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REVIEW

Sex-biased TGFβ signalling in pulmonary arterial hypertension

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Abstract

Pulmonary arterial hypertension (PAH) is a rare cardiovascular disorder leading to pulmonary hypertension and, often fatal, right heart failure. Sex differences in PAH are evident, which primarily presents with a female predominance and increased male severity. Disturbed signalling of the transforming growth factor-β (TGFβ) family and gene mutations in the bone morphogenetic protein receptor 2 (*BMPR2*) are risk factors for PAH development, but how sex-specific cues affect the TGFβ family signalling in PAH remains poorly understood. In this review, we aim to explore the sex bias in PAH by examining sex differences in the TGFβ signalling family through mechanistical and translational evidence. Sex hormones including oestrogens, progestogens, and androgens, can determine the expression of receptors (including BMPR2), ligands, and soluble antagonists within the TGFβ family in a tissue-specific manner. Furthermore, sex-related genetic processes, i.e. Y-chromosome expression and X-chromosome inactivation, can influence the TGFβ signalling family at multiple levels. Given the clinical and mechanistical similarities, we expect that the conclusions arising from this review may apply also to hereditary haemorrhagic telangiectasia (HHT), a rare vascular disorder affecting the TGFβ signalling family pathway. In summary, we anticipate that investigating the TGFβ signalling family in a sex-specific manner will contribute to further understand the underlying processes leading to PAH and likely HHT.

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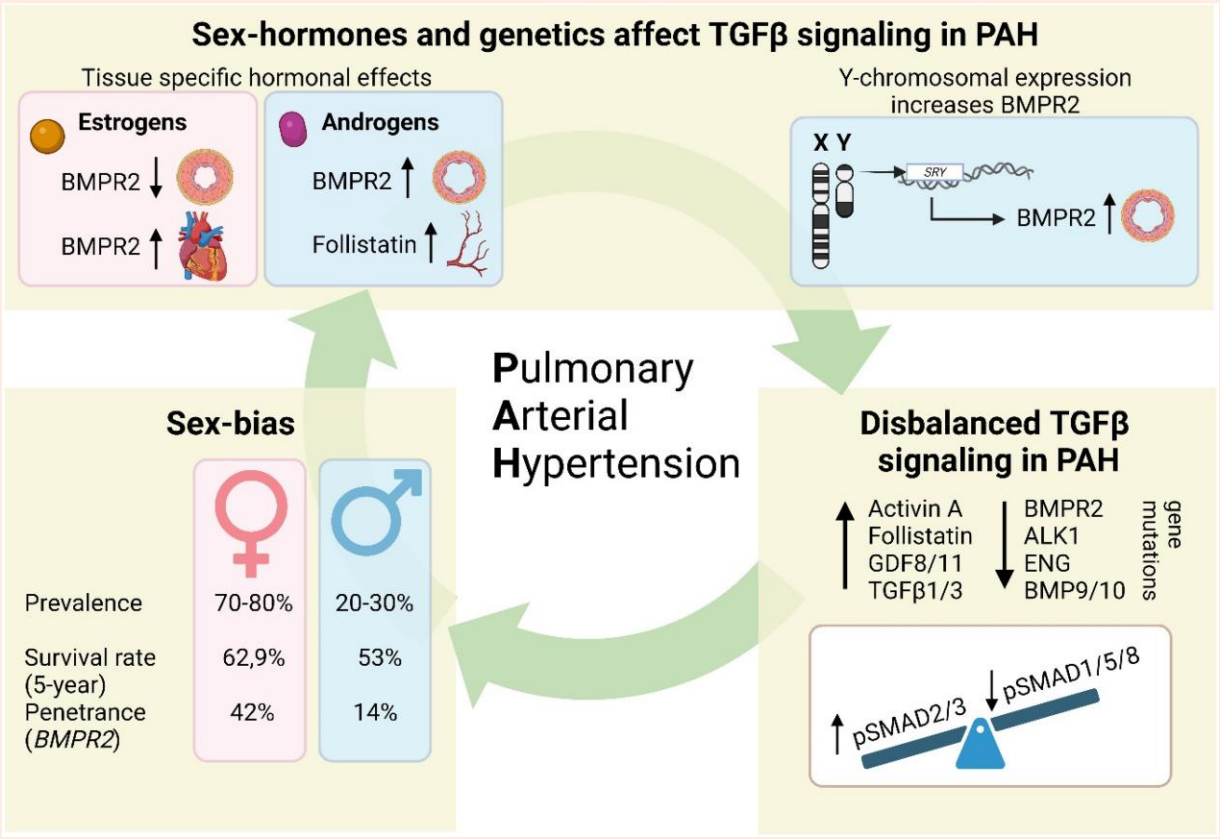
Graphical Abstract



Keywords Activin • Androgen • BMP • BMPR2 • Endothelial • Oestrogen • HHT • Hypertension • PAH • TGFβ

1. Introduction: pulmonary arterial hypertension

Pulmonary arterial hypertension (PAH) belongs to Group I in the total of five (I–V) groups of pulmonary hypertension. Group I is substratified in, among others, idiopathic PAH (IPAH) and heritable PAH (HPAH). HPAH has a known genetic origin, by either familial contribution or genetic correlation,[1] while IPAH has an un-familial cause at the time of diagnosis. As established in the 2022 ESC/ERS Guidelines for the diagnosis and treatment of pulmonary hypertension, pre-capillary PH (including PAH) is defined by a mean pulmonary arterial pressure (mPAP) of >20 mmHg, pulmonary arterial wedge pressure (PAWP) of ≤15 mmHg, and pulmonary vascular resistance (PVR) of >2 Wood Units (WU).[2] The increased workload on the right heart causes ventricular dilatation and hypertrophy, resulting in progressive right heart failure. Pulmonary vascular remodelling constitutes the main pathological event at the onset of PAH. Remodelling of the distal pulmonary arteries involves abnormal proliferation of endothelial cells (ECs), smooth muscle cells (SMCs), and fibroblasts; apoptosis resistance of ECs; excessive EC migration that becomes dysfunctional, in part due to endothelial-to-mesenchymal transition (EndMT) (distal); migration of SMCs (proximal); inflammatory influx of macrophages and lymphocytes; and the formation of plexiform lesions.[3–5]

Although PAH is a disease caused by remodelling of the pulmonary vasculature, end-stage patients ultimately die from right heart failure.[2] To date, there is no approved treatment curing or reversing disease progression. The current treatment of PAH mainly consists of the single or combined administration of pulmonary vasodilators acting on the guanylate cyclase, endothelin, or prostacyclin pathways,[6] only postponing further progression and eventually requiring lung transplantation in severe cases.[7] Recently, the Phase 3 clinical trial STELLAR has concluded excellent clinical outcomes in PAH patients using Sotatercept.[8]

Sex-related differences in disease prevalence and severity are known for PAH. The US REVEAL study showed that 80% of the PAH patients are women (4:1 ratio).[9,10] Comparably, multiple registries across Europe concluded a female bias in PAH of approximately 70% (2.3:1 ratio).[11–16] Interestingly, the disease bias towards women declines by age when comparing age groups 18–65 with >65 years old in IPAH patients.[12] In addition, PAH disease penetrance is also defined by sex, with a 42% in females over 14% in male HPAH patients.[17] Remarkably, diagnosed PAH male patients are more severely burdened, with nearly a 10% reduction in 5-year survival rate (53%) compared to females (62.9%).[9]

The underlying cellular and molecular causes of these sex-related differences in PAH have not yet been fully understood, although many hypotheses have been proposed. These often involve hormonal-based alterations, although metabolism, genetics, and/or the immune system might also play a role.[18–20] In general, androgens are considered vasculo-protective and a contributor to pulmonary vasodilation,[21] perhaps underlying the female predominance in PAH. On the other side, oestrogens have been reported to be vasculo-protective in coronary heart disease in women (reviewed in reference [22]). In PAH, oestrogens promote right ventricle adaptation in women,[23] which might lead to a less severe phenotype compared to men.[24] Further, chromosomal differences also play a role, for instance, the Y-chromosome is thought to have vascular protective

gene expression profiles in PAH.[25] In this review, we further discuss if sex determinants, i.e. sex hormones and -chromosomal effects, are a driver of PAH development by altering transforming growth factor-β (TGFβ) signalling.

2. Transforming growth factor-β signal transduction

Disturbances in the TGFβ signalling family contribute to PAH disease development and progression.[26–28] The TGFβ family pathway drives developmental processes and tissue homeostasis[29] within the cardiovascular system.[28,30] In mammals, the TGFβ family is comprised of 33 structurally related polypeptides, including the TGFβ1–3 isoforms, the bone morphogenetic proteins (BMP1–15), nodal, the growth and differentiation factors (GDFs), the activins and inhibins, and the anti-Müllerian hormone (AMH).[31–37] The TGFβ ligands exert pleiotropic effects by controlling cell proliferation, migration, and differentiation in a spatial and temporal manner.[29] Disturbed signalling can result in cancer,[38] musculoskeletal disorders,[39] fibrosis,[40] and cardiovascular diseases[28,41–43].

