The Estrogen Puzzle in Pulmonary Arterial Hypertension

Running title: Paulin et al.; The estrogen puzzle in PAH

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Stephen Hawking: Women. They are a complete mystery.

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The biology of Pulmonary Arterial Hypertension (PAH) is full of mysteries and one of its longer standing ones has also intrigued and inspired both scientists and artists throughout history: the female sex. While affecting patients of all ages and both genders, PAH preferentially affects young women, suggesting that the female gender is a risk factor for PAH. Even in heritable PAH (HPAH) associated with autosomal dominant mutations in the gene encoding the bone morphogenetic protein receptor type 2 (BMPR2), women after puberty are about 2.5 times more likely to develop PAH than males. In idiopathic PAH (IPAH) the female/male ratio ranges from 1.7:1 or 1.9:1 to 4.1:1 in three published PAH registries (the NIH registry in the 80s, the French registry and the REVEAL registry respectively). On the other hand, the disease can be more severe in men. Female animals with PAH tend to have lower pulmonary artery pressures and better outcomes compared to males and similarly, male PAH patients have higher mortality than females. The basis for this apparent paradox remains unknown.

Estrogens exert their effects through both genomic effects, by activating their cytosolic or membrane-bound receptors (ER), and nongenomic effects by activating membrane G-protein coupled ER receptors. For example, via the nongenomic effects estrogens promote the release of both prostacyclin and NO while via the genomic effects they decrease the expression of endothelin 1 under hypoxia, all promoting vasoconstriction in the pulmonary circulation. Yet the pathology of PAH is driven not by vasoconstriction but rather by a pro-proliferative and anti-apoptotic diathesis throughout the pulmonary arterial wall.
The main circulating estrogen in women, estradiol, has diverse effects in different vascular beds\(^5\), but in pulmonary arterial smooth muscle cells (PASMC) in vitro it enhances proliferation in a dose-dependent manner\(^7\). However, the same group showed that estradiol improved monocrotaline-induced PAH\(^8\) suggesting that in vivo its effects are more complex. The complexity increases by the observation that the effects of estrogens often depend on the animal model in which they were tested\(^5\).

Our understanding of the estrogen puzzle has improved by studies exploring the metabolites downstream of estrogens, by more than 15 human cytochrome P450 isoforms\(^9\). Dozens of metabolites are produced, with variable and often opposing biological effects, particularly in terms of cell proliferation. The P450-based enzyme CYP1B1 has received attention for its role in many diseases including cancer\(^10\). It catalyses principally the hydroxylation of estradiol at positions C2 and C4, forming 2-OH-estradiol and 4-OH-estradiol, the former being the dominant pathway (Figure 1). In the presence of COPT (catechol-O-methyltransferase) 2-Methoxy-estradiol (2-MeO-estradiol) is formed rapidly, a metabolite that has received attention for its potential therapeutic role in many diseases. When CYP1B1 is inhibited, metabolism can be shifted to the hydroxylation at C16 forming 16α-OH-estradiol and 16α-OH-estrone, metabolites that exhibit pro-inflammatory, pro-mitogenic and pro-angiogenic effects\(^5,11,12\). On the other hand, the 2-OH-estradiol metabolites have shown anti-proliferative and anti-angiogenic effects and when given exogenously in animal models of PAH have shown evidence of disease regression. For example, 2-MeO-estradiol has antiproliferative effects in PASMC and fibroblasts\(^13\), inhibits HIF1a and angiogenesis\(^14,15\) and attenuates PAH in animal models\(^13,16\).

Thus, individuals who metabolize a larger proportion of estrogen to 16α-OH-estradiol
and 16α-OH-estrone compared to 2-OH-estradiol may be at increased risk for proliferative and inflammatory diseases and this risk can be potentially assessed by measuring urinary 2-OH-estradiol/16α-OH-estrone ratio. Since CYP1B1 is the most efficient estradiol hydroxylase\textsuperscript{9,10}, the 2-OH-estradiol/16α-OH-estrone ratio is an indicator of CYP1B1 activity. CYP1B1 is highly expressed in various human hormone-regulated cancers compared to normal tissues. However, despite early indications that polymorphisms in the CYP1B1 would result in enzymatic activity changes increasing cancer risk in hormone-sensitive tissues like the breast\textsuperscript{17}, recent meta-analyses have failed to support this concept\textsuperscript{18}. Nevertheless, this idea has also been explored in PAH:

