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MODELS AND MOLECULAR MECHANISMS OF WHO GROUP 2 – 4 PULMONARY HYPERTENSION

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INTRODUCTION

Pulmonary hypertension (PH) is defined as a resting mean pulmonary artery pressure (mPAP) ≥25mmHg, which is roughly double the normal mPAP.[1] The breadth and inclusivity of this definition is problematic for several reasons. First, PH is a heterogeneous syndrome with variable etiologies, therapies and prognosis. Thus, a diagnosis of PH does not guide therapy; although it does portend poor prognosis. Second, mPAP is rarely measured directly, because this requires right heart catheterization. In clinical practice, right ventricular systolic pressure (RVSP) is usually estimated from noninvasive Doppler measurement of the peak velocity of a tricuspid regurgitation jet, using the Bernoulli equation. This estimate is fraught with difficulties including inaccuracy and an intrinsic inability to distinguish whether PH is due to pulmonary vascular disease versus left ventricular (LV) diastolic dysfunction. Although pulmonary vascular disease can be identified and mPAP calculated from the PA Doppler signal (by measuring pulmonary artery acceleration time, PAAT)[2] (Figure 1) this simple measurement is often omitted.

To facilitate clinical categorization and management of PH patients, the 1998 World Symposia on Pulmonary Hypertension divided PH into five groups. The latest update in 2013 modified the taxonomy; however the five major groups are unchanged.[1] Group 1 PH or pulmonary arterial hypertension (PAH) is an orphan disease, with an estimated prevalence of 10–52 cases/million adults.[3] Group 1 PH, defined as a mPAP≥25 mmHg with pulmonary capillary wedge pressure (PCWP) ≤15mmHg and pulmonary vascular resistance (PVR) >3 Wood units,[4] has received the most attention. Preclinically there are robust animal models of Group 1 disease and a related basic science knowledge base. Clinically these patients are

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DISCLOSURES

None.

managed in specialized clinics and are treated with 4 classes of approved PH-targeted therapies.[3] There has been a remarkable increase in our knowledge of the molecular mechanisms underlying Group 1 PH over the past decade.[5–7] In contrast, there are far fewer preclinical models and a less detailed understanding of the molecular mechanisms of the other PH groups. Most WHO Group 2–4 patients are not managed in specialized, Group-focused PH clinics, which is also an impediment to progress.

Group 2 is the most prevalent form of PH and is usually due to valvular or myocardial left heart disease.[3] An echocardiography-based epidemiologic study from Australia estimates the prevalence of Group 2 PH at 250 cases/100,000 population[8] and Group 3 PH at 37 cases/100,000 population.[8] Group 4 PH, also known as chronic thromboembolic pulmonary hypertension (CTEPH), has an incidence similar to Group 1 disease.[9] German and French PH registries suggest the annual incidence of CTEPH is 4 and 6 cases/million adults/year, respectively.[10] Since Group 5 PH is a heterogeneous collection of PH syndromes, the search for common intra-group mechanisms is most applicable to WHO Groups 1–4.

The WHO PH classification is based on clinical phenotype (hemodynamics, comorbidities and histology); rather than pathological cellular and molecular mechanisms, such as endothelial dysfunction, inflammation/immunity, and mitochondrial dysfunction.[11–14] Some molecular mechanisms, such as endothelial dysfunction, are shared across PH groups. For instance, in Group 2 PH caused by mitral stenosis (MS), the initiating pathophysiological stimulus is retrograde transmission of elevated left atrial (LA) pressure and the resulting pulmonary venous hypertension.[15] PH occurs in 78% of MS patients.[15] PH in MS may be proportional or disproportional. In a proportional PH response, the elevation of mPAP is at the minimum level required for antegrade circulation. In such cases, right heart catheterization (RHC) reveals a minimal gap between PA diastolic pressure (PAd) and PCWP. However, in MS patients with disproportionate PH there is a large PAd-PCWP gap and their hemodynamics have some commonality with Group 1 patients, notably elevated PVR. This reflects adverse pulmonary vascular remodelling and/or pulmonary vasoconstriction. Mitral valve replacement may not immediately cure PH in disproportionate responders because it does not address their residual pulmonary vascular disease. Indeed, 42–64% of MS patients have persistent PH after successful mitral valve surgery (Figure 2).[16]

Endothelin-1 (ET-1), a potent endothelial-derived vasoconstrictor that is elevated in Group 1 PH, is also elevated three-fold in patients with severe MS.[17] Indeed, Paul Wood showed the important role of pulmonary vasoconstriction in a subset of MS patients. He showed that PVR could be halved acutely with infusion of acetylcholine (Figure 3)[18], an endothelium-dependent vasodilator which causes synthesis of nitric oxide (NO)[19] and endothelium-derived hyperpolarizing factor[20]. In addition to vasoconstriction, some MS patients have disproportionate PH due to adverse pulmonary vascular remodelling, with thickening of the intima and media of small PAs (Figure 4)[21]. Thus, individual variations in endothelial function and vascular remodelling likely contribute to the susceptibility of patients with LHD to Group 2 PH.[17] The fact that these “disproportionate responders” have some physiologic similarities to Group 1 patients suggests that it may be profitable to examine

molecular pathways that are disordered in Group 1 PH for potential relevance to other PH Groups.

This review explores our current understanding of the preclinical models, underlying molecular pathways, and potential new therapeutic targets for Group 2, 3, and 4 PH. We will begin with a review of the preclinical models for each PH Group.

PRECLINICAL RODENT PH MODELS

Rodent models of PH are most frequently used due to their low cost, convenience, rapidity, and the greater societal acceptance of the ethics of using rodents versus large animals in research. Thus, most of our understanding of the molecular mechanisms responsible for PH is derived from rodent PH models.

Models of Group 1 PH

Although this review is focused on Group 2–4 PH, we briefly discuss the most widely used Group 1 rodent PH models, because Group 2–4 PH models share disorders of some of the same molecular pathways.

Monocrotaline-induced PAH—Monocrotaline (MCT) is derived from the plant *Crotalaria spectabilis*. CYP3A4-dependent hepatic metabolism creates a reactive MCT pyrrole, which causes endothelial damage. A single dose of MCT (60–100 mg/kg, subcutaneously) reliably causes PH in rats within 3–4 weeks and leads to death from RV failure within 6–8 weeks.[22] The MCT rodent model recapitulates many features of human PAH, such as early endothelial injury, generation of reactive oxygen species, adverse pulmonary vascular remodeling, increased mitochondria fission, and mitochondrial metabolic abnormalities in the pulmonary vasculature and RV.[22] However, critics of the model point out that MCT's systemic toxicities (hepatotoxicity, cardiac toxicity, immunotoxicity, and DNA crosslinking)[23] and the absence of plexiform lesions on lung histology, make it is an imperfect approximation of human PAH.[22] However, the MCT model does display all other histologic features of Group 1 PH, such as medial hypertrophy and intimal hyperplasia, and the animals die from RV failure, as do Group 1 patients.

Sugen 5416-hypoxia (SuHx) model—Sugen 5416 (SU) is an inhibitor of vascular endothelial growth factor (VEGF) receptor 2. A PAH model was initially created by subcutaneous injection of adult male Sprague-Dawley rats with SU5416 (200 mg/kg, 3-times per week for 3-weeks). At Denver, Colorado's altitude (~1600m) these rats developed PH (mPAP 32±2 mmHg).[24] The model has evolved to combine a single, lower dose, subcutaneous injection of SU5416 (20 mg/kg) with 3-weeks of hypoxia (10% oxygen). This reliably generates severe PAH (RVSP 96±11mmHg) that persist upon return to normoxia.[25] This model develops plexiform arteriopathy that resembles the histology in human PAH; however, it does not lead to RV failure as reliably as the MCT model.

Furthermore, gender and stain differences are important factors to consider when creating the SuHx model. Dean *et al.* showed that metformin reverses development of pulmonary hypertension via aromatase inhibition. However, this therapeutic response only occurs in

female SuHx rats, suggesting the importance of sex difference in the pathophysiology of PAH.[26] Jiang *et al.* showed marked strain-specific differences in the SU rat model.[27] Surprisingly, ~75% Sprague-Dawley (SD) rats from Charles River Laboratories (Montreal, QC, Canada) exhibit a severe PAH phenotype with SU5416 (20 mg/kg) subcutaneous (SC) injection alone, whereas the other 25% showed no response.[27] In comparison, SD rats from Harlan Laboratories (Indianapolis, Ind, USA) do not develop PAH phenotype with SU5416 (20 mg/kg, SC) alone.[27]

Models of Group 2 PH

Supracoronary aortic banding (SAB) and transverse aortic constriction (TAC) models—The SAB rat or TAC mice models are used to study Group 2 PH. The SAB model is created by banding the ascending aorta of a juvenile rat, using either a suture or a titanium clip, placed approximately 1 cm distal to the aortic valve.[28–30] Hemodynamic assessment is done 4–9 weeks after SAB[28–30]. Our preliminary data show that 8 weeks after banding and administration of Nω-Nitro-L-arginine methyl ester (L-NAME) 1.85 mM in drinking water, rats develop marked increase in pulmonary arteriole wall thickness (Figure 5 A & B) and decreased pulmonary artery acceleration time (PAAT) (Figure 5 C & D). (see Online Supplement for the materials and methods section.) In the murine TAC model a ligature is placed around the aortic arch between the brachiocephalic trunk and left common carotid artery[11, 31] and hemodynamic assessment is performed 1–4 weeks later[31, 32]. In animals that are not in heart failure, tighter aortic banding yields higher TBPG and LVSP (Figure 6); however, if the aortic banding is too severe animals die acutely of heart failure. Moreover, if the model progresses to the point of heart failure and cardiac output declines TBPG will fall in parallel. Although most studies using the SAB model do not report the TBPG[28–30], our preliminary data indicates that rise of TBPG is associated with increased RVSP. For example, a TBPG of approximately 100 mmHg generates a RVSP of ~30 mmHg 7–8 weeks post banding with an operative mortality of 5–10%. Chen et al. used a 26-gauge needle for banding and noted that this produced a mixed group of TAC mice, some with moderate and others with severe heart failure.[33] Subsequently, the same group used a smaller needle (27-gauge) to create more severe TAC and noted that these mice consistently developed severe heart failure.[34] This more severe TAC also caused greater pulmonary inflammatory changes and leukocyte infiltration.[33, 34] These data suggest that a tighter band does indeed create more severe left heart failure and a more severe PH phenotype.

The SAB and TAC rodent models have clinical relevance, mimicking aortic stenosis and aortic coarctation; however, they require technical expertise, develop only modest PH and do so relatively slowly. The modest PH that develops in SAB and TAC model is similar in magnitude to that seen in patients with isolated LV pressure overload due to diseases such as hypertension. A second, unidentified abnormality, likely some form of endothelial dysfunction, appears to be needed for the generation of severe PH.

Left atrial stenosis (LAS) model—Fujimoto *et al.* recently generated a novel Group 2 PH rat model by partially banding the left atrium with a metal clip in male Sprague-Dawley rat (5 weeks old) to increase the left atrial and pulmonary venous pressure.[35] The LAS model, relevant to mitral stenosis, displayed an increased RV/LV pressure ratio (0.52 vs.

sham 0.22), increased RV/body weight ratio (0.54 vs. sham 0.39 mg/g), and marked medial hypertrophy of the pulmonary veins (1.6 times that of sham rats).[35] Immunoblots revealed significantly elevated expression of transforming growth factor-β (TNF-β) and ET-1 levels in the lung of the LAS vs. sham rats.[35]

Models of Group 3 PH

Chronic hypoxia model—Exposing rodents to either normobaric or hypobaric hypoxia generates a Group 3 PH model. Rodents are acclimated to decreasing inspired oxygen concentrations over 3–5 days and then maintained at 10% oxygen for 3–4 weeks. They develop pulmonary artery medial hypertrophy, elevated mPAP, PVR, and right ventricular hypertrophy (RVH).[22] However, the chronic hypoxia model does not fully recapitulate Group 3 PH in humans, which usually results from chronic obstructive pulmonary disease (COPD).[36, 37] Specifically, chronic hypoxia does not cause abnormal lung parenchyma or obstructive PA intimal lesions and induces only mild PH.[36, 37] Both the PH and histologic changes reverse when the rodents are returned to normoxia.[22] Many preclinical therapies are effective in preventing and regressing PH in the chronic hypoxic rat model but most of these have failed to benefit Group 3 patients.

Bleomycin-induced PH—Bleomycin is a chemotherapeutic agent that creates DNA strand breaks. A rodent model of Group 3 PH due to pulmonary fibrosis is produced by endotracheal instillation of bleomycin (2–4 unit/kg) into rats[38] or mice,[39] respectively. Bleomycin causes fibrogenic cytokine release, resulting in pulmonary fibrosis, increased PA medial thickness, RVH, and PH.[38, 39] In a model using adult male C57/BL6 mice, survival is 74% 2-weeks post bleomycin.[39] Male Wistar rats instilled with bleomycin developed significant pulmonary fibrosis, accompanied by increased lung expression of hydroxproline, collagen, and connective tissue growth factor.[38]

Model of Group 4 PH

A Group 4 PH model has recently been created by tail vein injection of male Sprague-Dawley rats with 85μm polystyrene microspheres plus subcutaneous SU5416 (20 mg/kg).[40] All rats treated with microspheres+SU5416 developed sustained PH (RVSP of 62±13 mmHg and 53±14 mmHg at 3 and 6 weeks, respectively). They also developed significant RV dysfunction, RVH and RV fibrosis and had decreased exercise capacity. Lung sections of the microspheres+SU5416 rats demonstrated marked medial hypertrophy in small arterioles and intimal proliferation with obliteration of the lumen; however, as in humans with Group 4 disease there were no plexiform lesions. Computed tomography pulmonary vascular imaging showed lack of neovascularization and Ki-67 immunohistochemistry showed decreased cellular proliferation in the microspheres+SU5416 group vs. either microspheres or sham group. The microspheres+SU5416 rats also had evidence of increased inflammation and platelet activation.[40] In contrast, with microspheres alone only 10% of rats developed PH. Hence, a combined insult (pulmonary embolism plus inhibition of endothelial function) is required to routinely induce Group 4 PH.[40]

WHO GROUP 2–4 PH in PATIENTS

We will next discuss the definitions and disordered molecular pathways associated with Group 2–4 PH in patients.