Most TGFβ family members, with BMPs being the exception,[44] are secreted in an inactive form within a latent complex (reviewed in reference [45]). These large latent complexes include the mature TGFβ polypeptide shielded by latency-associated peptides and latent TGFβ binding proteins.[46] These additional factors also bind to the extracellular matrix (ECM) or the plasma membrane via receptors like glycoprotein-A repetitions predominant (GARP), creating an ECM storage of accumulated latent TGFβ. The mature TGFβ polypeptides are released via several mechanisms allowing a quick functional response on demand.[45]

Active TGFβ ligands signal via a heterotetrameric complex of Type I and II serine–threonine kinase receptors (*Figure 1*).[47] In vertebrates, seven activin like kinase (ALK)1-7 Type I receptors and five Type II receptors (TGFβ receptor 2 (TGFβR2), activin receptor 2A (ACVR2A), ACVR2B, bone morphogenetic protein receptor 2 (BMPR2), and anti-Müllerian hormone receptor 2 (AMHR2)) exist. Since the ligands of the TGFβ family bind with different affinities to their receptor complexes, the relative expression level of the TGFβ family receptors may determine sensitivity of a particular cell type or tissue to a TGFβ ligand.[48] Overall, TGFβs and activins bind with a high affinity to their Type II receptors, whereas BMPs and GDFs exhibit a high affinity for their Type I receptors.[49] Co-receptors like TGFβR3 (betaglycan) or endoglin (*Figures 1* and *2*) can enhance ligand binding to Type I/II receptors when membrane bound, but can act as ligand trap when secreted in a soluble form.[50] Next to these accessory proteins, soluble signalling modulators including Noggin, Gremlin, and Follistatin also exert regulatory effects on the TGFβ family signalling as ligand agonists or antagonists.[51]

Upon ligand–receptor interaction and receptor complex formation, the constitutively active Type II receptor phosphorylates and activates the Type I receptor. Next, the Type I receptor kinase initiates the signal transduction cascade by phosphorylating intracellular downstream proteins, i.e. receptor regulated-SMADs (R-SMADs) (*Figure 1*). Generally, TGFβ1–3 and Activins signal by SMAD2/3 phosphorylation whereas BMPs, GDFs, and AMH signal via phosphorylation of SMAD1/5/8. In the vasculature for instance, BMP9 and -10 are important factors necessary for endothelial homeostasis, exhibiting a high affinity for BMPR2/ALK1 receptor complexes, mainly expressed in ECs.[52,53] Both ALK1/SMAD1/5/8 and ALK5/SMAD2/3 signalling are co-regulated by endoglin in ECs.[54] Interestingly, the two splice variants short- and long-endoglin favour different Type I receptors, being S-endoglin pro-ALK5 and L-endoglin pro-ALK1 (*Figure 2*).[55]

Once phosphorylated, the R-SMADs bind to the co-SMAD SMAD4 and form heterotrimeric complexes. Furthermore, Inhibitory SMADs (I-SMADs, SMAD6 and 7) are transcriptional targets of the TGFβ superfamily and create a classical negative feedback loop interacting with and promoting the degradation of TGFβ receptors by e.g. SMURF1/2.[57,58]

SMAD4-containing heterotrimeric complexes translocate to the nucleus, where they associate with cell type- and pathway-induced transcription factors to modulate target gene expression.[59] Different DNA motifs on the regulatory regions of genes have been described for the SMAD4, SMAD2/3, and SMAD1/5/8.[57,60–62] The binding affinity of SMADs for DNA is relatively low and can be enhanced through association with other transcription factors, which may determine cell-type-specific TGFβ responses.[57] Therefore, the transcriptional activity induced by ligands of the TGFβ superfamily can be 'fine-tuned' at multiple levels, including the relative expression levels of ligands, (co)receptors, (ant)agonists, and nuclear transcription factors that are activated in a tissue and stimulus-dependent manner.[57,63] Many of the cell-type-specific responses to TGFβ ligands are attributed to the so-called non-canonical pathways. The non-canonical signalling may not require the Type I receptor kinase activity.[64] Furthermore, although the TGFβ Type I and II receptors are known serine/threonine kinases, they can also phosphorylate tyrosine residues and act as dual-specificity kinases. Therefore, tyrosine phosphorylation may be an alternative route to mediate SMAD-independent signalling.[65] TGFβ non-canonical signalling is often highly context dependent. For example in vascular settings, TGFβ-induced EndMT is also mediated through the activation of extracellular signal-regulated kinase (ERK)[66] and c-Jun *N*-terminal kinase (JNK).[67] Further, TGFβ-mediated inhibition of primary vascular smooth muscle cell proliferation has been demonstrated to be p38-dependent.[68] Unfortunately, much is still to be deciphered in the context of non-canonical TGFβ signalling and PAH. Accordingly, in this review, we mainly focus on canonical signalling of the TGFβ family.

3. The TGFβ signalling family in PAH

PAH is linked to disturbances within the TGFβ signalling family pathway. Mutations in genes encoding for components of the TGFβ signalling cascade have been identified, such as *ACVRL1* (encoding ALK1), *ENG* (encoding endoglin), *SMAD9* (encoding SMAD8),[69,70] *SMAD1*,[69] *SMAD4*,[69] and *GDF2* (encoding BMP9)[71] (*Figure 1*). The most relevant gene mutation by far involves the *BMPR2* gene, which is affected by loss of function mutations in 70–80% of the HPAH and in 10–20% of the IPAH patients.[72] Additionally, mutations in genes not part of the canonical TGFβ signalling cascade have also been reported (i.e. *CAV1*,[73] *TBX4*,[74] *EIF2AK4*,[75] and *KCKN3*[76]).

Currently, more than 650 different *BMPR2* mutations have been described.[77–79] These mutations may occur in non-coding regions but are mostly located in the coding regions containing the extracellular, transmembrane, kinase, and cytoplasmic functional domains. Noteworthy, approximately 50% of total mutations are found in the kinase domain of BMPR2.[77,80] The different gene mutations consist of single nucleotide substitutions, leading to non-sense, missense, or splice site mutations; and insertions or deletions causing small and partial insertions, deletions, or duplications. A study looking at 144 different *BMPR2* mutations from a broad international PAH patient cohort, predicted that around 70% of all the mutations result in non-mediated decay of the truncated transcripts.[80] Follow-up studies concluded similar findings.[77] The resulting haploinsufficiency is therefore the main cause of disrupted TGFβ signalling. Still, PAH penetrance is low in families with mutations causing haploinsufficiency. Comparing non-affected mutation carriers with PAH patients within the same family, Hamid *et al.*[81] showed that the expression levels from the wild-type *BMPR2* allele impact disease progression, with lower BMPR2 expression levels observed in more affected individuals. Therefore, next to loss of BMPR2 due to genetic mutations, additional triggers to reduce endogenous BMPR2 expression are needed to result in pathogenic TGFβ signalling.

In HPAH patients carrying a *BMPR2* mutation, the BMPR2 and phosphorylated SMAD1/5/8 expression are decreased in lung tissues,[42,82,83] consistent with a decreased expression of BMP transcriptional targets such as *ID3*.[84] Interestingly, BMPR2 expression is also decreased in idiopathic patients,[82] which might be due to (post)transcriptional inhibition

