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REVIEW

Pulmonary arterial hypertension: basis of sex differences in incidence and treatment response

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Pulmonary arterial hypertension (PAH) is a complex disease characterized by elevated pulmonary arterial pressure, pulmonary vascular remodelling and occlusive pulmonary vascular lesions, leading to right heart failure. Evidence from recent epidemiological studies suggests the influence of gender on the development of PAH with an approximate female to male ratio of 4:1, depending on the underlying disease pathology. Overall, the therapeutic strategy for PAH remains suboptimal with poor survival rates observed in both genders. Endogenous sex hormones, in particular 17β oestradiol and its metabolites, have been implicated in the development of the disease; however, the influence of sex hormones on the underlying pathobiology remains controversial. Further understanding of the influence of sex hormones on the normal and diseased pulmonary circulation will be critical to our understanding the pathology of PAH and future therapeutic strategies. In this review, we will discuss the influence of sex hormones on the development of PAH and address recent controversies.

LINKED ARTICLES
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Abbreviations
AR, androgen receptor; BMPR2, bone morphogenetic protein receptor 2; CYP19A1, aromatase; CYP1B1, cytochrome P450 1B1; DHEA, dehydroepiandosterone; DHT, dihydrotestosterone; ER, oestrogen receptor; ERα, oestrogen receptor alpha; ERβ, oestrogen receptor beta; ET-1, endothelin-1; GPER, G protein-coupled oestrogen receptor; PAH, pulmonary arterial hypertension; PASMC, pulmonary artery smooth muscle cell; SERT, serotonin transporter; Su-Hx, SUGEN hypoxic; Tph1, tryptophan hydroxylase 1

Pulmonary arterial hypertension (PAH) is a progressive disease leading to right heart failure. Recent epidemiological data reports an increased incidence of PAH among females compared to males. For example, in the UK/Ireland and the USA, the percentage of female patients is 70 and 80% respectively (Badesch *et al.*, 2010; Ling *et al.*, 2012). There is mounting evidence to suggest that oestrogen and its metabolites may influence the pathogenesis of PAH. Here, we will examine the pathology, current therapies and the basis for gender differences in PAH, considering evidence gathered from both patient data and animal studies.

Pulmonary arterial hypertension

PAH is defined by vascular remodelling and complex vascular lesion formation arising from the accelerated proliferation of pulmonary endothelial, smooth muscle and fibroblast cells (Rabinovitch, 2008). Clinically, the disease is defined as a mean pulmonary artery pressure of >25 mmHg at rest or >30 mmHg during exercise. Symptoms are often non-specific, including fatigue, exertional dyspnoea, oedema and syncope and as a result diagnosis is frequently delayed until the disease is well established.

The main genetic defect associated with PAH is a mutation in the gene encoding bone morphogenetic protein receptor 2 (BMPR2). Germ line mutations in BMPR2 were originally identified in patients with heritable PAH (HPAH; Deng *et al.*, 2000; Lane *et al.*, 2000). In these families, the disease segregates in an autosomal dominant fashion, with markedly reduced penetrance, approximately 20 to 30%. In addition, up to 25% of patients with apparently sporadic idiopathic PAH (IPAH) have been found to harbour similar mutations (Thomson *et al.*, 2000). A proportion of these

mutation carriers are examples of HPAH where the condition has not manifested in relatives due to low penetrance, while others are examples of *de novo* mutation (Newman *et al.*, 2001). The low penetrance of the disease among BMPR2 mutation carriers suggests other factors are important in the onset of clinical PAH and that at least one more genetic or environmental 'hit', in addition to a mutation in BMPR2, underlies established PAH. Indeed, a multitude of factors have been implicated in the pathobiology of the condition including endothelin-1 (ET-1; Giaid *et al.*, 1993), phosphodiesterases (Murray *et al.*, 2011), oestrogens (White *et al.*, 2011a) and serotonin (Maclean and Dempsie, 2010). Heterozygous mutations in the activin receptor-like kinase 1 and endoglin have also been associated with PAH (Trembath *et al.*, 2001; Chaouat *et al.*, 2004). The plethora of agents highlighted as potential mediators of PAH is consistent with the complex and multifactorial nature of the disease pathology.

Currently pulmonary hypertension (PH) is classified by the World Health Organization (WHO) into five main categories subject to underlying causes (Table 1). PAH, the focus of this review is categorized as group 1 PH and subdivided into IPAH, HPAH, drug- or toxin-induced PAH or PAH associated with diseases such as connective tissue diseases, HIV infection, portal hypertension, congenital heart disease, schistosomiasis and chronic haemolytic anaemia (APAH). Despite the wide spectra of initiating factors, the disease arises as a result of pulmonary vasoconstriction, vascular remodelling and thrombosis (Rabinovitch, 2008).

Maintaining a vasodilated pulmonary circulation is essential for oxygenation of the blood. The pulmonary arterial endothelium is a source of vasodilators such as NO and prostacyclins. In PAH there is an imbalance in the production of vasodilatory and vasoactive agents in the pulmonary artery ultimately favouring vasoconstriction. Elevated levels of ET-1, a potent pulmonary vasoconstrictor and a decrease in vasodilators such as NO have been reported in patients with the disease (Giaid and Saleh, 1995; Cacoub *et al.*, 1997). An imbalance in prostacyclin and thromboxane A_2 (TXA_2) levels in the favour of TXA_2 also leads to vasoconstriction, thrombosis and platelet activation (Christman *et al.*, 1992; Tuder *et al.*, 1999).

