task of raising a patient's EF from 40 to 60 for example is typically not within the range of effectiveness of any given therapy and the requirement to do so would seem to put the adjustment out of reach. However, here we show that EF is related to pressure via a sine transform. The frequency of sinusoidal oscillation over the EF axis is on the order of a complete cycle every 10 EF points. Thus, in this case, adjustments on the order of 5 EF points may be all that are required to beneficially change the phase of a pressure contribution. With reference to **Figure 8** we can appreciate that the magnitude of the combined modeled variables do not dramatically change over the PAPs range, whereas the phase changes of each individual component dominate the resultant PAPs and RV reserve. Thus, achieving a beneficial effect may be more feasible than previously thought.

4.6. Limitations

The study has a number of strengths and also many deficits and limitations, most notably the lack of measured PAPs in PH

and some PAH patients, the lack of a stress-test assessed measure of RV pulsatile reserve, the lack of a separate model generation and model assessment population and the use of clinically-obtained SBP. The PAH patients from the Complexa trial were well documented, but only a minority of the retrospectively obtained patients undergoing a functional CMR examination had an estimate of PAPs. However, assuming that data were missing at random, the distribution of estimated PAPs was sufficient to confirm the suspicion of elevated PAPs. Further, we lacked a normal PAPs population for model testing. The excellent correlation coefficient r^2 (0.89) for the model agreement with PAPs is gratifying, but as Bland and Altman indicate, a good correlation is necessary but not sufficient to warrant substitution of one test for another (91). The corresponding Bland-Altman analysis in **Figure 1** shows that the 95% limits of agreement are ±11 mm Hg. These limits, while not ideal, are lower than is traditionally achieved with echocardiography which are on the order of ±28 mm Hg (92). While we did not have sufficient data for test and prediction sub-sets, an estimate of the robustness of the model was obtained via bootstrapping, which indicated an excellent mean correlation r^2 of 0.92 with a standard deviation of 0.04 on the estimate of PAPs. The accuracy of model components can be estimated by comparing the modeled SBP with the directly measured SBP, but it is unknown to what extent the model may be adequate to screen for PH. Our assignment of component combinations to hydraulic pulsatile and hydraulic mean pressure was not confirmed by any direct measure of the hydraulic power distribution. The magnitudes of hydraulic pulmonary pulsatile and hydraulic systemic pulsatile are about one quarter of the magnitudes of hydraulic pulmonary mean and hydraulic systemic mean pressure when the constant offsets are assigned to the mean pressure components. This agrees with the observation that typically the oscillatory hydraulic power is about 30%–40% of the total hydraulic power (93). Our motivation for seeking a sinusoidal transform to relate cardiac features to the pressure data originated with an observation by the pioneers of cardiac energetics, Suga and Sagawa. Sinusoidal transforms allowed excellent fit of the data. The frequencies identified were not uniform, which in part is attributable to the different units for each variable and in part likely due to sensitivity to the fundamental as well as harmonic frequencies which are known to be present (94). Development of a schema that relates the sinusoidal transforms to a common axis might further add to the development of the model and further support its conceptual basis. **Figure 3A** shows that hydraulic pulmonary mean and hydraulic systemic mean pressures are almost perfectly antisymmetric. It might be thought that this is an artifact or natural consequence of the model. However, this is not the case since the relationship is not perfectly antisymmetric due to the differences in model coefficients between PAPs and SBP (**Table 3**): coefficients for age, RV mass and LV mass for SBP/PAPs are 0.45/−0.68, −0.26/0.39 and 0.17/0, respectively. Whether this relationship should be exactly antisymmetric is not known, but if it were, then adding this constraint to the model would further refine and improve it. One of the most gratifying aspects of the model is that it naturally suggests an upper limit for the RV and LV contractile function, i.e., contractile reserve. Indirect evidence that our identification of RV contractile reserve is related to the true reserve is that it significantly contributed to the 6MWD model (95). Further, clinical approaches to asses RV contractile reserve themselves have assumptions, and isolating which aspects relate specifically to RV contraction might be difficult to establish (96).

4.7. Summary of findings

In conclusion we have presented a pair of unified models of PAPs and SBP in PAH and PH patients with physiologically feasible assignment of components to hydraulic mean and hydraulic pulsatile pressures. The model indicated that both right and left ventricular variables contributed to right and left pressure either linearly or via sine transforms. It is possible that model refinements could be identified, such as incorporating a transform derived directly from time-resolved cardiac data. However, the fundamental connection between right and left pressure generation is expected to persist. Beyond modeling PAPs and SBP, successes of the model include natural emergence of the concept of RV contractile reserve which was shown to correlate with the 6MWD, the decrease in systemic perfusion pressure in advanced PAH patients, and the progressively poor performance of the LV in PAH patients despite a trend to increase LV mass. These features are consistent with a large body of clinical observations. Of primary importance, the model identified a very distinctive pattern of pulmonary pulsatile pressure that was different between PAH and PH patients. The pulsatile patterns were physiologically feasible given the pre vs. post capillary constrictions in PAH vs. PH, respectively. We regard the models as providing a universal framework with which to assess the results of therapy applied to PAH and PH patients.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by for entry into the Complexa trial, for which we were the CMR core lab, all patients provided written informed voluntary consent using forms and procedures approved by a third party IRB, Advarra (Title: Complexa, Inc./"Phase 2 Multicenter, Double-Blind, Placebo Controlled, Efficacy, Safety, and Pharmacokinetic Study of 2 Doses of CXA-10 on Stable Background Therapy in Subjects with Pulmonary Arterial Hypertension (PAH)", Protocol number CXA-10-301). Retrospective data was obtained from patients with a suspicion of PH or PAH without obtaining a signature. This approach was permitted by our institution's IRB under protocol entitled "Imaging and Patient Outcome Analysis

within the Allegheny Health Network System Cardiovascular MRI Unit". The patients/participants provided their written informed consent to participate in this study.

Author contributions

MD, RB, and GR contributed to the conceptualization and design of the study. MD drafted the initial manuscript and is guarantor of this work. All authors contributed to data abstraction and analysis, contributed to manuscript writing, and approved the submission of the final manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2023.1120330/full#supplementary-material.

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Light at the ENDothelium-role of Sox17 and Runx1 in endothelial dysfunction and pulmonary arterial hypertension

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Pulmonary arterial hypertension (PAH) is a progressive disease that is characterized by an obliterative vasculopathy of the distal pulmonary circulation. Despite significant progress in our understanding of the pathophysiology, currently approved medical therapies for PAH act primarily as pulmonary vasodilators and fail to address the underlying processes that lead to the development and progression of the disease. Endothelial dysregulation in response to stress, injury or physiologic stimuli followed by perivascular infiltration of immune cells plays a prominent role in the pulmonary vascular remodeling of PAH. Over the last few decades, our understanding of endothelial cell dysregulation has evolved and brought to light a number of transcription factors that play important roles in vascular homeostasis and angiogenesis. In this review, we examine two such factors, SOX17 and one of its downstream targets, RUNX1 and the emerging data that implicate their roles in the pathogenesis of PAH. We review their discovery and discuss their function in angiogenesis and lung vascular development including their roles in endothelial to hematopoietic transition (EHT) and their ability to drive progenitor stem cells toward an endothelial or myeloid fate. We also summarize the data from studies that link mutations in Sox17 with an increased risk of developing PAH and studies that implicate Sox17 and Runx1 in the pathogenesis of PAH. Finally, we review the results of recent studies from our lab demonstrating the efficacy of preventing and reversing pulmonary hypertension in animal models of PAH by deleting RUNX1 expression in endothelial or myeloid cells or by the use of RUNX1 inhibitors. By investigating PAH through the lens of SOX17 and RUNX1 we hope to shed light on the role of these transcription factors in vascular homeostasis and endothelial dysregulation, their contribution to pulmonary vascular remodeling in PAH, and their potential as novel therapeutic targets for treating this devastating disease.

KEYWORDS

pulmonary hypertension, endothelial dysfunction, transcription factors, SOX17, Runx1, pulmonary vascular remodeling, vasculogenesis

Introduction

The pulmonary circulation is a low-pressure circuit with a mean pulmonary arterial pressure (mPAP) of approximately 14 mmHg. Elevation of mPAP > 20 mmHg is defined as pulmonary hypertension and is common in patients with chronic heart and lung diseases (1, 2), occurring up to 50%–70% of patients. When pulmonary hypertension is accompanied by an elevation in pulmonary vascular resistance (PVR) and occurs in the

absence of elevated left sided filling pressures and significant heart or lung disease, it is referred to as pulmonary arterial hypertension (PAH). This term is used to distinguish the elevated PA pressure as being caused by a disease of the pulmonary arterial circulation as opposed to a consequence of chronic hypoxia, parenchymal lung disease or elevated pulmonary venous pressure. PAH is a complex, heterogeneous, and frequently fatal disease that results from progressive functional and structural changes in the pulmonary vasculature that lead to increased PVR, right ventricular failure and usually death. Although response to treatment can vary considerably between patients, overall survival from diagnosis averages only about 5 years.

The disease is rarely seen in the general population with an annual incidence of less than 1.5 per 100,000 healthy individuals but occurs in 1%–10% of patients with connective tissue disease, portal hypertension, or HIV infection (3). It is also associated with congenital left to right intra-cardiac shunts, and in patients with a history of fenfluramine/phentermine or methamphetamine use. When seen in conjunction with one of these conditions, the disease is described as associated PAH (APAH). There are also a number of gene mutations that greatly increase the risk of developing PAH and patients with one of these mutations and a family history of PAH are described as having heritable PAH (HPAH). PAH patients without APAH or HPAH are described as having idiopathic PAH (IPAH). Despite a comprehensive knowledge regarding clinical characteristics of PAH, the cellular injury or stressors and mechanisms responsible for disease development are not well understood.

At the turn of century, heterozygous germline mutations in the gene encoding bone morphogenetic protein receptor type 2 (BMPR2) were found to be associated with familial PAH. Mutations in BMPR2 have since been recognized as occurring in approximately 70% of HPAH (4, 5) and 10%–20% of IPAH cases (6, 7). However approximately 75% of cases of PAH cannot be explained by BMPR2 mutations alone and only about 1 in 8 people carrying one of the disease variant BMPR2 mutations will develop the disease (8). Thus, it is believed that PAH may be the result of a double hit phenomenon consisting of an initial injury to the pulmonary circulation combined with an impaired reparative response due either to a gene mutation or other disease conditions that facilitate abnormal pulmonary vascular remodeling. Since the initial reports of BMPR2 mutations, whole exome and whole genome sequencing has led to the identification of more than 16 genes that have been associated with PAH. Many of these (e.g., Krüppel-like factor 5 (KLF2), GATA-binding factor 2 (GATA2), T-box 4 (TBX4), SRY-box 17 (Sox17), eukaryotic initiation translation factor 2 αlpha kinase 4 (EIF2AK4)) are transcription factors or transcriptional coactivators/repressors that play important roles in lung and/or vascular development or vascular homeostasis (9–12).

Although the current understanding of PAH pathophysiology remains limited, an emerging hypothesis is that vascular injury in response to a variety of factors such as hypoxia, shear stress, inflammation, oxidative stress, or growth factors, leads to abnormal proliferation or differentiation of pulmonary vascular cells including endothelial cells, smooth muscle cells, and adventitial fibroblasts. Under normal conditions, cellular stress or injury may trigger downstream activation of genetic programs involved in angiogenesis and neovascularization that are important for restoring vascular integrity. These programs are mediated by specific transcription factors which if not properly expressed may lead to abnormal pulmonary vascular remodeling (**Figure 1**).

Currently approved medical therapies for PAH, have some ability to inhibit cellular proliferation and hypertrophy, but act primarily as pulmonary vasodilators and have limited ability to repress the pathogenic phenotypes of pulmonary vascular cells. Given their vital roles in angiogenesis and vascular homeostasis, several transcription factors have emerged as enticing therapeutic targets to potentially counteract and reverse the PAH vascular phenotype.

In this review we focus on two transcription factors with emerging roles in PAH: SOX17 and one of its downstream targets, RUNX1. We examine their normal structure and functions, and their roles in EHT, endothelial progenitor cell differentiation, and genetic mutations associated with PAH. By examining the role of SOX17 and RUNX1 in the pathogenesis of PAH we hope to further our understanding of the cellular mechanisms that drive remodeling of damaged lung vessels in pulmonary vascular disease and propose potential future therapeutic targets for the treatment of patients with PAH.

SOX17

In 1990, the SRY gene—sex-determining region of the Y-chromosome was first discovered in humans and mice as a testis-determining gene (13, 14). The identification of a highly conserved high-mobility group (HMG) domain led to the discovery of the SRY-box (SOX) transcription factor family. Over the following decades, gain and loss of function studies in the 20 members of the Sox transcription factor family revealed that they played a crucial role in the regulation of various developmental processes, organogenesis, cell fate determination and tissue homeostasis (15, 16).