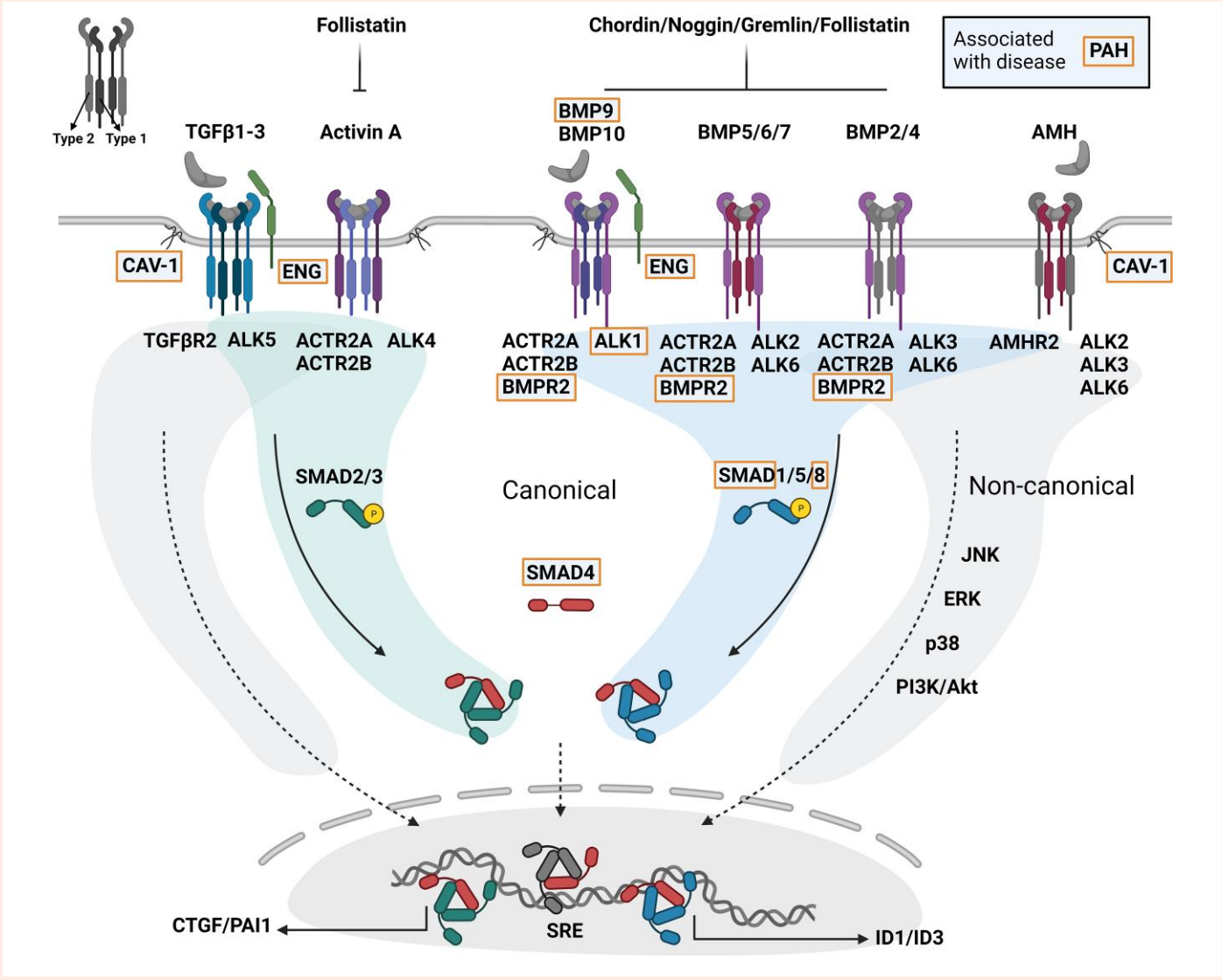


Figure 1 Schematic representation of the TGFβ signalling family. Ligands of the TGFβ family (TGFβ1–3, Activin A, BMP2/4/5/6/7/9/10, AMH) bind their type I (ALK1/2/3/4/5/6) and II (TGFβR2, ACTR2A/B, BMPR2, AMHR2) plasma membrane receptors. Soluble antagonists (Follistatin, Chordin, Noggin, Gremlin) can decrease ligand accessibility. Type III receptors (i.e., endoglin) can further regulate ligand–receptor complex formation. Upon Type I receptor activation, the intracellular signalling molecules (R-SMADs) are phosphorylated and form a heterotrimeric complex with SMAD4. ALK4/5 (stimulated by TGFβ/Activin A ligands) signal via SMAD2/3 whereas ALK1/2/3/6 (stimulated by BMP/AMH ligands) signal via SMAD1/5/8. R-SMAD/SMAD4 complexes translocate to the nucleus to regulate the activity of gene promoters. Also non-canonical signalling (JNK, ERK, p38, PI3K/Akt) can occur via TGFβ signalling. Mutations in genes encoding TGFβ factors have been linked to PAH development. Not all factors within the TGFβ signalling family have been incorporated in the figure for clarity purposes. PAH, pulmonary arterial hypertension; TGFβ, transforming growth factor-β; BMP, bone morphogenetic protein; AMH, anti-Müllerian hormone; CAV-1, caveolin-1; ENG, endoglin; ALK, activin receptor-like kinase; TGFβR2, TGFβ receptor 2; ACTR2, activin receptor Type II; BMPR2, BMP receptor Type II; SMAD, small mothers against decapentaplegic; JNK, c-jun N-terminal kinase; ERK, extracellular signal-regulated kinase; PI3K, phosphoinositide 3-kinase; SRE, SMAD responsive element.

of BMPR2 expression in inflammatory environments.[67,85] Serum and lung expression of TGFβ1 and TGFβ3 ligands are increased in PAH patients,[86,87] consistent with enhanced expression of a TGFβ target gene *SERPINE1*.[88] Additionally, Activin A and its natural antagonist Follistatin and Follistatin Like-3 are both increased in serum of HPAH and IPAH patients,[89,90] of which Activin A is known to be secreted by macrophages, bronchial epithelial cells, and lung microvascular ECs.[91] Given the counterbalance between BMP and TGFβ signalling, it is well accepted that increased TGFβ and Activin A signalling in PAH results from inactivating mutations in BMP pathway components.[26,92] However, recent publications have unveiled novel mechanisms triggered upon loss of BMPR2. Hiepen *et al.*[93] recently showed that loss of BMPR2 in ECs results in the formation of a mixed-tetrameric receptor complex TGFβ-TGFβR2-ALK5 including a Type I BMP receptor. The inclusion of a Type I BMP receptor allows the activation of pSMAD1/5/8 signalling, while this is prevented by BMPR2 over-expression. Earlier work by other groups further strengthens this hypothesis of mixed-TGFβ/BMP receptor complexes and subsequent activation of pSMAD1/5/8 upon stimulation with TGFβ or Activins.[94–97] This can be a very relevant mechanism in PAH, as not only TGFβ1, but also Activin A levels are increased in serum of IPAH and HPAH patients.[89,90]

Loss of function mutations in *ENG* have been found in familial PAH patients.[98] IPAH patients display increased circulating and non-circulating

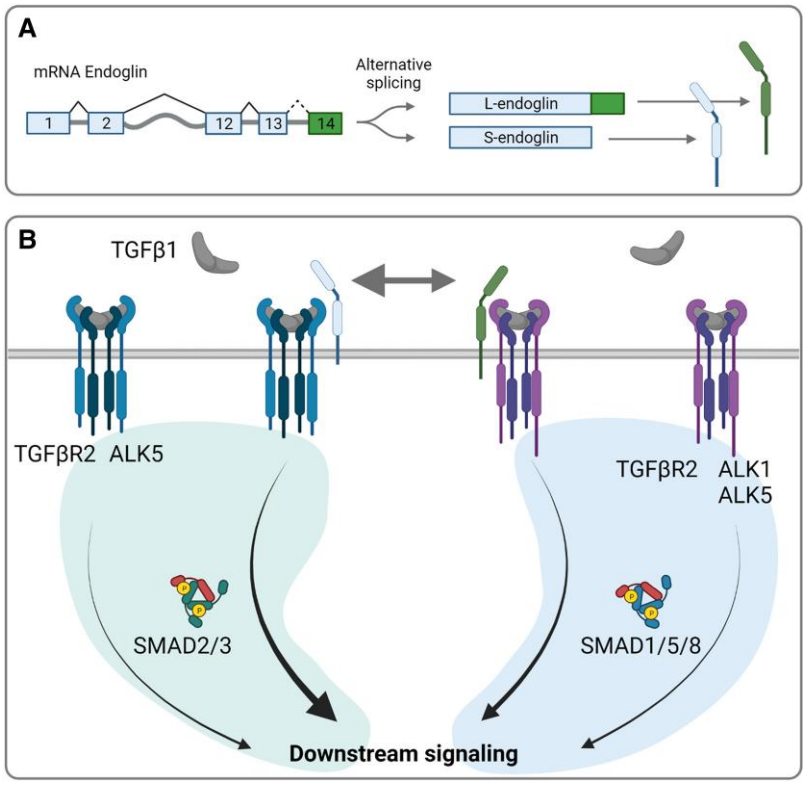


Figure 2 A schematic depiction of the splice variants (*A*) and signalling function (*B*) of endoglin on TGFβ1 signalling. The short (S-) and long (L-)endoglin variants are alternatively spliced by excluding or including exon 14, respectively (*A*). Both S- and L-endoglin increases TGFβ1 signalling; however, S-endoglin favours ALK5 signalling where L-endoglin favours ALK1 dependent signalling (*B*). Therefore, as observed by,[55,56] a balance shift towards S-endoglin increases TGFβ signalling by SMAD2/3 phosphorylation. TGF, transforming growth factor; ALK, activin-like kinase; SMAD, small mothers against decapentaplegic.

endoglin levels,[86] measured in serum and in isolated ECs, respectively. This increased soluble endoglin is related with disturbed EC function. Moreover, alternative splice variants of endoglin can shift the TGFβ/BMP signalling balance.[55] These variants differ in exon 14, and result in L-endoglin and S-endoglin variants, where L-endoglin displays a longer intracellular domain.[99] This intracellular domain contains phosphorylation sites for TGFβR2, ALK5, and ALK1.[100] As shown by Lee *et al.*,[56] increased short (S-)endoglin over long (L-)endoglin causes an increase in SMAD2/3 over SMAD1/5 phosphorylation in ECs (*Figure 2*). Interestingly, this disbalance may also occur in HPAH patients with mutations in exon 14 of the *ENG* gene, favouring the short splicing variant S-endoglin and therefore increasing TGFβ signalling.

Taken together, alterations in BMP receptor complexes due to, for example, loss of function mutations in *BMPR2* or *ENG*, can disbalance the cellular responses to the increased circulating levels of TGFβ/Activin ligands. Induction of BMP-driven pSMAD1/5/8 is often described as protective in PAH. However, pSMAD1/5/8 signalling resulting from TGFβ or Activins in the absence of BMPR2 may not be beneficial as well. One explanation might be that TGFβ and Activin may compete with canonical BMP ligands for the receptors, in this case inducing mixed-tetrameric receptor complexes. These mixed complexes may result in less potent or more transient pSMAD1/5/8 activation and different non-canonical signalling activation, compared with classical BMP-induced complexes. Further, it can lead to short-term signalling saturation (by e.g. SMAD4 competition). Therefore, comprehensive studies including not only BMPR2 downstream signalling but also other TGFβ branches in the context of PAH are needed, as all these different signalling branches may contribute to vascular remodelling and subsequent PAH development.[93]

In line with a prominent role of aberrant TGFβ signalling as underlying cause of PAH, the ACTR2A-Fc fusion molecule Sotatercept aims to counter this imbalance by trapping soluble TGFβ ligands (*Figure 3*) and thereby restoring pathogenic TGFβ signalling.[8,101] Indeed, *in vitro* evidence shows that ACTR2A-Fc treatment of pulmonary ECs reduces pSMAD2/3 while enhances pSMAD1/5/8 signalling. Further, pulmonary arterial thickening and cardiac hypertrophy were partially restored by only 2–4 weeks of Sotatercept treatment in PH rat models.[101] The Type II receptor ACTR2A is able to bind many different TGFβ ligands (*Figure 1*) with different affinities. High affinity ligands of ACTR2A include Activin A, GDF8, and GDF11,[49] which levels are all increased in PAH.[89,90,101] Due to the promiscuous role of ACTR2A in complex formation and binding capacity to many other ligands (also e.g. BMP10),[49] we stress that Sotatercept's success might rely on its unspecific targeting of TGFβ ligands. The balance of the combinatory levels of circulating TGFβ ligands in the patient and their differential affinities to Sotatercept therefore drives its pharmacological function. However, Sotatercept may also reduce BMP activity, which can underlie the undesirable side effects observed in PAH patients involved in a recent clinical trial (as reviewed in reference[102]). For instance, the inhibition of BMP10 by high doses of Sotatercept can interfere with BMP10 homeostatic function on the endothelium,[53] maybe resulting in telangiectasias (*Figure 3*). Furthermore, thus far this drug has been tested in patients on background therapy. Whether a therapeutic approach based on solely targeting ACTR2A ligands is successful, remains to be investigated.