Furthermore, in normal circumstances the diameter of the pulmonary artery wall is maintained by a balance between apoptosis and proliferation of the cells of the vessel wall. However in PAH proliferation is dominant, resulting in the muscularization of previously non-muscular pulmonary arterioles and a thickening of the medial layer of the vessel wall resulting in a narrowing of the lumen and the loss of small pre-capillary arteries (Rabinovitch, 2008). Ultimately, this vascular remodelling results in the formation of vascular lesions which eventually occlude the vessel. Plexiform lesions, comprised of a mass of disorganized vessels with proliferating endothelial cells (main component), smooth muscle cells, myofibroblasts and macrophages, also occur in PAH. These are angioproliferative lesions which arise from pre-existing pulmonary arteries and are often symptomatic of end-stage disease (Cool *et al.*, 1999).

In summary, the combination of vasoconstriction and arterial remodelling that occurs during PAH results in vascular pruning (the obliteration of small resistance arteries) and

Table 1

Dana Point classification of pulmonary hypertension

Dana Point clinical classification of pulmonary hypertension updated 2008	
1. PAH	
1.1 Idiopathic PAH	
1.2 Heritable	1.2.1 BMPR2
	1.2.2 ALK1, endoglin (with or without hereditary haemorrhagic telangiectasia)
	1.2.3 Unknown
1.3 Drug and toxin induced	
1.4 Associated with	1.4.1 Connective tissue diseases
	1.4.2 HIV infection
	1.4.3 Portal hypertension
	1.4.4 Congenital heart diseases
	1.4.5 Schistosomiasis
	1.4.6 Chronic hemolytic anaemia
1.5 Persistent pulmonary hypertension of the newborn	
1′ Pulmonary veno-occlusive disease (PVOD) and/or pulmonary capillary haemangiomatosis (PCH)	
2. Pulmonary hypertension owing to left heart disease	
3. Pulmonary hypertension owing to lung diseases and/or hypoxia	
4. Chronic thromboembolic pulmonary hypertension (CTEPH)	
5. Pulmonary hypertension with unclear multifactorial mechanisms	

Table summarizing the updated 2008 Dana Point classification of pulmonary hypertension with main focus on category 1 PAH. Adapted from Simonneau *et al.* (2009).

increases pulmonary vascular resistance and pressure. Thus, the disease pathology results in an excessive burden on the right ventricle due to the increased workload required to compensate for the elevated downstream pressure and eventually results in right-sided heart failure. The extent of right heart dysfunction is often used as predictor of prognosis in PAH.

Epidemiology, sex differences and survival

As evident by recent epidemiological registries, the incidence of PAH varies from 1.1, 2.0 and 2.4 per million of the adult population per year in the UK/Ireland, USA and France respectively (Humbert *et al.*, 2006; Frost *et al.*, 2011; Ling *et al.*, 2012). The reported incidence of IPAH in the USA and French registries of PAH is 46.2 and 39.2% respectively with APAH incidence also showing similarity at 50.7 and 52.7% respectively. The data documented from the UK and Ireland's PAH registries highlights different subcategory demographics with IPAH incidence at 92.9% and APAH at 1.7% (Ling *et al.*, 2012).

In the REVEAL registry, it was found that 83.1% of patients studied fell into the age category group 19–64 years with 12.8 and 4.1% in the 65–74 years and 75+ years group respectively (Badesch *et al.*, 2010). In this instance, the mean age of diagnosis appears similar between the sexes, 46 ± 19 years for men and 48 ± 17 years for women. However, it was documented that men present with significantly higher mean pulmonary artery pressure than women at baseline (Badesch *et al.*, 2010).

There is a general consensus that female gender is a risk factor for PAH, with recent studies showing a female to male ratio of 4.3:1 among the total PAH group (Walker *et al.*, 2006) and 4.1:1 in the IPAH subcategory (Badesch *et al.*, 2010). This female prevalence is much stronger compared to a previous report from the NIH registry where the female to male ratio was reported as 1.7:1 (Rich *et al.*, 1987). However, this ratio represented the total PAH population studied and was not further analysed into subcategories.

In some cohorts of patients, there has been considerable improvement in survival over the past two decades since the establishment of the NIH registry. This is likely to be due to changes in treatments and improved patient support strategies (Benza *et al.*, 2012). However, the current 3-year survival for patients with PAH managed with state-of-the-art multiple drug therapy remains troubling at approximately 58% (Humbert *et al.*, 2010a).

Although the incidence of PAH is higher in women than in men, the estimated 5-year survival rate from diagnosis in the REVEAL registry is ~52% in men, compared to 62% in women (Shapiro *et al.*, 2012). This suggests that either women respond better to treatment options or that female sex hormones are mediating protective effects. Interestingly, when age was accounted for, survival was only different between males and females after the age of 60 (Shapiro *et al.*, 2012). This again highlights the importance of considering gender and age differences in both the diagnosis of PAH and importantly, also in the treatment.

Current therapies for PAH

The current therapeutic strategy for PAH is suboptimal. The majority of therapies alleviate the symptoms and subsequently improve the quality of life of patients, but fail to address the underlying disease pathology. In order to make progress in the management and treatment of the condition, therapies that drastically regenerate the lost distal pulmonary arteries and prevent the extensive pulmonary vascular remodelling observed in PAH are urgently required. At present, patients will inevitably succumb to the disease, or are considered for lung transplantation. Although an immense amount of research effort has advanced our understanding of this once poorly characterized disease, the exact mechanisms that underlie the disease pathology are still being clarified. PAH is multifactorial and complex in nature and individual variability may also suggest personalized treatment options in the future. Furthermore, given the increased frequency of PAH in females, therapies specifically targeted to women may be a potential therapeutic strategy.

As PAH is a multifactorial process with varying aetiologies, treatment options therefore depend on the PAH classification. The majority of patients receive supportive therapies including diuretics, anticoagulants, oxygen and digoxin (Galie *et al.*, 2009b). Where possible therapies directed at the underlying cause of the PH are utilized. However, in WHO Group 1 PAH, the underlying cause of the disease is typically unknown, and therefore more advanced therapies which attempt to target the PAH are considered. If patients react positively to a vasoreactivity test (requiring right heart catheterization) calcium channel blockers are administered, and a certain percentage of patients will respond. The most commonly used are nifedipine and diltiazem (Galie *et al.*, 2009b).

