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INVITED REVIEW

Pulmonary arterial hypertension: Sex matters

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Pulmonary arterial hypertension (PAH) is a complex disease of multifactorial origin. While registries have demonstrated that women are more susceptible to the disease, females with PAH have superior right ventricle (RV) function and a better prognosis than their male counterparts, a phenomenon referred to as the 'estrogen paradox'. Numerous pre-clinical studies have investigated the involvement of sex hormones in PAH pathobiology, often with conflicting results. However, recent advances suggest that abnormal estrogen synthesis, metabolism and signalling underpin the sexual dimorphism of this disease. Other sex hormones, such as progesterone, testosterone and dehydroepiandrosterone may also play a role. Several non-hormonal factor including sex chromosomes and epigenetics have also been implicated. Though the underlying pathophysiological mechanisms are complex, several compounds that modulate sex hormones levels and signalling are under investigation in PAH patients. Further elucidation of the estrogen paradox will set the stage for the identification of additional therapeutic targets for this disease.

KEYWORDS
estrogen, pulmonary hypertension, right ventricle, sex chromosomes, sex hormones

1 | INTRODUCTION

Pulmonary hypertension (PH) encompasses a syndrome of diseases characterised by a progressive rise in pulmonary vascular resistance and pulmonary arterial pressure, eventually leading to right heart failure and death (Hassoun, 2021). From a hemodynamic perspective, PH is defined by a mean pulmonary arterial pressure of greater than 20 mmHg at rest (Simonneau et al., 2019). PH is classified into five clinical groups depending on aetiology and pathophysiology (Hoeper, Bogaard, et al., 2013; Humbert et al., 2023). This review focuses on pulmonary arterial hypertension (PAH), a rare but severe form of PH associated with extensive remodelling of the small pulmonary arteries (Tuder et al., 2013). PAH can be idiopathic (accounting for 50% of cases), heritable (with a family history of genetic mutation) or associated with connective tissue diseases (e.g. systemic lupus erythematosus), drugs and toxins (including anorexigens, such as **dexfenfluramine**), portal hypertension (portopulmonary hypertension), HIV infection or congenital heart disease (Simonneau et al., 2019). Outcomes vary between these subgroups, but overall 5-year survival is only 60% (Badesch et al., 2010; Gall et al., 2017).

While women are at greater risk of developing PAH than men, once the disease has manifested they present with a better prognosis (Humbert, Sitbon, Chaouat, et al., 2010) due, at least partially, to superior right ventricular adaptation (Jacobs et al., 2014). Beyond the right ventricle (RV), women with PAH also tend to have more favourable haemodynamic profiles (Ventetuolo et al., 2023). Sex also determines the response to approved PAH therapies, with women responding more

Abbreviations: E1, estrone; E2, 17β-estradiol, estradiol; E3, estriol, 16-hydroxyestradiol; ECs, endothelial cells; ERs, estrogen receptors; miRNA, microRNA; PAH, pulmonary arterial hypertension; PH, pulmonary hypertension; RV, right ventricle; RVEF, right ventricular ejection fraction; RVH, right ventricular hypertrophy; SMCs, smooth muscle cells; SuHx, Sugen–hypoxia; TMS, 2,3′,4,5′-tetramethoxystilbene.

favourably to **endothelin receptor** antagonists (Gabler et al., 2012) and men responding more favourably to the **phosphodiesterase type 5** inhibitor **tadalafil** (Mathai et al., 2015; Rusiecki et al., 2015).

A growing body of evidence suggests that sex hormones, especially estrogens and their metabolites, influence and drive the development of PAH (Lahm et al., 2014). However, manipulation of **17β-estradiol (E2; estradiol, estrogen)** in pre-clinical PH models has produced conflicting and contradictory results (Hester et al., 2019), highlighting the complexity of this pathway and/or the involvement of non-hormonal mechanisms. Estrogens are an attractive therapeutic target in PH, as their activity is readily modulated by drugs already approved for the treatment of estrogen-sensitive breast cancers (Lumachi et al., 2015). Indeed, several clinical trials have been recently completed or are ongoing to evaluate therapies that antagonise estrogen production (**anastrozole**; ClinicalTrials.gov identifier: NCT03229499) or signalling (**tamoxifen** and **fulvestrant**; Kawut et al., 2019; ClinicalTrials.gov identifier: NCT03528902) in PAH patients. The effects of the endogenous sex hormone precursor **dehydroepiandrosterone (DHEA)** on right ventricle (RV) function are also under investigation in a cohort of PAH patients (ClinicalTrials.gov identifier: NCT03648385).

The aim of this review is to summarise current evidence and understanding of sexual dimorphisms in PAH, focusing on (i) altered estrogen synthesis, metabolism and signalling; (ii) other sex hormones; and (iii) non-hormonal factors, and the potential for targeting these pathways for therapeutic benefit in PAH.

2 | A TALE OF SEX, SUSCEPTIBILITY AND SURVIVAL

PAH registries consistently report a higher female to male ratio, ranging from 1.4:1 in the UK/Ireland registry (Ling et al., 2012) to 1.6:1 in the European COMPERA registry (Hoeper, Huscher, et al., 2013), all the way up to 4.1:1 in the North American REVEAL registry (Badesch et al., 2010). Notably, COMPERA found that the female to male ratio of PAH patients varies dramatically with age, declining from 2.3:1 in subjects aged 18–65 to 1.2:1 in those older than 65 (Hoeper, Huscher, et al., 2013). While women are at greater overall risk of developing PAH, men consistently demonstrate poorer outcome (Benza et al., 2010; Humbert et al., 2010; Olsson et al., 2014), as evidenced by a 10% reduction in 5-year survival (Kjellstrom et al., 2019; Shapiro et al., 2012). These seemingly opposing observations regarding female sex and PAH susceptibility and survival have come to be described as the 'estrogen paradox'.

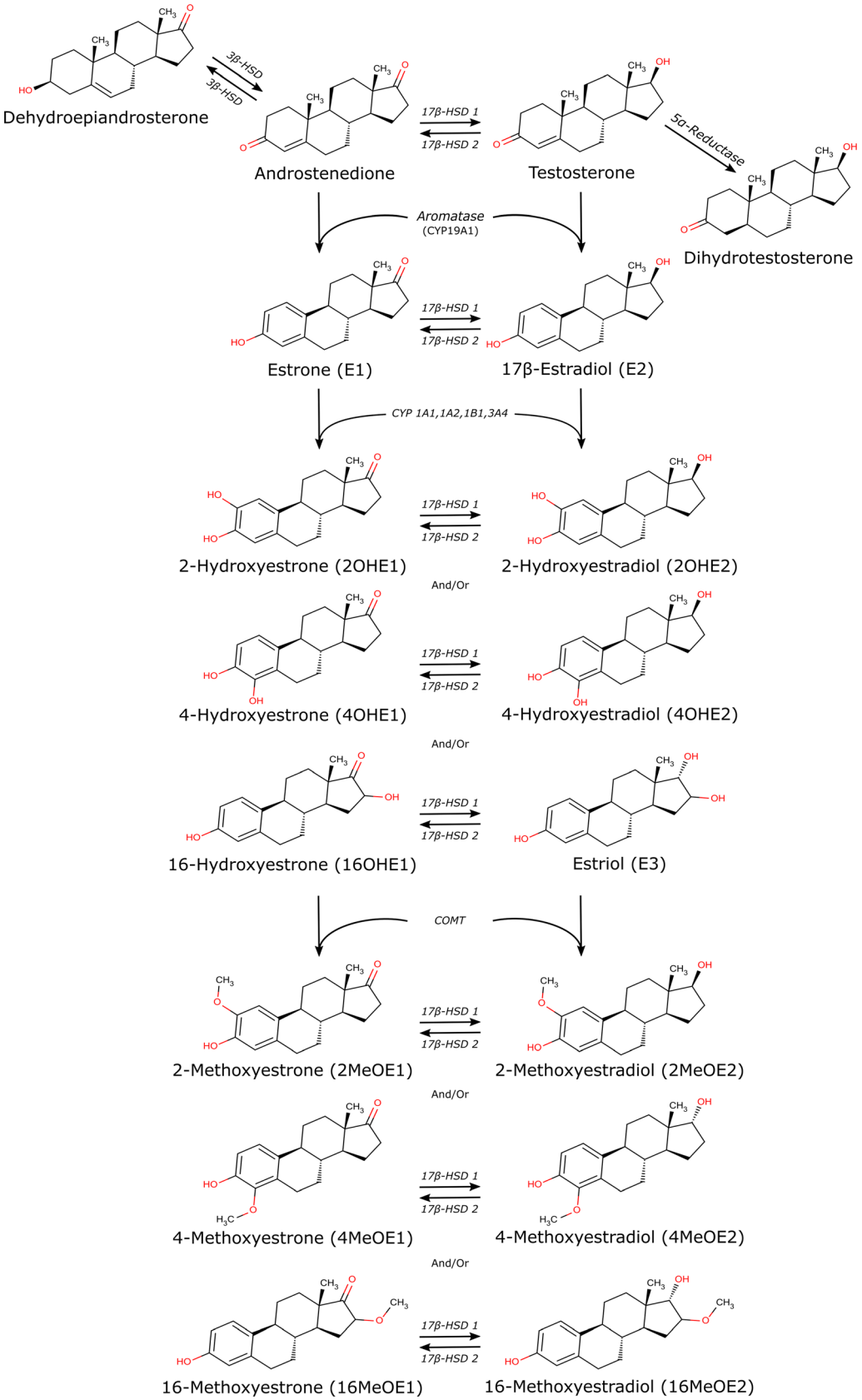
3 | PATHOPHYSIOLOGY OF PULMONARY ARTERIAL HYPERTENSION (PAH)

Our understanding of the cellular changes and molecular regulators responsible for PAH pathobiology has improved tremendously in recent years (de Jesus Perez, 2016). The central dogma is that PAH pathogenesis is driven by one or more 'hits', injurious environmental factors such as hypoxia, inflammation, drugs/toxins, viruses and shear stress, on a background of genetic predisposition (Hemnes & Humbert, 2017). Altered expression of growth factors, ion channels, hormones and cytokines impact downstream signalling cascades, fuelling the recruitment and activation of transcription factors that promote endothelial dysfunction, inflammation, metabolic dysfunction and oxidative stress in the pulmonary vascular wall (de Jesus Perez, 2016; Humbert et al., 2004, 2019). These changes ultimately confer a proliferative, apoptotic-resistant phenotype on pulmonary vascular cells, including endothelial cells (ECs), smooth muscle cells (SMCs), adventitial fibroblasts and inflammatory cells (Voelkel et al., 2012). The gradual obstruction and obliteration of small pulmonary arteries is thought to drive the progressive increase in pulmonary vascular resistance and pulmonary arterial pressure (Tuder et al., 2013). Pathological hallmarks of PAH include intimal and adventitial fibrosis of the pulmonary arteries, increased medial thickness, in situ thrombosis, perivascular inflammation and occlusive plexiform lesions (Hu et al., 2020; Tuder et al., 2013). As lung samples are typically acquired during transplantation or post-mortem, these pathological changes reflect severe, terminal PAH, rather than the initial stages of the disease (Hamid & Austin, 2019).