The high-mobility group (HMG) domain in SOX genes allows for DNA binding in a sequence-specific manner. Each of the SOX family of genes encodes an HMG domain of 79 amino acids that is highly conserved throughout eukaryotic species and enables their binding to a specific DNA sequence (A/T A/T CAA A/T) (17). SOX genes are classified into groups A-H based on phylogenetic differences in their HMG box sequences, protein structure and involvement in developmental processes (**Figure 2**) (18). Within SOX subfamilies, the structural domains of the proteins outside the HMG domain are similar but not identical. These domains include the transactivation domain (TAD), the transrepression domain (TRD), coiled-coil domain (CC) and the dimerization domain (DIM). The TAD or TRD are domains that contain binding sites for other proteins (i.e., transcription coregulators) to bind, whereas DIM and CC allow for dimerization of Sox proteins (19). In contrast to most transcription factors, Sox proteins introduce strong bends into DNA, thereby allowing multiple

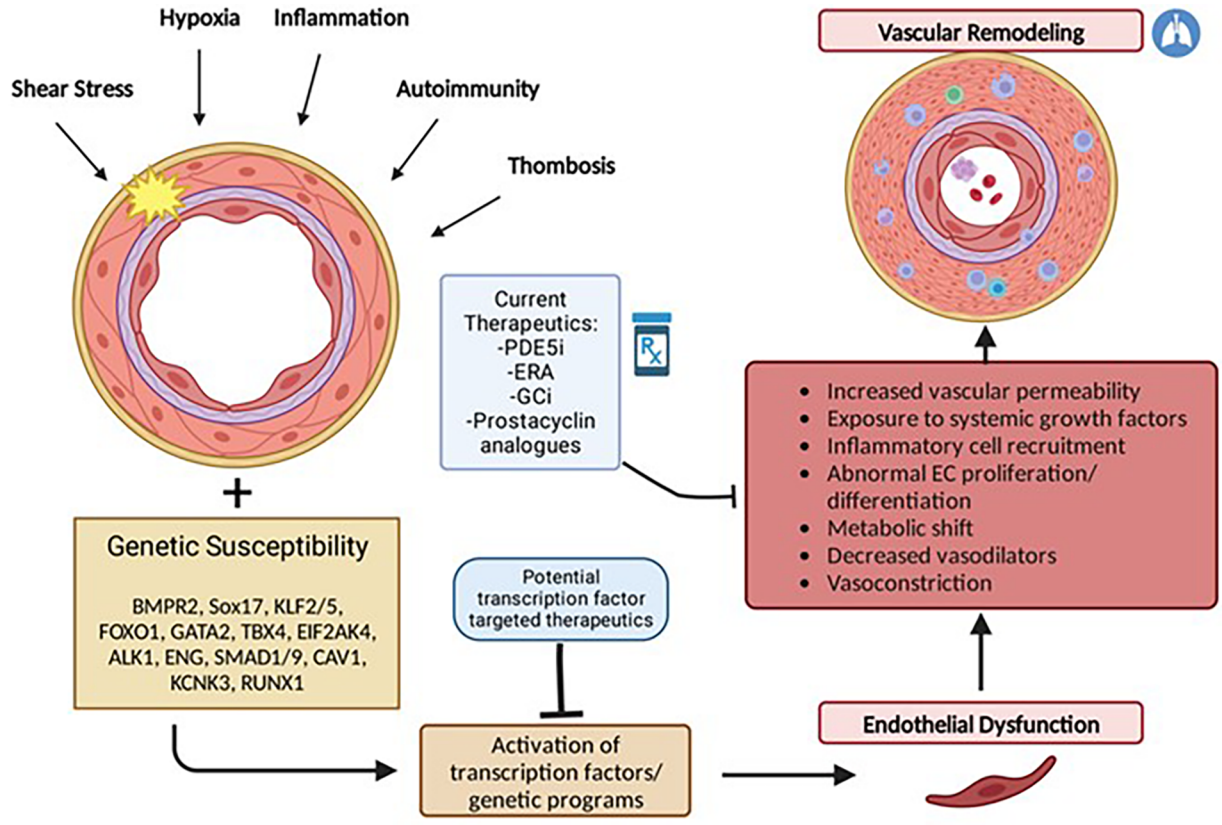

FIGURE 1
Multiple hit model of pulmonary arterial hypertension development. PAH results from endothelial dysfunction and subsequent vascular remodeling triggered by activation or suppression of transcription factors in response to repetitive stressful stimuli. Individual genetic susceptibility (i.e., genetic mutations, gender and epigenetic factors) predisposes endothelial cells to cellular dysregulation. Current therapeutics act primarily in response to vascular remodeling and as pulmonary vasodilators but largely do not reverse pulmonary vascular remodeling characteristic of PAH.

regulatory proteins and transcription factors to bind by colocalization with enhancers or gene promoters (20). Importantly, many Sox family proteins are pioneer factors, a subset of transcription factors that serve as master regulators that can bind to target DNA sequences even when they are buried inside condensed chromatin and can thereafter initiate opening of closed chromatin and activation of transcription (21). Interestingly, SOX genes appear to play critical roles in the generation and induction of pluripotent stem cells (PSCs) and embryonic stem cells (ESCs)—regulating pluripotency and mediating self-renewal and differentiation to endothelial cells (21, 22).

SRY-box transcription factor 17 (SOX17) is a member of the SOXF subfamily along with SOX7 and SOX18. All members of this subfamily contain a TAD in the C-terminal and a short functional motif (DxxEFD/EQYL) thought to be involved in the interaction with Beta-catenin (18). Members of this group have been shown to be crucial in early cardiovascular and hematopoietic cell development in mice and humans and are heavily expressed in vascular endothelial precursors and progenitor cells (see section below on endothelial progenitor cells). All members of the SOXF subgroup are expressed in endothelial cells during development and frequently have overlapping roles in the development of the cardiovascular system (21).

The human *SOX17* gene contains two exons expressed in human heart, lung, spleen, testis, ovary and placental and in fetal lung, and kidney tissue. Although widely expressed during development, *SOX17* displays a largely endothelial-specific expression profile in healthy adult tissues (23). In mouse embryonic tissue, *Sox17* expression is initially localized to the endoderm but subsequently increases in the dorsal aorta during vascular development. In adult mice, expression is largely contained to arterial vascular endothelium rather than venous endothelium (24).

SOX17 is a crucial endothelial-specific transcription factor involved in arteriovenous differentiation, pulmonary vascular morphogenesis, angiogenesis, and pulmonary endothelial regeneration following vascular injury. *Sox17* and *Sox18* both appear to be necessary for vascular endothelial postnatal angiogenesis, whereas low *Sox17* and high *Sox7*/*Sox18* expression are necessary for systemic vein development (25). *Sox17* is necessary for arterial differentiation and vascular development and promotion of arterial identity appears to be mediated via downstream activation of the Notch pathway (**Figure 3**). Endothelial cell-specific deletion of *Sox17* in mice leads to death *in utero* associated with lack of large artery formation, formation of defective vascular networks including arterio-venous

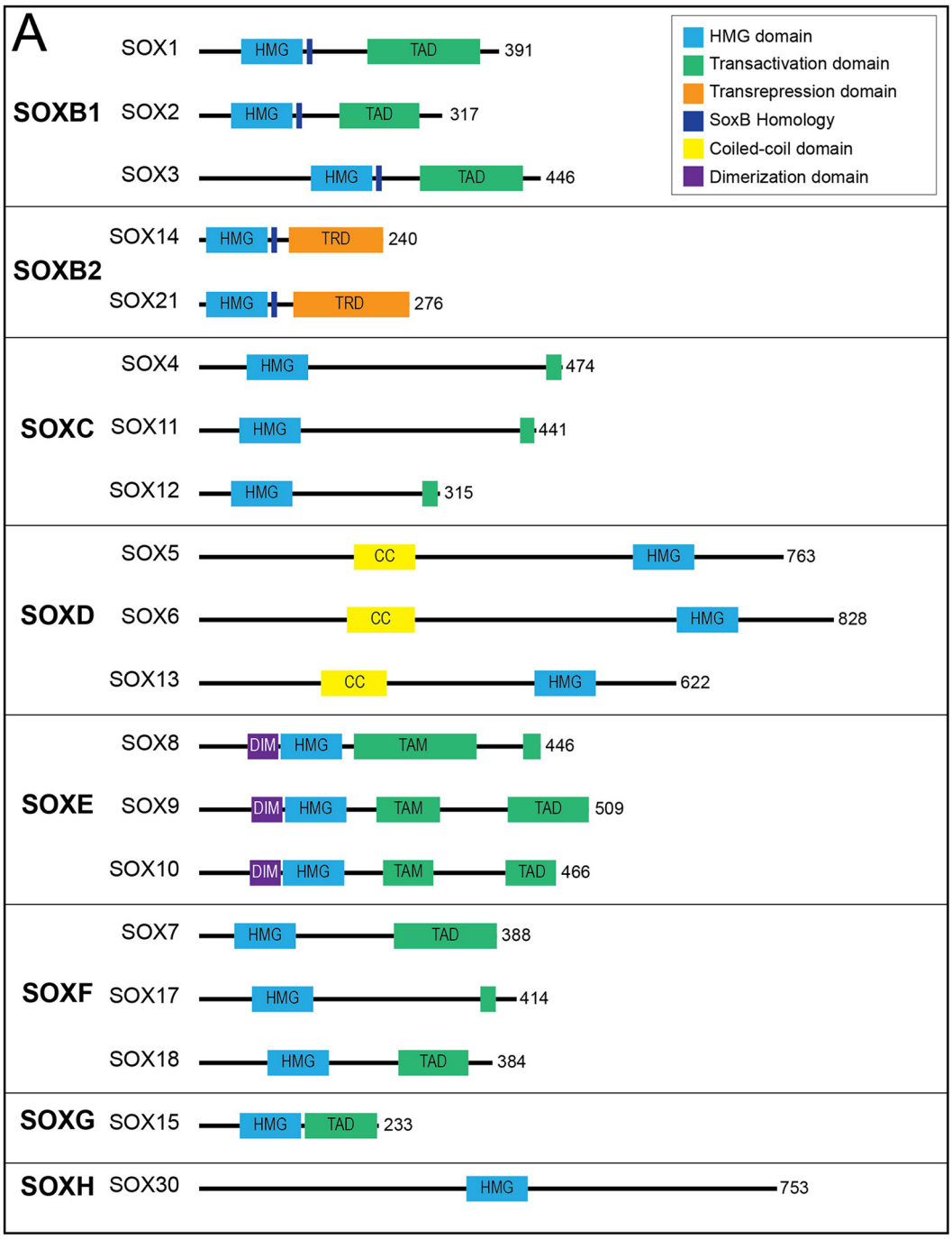

FIGURE 2
Sox transcription factor subfamilies and their functional domains. SOX proteins grouped by subfamily. Major protein functional domains are depicted as colored boxes: High-mobility group (HMG) domain (light blue), transactivation domain (TAD; green), transrepression domain (TRD; orange), SoxB homology domain (dark blue), coiled-coil (CC) domain (yellow), and dimerization (DIM) domain (purple). Reproduced from Schock EN, LaBonne C. Sorting Sox: Diverse Roles for Sox Transcription Factors During Neural Crest and Craniofacial Development. Front Physiol. (2020 Dec 8) 11:606889. doi: 10.3389/fphys.2020.606889. PMID: 33424631; PMCID: PMC7793875.

malformations, and lack of arterial and venous differentiation (21). Cell-specific knockout of SOX17 in developing mouse pulmonary vascular endothelium causes the development of pulmonary vein varices, dilated pulmonary arteries and abnormal lung perfusion along with biventricular hypertrophy (23).

SOX17 expression remains high in arteries of mature mice, supporting a role for not only induction of artery formation but also maintenance of mature vasculature identity. Interestingly, single nucleotide polymorphisms in the *SOX17* gene have been associated with increased risk of intracranial aneurysm development in humans (26). In animal models, endothelial-specific SOX17 loss of function has been shown to result in impaired endothelial integrity, proliferation, paracrine regulation and increased development of intracranial aneurysms in response to hypertensive stress (27). Loss

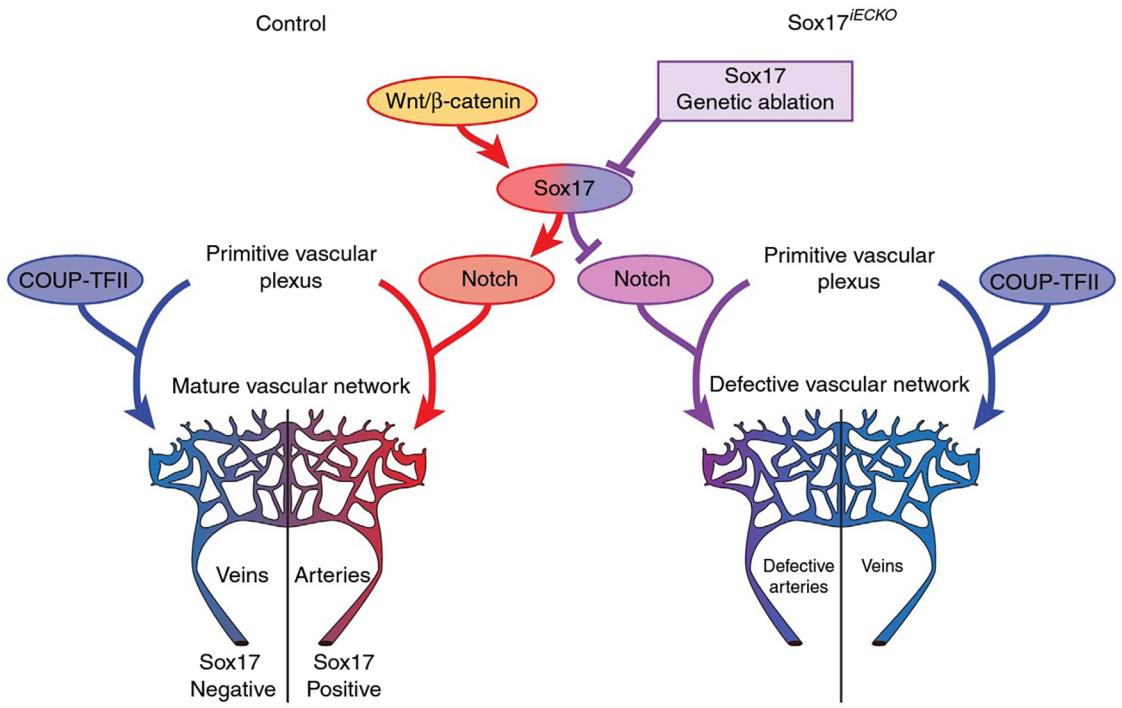

FIGURE 3
Representation of potential signaling pathways regulating arterial/venous specification. Wnt/Beta-catenin signaling activates Notch signaling and arterial differentiation through Sox17. Endothelial-specific inactivation of Sox17 prevents Notch signaling and acquisition of arterial identity by endothelial cells. COUP transcription factor II (COUP-TFII—important for venous determination), Sox17 iECKO (Sox17 endothelial cell specific knock out at P1) Wnt. Reproduced from Corada et al., 2016 2609.

of Sox17 function appears to disrupt cell to cell adhesion via decreased VE-cadherin expression—a possible explanation for both intracranial aneurysm development and non-functional vascular networks with decreased Sox17 function (22).