Prostacyclin is a potent vasodilator and inhibitor of platelet aggregation. Dysregulation of prostacyclin metabolic pathways have been reported in patients with PAH (Tuder *et al.*, 1999), and as such, several prostacyclin analogues including epoprostenol, iloprost and treprostinil are used clinically in the treatment of PAH (Galie *et al.*, 2009b). Although these drugs improve symptoms and exercise capacity, many require administration by intravenous infusion due to the short half-life of prostacyclin. There are serious adverse effects related to this delivery system, such as local site infection, catheter obstruction and in severe instances sepsis. Treprostinil is currently available in a subcutaneously administered form, while oral and aerosol forms of iloprost and treprostinil have now been developed (Olschewski, 2009; Channick *et al.*, 2012; Tapson *et al.*, 2012). Inhaled drugs, which target PAH, are an attractive possibility as this would potentially make them selective for the pulmonary circulation.

ET-1 is abundantly over-expressed in endothelial cells from patients with PH (Giaid *et al.*, 1993) and stimulates proliferation of pulmonary artery smooth muscle cells (PASMC; Wort *et al.*, 2001) and vasoconstriction. ET-1 receptor antagonists, bosentan, sitaxsentan and ambrisentan are currently approved for the treatment of PAH, preventing the aberrant activity of ET-1 observed in patients (Oudiz *et al.*, 2009; Dwyer and Kilpatrick, 2011; Rubin, 2012). Precautions must also be taken when administering this class of

compounds as they have been reported to display marked liver toxicity, and as a result, regular liver function test are required (McGoon *et al.*, 2009).

Finally, inhibitors of PDE5 have also been licensed for the treatment of PAH, including sildenifil and tadalafil (Galie *et al.*, 2009a). PDE5 inhibitors prevent the breakdown of cGMP and mediate vasorelaxation as well as having antiproliferative properties.

In instances where response to a single therapy is inadequate, combination therapy, utilizing more than one PAH-specific class of drug has become standard (Chen *et al.*, 2009; Galie *et al.*, 2009c). There are many questions regarding combination therapy including which combinations to use and whether a combination of drugs should be used initially or sequentially depending on response to the first drug.

Despite PAH being more frequently observed in females, a personalized medicine approach is not yet possible. Recently, it has been reported that women with PAH exhibit a greater clinical benefit from ET-1 receptor antagonists than men, as measured by a 44.1 metre improvement in the 6 min walk distance compared to 16.7 metre improvement in men (Gabler *et al.*, 2012). As men often present with significantly higher mean pulmonary arterial pressure than women at baseline (Shapiro *et al.*, 2012), this could be the reason as to why women respond better to treatment as their phenotype is less severe. There may also be differing circulating ET-1 levels between women and men with men having higher ET-1 compared to women (Miyauchi *et al.*, 1992; Polderman *et al.*, 1993). This heterogeneity in treatment response may reflect pathophysiological differences between sexes or distinct disease phenotypes.

PAH in females

Some studies have linked the use of the contraceptive pill and hormone replacement therapy to the increased incidence of PAH in females (Masi, 1976; Thorne *et al.*, 2006; Sweeney and Voelkel, 2009). However, a clear link between hormonal-based therapies and PAH has not been established. Despite this, the increased frequency of PAH in women has given rise to the hypothesis that oestrogen may play a role in disease development and progression. This idea may not only suggest why more women appear to be affected by PAH but may also allow us to propose possible mechanisms involved in disease development.

Other risk factors associated with being female such as increased autoimmunity may also play a role in the development of PAH. For instance, PAH is a devastating complication in patients with the autoimmune diseases such as systemic sclerosis and systemic lupus erythematosus (Ruiz-Irastorza *et al.*, 2012). These conditions are well documented to be more prevalent in females (Rubtsov *et al.*, 2010). Moreover, female gender and autoimmune hepatitis are also associated with an increased risk for portopulmonary hypertension, a subcategory of PAH (Kawut *et al.*, 2008).

Most studies, registries or clinical trials will report the effects of treatment on PAH indices and patient survival but not necessarily divide this into female and male cohorts. Retrospective analysis of this data would provide valuable information. However, some studies have reported gender differences in treatment response and in doing so these findings may eventually challenge the therapeutic decision made by clinicians. Further analysis of the longitudinal REVEAL study shows that women, in the particular cohort studied, have improved survival rates compared to men (Shapiro *et al.*, 2012). However, this analysis does not declare which, if any, treatments were better than others in causing this improvement. The underlying factors responsible for this remarkable difference are unclear. The study does suggest that in certain cohorts there may be male/female differences in the pathology of PAH.

Although the incidence of PAH is greater in female patients, numerous studies have demonstrated better outcome in female animals using classical PAH models such as chronic hypoxia and monocrotaline-induced PAH (Rabinovitch *et al.*, 1981; Umar *et al.*, 2011). This coupled with the fact that oestrogen is a potent pulmonary vasodilator (Lahm *et al.*, 2008) and that removal of female sex hormones by ovariectomy induces a more severe PAH phenotype (Ahn *et al.*, 2003; Nadadur *et al.*, 2012) has been coined the 'oestrogen paradox' of PAH (Umar *et al.*, 2012). Research into why PAH occur more frequently in women has therefore been hampered by lack of appropriate models.