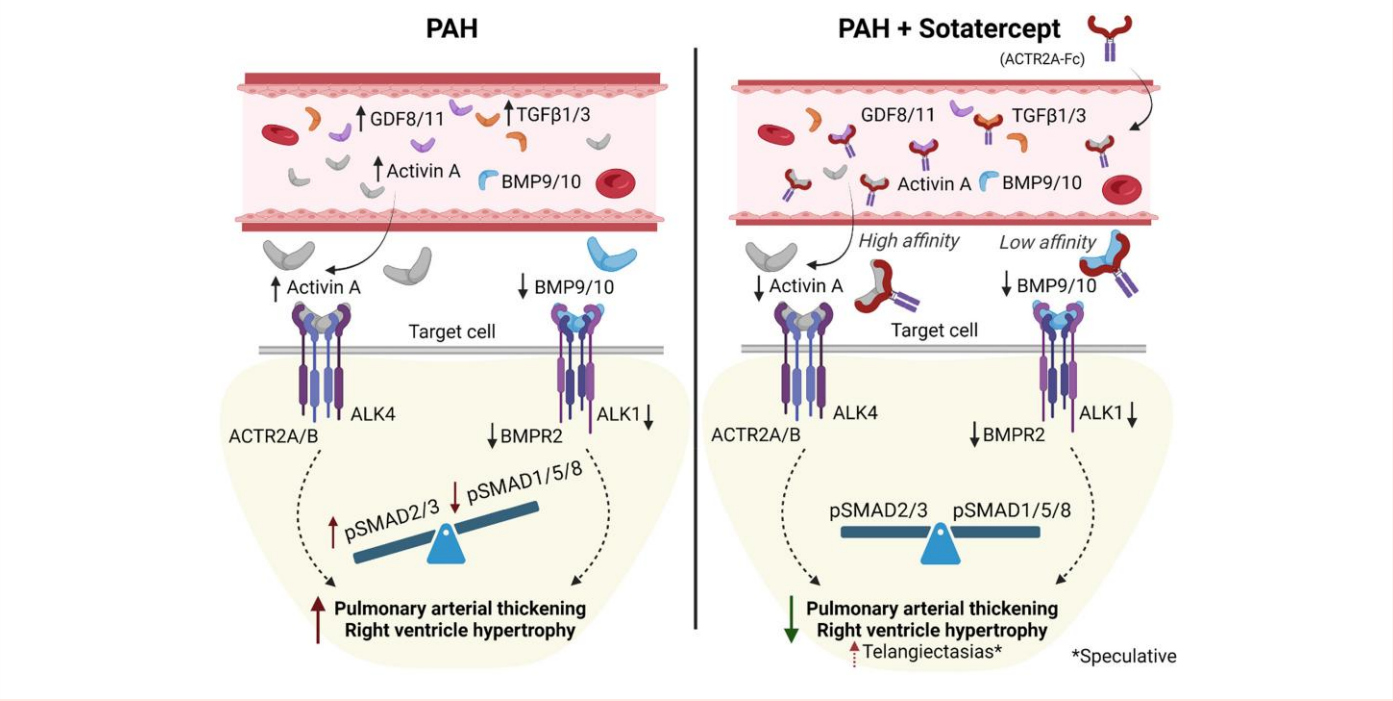


Figure 3 Sotatercept (ACTR2A-Fc) sequesters TGFβ ligands to restore the disbalanced signalling in PAH. The soluble ligands activin A, GDF8/11 and TGFβ1/3 are elevated in PAH causing increased SMAD2/3 phosphorylation over SMAD1/5/8 signalling. This disturbed TGFβ signalling underlies increased pulmonary arterial thickening with a subsequent rise in pulmonary arterial pressure and right ventricle hypertrophy. Treatment with Sotatercept normalizes the signalling imbalance by shielding soluble TGFβ ligands, resulting in a decrease in pulmonary arterial thickening and right ventricle hypertrophy. *Low affinity inhibition of BMP10 by Sotatercept might disturb endothelial homeostasis and subsequently causing telangiectasias. TGF, transforming growth factor; GDF, growth differentiation factor; BMP, bone morphogenetic protein; ALK, activin receptor-like kinase; ACTR2, activin receptor Type II; BMPR2, BMP receptor Type II; SMAD, small mothers against decapentaplegic.

4. Sex hormones and the TGFβ signalling family

As aforementioned, disturbed signalling induced by TGFβ family members constitutes a hallmark in PAH. Given the sex bias observed in this disease, it becomes key to understand the mechanisms by which sex-specific cues may fine-tune the TGFβ family signalling. Sex hormones are derived from cholesterol. Female sex hormones are oestrogens and progestogens, including oestradiol and progesterone, respectively. Male hormones are androgens, of which testosterone is the dominant effector. Sex steroids induce signal transduction by binding to their soluble nuclear receptors; oestrogen receptor (ER), progesterone receptor (PR), and androgen receptor (AR). These receptors act as signal transducer and transcription factors by binding to DNA responsive elements (RE, ERE, PRE, ARE).[103–105] In addition, membrane bound G-protein-coupled receptors for all these sex hormones exist[106] which modulate non-canonical TGFβ signalling pathways.

Oestrogens have strong implications in vascular diseases and promote cardiovascular protection.[107,108] Frump *et al.*[109] showed that 17β-oestradiol substantially improves right ventricular function in the Sugen-Hypoxia (SuHx) PH rat model, and they further linked ERα signalling in the right ventricle to protective adaptation in PAH in a BMPR2-dependent manner.[110] Although less characterized than oestrogens, progestogens, and androgens are also cardiovascular active, and play a substantial role in vascular health and disease.[111–114] While the effect of sex hormones on the (pulmonary) vasculature is well appreciated,[111,115,116] the molecular mechanisms underlying their functions remain elusive. Both sex hormones and TGFβ family members exert a tight control of the vasculature also in pathogenic conditions like PAH.[26,116,117] For comprehensive understanding of the TGFβ and sex-hormone crosstalk, we will summarize the molecular mechanisms described so far, mainly in vascular cells. Unfortunately, most mechanistic studies have been performed in non-vascular settings. Given that sex hormones act on many non-cardiovascular tissues, influencing systemic levels of circulating TGFβ components and hence indirectly the cardiovascular system, we will learn from studies performed in non-vascular tissues and discuss how the crosstalk between TGFβ signalling and sex hormones may be applicable to vascular biology and PAH.

4.1 Oestrogens

Oestrogen signalling involves several members of the TGFβ family pathway in a vascular context (*Table 1*). As such, transcriptome analysis of human umbilical vein endothelial cells (HUVECs) showed that the expression of *ACVRL1* (encoding ALK1), and latent-transforming growth factor beta-binding protein 3 (*LTBP3*) are increased in response to exogenous oestradiol, while *CAV2* (caveolin-2), a negative regulator of TGFβ1-induced ALK5/SMAD2/3 signalling in ECs,[132] and *SMURF2* are decreased, partially overlapping the transcriptome of TGFβ1-stimulated cells.[119] Additionally, administration of the selective oestrogen receptor modulator (SERM) Raloxifene increased the protein expression of ALK1 and endoglin in ECs,[118] hence favouring SMAD1/5/8 over SMAD2/3 signalling. SERMs can have an agonistic and antagonistic effect, depending on the tissue type and availability of oestrogen receptors.[133] These effects have been extensively studied in mammary and skeletal tissues but are underexplored in the cardiovascular system, which is evidently necessary in the context of PAH therapy.

The plasma membrane G-protein-coupled oestrogen receptor (GPER, or GPR30) is an important mediator of oestrogen-induced signalling in vascular aetiologies.[134,135] Interestingly, GPER activation by oestradiol or the GPER agonist G1 increased SMAD1/5/8 phosphorylation and the downstream

Table 1 An overview of studies investigating transcriptional effects of the different sex hormones on targets within the TGFβ signalling cascade. The table shows increased or decreased expression, at which level it has been investigated, in which model or cell type and the specific metabolite used

Hormone	Expression ↑/↓	Level of expression	Model (tissue)/cell type	Metabolite	Ref.
Estrogens	↑ ALK1	mRNA and protein mRNA	HMEC-1 HUVECs	Raloxifene 17β-oestradiol	118,119
	↑ ALK5	Promoter	Rat osteoblasts	Oestradiol	120
		Protein			
	↑ BMP2	mRNA	Mouse MSCs	17β-Oestradiol	121
	↑ BMP6	Promoter	Osteoblasts/MCF-7	17β-Oestradiol	122
	↑ BMPR2	Protein	RV Su-Hx rat	17β-Oestradiol	110
			RVCM WT/Su-Hx rats	PPT	
	↑ endoglin	mRNA and protein	HMEC-1	Raloxifene	118
	↑ LTBP3	mRNA	HUVECs	17β-Oestradiol	119
	↑ TGFβ3	Promoter and mRNA	Rat (bone)	17β-Oestradiol	123
				Raloxifene	
	↓ BMPR2	mRNA	Wild-type mice	17β-Oestradiol	124–126
		Protein	HPASMC	17β-Oestradiol	
		Protein	Su-Hx rat	Anastrozole	
	↓ ID	Protein	HPASMC	17β-Oestradiol	125
	↓ SMURF2	mRNA	HUVECs	17β-Oestradiol	119
Progestogens	↓ CTGF	Promoter	A549 (lung epithelial cells)	Progesterone	127
	(TGFβ1 induced)	mRNA			
		Protein			
	↓ PAI-1	Promoter	MLECs (mink lung epithelial cells)	Progesterone	127
	(TGFβ1 induced)				
	↓ TAGLN	Promoter	A549	Progesterone	127
	(TGFβ1 induced)	mRNA			
		Protein			
Androgens	↑ BMPR2	mRNA	PAH HPASMC	DHEA	128
	↑ BMP7	mRNA	Stellate cells	Testosterone	129
	↑ Chordin	mRNA (array)	Stellate cells	Testosterone	129
	↑ FST	Protein	Stellate cells	Testosterone	129
	↑ Noggin	mRNA (array)	Stellate cells	Testosterone	129
	↑ SMAD7	mRNA	Stellate cells	Testosterone	129
	↓ ACVR2A	mRNA	Stellate cells	Testosterone	129
	↓ BMP2	mRNA (array)	Stellate cells	Testosterone	129
	↓ BMP4	mRNA (array)	Stellate cells	Testosterone	129
	↓ Nodal	mRNA (array)	Stellate cells	Testosterone	129
	↓ PAI-1	mRNA (array)	Stellate cells	Testosterone	129
	↓ SMAD2/3	Protein	Rat (kidney)	Testosterone propionate	130
	↓ SMAD4	Protein	Rat (kidney)	Testosterone propionate	130
	↓ SMURF1	mRNA (array)	Stellate cells	Testosterone	129
	↓ TGFβ1	mRNA	Stellate cells	Testosterone	129,130
		Protein	Rat (kidney)	Testosterone propionate	
	↓ TGFβR2	mRNA	Stellate cells	Testosterone	129
AMH	↓ ALK2	Protein	Lung epithelial cells	AMH (expressed)	131
	↓ ALK3	Protein	Lung epithelial cells	AMH (expressed)	131
	↓ BMPR2	Protein	Lung epithelial cells	AMH (expressed)	131