WHO Group 2 (Table 1)

PH-LHD is defined as a mPAP ≥25 mmHg with elevated left heart filling pressures (PCWP >15 mmHg or LVEDP >18 mmHg).[41] PH-LHD is sub-classified as *isolated post-capillary PH* (IpcPH) or *combined post- and pre-capillary PH* (CpcPH). In IpcPH the diastolic pulmonary vascular gradient (DPG), defined as $PAP_{diastolic}$–PCWP, is <7mmHg and the PVR≤3WU; whereas in CpcPH the DPG is ≥7mmHg and the PVR>3WU.[42] The major causes of elevated left heart filling pressures and heart failure that give rise to PH are: 1) mitral and aortic valvular disease and 2) LV myocardial diseases. Heart failure is further subdivided into heart failure with reduced ejection fraction (HFrEF) or preserved ejection fraction (HFpEF).[41] (Figure 7) Most patients with HFrEF or HFpEF develop PH. A community-based study showed that 83% of HFpEF patients (n=244; mean age 76±13 years; 45% male) have PH.[43] The incidence of PH in HFrEF patients is similar (72–79%).[44, 45]

The treatment of Group 2 PH focuses on symptomatic care (diuretics, sodium restriction) and correction of the underlying LHD. For HFpEF this often involves control of systemic hypertension to a target pressure of 120/80 mmHg. For HFrEF this involves the use of angiotensin converting enzyme inhibitors or angiotensin receptor antagonists as well as β-adrenergic receptor antagonists. For patients with valvular LHD surgical correction of the valve abnormality is recommended. However, Group 2 PH cannot always be completely reversed by treating the LHD[16], as discussed previously (Figure 2). We next review molecular mechanisms that may be responsible for the pathological remodeling and vasoconstriction of the pulmonary vasculature that underlies disproportionate PH in some Group 2 patients.

Disordered Pathways in Group 2 PH

Endothelial Dysfunction—PH-LHD is associated with endothelial dysfunction, characterized by an increase in levels of vasoconstrictors, such as endothelin-1 (ET-1) and a decrease in vasodilators, such as nitric oxide (NO).[46, 47] (Figure 8)

Endothelin-1 (ET-1): ET-1 is a potent vasoconstrictor identified by Yanagisawa in 1988.[48] ET-1 levels are markedly elevated in PH, including Group 2 patients.[49] Exposing endothelial cells to increased pressure increases ET-1 production by a phospholipase C (PLC) and protein kinase C (PKC)-dependent mechanism.[50] Pressure-induced elevations of ET-1 likely contribute to Group 2 PH.

ET-1 binds to ET_A and ET_B receptors.[51] ET_A receptors are expressed mainly on vascular smooth muscle cells (VSMCs) and cardiac myocytes. Binding of ET-1 to ET_A receptors activates PLC, which generates diacylglycerol (DAG) and inositol triphosphate (IP_3).[52] These secondary messengers trigger the release of intracellular calcium, which leads to activation of myosin light chain kinase, phosphorylation of myosin light chain, and

vasoconstriction.[52] ET-1 also activates the RhoA/Rho kinase pathway, which leads to calcium sensitization and sustained vasoconstriction.[53] ET_B receptors are expressed on vascular endothelial cells, and upon binding of ET-1, promote production of NO and prostacyclin, resulting in vasodilation.[54] The ratio of ET_A to ET_B receptors in humans is approximately 9:1 and the net effect of ET-1 on pulmonary arteries is vasoconstriction.[55]

In heart failure, there is not only elevation of ET-1 levels but also increased vasoconstrictor responsiveness to ET-1.[56] In a canine HF model induced by rapid ventricular pacing, Tadano *et al.* demonstrated a functional shift of ET receptor subtypes favoring vasoconstriction.[57] While there is biological plausibility that ETRAs (endothelin receptor antagonists), such as bosentan or macitentan, might benefit PH-LHD patients many clinical trials indicate a lack of benefit.[58–61] In examining putative PH-targeted therapies one must not only consider their pulmonary vascular interactome but also their effect on the RV, which is a key determinant of prognosis in PH. One potential mechanism for the lack of benefit from ETRA in Group 2 PH is the preclinical observation that some ETRAs are negative RV inotropea.[62]

NO-soluble guanylate cyclase (sGC)-cyclic guanosine monophosphate (cGMP) pathway: NO is an endothelium-derived vasodilator produced from L-arginine by the endothelial NO-synthase (eNOS).[63] NO is a nitrogen based radical and has a very short half-life of only 0.05–1 second.[64] NO is synthesized in the endothelium and diffuses to subjacent SMC.[65] The effects of this gaseous vasodilator are largely exerted by activation of its molecular receptor, sGC and subsequent generation of cGMP.[19] NO production can be triggered by various endogenous factors, such as acetylcholine (Ach) and bradykinin, or mechanical stimuli, such as shear stress and stretch.[66–68] sGC stimulators, such as riociguat, compensate for deficiency in biologically active NO and for sGC dysfunction, resulting from sGC oxidation or loss of sGC's heme moiety.[69] Riociguat is approved for treatment of Group 1 and Group 4 PH.[70] However, in the LEPHT trial, which studied 201 Group 2 patients, riociguat (2mg three-times daily for 16-weeks) failed to reduce mPAP, the study's primary endpoint.[71] Riociguat did improve cardiac index and reduced pulmonary and systemic vascular resistance.[71]

Both decreased NO production and reduced vasodilator responsiveness to NO occur in PH-LHD.[72] SAB rats have impaired pulmonary endothelium-dependent vasodilation and reduced lung capillary endothelial NO synthesis.[73] The impaired NO synthesis reflects a decrease in intracellular calcium oscillations in endothelial cells, rather than down regulation of eNOS.[73] Calcium oscillations were attenuated in SAB rats in response to mechanical stress, ACh, histamine, and thapsigargin, an inhibitor of sarcoplasmic reticulum Ca^{2+} ATPase.[73] The Ca^{2+} ionophore, A-23187 restored endothelial calcium oscillations and NO production.[73] Thus, the underlying cause of endothelial dysfunction and decreased NO production in this preclinical Group 2 PH model is dysfunction of endothelial calcium signaling pathways. The cause of impaired calcium signaling in Group 2 PH and its relevance to human PH requires further study.

The endothelium has many functions beyond NO synthesis. Simply eliminating NO, by exposing rats to N_ω-Nitro-L-arginine methyl ester (L-NAME ,1.85 mM in drinking water for 3-weeks) failed to cause Group 2 PH, despite induction of severe systemic hypertension.[74]

Phosphodiesterase 5 (PDE5) Dysregulation: PDE5 catalyzes the hydrolysis of cGMP. Although PDE5 inhibitors, such as sildenafil, are approved therapy for Group 1 PH, off-label use of sildenafil in Group 2 PH patients shows some potential clinical and hemodynamic benefits.[75, 76]

In the SAB rat model, sildenafil (60 mg/kg daily for 8-weeks) decreased endothelial dysfunction (Figure 9A), restored NO production (Figure 9B), and restored pulmonary arteriole vasorelaxation (Figure 9C).[29] Interestingly, the PDE5 expression in SAB rats lung homogenate was decreased by ~50%.[29] This differs from Group 1 PH rat models in which lung PDE5 expression is increased.[77, 78] Nonetheless, plasma cGMP levels were reduced in SAB rats and sildenafil significantly increased cGMP levels.[29]

Clinical trials of sildenafil in Group 2 PH are promising. A review by Hansdottir *et al.* summarized three small trials showing patients with PH secondary to HFrEF and HFpEF treated with sildenafil (25 to 75 mg orally 2 to 3 times daily for 12 weeks to 1 year) had reduced hospitalizations for HF, improved exercise capacity, enhanced quality of life, reduced mPAP, and decreased breathlessness score versus control subjects.[79] Two multicenter clinical trials testing PDE5 inhibitors in Group 2 PH are currently underway (PITCH-HF trial for Tadalafil; NCT01910389 and the Sil-HF trial[80] for sildenafil; NCT01616381).

Mitochondrial dysfunction—Mitochondria are not only generators of ATP but also regulate processes such as cell proliferation and apoptosis.[81] Mitochondria have noncanonical function that in aggregate are referred to as mitochondrial dynamics.[81] These properties include division (fission), union (fusion), movement (translocation), biogenesis and quality control (mitophagy). Using confocal imaging, and mitochondrial-targeted potentiometric dyes and fluorescent probes, one can visualize dynamic networks of mitochondria in live cells.[81] Acquired disorders of mitochondrial metabolism and dynamics contributes to the pathogenesis of complex diseases such as cardiovascular disease, neurodegenerative disease, and cancer.[82]

In Group 1 PH, there are acquired abnormalities of mitochondrial metabolism and dynamics[81] in both the pulmonary vasculature[83] and RV[84]. The mitochondrial phenotype in PAH includes suppression of glucose oxidation, due to increased expression and activation of pyruvate dehydrogenase kinase (PDK)[81, 84, 85], and mitochondrial fragmentation, due to increased mitochondrial fission and impaired mitochondrial fusion.[86]

The metabolism of glucose normally involves close coupling of cytosolic glycolysis with mitochondrial glucose oxidation (which relies on the pyruvate generated by glycolysis). However, PDK activation can disrupt this coupling by phosphorylating and inhibiting pyruvate dehydrogenase (PDH)[85]. This metabolic shift (called the Warburg phenomenon) is reversed by PDK inhibitors, such as dichloroacetate, with therapeutic benefit to the pulmonary vasculature and RV.[85, 87, 88] Uncoupled glycolysis promotes cell proliferation and suppresses mitochondria-mediated apoptosis.[85] This favors survival of abnormal cells in the pulmonary circulation and impairs the bioenergetic reserve of the RV. In models of Group 1 PH, such as the fawn hooded rat (a Group 1 PH model caused by epigenetic

silencing of superoxide dismutase 2, SOD2) and MCT rats, PDK inhibition regresses established pulmonary vascular disease.[87, 88] In the PAH RV, the metabolic shift to Warburg metabolism causes RV hypokinesis, and this too is improved by dichloroacetate.[84, 85]

Although there are parallels in the metabolic alterations in the PASMC and RV cardiomyocytes the initiating factors and their functional consequences differ. In PASMC, the mitochondrial morphologic changes reflect enhanced mitotic fission, which is initiated by the kinases that regulate both the activation of the fission mediator, dynamin related protein 1 (Drp1) and mitosis[83] (e.g. cyclin B-CDK1), as well as by elevation of cytosolic calcium[86]. The consequences of the mitochondrial metabolic phenotype in the vasculature is accelerated cell proliferation and migration and apoptosis-resistance.[81] In the RV myocytes changes in metabolism and dynamics are likely initiated by RV ischemia and result in myocardial hibernation and reduced RV function.[84]

Cell division requires a coordination of nuclear and mitochondria division.[81]. Mitochondrial fission that is coordinated with mitosis is called mitotic fission. The coordination reflects a shared reliance on mitosis promoting Drp1-activating kinases, including cyclin B1-CDK1.[81] Mitochondria in PAH PASMC are fragmented due to increased fission (due to activation of dynamin related protein 1 (Drp1)) and impaired fusion (due to decreased function of the fusion mediator, mitofusion-2).[85] This fission/fusion imbalance promotes excessive cell proliferation and impairs apoptosis.[81] Preventing mitotic fission, by inhibiting Drp1 or enhancing mitofusin-2, causes cell cycle arrest and can regress vascular disease and improve hemodynamics in experimental models of PAH.[81]

In PAH, RV ischemia, related to microvascular rarefaction and impaired coronary perfusion pressure, promotes Drp1 activation and mitochondrial fission, which increases ROS production.[81] Inhibiting fission, using the Drp1 inhibitor, mitochondrial division inhibitor-1, Mdivi-1, or P110 (a competitive peptide inhibitor of the interaction between Drp1 and its binding partner, fission protein 1, Fis1), preserves RV function in an ischemia-reperfusion injury model.[89]

Interaction between the changes in metabolism and altered mitochondrial dynamics in PH is poorly understood. In Group 1 PH impaired expression and function of the mitochondrial calcium uniporter complex (MCUC) constitutes one link between metabolism and dynamics.[86] The MCUC is the major route of calcium entry into the mitochondrial matrix.[86] Loss of MCUC function (due to a downregulation of the pore forming subunit, called MCU, and upregulation of the negative regulatory element MICU1) impairs mitochondrial calcium uptake and causes accumulation of calcium in the cytosol.[86] Reduced intramitochondrial calcium inhibits calcium-sensitive dehydrogenases within the mitochondria, including PDH. This inactivates Krebs' cycle, contributing to the shift to uncoupled glycolysis.[86] The elevation of cytosolic calcium, resulting from impaired MCUC function promotes vasoconstriction and mitochondrial fission.[86] The down regulation of MCUC in PAH results from increased expression of microRNAs (miRs) miR-138 and miR-25. Anti-miRs against these epigenetic mediators reduces PASMC proliferation, migration, and apoptosis-resistance and regress experimental PAH.[86] The potential role of the MCUC is Group 2 PH has not been assessed.

Data regarding mitochondria in PH-LHD is scarce. In dogs with CHF produced by intracoronary microembolizations the number of mitochondria in cardiomyocytes was increased but their average size was smaller, both in the LV and RV (Table 1).[90] These findings are consistent with increased fission. Although RVSP or mPAP was not reported, mean LVEDP (23±3 mmHg) and PAWP (13±1 mmHg) were markedly elevated compared to control. Dogs with the highest plasma norepinephrine (>600 pg/mL), had smaller mitochondria and worse heart failure.[90]

In the TAC model, Mdivi-1 improves LV function and prevents ventricular remodeling by preventing cardiomyocyte apoptosis and promoting angiogenesis.[12] Inhibiting mitochondrial fission also preserves LV function in a global ischemia model and, in a rodent cardiac arrest model, enhances the success of resuscitation.[91, 92] However, the pulmonary hemodynamics were not reported in these studies. Thus, it is uncertain whether inhibiting mitochondria fission in Group 2 PH can improve RV function or clinical outcomes.