However, more recently, transgenic mouse models have been characterized that demonstrate female susceptibility. These include mice over-expressing the human serotonin transporter ($SERT^+$ mice; White *et al.*, 2011a), those that over-express the calcium-binding protein S100A4/Mts1 (Dempsie *et al.*, 2011) and dexfenfluramine-treated mice (Dempsie *et al.*, 2009). What all these models have in common is over-activity of the serotonin system. Indeed, synthesis of serotonin has also been associated with the development of the SUGEN/hypoxic model of PAH. Inhibition of the VEGF receptor by SUGEN combined with hypoxia (Su-Hx) generates a preclinical model of PAH that recapitulates human PAH more closely than previously characterized models (Gomez-Arroyo *et al.*, 2012). This model is associated with increased expression of tryptophan hydroxylase 1 (Tph1), the rate-limiting enzyme in serotonin synthesis (Ciuclan *et al.*, 2011, 2013). It has also recently been reported that unlike classical PAH models, female Su-Hx rats develop a more severe disease phenotype than males suggesting oestrogen may be a risk factor (Tofovic *et al.*, 2012). However, this difference is not observed in the Su-Hx mouse (White *et al.*, 2012). Models which demonstrate the development of PAH in females rather than males are already yielding important information and may give rise to novel therapeutic strategies (Dempsie *et al.*, 2011; White *et al.*, 2011a,b).

Role of oestrogens in PAH

Oestrogens are steroid hormones, which in addition to their key role in the development of secondary sex characteristics, also play a role in memory, bone density and have been show to have cardiovascular effects. The three major oestrogens are oestrone, oestradiol and oestriol. Aromatase (CYP19A1), a member of the cytochrome P450 superfamily, synthesizes oestrogens through the aromatization of androgens, specifically testosterone and androstenedione (Figure 1). Oestrogen synthesis occurs mainly in the ovarian follicles and corpus

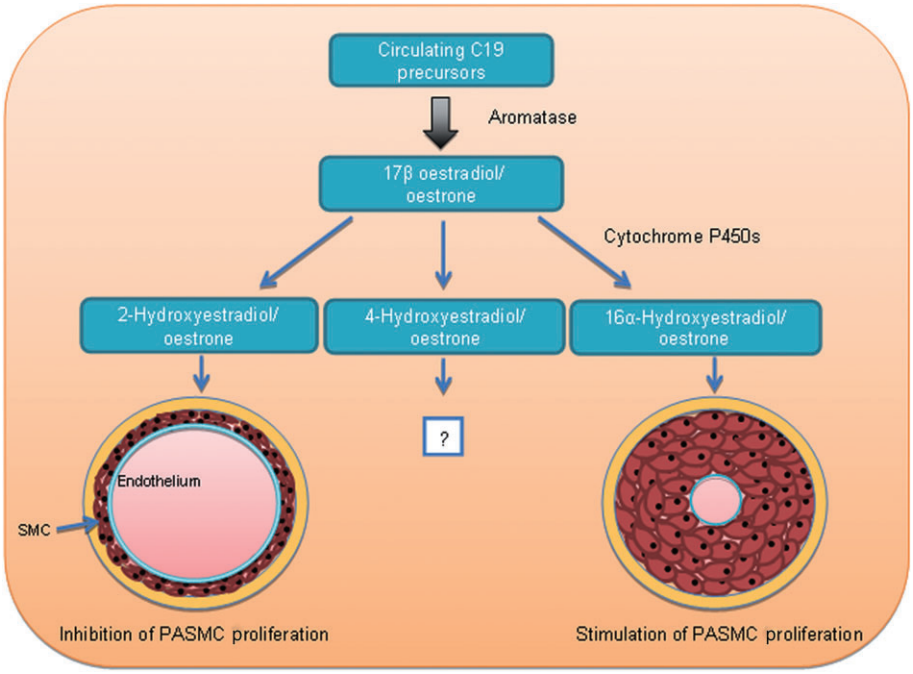

Figure 1

Oestrogen metabolism in PAH. The circulating C19 precursors, testosterone and androstenedione are converted by CYP19A1 to 17β oestradiol and oestrone respectively. These metabolites then undergo hydroxylation at the C2, C4 or C16 positions by activity of CYP enzymes, including CYP1B1. While hydroxylation at C2 produces anti-proliferative metabolites, C16 hydroxylation induces proliferative metabolites. The CYPs are therefore key determinants of metabolite formation and can perturb the pathway towards an anti- or pro-proliferative state. Hydroxylated oestrogens can be further metabolized to the methoxyestrogens, which may also influence PASMC proliferation.

luteum from where oestrogen is released into the circulation. Synthesis also occurs to a lesser extent in non-glandular tissues such as adipose tissue, liver, skin, muscle and brain (Simpson *et al.*, 2005). MacLean *et al.*, have recently demonstrated that CYP19A1 is expressed in the medial layer of pulmonary arteries from PAH patients, providing evidence of *de novo* synthesis (unpublished).

17β oestradiol, the main circulating premenopausal hormone, mediates protective effects in models of PAH. For example, 17β oestradiol attenuates the development of a PAH phenotype in both the chronic hypoxic model of PAH (Xu *et al.*, 2010; Lahm *et al.*, 2012) and in the inflammatory monocrotaline model of the disease (Farhat *et al.*, 1993). These studies all used male rats; however, as females have much higher circulating levels of oestrogen with cyclical fluctuations, comparative studies using females would be of interest. However, there is evidence that oestrogen may also attenuate the development of PAH in female animals. These studies used monocrotaline or the chronic hypoxic model, which as discussed previously are more effective at inducing PAH in males (Resta *et al.*, 2001; Nadadur *et al.*, 2012; Yuan *et al.*, 2012).

In contrast, there is evidence that oestrogen is a causative factor in novel models that demonstrate the development of PAH in females but not males. For example, the PAH disease phenotype in the SERT+ female mice can be prevented by ovariectomy and recovered by chronic administration of 17β oestradiol, indicating an essential function of this hormone in the development of a PAH phenotype (White *et al.*, 2011a). These findings suggest that serotonin is facilitating and amplifying the effects of oestrogen in the pulmonary circulation. Indeed, it has been shown that oestrogen increases expression of Tph1, SERT and the 5-hydroxytryptamine 1B receptor in human PASMCs (White *et al.*, 2011a).