target *ID1* in HUVECs.[136] These effects can be inhibited by a G-protein pathway inhibitor, indicating a specific role for canonical GPER signalling. This study suggests for the first time a crosstalk between GPER and canonical TGFβ signalling in ECs, and therefore more research is encouraged. Activation of GPER induces Src, MAPK, and PI3K/Akt signalling via transactivation of the epidermal growth factor receptor (EGFR) pathway.[137] GPER modulates hypoxia-inducible factor (HIF) and vascular endothelial growth factor (VEGF) signalling, which makes it an interesting receptor to target in the endothelium.[106] In addition, oestrogen-GPER signalling enhances Notch-mediated epithelial-to-mesenchymal transition (EMT),[106,138] a process resembling EndMT (functionally relevant in PAH, as described above). Importantly, all these non-canonical TGFβ signalling routes (*Figure 1*) have shown to impact PAH development.[139–142]

Oestrogens influence PAH disease development and are thought to be an important driver causing the sex bias in PAH. As such, decreased expression of an important 2-hydroxyestrogen (2-OHE) catalyst, CYP1B1,

may be a second-hit favouring PAH development in female HPAH patients.[143] In blood isolated lymphoblastoid cells, this enzyme showed a 10-fold decreased expression in affected compared to unaffected female *BMPR2* mutation carriers.[143] As a follow-up, Austin *et al.*[144] showed that female *BMPR2* mutation carriers have a 4-fold decreased disease penetrance when expressing the N453S CYP1B1 variant compared to wild-type. Further, they observed a decreased urinary 2-OHE/16α-OHE metabolite ratio in affected female *BMPR2* mutation carriers. Unexpectedly, the enzymatic function of CYP1B1 was unrelated to 2-OHE levels but predominantly caused by increased levels of 16α-OHE (although highly variable).[144] This study therefore demonstrates the importance of oestrogen metabolites in PAH disease penetrance in women.

Indeed, Mair *et al.*[125] found that basal BMPR2 protein levels in female non-PAH hPASMCs are lower compared to male cells. BMP4-induced pSMAD1/5/8 and *ID1/3* expression was lower in female than in male hPASMCs. Interestingly, administration of exogenous oestradiol to male hPASMCs decreased *ID1/3* expression to levels comparable to female cells.[125] Consistently, oestrogen-ERα activation was reported to downregulate *BMPR2* expression in pulmonary microvascular ECs (MVECs) via an ERE in the promoter of *BMPR2*.[124] Moreover, inhibition of oestrogen synthesis by the aromatase inhibitor anastrozole alleviated experimental PAH in a SuHx rat model by restoring BMPR2 expression.[126] Conversely, in the right ventricle of multiple PH rat models and cultured rat right ventricle cardiomyocytes, E2-ERα signalling increased *BMPR2* expression.[110] Further, basal BMPR2 levels were higher in female right ventricle samples compared to males. Interestingly, they showed a direct interaction between ERα and BMPR2, which improved cardiac function via Apelin upregulation. In this study, Frump *et al.* also showed a protective effect of E2, or an ERα agonist, by preventing PH disease development in multiple PH rat models, driven via this BMPR2/Apelin-axis. Compared to human control samples, IPAH patients showed decreased ERα levels in the right ventricle.[110] Taken together, oestrogens decrease *BMPR2* expression in the vasculature but promote BMPR2 levels in the right heart. This cell type-dependent effect can explain female predominance and increased male severity in PAH.

Circulating sex hormones may be also secreted by and affect non-cardiovascular tissues, which in turn may impact the cardiovascular system indirectly. Through this angle, multiple studies have been performed using non-vascular cell models like MCF-7 and HEK293 that could help us to unveil the mechanistic crosstalk between TGFβ and sex hormones (summarized in *Table 1*). Researchers have shown that ERα/β can directly bind, inhibit, and recruit protein degradation systems (by e.g. SMURF1) to SMAD2/3 in an oestrogen-dependent manner (*Figure 4*).[145–148] BMP stimulated SMAD1/5/8 phosphorylation was also reduced by oestrogen treatment in the same non-vascular cell lines.[149] To add complexity to this oestrogen-TGFβ crosstalk, SMADs can also be a cofactor for sex-hormone receptor-mediated transcription.[150,151] Evidently, as these studies made use of non-vascular cells, there is a need to confirm their findings towards vascular biology in the context of PAH.

In conclusion, accumulating evidence indicates that oestrogens can regulate canonical TGFβ signalling by directly altering the expression of TGFβ receptors and signalling modulators, at the transcriptional and protein level. Moreover, oestrogen signalling via GPER may indirectly modulate TGFβ non-canonical routes (*Figure 4*).

4.2 Progestogens

Progestogens may positively impact the cardiovascular system,[152] by negatively regulating the hyperproliferation of ECs and SMCs.[112,153,154] Progesterone induces a strong vasodilating response compared to oestradiol and testosterone in male and female rat coronary and pulmonary arteries *ex vivo*.[114] Congruently, low progesterone levels correlate with increased risk of PAH in men.[155] To date, a direct link between progestogens and TGFβ signalling (including BMPR2 regulation) in cardiovascular cells is underexplored. In epithelial cells, progesterone inhibits TGFβ1-induced SMAD3 phosphorylation in a dose-dependent manner,[127] and antagonizes TGFβ1-mediated upregulation of the target genes *CTGF*, *transgelin*, and *PAI-1*. In human granulosa cells, BMP-15-induced signalling via BMPR2 and ALK6 was shown to suppress progesterone production,[156] although likely indirectly. In addition, Activin A repressed progesterone synthesis in the reproductive system,[157,158] which might explain low progestogen levels in male PAH patients,[155] as Activin A plasma levels are increased.[89] Similarly, BMP4 and BMP7 also suppressed progesterone synthesis in Granulosa-Lutein cells.[159] The crosstalk between progesterone and TGFβ signalling is most likely cell type and context dependent.

In summary, although functional progesterone responses on vascular cells are well described, data regarding crosstalk between progestogens and TGFβ signalling in this context is lacking, and more research is needed to further understand the sex-related differences in PAH.

4.3 Androgens

Androgens have been proposed as a therapeutic treatment for PH,[116,160] because of its quick beneficial vasodilatory effect on the pulmonary vasculature[21] and its protective effect on right ventricle adaptation and function.[160,161] Androgens classical mode of action involves gene transcriptional responses through intracellular binding to AR,[113,162,163] expressed in PASMCs and ECs. The androgen-induced vasodilation response occurs within 20 minutes after androgen administration.[21,114] As a direct effector, testosterone can antagonize calcium channels in SMCs, thereby triggering a fast cellular response, not mediated by classical AR-dependent gene transcription. The androgen metabolite DHEA is shown to restore cardiac remodelling and increase right ventricular function in rat models for experimentally induced PAH.[128,160] Further, DHEA treatment of PAH patient-derived PASMCs increased *BMPR2* mRNA expression,[128] explaining an increased disease penetrance in individuals with low DHEA-S levels.[164–166] Therefore, DHEA (or DHEA-sulphate, -S) treatment is currently investigated in a clinical setting.[161]

Beyond the vasculature, androgens are described to modulate TGFβ signalling at multiple levels (*Figure 4* and *Table 1*). Also mechanistically, in prostate cancer cell lines such as LNCaP and PC3 cells, dihydrotestosterone (DHT)-induced AR transactivation can form a complex with SMAD3 and SMAD4, where SMAD3/AR complexes promote transcription via DNA binding to AREs, while SMAD3/SMAD4/AR complexes inhibit androgen target gene expression.[150] Hayes *et al.*[167] observed a repression of androgen target gene expression by SMAD3/AR complexes, by direct binding of the MH2 domain of SMAD3 with the transcription activation domain of the AR. Interestingly, the androgen-driven inhibitory effects on gene transcription are not specific for the TGFβ branch of the family, but also BMP signalling and its downstream targets are inhibited upon DHT treatment in e.g. intestinal stromal cells.[168] Furthermore, phosphorylated SMAD1 interacts with AR to suppress its transcriptional function,[169] indicating that androgens may regulate both TGFβ and BMP signalling pathways and vice versa (*Figure 4*).