RhoA/Rho-kinase pathway—The RhoA/Rho-kinase pathway is implicated in PH-LHD.[28, 30] Rho-kinase (ROCK) is a serine/threonine kinase that its activated when it binds GTP-bound RhoA.[93] RhoA, a small GTPase, is activated by guanine nucleotide exchange factor (GEF) and inactivated by GTPase-activating protein (GAP).[93] Normally a balance between GEF and GAP determine RhoA activity, which regulates the two ROCK isoforms.[94] ROCK1 is expressed in circulating inflammatory cells whilst Rho-kinase 2 (ROCK 2) is found in VSMC.[95, 96] The ROCKs produce sustained vasoconstriction due to calcium sensitization, a result of their interactions with substrates, including myosin light chain (MLC) and myosin phosphatase target subunit-1 (MYPT-1).[97] Relevant to PH, ROCK activation also increases VSMC proliferation, inflammatory cell migration, platelet activation, ROS production, and endothelial dysfunction.[97] MYPT-1 is activated in the lungs of rats with SU5416-hypoxia induced PAH and this is reversed by the rho kinase inhibitor, fasudil.[98] Rho kinase inhibition regresses SU5416-hypoxia-induced PAH, suggesting that severe sustained vasoconstriction is important.[98] RhoA/Rho-kinase inhibition also attenuate PH-LHD induced by SAB.[28, 30] Fasudil (30 mg/kg/day administered from day 29–42 post-banding), decreased mPAP by 65%, RVH by 30%, and PA medial thickness by 50%.[28] On a molecular level, fasudil decreased expression of ROCK2 expression by 28% (without altering ROCK1) while increasing lung eNOS.[28]

Drugs that indirectly inhibit the RhoA/Rho-kinase pathway also attenuate PH-LHD in rodent models. Atorvastatin (10 mg/kg/day orally for 9-weeks), an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase[30], significantly reduced RhoA and ROCK2 expression in SAB rats by impairing production of isoprenoids, which are required for isoprenylation of Rho-kinase.[30] However, this same statin failed to regress Group 1 PH in both rats and humans[99, 100].

Metabolic Syndrome—Metabolic syndrome (MetS) has emerged as a risk factor for both Group 1[101, 102] and Group 2 PH[103]. In mice, there is a link between genetic background and susceptibility to PH induced by a high fat diet (HFD). In a genome-wide association study (GWAS) of 36 mouse strains challenged with 20-weeks of HFD[104], variants in the endothelial growth factor receptor (EGFR) gene were associated with increased

susceptibility to Group 2 PH.[105] EGFR expression was increased in the lungs of mice with the most severe HFD-PH (although the severity of PH in this study was mild).[105] Previous studies have identified the critical role of EGFR in PASMC growth, vascular remodeling, and survival in experimental model of Group 1 PH.[106] Based on the GWAS and supporting data, Kelly *et al.* concluded that higher baseline level of EGFR expression may increase susceptibility to HFD-induced Group 2 PH.[105]

Type 2 diabetes mellitus (type 2 DM) is a component of MetS. Chronic hyperglycemia inhibits adenosine monophosphate-activated protein kinase (AMPK).[107] AMPK is a heterotrimeric enzyme with α, β and γ subunits. AMPK serves as a fuel gauge, generating ATP as required to correct energy depletion. When AMP levels are increased AMPK is activated. There are 12 AMPK variants with tissue specific distribution.[108] AMPK is activated by phosphorylation of its α-subunit at threonine 172. This phosphorylation is regulated by competitive binding of adenosine monophosphate (AMP) versus adenosine triphosphate (ATP) to the γ subunit.[108] Metformin, which is used to treat type 2 DM, is an AMPK activator.[109] In experimental Group 2 PH metformin decreases RVSP and adverse vascular remodeling.[110] Lai *et al.* developed a model of MetS and PH-HFpEF by injecting an obese rat strain (obese ZSF1) with SU5416.[110] In this model, metformin decreased hyperglycemia, activated AMPK in PASMC and caused beneficial vascular remodeling. Metformin[111] and a more selective AMPK activator, 5'-aminoimidazole-4-arboxamide ribonucleoside (AICAR),[112] also decrease PASMC proliferation and improve survival in rodent PAH models.

Lung Inflammation and Fibrosis—Chen *et al.* showed that male TAC mice (C57B6J) have a ≤5.3 -fold increase in lung wet weight that reflects massive pulmonary fibrosis and significant pulmonary leukocyte infiltration, rather than pulmonary edema. Thus, lung fibrosis and inflammation may play an important role in the pathophysiology of Group 2 PH.[33] Wang *et al.* used either CD28 or B7 knockout mice, which have impaired T-lymphocyte activation, to show that activated effector T cells ($CD3^{+}CD44^{high}$ cells) play a central role in the inflammatory response associated with TAC-induced HF.[34] Knockout mice lacking either CD28 or B7 had dramatic reduction in the accumulation of activated effector T cells in both the heart and the lung, and had better preserved LV function post-TAC. Although RVSP was not measured in this study, the ratio of RV/body weight was significantly reduce in both the CD28 or B7 knockout TAC mice[34] These data suggest targeting T cell activation may be useful in treating Group 2 PH.[34]

WHO Group 3 PH (Table 2)—Group 3 PH is defined as mPAP ≥25mmHg and PCWP pressure ≤15 mmHg associated with COPD, interstitial lung disease (ILD), sleep disordered breathing, alveolar hypoventilation disorders, or chronic exposure to high altitude.[113] RV failure associated with Group 3 PH is referred to as "cor pulmonale". Group 3 patients may receive long-term supplemental oxygen and supportive care with diuretics; however, PH-targeted therapies are not recommended.[42, 113] The nonselective nature of the vasodilatation caused by these agents often impairs hypoxic pulmonary vasoconstriction and exacerbates hypoxemia. For example, when Group 3 patients (mPAP >30mmHg) with COPD exacerbations requiring mechanical ventilation, were randomized to receive flolan

(prostacyclin, 2–12 ng/kg/min, intravenously) vs placebo PVR was reduced but there was no improvement in respiratory status.[114] Patients receiving flolan developed more severe hypoxemia due to the nonselective vasodilatation. Likewise, ETRAs are not recommended for Group 3 PH.[115, 116] Although small open-label studies suggest that PDE5 inhibitors,[117, 118] sGC stimulators,[119] and intravenous prostacyclins[120] may be beneficial in selected Group 3 patients larger, double-blinded trials would be required to change practice.

Disordered Pathways in Group 3 PH

Endothelial AMPK—AMPK is a putative therapeutic target in PH.[26, 121] Dean et al. showed that metformin can reduce the development of PH in the SU5416/hypoxia female rat model.[26] While this is considered a Group 1 model there is some relevance to Group 3 PH, in that hypoxia is a key contributor to the model. Metformin's mechanism of action is complex. They propose that the metformin activates AMPK, which in turn inhibits aromatase.[26] Aromatase converts testosterone to estrogen, which in turn is metabolized via cytochrome p450 1B1 to generate 16a-hydroxyestrone, a metabolite known to cause PH.[26] Interestingly, inhibiting aromatase activity is only effective in the female PH rat models.[26] Epidemiological studies reveal a higher percentage of female than male COPD patients have PH.[122, 123] Thus, both in humans and in rodent models there are important sex difference in the pathophysiology of Group 3 PH.

In addition to responding to bioenergetic perturbations AMPK can be activated by inhibition of Rho-kinase.[124] AMPK activation has differential effects in endothelial versus SMC. In endothelial cells AMPK activates eNOS and has an antiapoptotic effect; whereas, in VSMCs, it reduces secretion of various growth factors and has a proapoptotic effect.[125–127]

Omura *et al.* showed that AMPK is downregulated in a hypoxia-induced Group 3 PH mouse model.[121] Moreover, they created endothelial-specific AMPK-knockout mice (eAMPK$^{-/-}$).[121] The eAMPK$^{-/-}$ mice have no basal increase in % muscularized distal PA; however, compared to control mice, they develop more severe PH after 4 weeks of hypoxia.[121] The eAMPK$^{-/-}$ mice have impaired endothelial function (due to reduced eNOS activity) and increased PASMC proliferation due to upregulation of growth factors [platelet derived growth factor-BB (PDGF-BB) and fibroblast growth factor-2 (FGF-2)].[121] Metformin, attenuates hypoxia-induced PH in these mice.[121]

Li et al. showed that AMPK activation in endothelial cells prevents Drp1-mediated mitochondrial fission and improves endothelial function.[128] Pretreating rat aortic endothelial cells with AMPK activators, salicylate or AICAR, prevents palmitate-induced mitochondrial fission, with an efficacy that is comparable to Mdivi-1.[128] This study suggests that endothelial AMPK activation inhibits Drp1 recruitment, prevents mitochondrial fission and provides an intriguing link between the disorders of mitochondrial dynamics and bioenergetics that may be relevant to Group 3 PH. However, metformin has many actions in addition to AMPK activation, including an anti-inflammatory effect evident in its ability to inhibit of endoplasmic reticulum stress-associated inflammasome activation in the adipose tissue of diabetic mice.[129]

However, there are also data suggesting AMPK activation may be harmful in Group 3 PH. Ibe *et al.* showed that inhibition of AMPK with Compound C significantly reduced RVH, RVSP, and pulmonary vascular remodeling in chronically hypoxic C57BL/6 mice.[130] The opposing data on the role of AMPK inhibition may reflect the different animal models used, off target effects of AMPK modulating drugs, the cellular compartment in which the AMPK dysregulation occurs (SMC vs. endothelium) and/or tissue heterogeneity in AMPK isoform expression.

RhoA/Rho-kinase pathway—Hemnes *et al.* showed that sildenafil inhibits ROS generation and RhoA/Rho-kinase activation thereby attenuating bleomycin-induced PH.[39] Male C57/BL6 mice were treated with intratracheal bleomycin (4 U/kg).[39] Sildenafil (100 mg/kg/day orally) improved survival by 10.5%, reduced RVH and fibrosis, and pulmonary vascular medial hypertrophy.[39] PDE5 inhibition decreased RhoA and ROCK activity, increased PKG activity, enhanced eNOS activity and inhibited inducible nitric oxide synthase (iNOS).[39]

Hypoxia-induced PH is attenuated in heterozygous ROCK2-deficient ($ROCK2^{+/-}$) mice and exacerbated in VSMC-specific ROCK2-overexpressing transgenic mice (ROCK2-Tg).[96] ROCK activity exacerbates PH by increasing cell proliferation. In $ROCK2^{+/-}$ mice both the expression of the activated form of the mitosis-promoting kinase extracellular signal-regulated kinase (ERK1/2) and the number of Ki67-positive proliferating cells in the lung were reduced; whereas, in the ROCK2-Tg mice opposite results were found.[96]

Hypoxia-inducible factor (HIF-1α)—The transcription factor, HIF-1α, plays an important role in the pathogenesis of PAH.[88] Patients with Group 3 PH are exposed to chronic hypoxia either secondary to diseased lung parenchyma (COPD), intermittent apnea (sleep apnea) or environmental hypoxia (altitude). Thus, HIF-1α also plays a key role in the pathogenesis of Group 3 PH.[131] HIF-1α is activated in the rodent hypoxia PH model.[6] HIF-1α haploinsufficiency protects mice from Group 3 PH, reducing polycythemia, RVH, mPAP, and adverse pulmonary vascular remodeling.

In chronic hypoxia HIF-1α-dependent transcriptional activation of the *TRPC1* (transient receptor potential channel 1) and *NHE1* (sodium-hydrogen antiporter 1) genes increases intracellular calcium concentration ($[Ca^{2+}]_i$) and intracellular pH (pH_i) in PASMC, respectively.[132, 133] Inhibiting HIF-1α, using digoxin, decreases $[Ca^{2+}]_i$ and pH_i, reduces RVH, and attenuates pulmonary vascular remodeling in this model (Figure 10).[134]

HIF-1α also regulates the expression of voltage-gated, oxygen-sensitive potassium channels.[88] Normoxic activation of HIF-1α in fawn hooded rat inhibits the expression of Kv1.5. This depolarizes PASMC, which activates the voltage-dependent, large conductance calcium channel and elevates $[Ca^{2+}]_i$. Conversely, augmenting SOD2 inhibits HIF-1α, restores Kv1.5 expression and regresses PH.[88] Consistent with this, Whitman *et al.* found that Kv1.5 and Kv2.1 expression is preserved in HIF-1α haploinsufficient mice exposed to chronic hypoxia.[135] These ionic mechanisms are relevant both for control of pulmonary vascular tone and structural remodeling.[6]

Mitochondria-ROS-HIF-1α-Kv channel pathway—Hypoxia stabilizes HIF-1α, increasing its accumulation in the nucleus and its activity. The regulation of HIF-1α involves oxygen-sensitive prolyl hydroxylases and von Hippel-Lindau factor-mediated ubiquitination.[136] However, the cell's redox state, which is, largely determined by mitochondria, also regulates HIF-1α.[6] SOD2, converts toxic, short-lived ROS, produced by the mitochondrial electron transport chain, into diffusible H_2O_2, a redox-signaling molecule that regulates HIF-1α.[6] Mitochondrial ROS can be regulated by pO_2 or by alteration in SOD2 expression. Hypoxia alters mitochondrial ROS production.[137–139] Controversy exists as to whether hypoxia increases or decrease mitochondrial ROS production. However, we consistently find that ROS production in whole lungs, PASMC and PASMC mitochondria decreases in response to physiologic hypoxia. In FHR PASMC, a deficiency in normoxic H_2O_2 production, triggered by epigenetic SOD2 downregulation, creates a "pseudo hypoxic milieu, in which normoxic activation of HIF-1α promotes PH[88]