Given that right ventricular dysfunction is the best predictor of prognosis in PAH and females are afflicted less severely than males with regard to a failing right ventricle, it is conceivable that oestrogen is cardioprotective. Indeed, in an animal model of right ventricular failure, oestrogen therapy is shown to completely reverse PH-induced right ventricular remodelling associated with right ventricular dysfunction (Lahm *et al.*, 2012; Nadadur *et al.*, 2012). Moreover, higher right ventricular ejection fraction and survival rates in females correlate with oestradiol levels (Kawut *et al.*, 2008; Ventetuolo *et al.* 2011). Cardioprotective properties of oestradiol perhaps contribute to improved survival in female patients with PAH despite the higher female prevalence in the disease. In support of this, female Su-Hx rats display a significantly smaller increase in right ventricular mass compared to males even although they develop a more severe disease phenotype (Tofovic *et al.*, 2012). Thus, the potential diverging effects of oestrogens in the cardiopulmonary unit merit further investigation before we consider treatment options. However, these findings highlight the importance of incorporating gender differences into basic research and may go some way to explaining the 'oestrogen paradox' whereby exogenous oestrogens appear protective in male animals.

Currently, two promoter single nucleotide polymorphisms in the gene coding for CYP19A1 that result in elevated oestrogen production have been associated with an increased risk of portopulmonary hypertension (Roberts *et al.*, 2009). Furthermore, preliminary unpublished studies from MacLean *et al.*, indicate that CYP19A1 is present in both smooth muscle cells and endothelial cells of the pulmonary vasculature. Locally synthesized oestrogen could therefore act in a paracrine fashion on smooth muscle cells and exert a more powerful influence than circulating oestrogens. Furthermore, expression of CYP19A1 is regulated, in part, by tissue-specific promoters and by alternative splicing mechanisms (Simpson *et al.*, 2005). Further investigation into the specific mechanisms that regulate CYP19A1 expression in the lung will contribute to improved understanding of the role of CYP19A1 and oestrogen in the lung under both physiological and pathophysiological conditions.

Oestrogen receptors

Oestrogen activates three oestrogen receptors (ER), ERα, ERβ and G protein-coupled oestrogen receptor (GPER). ERα and ERβ mainly mediate the genomic effects of oestrogen; however, some non-genomic effects have been reported (Lahm *et al.*, 2008). GPER signals rapidly through non-genomic mechanisms (Filardo *et al.*, 2000). All three receptor types are present in the human pulmonary artery. In the hypoxic rat model, the beneficial effects of oestrogen on the pulmonary circulation are mediated by genomic ERs, ERα and ERβ (Lahm *et al.*, 2012). Additionally, the ERβ agonist, diarylpropionitrile, has been shown to rescue severe monocrotaline-induced PAH to a similar extent as 17β oestradiol (Umar *et al.*, 2011).

Transcript levels of ESR1, the gene encoding ERα are increased in both male and female PAH patients (Rajkumar *et al.*, 2010), raising the novel paradigm of ER-positive PAH. This would certainly impact the way we consider individual patient treatment options. Recently, evidence has emerged that ERα has an evolutionary conserved binding site on the BMPR2 promoter and can thereby reduce its expression (Austin *et al.*, 2012), giving evidence for a functional role of ERα in PAH. Furthermore, BMPR2 expression was found to be decreased in both lymphocytes from female patients and in whole lungs from female mice compared with their male counterparts. It is plausible that the increased endogenous oestrogen levels in females compared to males results in the decrease in BMPR2 expression observed and contributes to the increased incidence of PAH in females.

While evidence suggests that ERα may mediate the detrimental effects of oestrogens in PAH development, clinical data regarding ERβ in PAH is lacking. ERβ agonists have been shown to be protective in animal models of PAH (Umar *et al.*, 2011). The phytoestrogen, genistein has much higher affinity for ERβ than ERα and reverses severe PAH in rats (Matori *et al.* 2012). However, lung dysfunction has been reported in ERβ knockout mice. Loss of ERβ in female mice leads to abnormal lung structures and a hypoxic environment, which ultimately contributes to ventricular hypertrophy (Morani *et al.* 2006).

Similarly, changes in the receptor expression profile of GPER in the pathogenesis of PAH are yet to be investigated. Detailed studies to determine the precise function of all three ERs in the healthy lung compared to PAH may yield vital information on the contribution of oestrogen signalling in the disease process. Furthermore, teasing out differences in changes in the receptor expression profile between males and females may prove useful in identifying underlying mechanisms responsible for the female predominance in PAH patients and contribute to more targeted therapies for the condition.

Oestrogen metabolites and PAH

Oestrogen metabolites and alterations in oestrogen metabolism have also been implicated in the pathobiology of PAH. 17β oestradiol is readily metabolized by cytochrome P450 (CYP) enzymes, which are abundantly expressed in the lung. CYPs catalyse the oxidation of 17β oestradiol, in the presence of NADPH and oxygen, to the 2-, 4- and 16α hydroxyestradiols. 17β oestradiol can also be inter-converted to oestrone by enzymatic activity of the 17β hydroxysteroid dehydrogenases, which can subsequently be converted to the 2-, 4- and the 16α hydroxyestrones (Figure 1). The 2- and 4-hydroxyestrogens can then undergo methylation by the activity of catechol O-methyltransferase (COMT) to the respective methoxyestrogens.