Sox17 also appears to actively prevent the transition from hematopoietic fate by repression of key hematopoietic transcription factors, thereby allowing cells to maintain arterial identity (See section below on endothelial to hematopoietic transition). Expression of SOX17 appears to be crucial in maintenance of endothelial rather than hematopoietic cell fate, as studies have demonstrated that tissue-specific, temporally controlled, knockout of arterial genes (SOX17 and NOTCH1) causes an increase in hematopoietic cells due to loss of Sox17 dependent repression of hematopoietic transcription factors Runx1 and Gata2 in both human and mice stem cells (28). *Sox17* is also crucial for maintaining a population of intra-aortic hematopoietic cluster (IAHCs) and fetal liver hematopoietic stem cells (HSCs) during development (28). Importantly, when SOX17 is downregulated during development in mouse embryonic days 9–11, hematopoietic cell differentiation is increased in cells in which *Sox17* is downregulated. So, although *Sox17* is crucial to the development of the endothelium, it also appears to be important in suppressing differentiation of embryonic endothelial cells towards a hematopoietic fate (28). Interestingly, while Sox17 binds directly to the *Runx1* and *Gata2* promotors to suppress their expression (28), Runx1 appears to repress Sox17 expression, indicating the presence of a negative feedback loop which appears to be important in directing endothelial progenitor cells toward an endothelial or hematopoietic fate (28).

Role of *Sox17* in vascular homeostasis and neovascularization

While *Sox17* is responsible for endothelial development, differentiation and identity, it also plays a critical role in endothelial regeneration and homeostasis in response to injury. Activation of developmental pathways is key to tissue regeneration in response to tissue injury. Accordingly, Sox17 has been shown to be upregulated following inflammatory induced vascular injury and is necessary for endothelial regeneration thereafter. For example, SOX17 expression was diminished in intracranial aneurysms of adults undergoing microsurgical clipping, whereas it was highly expressed in intracranial arteries from controls (27). Similarly, *Sox17* has been shown to be necessary for vascular regeneration in response to vessel wall injury in adult mice (29). Activation of *Sox17* in response to inflammation induced vascular injury appears to be dependent on hypoxia inducible factor 1α (HIF-1α) signaling (29). *Sox17* overexpression in endothelial cells via liposomal cDNA delivery enhances endothelial cell and mouse survival in response to LPS-induced lung injury (29).

Sox17 may also promote the development of progenitor cell behavior in multiple adult cell types (30). Sox17 has been demonstrated to play a key role in maintenance of pluripotency and endothelial differentiation potential. It has been shown to be central in trans-differentiation of fibroblasts to endothelial cells via dedifferentiation into CD34 + progenitor cells in response to

lung injury (29). Endothelial-specific deletions of Sox17 result in impaired endothelial cell junctional assembly, cell matrix adhesion and proliferative/regenerative capacity (28). *SOX17* overexpression has also been found to give rise to fetal-like HSCs with high self-renewal capacity (31). Similarly, *SOX17* appears to be crucial for maintenance of endothelial potential/differentiation in human pluripotent stem cells (hPSCs) (32). SOX17 has also been shown to upregulate tumor angiogenesis via increased VEGFR2 expression and inhibition of Sox17 leads to marked decreases in tumor progression, angiogenesis, and vascular density in multiple tumor models (33).

The signaling pathways by which SOX17 promotes progenitor cell and endothelial identities is not well understood. SOX17 appears to suppress SMAD3/TGF-β signaling—a pathway known as a potent inhibitor of epithelial cell proliferation. Interestingly, SOX17 mediated repression of hematopoietic lineage in EHT also appears to happen through TGF-β signaling (30). Recent studies have shown that SOX17 knockdown reduces BMPR2 expression and BMP9-induced phosphorylation of Smad1/5/9 (34) supporting a role for Sox17 in enhancing BMP signaling. Conversely, BMP signaling has been shown to repress Sox17 expression in zebrafish endoderm, whereas TGF-β signaling activates Sox17 signaling (35). Thus, BMP and Sox17 appear to form a feedback loop that modulates pluripotent stem cells differentiation. However, BMPs and SOX17 have significant overlap in their signaling effector targets in vascular cells, including VEGF, Notch, SMAD and Wnt signaling (36).

Role of SOX17 in PAH

The healthy pulmonary vascular endothelium is designed to dynamically adapt to stressful stimuli such as shear stress, inflammation, or hypoxia. Under circumstances with repetitive injury and/or enhanced genetic susceptibility, the protective endothelial layer breaks down and can take on a proliferative, vasoconstrictive, and proinflammatory phenotype. This dysfunctional endothelium is thought to be an early trigger in PAH. Recent, genome-wide association data have demonstrated significant increased risk of PAH from genetic variants in *SOX17* enhancer regions (37). Similarly, prior data also demonstrated an association of deleterious mutations in *SOX17* with PAH (38, 39). Considering the endothelial cell-specific nature of SOX17, its role in neovascularization and maintenance of arterial identity, the increase risk of PAH in patients with loss of function mutations in *SOX17,* strongly suggest that this transcription factor plays a pivotal role in the pathogenesis of the disease.

In 2018, Graf et al., first identified *SOX17* mutations as being significantly over-represented in whole-genome sequencing of 1,038 PAH patients compared to 6,385 control subjects (38). Missense mutations and protein truncating variants in *SOX17* were heavily over-represented in PAH patients (**Figure 4**). Interestingly, patients with *SOX17* mutations were significantly younger at the time of diagnosis compared to other PAH patients. Mutations identified in *SOX17* gene were predicted to lead to loss of function of the beta-catenin region or suppress beta-catenin or Oct4 binding (38). Furthermore, SOX17 expression was isolated largely to the pulmonary endothelium in the lungs of healthy people and to the endothelial cells within plexiform lesions in patients with PAH. Shortly after Graf et al.'s work, Zhu et al., demonstrated an enrichment of rare deleterious *SOX17* mutations in patients with PAH associated with congenital heart disease (PAH-CHD) (3.2% of patients) and in patients with IPAH/HPAH (0.7% of patients). Interestingly, the majority of the missense mutations occurred in the highly conserved HMG-box protein domain responsible for DNA binding (39). Additionally, they also demonstrated enrichment of missense mutations in downstream genes targeted by SOX17 in PAH patients (39). More recently, a genome-wide association study of 11,744 European individuals (2,085 patients) identified 2 independent risk variant-containing signals (Sox17-signal 1 and Sox17-signal 2) in an enhancer region (**Figure 5**) near *SOX17* that were associated with PAH despite the relatively rare prevalence of mutations in the coding region of the *SOX17* gene itself—implicating a more common causative role for Sox17 in PAH development than previous thought (39). In that study, 59% of patients with PAH were homozygous for both risk alleles compared to 47% of controls. Furthermore, PAH patients with *SOX17* mutations demonstrate high rates of dilated and tortuous pulmonary vessels, hemoptysis and atrial and ventricular septal defects (**Figure 6**). These patients frequently present with severely compromised hemodynamic parameters at the time of diagnosis with a mean mPAP of 67 mmHg and PVR of 14 WU in one European cohort (40).

Potential pathogenic mechanisms for SOX17 in PAH

Since the discovery of *SOX17* mutations and their association with PAH, investigators have sought to understand the mechanism by which impaired *SOX17* expression predisposes patients to PAH. Animal models and in-vitro studies have replicated many of the pathophysiologic hallmarks observed in human PAH patients in Sox17 deficient mice (**Table 1**). Studies using endothelial specific knockdown of SOX17 show either no pulmonary hypertension or trends toward increases in basal RV systolic pressure and RV hypertrophy compared to wild-type mice but more severe pulmonary hypertension or earlier development of pulmonary hypertension in response to chronic hypoxia or sugen/hypoxia (Su/Hx-PH), suggesting that loss of endothelial SOX17 expression exacerbates pulmonary hypertensive responses (41, 43, 44). In contrast to other animal models of PAH, the pulmonary remodeling and right ventricular hypertrophy observed in these mice does not reverse following return to normoxia (41). *Sox17* deficient mice also have increased pulmonary inflammation as exemplified by increased perivascular infiltration of CD11b + cells (41). Pulmonary endothelial cells from *Sox17* knockout mice also demonstrate marked hyperproliferation and upregulation of inflammatory gene expression (42). In line with these results, autopsies from 4 out of 15 patients with PAH demonstrated marked decreases in

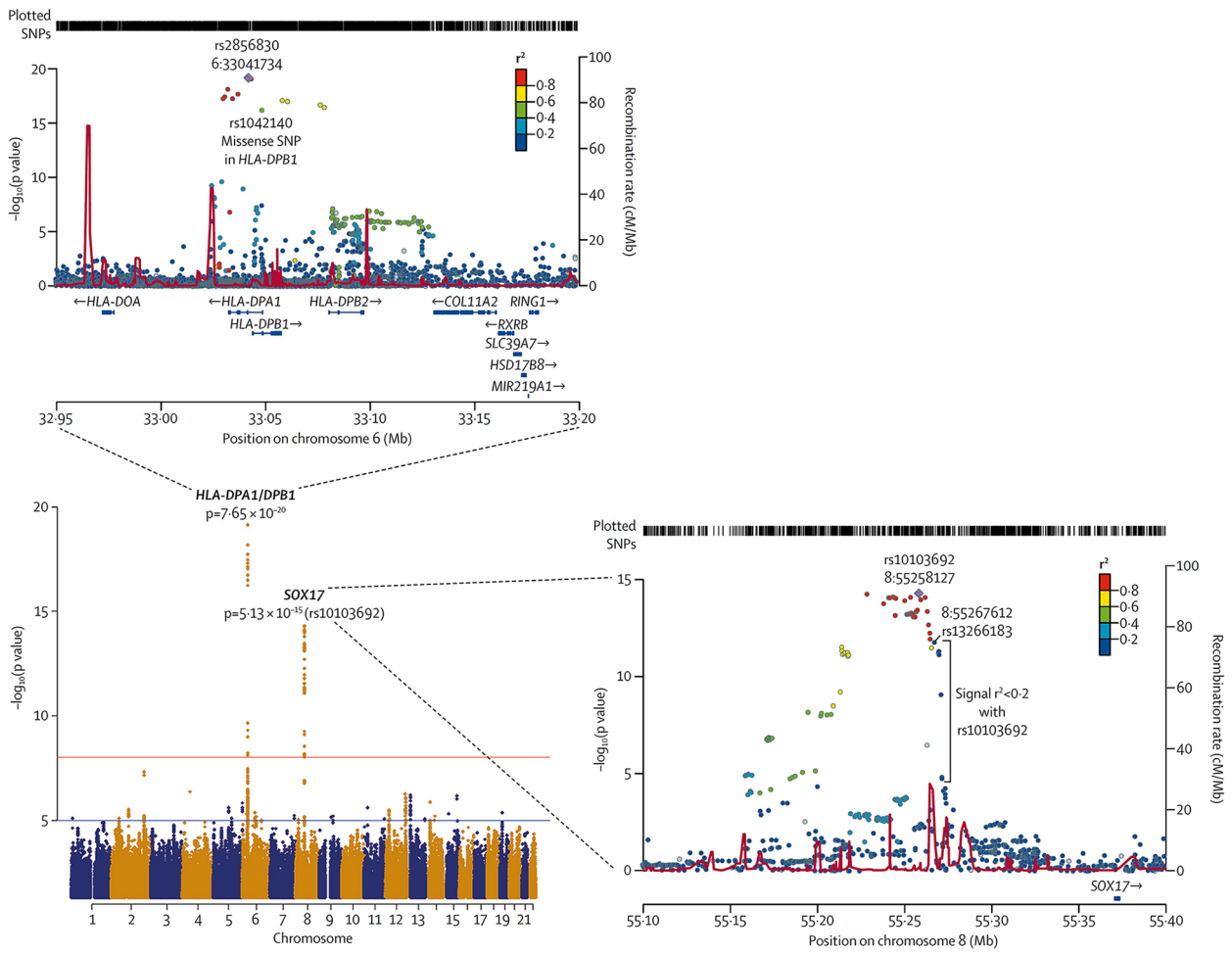

FIGURE 4
Meta-analysis of all cohorts (total 2,085 PAH patients and 11,744 controls) and regional plots of novel loci identified as being more frequently associated with PAH. Regional plots indicate variant location and linkage disequilibrium structure at SOX17 locus. Several variants associated with pulmonary arterial hypertension are in very weak or no linkage disequilibrium ($r2 < 0{\cdot}2$) with the lead single-nucleotide polymorphism (SNP), rs10103692. Variants are referred to as SOX17 signal 1 and the most significant variant, rs13266183. The variants coloured as in linkage disequilibrium with rs10103692 comprise signal 2. Reproduced from Rhodes et al., 2019.