In conclusion, androgens and TGFβ crosstalk via direct AR and SMAD interactions and indirectly via transcriptional regulation through AREs (*Figure 4*). The vast majority of these data result from studies using prostate cancer or other non-vascular models but may very well be applicable to PAH. For example, testosterone administration increased the expression of the circulating TGFβ regulators Follistatin, Chordin, and Noggin expression in muscle stellate cells[129] (*Table 1*), which may impact distant organs, including the heart and the pulmonary vasculature. PAH patients exhibit increased Activin A and Follistatin circulating levels,[89] and Activin A levels correlate with increased mortality. Higher androgen-mediated Follistatin in males could potentially suppress high amounts of Activin A in PAH and might contribute to the lower prevalence in men.[170] The decrease in androgens with age would lead to decreased Follistatin levels with increased active Activin A levels and disturbed TGFβ and BMP signalling balance as consequence. In line, the sex-biased disease prevalence in PAH also decreases upon ageing.[12] Following this hypothesis, one might warrant the prescription of (Activin A) ligand traps like Sotatercept. Indeed, as described earlier, clinical trials have been performed treating Sotatercept to PAH patients with striking results.[8,171]

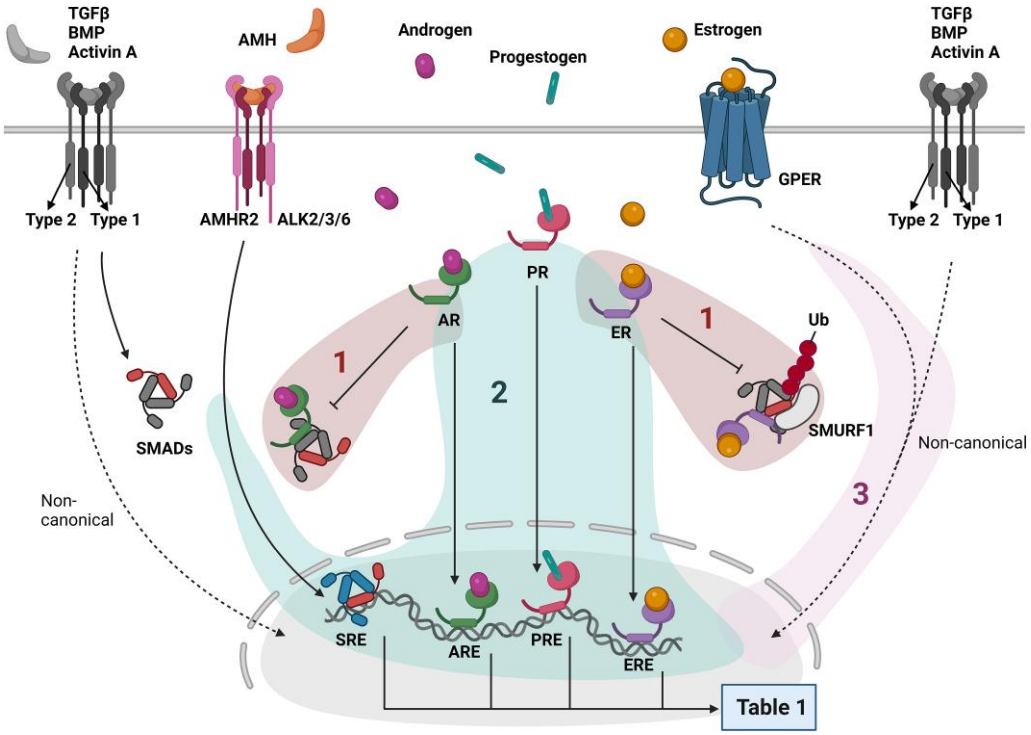


Figure 4 Signalling crosstalk of sex hormones and TGFβ signalling. The membrane permeable sex hormones androgens, progestogens, and oestrogens bind their nuclear receptors androgen receptor (AR), progestogen receptor (PR), and oestrogen receptor (ER), respectively. Oestrogens also bind the membrane receptor G-protein-coupled oestrogen receptor (GPER). Sex-hormones crosstalk on three different levels with TGFβ signalling. (1) The activated nuclear receptors can directly interact with SMADs to inhibit downstream signalling. Oestrogen-ER signalling has been associated with SMURF1-mediated proteasomal degradation of SMADs. (2) All sex-hormones have shown to regulate TGFβ target genes, via their corresponding responsive elements. (3) The oestrogen-GPER signalling cascade includes routes overlapping non-canonical TGFβ signalling routes. TGFβ, transforming growth factor-β; BMP, bone morphogenetic protein; AMH, anti-Müllerian hormone; AR/PR/ER, androgen/progestogen/oestrogen receptor; GPER, G-protein-coupled oestrogen receptor; SRE/ARE/PRE/ERE, SMAD/androgen/progestogen/oestrogen responsive element; SMAD, small mothers against decapentaplegic; SMURF, SMAD specific ubiquitin ligase.

Taking into consideration the TGFβ/BMP balance and the effects sex hormones have on TGFβ signalling components, including BMPR2, one could assume that BMPR2 expression levels are higher in men compared to women. Low androgen levels with a corresponding drop in *BMPR2* expression could initiate PAH development, as low DHEA-S levels are correlated with worse disease outcome in male PAH patients.[166] Further, high androgen-driven Follistatin levels in men might protect from pathogenic signalling by e.g. Activin A in PAH. Taken together, this delineates a higher incidence in PAH development in predominantly younger women but also a more severe disease outcome in men with low DHEA levels.[166]

4.4 Anti-Müllerian hormone

AMH is expressed in follicular sertoli and ovarian granulosa cells and is known to be a circulating hormone throughout life, although declining with age. AMH is a TGFβ family member that binds its dedicated TGFβ Type II receptor AMHR2,[172] also expressed in the human heart.[173] Associated Type I receptors include ALK2, -3 and -6, thereby involving BMP-like downstream signalling (*Figure 1*).[37,172] Although typically linked with sexual dimorphisms[174] and female fertility, other studies indicate AMH to have cardiovascular regulatory properties. Since 2012, high levels of AMH have been correlated with cardiovascular protection,[175] decreased plaque diameter in non-human primates,[176] and decreased male aortic diameter, which are all risk factors for aneurysm.[177] More recently, in the Doetinchem Cohort Study, they found that decreasing AMH trajectories are associated with a substantial elevated risk of CVD in women.[178]

A potential role of AMH in PAH was recently suggested in a case report study[179] describing a novel loss-of-function *BMPR2* mutation in exon 2 associated with IPAH development. The resulting BMPR2 mutant protein is unable to translocate to the plasma membrane. Comprehensive analysis of the TGFβ/BMP signalling signature in peripheral blood mononuclear cells (BPMCs) of this patient confirmed low BMPR2 expression levels, and increased expression of AMHR2, ALK1, ALK3, and ALK6 protein levels, whereas TGFβ receptors remained unchanged.[179] Noteworthy, increased SMAD1/5 and SMAD2/3 phosphorylation was observed upon BMP2 and TGFβ stimulation. Furthermore, mRNA expression of the BMP target genes *ID1*, *SMAD6*, and *STAT1* was increased, suggesting that BMP signalling was not compromised due to the *BMPR2* mutation, at least in PBMCs. The expression of AMHR2 in PBMCs supports the hypothesis that AMH affects inflammation responses and therefore influences PAH. Indeed, higher circulating AMH levels has been correlated with the reduced inflammation marker C-reactive protein in men.[180] Disturbed inflammatory responses have been proposed as an additional driver of PAH development,[181] therefore, reducing inflammation via increased AMH signalling in *BMPR2* mutant carriers might be beneficial in PAH. In this case report however, increased AMHR2 not necessarily proves increased signalling as functional AMHR2 ligands activity was not quantified.

Studies using lung cancer epithelial cells reported a crosstalk between AMHR2 and BMPR2 causing enhanced SMAD2/3 phosphorylation upon loss of AMH or AMHR2,[131] possibly via mixed-heteromeric receptor complexes driven by BMP ligands.[93] Correspondingly, in these cancerous epithelial cells, siRNA depletion of *AMH* or *AMHR2* drives EMT,[131] suggesting inhibitory functions of AMH in EMT. Early in life, males show higher AMH levels than females, but women have higher AMH levels throughout life.[177] To date, relevant data in relation to the pulmonary vasculature are lacking, but if the mechanisms described above for AMH are applicable to vascular cells too, unravelling the role of AMH in the vasculature might help understand PAH disease development.

4.5 Sex hormonal therapy and the clinic

The crosstalk between oestrogens and androgens and the TGFβ signalling family is relatively well described in the vascular system. The findings described in previous chapters indicated a protective effect of androgens, by increasing *BMPR2* expression and circulating Follistatin levels, and oestrogens being an additional risk factor, by decreasing BMPR2 levels in the vasculature but cardioprotective in the heart. Correspondingly, targeting sex-hormone signalling in PAH is a strategy applied within the clinic by multiple groups.

Baird *et al.* showed that lower levels of dehydroepiandrosterone-sulphate (DHEA-S, a prohormone for androgens and oestrogens) and higher levels of E2 were associated with severe PAH in men[164] and in postmenopausal women.[165] This profile caused a worsened disease outcome, suggesting substantial roles of these sex hormones in disease progression and response.[164] In a recent study analysing a large Dutch PAH cohort, low DHEA-S levels in male and female PAH patients were confirmed.[166] These studies validated a clinical trial to evaluate the effect of DHEA-S administration in PAH (EDIPHY: NCT03648385).[161] Targeting high oestrogen levels also seems a possible treatment option for PAH, as oestrogen inhibition by anastrozole (aromatase inhibitor) and fulvestrant (ER antagonist) prevented and reversed PAH development in BMPR2 mutant mice.[182] A small proof-of-concept trial using fulvestrant on five PAH patients showed an increasing trend of the primary outcome 6-minute walking distance comparing baseline with 9 weeks of treatment, although not significant (NCT02911844).[183] Two clinical studies are being conducted using anastrozole in PAH. The first small Phase 2 clinical trial of anastrozole in PAH patients showed a 40% reduction of oestrogen plasma levels, a good safety profile and a significant increased 6-minute walking distance. However, other PAH clinical outcome measures remained unchanged (NCT01545336).[184] A larger follow-up trial has been recently performed (PHANTOM: NCT03229499). While we still wait for the final data to be published, the preliminary results presented at the American Thoracic Society International Conference 2023 revealed no significant improvement in 6-minute walking distance after 6 months, NT-proBNP levels or echocardiographic parameters in individuals treated with anostrozole.[185] Importantly, oestrogens show a protective effect on the right heart by increasing BMPR2 levels.[110] Therefore, this might raise concerns when applying anti-oestrogen therapies. However, PHANTOM showed that decreasing oestrogen levels did not have adverse effects on the right heart of PAH patients. Of course, potential systemic effects of anti-oestrogen therapy should be carefully evaluated, particularly when treating reproductive aged women.