Heterozygous mutations in the **bone morphogenetic protein receptor 2 (BMPR2)** gene are the principal genetic risk factor for PAH (Machado et al., 2009). More than 400 mutations predicted to reduce the expression and activity of the BMPR2 protein have been described in PAH patients (Best et al., 2014). These mutations are present in 70% to 80% of hereditary PAH cases and 10% to 20% of idiopathic PAH cases (Evans et al., 2016). Intriguingly, BMPR2 expression is decreased even in peripheral lung tissue and blood outgrowth ECs from PAH patients without mutations (Atkinson et al., 2002; Lavoie et al., 2014), and in the pulmonary vasculature and lungs of several non-transgenic experimental models of PH (Long et al., 2009; Morty et al., 2007; Takahashi et al., 2006).

BMPR2 is a transmembrane serine/threonine receptor kinase belonging to the **transforming growth factor beta-1** superfamily of receptors. BMPR2 signals via the activation of small mothers against decapentaplegic (**Smad**) transcription factors, which translocate to the nucleus to regulate the expression of target genes (Tielemans et al., 2019). Molecular studies have positioned BMPR2 as a key opponent of pulmonary vascular remodelling, enhancing pulmonary artery EC growth and survival in pro-apoptotic conditions (Masri et al., 2007; Teichert-Kuliszewska et al., 2006), and neutralising the mitogenic effects of growth factors on pulmonary artery SMCs (Davies et al., 2012; Morrell et al., 2001; Yang et al., 2005). Despite the importance of BMPR2 to PAH pathobiology, the penetrance of BMPR2 mutations is low, with only 20% to 30% of carriers developing the disease (Hamid et al., 2009; White & Morrell, 2012). Notably, when stratified by sex, penetrance is approximately 15% for males and 40% for females (Larkin et al., 2012), which had led to speculation that female sex hormones act as a second hit to initiate PAH pathogenesis.

Sotatercept is a fusion protein that traps **activins** and other growth factors from the transforming growth factor beta superfamily,



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restoring the balance between these growth promoting pathways and the anti-mitogenic bone morphogenetic protein pathway (Yung et al., 2020). Milestone clinical trials of male and female PAH patients have reported favourable effects of sotatercept on exercise capacity, pulmonary vascular resistance and time to death or clinical worsening (Hoeper et al., 2023; Humbert et al., 2021). Further phase 3 studies are ongoing to determine the efficacy of sotatercept in newly diagnosed patients (ClinicalTrials.gov identifier: NCT04811092) and individuals with a high risk of mortality (ClinicalTrials.gov identifier: NCT04896008).

4 | ESTROGENS: AN OVERVIEW

Sex hormones, including the estrogens and androgens, are circulating steroid hormones that govern sexual differentiation and reproduction. In humans, there are three main estrogens:- **estrone (E1)**, 17β-estradiol (E2) and **estriol (E3, 16-hydroxyestradiol [16OHE2])**. E2 is predominantly secreted by the ovaries of premenopausal women and is primarily responsible for reproductive function and the development and maintenance of secondary sexual characteristics (Tang et al., 2019). The significance of the less potent E1 increases dramatically after the menopause, when the major source becomes adipose tissue (Cui et al., 2013). Peripheral tissues, including adipose, skin and the cardiovascular system, are also the main source of estrogens in men, with the gonads making a smaller contribution (Harada et al., 1999; Park, 2000). During pregnancy, the placenta uptakes steroid precursors from the foetus and converts them into E3, the least potent of the major estrogens, which is then released into the maternal circulation (Morel et al., 2016). Outside of the reproductive system, sex hormones also hold important roles in metabolism, immunity and cardiovascular function (Bouman et al., 2005; Faulkner & Belin de Chantemele, 2019; Lim, 2021; Willemars et al., 2022). The major sex hormone pathways are summarised in Figure 1.

5 | SEX AND THE PULMONARY VASCULATURE: LESSONS FROM ANIMAL MODELS

The so-called 'classical' models of PH include exposure to chronic hypoxia or the pyrrolizidine alkaloid plant toxin monocrotaline. It has long been known that female rats exposed to either monocrotaline or chronic hypoxia develop less severe PH than their male counterparts (Farhat et al., 1993; Kiyatake et al., 1994). Furthermore, ablation of endogenous E2 production by ovariectomy exacerbates PH in these models (Ahn et al., 2003; Earley & Resta, 2002; Farhat et al., 1993; Umar et al., 2011) and increases mortality in a model of PH secondary to pulmonary fibrosis (Ahn et al., 2003; Earley & Resta, 2002; Farhat et al., 1993; Umar et al., 2011). Importantly, E2 supplementation attenuates PH and improves survival in male and ovariectomised female rats treated with monocrotaline (Tofovic, Zhang, Jackson, et al., 2009). Taken together, these observations suggest that E2 exerts favourable effects on the pulmonary circulation, a notion at odds with the female predominance of clinical PAH.

It is important to note that the degree of pulmonary vascular remodelling in the classical models of PH is relatively mild, largely affects the medial layer and is accompanied by little change in endothelial architecture (Dignam et al., 2022). In contrast, combining **vascular endothelial growth factor receptor 2** inhibition with Sugen along with hypoxia (SuHx) results in endothelial disruption and occlusive pulmonary vascular lesions reflective of clinical PAH (Abe et al., 2010; Taraseviciene-Stewart et al., 2001). Given that E2 has direct pro-mitogenic, pro-angiogenic and anti-apoptotic effects on ECs (Chakrabarti et al., 2014), this model may be more appropriate for studying the effects of E2 on PAH. Supporting studies have found that female SuHx rats have higher cardiopulmonary pressures, greater medial thickening, more occlusive intimal lesions and yet superior survival versus male animals (Rafikova et al., 2015; Tofovic et al., 2012; Tofovic & Rafikova, 2009). In this clinically relevant model, the detrimental effects of endogenous E2 on the pulmonary vasculature model of PH can be antagonised with anastrozole, which also reverses PH in several other non-classical PH models (Chen et al., 2017; Mair et al., 2014, 2019). However, findings have not been concordant; others have found that ovarian-derived and exogenous E2 attenuate PH in the SuHx rat (Frump et al., 2015; Lahm et al., 2016; Philip et al., 2020).

Interestingly, evidence from animal models suggests that the **5-hydroxytryptamine (5-HT; serotonin)** pathway plays an important role in PH development in females. Administration of dexfenfluramine, a 5-HT based anorectic drug associated with clinical PAH (Kramer & Lane, 1998), induces PH in female mice only (White, Dempsie, et al., 2011). Furthermore, overexpression of the human genes for the **serotonin transporter (SERT+)** or S100A4/Mts1 (which functions downstream of 5-HT) results in a spontaneous PH that is specific to female animals (Dempsie et al., 2011; White, Dempsie, et al., 2011). In the SERT+ mouse, PH can be abolished by

FIGURE 1 Sex hormone pathways. The 3β-hydroxysteroid dehydrogenase (3β-HSD) family of enzymes catalyse the interconversion of precursor steroid dehydroepiandrosterone and the intermediate androstenedione. The 17β-hydroxysteroid dehydrogenase (17β-HSD) enzymes catalyse the interconversion of androstenedione and testosterone, which can also be reduced by 5α-reductase, forming dihydrotestosterone. Aromatase converts androstenedione and testosterone to estrone and estradiol, respectively. Several cytochrome P450 (CYP) enzymes (CYP1A1, CYP1B1, CYP1A2 and CYP3A4) catalyse the oxidation of E1 and E2 to hydroxy-estrogens, including 16-hydroxyestrone and 16-hydroxyestradiol. The 17β-HSD enzymes catalyse the interconversion of E1 (estrone) and E2 (17β-estradiol, estradiol) and their respective metabolites. The terminal step of this process involves the O-methylation of hydroxy metabolites by catechol-*O*-methyltransferase (COMT), forming methoxy metabolites such as 2-methoxyestrone and 2-methoxyestradiol. *Created using Inkscape v1.2.1 and MarvingSketch v20.15.*