SOX17 expression in pulmonary arterial endothelial cell (PAEC) (41). Recent studies have also shown decreased *SOX17* gene expression and protein levels in pulmonary vascular endothelial cells (PVEC) isolated from PAH patients compared to failed donor lungs (34). Similarly, *SOX17* silencing mutations in human PAECs result in increased apoptosis, proliferation and adhesion (41). Conversely, Sox17 overexpression in mice attenuates hypoxia-induced PH and suppresses vascular remodeling, proliferation and inflammation in Sugen-hypoxia induced PH in an autocrine manner (44).

In addition to animal models using conditional deletion of endothelial SOX17, transgenic mice engineered to resemble SOX17 mutations associated with increased risk of PAH are also more susceptible to pulmonary hypertension. *Sox17* enhancer knockout mice (*Sox17eKO*) generated by CRISPR-mediated knockdown of the *SOX17* enhancer regions initially detected in PAH patients (rs10958403 and rs765727) reduces Sox17 expression in mouse lung tissue and increases human pulmonary artery endothelial cell (PAEC) permeability while decreasing endothelial cell adhesion and VEGF-induced proliferative capacity (42). *Sox17eKO* mice develop normally but exhibit greater right ventricular hypertrophy and higher elevation of right ventricular systolic pressure (RVSP) compared to controls when exposed to hypoxia (44). They also demonstrate increased susceptibility to Sugen/hypoxia-pulmonary hypertension (42). In that study, low dose Sugen 5,416 (5 mg/kg) and mild hypoxia (12% oxygen) had no effect on wild-type mice but induced severe PH in *Sox17eKO* mice (**Figure 7**). These findings suggest that even when SOX17 is expressed in endothelial cells, mutations in the enhancer region that may impair normal activation of the SOX17 gene can increase the risk or severity of pulmonary hypertension.

SOX17 has been shown to be vital in numerous molecular signaling pathways important to PAH pathogenesis (**Table 1**, column 4). Important SOX17 downstream targets such as Notch signaling, BMPR2, estrogen receptor signaling, prostacyclin

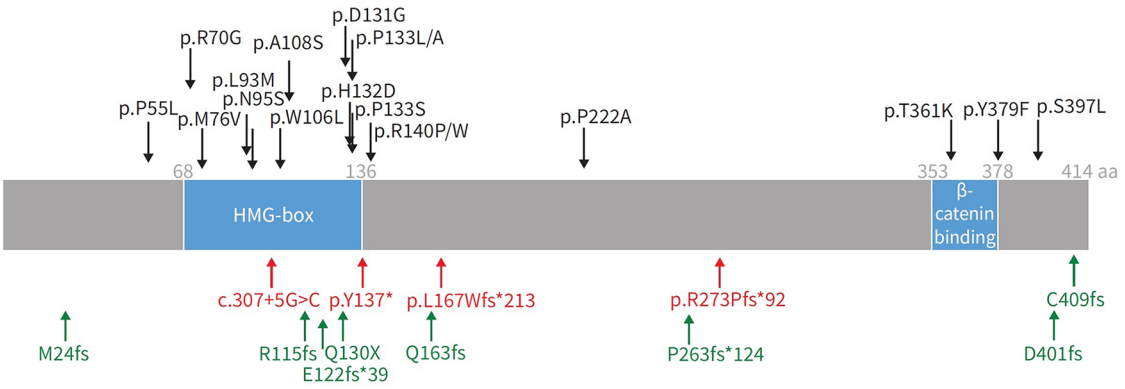

FIGURE 5
Representation of SOX17 gene with overlying variants identified in PAH patients. aa: amino acids; HMG: high mobility group. Black: missense mutations; red: protein-truncating variants; green: likely gene-disrupting variants. Reproduced from Wu, Y., Wharton, J., Walters, R., Vasilaki, E., Aman, J., Zhao, L., Wilkins, M. R., & Rhodes, C. J. (2021). The pathophysiological role of novel pulmonary arterial hypertension gene SOX17. The European respiratory journal. 58(3): 2004172. doi: 10.1183/13993003.04172-2020.

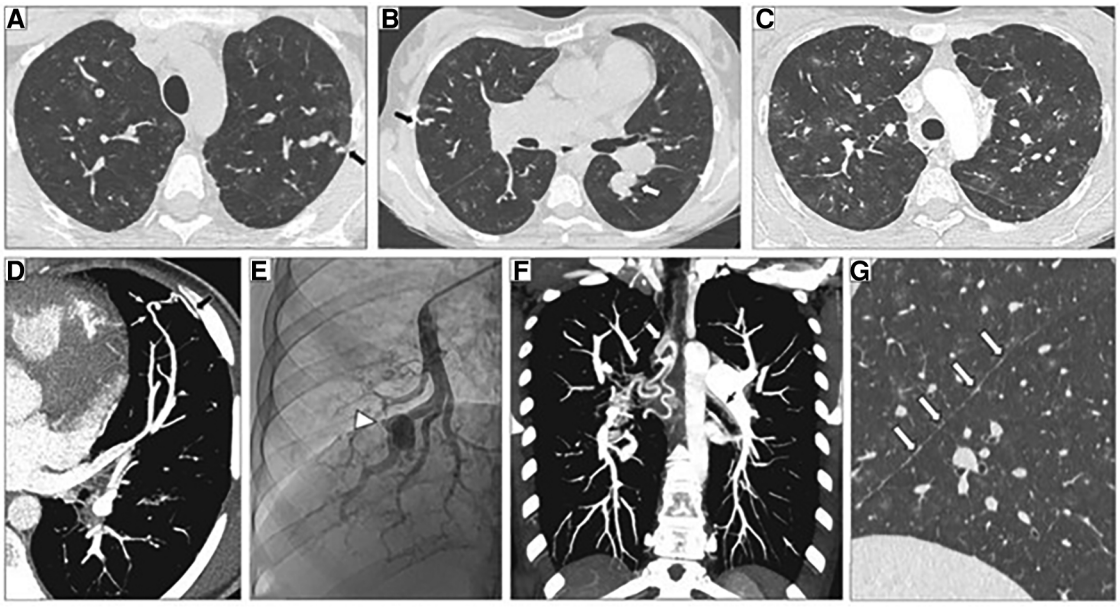

FIGURE 6
Representative high-resolution computed tomography (HRCT) of the chest, CT pulmonary angiography (CTPA) and pulmonary angiogram of pulmonary arterial hypertension patients carrying a SOX17 pathogenic variant. (**A–C**) Thin-collimated HRCT of the chest showing (**A**,**B**) subpleural dilated and tortuous pulmonary vessels (black arrows) and (**C**) ground-glass opacities. (**D**) The black arrow points to direct communication between a dilated distal pulmonary artery and a dilated intercostal artery. (**B**,**E**) Aneurysmal dilatation identified on b) CTPA (white arrow) and (**E**) pulmonary angiography (arrowhead). (**F**) Marked dilatation of proximal bronchial arteries is frequently observed (white and black arrows). (**G**) The presence of numerous fissural irregularities (white arrows) suggests the additional presence of dilated systemic vessels at the pleural surface. Reproduced from Montani et al., 2022.

synthase and c-Met/HGF have been implicated in the endothelial dysfunction underlying Sox17's involvement in PAH.

SOX17 deficiency appears to upregulate endothelial cell hyperplasia and proliferation through upregulation of growth factor signaling in response to hypoxia or cellular stress. Interestingly, increased pulmonary vascular endothelial cell proliferation is observed in Sox17 deficient mice under hypoxic conditions (34). This appears to be due in part due to upregulation of Hepatocyte growth factor (HGF)/c-Met signaling. Along these lines, PAH can be reversed by suppression of c-Met via a small molecule inhibitor, crizotinib, in Sox17 knockout/hypoxic mice (41). Similarly, *SOX17* endothelial specific knockdown and deletion in both human and mice pulmonary vascular endothelial cells results in increased endothelial cell

TABLE 1 Animals models of PAH with Sox17 inhibition.

Animal model	Cell type/knockout condition	Histologic/morphologic changes observed	Signaling pathways affected	Reference
– Adult Mice exposed to hypoxia	Sox17 deletion in pulmonary endothelial cells	Hypermuscularization, endothelial cell hyperplasia/proliferation, lung arteriolar inflammation, elevated RAP and RVH (by RVSP and RV/LV + septum ratio)	– Upregulation of HGF in Sox17 deficient EC after hypoxia – Inhibition of c-Met/HGF signaling reverses and prevents PH	Park et al., Circulation Research (41)
– In-silico electromobility shift assays of human PAECs – Adult Mice exposed to hypoxia and Su5416/hypoxia	Common human PAH risk *Sox17* enhancer region knockout in both hPAECs and adult mice	– Increased apoptosis, proliferation and disturbance of barrier function in hPAECs – Severe PH (by RVSP and RV/LV + septum ratio) in hypoxia and Su/hypoxia *Sox17ko* mice along with increased vessel muscularization and permeability	– Noted decreased binding of RORα and HOXA5 TFs with Sox17 enhancer mutations	Walters et al., Circulation (42)
– Adult mice/rat exposed to hypoxia, MCT or 16α-hydroxyestrone (16 αOHE-estrogen metabolite)	– Conditional *Sox17* knockout or conditional transgenic overexpression	– Increased muscularization in pulmonary arterioles, elevated PA pressures /thickness/RVH and PH in *Sox17* knockout mice/rats exposed to hypoxia (by RVSP and RV/LV + septum ratio)	– HIF2α concentrations increased in lungs of *Sox17* knockout mice – Increased Sox17 expression promoted oxidative phosphorylation and mitochondrial function in PAECs – Male rats had higher Sox17 expression than female counterparts – Sox17 expression decreases with treatment with 16 αOHE and is increased in estrogen receptor knockout rats	Sangam et al., AJRCCM (43)
– Human endothelial cells from IPAH patients – Adult mice exposed to hypoxia	– Endothelial specific temporally controlled *Sox17* knockdown in mice – Knockout of *Sox17* in embryonic stages	– Increased spontaneous pulmonary artery muscularization and PH (by RVSP and RV/LV + septum ratio) in both embryonic and adult *Sox17* knockout mice and exaggerated hypoxia-induced PH in adult *Sox17* knockout mice (by RVSP and RV/LV + septum ratio) – Marked proliferation of endothelial cells with Sox17 deficiency both in HPVEC and mouse knockouts	– Increased paracrine mediated PASMC proliferation in-vitro in *Sox17* knockouts – Increased EC dysfunction in HPVEC that is mediated through E2F1 transcription factor (in-vitro dysfunction/proliferation and *Sox17* knockout PH rescued with E2F1 inhibition)	Yi et al., bioRxiv (34)
– Adult mice exposed to Su/Hypoxia	– Endothelial cell-specific knockdown or overexpression of *Sox17* via adenoviral vector siRNA	– Increased muscularization, RVH and RVSP in *Sox17* knockdown mice (by RVSP and RV/LV + septum ratio)	– SOX17 overexpression in HPAECs blocked VEGF-induced proliferation, serum free apoptosis and hypoxia/TNFalpha induced inflammation in a autocrine fashion – Exosomes from SOX17 overexpression in HPAECs prevented Su/hypoxia PH and reduced endothelial proliferation/inflammation – Protective affects appear to be in part mediated through micro RNA inhibition of NR4A3 and PCSK9	Zou et al., (44)
– HPAECs and HPASMCs – BMPR2 mutant mice	– Pulmonary artery on-a-chip model with HPAECs and HPASMCs separated by a membrane with ongoing pulsatile flow – Treated HPAECs with adenoviral mediated BMPR2 silencing RNA and hypoxia		– Decreased endothelial Sox17 expression observed in BMPR2 deficient HPAECs and endothelial cells of BMPR2 knockout mice. Associated with increased PASMC proliferation – Sox17 overexpression rescued PASMC proliferation and was associated with a significant increase in Prostacyclin synthase expression	Ainscough et al., (45)

proliferation and smooth muscle cell proliferation. This endothelial cell dysfunction appears to act through E2F1, as its expression is upregulated in response to *SOX17* knockout and siRNA/small molecule inhibitor induced E2F1 knockdown rescues both endothelial dysfunction and *SOX17* deficient hypoxia-induced PH (34). This knockdown of SOX17 expression reduces BMPR2 expression and BMP9-induced phosphorylation of Smad1/5/9. Reciprocal results were also observed in BMPR2 deficient