HIF-1α activation increases transcription of PDK, which creates a pathologic metabolic shift from oxidative glucose metabolism to reliance on uncoupled glycolytic metabolism. This form of metabolism is also observed in cancer and is called the Warburg effect.[6, 85] Hence, there is biologic plausibility that drugs that modulate mitochondrial metabolism may have therapeutic potential in Group 1 and Group 3 PH. Michelakis *et al.* showed that dichloroacetate, a metabolic modulator that inhibits all 4 isoforms of PDK, regresses chronic hypoxic PH in rats (Figure 11).[140] PDK inhibition increases glucose oxidation and mitochondrial NADH (nicotinamide adenine dinucleotide) thereby increasing mitochondrial electron flux and restoring physiologic ROS production. Dichloroacetate also regresses PH in the FHR model.[88]

Cyclophilin A (CyPA)-Basigin (Bsg) Pathway—Bsg, also known as CD147 or extracellular matrix metalloproteinase (MMP) inducer (EMMPRIN), is a ubiquitous transmembrane glycoprotein.[14, 32] Bsg is activated by CyPA (an immunophilin), mechanical stretch, soluble Bsg (sBsg), or angiotensin II.[14, 32] Bsg regulates many intracellular pathways involved in VSMC proliferation, including MMP activation, autophagy, and platelet activation.[14, 32]

Haploinsufficiency of CyPA ($CyPA^{+/-}$) or $Bsg^{+/-}$ reduces chronic hypoxic PH and pulmonary artery remodeling in mice.[14] Furthermore, $Bsg^{+/-}$ PASMC had reduced rates of proliferation.[14] $CyPA^{+/-}$ mice exposed to hypoxia had lower levels of inflammatory cytokines, especially chemokine ligand 2, macrophage colony-stimulating factor, interleukin (IL)-2 and IL18.[14] Proliferative signaling was also reduced in PASMC from hypoxic $Bsg^{+/-}$ mice, with reduced expression of PDGF-BB (platelet derived growth factor BB) and decreased phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2).[14] These data suggest CyPA/Bsg signaling regulates inflammatory cell recruitment and growth factor secretion and may be a novel therapeutic target for treating Group 3 PH. In PAH patients, plasma CyPA levels directly correlate with PVR.[14]

WHO Group 4 PH (CTEPH, Table 3)—CTEPH occurs when pulmonary emboli fail to resolve and an obstructive neo-intima reduces perfusion to a critical volume of the pulmonary circulation.[141] The incidence of CTEPH following acute pulmonary embolism

ranges from 0.1–9.1% (average 4%).[9, 142–147] A clinically diagnosed pulmonary embolic event is absent in 1/3 of CTEPH patients.[141] Once established CTEPH gradually worsens, even without additional clinical embolic events.[141] The preferred treatment is pulmonary thromboendarterectomy (PTE).[141] In the United States, the estimated annual incidence of CTEPH is around 5000 cases, but only 300–400 PTEs/year are performed, suggesting patients are being deprived of an effective therapy.[141] In 2013, riociguat, a soluble guanylate cyclase stimulator, was approved for inoperable CTEPH patients.[70] In CHEST-2, riociguat improved 6-min walk test duration and reduced PVR and N-terminal pro-brain natriuretic peptide in PTE ineligible patients.[70, 148]

Disordered Pathways in Group 4 PH

Endothelial Dysfunction—In creating a novel CTEPH animal model, Neto-Neves *et al.* showed embolization with polystyrene microspheres (PE) alone was usually insufficient to create CTEPH.[40] However, combining embolization with SU5416 reliably produced sustained PH (Figure 12).[40] Likewise, plasma levels of asymmetric dimethylarginine (ADMA), a potent endogenous NOS inhibitor, are significantly elevated in CTEPH patients and eNOS expression is reduced.[149] Thus, there is an important synergistic, role of endothelial dysfunction in promoting CTEPH in PE patients.

ET-1: ET-1 levels are increased in dogs with CTEPH (induced by repeated embolization with ceramic beads).[141] Bosentan attenuated pulmonary vascular remodeling, reduced mPAP and PVR in this model.[150] Reesink *et al.* found serum ET-1 levels were increased in 35 CTEPH patients (1.62±0.21 pg/mL) compared with controls (0.75±0.06 pg/mL).[151] Furthermore, ET-1 levels correlated well with measures of CTEPH severity, including mPAP (r=0.70), cardiac index (r=−0.76), total pulmonary resistance (r=0.72), and 6-min walk test (r=0.59). ET-1 levels>1.77 pg/mL are associated with a bad postoperative outcome from PTE.[151] Recently, Southwood *et al.* found there was a greater expression of ET_A than ET_B receptors in the chronic thrombus from PTE patients.[152]

However, clinical trials do not support the use of ETRAs for CTEPH. In the BENEFiT trial, patients with inoperable CTEPH were randomized to placebo (80) or bosentan (77), (62.5 mg twice a day for 4-weeks, then 125 mg twice a day for 12-weeks). Bosentan significantly reduced PVR (−24.1) and improved cardiac index (+0.3 L/min/m^2) but did not improve 6-min walk distance (6MWD) (+2.2 m) or reduce mPAP (−2.5 mmHg).[153] In MERIT-1 (NCT02021292), a randomized, double-blind, placebo-controlled study, macitentan (10 mg/ daily for 24-weeks) significantly improved PVR and 6MWD in CTEPH patients.[154] However, the validity of the unpublished data awaits peer review.

Platelets in CTEPH—Surprisingly, the prevalence of hereditary thrombophilia, such as factor V Leiden, factor II mutations, protein C and S deficiencies is not significantly different in PAH or CTEPH, versus control subjects[155], reviewed by Mehta *et al.*.[156] However, other prothrombotic mechanisms, including enhanced platelets activation and reduced fibrinolytic function, occur in CTEPH.[157, 158] Platelets from CTEPH patients are hypersensitive to thrombin stimulation.[157] P-selectin and PAC-1 are markers of activated platelets. After thrombin stimulation, the percentage of P-selectin-positive and PAC-1-

positive platelets in CTEPH patients (14.7% and 53.9% respectively) was significantly higher versus controls (2.09% and 20.86% respectively).[157] The activated platelets induce the expression of vascular cell adhesion molecule-1 (VCAM-1)[157], an important mediator of immune cells adhesion to the vascular endothelium. Increases in VCAM-1 are associated with development of PH.[159] After thrombin stimulation, the relative increase in platelet VCAM-1 expression was significantly higher in CTEPH patients (17.6 fold) than controls (2.4 fold).[157] These findings suggest platelet dysfunction may be involved in the pathogenesis of CTEPH. If confirmed this would change the therapeutic approach to this PH Group, which currently focuses on inhibiting of the clotting cascade.

Fibrinolytic abnormalities—Thrombin also activates a plasma carboxypeptidase inhibitor, TAFI, the thrombin-activatable fibrinolysis inhibitor.[160] TAFI removes the C-terminal lysines from fibrin and decreases its binding by tissue-type plasminogen activator and plasmin.[161] Thus, TAFI activation impairs fibrinolysis.[162] Plasma TAFI levels were significantly higher in CTEPH patients versus controls.[158] Furthermore, impairment of fibrinolysis in patients was improved with a TAFI inhibitor, CPI-2KR, suggesting TAFI as a potential therapeutic target in CTEPH.[158] TAFI can also be released from activated platelets.[157, 163] In CTEPH patients, TAFI released from activated platelets was markedly higher compared to controls.[158] Yaoita et al. demonstrated that an inhibitor of platelet activation, prostaglandin E_1, indirectly lowers TAFI levels in CTEPH patients.[158]

CONCLUSION

There remains inadequate preclinical focus on basic mechanisms and novel therapeutics for Group 2–4 PH. The human experience to date suggests little therapeutic overlap of existing PH-targeted therapeutic agents amongst groups. Thus, the search for animal models that accurately recapitulate the disease phenotype for each PH group is important as a means of discovering new Group specific therapies and biomarkers. We have identified some knowledge gaps and listed corresponding future research directions in Table 4.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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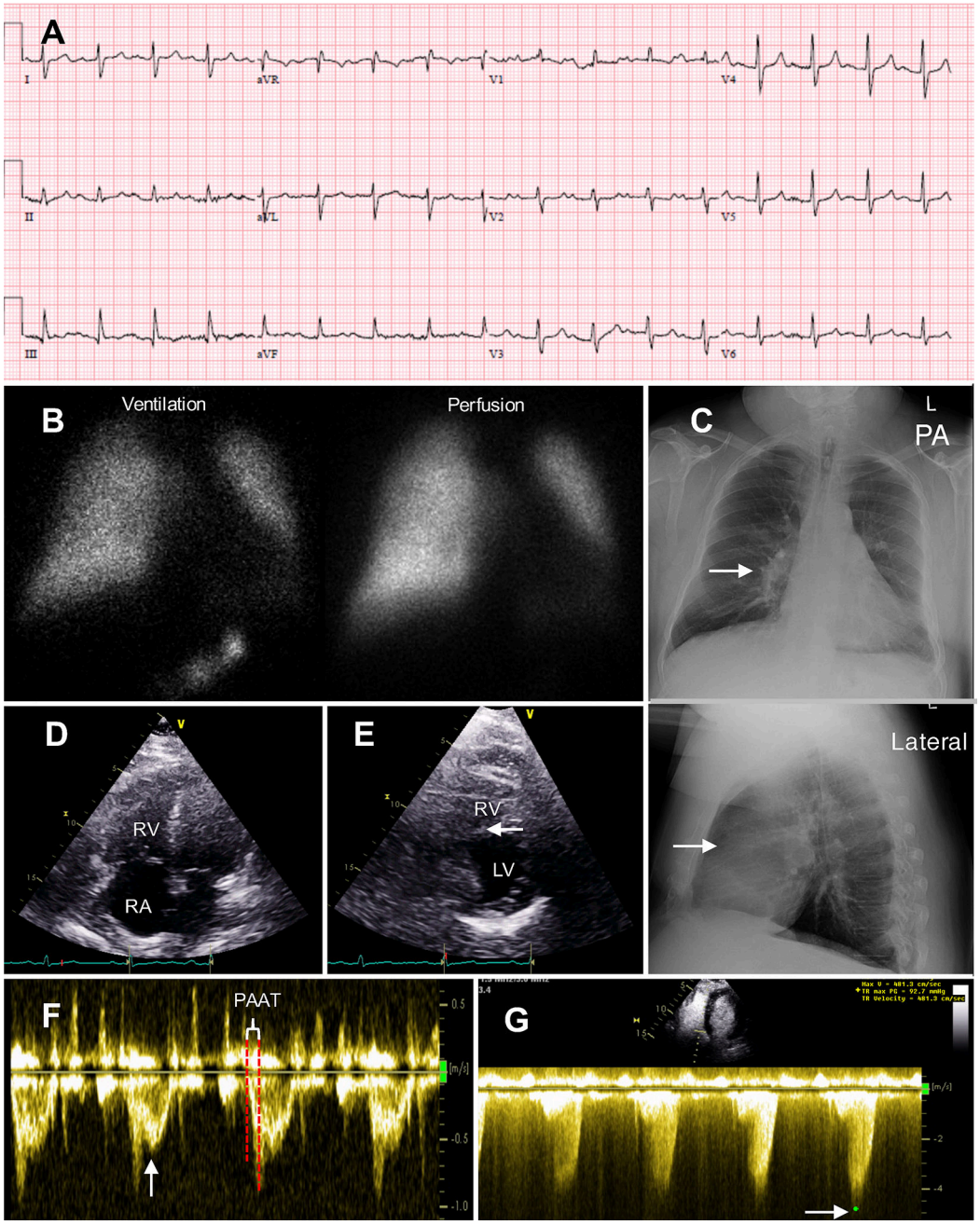

Figure 1. Group 3 Pulmonary Hypertension case: 68 year-old male with moderately severe chronic obstructive pulmonary disease and obstructive sleep apnea

A. Electrocardiogram showed sinus tachycardia with first degree atrial ventricular block. Note right axis deviation and right ventricular hypertrophy with S1Q3T3 pattern.

B. Ventilation perfusion (VQ) scan did not demonstrate VQ mismatch. No evidence of pulmonary embolism.

C. Posterior anterior (PA) and lateral chest x-ray. Evidence of right ventricle (RV) enlargement (arrows indicate enlarged right pulmonary artery on PA view and loss of retrosternal airspace on lateral view).

D. Apical four chamber view on ultrasound demonstrating RV and right atrial (RA) enlargement.

E. Parasternal short axis view on ultrasound showing mild septal flattening (arrow), a sign of RV pressure/volume overload.

F. Pulse wave Doppler of the pulmonic valve demonstrates severe pulmonary hypertension. The Doppler signal is early peaking and there is a short pulmonary artery acceleration time (PAAT) with mid-systolic notching (arrow), reflective of a noncompliant pulmonary vascular bed.

G. Tricuspid regurgitation (TR) continuous wave Doppler [using Definity® contrast (Lantheus Medical Imaging, N. Billerica, Massachusetts) to enhance the signal]. TR peak velocity (V) (arrow) was used to calculate right ventricular systolic pressure (RVSP) of 93 mmHg using Bernoulli equation ($RVSP=4V^2$).