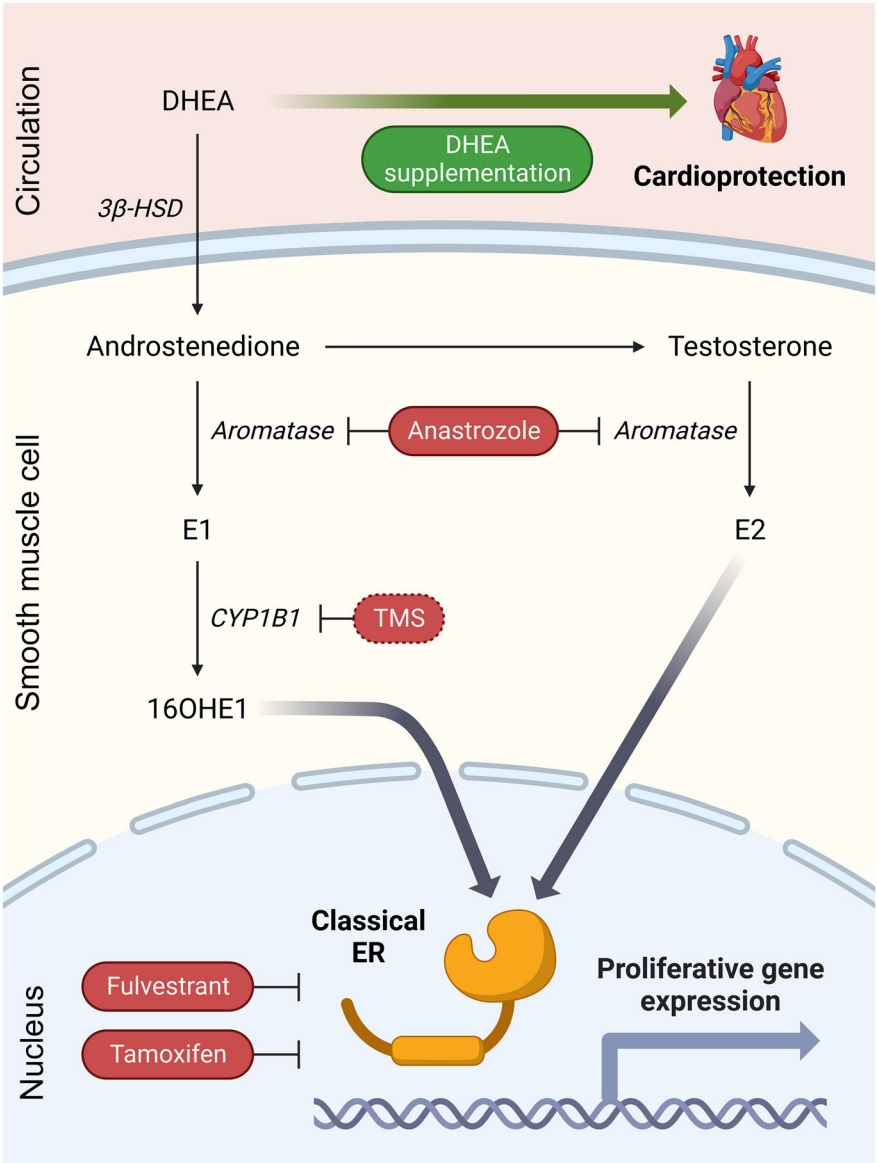

FIGURE 2 Sex hormone-targeted therapies under investigation in pulmonary arterial hypertension (PAH). Several compounds are currently under evaluation in clinical trials of PAH patients. The aromatase inhibitor anastrozole blocks estradiol (E2) synthesis. Fulvestrant and tamoxifen reduce estrogenic signalling by down-regulating or modulating classical estrogen receptors (ERs), respectively. Dehydroepiandrosterone (DHEA) is an endogenous sex hormone precursor with cardioprotective properties. The cytochrome P450 1B1 (CYP1B1) inhibitor 2,3′,4,5′-tetramethoxystilbene (TMS) has yielded promising results in pre-clinical studies. It functions by preventing the formation of 16-hydroxyestrone (16OHE1), an estrone (E1) metabolite with mitogenic properties in pulmonary vascular cells. 3β-HSD, 3β-hydroxysteroid dehydrogenase (AKR1C3). *Created in BioRender*.

ovariectomy and re-established by E2 repletion (White, Dempsie, et al., 2011). Taken together, these results indicate a key role for the 5-HT pathway in the sexual dimorphism of PAH.

6 | TARGETING ESTROGEN SYNTHESIS

Estrogen biosynthesis is mediated by the enzyme **aromatase (CYP19A1)**, a member of the cytochrome P450 (CYP) superfamily (Simpson & Davis, 2001). Aromatase converts **androstenedione** and **testosterone** to E1 and E2, respectively (Hemsell et al., 1974). The major site of aromatase expression in premenopausal women is the ovarian granulosa cells (Grodin et al., 1973), transitioning to adipose tissue after the menopause (Bulun & Simpson, 1994). Downstream of aromatase, estrogenic activity is highly influenced by **17β-hydroxysteroid dehydrogenase (AKR1C3/17β-HSD)** enzymes, which catalyse the interconversion of E1 and E2 (and their respective metabolites), maintaining a constant equilibrium (Figure 1).

6.1 | Altered E2 production in PAH

Extensive clinical data indicate that increased extra-gonadal E2 synthesis plays a role in the pathogenesis of PAH. In postmenopausal females and men with idiopathic PAH, circulating E2 is elevated and correlates with disease severity (Baird et al., 2018, 2021; Denver et al., 2020; Ventetuolo, Baird, et al., 2016; Wu et al., 2018). Recent findings suggest that plasma E2 levels are also elevated in premenopausal women with PAH (Baird et al., 2021). Furthermore, genetic variation in aromatase is associated with elevated circulating E2 and PAH risk in liver disease patients, irrespective of sex (Al-Naamani et al., 2021; Roberts et al., 2009).

TABLE 1 Pre-clinical studies exploring pharmacological manipulation of sex hormone pathways in experimental PH models.

Target	Drug	Class	Species	Model	Sex	Major findings	References
Estrogen synthesis	Anastrozole	Aromatase inhibitor	Mouse	CH	Both	↓ PH (females only)	Mair et al. (2014)
			Rat	SuHx	Both	↓ PH, including occlusive neointimal lesions (females only)	Mair et al. (2014)
			Mouse	Obese ob/ob	Both	↓ PH, particularly under hypoxic conditions; ↓ ROS production in the lung	Mair et al. (2019)
Estrogenic signalling	MPP dihydrochloride	Selective ER-α antagonist	Mouse	SERT overexpression	Female	↓ PH, particularly under hypoxic conditions	Wright et al. (2015)
	Fulvestrant	Selective ER down-regulator	Mouse	BMPR2 mutant	Female	↓ PH (fulvestrant + anastrozole > tamoxifen); ↓ oxidised lipid deposition in the pulmonary vascular wall; reversal of insulin resistance	Chen et al. (2017)
	Tamoxifen	Selective ER modulator					
	Ormeloxifene	Selective ER modulator	Rat	MCT	Both	↓ PH; ↓ inflammation, mitochondrial dysfunction, apoptosis-resistance and ROS production; normalisation of pulmonary vasoreactivity	Abdulkareem et al. (2023)
Estrogen metabolism	TMS	CYP1B1 inhibitor	Mouse	CH, SuHx	Both	↓ PH	White et al. (2012)
			Mouse	SERT overexpression	Female	↓ PH; no effect on oxidative stress in the lung or RVH	Johansen et al. (2016)
			Rat	MCT	Both	No effect on PH; ↓ RVH and mortality in males	Johansen et al. (2016)
			Mouse	Obese ob/ob	Male	↓ PH	Mair et al. (2019)
Other sex hormone pathways	DHEA	Steroid hormone supplement	Rat	CH	Male	↓ PH; normalisation of pulmonary vasoreactivity; ↑ soluble guanylate cyclase expression in pulmonary arteries; ↑ PASMC BK_{Ca} activation	Bonnet et al. (2003); Hampl et al. (2003); Oka et al. (2007)
			Rat	MCT + PNX	Male	↓ PH (including occlusive neointimal lesions) and mortality; normalisation of RhoA/ROCK signalling in the lung	Homma et al. (2008)
			Rat	MCT	Unknown	↓ PH; ↑ exercise capacity; ↓ Src/STAT3 activation in the lung and distal pulmonary arteries	Paulin et al. (2011)
			Rat	CH + reoxygenation	Male	Improvement in RV systolic and diastolic dysfunction; ↑ RV myocyte density and ↓ mitochondrial fragmentation	Dumas de La Roque, Bellance, et al. (2012)
			Rat (juvenile)	CH	Unknown	↓ PH; pulmonary vasodilatation; ↑ PASMC BK_{Ca} activation	Dumas de La Roque et al. (2013)
			Rat	SuHx	Male		Alzoubi et al. (2013)

(Continues)

TABLE 1 (Continued)

Target	Drug	Class	Species	Model	Sex	Major findings	References
						Moderate reduction in PH; normalisation of cardiac index; ↓ RV capillary rarefaction, apoptosis, fibrosis and oxidative stress; ↓ activity of ROCK, STAT3 and NFATc3 in the RV	

Abbreviations: BK_{Ca}, large conductance Ca^{2+}-activated potassium channel; BMPR2, bone morphogenetic protein receptor 2; CH, chronic hypoxia; DHEA, dehydroepiandrosterone; ER, estrogen receptor; MCT, monocrotaline; NFATc3, nuclear factor of activated T-cells/cytoplasmic 3; PASMC, pulmonary artery smooth muscle cell; PH, pulmonary hypertension; PNX, pneumonectomy; RhoA, Ras homologue family member A; ROCK, Rho-associated protein kinase; ROS, reactive oxygen species; RV, right ventricle; RVH, right ventricular hypertrophy; SERT, serotonin transporter; STAT3, signal transducer and activator of transcription 3; SuHx, Sugen plus hypoxia; TMS, 2,3′,4,5′-tetramethoxystilbene.

Beyond circulating E2, local production of the hormone in the lungs may also contribute to PAH pathobiology. In the pulmonary circulation aromatase is expressed in the arteries, localising to the medial layer. Furthermore, aromatase is highly expressed in control human pulmonary artery SMCs from postmenopausal women versus those of male origin. However, aromatase expression is not further elevated in the human pulmonary artery SMCs of female subjects with PAH (Mair et al., 2014). Control human pulmonary artery SMCs isolated from women also show a greater proliferative response to E2 than those isolated from male donors, which may be driven by reduced basal BMPR2 expression and signalling in the female cells (Mair et al., 2015). Taken together, these observations suggest that locally elevated E2 levels in the lungs of females may enhance human pulmonary artery SMC proliferation.

6.2 | Aromatase inhibition

Pharmacological inhibition of E2 synthesis has been explored as a therapeutic strategy in several experimental models of PH, using the aromatase inhibitor anastrozole (Figure 2 and Table 1). This drug reduces plasma E2 levels in female hypoxia-adapted mice and SuHx rats, alleviating cardiopulmonary pressure overload, pulmonary vascular remodelling and right ventricular hypertrophy (RVH), but has no effect in male animals. These changes are associated with a normalisation of BMPR2 signalling in the lung (Mair et al., 2014). Obese ob/ob mice develop mild PH spontaneously and have increased reactive oxidative species production in the lung. These effects are also reversed by anastrozole (Mair et al., 2019). In addition, the propensity of the anti-diabetic drug **metformin** to reverse PH in female SuHx rats is also dependent on aromatase inhibition (Dean et al., 2016).

Based on this promising pre-clinical data, anastrozole was evaluated in a small randomised, double-blind, placebo-controlled 'proof-of-principle' study of men and postmenopausal women with PAH (Table 2). Anastrozole was well tolerated, reduced circulating E2 by 40% and improved 6-min walk distance (Kawut et al., 2017), a measure of functional exercise capacity (Agarwala & Salzman, 2020). Results are now eagerly awaited from a multicentre double-blind, controlled phase 2 trial of anastrozole in men and postmenopausal women with PAH (PHANTOM; ClinicalTrials.gov identifier: NCT03229499), which has a primary endpoint of change in 6-min walk distance.

7 | TARGETING ESTROGEN METABOLISM

CYP enzymes are haem-thiolate monooxygenases that mediate the oxidative metabolism of endogenous and exogenous compounds, including steroid hormones and lipids (Nebert & Dalton, 2006). **CYP1** and **CYP3** enzymes (**CYP1A1**, **CYP1A2**, **CYP1B1** and **CYP3A4**) convert E1 and E2 to hydroxy-estrogens (OHEs). Oxidation of E1 at position 2, 4 or 16 of the carbon skeleton results in the production of 2-hydroxyestrone (2OHE1), 4-hydroxyestrone (4OHE1) or

TABLE 2 Clinical trials investigating pharmacological manipulation of sex hormone pathways in PAH patients.