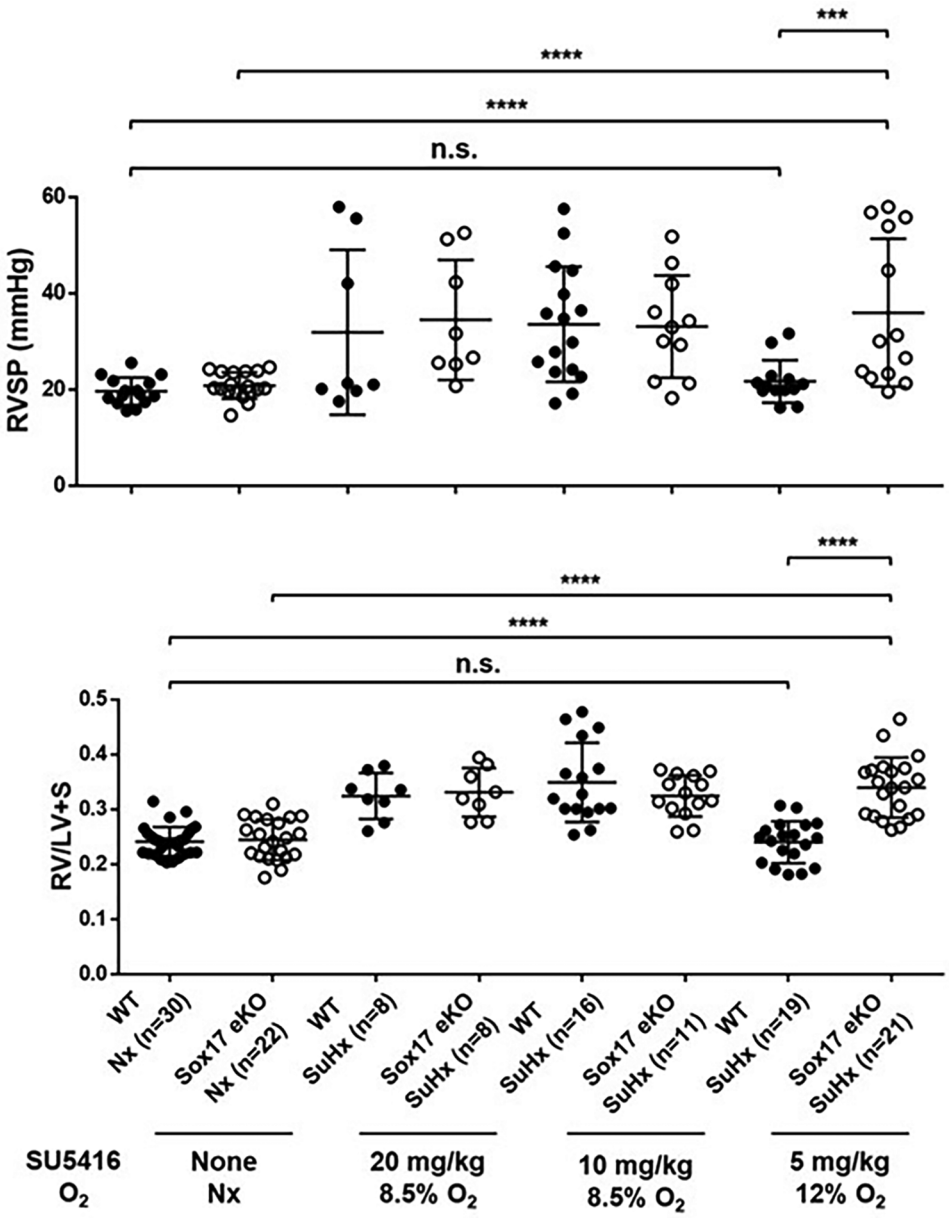

FIGURE 7
Exposure of SOX17 enhancer knockout mice model (eKO) to different levels of Sugen-5416 and hypoxia. (**A**) Right ventricular hypertrophy index (RV/LV + S). (**B**) Right ventricular systolic pressure (RVSP mmHg). SuHx, Sugen-5416 Hypoxia. O2, oxygen (Nx = normoxia). Numbers are shown on graphs. Ordinary One-way ANOVA. WT, wild type. KO, knockout. Reproduced from Walters et al., 2023.

HPAECs, as decreased endothelial expression of SOX17 was observed in these cells and was associated with a paracrine mediated increase in pulmonary artery smooth muscle cell (PASMC) proliferation (45). Interestingly, in these experiments, overexpression of SOX17 in HPAECs rescued HPASMC proliferation and was associated with a significant increase in prostacyclin synthase. Similarly, hypoxia-inducible factors are transcription factors that upregulate angiogenesis, apoptosis and glycolysis in response to hypoxic stimuli. In *Sox17* knockout mice, HIF2alpha concentrations are increased in response to hypoxia and overexpression of Sox17 upregulates oxidative phosphorylation and mitochondrial function in endothelial cells (43). Interestingly, male rats appear to have increased Sox17 expression compared to their female counterparts, a finding which in part, may be explained by decreased Sox17 expression in the presence of estrogen metabolites. It is unlikely that sex related differences in SOX17 explain the higher incidence of PAH in woman compared to men, but raises an intriguing potential mechanism that may contribute to this well-known observation. Other potentially important roles for Sox17 include its ability to bind VE-cadherin and endothelial cell-selective adhesion molecules (ESAM) which are required to maintain blood vessel wall stability and permeability in the lung (46). Missense mutations in the highly conserved DNA-binding domain region of *SOX17* HMG box —similar to those described in humans, have been shown to impair direct DNA-binding and

Beta-catenin protein complex interactions vital to its Beta-catenin, TGF-β and Notch signaling (47). Similarly SOX17 has been shown to be involved in other cellular pathways important to PAH pathogenesis such as cyclin, VEGF, Wnt/B-catenin, and endothelin signaling in the context of embryonic and arterial development. Evidence for Sox17's direct influence on these pathways in the context of PAH remains however limited or unexplored.

SOX17 expression is maintained in a restricted fashion in the adult endothelium, but increases in response to inflammatory or hypoxic signaling and deficiencies in expression appear to increase the likelihood of endothelial dysfunction and subsequent PAH. Given that relative deficiencies in SOX17 expression may predispose patients to PAH, increasing SOX17 expression or manipulating its downstream targets may be a novel approach to treating PAH. In-vitro and animal models have given rise to promising potential future therapies to rescue loss of SOX17 function in PAH. For example, in hPAECs with enhancer knockouts characteristic of PAH patients, Sirolimus and YK4279 [identified initially through high-throughput omics signatures using connectivity map (CMap)] were found to reverse *Sox17* enhancer knockout mediated repression (42). Similarly, inhibition of E2F1, a downstream target of Sox17, with a small molecular inhibitor (HLM) rescues PH in Sox17 deficient mice, reducing RSVP, pulmonary artery muscularization and pulmonary wall thickness (34).

Collectively, these results point to impaired SOX17 signaling as an important contributor to the pathogenesis of PAH. The major roles of SOX17 in vascular homeostasis, neovascularization and maintenance of arterial/endothelial identity as well as its importance in response to vascular injury and stress, make it a promising therapeutic target for PAH. At the same time, SOX17 knockout models may serve as important new preclinical models for investigating PAH.

RUNX1

Runt-related transcription factor 1 (RUNX1) is a downstream target of SOX17 and may play a role in mediating the effect of impaired Sox17 expression on PAH. The Runt gene was discovered in 1980 by Nusslein-Volhard and Wieschaus in a screen conducted to identify mutations affecting Drosophila larvae segment number, and polarity (48). One mutation resulted in pre-segmentation patterning defects that resulted in runted embryos—thus the Runt name was given to the mutated gene (**Figure 8**). Subsequent cloning revealed that the protein it encoded for was a transcription factor. RUNX1 was first identified in 1991 as a gene involved in the chromosome rearrangement t (8;21) associated with acute myeloid leukemia (49). Runx1 belongs to a transcription factor family called core-binding factors (CBF), which includes sequence-specific DNA-binding proteins Runx1-3. All Runx proteins share a non-DNA binding protein CBF-beta that helps them effectively bind DNA. RUNX1 is encoded by 12 exons and has various isoforms that can be synthesized by alternative splicing. Among these exons are two well defined domains: the runt homology domain (RHD) and the transactivation domain that bind DNA and facilitate protein-protein interactions, respectively. Runx1 partners with CFB-beta subunit to form a transcriptionally active heterodimer that can either activate or repress gene expression (50).

RUNX1 regulates the differentiation of HSCs into mature blood cells and is indispensable for the establishment of definitive hematopoiesis in vertebrates. RUNX1 is expressed in cells from all hematopoietic sites that contribute to formation of HSCs during development. HSCs are generated via a specialized subset of vascular endothelial cells known as the hemogenic endothelium that can differentiate into hematopoietic cells in a process called endothelial to hematopoietic transition (EHT). Developmentally, the endothelium arises in close contact with the hematopoietic system, and they share a common lineage in hemogenic endothelium. The adult hematopoietic system and long-term hematopoietic cells that give rise to life-long blood cell production are derived from hemogenic endothelial cells (HECs) in mid-gestational embryos. HECs are generated from embryonic endothelial cells after upregulation of Runx1 and activation of the Notch and TGF-β pathways during a brief developmental window in embryogenesis (Embryonic days 9.5–10.5). Runx1 is essential for EHT and its expression distinguishes HECs from non-HECs (51). HECs are located within the ventral aspect of the dorsal aorta and other large vessels. During EHT, HECs lose their tight junctions and demonstrate increased migratory behavior, characteristic of HSPCs. The HSPCs generated during EHT then seed the fetal liver and later the bone marrow (BM) to sustain hematopoiesis. EHT is a highly conserved stepwise process regulated by a complex interplay of a specific set of transcription factors, including Runx1, GATA1, GATA2, Lmo2, Scl/Tal1.

Homozygous RUNX1 mice lack hematopoiesis and are unable to survive past an early embryonic stage (E11.5-E12.5) (52) due to CNS hemorrhage. Heterozygous germline mutations in *RUNX1* are associated with Familial Platelet Disorder, a mild bleeding disorder associated with thrombocytopenia and high rates of myeloid leukemia (53). Other mutations in *RUNX1* are also associated with acute myeloid leukemia, T-cell acute lymphoblastic leukemia (present in ~15% of cases), pancreatic cancer, myelodysplastic disorders, and a variety of other tumors (54).

Although EHT has been considered a developmental process that becomes inactive in adult life, under pathogenic conditions endothelial progenitor cell reservoirs may reactivate and contribute to adult hematopoiesis (55, 56). Furthermore, recent single-cell transcriptome expression analysis suggests that definitive hematopoiesis represents a continuum of phenotypes from endothelial cells to fully determined HSCs that undergo a continuous gain of specific lineage characteristics (57, 58).

Although initially thought to only be expressed in areas of hematopoiesis, in 2011 Heley et al., found that RUNX1 is also highly expressed in the developing human lung (59). Future work also uncovered that Runx1 is expressed in postnatal and adult murine lung and is highly upregulated in response to inflammation (60). Runx1 was also found to upregulate NF-kB and LPS-induced increases in macrophage IL-1B and IL-6 expression (61). At the same time, increased expression of Runx1 leads to enhanced endothelial cell proliferation and migration (62, 63).

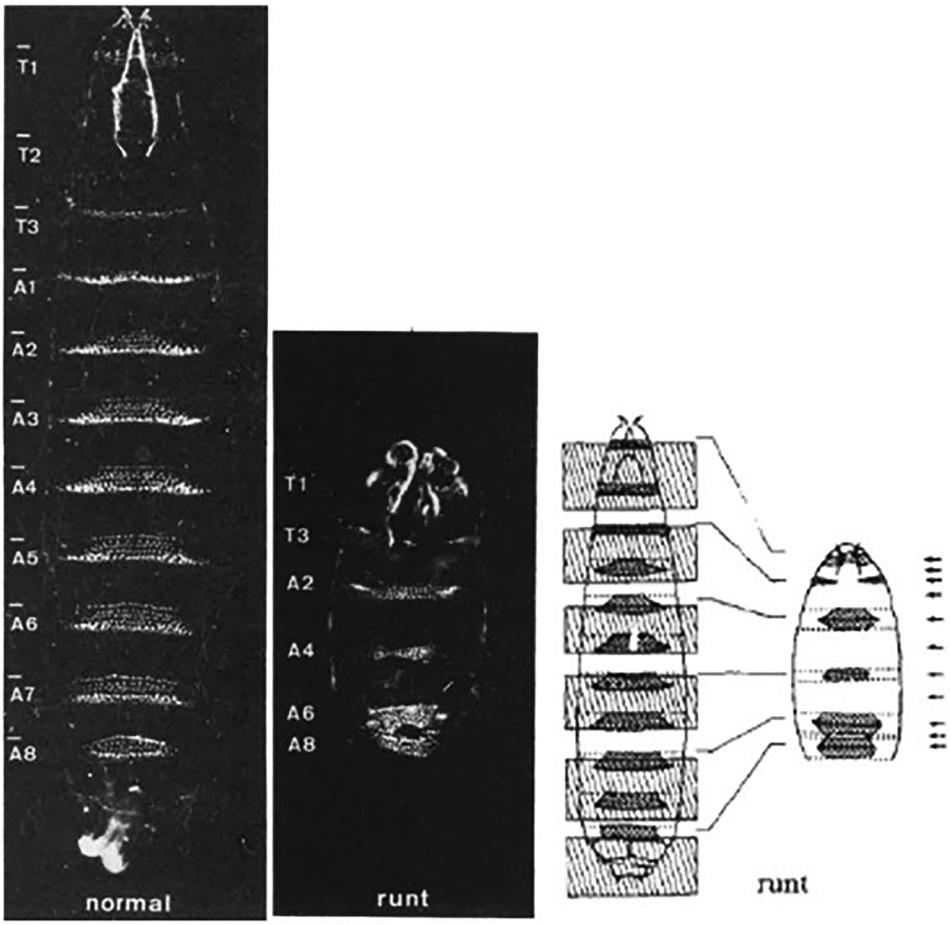

FIGURE 8
Representative images from Nusslein-Volhard's experiments on drosophila larvae. Normal larvae (left), Runx mutant larvae (middle) and authors representative depiction of inverted segment polarity and short segments (right). Reproduced from Nüsslein-Volhard et al., 1980.