RA (mmHg)	12
PAP (mmHg)	92/33
Mean PA (mmHg)	58
Wedge (mmHg)	25
CO (L/min)	4.1
Heart rate (beats/min)	77
Mean gradient across mitral valve (mmHg)	18

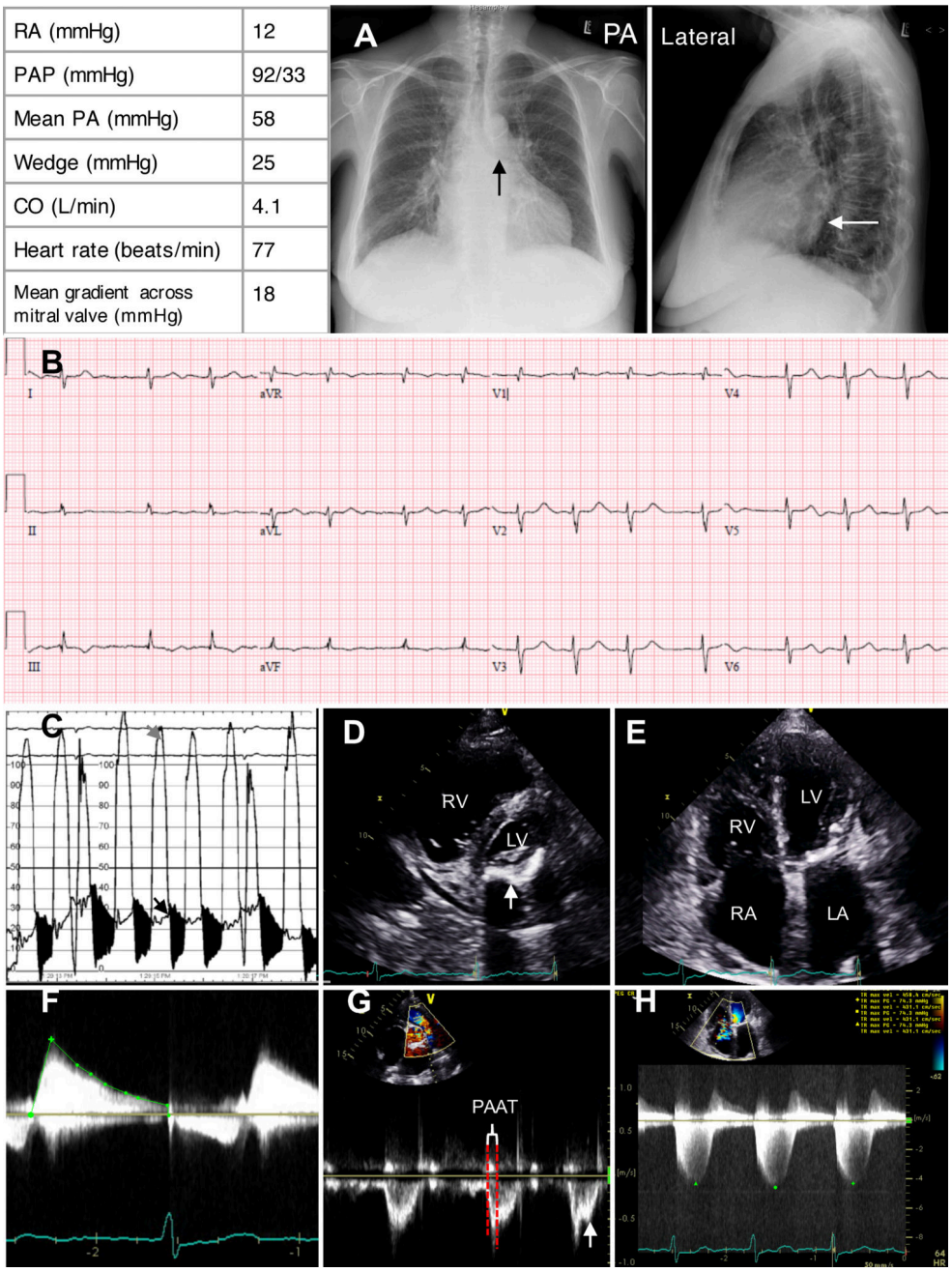

Figure 2. Group 2 pulmonary hypertension case: 73 year-old female with severe mitral valve stenosis and scleroderma

Table displays typical right heart catheterization parameters of a Group 2 PH patient, which is characterized by increased right atrial (RA) pressure of 12mmHg, elevated pulmonary artery pressure (PAP) of 92/33 mmHg, mean pulmonary artery pressure (mPAP) of 58 mmHg, increased pulmonary capillary wedge pressure (PCWP) of 25 mmHg, cardiac output (CO) of 4.1 L/min, heart rate of 77 beat per minute and mean gradient across the mitral valve of 18 mmHg.

A. Chest x-ray demonstrates left atrial enlargement with elevation of left main stem bronchus and splaying of carina on posterior anterior (PA) view (black arrow) and posterior prominence of left atria on lateral view (white arrows).

B. Electrocardiogram demonstrates atrial fibrillation with right axis deviation, S1Q3T3 pattern and right ventricular hypertrophy. There is also low voltage in limb and precordial leads and poor R wave progression, consistent with lung disease.

C. Left ventricular (grey arrow) and wedge pressure (black arrow) tracings superimposed. Mitral valve was severely stenotic with a gradient (in black) of 18 mmHg (heart rate 77 bpm).

D. Parasternal short axis view on ultrasound with severe posterior mitral annular calcification (arrow).

E. Apical four chamber view on ultrasound shows right ventricle (RV), right atrium (RA), left atrium (LA) enlargement with normal left ventricle (LV).

F. Continuous wave Doppler across the mitral valve displays a mean gradient of 10 mmHg characteristic of severe mitral valve stenosis.

G. Pulse wave Doppler through pulmonic valve demonstrates early peaking signal with short PAAT and mid systolic notching (arrow), consistent with severe PH

H. Continuous wave Doppler across tricuspid valve displayed tricuspid regurgitation permitted calculation of a peak (green dot) RV-RA gradient (averaged) of 75 mmHg.

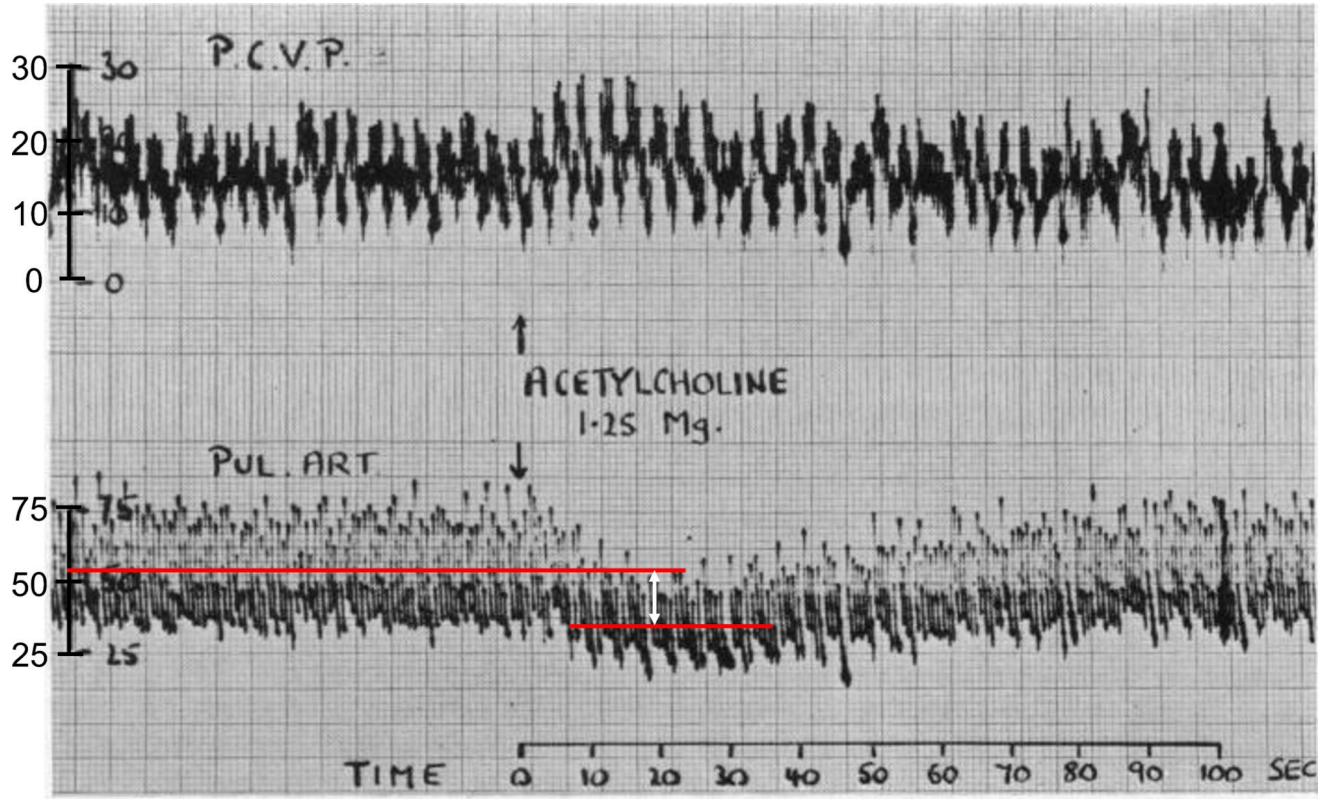

Figure 3. Impaired pulmonary vasodilation in response to acetylcholine in a patient with mitral stenosis-induced Group 2 PH

Simultaneous tracing of pulmonary capillary venous pressure (PCVP; upper trace) and pulmonary artery pressure (PAP; lower trace) of group 2 PH patient with mean PAP (mPAP) around 50 mmHg and PCVP of 25 mmHg. PCVP is an indirect measure of left atrial pressure. Acetylcholine (Ach) injection (1.25 mg) significantly decrease PAP and increase PCVP. The red lines indicate the mPAP pre- and post-Ach injection. The white arrow indicates the drop in mPAP around 20 mmHg. Reproduced from Wood *et al.* with permission.[18]

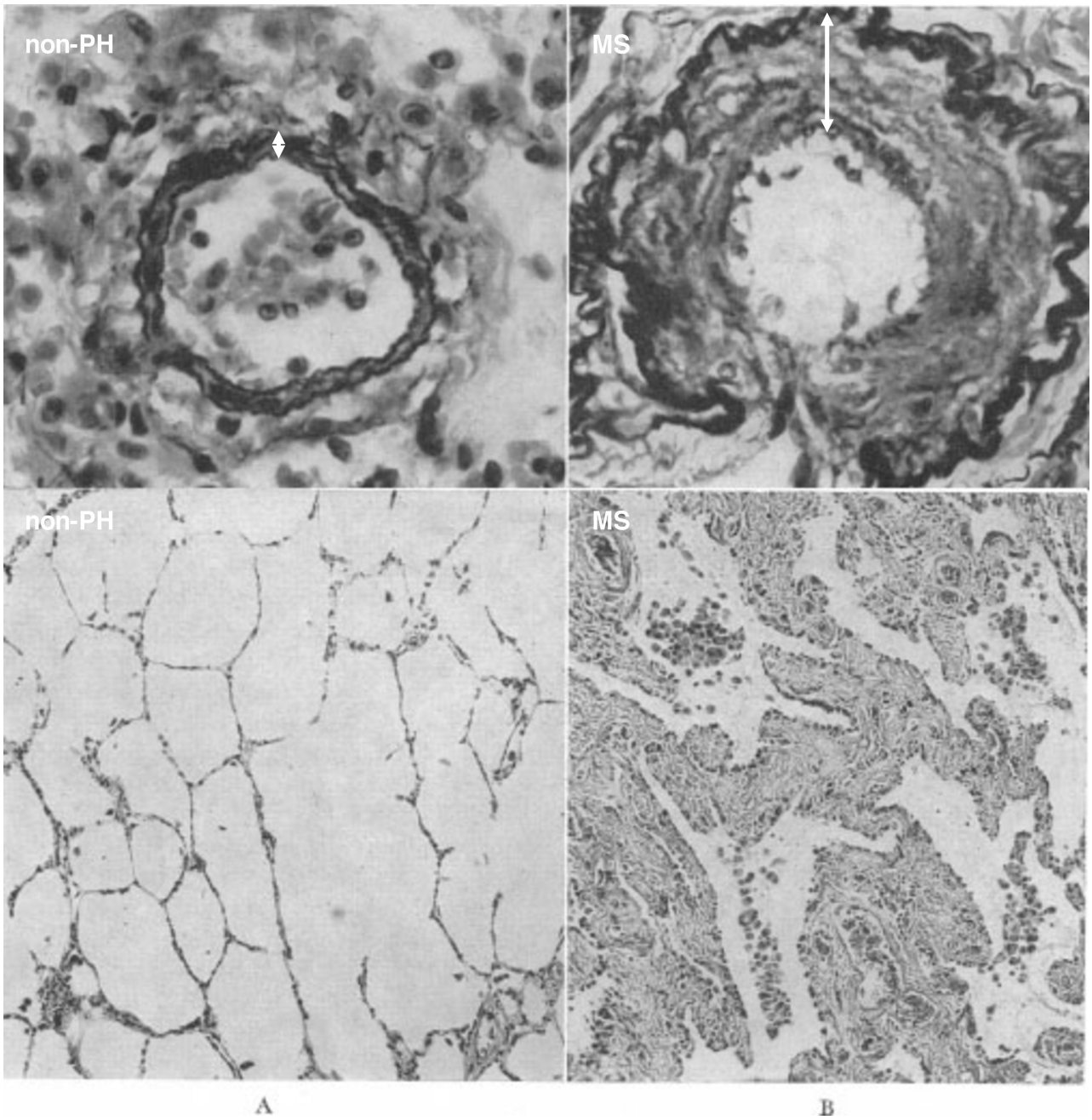

Figure 4. Structural remodeling of pulmonary circulation in mitral stenosis-induced Group 2 PH

Pulmonary arteriole (top row) and section of the lung (bottom row) from a non-pulmonary hypertension (non-PH) patient (A) compared with that from a patient with mitral stenosis (MS) (B). Top row: both vessels being less than 100 microns in diameter (Verhoeff Elastic-tissue Stain × 500). Note how marked intimal thickening (arrow) partially occludes the lumen in B. Bottom row: showing severe thickening of the alveolar walls (Hematoxylin and Eosin Stain × 125). Reproduced from Goodale *et al.* with permission.[21]