Drug	Class	Trial design	Eligible sexes	Enrolment	Primary endpoints	Secondary endpoints	Status	Major findings	Identifiers/ references
Anastrozole	Aromatase inhibitor	Phase 2, double-blind, placebo-controlled	Men and postmenopausal women	18	Plasma E2, TAPSE	6MWD	Completed	↓ plasma E2, ↑ 6MWD	NCT01545336 (Kawut et al., 2017)
		Phase 2, double-blind, placebo-controlled	Men and postmenopausal women	84	6MWD	RV function, plasma NT-proBNP, biomarkers, QoL, physical activity, TTCW, bone mineral density, side effects	Completed	Not yet reported	NCT03229499 (PHANTOM) (Kawut et al., 2023)
Fulvestrant	Selective ER down-regulator	Phase 2, single group, open-label, single-centre	Postmenopausal women	5	Plasma E2, TAPSE, 6MWD, plasma NT-proBNP	None	Completed	No effect on primary outcome measures	NCT02911844 (ERA-PAH) (Kawut et al., 2019)
Tamoxifen	Selective ER modulator	Phase 2, single-centre, double-blind, placebo-controlled	Both	24 (estimated)	TAPSE	6MWD, QoL, plasma BNP, plasma HgbA1c	Recruiting	N/A	NCT03528902 (T3PAH)
DHEA	Steroid hormone supplement	Phase 2, double-blind, placebo-controlled, crossover	Both	24 (estimated)	RV longitudinal strain	RVEF, serum NT-proBNP, sex hormone levels, 6MWD, WHO functional class, QoL, side effects and adverse events	Recruiting	N/A	NCT03648385 (EDIPHY)

Note: Trial status correct as of publication of this review.

Abbreviations: 6MWD, 6-min walk distance; BNP, B-type natriuretic peptide; DHEA, dehydroepiandrosterone; E2, 17β-estradiol; ER, estrogen receptor; HgbA1c, glycated haemoglobin; NT-proBNP, N-terminal pro B-type natriuretic peptide; PAH, pulmonary arterial hypertension; QoL, quality of life; RV, right ventricle; TAPSE, tricuspid annular plane systolic excursion; TTCW, time to clinical worsening.

16-hydroxyestrone (16OHE1), respectively. The equivalent E2 metabolites are 2-hydroxyestradiol (2OHE2), 4-hydroxyestradiol (4OHE2) and E3 (Figure 1) (Lee et al., 2003; Sissung et al., 2006). As their formation does not involve the genesis of unstable intermediates (Jellinck & Fishman, 1988), the 2-OHEs are the most abundant OHEs (Jellinck et al., 1986; Lee et al., 2003). The enzyme **catechol-O-methyltransferase (COMT)** methylates OHEs into methoxy-estrogens (Figure 1), most notably **2-methoxyestradiol (2MeO2)**.

Emerging evidence suggests that it is the relative abundance of parental estrogens and metabolites that ultimately determines the overall biological effect of this pathway in a given tissue, rather than the absolute levels of a given hormone (Han et al., 2018). Indeed, it is postulated that altered estrogen metabolism is central to the estrogen paradox of PAH, favouring the accumulation of mitogenic 16OHEs versus anti-proliferative methylated estrogen metabolites. Differential metabolism may also explain contradictory findings regarding E2 inhibition or supplementation in different animal models of PH.

7.1 | 2/4-Hydroxy-estrogens

The 2/4-OHEs have relatively little estrogenic activity, with fourfold lower binding affinity at **ERs** than E2 (Dubey et al., 2004). These metabolites are detected in human lung tissue with the following order of abundance:- 2OHE1 > 2OHE2 > 4OHE1 > 4OHE2 (Peng et al., 2017). There is a paucity of data describing the actions of the 2/4-OHEs in PAH, though some preliminary studies have been conducted.

2OHE2 was found to attenuate monocrotaline-induced PH in male rats (Tofovic et al., 2005). Furthermore, 4OHE2 increases BMPR2 signalling in male human pulmonary artery SMCs, impairing the expansion of these cells. In contrast, 4OHE2 treatment reduces BMPR2 signalling in female control human pulmonary artery SMCs but does not influence proliferation (Mair et al., 2015). Supporting this, 4OHE2 does not offset the development of PH in female SuHx rats (Bilan et al., 2013). These sex-dependent effects of 4OHE2 on the BMPR2 pathway may explain why basal BMPR2 signalling is restrained in control human pulmonary artery SMCs isolated from females compared with those from male donors (Mair et al., 2015). 2OHE1 and 4OHE1 have not been studied in the context of PAH, however urinary 2OHE1 levels are known to be decreased in breast cancer patients (Ho et al., 1998) and this OHE has anti-proliferative effects in experimental tumour models (Bradlow et al., 1996).

7.2 | 16-Hydroxy-estrogens

An extensive body of evidence suggests that 16OHE synthesis is elevated in patients with PAH. To date, studies have overwhelmingly focused on 16OHE1, which has comparable estrogenic activity to E2 (Fishman & Martucci, 1980). In a small cohort of idiopathic PAH patients, serum 16OHE1 was elevated in male patients and correlated with disease severity, while E3 was increased in female patients (Denver et al., 2020). Furthermore, E3 also accumulates in the blood of patients with portopulmonary hypertension and these individuals also exhibit a lower urinary 2OHE/16OHE1 ratio (Al-Naamani et al., 2021), indicative of increased 16OHE1 activity. Also, E3 is strongly associated with pregnancy, a risk factor for PAH (Hemnes et al., 2015).

The role of 16OHE1 in PAH pathophysiology has been further elucidated by studies using animal models of PH. Urinary 16OHE1 is increased in female hypoxia-adapted mice and 16OHE1 administration evokes pulmonary vascular remodelling and RVH in these animals (White et al., 2012). 16OHE1 also promotes the growth of human pulmonary artery SMCs from PAH patients (Hood et al., 2016; White et al., 2012), which is mediated, at least partially, by up-regulation of **NADPH oxidase 1 (NOX 1)** and increased production of reactive oxygen species. Accordingly, mice lacking NOX1 demonstrate resistance to hypoxia-induced PH (Hood et al., 2016).

Emerging evidence supports an influential role for 16OHE1 in BMPR2 mutation penetrance. In a study by Austin et al. (2009), the urinary 2OHE/16OHE1 ratio of BMPR2 mutation carriers with hereditary PAH was 2.3-fold lower than that of unaffected carriers. Supporting this, BMPR2 expression and signalling is suppressed in wild-type mice treated with 16OHE1 (Fessel et al., 2013). Furthermore, 16OHE1 administration increases the penetrance and severity of PH in BMPR2 mutant mice of both sexes (Chen, Talati, et al., 2016; Fessel et al., 2013). The underlying mechanism is thought to involve pulmonary up-regulation of microRNA (miRNA, miR)-29, which is also up-regulated in the lungs of subjects with hereditary PAH and metabolic dysfunction (Chen, Talati, et al., 2016), which is known to contribute to clinical PAH (Chan & Rubin, 2017).

The full pharmacological effects of E3 on the pulmonary circulation have yet to be elucidated. Compared with 16OHE1, E3 is only weakly estrogenic (Fishman & Martucci, 1980), yet preliminary evidence suggests this metabolite can induce migration and proliferation of blood outgrowth ECs and human pulmonary artery SMCs from female PAH patients (Denver et al., 2020), decrease BMPR2 expression in normoxic male rat pulmonary artery SMCs and decrease markers of fibrosis in the mouse lung (unpublished observations).

7.3 | CYP1B1 inhibition

Of the CYPs, CYP1B1 exerts the greatest influence on estrogen hydroxylation (Hanna et al., 2000), with the human enzyme preferentially forming 4OHE2 and the mouse and rat homologues favouring production of 2OHE2 (Nishida et al., 2013). Of the 16OHEs, it is reported that CYP1B1 makes a relatively minor contribution to 16OHE1 production (Cribb et al., 2006) but actively forms E3 (Hanna et al., 2000). Unlike most CYPs, CYP1B1 is not present in the human liver, but expression is noted in many other tissues, including the lungs, heart and adipose tissue (Choudhary et al., 2005; Ellero et al., 2010; Li et al., 2017).

Consistent with a role for this enzyme in PAH, disease penetrance is decreased fourfold in female BMPR2 mutation carriers with a gene

variant that decreases CYP1B1 activity (Austin et al., 2009). Crucially, CYP1B1 expression is elevated in the lungs and pulmonary arteries of rodents and humans with PH (Dean et al., 2016; White et al., 2012; White, Loughlin, et al., 2011), which may cause 16OHEs to accumulate. On the other hand, CYP1B1 expression is decreased 10-fold in Epstein–Barr virus-immortalised cultured B-cells isolated from female heritable PAH patients (West et al., 2008).

Pre-clinical observations suggest that inhibiting the activity of CYP1B1 enzyme mitigates aberrant proliferative responses and alleviates PH (Figure 2 and Table 1). Administration of either dexfenfluramine or E2 promotes CYP1B1 expression in human pulmonary artery SMCs from female PAH patients and these two compounds act synergistically to promote the proliferation of these cells. Critically, the CYP1B1 inhibitor 2,3′,4,5′-tetramethoxystilbene (TMS) impairs proliferative responses to dexfenfluramine or E2 (Dempsie et al., 2013; White et al., 2012), and female mice with a global CYP1B1 deficiency are protected against dexfenfluramine-induced PH (Dempsie et al., 2013). TMS also inhibits E2-mediated reactive oxygen species production in human pulmonary artery SMCs isolated from female control donors and PAH patients (Hood et al., 2016). Pharmacological intervention with TMS attenuates the development of PH in male and female hypoxia-adapted and SuHx mice (White et al., 2012). In addition, TMS also alleviates PH in female SERT+ mice and restrains RVH and mortality in male monocrotaline rats (Johansen et al., 2016).

7.4 | Estrogen metabolism and obesity

Given that adipose tissue is actively involved in estrogen synthesis and metabolism (Ellero et al., 2010) it is notable that 30% to 50% of PAH patients are obese (Badesch et al., 2010; Taraseviciute & Voelkel, 2006). Furthermore, retrospective analyses have found that a significant proportion of obese subjects display medial thickening of the pulmonary arteries, consistent with subclinical PH (Blankfield et al., 2000; Haque et al., 2008), and body mass index (BMI) was recently found to be associated with severity (MacLean et al., 2022) and female exercise capacity (Ventetuolo et al., 2023) in PAH cohorts.