In a study using Affymetrix arrays to compare asymptomatic BMPR2 mutation carriers (unaffected by PAH) to carriers affected by PAH, CYP1B1 expression was almost tenfold decreased in affected female BMPR2 carriers\textsuperscript{19}. In order to address the “background noise” caused by environmental and drug-dependent factors, the investigators used patient-derived immortalized circulating B cells, instead of pulmonary vascular or other PAH-relevant tissues. These authors also showed a higher frequency of a polymorphism on the CYP1B1 gene (N453S) that has been associated with decreased expression in cancer tissues. In a study of 140 BMPR2 mutation carriers (86 females and 54 males) a 4-fold higher PAH penetrance was found in females homozygous for the wild-type genotype (N/N), than those with N/S or S/S genotypes\textsuperscript{20}. Moreover, in a small subgroup of these patients, the 2-OH-estradiol/16α-OH-estrone ratio was 2.3-fold lower in affected BMPR2 mutation female carriers compared to unaffected carriers\textsuperscript{20} (Figure 2A). Taken together these results suggest that the decreased level of CYP1B1 may explain the low ratio of “protective vs harming metabolites” which would promote the development of PAH in female BMPR2 mutation carriers. Although these authors did not study
PAH patients without BMPR2 mutations, extrapolation of their results would suggest that mice lacking CYP1B1 may develop worse pulmonary hypertension when exposed to a disease “trigger” like hypoxia, due to relative lack of the “protective” metabolites like 2-MeO-estradiol.

In this issue of Circulation, White et al.\textsuperscript{21} showed that CYP1B1 expression is specifically increased in the pulmonary arteries of two mice PAH models (chronic hypoxic and chronic hypoxia+SU5416 mice). This increase appeared to be selective for the pulmonary arteries; for example it did not take place in the right ventricle. They also showed that CYP1B1 is over-expressed in the pulmonary arteries of a few IPAH and HPAH, compared to non-PAH, human lungs. They then showed that deletion of the CYP1B1 gene in mice (CYP1B1\textsuperscript{-/-}) attenuated the degree of hypoxia-induced PAH compared to wild-type (WT) male but not female mice. Female CYP1B1\textsuperscript{-/-} mice developed PAH similarly to WT mice, with a similar increase in right ventricular systolic pressure and vascular remodeling. However, despite the same increase in pressure, the CYP1B1\textsuperscript{-/-} mice had less right ventricular hypertrophy compared to the WT mice. While the levels of right ventricular CYP1B1 did not change in the hypoxia-induced PAH model, it is possible that the CYP1B1\textsuperscript{-/-} mice had different circulating estrogen metabolites that could affect the right ventricle directly. The dissociation between the increase in pressure and right ventricular hypertrophy is intriguing, but it cannot be interpreted fully since the authors did not measure the effects of estrogens on the right ventricle ex vivo and did not measure mean pulmonary artery pressure and cardiac output in order to report pulmonary vascular resistance. For example, a decrease in right ventricular systolic pressure may reflect a primary decrease in right ventricular contractility and not a decrease in right ventricular afterload. The authors did not measure estrogen metabolite levels in these mice, limiting our ability to extrapolate to PAH patients with decreased CYP1B1 levels and 2-OH-estradiol/16\alpha-OH-estrone ratios. Nevertheless,
although the results of the current study appear in conflict with the human genetic CYP1B1 studies, it is important to remember that in the former, expression of protein in the pulmonary arteries is measured while in the later gene expression in immortalized B cells was reported.

The authors also confirmed convincingly that the proliferative effects of 16α-OH-estrone in vitro and in vivo are promoting PAH. Administration of 16α-OH-estrone during 28 consecutive days in mice induced an increase in right ventricular systolic pressure, pulmonary vascular remodeling and right ventricular hypertrophy, compared to vehicle treated mice. They then showed that 2,3′,4,5′-tetramethoxystilbene (TMS) was beneficial in two models of mice PAH under disease prevention protocols. While the effects were modest, they were convincing. However, although TMS is frequently used as a CYP1B1 inhibitor, it is difficult to interpret its effects based on CYP1B1 inhibition. This is because the effects of the drug on CYP1B1 levels or activity in the pulmonary arteries of these mice were not measured. Moreover, it is difficult to understand how complete absence of an enzyme (as is the case in CYP1B1−/− mice) has less effect than a putative inhibitor of the enzyme. In other words, it is difficult to understand that while hypoxic female CYP1B1−/− mice had the same degree of pulmonary hypertension with the hypoxic female WT mice (Figure 3B of the White et al manuscript), TMS attenuated pulmonary hypertension in the same hypoxic female WT mice (Figure 4B of the White et al manuscript). This suggests that the drug has additional, off target effects. This possibility could have been addressed by showing absence of TMS effects on CYP1B1−/− mice.