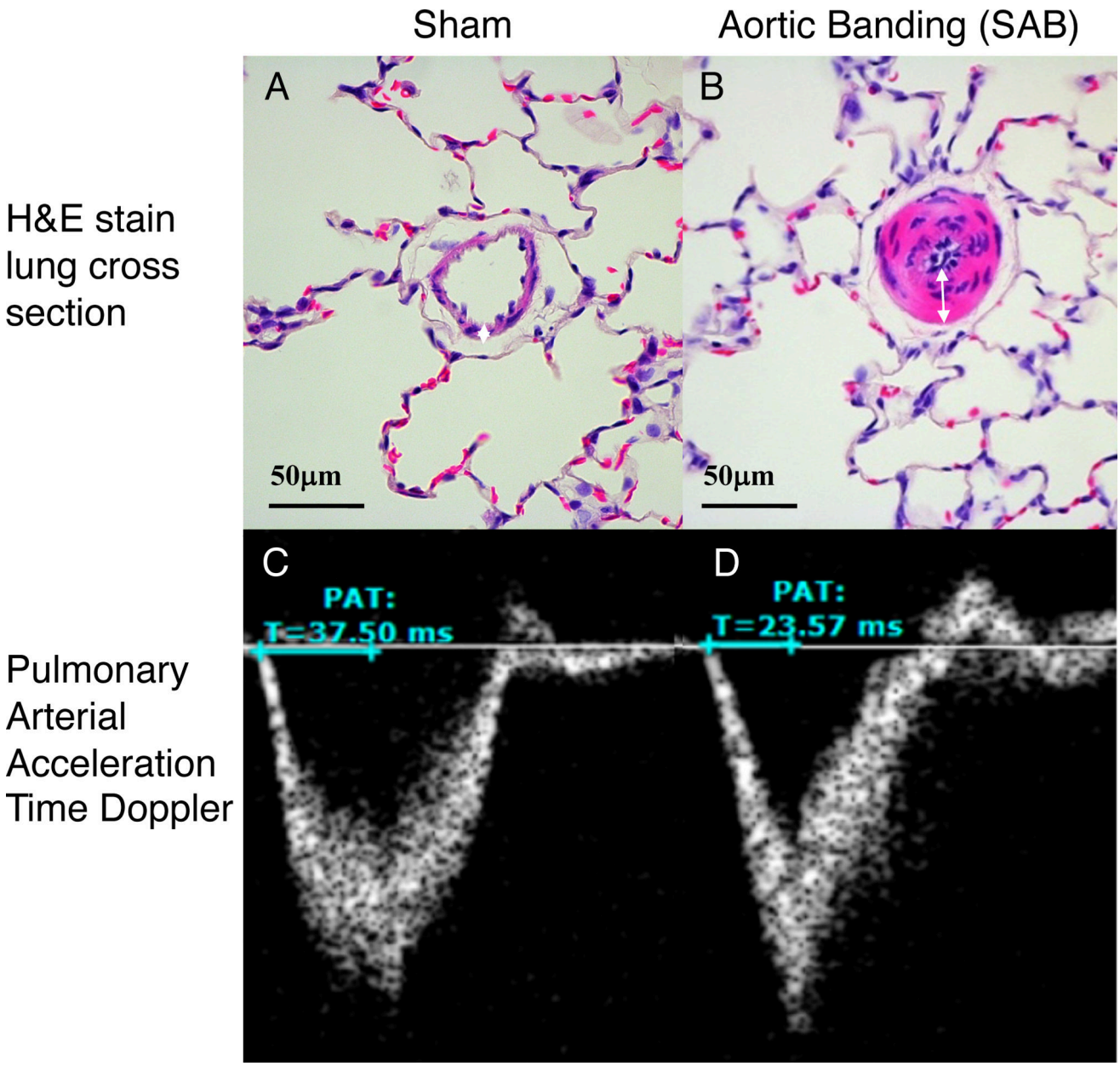

Figure 5. Increased pulmonary arteriole wall thickness and decreased pulmonary arterial acceleration time (PAAT) in rats 8 weeks post supra-coronary aortic banding (SAB) surgery

(A–B) Haemotoxylin and Eosin (H&E) stain of lung cross-sections show increased pulmonary arteriole wall thickness (white arrows) in SAB animal (B) compared to sham (A). (C–D) Pulse wave Doppler through pulmonic valve demonstrates decreased pulmonary artery acceleration time (mentioned as PAT) in SAB rats (D) compared to sham rat (C). Both sham rats and aortic banding rats were fed with Nω-Nitro-L-arginine methyl ester (L-NAME) 1.85 mM in drinking water for 7–8 weeks, before tissue collection.

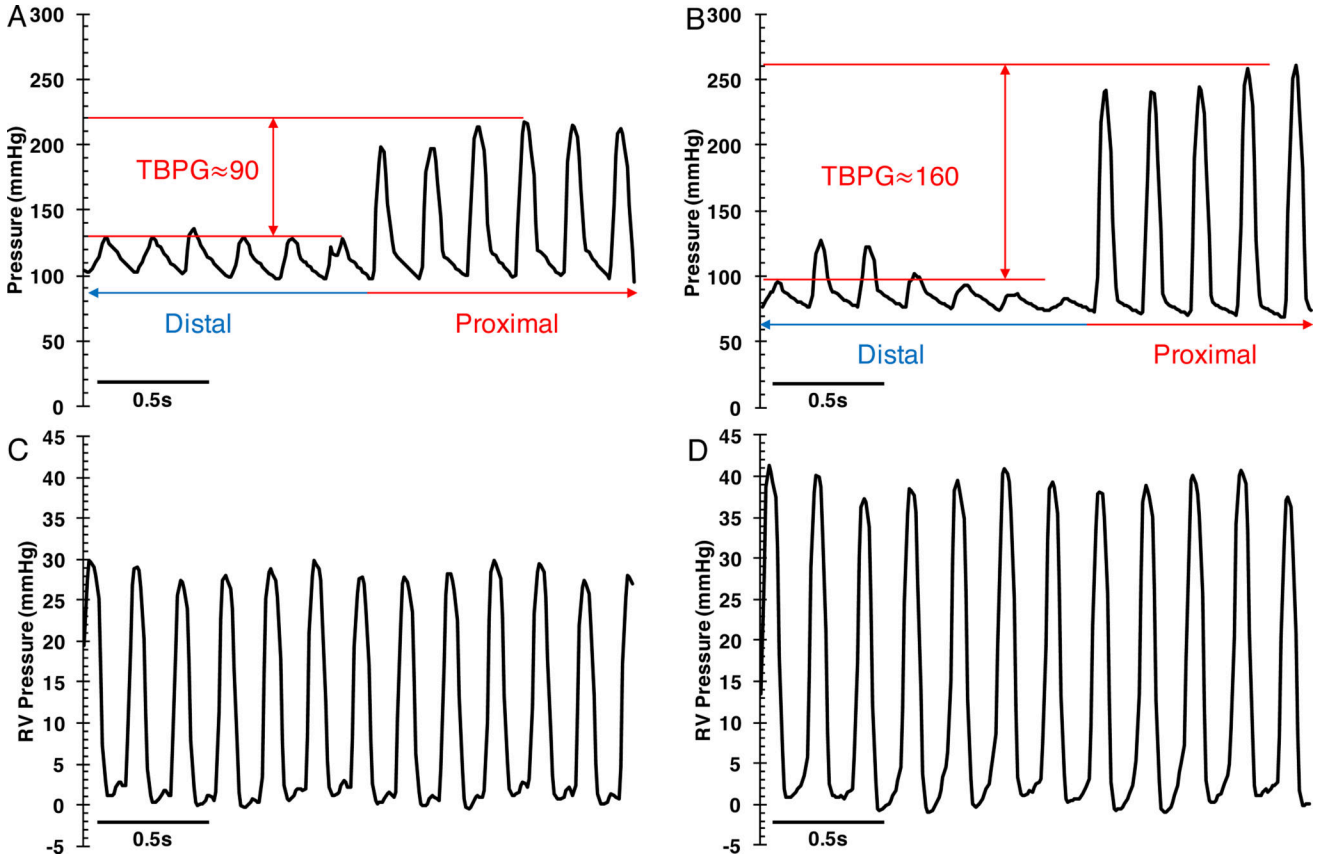

Figure 6. Hemodynamic assessment of the left and right heart in supra-coronary aortic banding (SAB) rats

(A) and (C) are the left and right heart pressure traces respectively from one rat. (B) and (D) are the left and right heart pressure traces respectively from another rat. Trans-banding pressure gradient (TBPG) is equal to systolic pressure proximal to the band (red arrow) minus systolic pressure distal to the band (blue arrow). Higher TBPG is associated with higher right ventricle (RV) pressure.

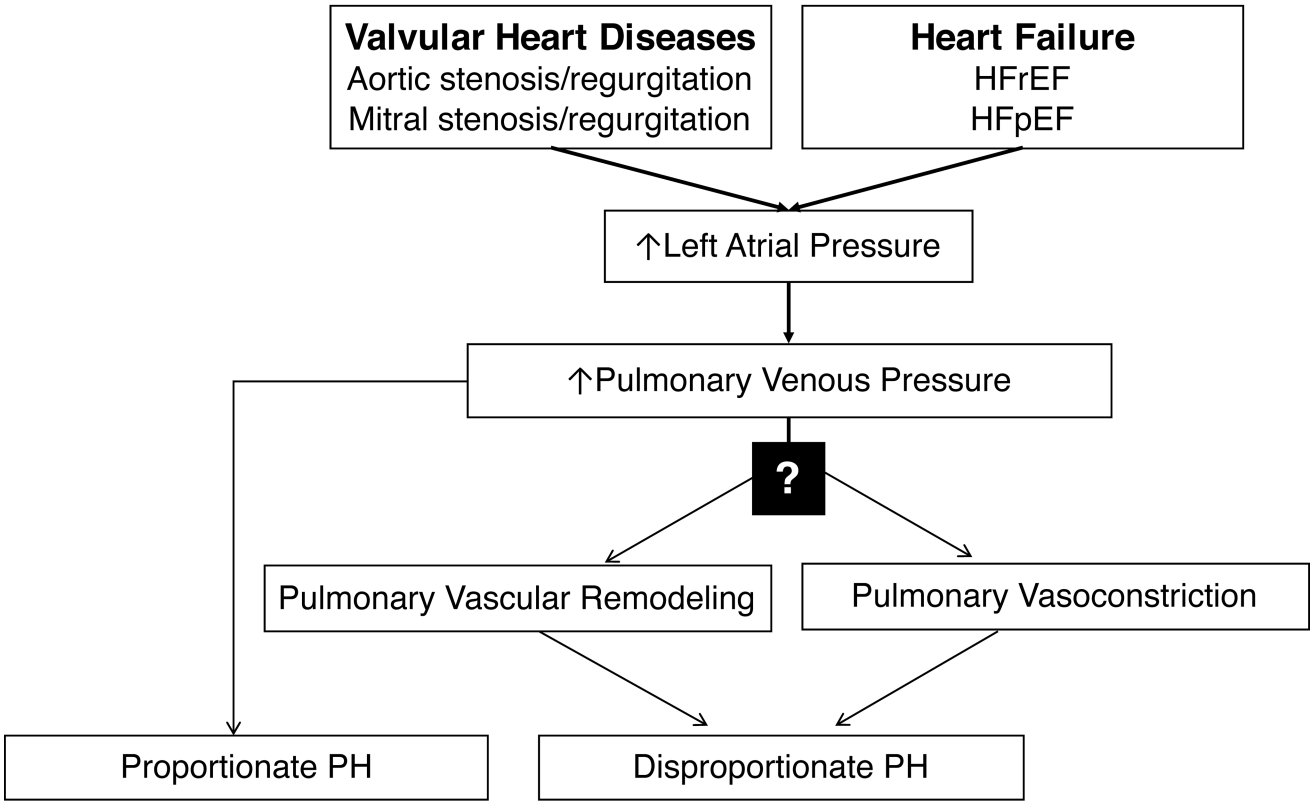

Figure 7. Pathophysiology of Group 2 Pulmonary Hypertension (PH)

In most patients left heart disease increases pulmonary venous pressure and causes a proportionate PH (left). However, for unknown reasons, in a minority of patients with LHD, elevation of pulmonary venous pressure leads to adverse pulmonary vascular remodeling, and/or pulmonary vasoconstriction. This causes disproportionate PH, which may not fully resolve with relief of elevated pulmonary venous pressure. HFrEF: heart failure with reduced ejection fraction; HFpEF: heart failure with preserved ejection fraction.