The compounds formed during this metabolic process can play differential roles in PAH. For instance, 2-hydroxyestradiol, its methylated metabolite, 2-methoxyestradiol and the synthetic analogue 2-ethoxyestradiol mediate protective effects in experimental models of PAH (Tofovic *et al.*, 2008). In contrast, 16α hydroxyestrone induces significant proliferation of human PASMCs and administration of this oestrogen metabolite can induce PAH in mice (White *et al.*, 2012). Furthermore, 16α hydroxyestrone may be actively involved in the pathogenesis of PAH, as indicated by increased urinary concentrations in experimental PAH (White *et al.*, 2012). Importantly, female urinary 16α hydroxyestrone levels are also increased in patients with BMPR2-associated PAH (Austin *et al.*, 2009). At present, the precise function and relationship between oestrogen and its metabolites in PAH is still largely unknown. A metabolic shift towards the formation of pro-proliferative metabolites by altered expression of enzymatic proteins could have potentially detrimental effects on the pulmonary circulation. For example, increased expression of the oestrogen-metabolizing enzyme, CYP4501B1 (cytochrome P450 1B1; CYP1B1), altering the metabolic pathway, is associated with the development and progression of PAH, potentially through the increased formation of 16α hydroxyestrone (White *et al.*, 2012).

CYP1B1 metabolizes oestrogen predominantly to the 4-hydroxyestrogens and to a lesser extent to the 2- and 16α-hydroxyestrogens (Badawi *et al.*, 2001; Lee *et al.*, 2003). Under basal conditions, CYP1B1 expression is low. However CYP1B1 expression is up-regulated in animal models of PAH and critically, it is also consistently up-regulated in both IPAH and HPAH (White *et al.*, 2012). In line with this, loss of function and inhibition of CYP1B1 is protective in preclinical models of PAH, giving evidence that CYP1B1 is involved in the pathogenesis of PAH (White *et al.*, 2012). In this study,

CYP1B1 was integral to the pathogenesis of PAH in both male and female animals, with no major gender differences reported, emphasizing the potential impact of locally synthesized oestrogens (White *et al.*, 2012). Additionally, CYP1B1 is also involved in other metabolic pathways, such as metabolism of arachidonic acid, which has been implicated in PAH (Choudhary *et al.*, 2004; Zhu and Ran, 2006). Thus, additional protective effects may be mediated by other pathways. Consistent with the hypothesis that serotonin may amplify the effects of oestrogen, serotonin has been found to increase CYP1B1 expression in human PASMCs and CYP1B1 expression is also increased in pulmonary arteries from female SERT+ mice (White *et al.*, 2011b). These finding suggest serotonin may alter oestrogen metabolism and promote the formation of pro-proliferative oestrogen metabolites leading to the development of PAH.

In addition, oestrogen metabolites may interact and signal through the ERs. In a comprehensive analysis, the binding affinity of natural and synthetic oestrogen metabolites for the different receptor subtypes was studied. Interestingly, 17β oestradiol, 4-hydroxyestradiol and 4-hydroxyestrone and 2-hydroxyestradiol have an equal binding affinity for both ERα and ERβ receptors, whereas oestrone and 2-hydroxyestrone preferentially bind at ERα (Zhu *et al.*, 2006). In contrast both 16α hydroxyestradiol and 16α hydroxyestrone have a higher binding affinity for ERβ (Zhu *et al.*, 2006). Taken together, these findings suggest that modulating oestrogen metabolism, for example, by targeting CYP1B1 may be a novel therapeutic strategy for the treatment of PAH.

Androgens and PAH

Despite females developing PAH more frequently, male patients are consistently shown to have poorer survival even with treatment (Benza *et al.*, 2010; Humbert *et al.*, 2010a; 2010b). Androgens may therefore play a role in the gender differences observed in PAH. There is a paucity of data on male hormones in the pulmonary vasculature and the exact role of androgens in the physiology and pathophysiology in PAH remains uncertain.

Testosterone is the main secreted androgenic steroid. It is primarily biosynthesized in the testes and by the ovaries in females, although small amounts are also secreted from the adrenal cortex. The effects of testosterone are mediated via the androgen receptor (AR), which is located in a variety of tissues including vascular smooth muscle and endothelial cells (Dubey *et al.*, 2002; Liu *et al.*, 2003) and also in the lung (Mikkonen *et al.*, 2010). Testosterone can activate the AR directly, or following its conversion by the enzyme 5α-reductase to the more potent androgen dihydrotestosterone (DHT; Figure 2). In general, testosterone plays a crucial role in the development, growth and function of the male reproductive tissues although it is becoming increasingly apparent that androgens also play a pivotal role in cardiovascular disease (Dubey *et al.*, 2002).

As previously mentioned, in PAH, right ventricular dysfunction is the most important prognostic factor and indicator of survival (D'Alonzo *et al.*, 1991). ARs have been identified in both the right and left ventricle (Lizotte *et al.*, 2009); however, to date, the left ventricle has been more extensively studied. Both testosterone and its primary metabolite, DHT, mediate genomic effects through the AR, although DHT is 10 times more potent in activation of the AR than testosterone itself (Liu *et al.*, 2003). Furthermore, there is strong evidence that testosterone does indeed cause structural and morphological changes in human hearts (Achar *et al.*, 2010) and both testosterone and DHT initiate cardiac hypertrophy (Hayward *et al.*, 2001). In addition, metabolism of testosterone is significantly changed in the hypertrophic heart, with elevated levels of DHT described in human left ventricular hypertrophy (Thum and Borlak, 2002) and this is thought to exacerbate cardiac hypertrophy. As survival is worse in males, and survival is closely linked to right ventricle function, a correlation between testosterone and the right ventricle is proposed. The degree of right ventricular hypertrophy in rats exposed to high altitude is greater in castrated males treated with testosterone (Vander *et al.*, 1978) and the effect of testosterone and hypoxia appear additive. Recently, Hemnes *et al.* provided the first evidence for testosterone-induced right ventricular fibrosis and increased myocyte size in a model of PAH. In this study, it was observed that there were no alterations in haemodynamic properties resulting from testosterone manipulation suggesting that the effects of testosterone are primarily involved in dysfunctional right ventricular (RV) hypertrophy (Hemnes *et al.*, 2012). Therefore, following increased afterload inflicted by elevated pulmonary pressures in PAH, testosterone may be the underlying cause of some of the differences in survival between males and females.