In the pulmonary vasculature, injury to the endothelium followed by upregulation of inflammatory signals and recruitment of macrophages, neutrophils and inflammatory cells are thought to be sentinel events in the development of pulmonary hypertension. In accordance with this, lung inflammation precedes pulmonary vascular remodeling in experimental models of PAH and failure to resolve progression of this inflammatory response is thought to underlie the development of PAH (64). Pulmonary vascular lesions in PAH patients and animal models are characterized by progressive degrees of inflammatory/myeloid infiltrates including mast cells, dendritic cells, macrophages, T cells, B cells and lymphocytes. These infiltrates have corresponding increases in inflammatory cytokine and chemokine secretion, including IL-1, IL-6, IL-8, CCL5 and TNF-α; all of which are correlated with poor clinical outcomes in PH (65). In response to inflammatory signals and cells, pulmonary vascular cells can change their phenotype and cell fate and contribute to pulmonary vascular remodeling. Thus, transcription factors that direct stem cell differentiation from an endothelial to a myeloid fate may play important roles in the pulmonary vascular remodeling found in PAH.

Although the role of RUNX1 in the pathophysiology of PAH is not yet known, one hypothesis is that disruptions in endothelial progenitor cell (EPC) fate from definitive endothelium to hematopoietic or myeloid fate may explain inadequate vascular repair and perivascular infiltrates in patients with PAH. Interestingly, increased myeloid derived cells and increased myeloid specific transcription factors are seen in the perivascular infiltrates of PAH patients and animal models of PAH (66). Activation of developmental pathways is key in tissue regeneration in response to tissue injury. During injury, generation or dedifferentiation of cells into EPCs appears to be crucial for neovascularization. Maintenance of a differentiated endothelial fate for these progenitor cells however requires constant signaling. Defects in the maintenance of this fate or a predisposition to development of EHT and a shift toward myeloid differentiation may play a significant role in the abnormal pulmonary vascular remodeling that occurs in PAH.

Runx1 in PAH

Emerging data suggest that Runx1 plays a major pathogenic role in mouse models of PAH. Using conditional labeling of adult endothelial cells and lineage tracing (via VE-cadherin-cre;

Zsgreen mice) in Su/Hx mice, we demonstrated that adult endothelial cells undergo hematopoietic transformation suggesting reactivation of EHT, and migrate from the BM to the pulmonary vasculature (55). We also found that RUNX1 expression is higher in circulating EPCs in mice with Sugen/hypoxia-induced pulmonary hypertension (SuHx-PH) and in patients with PAH, compared to EPCs obtained from control mice or healthy volunteers, respectively (**Figure 9**) (55). Other studies from our lab showed that Runx1 inhibition via small molecule inhibitor (Ro5-3335) blocks EHT *in vivo* and prevents both migration of EPCs from BM and the development of Su/Hx-PH- and monocrotaline-induced PH (MCT-PH) in mice (**Figure 10**) (55, 56). The prominent role of Runx1 in the pathogenesis of PH was further supported by recent studies in our lab that utilized lineage tracing of adult endothelium and conditional cell-specific Runx1 knockout mice. We found that targeted deletion of Runx1 in either myeloid or endothelial cells prevents the development of SuHx-PH (**Figure 11**). Interestingly, inhibition of Runx1 via Ro5-3335 also appeared to reduce perivascular macrophage recruitment and reverse established Su/Hx-PH in mice while dampening macrophage activation *in vitro* (55).

Collectively, these results indicate that Runx1 may play a crucial pathologic role in the development and progression of PH. Considering that Runx1 is a downstream target of Sox17, it is tempting to speculate that mutations in the Sox17 enhancer that are associated with increased risk of PAH, may predispose to PH by failing to suppress Runx1 expression. Studies in our lab aimed at testing this hypothesis by examining the effect of suppressed Sox17 expression on the expression of Runx1 in the lung and in progenitor cells of animals with pulmonary hypertension and patients with PAH are ongoing. If the findings further studies support this hypothesis, Runx1 inhibition may prove an effective approach to treating PAH.

Bone marrow-derived progenitor cells

Although data from our lab suggests that Sox17 and Runx1 play an important role in the pathogenesis of PAH, the mechanism by which they do so is not clear. One possibility may be their role in driving HSCs toward either an endothelial or myeloid fate. The healthy endothelial monolayer of pulmonary arteries regulates the influx of fluid, proteins, and blood cells into

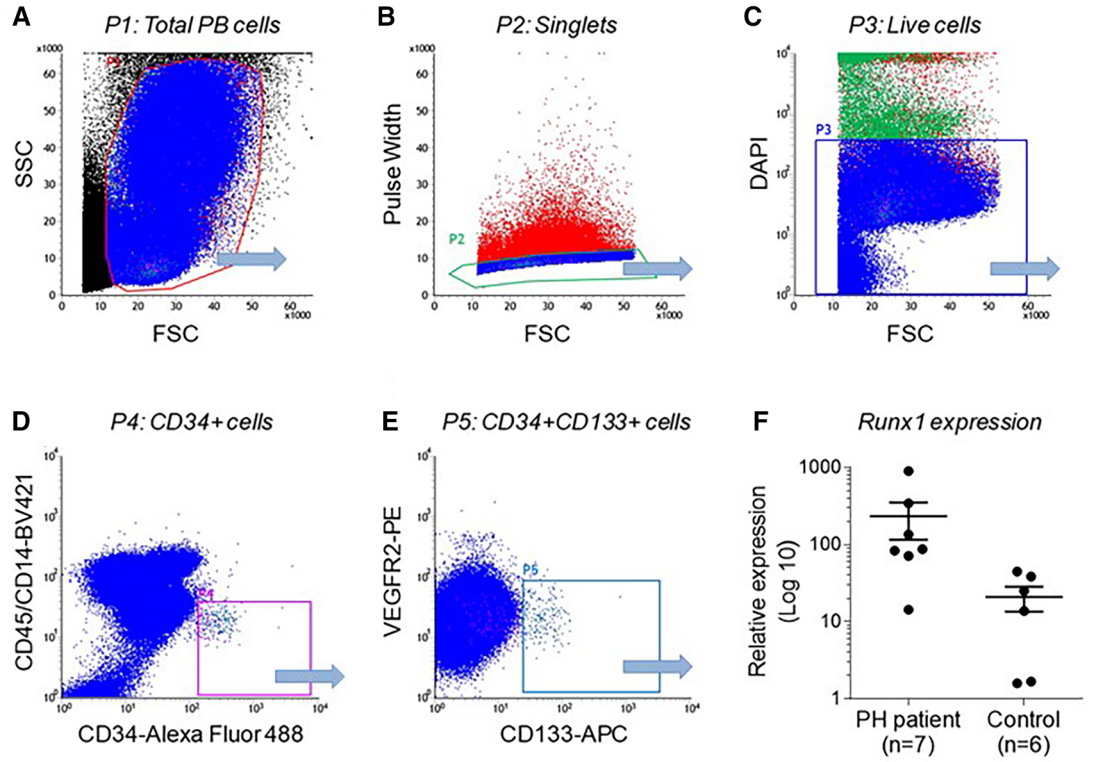

FIGURE 9
High expression of Runx1 in circulating CD34 + CD133 + EPCs from PH patients. Flow cytometry data showing (A) total peripheral blood (PB) cells (B) Single cells after doublet discrimination (C) Live cells (D) CD 34 + expression (E) CD34+, CD133+ and VEGF2R + cells and (F) Relative Runx1 expression in CD34+, CD133+, VEGF2R + cells in PH patients and controls. Reproduced from Liang et al., 2017.

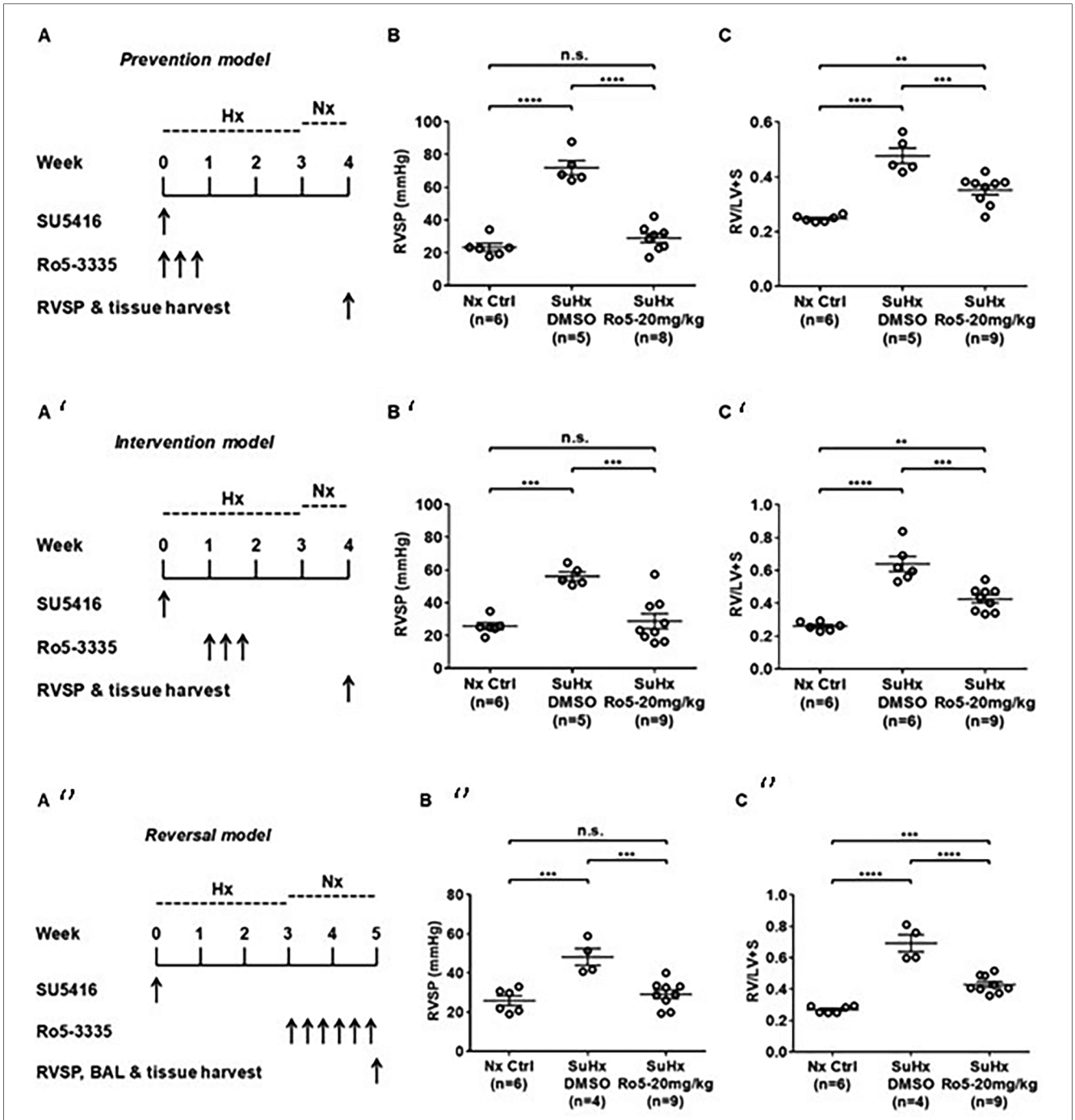

FIGURE 10
Runx1 inhibition prevents the development of and reverses established Su/Hx-PH. (**A**) Prevention model, SU5416 given along with Runx1 inhibitor (Ro5-3335) followed by subsequent measurement of (**B**) RVSP and C) RV/LV + S. (**A'**)Intervention model, SU5416 given followed by subsequent Runx1 inhibitor followed by subsequent measurement (**B'**) RVSP and (**C'**) RV/LV + S. (A'')Reversal model SU5416 given followed by delivery of Runx1 inhibitor after development of PH followed by measurement of (**B''**) RVSP and (**C''**) RV/LV + S. RVSP, Right ventricular systolic pressure. RV/LV + S, Right ventricular hypertrophy index. BAL, bronchial alveolar lavage. SuHx, Sugen-5416. Hx, Hypoxia. Nx, normoxia. Reproduced from Jeong et al., 2022.