A growing body of evidence supports the interconnection between obesity, estrogen metabolism and PAH. Indeed, obese female BMPR2 mutant mice have higher adipose CYP1B1 expression and increased PH penetrance versus their male counterparts (Labazi et al., 2022). In addition, male ob/ob mice have increased CYP1B1 expression in their visceral adipose tissue and elevated urinary 16OHE1 levels; TMS attenuates spontaneous PH development in these animals (Mair et al., 2019). These results suggest that CYP1B1 inhibition may be particularly efficacious in obese individuals with PAH.

7.5 | Methylated estrogen metabolites

There is a paucity of data around COMT in PAH development and progression. Still, this enzyme is more active in hepatic tissue from male subjects than in samples from females individuals (Boudikova et al., 1990) and is reportedly down-regulated by E2 *in vitro* (Xie et al., 1999). COMT catalyses the conversion of OHEs into terminal metabolites, including 2MeOE1 and 2MeOE2, which have little activity at ERs (Dubey et al., 2000).

Of the methylated estrogen metabolites, 2MeOE2 has been most extensively studied in experimental PH. 2MeOE2 offsets the development of PH in hypoxia-adapted rats of either sex (Docherty et al., 2019; Hao et al., 2019) and delays disease progression in male monocrotaline rats (Tofovic et al., 2005). This metabolite also improves survival in ovariectomised rats with monocrotaline-induced PH (Tofovic et al., 2006) and PH secondary to pulmonary fibrosis (Tofovic, Zhang, Jackson, et al., 2009). Finally, 2MeOE2 has both preventive and therapeutic effects in intact and ovariectomised female SuHx rats (Tofovic & Rafikova, 2009). The protective effects of endogenous 2MeOE2 may be diminished in clinical PAH, as the urinary 16OHE1/2MeOE2 ratio is elevated in male heritable PAH patients. 2MeOE2 treatment does not protect BMPR2 mutant mice against spontaneous PH but tends to offset further increases in cardiopulmonary pressure and pulmonary vascular resistance induced by 16OHE1 (Fessel et al., 2013).

The protective effects of 2MeOE2 are thought to be mediated by inhibition of hypoxia-inducible factor 1α in the lung (Docherty et al.,;2019; Wang et al., 2017). Supporting this, 2MeOE2 lowers hypoxia-inducible factor 1α expression in female human pulmonary artery SMCs from PAH patients and exerts both anti-proliferative and pro-apoptotic effects on these cells (Docherty et al., 2019). Anti-proliferative effects have also been reported in control human pulmonary artery ECs, at physiological 2MeOE2 concentrations (Borahay et al., 2014; Salama et al., 2009). This metabolite also promotes **nitric oxide (NO)** synthesis resulting in vasodilatation (Chen et al., 2015; Fenoy et al., 2010). Notably, several approved pharmacological therapies for PAH function by enhancing deficient NO signalling in the pulmonary vasculature (Lazar et al., 2020). 2-Ethoxyestradiol, a synthetic analogue of 2MeOE2 with 10-fold greater anti-mitogenic potency against control human pulmonary artery ECs and human pulmonary artery SMCs, also improves monocrotaline-induced PH and the associated mortality in male rats (Tofovic et al., 2008). 2MeOE1 also attenuates PH in male monocrotaline rats (Rafikova et al., 2008), though it remains unclear if this effect is mediated by conversion to 2MeOE2 (Tofovic, 2010).

The effects of exogenous 2MeOE2 in patients with PAH are unknown. However, this compound has been pursued as a potential anti-cancer agent, owing to its anti-proliferative effects. While well tolerated, the clinical use of this compound has thus far been hampered by formulation issues and low bioavailability (Ba & Duan, 2020).

7.6 | CYP1A1 and the aryl hydrocarbon receptor

Compared with the other CYPs, CYP1A1 shows considerable activity with regard to 16-hydroxylation of E1 (Cribb et al., 2006) and can also

form E3 (Badawi et al., 2001). Both CYP1A1 and CYP1B1 are stimulated by the **aryl hydrocarbon receptor (AhR)**, a transcriptional regulator highly expressed in the lung (Murray et al., 2014). The aryl hydrocarbon receptor is up-regulated in human pulmonary artery SMCs from PAH patients (Dean et al., 2016) and this pathway promotes proliferation in control human pulmonary artery SMCs (Huang et al., 2014). *In vivo*, the aryl hydrocarbon receptor promotes diet-induced obesity (Xu et al., 2015) and is highly expressed in the small occluded pulmonary arteries of SuHx rats (Dean et al., 2016), coalescing with increased CYP1A1 expression (Dean et al., 2018). Curiously, Sugen is an agonist at the aryl hydrocarbon receptor (Baba et al., S2005) and the nuclear translocation of this receptor promotes pulmonary E2 synthesis and EC apoptosis in female SuHx rats (Dean et al., 2018), which may contribute to the pathobiology of this model.

Pharmacotherapy with an aryl hydrocarbon receptor antagonist (CH-223191) normalises CYP1A1 and aromatase expression and E2 levels in the lung of female SuHx rats, and these changes are associated with reversal of cardiopulmonary pressure overload, pulmonary vascular remodelling and RVH (Dean et al., 2018). The expression of the other estrogen metabolising CYP isoforms, CYP1A2 and CYP3A4, has yet to be investigated in the context of PAH.

8 | TARGETING ESTROGEN RECEPTORS (ERS)

8.1 | Estrogenic signalling

Sex hormone receptors are ligand-activated transcription factors that belong to the nuclear hormone receptor family (Hager et al., 2000). Classical E2 signalling is characterised by activation of **ER-α (NR3A1)** and/or **ER-β (NR3A2)**, which are encoded by the *ESR1* and *ESR2* genes, respectively (Tang et al., 2019). The abundance and distribution of these receptors varies greatly depending on tissue and cell type; ERα is predominantly expressed in the uterus, ovaries and breasts, while ER-β is mainly found in the male reproductive system, ovaries, nervous system and cardiovascular system (Jia et al., 2015). Receptor activation is influenced by other differentially expressed factors, including co-activators and co-inhibitors, non-estrogen ligands and estrogen metabolising enzymes (Menazza & Murphy, 2016; Murphy, 2011).

Unlike other nuclear receptors, inactive ERs are mainly found in the nucleus (Hager et al., 2000), though ER-α can also localise to the plasma membrane, where it functions as an atypical G protein-coupled receptor (Evinger & Levin, 2005; Marino et al., 2006). In the nucleus, activated ERs can bind directly to the estrogen response elements of genes or may indirectly bind to DNA by forming complexes with other transcriptional regulators (Hewitt & Korach, 2018); target genes are determined by the tissue, ligand and receptor type (Burris et al., 2013; Charn et al., 2010). Due to the multifaceted nature of estrogenic signalling, E2 and its metabolites have diverse and conflicting effects, even within a single-organ system (Lahm & Kawut, 2017).

Besides the classical nuclear ERs, E2 is also reported to engage in rapid non-genomic intracellular signalling events, including **3′,5′-cyclic adenosine monophosphate (cAMP)** production, Ca^{2+} mobilisation and the activation of protein kinases, including **phosphoinositide 3-kinases (PI3K)** and **mitogen-activated protein kinases (ERKs)** (Luo & Liu, 2020; Prossnitz & Barton, 2011). These events are thought to be mediated by a third estrogen receptor, **G protein-coupled estrogen receptor 1 (GPER/GPR30)**. In addition to mediating rapid signalling events, this cell surface receptor is also able to regulate the expression of genes, including c-fos, cyclin D1 and **VEGF** (Albanito et al., 2007; De Francesco et al., 2014; Vivacqua et al., 2006). It remains unclear if ER-α, ER-β and GPER function synergistically or antagonistically. The overall effect of an estrogen in a given tissue is likely to be decided by the interaction and balance of multiple signalling pathways.

8.2 | ER inhibition

All three ERs are prominently expressed in the pulmonary arteries of both healthy donors and PAH patients. The classical ERs are found in all three layers of the artery, while ER-α is mainly localised to smooth muscle, and ER-β expression is largely endothelial. Like ER-α, GPER is primarily localised to smooth muscle, though limited endothelial expression is also noted (Wright et al., 2015).

It was recently shown that the expression of both classical ERs is decreased in hypoxic control human pulmonary artery SMCs and the lungs of female monocrotaline rats (Abdulkareem et al., 2023). On the other hand, ER-α is exclusively up-regulated in human pulmonary artery SMCs isolated from females with PAH, while ER-β expression is increased in cells from male donors. Interestingly, agonism of ER-α, but not ER-β or GPER, evokes proliferation in female cells (Wright et al., 2015). Dovetailing with this finding, the proliferative response of male and female control human pulmonary artery SMCs to E2 is inhibited by the ER-α antagonist **MPP dihydrochloride**, whereas ER-β and GPER antagonists have no effect (Mair et al., 2015). Supporting this, MPP dihydrochloride attenuates the development of PH in female SERT+ mice (Wright et al., 2015).16OHE1-driven human pulmonary artery SMC growth is also thought to be mediated via ER-α (Hood et al., 2016; Johansen et al., 2016). These mitogenic responses are driven by direct binding of ER-α to a conserved ER binding site in the BMPR2 promoter, which supresses BMPR2 signalling (Austin et al., 2012).

In vivo, deletion of *ESR2* and to a lesser extent *ESR1* protects BMPR2 mutant ($BMPR2^{R899X}$) mice (of either sex) against pulmonary vascular remodelling and PH (Chen et al., 2017). Interestingly, genetic deletion of nuclear ERs is accompanied by increased GPER expression (Chen et al., 2017) and drugs that inhibit the classical ERs (i.e. fulvestrant and tamoxifen) appear to have agonist activity at GPER (Barton, 2016). This is relevant as GPER activation ameliorates PH and RV dysfunction in male monocrotaline rats (Alencar et al., 2017).

Fulvestrant is a selective down-regulator of classical ERs that competitively binds to the receptor and impairs dimerisation, leading to rapid degradation (Osborne et al., 2004) (Figure 2). In combination with anastrozole, fulvestrant prevents and reverses PH in female BMPR2 mutant mice (Chen et al., 2017) (Table 1). In a small, proof-of-concept, open-label study of five postmenopausal women with PAH, fulvestrant was well tolerated, but due to the small number of participants, no conclusions could be drawn, despite trends towards reduced E2 and E3 levels, and improved stroke volume (Kawut et al., 2019) (Table 2). A larger, randomised, controlled phase 2 study of fulvestrant in PAH is warranted.