TMS is a resveratrol analogue (Figure 2B), a natural compound that activates the deacetylase SIRT1, that has received a lot of attention due to its involvement in the biology of aging. Although resveratrol also has multiple effects, by activating SIRT1 it can regulate the activity of both members of the PGC1-α/Estrogen-related receptor-α (ERR-α) complex, which
are essential metabolic regulatory transcription factors. Although the ERRs do not directly respond to 17β-estradiol, they can bind to estrogen response elements (ERE) thus regulating mitochondrial and metabolic functions. Indeed, estrogens have been shown to regulate multiple aspects of mitochondrial biology. Thus the possibility that TMS (like resveratrol) could have off-target effects on mitochondrial function has to be considered as it is now increasingly being recognized that metabolism and mitochondria may be central to PAH biology. In fact, resveratrol has been shown to prevent monocrotaline PAH in rats. In keeping with off-target, non-CYP1B1-mediated effects of TMS are studies in cancer showing TMS-dependent inhibition of activated focal adhesion kinase (FAK), Akt, and mTOR. In summary, TMS may have therapeutic value for PAH but it is likely that this is not based on CYP1B1 inhibition alone.

The authors brought to our attention the CYP1B1 mice as a new model in which to study many aspects of the estrogen puzzle in PAH. While it is not clear that CYP1B1 can be a therapeutic target in PAH, it may be an important disease modifier due to its ability to regulate local estrogen metabolism in the pulmonary arteries, and may lead to biomarker discoveries. More work is needed to determine whether TMS is a potential therapy for PAH. It is important for a mechanism to be well characterized before experimental therapies can be translated to humans.

Future studies in this important field need to provide comprehensive data on levels of estrogen metabolites in animals or patients and provide careful comprehensive hemodynamic assessment of the Pulmonary Artery - Right Ventricle unit. The possibility that estrogens and their metabolites may have direct effects on the right ventricle has to be considered since it is the right ventricle that primarily drives morbidity and mortality in PAH. For example, due to higher estrogen levels, females may have more vulnerable pulmonary circulation for the development of
PAH but stronger right ventricles. Indeed, both in PAH patients and healthy subjects, right ventricular ejection fraction is lower in males compared to females\textsuperscript{27, 28}.

Until then, the estrogen mysteries will continue to puzzle us. At the end, if one of the brightest minds of our century, the cosmologist Stephen Hawking, still struggles to understand women, who are we to solve the estrogen puzzle in a disease already full of mysteries?

**Conflict of Interest Disclosures:** None.

**References:**


Figure Legends:

**Figure 1.** Estrogen Metabolites: The P450-based enzyme CYP1B1 is critical for the balance of “good” and “bad” metabolites in terms of cellular proliferation, inflammation or angiogenesis and thus in their ability to promote proliferative and inflammatory diseases like cancer or PAH (see text for details).

**Figure 2. A.** (Left) Ratio of urinary 2-hydroxyoestrogen (2-OH-estradiol) to 16α-hydroxyoestrone (16α-OH-estrone) in female affected BMPR2 mutation carriers with PAH (AMCs) versus unaffected BMPR2 mutation carriers without PAH (UMCs). AMCs have a significantly reduced ratio compared to UMCs (p=0.006). This decrease in the ratio in the affected patients may be due to the fact they have a markedly decreased expression of CYP1B1 in their circulating B cells, compared to unaffected mutation carriers (Right, SpringerOpen original publisher) as discussed in the text. These figures are taken from references 19 and 20 with permission. **B.** The structure of TMS is impressively similar to the structure of Resveratrol (see text).
A. Graph showing the ratio of 2-OHE/16α-OHE for AMC and UMC.

B. Comparison of CYP1B1 expression levels between male and female non-carriers, unaffected BMPR2 mutation carriers, and affected BMPR2 mutation carriers. TMS and Resveratrol structures are also shown.
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