In this regard, pregnancy has been associated with increased risk of PAH development in *BMPR2* mutation carriers, as patients have been diagnosed with PAH after pregnancy.[186] Disease severity is also higher peri- and postpartum,[187] resulting in a mortality of pregnant PAH patients of around 11–25%.[2] These observations can easily be linked to drastic haemodynamic changes during pregnancy,[187] but the long-term effects of hormonal changes are often not considered. As such, oestrogens and progestogens rise dramatically during pregnancy. As already described, this affects the TGFβ family signalling pathway in different manners. Hence, sex-hormonal changes during pregnancy might enhance TGFβ signalling dysregulation (by an additional drop of BMPR2 levels in the vasculature) and subsequent PAH development and severity.

Taken together, these studies underline the importance of sex hormones in PAH disease initiation and progression (in pregnancy) and set the stage for clinical (anti-)hormone therapies for PAH, although context-dependent cellular and molecular mechanisms driving these effects are still incompletely understood.

5. Genetic-related sex differences and the TGFβ signalling family

The X and Y sex chromosomes contain specific genetic information which might differentially regulate the TGFβ signalling family in males and females. Although most of the genes expressed from the Y-chromosome encode for proteins required during gonad development, some factors also have roles outside the reproductive system. In females, expression levels of genes located on the X-chromosome are regulated by the inactivation of one of the two X-chromosomes. As we will discuss below, in some occasions this process can be disturbed, leading to enhanced gene expression due to increased genetic load. In this section, we elaborate on X- and Y-linked genes in relation to the TGFβ signalling family in PAH.

5.1 Y-chromosomal expression

The Y-chromosome is a relatively small chromosome containing a low number of genes in comparison with other mammalian chromosomes. There are 568 genes harboured on the Y-chromosome, of which only 71 have protein encoding potential.[188] Multiple genes encode proteins of the same protein families, leaving only 27 non-related proteins encoded on the Y-chromosome. In a mouse model for PAH, Umar *et al.*[25] found that the Y-chromosome protects disease development, unrelated to gonadal sex (testes or ovaries), suggesting an important role for Y-chromosomal expression in preventing PAH development. Of all Y-chromosomal genes, the sex-determining region Y (*SRY*) gene is the most studied.[189] SRY is a DNA-binding transcription factor regulating gene expression at the early initiation of testes development, but SRY also functions outside the reproductive system.[190] As such, SRY directly binds the promoter of *BMPR2* to upregulate *BMPR2* expression in PAH fibroblasts.[191] As females lack SRY, this *BMPR2* transcriptional regulation does not occur. Correspondingly, *BMPR2* mRNA levels in male PAH patient-derived lymphocytes are higher compared to female equals.[124] Further, SRY may indirectly modulate the TGFβ family signalling by interacting with AR thereby dampening testosterone-induced transcription.[192]

Of all the genes found on the Y-chromosome in PAH patients, eight genes showed decreased expression in diseased lung tissues.[25] One of these genes is *USP9Y*, a ubiquitin-associated hydrolase preventing ubiquitin-dependent degradation of proteins including SMAD4, thereby increasing TGFβ signalling (see reference [193] and ENSG00000114374). Another downregulated Y-linked gene in PAH lungs is the ATP-dependent RNA helicase *DDX3Y*.[25] Although DDX3Y interacts with SMAD2 and SMAD3,[194] the functional consequence of this interaction is unknown. In summary, Y-specific expression profiles may alter the signal transduction induced by TGFβ family members (*Figure 5B*) and might prevent the initiation and progression of PAH. How these interactions with the TGFβ family results in changes of cellular behaviour needs still to be deciphered.

5.2 X-chromosome inactivation

The X-chromosome contains over 1200 genes. In females, the expression of X-linked genes is tightly regulated by X-chromosomal inactivation. This process is necessary for genetic dosage, leading to similar gene expression levels of X-linked genes in female XX cells compared to XY male cells.[195] Silencing of the X-chromosome is mediated by the long non-coding RNA (lncRNA) antisense pair X-inactive specific transcript (XIST) and TSIX (XIST, opposite strand). While *XIST* shields (thereby silences) one of the

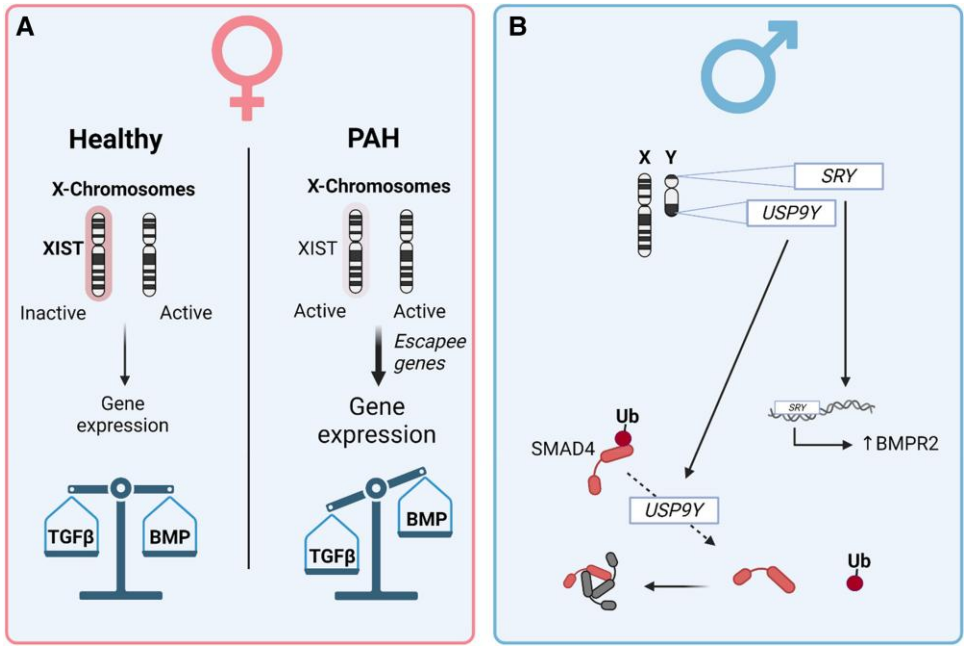


Figure 5 Genetics sex-related differences on the TGFβ signalling family in health and PAH. (*A*) In females, proper X-chromosome inactivation results in healthy genetic output leading to a balanced TGFβ/BMP signalling. However, disturbances in X-chromosome inactivation results in dysregulated genes (*escapees*) and increased genetic output which might cause a diseased disbalance in TGFβ/BMP signalling. (*B*) In males, SRY has been linked to increased BMPR2 expression, while USP9Y is an ubiquitin-dependent hydrolase that targets SMAD4. TGFβ, transforming growth factor-β, BMP, bone morphogenetic protein; SMAD, small mothers against decapentaplegic; SRY, sex-determining region of Y; USP9Y, ubiquitin specific peptidase 9 Y-linked; BMPR2, BMP receptor Type 2.

X-chromosomes, *TSIX* impairs the inactivation of the active X-chromosome through complementary binding to XIST. Furthermore, epigenetic modifications of the *XIST* locus can cause XIST silencing.[196] In addition, the lncRNA X-active specific transcript (XACT) coats the active X-chromosome and also antagonizes *XIST*.[197] Most genes on the inactivated X-chromosome remain silenced; however, 15–25% of X-linked genes escape this silencing process (known as 'escapees').[198] These escapees have been linked to sex differences in diseases like auto-immune diseases and cancers.[199]

Recently, in the EH_{itsn}-$KO^{ITSN+/-}$ PAH mouse model for plexiform arteriopathy, Xist expression levels were increased in female PAH mice compared to the male mice or female WT mice.[200] Noteworthy, female EH_{itsn}-$KO^{ITSN+/-}$ mice showed worsened vascular remodelling compared to their male equals. While no difference in Xist levels were observed in the SuHx PAH rat model, increased Xist expression was observed in human female PAH lungs compared to healthy subjects. Taken together, the upregulations of the lncRNA Xist/XIST may explain the sexual dimorphism in vascular remodelling and therefore highlights the importance of X-chromosome inactivation in the sex bias in PAH.

Several studies suggest an interplay with Xist and BMP/TGFβ signalling. Genetic knockdown of *ACVR1B* (ALK4), *BMPR2*, and *SMAD2* inhibits the expression of *Xist* in mouse fibroblasts.[201] BMP signalling was found to induce and maintain the expression of XIST, while TGFβ signalling served as an antagonist. Furthermore, TGFβ signalling induced TSIX expression in dermal fibroblasts.[202] Although specific XIST/TSIX expression levels are suggestive for X-chromosomal silencing, deeper comprehensive studies are needed for conclusive results. Nevertheless, dysregulation of TGFβ/BMP signalling could impact the chance of genes on the X-chromosome to escape gene silencing, thereby contributing to sex differences in PAH pathology.