Tamoxifen is a selective modulator of classical ERs that functions as a partial agonist or antagonist depending on the tissue in question (Jordan, 2003) (Figure 2). Tamoxifen was found to be less efficacious at reversing PH in BMPR2 mutant mice than dual therapy with anastrozole and fulvestrant (Chen et al., 2017) (Table 1) but remains attractive from a clinical perspective as it can be used in fertile women without inducing menopause (Colleoni & Munzone, 2015). Ormeloxifene, another selective ER modulator, was recently reported to have protective effects in male and female monocrotaline rats (Abdulkareem et al., 2023). A small randomised controlled trial of tamoxifen in PAH patients of both sexes is now recruiting (T3PAH; ClinicalTrials.gov identifier: NCT03528902) (Table 2), with a primary endpoint of change from baseline on tricuspid annular plane systolic excursion (TAPSE; an echocardiographic measure of RV systolic function).

9 | OTHER SEX HORMONE TARGETS IN PAH

9.1 | Progesterone

Progesterone is an endogenous steroid hormone produced by the adrenal cortex, ovaries and, to a lesser extent, the testes. This hormone has important roles in the female menstrual cycle, reproduction and pregnancy (Nagy et al., 2021). Despite its role as a major female sex hormone, very few studies have investigated the role of progesterone in PAH. Lower circulating levels of the hormone are associated with increased PAH morbidity and mortality in premenopausal females (Zhang et al., 2020) and increased PAH occurrence and severity in men (Wu et al., 2018). In contrast, circulating progesterone levels are unaltered in postmenopausal women with PAH and do not associate with disease severity (Baird et al., 2018).

It has long been known that progesterone also causes vasodilatation and reduces blood pressure (Thomas & Pang, 2013), which may be related to activation of **endothelial NO synthase (eNOS)** and NO production (Selles et al., 2001; Simoncini et al., 2007). It was recently demonstrated that progesterone inhibits the proliferative response of human pulmonary artery SMCs to **interleukin (IL)-6**, by inhibiting STAT3 and the expression of downstream proliferative genes (Hu et al., 2022). There is also anecdotal evidence of progesterone receptor expression in the plexiform lesions of a PAH patient, localised to myofibroblasts and modified ECs (Barberis et al., 1995). Furthermore, progesterone offsets the development of PH in ovariectomised female monocrotaline rats, by inhibiting vascular and cardiac remodelling and improving survival (Tofovic, Zhang, & Petrusevska, 2009). Further studies of role of progesterone in PAH pathobiology are clearly warranted.

9.2 | Gonadotropins

Luteinising hormone (LH) and **follicle stimulating hormone (FSH)** are pituitary-derived gonadotropin peptides that regulate growth, sexual development and reproduction (Anderson et al., 2018). Data on circulating FSH in male subjects with PAH are conflicting, with one study reporting no change (Wu et al., 2018) and another reporting an increase, associated with poor survival (Wang et al., 2022). The latter study dovetails with observations in females; increased FSH in fertile women with PAH is associated with a lower survival rate (Zhang et al., 2020). In contrast, LH is unaltered in men, but increased in fertile women, albeit with no association with mortality (Zhang et al., 2020). FSH regulates angiogenesis and EC proliferation in various tissues (Alam et al., 2009; Radu et al., 2010; Stilley et al., 2014) and promotes vascular remodelling in an experimental model of atherosclerosis (Piao et al., 2022). Of note, FSH is a key activator of aromatase (Erickson & Hsueh, 1978; Lambert et al., 2000), raising the possibility that its biological actions in the cardiovascular system are dependent upon the conversion of testosterone to E2.

10 | TARGETING THE RIGHT VENTRICLE (RV)

It is increasingly recognised that women with PAH have lower RV mass, lower RV volume, superior right ventricular ejection fraction (RVEF) and enhanced RV–pulmonary artery coupling compared with men (Kawut et al., 2011; Swift et al., 2015; Tello et al., 2020). The association between female sex and greater RVEF is evident even when adjusted for pulmonary vascular resistance and left ventricular function (Kawut et al., 2009). Importantly, RV function is a critical determining factor of outcome in PAH (van de Veerdonk et al., 2011) and the female survival advantage has been linked with superior RVEF following therapeutic intervention (Jacobs et al., 2014). Numerous studies suggest that sex differences in the mortality of PAH patients are mediated by the effects of sex hormones on the RV.

10.1 | Estrogens

Circulating E2 correlates positively with RVEF in postmenopausal women on hormone replacement therapy (Ventetuolo et al., 2011). Moreover, females with PAH also demonstrate higher cardiac index compared with their male counterparts, but this functional advantage dissipates once the age of menopausal transition (>45 years) is

reached (Ventetuolo et al., 2014). These clinical observations are also replicated in pre-clinical models of PH. The cardiac index of male SuHx rats is lower than that of female animals but is improved with E2 supplementation (Frump et al., 2015). Likewise, ovariectomy exacerbates RV dysfunction and exercise intolerance in SuHx rats, but these deficits can be normalised with E2 repletion (Lahm et al., 2016).

Preliminary evidence suggests that altered estrogen metabolism also impacts RV function. Ventetuolo et al. identified a second CYP1B1 single-nucleotide polymorphism (SNP; tightly linked to the SNP associated with disease penetrance in hereditary PAH; Austin et al., 2009) that correlates with higher RVEF in African American and white women free of cardiovascular disease. Furthermore, this study found that higher urinary 2OHE1 and 16OHE1 levels associate with increased RVEF function in female Caucasians and higher urinary 16OHE1 associates with decreased RVEF in female Hispanics. As such, the consequences of altered estrogen metabolism in the heart appear to be both sex and race dependent (Ventetuolo, Mitra, et al., 2016). Also, 4OHE2 markedly reduces RVH *in vivo*, independent of changes in cardiopulmonary pressure (Bilan et al., 2013), suggesting that the cardioprotective effects of E2 may be mediated via conversion to this OHE. Based on these observations, it is theorised that estrogens and their metabolites have pleiotropic effects on the pulmonary circulation and RV, giving rise to the unique sex-specific phenotypes observed in PAH.

It is thought that E2 supports RV adaptation and delays the transition to maladaptive RVH through activity at ER-α and, to a lesser extent, ER-β (Frump et al., 2015, 2021; Lahm et al., 2012; Umar et al., 2011). The resulting cardioprotection is posited to rely on attenuation of RVH, inflammation, apoptosis, oxidative stress (Frump et al., 2015), together with preservation of mitochondrial function (Liu et al., 2017). The overall outcome of these effects is a reduction in RV fibrosis, mediated by beneficial effects on collagen I/III ratio (Lahm et al., 2016). This observation is supported by a study in rat cardiac fibroblasts, which reported a female-specific down-regulate of collagen types I and III following E2 treatment, mediated by ERα binding to collagen promoters (Dworatzek et al., 2019). Interestingly, the same study found that E2 increased the expression of **collagen type I (COL1A1)** and **collagen type III (COL3A1)** in male rat cardiac fibroblasts, in an ER-β-dependent manner. Higher levels of pro-angiogenic factors are expressed in the RV of female hypoxia-adapted mice, which may facilitate adaptation to pressure overload (Bohuslavova et al., 2010). E2 may also have indirect protective effects on the heart, by attenuating collagen accumulation in the proximal PA, improving arterial compliance and reducing RV afterload (Liu et al., 2014, 2015).

One limitation of using the models mentioned thus far (chronic hypoxia, monocrotaline and SuHx) to study the effects of estrogens on RV adaptation is the confounding influence of estrogenic signalling in the pulmonary vasculature. This issue can be circumvented experimentally using the pulmonary artery banding model, in which afterload is elevated by mechanical constriction of the pulmonary trunk, rather than increased pulmonary vascular resistance (Anderson et al., 2018). Using this model, Cheng et al. (2020) found that the presence of a loss-of-function mutation in ER-α leads to RV–pulmonary artery uncoupling, RV diastolic dysfunction and fibrosis, in female animals only. Consistent with this, Frump et al. (2021) recently described a novel pathway by which ER-α activation promotes BMPR2 signalling to up-regulate the cardioprotective molecule apelin in the RV.

10.2 | The right drug for the RV

Further investigation of the varied and contradictory influences of estrogenic signalling in the cardiopulmonary circulation is clearly required. Due to these complexities, it remains unclear whether the beneficial effects of estrogens in the RV can be harnessed without harming the pulmonary vasculature. Similarly, drugs that inhibit estrogen production or signalling have the potential to precipitate cardiac dysfunction and thus clinical trials evaluating these agents in PAH patients have integrated echocardiographic measures of RV function and biochemical markers of cardiac dysfunction (circulating N-terminal pro B-type natriuretic peptide) as efficacy and safety endpoints (Table 2).

Large cohort, long-term observational studies comparing aromatase inhibitor use with tamoxifen in women with breast cancer have shown conflicting results with regard to cardiovascular outcome, with some studies reporting an increased risk of left heart failure, myocardial infarction, stroke and cardiovascular mortality, and others finding no difference in the incidence of serious cardiovascular events (Abdel-Qadir et al., 2016; Haque et al., 2016; Kamaraju et al., 2019; Khosrow-Khavar et al., 2020). In addition, randomised, controlled trials have shown that long-term tamoxifen use is associated with decreased lipid concentrations (Dewar et al., 1992; Love et al., 1994) and reduced cardiovascular risk and morbidity (Rosell et al., 2013), which may have biased the results of these studies. However, the specific effect of aromatase inhibitors on the heart also remains unclear, as there are reports of both increased and unaltered risk of cardiovascular events in chronic users of these drugs versus untreated individuals (Jacobse et al., 2021; Ligibel et al., 2012; Matthews et al., 2021). The results of one recent study suggests that negative cardiovascular outcomes with aromatase inhibitors are limited to patients treated continuously for more than 4 years and patients over 75 years old (Sund et al., 2021). It is important to note that that there are no published reports of RV dysfunction in women treated with aromatase inhibitors.

It is clear that an informed and focused approach will be needed to target estrogenic signalling safely and effectively in PAH patients. Larger randomised controlled trials will be essential to identify populations most likely to benefit from estrogen-directed therapies. These drugs may need to be deployed with a precision medicine approach, considering age, sex, menopausal status, RV function, genetic variants, E2 and metabolite levels or cardiopulmonary ER expression (Lahm & Kawut, 2017).