Testosterone is also a potent vasodilator in isolated human pulmonary vasculature, an effect that is independent of gender (Smith *et al.*, 2008; Rowell *et al.*, 2009). It is in fact a more potent vasodilator than oestrogen in this vascular bed (English *et al.*, 2001). Importantly, this action of testosterone is generally accepted to be a rapid, non-genomic effect and therefore is independent of the AR (Yue *et al.*, 1995; Jones *et al.*, 2002). The mechanism of vasodilation may be independent of the endothelium and NO (Yue *et al.*, 1995; Jones *et al.*, 2002) and due to inhibition of Ca^{2+} entry via voltage-gated calcium channels (Scragg *et al.*, 2004; Hall *et al.*, 2006). Acute and long-term vasodilation by testosterone is often suggested to be due to conversion to oestrogen. However, several studies have demonstrated that the vasodilator effect of testosterone is not inhibited by CYP19A1 inhibition or by ER antagonism (Teoh *et al.*, 2000; Deenadayalu *et al.*, 2001; Tep-areenan *et al.*, 2002). Thus, the protective vascular effects of testosterone could provide rationale for a lower incidence of PAH in men. However, as PAH becomes established, the disease progress may be more exaggerated in men due to the negative effects of testosterone on the right ventricle leading to subsequent right ventricular failure. For therapeutic intervention in men, treatments reversing right ventricular remodelling may be more beneficial in prolonging survival compared to the currently available vasodilator options.

Another interesting androgen target, which is becoming increasingly studied in the setting of PAH, is dehydroepiandosterone (DHEA). DHEA is a naturally occurring steroid derived from the adrenal glands and is the most abundantly secreted circulating steroid. The sulphated ester, DHEA-S, serves as an inactive reservoir with conversion by sulfotransferases occurring in a wide range of tissues. Previously,

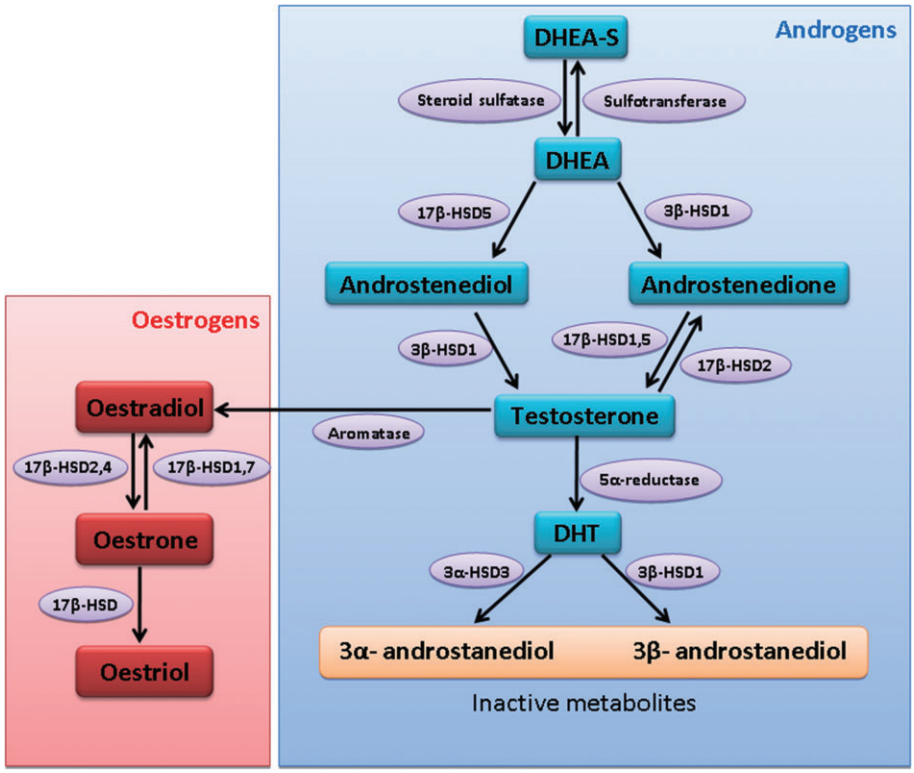

Figure 2

Schematic representation of androgen and oestradiol metabolism. Sex hormones are derived from cholesterol and converge on the circulating precursor DHEA and its sulphated form (DHEA-S). Both males and females possess the enzyme 17β-hydroxysteroid dehydrogenase (17β-HSD) that enables the conversion androgens to testosterone. The enzyme 5α-reductase then converts testosterone into the more potent metabolite DHT, which is subsequently deactivated by HSD enzymes. Testosterone can also be metabolized by the cytochrome P450 enzyme CYP19A1 to oestradiol which is further metabolized by HSD enzymes to oestrone and oestriol.

age-related declines in serum DHEA and DHEA-S have been attributed to development of cardiovascular diseases (Barrett-Connor *et al.*, 1986; Baulieu, 2002) and epidemiological and animal studies support a beneficial role for DHEA (Barrett-Connor and Goodman-Gruen, 1995; Williams, 2000). In addition, clear gender differences have been described in circulating levels of DHEA-S with levels twice as high in men as in women (Parker, 1999). DHEA has recently been described as having a multifunctional protective role in PAH. Pulmonary vasodilator properties of DHEA have been demonstrated in chronic hypoxic male rats and monocrotaline models associated with both opening of voltage-gated potassium channels (Farrukh *et al.*, 1998; Gupte *et al.*, 2002) and increased expression and function of pulmonary artery Ca^{2+}-activated K^+ channels (Bonnet *et al.*, 2003; Hampl *et al.*, 2003).