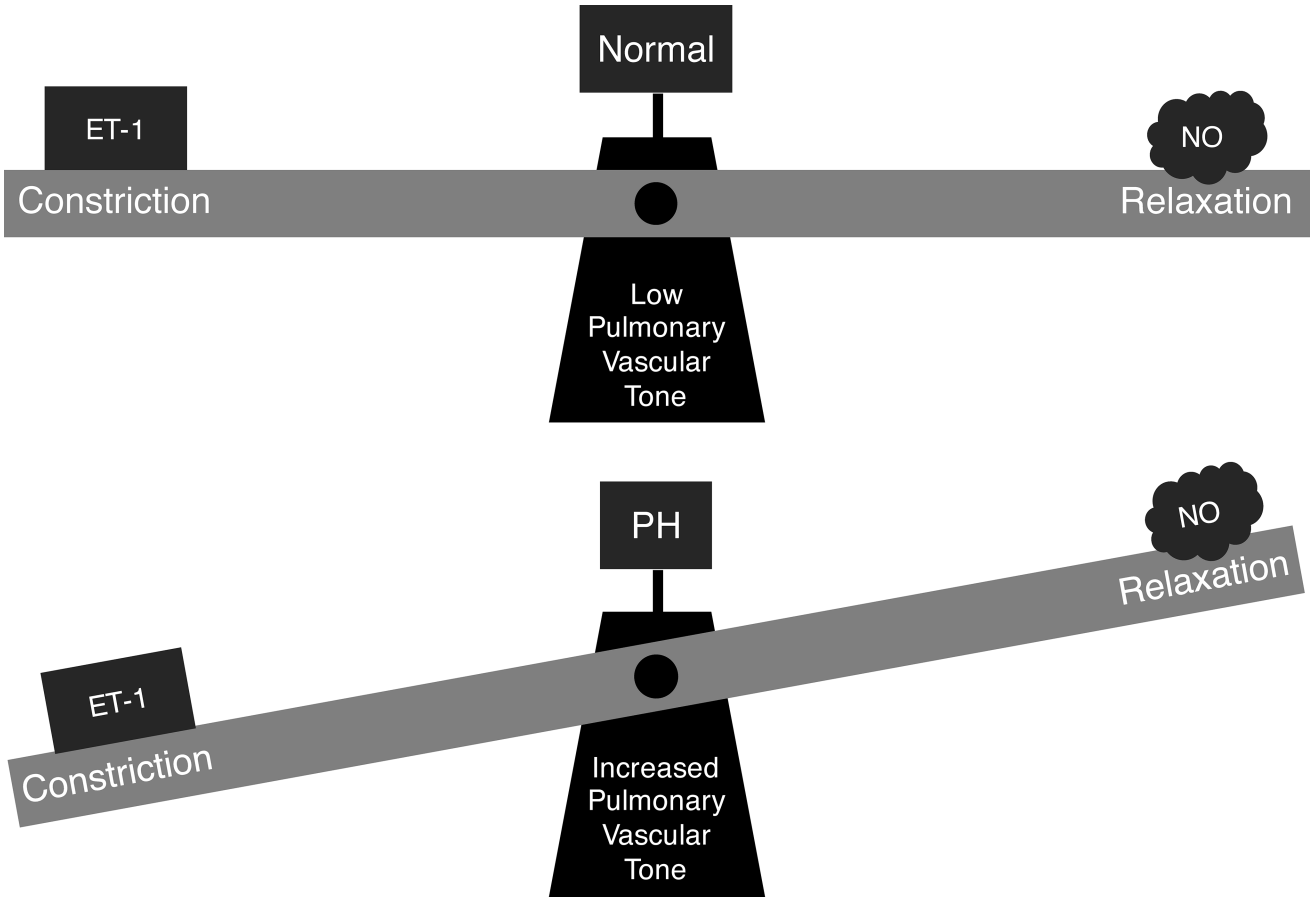

Figure 8. Imbalance in endothelin-1 (ET-1) and nitric oxide (NO) homeostasis in pulmonary hypertension favours pulmonary vasoconstriction

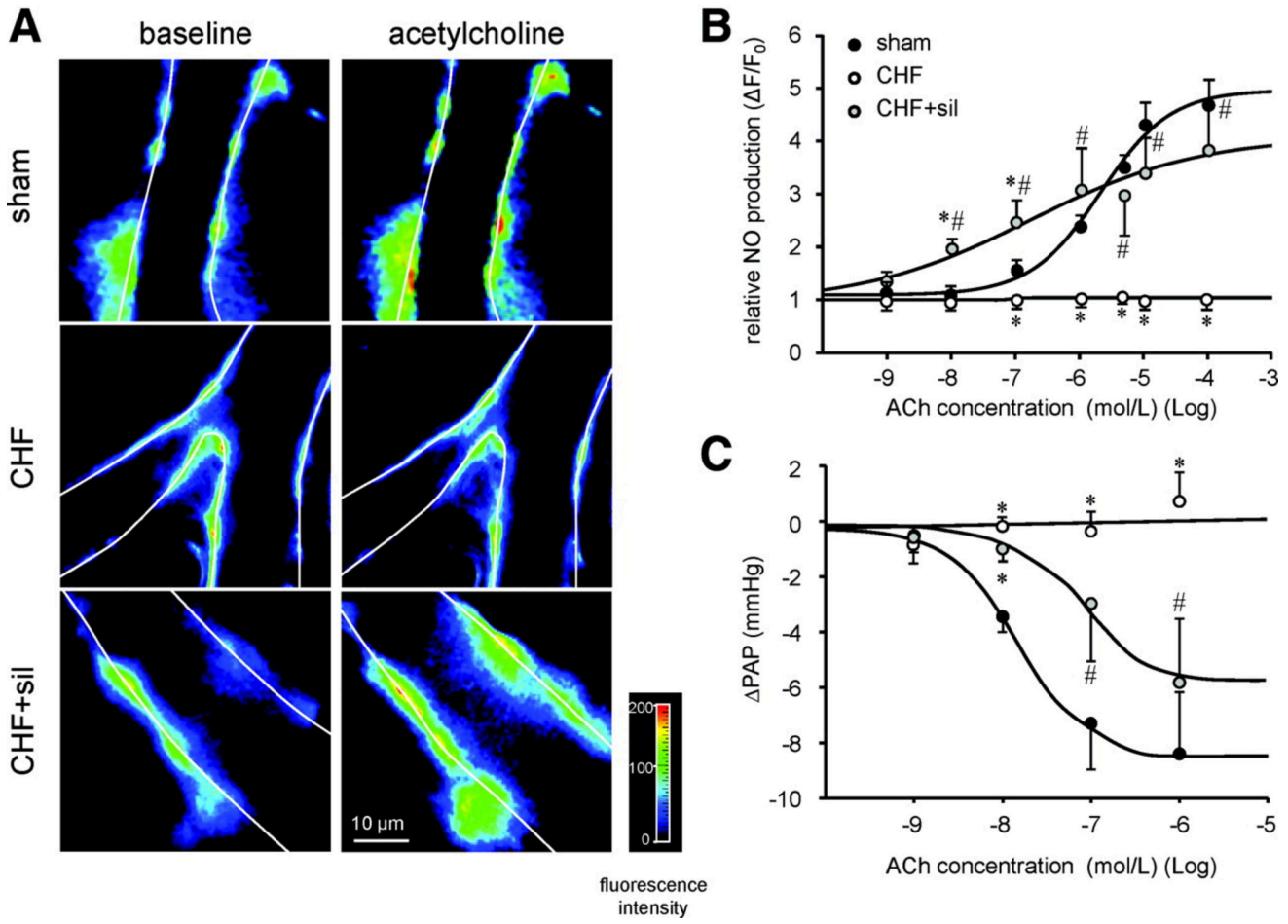

Figure 9. Impaired endothelial function in SAB (Group 2) PH

A. Representative images of 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM)–loaded capillaries show endothelial cells color-coded for NO production in response to acetylcholine (ACh)-note that CHF=SAB rats.

B. Impaired capillary DAF-FM fluorescence in response to stimulation with ACh in lungs from SAB rats.

C. Endothelium-dependent vasorelaxation response to ACh in isolated, perfused rat lungs preconstricted by U46619 (is impaired in SAB rats and this is partially restored by sildenafil. Reproduced from Yin *et al.* with permission.[29]

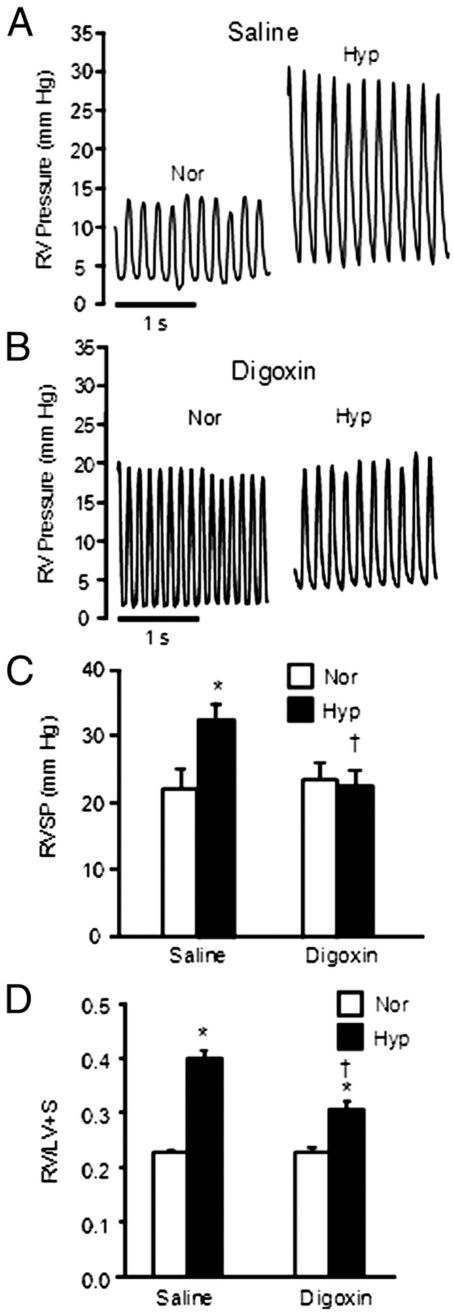

Figure 10. Digoxin reduces Group 3 PH in rats

Representative tracings (A-B) and mean data (C) showing that digoxin (1 mg/kg digoxin) attenuates PH induced by chronic hypoxia (3 weeks at 10% O_2) and (D) reduces RVH. Bar graphs (mean±SEM) Reproduced from Abud *et al.* with permission.[134]

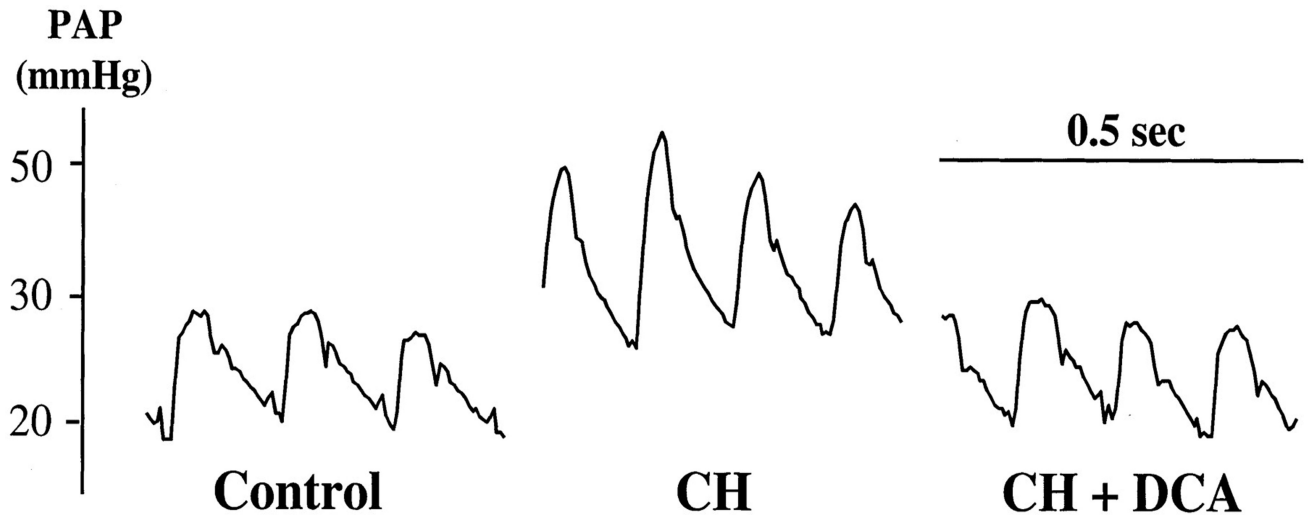

Figure 11.

Representative trace PAP in 3 rat groups with Group 3 PH treated with the PDK inhibitor Dichloroacetate (DCA). DCA in drinking water reverses rise in PA pressure caused by CH. Reproduced from Michelakis *et al.* with permission.[140]

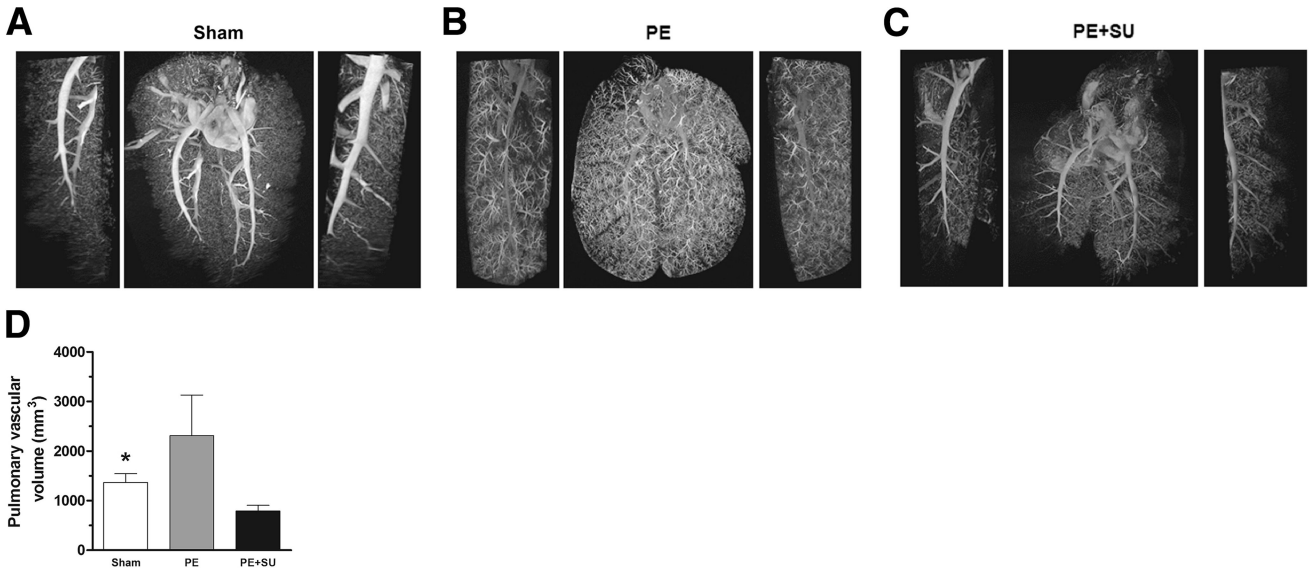

Figure 12. Lung computed tomography imaging in experimental CTEPH

A. Representative three-dimensional micro–computed tomographic images and magnifications (left and right sides) of sham rats injected with vehicle only.