10.3 | DHEA

DHEA is an endogenous precursor for androgens and estrogens that is posited to play a role in RV adaptation. From a clinical perspective,

circulating DHEA-sulphate (DHEA-S; a metabolite of DHEA) levels are reduced in men and women with PAH and are associated with poorer RV function, exercise capacity and survival (Baird et al., 2018, 2021; Rhodes et al., 2017; van Wezenbeek et al., 2022; Ventetuolo, Baird, et al., 2016; Wu et al., 2018). It remains unclear whether the DHEA-mediated cardioprotection is the result of direct RV-specific effects or conversion to downstream hormones (Figure 2). However, it was recently reported that the relationship between lower circulating DHEA-S and shorter 6-min walk distance was moderated by 2MeOE1 (Baird et al., 2021).

In vitro, treatment of human pulmonary artery SMCs isolated from PAH patients (two male donors and one female donor) with DHEA decreases activation of **signal transducer and activator of transcription 3 (STAT3)**, a transcription factor that promotes pulmonary vascular remodelling and concomitantly up-regulates BMPR2 (Paulin et al., 2011, 2012). Furthermore, DHEA prevents and reverses the development of PH in hypoxia-adapted rats (Bonnet et al., 2003; Dumas de La Roque et al., 2013; Hampl et al., 2003; Oka et al., 2007) and intact or pneumonectomised monocrotaline rats (Homma et al., 2008; Paulin et al., 2011). Several of these studies attribute the favourable effects of DHEA administration to increased expression and activation of large conductance Ca^{2+}-activated potassium channels (BK_{Ca}) (Bonnet et al., 2003; Dumas de La Roque et al., 2013; Hampl et al., 2003).

In SuHx rats, DHEA has only moderate effects on cardiopulmonary pressure and pulmonary vascular remodelling but has profound effects on RV function. Indeed, DHEA treatment inhibited capillary rarefaction, apoptosis, fibrosis and oxidative stress in the RV; these changes were associated with reduced Rho kinase activity and inhibition of STAT3 and nuclear factor of activated T-cells/cytoplasmic 3 (NFATc3) (Alzoubi et al., 2013). A supporting study found that DHEA increases RV myocyte density, reduces mitochondrial fragmentation and abolishes RV systolic and diastolic dysfunction in re-oxygenated hypoxia-adapted rats (Dumas de La Roque, Bellance, et al., 2012) (Table 1).

In a small study of patients with chronic obstructive pulmonary disease PH, DHEA treatment reduced pulmonary vascular resistance and mean pulmonary arterial pressure, improving 6-min walk distance and diffusion capacity, without compromising gas exchange (Dumas de La Roque, Savineau, et al., 2012). Recruitment is ongoing for a small crossover study of DHEA in PAH patients of both sexes (EDIPHY; ClinicalTrials.gov identifier: NCT03648385); the primary outcome will be change in RV longitudinal strain determined by cardiac magnetic resonance imaging (Table 2).

10.4 | Testosterone

Testosterone (17-beta-hydroxy-4-androstene-3-one), the principal androgen hormone, governs male secondary sexual characteristics, sperm production, skeletal muscle mass and bone density (Kelly & Jones, 2013; Mohamad et al., 2016; Rastrelli et al., 2018). This hormone is predominantly secreted by the testes in men and is produced in smaller amounts by women, via androstenedione conversion in the ovaries, adrenal glands and peripheral tissues (Kanakis et al., 2019; Lobo, 2001). Much of the bioactivity of testosterone is attributable to enzymatic conversion (mediated by 5α-reductase) to the active metabolite dihydrotestosterone, which has much greater potency (Figure 1) (Swerdloff et al., 2017). The actions of testosterone in the cardiovascular system are complex, with both protective and detrimental effects reported across various cardiovascular diseases (Diaconu et al., 2021). Compared with estrogens, relatively little is known about the influence of androgens on PAH.

Higher testosterone levels are associated with greater RV mass and volume in healthy men (Ventetuolo et al., 2011), indicative of a detrimental effect on RV function. Supporting this notion, genetic variation in the androgen receptors is associated with poorer RV function in men free of cardiovascular disease (Ventetuolo, Mitra, et al., 2016). Furthermore, though circulating testosterone is unaltered in men with PAH (Ventetuolo, Baird, et al., 2016; Wu et al., 2018), a recent study found that levels of this hormone correlate negatively with RVEF in these patients (Zhang et al., 2020). Overall, while testosterone levels are unchanged in men with PAH, it seems that even physiological levels of this hormone have negative effects on RV function in these patients.

On the other hand, circulating testosterone levels were found to be reduced in a mixed cohort of females of reproductive and postmenopausal age with PAH, albeit with no impact on RV function (van Wezenbeek et al., 2022). An earlier study reported that lower plasma testosterone levels are associated with increased PAH risk and severity in premenopausal females with idiopathic PAH (Zhang et al., 2020), though RV function was not assessed. Lower testosterone levels were also found to increase the occurrence of PAH in postmenopausal women, though no consistent associations of testosterone with echocardiographic RV structure and function, circulating N-terminal pro B-type natriuretic peptide or 6-min walk distance were noted in these patients (Baird et al., 2018).

From a pre-clinical perspective, testosterone readily relaxes isolated human and rat pulmonary arteries from donors of both sexes, though pulmonary arteries isolated from males are more sensitive to the hormone (English et al., 2001; Rowell et al., 2009; Smith et al., 2008). While testosterone-evoked vasodilatation would theoretically be protective against PAH, the effect of the hormone on more clinically relevant processes (i.e. proliferation and vascular remodelling) has not been investigated (Hester et al., 2019).

At a cellular level androgen receptors are present in human cardiomyocytes and evoke a hypertrophic response following activation by testosterone (Marsh et al., 1998). Accordingly, testosterone appears to have detrimental effects on RV remodelling, as RVH is accentuated in hypoxia-adapted castrated male rats treated with the hormone (Moore et al., 1978). In addition, pulmonary artery banded castrated male mice have reduced RVH and fibrosis and improved survival; these advantages are mitigated by testosterone repletion (Hemnes et al., 2012).

11 | NON-HORMONAL FACTORS

Despite their significant contribution to PAH pathophysiology, the influence of sex hormones alone cannot fully explain the sexual dimorphisms observed in PAH patients. Indeed, emerging evidence suggests that non-hormonal factors such as sex chromosomes, epigenetic modifications and immune cell regulation may also contribute to sex differences in PAH risk and outcome. It remains unclear whether these components have direct effects on the pulmonary vasculature and RV or act by altering the equilibrium between pro- and anti-inflammatory/mitogenic estrogen metabolites. Similarly, the influence of estrogen metabolites on non-hormonal factors remains uncertain (Figure 3).

11.1 | Sex chromosomes

The sex chromosomes (X and Y) are known to contribute to sexual dimorphisms across various pathologies, including neurological, autoimmune and cardiovascular diseases, irrespective of gonadal sex (Arnold et al., 2016; Du et al., 2014). As such, it is likely that the actions of sex hormones alone are insufficient to explain the marked sex bias observed in PAH. Genes encoded by the sex chromosomes have direct actions on non-gonadal cells that can modulate disease pathology (Burgoyne & Arnold, 2016). In spite of this, most studies investigating the influence of sex on PAH ignore these clear chromosomal differences.

In mammals, gonadal sex differences are mediated by *Sry* (sex-determining region Y), a Y-linked gene and transcriptional activator that initiates male sexual determination. Outside of sexual development, *Sry* regulates multiple signalling pathways relevant to PAH, including Wnt signalling (Awad et al., 2016; Wissmuller et al., 2006). Furthermore, by binding to the BMPR2 promoter, this protein is able to promote expression of the BMPR2 gene (Umar et al., 2018). These findings may explain the lower prevalence of PAH in males.

Using murine models in which the number and type of sex chromosomes are independent of gonadal sex, Umar and colleagues demonstrated that hypoxia-adapted XY mice develop less severe pulmonary vascular remodelling than XX animals, suggesting that presence of Y chromosome protects against PH (Umar et al., 2018). In a recent study, the Y-chromosome gene *Uty* (ubiquitously transcribed tetratricopeptide repeat containing, Y-linked) was shown to be protective in gonadectomised and hypoxia-adapted male mice. In these animals, reduced *Uty* expression up-regulates the pro-inflammatory chemokines **CXCL9** and **CXCL10**, which are also up-regulated in lungs of humans, particularly women, with PAH. In female SuHx and monocrotaline rats, blocking the activity of these two chemokines dampens pulmonary artery ECapoptosis and reverses PH (Cunningham et al., 2022).

11.2 | Other epigenetic factors

Besides genetic mutations, DNA modifications can also alter gene expression and contribute to pulmonary vascular remodelling in PAH. Epigenetic regulatory mechanisms such as DNA methylation, histone modification and non-coding RNAs have been associated with PAH pathogenesis. DNA methylation studies using human pulmonary artery ECs from idiopathic PAH and hereditary PAH patients identified dysregulated genes involved in the lipid transport pathway (Hautefort et al., 2017). At present, there are limited studies to correlate the methylation status with sex; however, abnormal methylation pattern in males is reported in the context of cancer (Zhang et al., 2011) and metabolic disorders (Hall et al., 2014).

Histone deacetylases (HDACs) are enzymes that strengthen the interaction between DNA and histones. In the past decade, several studies have shown that HDACs play a crucial role in cardiac remodelling. In the monocrotaline and pulmonary artery banding experimental models, a small molecule HDAC inhibitor (valproic acid [VPA]) was shown to temper RVH and improve RV systolic function in male rats (Cho et al., 2010). On the other hand, a broad-spectrum HDAC inhibitor (**trichostatin A [TSA]**) had no effect on RVH or fibrosis in pulmonary artery banded male rats and promoted RV dysfunction by a mechanism attributed to suppression of pro-angiogenic factors, such as VEGF and **angiopoietin-1** (Bogaard et al., 2011). Additional studies are needed to understand how HDAC inhibition influences PH and whether the effects of HDAC inhibitors are sex dependent.

miRNAs are short non-coding nucleic acid sequences that regulate the expression of various genes by directing degradation of mRNA transcripts or silencing mRNA translation. Several miRNAs that affect signalling cascades involved in PAH development have been identified. Overexpression of miR-17 reduces BMPR2 expression (Brock et al., 2009) and promotes human pulmonary artery SMC proliferation *in vitro* (Chen, Zhou, et al., 2016; Lu et al., 2016; Pullamsetti et al., 2012). *In vivo*, miR-17 inhibition improves PH and RV dysfunction in monocrotaline rats by up-regulating p21 in the lung (Pullamsetti et al., 2012). Two further miRNAs, miR-143 and miR-361, induce proliferation and survival in human pulmonary artery SMCs under hypoxic conditions (Yue et al., 2018; Zhang et al., 2018).