surrounding parenchyma and maintains vascular tone and integrity. The gradual disruption of the endothelial layer in response to stress is thought to be one of the precipitating events in PAH. Loss of endothelial integrity eventually leads to the accumulation of pro-inflammatory cells, altered cell viability, smooth muscle cell hyperplasia, fibroblast proliferation and eventually occlusive vascular lesions, and increased vascular tone. At the same time, BM-derived myeloid cells play a prominent role in the perivascular inflammation that leads to remodeling of the extracellular matrix and adventitia. Emerging data suggest that local and BM-derived progenitor cells play important roles in homeostasis of the pulmonary endothelium and circulation that may be particularly important after injury that leads to PAH. Interestingly, SOX17 and RUNX1 are closely linked in

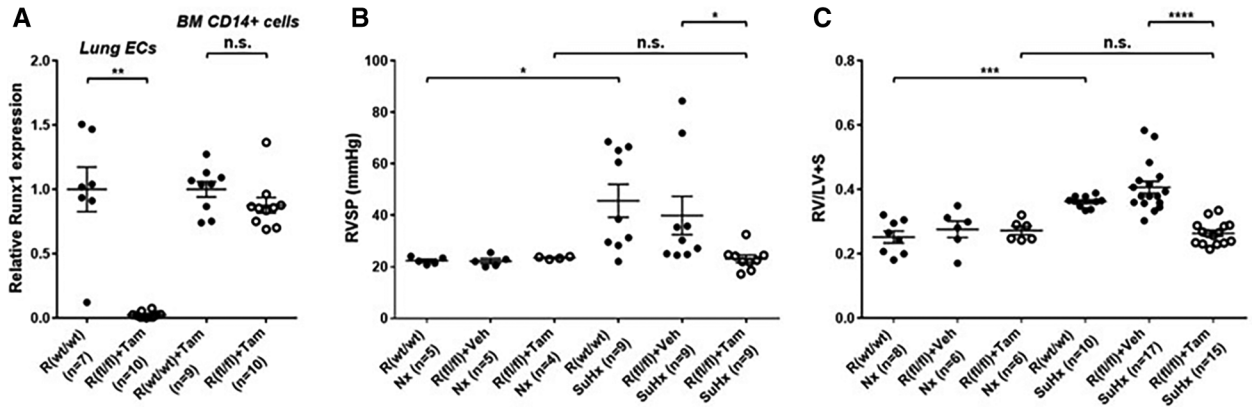

FIGURE 11
Genetic deletion of Runx1 in adult ECs/EPCs prevents SuHx-PH development in mice. (**A**) In 10- to 13-week-old mice, the loss of Runx1 in lung endothelial cells (ECs) upon Tam induction was verified per qRT–PCR. No changes in Runx1 gene expression in BM-derived CD14 + cells were found, demonstrating the endothelial specificity of Runx1 deletion in these mice. (**B**,**C**) Under Nx conditions, Cdh5-CreERT2; Runx1(wt/wt) mice, Cdh5-CreERT2; Runx1(fl/fl) mice treated with corn oil, and Cdh5-CreERT2; Runx1(fl/fl) mice treated with Tam all exhibited normal RVSP (**B**) and RV/LV + S ratio (**C**) Under SuHx conditions, Cdh5-CreERT2; Runx1(wt/wt) mice and Cdh5-CreERT2; Runx1(fl/fl) mice treated with corn oil exhibited significantly elevated RVSP (**B**) and RV/LV + S ratio (**C**) When the Cdh5-CreERT2; Runx1(fl/fl) mice were treated with Tam and placed under SuHx conditions, they exhibited normal RVSP (**B**) and RV/LV + S ratio (**C**) $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, n.s.: not significant, unpaired two-tailed Student's *t*-test (**A**) and ordinary one-way ANOVA with multiple comparisons (**B**,**C**), *n* = number of animals in each experimental group. Reproduced from Jeong et al., 2022.

EHT, as SOX17 has been shown to directly repress RUNX1 expression and induce human embryonic stem cells towards a hemogenic fate rather than an endothelial one (33). The ability of the Sox17/Runx1 axis to drive EPCs toward an endothelial or myeloid fate, may be one mechanism to explain how disrupted Sox17/Runx1 signaling results in pulmonary vascular remodeling.

The discovery of EPCs in peripheral blood during the late 1990s introduced the idea that vasculogenesis and angiogenesis did not occur only during fetal development but could arise in adulthood (67). The intense research into the origin and function of these cells opened a new era in theoretical regeneration of the cardiovascular (CV) system. However, confusion regarding the origin and identity of these cells quickly arose due to significant technological differences in how these cells were isolated, characterized and sustained in culture. Even today, there is no specific marker that clearly distinguishes a unique population of cells that can consistently be identified as EPCs. Nonetheless, their role in cardiovascular disease continues to be an active area of research and their role in the pathogenesis of PAH continues to be explored.

In 1997, Asahara et al. (68) characterized a population of progenitor cells expressing the cell surface markers CD34 and VEGFR2 which mobilized from the BM to the endothelium after ischemic injury and were capable of differentiating into endothelial cells that appeared to participate in vessel repair. These cells were termed EPCs. Traditionally, it was thought that injured endothelial cells in the pulmonary arteries were replaced by resident progenitor cells. However, since Asahara et al.'s findings, mounting evidence has indicated that circulating BM-derived EPCs have a prominent and pathologic role in PAH (69–71).

At the same time, our understanding of EPCs has changed significantly. Importantly, identification of EPCs in peripheral circulation via monoclonal antibodies and flow cytometry has been challenging as labeling of EPCs via CD34 positivity with endothelial antigens (i.e., VEGFR2, CD146, CD144, CD31, Tie-1, Tie-2) has shown significant overlap with markers on primitive HSCs. Furthermore, once isolated, these cells behave like hematopoietic colony forming cells and do not form endothelial cells (72). An alternative approach to isolating EPCs is based on isolation of circulating peripheral blood mononuclear cells (PBMCs) and growing them in pro-angiogenic cell culture media. Interestingly, EPCs obtained through this method comprise two relatively discrete populations. First described by Kalka et al. as "early" and "late" EPCs based on the time of the appearance of endothelial cells *in vitro* (73). Early EPCs are characterized by detection of endothelial cells after 7–10 days of growth and display limited endothelial proliferative capacity but have strong pro-angiogenic paracrine activity. The majority of these early EPCs display markers similar to those of monocyte/macrophage and lymphocyte lineage. Conversely, late EPCs develop endothelial cell characteristics after three weeks of growth and display high endothelial proliferative potential and express endothelial cell lineage markers.

The means by which EPCs influence PAH remains unclear. Current data support 4 main mechanisms: (1) localization to areas of vascular injury and restoration of vascular integrity; (2) paracrine effects—potentially mediated by secretions of proangiogenic growth factors; (3) support/restoration of other cells, and (4) differentiation into proinflammatory cells with subsequent immunomodulatory effects.

Early studies on the vascular reparative effects of EPCS were conducted in the systemic circulation. These studies demonstrated that BM derived EPCs were recruited to the site of vascular injury following wire disruption of the femoral artery in mice (74). In 2005, Hayashida et al. used green fluorescent protein (GFP)-labeled BM cells to demonstrate a similar recruitment of BM-derived progenitor cells to the pulmonary

circulation during the development of PH. They showed a striking increase in BM-derived cells in the pulmonary arteries of mice after induction of hypoxic PH. GFP positive cells were recruited to the distal pulmonary circulation and appeared to be involved in pulmonary vascular remodeling (75). Similarly, in calves with hypoxic pulmonary hypertension, a marked increase in BM derived c-kit + stem cells was seen in peripheral blood with a corresponding decrease in c-kit + cells in BM (76). BM-derived EPCs have also been identified in the pulmonary vasculature in other pre-clinical models of PAH including MCT and Sugen/hypoxia (76, 77), as well as in patients with PAH (78). Recruitment of BM-derived progenitor cells to areas of vascular injury is driven in part by chemokine gradients. In particular, vascular injury is associated with increased expression of CXCL12, the ligand for the CXCR4 receptor expressed by HSC (79). Pulmonary vascular expression of both CXCL12 and CXCR4 has been demonstrated in lungs from rats with Sugen/hypoxia PH and in patients with PAH (80) and inhibition of the expression or binding of CXCR4/CXCL12 reverses MCT-induced PH in rats (81). Interestingly, EPCs from patients with PAH have increased proliferative capacity compared to control patients (82). Furthermore, the number of EPCs in PAH patients with BMPR2 mutations are increased and when grown *in vitro* demonstrate a hyperproliferative phenotype and an inability to form vessel networks (83).

A pathogenic role of BM-derived EPC has been suggested by a number of studies in which these cells have been shown to induce PH when transplanted into healthy animals. For example, our lab demonstrated that whole BM obtained from mice with MCT-PH induces PH when transplanted into healthy mice (84, 85). Transplanting BM from control mice into healthy mice had no effect. Similarly, BM from pulmonary hypertensive BMPR2 heterozygote mice induce PH in a dose-dependent manner and increase the number of donor-derived inflammatory cells in the pulmonary vasculature when transplanted into healthy control mice (81). In contrast, transplantation of BM from wild-type mice into BMPR2 heterozygotes attenuates the severity of PH. Whether the latter result is due to a healing effect of the BM cells from wild-type mice or the destruction of pathogenic EPC in the PH mice is unclear, but these transplantation experiments require non-lethal radiation prior to transplant which destroys most of the native BM cells. Our group has also demonstrated that non-lethal irradiation alone (i.e without subsequent BM transplant) can partially reverse SuHx-PH (86). Whether or not it is the EPC fraction that was responsible for BM inducing PH in the above experiments is uncertain. However, we demonstrated that depletion of EPC, defined as ckit+/sca1+/vegfr2+, from the BM of MCT-PH mice prior to transplantation prevented induction of PH in the healthy mice (85). Finally, EPCs defined as CD133 + obtained from the BM of PAH patients cause pulmonary vascular endothelial injury, *in situ* thrombi, right ventricular hypertrophy and increased mortality when xenografted into immunodeficient mice, whereas EPCs obtained from patients without PAH do not. Interestingly, these CD133 + cells expressed increased myeloid-specific

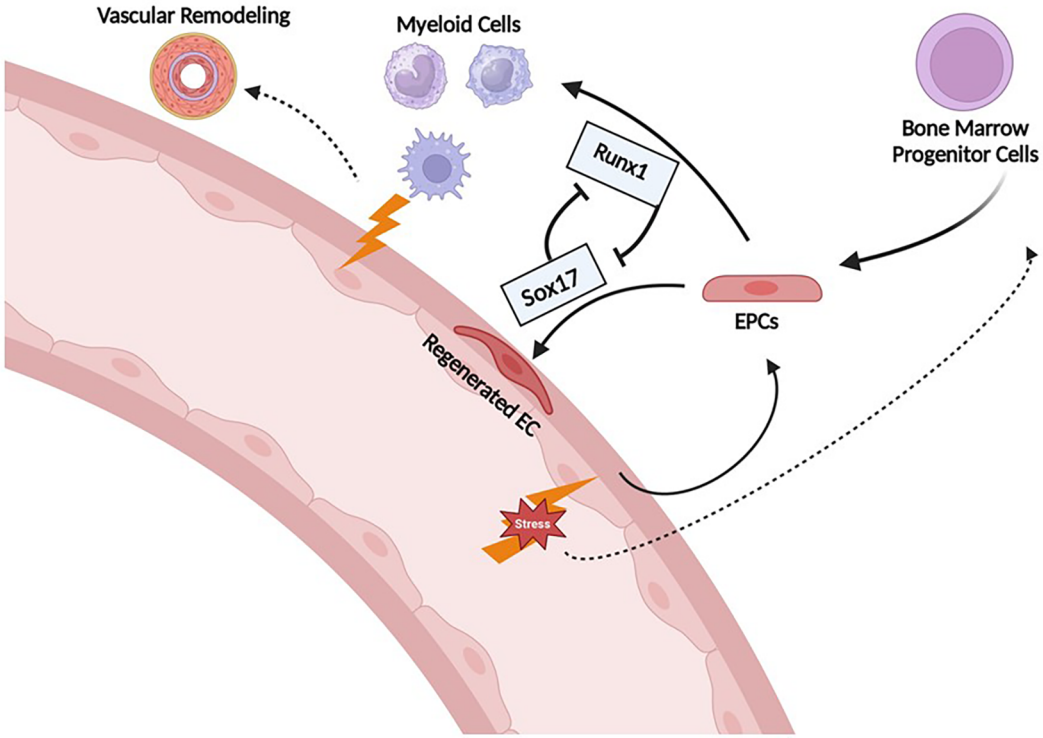

FIGURE 12
Proposed mechanism of Runx1 and Sox17 involvement in endothelial cell regeneration after injury. Sox17 is necessary for EC regeneration following injury. Runx1 skews differentiation towards HSC/myeloid lineage which may contribute to further inflammation/propagation of PAH. EPCs, endothelial progenitor cells; EC, endothelial cells.

transcription factors (GATA1, EKLF, Fli) compared to controls (85, 87). Collectively, these results demonstrate that EPCs obtained from animals with PH and patients with PAH are capable of inducing PH, and causing pulmonary vascular remodeling. However, the mechanisms responsible for driving EPCs from a reparative response toward pathologic remodeling is not clear. Previous studies have shown that SOX17 plays a key role in the induction and expansion of arterial endothelial precursors derived from $CD34^+$ progenitors in human umbilical cord blood or adult BM (88) and we have shown that Runx1 expression is higher in circulating EPCs obtained from PAH patients compared with controls. Together, these findings support the hypothesis that aberrant pulmonary vascular remodeling in PAH may be due in part to imbalances in SOX17 and RUNX1 expression in EPCs that leads to suppression of endothelial differentiation and instead drives them toward a hematopoietic fate (**Figure 12**).