B. Pulmonary embolism (PE) rats injected with microspheres only.

C. PE + SU6416 rats injected with both microspheres and SU5416 (20 mg/kg).

D. Quantification of the pulmonary vascular volume in sham, PE, and PE+SU groups. N-4 per group. *P<0.05 for sham versus PE+SU group. Reproduced from Neto-Neves *et al.* with permission.[40]

Table 1

Molecular pathways implicated in Group 2 pulmonary hypertension.

Pathway	Molecular Mechanism	Consequence	Animal Model-Species	Author (year)[ref]
ET-1	A functional shift of ET receptor subtype towards ET_A occurs in Group 2 PH	In CHF, responses via the ET_B receptor are reduced and responses via the ET_A receptors are enhanced in LV and PA	CHF induced by rapid ventricular pacing-dogs	Tadano (2004)[57]
NO-sGC-cGMP	Impaired endothelial $[Ca^{2+}]_i$ oscillation	Reduced NO synthesis, decreased endothelium-dependent vasodilation without alteration of eNOS expression	SAB-rats	Kerem (2010)[73]
PDE5 dysregulation	Despite down-regulation of PDE5 in SAB rats, chemical inhibition of PDE5 preserves endothelial function	Sildenafil, a PDE5 inhibitor, attenuates PH, RVH, and dysfunction in SAB rats	SAB-rats	Yin (2011)[29]
Mitochondrial dysfunction	Smaller mitochondria (area $<0.4\mu m^2$) are more abundant and larger ones (area$>1.6\mu m^2$) rarer in cardiac myocytes of CHF dogs	Profound mitochondria structural abnormalities are present throughout cardiomyocytes in dogs with CHF. Smaller mitochondria portend worse H, indicated by lower EF, higher LVEDP, higher PAWP, and higher plasma NE level	CHF induced by intracoronary multiple embolization with microsphere-dogs	Sabbah (1992)[90]
Mitochondrial dysfunction	Inhibition of mitochondrial fission, promotes angiogenesis, and decreases mitophagy	Mdivi-1 improves cardiac function and decreases cardiac fibrosis in TAC mice	TAC-mice	Givvimani (2012)[12]
RhoA/Rho-kinase inhibition (direct)	Fasudil decreased Rho-kinase activity, enhanced eNOS expression, increased NO and cGMP, and decreased ET-1 level	Fasudil 30 mg/kg/day decreases mPAP, attenuates RVH and pulmonary arteriolar medial hypertrophy	SAB-rats	Dai (2011)[28]
RhoA/Rho-kinase inhibition (indirect)	Inhibition of HMG-CoA reductase decreases isoprenoids production and subsequent isoprenylation (and inhibition) of Rho-kinase	Atorvastatin 10 mg/kg/day decreases mPAP, RVH, and pulmonary arteriolar medial thickness by down-regulating RhoA/Rho kinase expression	SAB-rats	Wang (2016)[30]
Metabolic syndrome	Genome-Wide association study identified increased EGFR expression in lungs of mice that develop the most severe PH after exposure to a high fat diet	Increased EGFR expression is associated in the susceptibility to develop PH with metabolic syndrome (increased RVSP)	High fat diet-mice	Kelly (2017)[105]

Abbreviations: ET=Endothelin; CHF=congestive heart failure; LV=left ventricle; PA=pulmonary artery; NO=nitric oxide; sGC=soluble guanylate cyclase; cGMP=cyclic guanosine monophosphate; $[Ca^{2+}]_I$=intracellular calcium concentration; eNOS=endothelial nitric oxide synthase; SAB=supracoronary aortic banding; PDE5=phosphodiesterase 5; PH=pulmonary hypertension; RVH=right ventricle hypertrophy; EF=ejective fraction; LVEDP=left ventricle end-diastolic pressure; PAWP=pulmonary artery wedge pressure; NE=norepinephrine; TAC=transverse aortic

constriction; mPAP=mean pulmonary arterial pressure; HMG-CoA=3-hydroxy-3-methyl-glutaryl-coenzyme A reductase; EGFR=endothelin growth factor receptor; RVSP=right ventricle systolic pressure;

Table 2

Molecular pathways implicated in Group 3 pulmonary hypertension.

Pathway	Molecular Mechanism	Consequence	Animal Model-Species	Author (year)[ref]
Endothelial AMPK inhibition	Inducible knockdown of eAMPK (generated by crossing AMPK floxed mice with Tie2-Cre mice on a C57BL/6 background) increases VEGF, IL-6, PDGF-BB, and FGF-2; decreases expression of p53 and eNOS in hypoxic lung	eAMPK knockdown mice are more susceptible to hypoxia induced PH than WT mice. Metformin up-regulates eAMPK and ameliorates hypoxia-induced PH	Chronic hypoxia-mice	Omura (2016)[121]
PDE5 activation	Sildenafil decreases RhoA/ROCK, iNOS activity, and ROS generation whilst increasing PKG and eNOS activity	Sildenafil improves survival, reduces RVH and fibrosis, and pulmonary vascular medial hypertrophy in bleomycin-induced pH	Bleomycin-induced PH -mice	Hemnes (2008)[39]
HIF-1α activation	Genetic haploinsufficiency of HIF-1α decreases NHE1 and TRPC1 expression, which prevents hypoxia induced alkalinization and elevation of $[Ca^{2+}]_I$ in PASMC, respectively	Reduced HIF-1α activation decreases polycythemia, RV hypertrophy, PH, and adverse pulmonary vascular remodeling in HIF1a$^{+/-}$ mice	Chronic hypoxia-mice and rats	Shimoda (2006)[132] Wang (2006)[133]
HIF-1α activation	inhibition of HIF-1α transcriptional activity by digoxin has therapeutic benefit	Treatment with digoxin (0.2 or 1 mg/kg/d, IP) prevented attenuates RV hypertrophy, prevented increases in RV pressure, PASMC $[Ca^{2+}]_i$ and pH_i	Chronic hypoxia-mice	Abud (2012)[134]
Mitochondria-HIF-1α-Kv	Hypoxia decreases mitochondria ROS production and activatesHIF-1α, which in turn suppresses PASMC and cardiac myocytes Kv channel expression and alters metabolism (favoring uncoupled glycolysis). This increases cell proliferation and vasoconstriction (in vessels) and promotes hypertrophy and impairs contractility (in cardiomyocytes).	Dysfunction in this pathway promotes adverse pulmonary vascular remodeling and RV dysfunction in PH. DCA, a mitochondrial PDK inhibitor, shifts redox balance towards oxidized state and restored Kv channel expression and activates PDH restoring glucose oxidation. This latter change regresses vascular obstruction by increasing PASMC apoptosis and enhances RV function.	Chronic hypoxia-rats and the spontaneously pulmonary hypertensive fawn hooded rat	Michelakis (2002)[140] Bonnet (2006)[88]
Mitochondria-HIF-1α	Chronic hypoxia decreases mitochondria ROS production and promotes HIF-1α activation	HIF-1α activation facilitates development of PH and exacerbated the associated pulmonary vascular remodeling	Chronic hypoxia-mice	Nakada (2017)[139]

Pathway	Molecular Mechanism	Consequence	Animal Model-Species	Author (year)[ref]
CyPA-Basigin	CyPA promotes inflammatory cytokine release and Bsg increases CyPA, PDGF-BB, and ERK1/2 phosphorylation	Mice engineered to have partial CyPA or Bsg deficiency in $CyPA^{+/-}$ or $Bsg^{+/-}$ have reduced RVSP, pulmonary arterial remodeling, and RV hypertrophy compared $CyPA^{+/+}$ or $Bsg^{+/+}$ mice in response to chronic hypoxia.	Chronic hypoxia-mice	Satoh (2014)[14]

Abbreviations: AMPK: adenosine monophosphate-activated protein kinase; eAMPK: endothelial AMPK; PDE5=phosphodiesterase 5; ROCK=Rho-associated protein kinase; iNOS=inducible nitric oxide kinase; PKG=cGMP-dependent protein kinase; eNOS=endothelial nitric oxide kinase; HIF=hypoxia inducible factor; NHE1=sodium-hydrogen antiporter 1; TRPC1=transient receptor potential channel 1; IP=intraperitoneal; RV=right ventricle; PASMC=pulmonary arterial smooth muscle cell; $[Ca^{2+}]_I$=intracellular calcium concentration; pH_i=intracellular pH; ROS=reactive oxygen species; CyPA=cyclophilin A; Bsg=basigin; PDGF-BB=platelet-derived growth factor-BB; ERK1/2=extracellular signal-regulated kinase 1 and 2; Kv=voltage-gated potassium channel; FHR=Fawn Hooded Rats: DCA=dichloroacetate; PDK=pyruvate dehydrogenase kinase

Table 3

Molecular pathways implicated in Group 4 pulmonary hypertension.

Pathway	Molecular Mechanism	Consequence	Species	Author (year)[ref]
NO-sGC-cGMP	CTEPH patients have elevated plasma ADMA levels, a potent endogenous NOS inhibitor and lower eNOS expression compared to controls	Endogenous NOS inhibition by ADMA is associated with development of CTEPH	Human	Skoro-Sajer (2007)[149]
ET-1	Plasma ET-1 levels are increased in dogs with CTEPH induced by repeated embolization with ceramic beads	Treatment with bosentan, an endothelin receptor antagonist, decreases PAP, PVR, and pulmonary arterial medial hypertrophy in dogs with CTEPH	CTEPH embolization model-dogs	Kim (2000)[150]
ET-1	CTEPH patients have elevated plasma ET-1 levels	Plasma ET-1 levels correlated positively with severity of CTEPH. ET-1 levels > 1.77 pg/mL are associated with a poor PTE postoperative outcomes	Human	Reesink (2006)[151]
Platelet dysfunction	Platelets in CTEPH patients are hypersensitive to thrombin stimulation compared to platelets in controls	Activated platelets have higher expression of VCAM-1, which is an important mediator of immune cells adhesion to vascular endothelium and is associated with development of PH	Human	Yaoita (2014)[157]
Fibrinolytic abnormalities	TAFI, a plasma carboxypeptidase, decreases binding capacity of plasminogen activator and plasmin to fibrin by removing the C-terminal lysines from the fibrin	TAFI plasma levels are significantly higher in CTEPH patients compared to controls. Impairment of fibrinolysis is improved with a TAFI inhibitor, CPI-2KR	Human	Yaoita (2016)[158]

Abbreviations: NO=nitric oxide; sGC=soluble guanylate cyclase; cGMP=cyclic guanosine monophosphate; CTEPH=chronic thromboembolic pulmonary hypertension; ADMA=asymmetric dimethylarginine; N/A=not applicable (human subjects); ET=endothelin; PTE=pulmonary thromboendarterectomy; PAP=pulmonary arterial pressure; PVR=pulmonary vascular resistance; VCAM-1=vascular cell adhesion protein-1; PH=pulmonary hypertension; TAFI=thrombin activatable fibrinolysis inhibitor; CPI-2KR=carboxypeptidase R inhibiting peptide-2KR;N/A=not applicable

Table 4

Key knowledge gaps and future directions for Group 2, 3, and 4 pulmonary hypertension research.

Key knowledge gaps
• Optimal pre-clinical models that recapitulate many forms of Group 2 PH (e.g. mitral stenosis), Group 3 PH (e.g obstructive sleep apnea), and Group 4 PH do not exist or are not well standardized and easy to use.
• Preclinical therapeutic trials require greater rigour in terms of duration, endpoints and characterization of cardiopulmonary function and structure. Optimal assessment of PH should include characterization of the pulmonary vasculature and right ventricle and careful measurement of diastolic function of the left ventricle. This requires advanced hemodynamic studies in closed-chest animals. Broader application of high fidelity ultrasound, cardiac catheterization, micro-CT, positron emission tomography and magnetic resonance imaging is required to better phenotype Groups 2–4 PH and measure response to experimental therapeutics.
• The importance of age, sex, diet and strain differences on hemodynamics, lung and heart histology, and mortality in preclinical models of Group 2–4 PH is poorly understood.
• The mechanism by which disproportional PH develops in Group 2 and 3 patients is not well understood. It is unclear, for example, why similar elevations of LVEDP or LA pressure elicits severe PH in some patients and experimental animals while only eliciting a modest increase in PVR in others.
• Can disordered molecular pathways that are shared by several PH groups be therapeutically exploited to create more universally applicable PH-targeted therapies?
• The importance of changes in mitochondrial metabolism and mitochondrial dynamics in Group 2–4 PH is unknown.
• The role of genetic and epigenetic dysregulation in Group 2–4 PH is largely unknown.
• It is poorly understood why a second insult, in addition to aortic banding, hypoxia, and pulmonary embolism, is often needed to generate severe PH in preclinical models. The role of adverse stimuli, such as endothelial dysfunction, inflammation, impaired angiogenesis or metabolic syndrome, in the genesis of Group-4 PH patients remains poorly characterized. These PH-promoting abnormalities are not factored into the WHO classification system nor are they included in the standard operating procedures for creation of most preclinical PH models.
• The prevalence and prognostic impact of adaptive versus maladaptive right ventricular responses to Group 2–4 PH is poorly understood.
• Most preclinical studies of PH mechanisms focus on a single molecular pathway. A systems biology approach that addresses the interaction of pathways is needed.
• Therapies for Group 1 PH are ineffective in Group 2–3 PH and the optimal molecular targets for Group 2–4 PH remain poorly defined. Therefore, better understanding of the basic mechanisms underlying each of these PH groups is required to identify new therapeutic targets. This will require integrated use of preclinical models and human cells. The value of blood out growth endothelial cells (BOECs) as readily accessible, disease-relevant cells remains unknown.
• The potential value of modifying the current PH classification system based on better understanding of molecular mechanisms is unexplored. Could knowledge of pathologic mutations, epigenetic modifications and altered signaling pathway function refine the WHO classification system to better support a more personalized medicine approach to diagnosis and treatment?

Abbreviations: PH=pulmonary hypertension; PAH=pulmonary arterial hypertension; LVEDP=left ventricle end diastolic pressure; LA=left atrium;