Importantly, estrogens are known to regulate the expression of some miRNAs (Bhat-Nakshatri et al., 2009). For example, E2 can down-regulate miR-96, which in turn regulates the gene for the **5-hydroxytryptamine 1B (5-HT_{1B}) receptor**, a mediator of pulmonary artery SMC. The expression of miR-96 is reduced in SMCs from female BMPR2 mutant mice and female patients with PAH, resulting in increased 5-HT_{1B} expression and 5-HT-driven proliferation (Wallace et al., 2015). *In vivo*, administration of a miR-96 mimic offsets the development of PH in female hypoxia-adapted mice and SuHx rats (Docherty et al., 2020; Wallace et al., 2015). Targeting miRNAs influenced by E2 may provide a means of treating PAH without the need to target sex hormones directly, avoiding potential adverse effects.

Female susceptibility to PAH may also be mediated by long non-coding mRNAs (lncRNAs) such as X-inactive-specific transcript (Xist), one of the first identified mammalian lncRNAs. Xist plays an

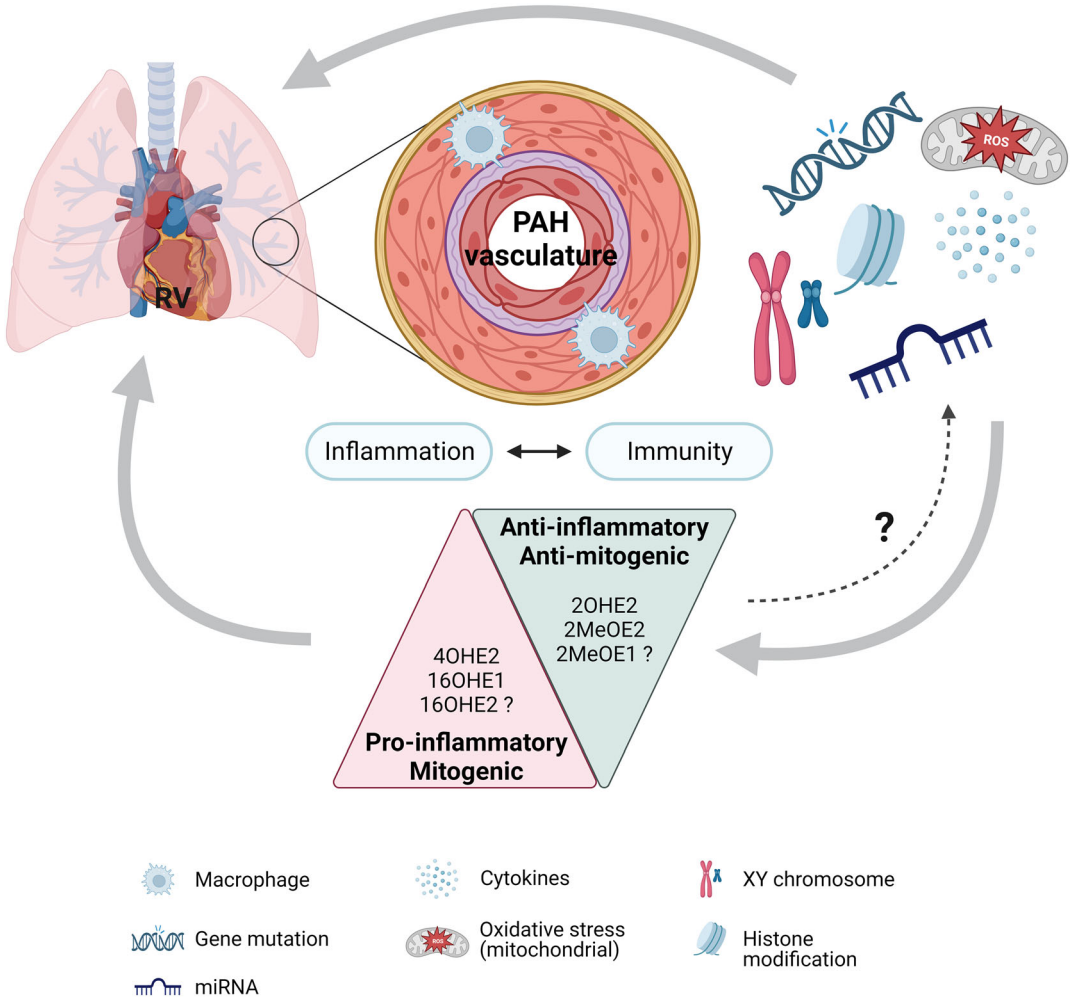

FIGURE 3 Non-hormonal mediators influencing vascular remodelling in PAH. Various non-hormonal mediators, including XY chromosomes, miRNAs, oxidative stress, inflammation and epi-(genetics), may alter the balance between pro- and anti-mitogenic/anti-inflammatory estrogen metabolites, impacting remodelling in the pulmonary vasculature and RV. Non-hormonal components may exert direct effects on these tissues and/or act indirectly via estrogen metabolites. 16OHE1, 16-hydroxyestrone; 16OHE2 16-hydroxyestradiol; 2OHE2, 2-hydroxyestradiol; 2MeOE1, 2-methoxyestrone; 2MeOE2, 2-methoxyestradiol; 4OHE2, 4-hydroxyestradiol; miRNA, microRNA; PAH, pulmonary arterial hypertension; RV, right ventricle. *Created in BioRender*.

essential role in X chromosome inactivation and dosage compensation of X-linked genes. Abnormal Xist expression has been reported in several cancers (Yang et al., 2018) and in neurological disorders (Ji et al., 2015). Using the intersectin-1s heterozygous knockout mouse, a model of PAH-like plexiform/obliterative arteriopathy (Patel et al., 2017), Qin et al. found that Xist is preferentially up-regulated in females. While sex-based differences in pulmonary Xist expression are not observed in SuHx rats, human pulmonary artery ECs isolated from female idopathic PAH patients also demonstrate higher Xist expression versus male cells (Qin et al., 2021).

Considerable progress has been made with regard to our understanding of the epigenetics of PAH. However, further mechanisms, including the influence of sex on these pathways, must be unravelled before they can be targeted therapeutically.

11.3 | Inflammation and immune cell regulation

The immune system plays a key role in PAH pathogenesis (Ichimura et al., 2018; Nicolls & Voelkel, 2017; Seropian et al., 2018). Pulmonary vascular injury triggers inflammatory cytokine release, resulting in the recruitment and activation of immune cells, which drive vascular remodelling (Simpson et al., 2020; Wang et al., 2020). The intensity of immunological responses is known to vary considerably between the sexes and accounts for differential susceptibility to infectious diseases and cancer (Klein & Flanagan, 2016). As such, it is conceivable that immunological sexual dimorphisms play a role in PAH.

It was recently shown that exposing female rats with a deficiency in regulatory T-cells (Tregs) to Sugen or hypoxia results in more severe PH, characterised by extensive pulmonary inflammation and RV fibrosis (Tamosiuniene et al., 2018). While this finding suggests that

females are more reliant on regulatory T-cells to counteract vascular insults than males, regulatory T-cell reconstitution is protective against PH in animals of both sexes. The same study found that T regulatory T-cells mediate vasoprotection by up-regulating anti-inflammatory cytokines (**IL-10**) and interacting with ERs on the surface of ECs, indicating crosstalk between the immune system and estrogenic signalling. Favouring this observation, immune disorders associated with PAH share similar sex ratios to those seen in idiopathic PAH cohorts (Batton et al., 2018). Furthermore, subjects with systemic lupus erythematosus often present with increased 16-hydroxylation of E1 and E2, mirroring PAH patients. Indeed, 16OHE1 is elevated in male and female systemic lupus erythematosus patients, and E3 is increased in women with systemic lupus erythematosus. This is curious given that patients with systemic lupus erythematosus often develop PAH and 95% of these are women (Chung et al., 2010).

In pre-clinical models, inflammatory involvement in pulmonary vascular remodelling seems to be greater in males compared with females. Using the monocrotaline model, enhanced endothelial barrier dysfunction has been shown to accentuate pulmonary vascular leak in male animals (Rafikova et al., 2020), which may promote inflammation. Furthermore, Rafikova et al. (2015) have demonstrated that perivascular inflammation of the small pulmonary arteries is exclusive to male SuHx rats, corresponding with the enhanced degree of adventitial and medial fibrosis in these animals. Supporting this finding, increased pro-inflammatory **toll-like receptor 4** signalling has been linked to the specificity of vascular necrosis to male SuHx rats (Rafikov et al., 2019; Zemskova et al., 2020). Interestingly, a male bias has also been noted in the contribution of renal inflammation and necrosis to blood pressure elevation in spontaneously hypertensive rats (Abdelbary et al., 2019). These animals also exhibit spontaneous medial thickening of the pulmonary arteries and elevated pulmonary vasoreactivity to endothelin (Aharinejad et al., 1996; Gomart et al., 2014). The contribution of inflammatory processes to this PH phenotype remains unclear. The influence of immune cells and inflammation on sex-dependent outcomes in PAH patients requires further investigation; sex-based stratification may be warranted for clinical trials investigating anti-inflammatory therapies.

12 | CONCLUSIONS

Sex is a major disease modifier for PAH, highlighting the key role of sex hormones in the pathophysiology of this disease. The higher incidence but superior outcome of PAH in females has been linked to sexually dimorphic effects of estrogens in the pulmonary circulation and RV. A significant body of evidence suggests that estrogen synthesis, metabolism and signalling are dysregulated in both women and men with PAH. In particular, the estrogen metabolite 16OHE1 increases the penetrance of BMPR2 mutations, while 2MeOE2 has powerful anti-mitogenic effects on pulmonary vascular cells. Estrogen modulation has been explored extensively in animal models of PH to establish new targeted treatments. Several clinical trials evaluating therapies that block estrogen synthesis or signalling are ongoing or have recently been completed. Data on the role of other sex hormones, such as progesterone and testosterone, are limited, though the sex hormone precursor DHEA is under investigation for the treatment of RV dysfunction. Non-hormonal factors influenced by sex, including Y-linked gene expression, epigenetics and inflammation, have also garnered interest. A better understanding of sex hormone signalling and sexually dimorphic non-hormonal influences may ultimately lead to new targeted therapeutic approaches for PAH patients of either sex.

12.1 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander, Christopoulos, et al., 2021; Alexander, Cidlowski et al., 2021; Alexander, Fabbro et al., 2021).

AUTHOR CONTRIBUTIONS

All authors contributed to the conceptualisation, writing, review and editing of the manuscript. All authors approved the submitted version.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

N/A-Review.

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