Moreover, antioxidant properties of DHEA have also been demonstrated in human PASMCs, decreasing proliferation and resistance to apoptosis by modulating mitochondrial functions (Dumas de la Roque *et al.*, 2010). Effects of DHEA on proliferation and apoptosis appear directly mediated as they are independent of both AR and ERs (Williams *et al.*, 2002; 2004; Oka *et al.*, 2007; Bonnet *et al.*, 2009). In line with this, DHEA reverses pulmonary arterial remodelling by various mechanistic pathways. Normalizing RhoA/ROCK activity in hypoxia was associated with prevention of vascular remodelling (Homma *et al.*, 2007), decreased Src/STAT3 activation with restored BMPR2 (Paulin *et al.*, 2011) and decreased accumulation of hypoxia-inducible factor α in PASMC during hypoxia (Dessouroux *et al.*, 2008) contribute to decreased PASMC proliferation and remodelling by DHEA.

In summary, it is possible that the lower DHEA levels in women compared to men contributes to the higher frequency of PAH in women. The ability of DHEA to inhibit proliferation and induce significant vasodilation in pulmonary vasculature makes it an attractive drug target for treatment of PAH. Although there is yet no data on the clinical effect of DHEA treatment of PAH, DHEA has been shown to reverse PAH in chronic hypoxic and monocrotaline male rat models (Hampl *et al.*, 2003; Homma *et al.*, 2007; Oka *et al.*, 2007; Dumas de la Roque *et al.*, 2010). Interestingly, DHEA has also been found to act as a potent suppressor of CYP1B1 expression in cancer cells (Ciolino *et al.*, 2003; Mikstacka *et al.*, 2008). Data regarding the effect of DHEA in females are lacking. However, DHEA treatments in females should be approached with caution given that metabolism of DHEA to oestrogen may augment PAH (Dempsie *et al.*, 2011; White *et al.*, 2011a) and long-term exposure could promote hormone-dependent breast cancer.

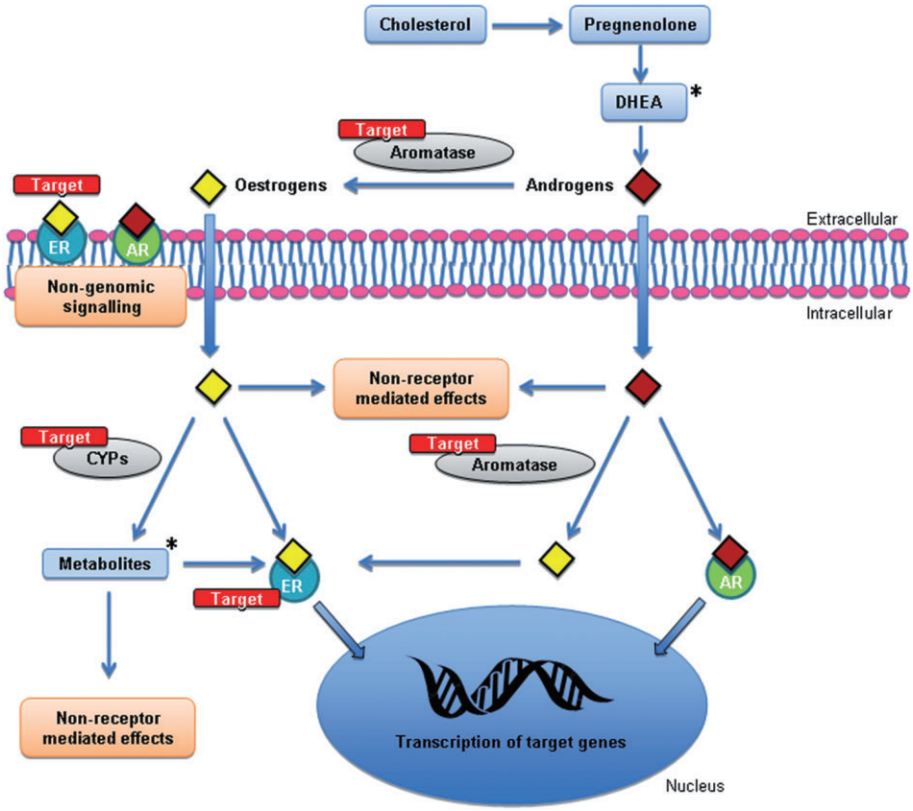

Figure 3

A schematic representation highlighting the potential therapeutic targets within sex hormone pathways in pulmonary arterial smooth muscle cells. *Metabolites of oestrogens and DHEA may also be useful in the treatment of PAH.

Conclusion

Despite the female predominance in patients with PAH, no sex-based treatments are currently offered. Most issues specifically related to the care of women with PAH are related to birth control and pregnancy, as pregnancy in PAH carries a significant risk to maternal health (Weiss *et al.*, 1998). Recently, it has been reported that women with PAH exhibit a greater clinical benefit from endothelin receptor antagonists than men (Gabler *et al.*, 2012); this heterogeneity in treatment response may reflect underlying gender-specific pathophysiological differences in the development of PAH.

In PAH, sex hormones appear to influence the development and progression of the disease. The evidence suggests that oestradiol promotes cardioprotection while testosterone contributes to cardiac hypertrophy and consequently RV failure. Where other mediators of PAH such as serotonin are elevated, oestrogen may actually promote PASMC proliferation via accumulation of damaging oestrogen metabolites. The differential effects of male and female hormones in the pulmonary circulation highlight the advantages of incorporating comparative studies on males and female gender into both basic and clinical research.

Furthermore, drugs that target components of the oestrogen pathway such as CYP19A1 (i.e. anastrozole), CYP1B1 (2, 4, 3′, 5′-tetramethoxystilbene) and ERs such as selective oestradiol receptor modulators (tamoxifen) may prove to be novel therapeutic strategies for the treatment of PAH (Figure 3). As these drugs are already widely used in the treatment of cancer, this improves the translational potential of preclinical research.

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Conflict of interest

None.

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