Future directions

Evidence is accruing to support a role for SOX17 and RUNX1 in the pathogenesis of PAH. Further research is needed to elucidate the mechanisms by which these developmentally important transcription factors may modulate pulmonary vascular remodeling and the development or progression of PAH. Whether or not the increased risk of PAH associated with mutations in the *SOX17* gene is due to insufficient repression of its downstream target *RUNX1* is not known. RUNX1 expression in the lung or circulating progenitor cells has not been examined in patients with PAH associated with SOX17 mutations, although this is an ongoing area of research in or lab and by other investigators. In addition to RUNX1, numerous other targets of SOX17 including HGF/c-Met, E2F1, BMPR2, and estrogen metabolism have been shown to play significant roles in modulating pulmonary hypertensive responses, and perturbations in their expression may play important roles in PAH associated with impaired SOX17 expression. Furthermore, if insufficient suppression of RUNX1 by impaired SOX17 expression causes pulmonary hypertension, it is not clear whether impaired SOX17 and RUNX1 signaling fails to maintain pulmonary vascular endothelial cell homeostasis or drives differentiation of BM-derived progenitor cells toward a myeloid fate. However, there is strong and accruing evidence to support the pathogenic role of SOX17 and RUNX1 in PAH and the potential of targeting the SOX17 and RUNX1 axis as a novel therapeutic approach in the management of PAH. Future studies are needed to determine if enhanced expression of SOX17 or inhibition of its downstream targets such as RUNX1, HGF/c-Met, or E2F1 can reverse PAH associated with the rare or common variants of *SOX17* mutations or in patients with PAH that are not associated with impaired SOX17 expression. To this end, RUNX1 inhibitors have been developed for the treatment of hematologic malignancies and may be repurposed for the treatment of PAH and recent studies have suggested several drugs that have potential to enhance SOX17 expression despite mutations in enhancer signal 1 and 2 (42). Targeting transcription factors that are intimately involved in endothelial generation and repair such as SOX17 and RUNX1 has the potential to not only shed new light on the pathogenesis of PAH but to significantly change the approach to treating this devastating disease.

Author contributions

RS: Conceptualization, Data curation, Methodology, Supervision, Writing – original draft, Writing – review & editing. JK: Conceptualization, Investigation, Writing – review & editing. OL: Data curation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Inclusion of mobile phone usage guidelines in universal hypertension management protocol: an opinion

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hypertension, blood pressure, mobile phone, NCD (non-communicable disease), epidemiologic transition, diabetes, hypertension

Burden of hypertension

According to a global analysis published in *The Lancet* in 2019, the estimated global prevalence of hypertension among adults was approximately 1.13 billion, or 26.1% of the population aged 20 years and older (1). This prevalence has increased from 594 million in 1975, indicating a substantial rise over the years (1). Hypertension is a leading risk factor for cardiovascular diseases, including heart disease and stroke. The Global Burden of Disease study estimated that high blood pressure (BP) was responsible for approximately 10.8 million deaths worldwide in 2019, accounting for 19.4% of all deaths. Disability-adjusted life years (DALYs) represent the burden of disease, combining the years of life lost due to premature mortality and the years lived with disability. In 2019, hypertension was responsible for 182.6 million DALYs globally, indicating a significant impact on disability and overall health (2).

The burden of hypertension in India is a significant public health concern due to its high prevalence and association with cardiovascular diseases. According to a large-scale national study, the India State-Level Disease Burden Initiative, published in *The Lancet Global Health* in 2018, the prevalence of hypertension among adults in India was estimated to be approximately 29.8%. This study reported that approximately 199.5 million individuals in India were affected by hypertension (3). Hypertension-related mortality contributes to a substantial burden in India. According to the Global Burden of Disease study, in 2019, high blood pressure was responsible for approximately 1.6 million deaths in India, accounting for 14.1% of total deaths (4). Hypertension also has a significant impact on disability and overall health in India. The India State-Level Disease Burden Initiative estimated that hypertension accounted for 2.4 million DALYs in India in 2017, representing a considerable burden of disease (5). A study published in 2020 examined the risk factors associated with hypertension in India. It found that factors such as older age, urban residence, higher body mass index (BMI), and diabetes were significantly associated with hypertension (3). Another study also highlighted the low awareness and control rates of hypertension in India, emphasizing the need for improved screening and management (6).

Blood pressure is typically checked using a device called a sphygmomanometer. There are two main types of blood pressure measurements: manual (using an aneroid or mercury sphygmomanometer) and automated (using an electronic or digital device).

As per standard guidelines, the person having their blood pressure checked should be seated comfortably in a quiet environment; the guidelines also recommend refraining from talking as talking may include the use of mobile phones for a few minutes before the blood pressure measurement. The individual should avoid smoking, caffeine, and exercise for at least 30 min before the blood pressure measurement (7).

Problem statement

It is not uncommon for patients to use their mobile phones to surf the Internet while waiting in queues, including those at doctor's offices. Mobile devices provide easy access to various forms of entertainment, information, and communication, making them a convenient way to pass the time. The use of social media and mobile devices has become pervasive in today's society, and although they offer many benefits, they can also contribute to mental health disorders such as stress and anxiety in individuals. A study identified different profiles of social media addiction among college students and found a positive association between social media addiction and symptoms of anxiety (8).

Another study revealed that problematic social media use was associated with poor sleep quality, attention-deficit hyperactivity disorder (ADHD), and lower self-esteem among adolescents (9). A study on a similar topic analyzed online information related to anxiety and depression and found that the quality of information on social media platforms was variable, and inaccurate or misleading content could contribute to increased stress and anxiety (10). Another study explored the relationship between digital technology use and wellbeing in adolescents and found that the association between digital technology use and mental health outcomes, including stress and anxiety, was small but statistically significant (11). To our knowledge, the association between increase in mobile phone usage and increase in BP is very scarce, and the available evidence is indirect, which is commonly interacted with mental disorders such as stress and anxiety.

A study involved participants with a mean age of 54 years, consisting of 62% women and 88% mobile phone users. Over a median follow-up period of 12 years, 13,984 (7%) participants developed hypertension (12). Mobile phone users had a 7% increased risk of developing hypertension compared to non-users. Among mobile phone users, those who spent 30 min or more per week on phone calls had a 12% higher likelihood of developing high blood pressure compared to participants who spent less than 30 min on calls. These associations were observed in both women and men. Further analysis revealed more detailed findings regarding the relationship between weekly usage time and the risk of high blood pressure. Compared to participants who spent less than 5 min per week on phone calls, those who spent 30–59 min, 1–3 h, 4–6 h, and more than 6 h had an 8%, 13%, 16%, and 25% higher risk of developing hypertension, respectively (9). However, among mobile phone users, the number of years of use and the use of hands-free devices or speakerphones did not significantly affect the likelihood of developing hypertension. The researchers also investigated the impact of usage time (less than 30 vs. 30 min or more) on new-onset hypertension in relation to participants' genetic risk levels for developing hypertension. Genetic risk was determined using data from the UK Biobank. The analysis demonstrated that individuals with a high genetic risk who spent at least 30 min per week on mobile phone calls had a 33% higher likelihood of developing hypertension compared to those with a low genetic risk who spent less than 30 min per week on the phone (13).

In this study, a significant decreasing trend was found between systolic blood pressure (SBP), diastolic blood pressure (DBP), and heart rate and higher mobile phone usage in women. Based on a regression analysis, SBP, DBP, and duration of mobile phone use were associated negatively in those who used their phones for at least 8 h, which is in contrast with our previous study. The type of use and content may determine the impact on blood pressure. For example, if someone is scrolling funny videos, their BP may decrease; on the other hand, if a person is constantly looking at the stock market, their BP may be on the higher side (14).

In our opinion, using a mobile phone before a blood pressure checkup can potentially influence the blood pressure reading. This phenomenon may be called a "confounding effect in hypertension" or "mobile phone error in hypertension measurement," where a person's blood pressure may be higher when measured in a clinical setting due to anxiety or stress associated with the medical environment or mobile phone-induced stress.

Few studies have explored the impact of mobile phone use on blood pressure measurements. For example, the "European Society of Hypertension guidelines for blood pressure monitoring at home: a summary report of the Second International Consensus Conference on Home Blood Pressure Monitoring" mention that activities such as smoking, exercise, and using a mobile phone should be avoided for at least 30 min before blood pressure measurements (15). Still, this guideline is yet to be widely accepted and implemented. Another study investigated the acute effects of exposure to mobile phone electromagnetic fields on blood pressure and found that it had a small but significant effect on increasing blood pressure measurements (16).

The exact mechanisms underlying how using a mobile phone before a blood pressure checkup may influence blood pressure readings are not yet fully understood. Several factors associated with mobile phone use can impact blood pressure measurements. First, engaging in activities such as checking work emails or receiving distressing news on a mobile phone can induce psychological stress and anxiety, both recognized as temporary elevators of blood pressure levels. In addition, the act of using a mobile phone itself can lead to distraction, diverting attention from relaxation—an essential state for accurate blood pressure readings. Poor posture during mobile phone use, characterized by bending the neck and shoulders forward, can induce

temporary changes in blood flow dynamics and blood pressure regulation, potentially affecting measurements. Moreover, some studies suggest that exposure to electromagnetic fields emitted by mobile phones may directly influence the autonomic nervous system, responsible for blood pressure regulation. However, the evidence supporting this specific physiological arousal mechanism is currently limited and inconclusive.

It is important to note that the impact of mobile phone use on blood pressure measurements may vary between individuals and depend on factors such as their baseline blood pressure, level of stress, and the duration and intensity of mobile phone use.

Recommendations

Future research in the area of mobile phone use and its influence on blood pressure measurements could explore the following avenues: conducting long-term studies that follow individuals over an extended period could provide valuable insights into the long-term effects of mobile phone use on blood pressure. This would help determine whether any observed influences are transient or persistent. In addition, using experimental designs, researchers can manipulate variables such as mobile phone use duration, content type, and posture to better understand their impact on blood pressure. Controlled experiments can provide more precise insights into the causal relationships between mobile phone use and blood pressure changes. We can also investigate whether studies on the potential effects of mobile phone electromagnetic field exposure on blood pressure regulation. Research could involve assessing physiological responses to electromagnetic field exposure and exploring potential mechanisms through which it might influence blood pressure. Studying the effectiveness of smartphone applications or interventions specifically designed to mitigate the potential negative effects of mobile phone use on blood pressure could be another interesting area of research. This could involve developing and testing apps that promote relaxation, stress reduction, and proper posture during mobile phone use. Investigating individual factors that may modulate the impact of mobile phone use on blood pressure could be valuable. Factors such as age, baseline blood pressure, stress levels, and psychological factors could be considered to better understand why some individuals may be more susceptible to blood pressure changes due to mobile phone use. Operational research conducted in healthcare clinics or workplaces could provide insights into the practical implications of mobile phone use on blood pressure measurements. Understanding the influence of environmental factors and contextual variables on the relationship between mobile phone use and blood pressure would be important. By exploring these research avenues, we can enhance our understanding of the complex relationship between mobile phone use and blood pressure and develop evidence-based guidelines for restricting mobile phone usage before measuring accurate BP cases in various settings.

Author contributions

SB: Writing – original draft, Writing – review & editing. AG: Writing – original draft, Writing – review & editing. SG: Writing – original draft, Writing – review & editing. SS: Writing – original draft, Writing – review & editing. AB: Writing – original draft, Writing – review & editing. VL: Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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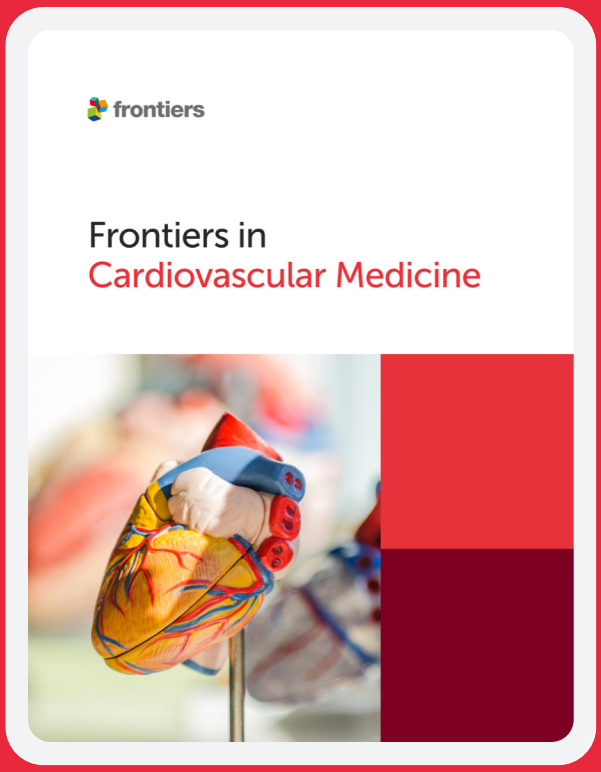

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