The genetic impact on PAH development suggest a protective role for specific genes expressed from the Y-chromosome.[25] The Y-chromosomal expressed SRY transcription factor upregulates *BMPR2* expression in PAH fibroblasts.[191] As discussed above, TGFβ signalling can influence X-chromosomal inactivation in females, further enhancing TGFβ signalling disbalance in PAH. These observations strengthen the link between sex hormones, sex-related genetics, disturbed TGFβ signalling, and PAH disease development.

6. Hereditary haemorrhagic telangiectasia

The genetic background and disease aetiology in Hereditary Hemorrhagic Telangiectasia (HHT) (or Rendu–Osler–Weber syndrome) and HPAH patients sometimes overlap.[203] Interestingly, there is also a sex bias observed in HHT although this is less pronounced compared to PAH. Therefore, many findings in this review are also relevant in a HHT context, which we shortly highlight in this section.

HHT is a vascular disorder presenting with malformed vessels leading to telangiectasia (*spider veins*), haemorrhages, and arteriovenous malformations (AVMs).[204] Similarly as HPAH, HHT originates in people harbouring loss-of-function mutations in genes encoding BMP receptors, i.e. *ACVRL1* (ALK1: HHT2) and *ENG* (endoglin: HHT1).[98,205] It is thought that decreased BMP signalling causes endothelial dysfunction, leading to the malformed vasculature in HHT.[206,207] Sex differences in HHT present mainly by more severe symptoms in women compared to men (increased pulmonary and hepatic AVMs),[208,209] although some small registry studies describe a female predominance.[210–212]

In this review, we explored sex differences in the TGFβ signalling family in PAH, but our discussion may have implications for HHT too. For instance, administration of Raloxifene increases ALK1 and ENG expression in ECs[118] and is therefore proposed as treatment option for HHT (reviewed in reference [213]). Another SERM, Tamoxifen, showed promising effects in a clinical trial reducing severe epistaxis.[214] There is a marked

influence of sex in pulmonary and hepatic vascular malformations in HHT, suggesting organ or tissue-specific features in comparison with other organs.[215] It might be that expression levels of sex-hormone receptors in hepatic or pulmonary ECs makes these cells more sensitive to circulating sex hormones. This review highlights three levels on which sex hormones can alter TGFβ signalling (*Figure 4*). Further research on these organ-specific endothelial effects is warranted to delineate the sex bias in HHT.

7. Discussion and concluding remarks

PAH is a cardiovascular disease with a clear sex bias towards increased female predominance and more severe male phenotype. The molecular causes of this bias are incompletely understood. This review therefore explored sex differences in the TGFβ signalling family to understand the sex bias in PAH (and by extension in HHT).

We have emphasized that hormonal and genetic sex differences may regulate the TGFβ signalling family in different ways to contribute to PAH. Noteworthy, many of the mechanistic findings described above originate from non-vascular cell models, hence translation into PAH should be done carefully. Future studies should be performed aiming to investigate sex-specific effects on the TGFβ signalling family in a cardiovascular setting. Often, sex-related genetics are not taken into account while investigating sex hormonal effects on TGFβ signalling. For instance, researchers should include karyotypes of the cells or tissues studied. We further stress the importance of implementing sex-related genetics in sex-hormone-based studies.

In the meantime, we can anticipate that personalized treatments will progressively become more relevant in clinical decision-making, and therefore sex-related components need to be addressed accordingly. We highlight sex-specific features like hormones and genetic differences in relation to the TGFβ signalling pathway in pulmonary vascular diseases. These findings could implicate differential treatments based on sex, e.g. hormonal therapy like tamoxifen, raloxifene, anastrozole, or DHEA-S, of which the latter two clinical trials are discussed in this review (Section 4.5). These trials are eligible for all sexes although, depending on the study outcomes, sex-customized treatments should not be overlooked. Adverse effects of hormone therapies might be overcome by the development of next-generation SERMs like LY2066948.[133,216] Unfortunately, anastrozole (anti-oestrogen) therapy in PAH showed lack of efficacy following the preliminary clinical data.[185] Conversely, pre-clinical evidence shows that oestrogen administration also ameliorates PAH outcome in a tissue-specific manner, by targeting the right heart.[110] Oestrogen therapy targeting the heart, as an organ-specific treatment, might therefore be a promising treatment option, especially in men showing less right ventricular adaptation.

Overall, sex-specific differences in the TGFβ signalling family potentially explain sex differences in PAH. Many aspects of sex-related crosstalk with the TGFβ signalling family within the cardiovascular system are incompletely understood and more research is therefore warranted. Sex-specific determinants are becoming increasingly important for biomarker identification, drug development and therefore, to find a definitive cure for PAH.

Authors' contributions

M.W. and C.B. wrote the initial draft of the manuscript and performed the literature search. G.S.D., F.d.M., and M.J.G. critically revised the work. G.S.D. supervised and coordinated the writing. M.W. finalized the manuscript. M.J.G. and G.S.D. provided funding. All authors have approved the manuscript for publication.

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References

1. Morrell NW, Aldred MA, Chung WK, Elliott CG, Nichols WC, Soubrier F, Trembath RC, Loyd JE. Genetics and genomics of pulmonary arterial hypertension. *Eur Respir J* 2019;**53**:1801899.
2. Humbert M, Kovacs G, Hoeper MM, Badagliacca R, Berger RMF, Brida M, Carlsen J, Coats AJS, Escribano-Subias P, Ferrari P, Ferreira DS, Ghofrani HA, Giannakoulas G, Kiely DG, Mayer E, Meszaros G, Nagavci B, Olsson KM, Pepke-Zaba J, Quint JK, Rådegran G, Simonneau G, Sitbon O, Tonia T, Toshner M, Vachiery J-L, Noordegraaf AV, Delcroix M, Rosenkranz S; ESC/ERS Scientific Document Group. 2022 ESC/ERS Guidelines for the diagnosis and treatment of pulmonary hypertension. *Eur Respir J* 2022;**43**:3618–3731.
3. Humbert M, Guignabert C, Bonnet S, Dorfmüller P, Klinger JR, Nicolls MR, Olschewski AJ, Pullamsetti SS, Schermuly RT, Stenmark KR, Rabinovitch M. Pathology and pathobiology of pulmonary hypertension: state of the art and research perspectives. *Eur Respir J* 2019;**53**:1801887.
4. Yeager ME, Halley GR, Golpon HA, Voelkel NF, Tuder RM. Microsatellite instability of endothelial cell growth and apoptosis genes within plexiform lesions in primary pulmonary hypertension. *Circ Res* 2001;**88**:E2–E11.
5. Ranchoux B, Antigny F, Rucker-Martin C, Hautefort A, Péchoux C, Bogaard HJ, Dorfmüller P, Remy S, Lecerf F, Planté S, Chat S, Fadel E, Houssaini A, Anegon I, Adnot S, Simonneau G, Humbert M, Cohen-Kaminsky S, Perros F. Endothelial-to-mesenchymal transition in pulmonary hypertension. *Circulation* 2015;**131**:1006–1018.
6. Mandras SA, Mehta HS, Vaidya A. Pulmonary hypertension: a brief guide for clinicians. *Mayo Clin Proc* 2020;**95**:1978–1988.
7. Galiè N, Humbert M, Vachiery JL, Gibbs S, Lang I, Torbicki A, Simonneau G, Peacock A, Vonk Noordegraaf A, Beghetti M, Ghofrani A, Gomez Sanchez MA, Hansmann G, Klepetko W, Lancellotti P, Matucci M, McDonagh T, Pierard LA, Trindade PT, Zompatori M, Hoeper M, Aboyans V, Vaz Carneiro A, Achenbach S, Agewall S, Allanore Y, Asteggiano R, Paolo Badano L, Albert Barberà J, Bouvaist H, Bueno H, Byrne RA, Carerj S, Castro G, Erol Ç, Falk V, Funck-Brentano C, Gorenflo M, Granton J, Iung B, Kiely DG, Kirchhof P, Kjellstrom B, Landmesser U, Lekakis J, Lionis C, Lip GYH, Orfanos SE, Park MH, Piepoli MF, Ponikowski P, Revel MP, Rigau D, Rosenkranz S, Völler H, Luis Zamorano J, Myftiu S, Bonderman D, Firdovsi I, Lazareva I, Sokolović Š, Velchev V, Čikeš M, Moutiris JA, Jansa P, Nielsen-Kudsk JE, Anton L, Jääskeläinen P, Bauer F, Chukhrukidze A, Opitz C, Giannakoulas G, Karlócai K, Oddsson H, Gaine S, Menachemi D, Emdin M, Sooronbaev T, Rudzītis A, Gumbiene L, Lebrun F, Micallef J, Botnaru V, Oukerraj L, Andreassen AK, Kurzyna M, Leite Baptista MJR, Coman IM, Moiseeva O, Stefanović BS, Šimková I, Wikström G, Schwerzmann M, Srbinovska-Kostovska E. 2015 ESC/ERS Guidelines for the diagnosis and treatment of pulmonary hypertension. *Eur Heart J* 2016;**37**:67–119.
8. Hoeper MM, Badesch DB, Ghofrani HA, Gibbs JSR, Gomberg-Maitland M, McLaughlin VV, Preston IR, Souza R, Waxman AB, Grünig E, Kopeć G, Meyer G, Olsson KM, Rosenkranz S, Xu Y, Miller B, Fowler M, Butler J, Koglin J. Phase 3 trial of sotatercept for treatment of pulmonary arterial hypertension. *N Engl J Med* 2023;**388**:1478–1490.
9. Farber HW, Miller DP, Poms AD, Badesch DB, Frost AE, Rouzic EML, Romero AJ, Benton WW, Elliott CG, McGoon MD, Benza RL. Five-year outcomes of patients enrolled in the REVEAL Registry. *Chest* 2015;**148**:1043–1054.
10. McGoon MD, Miller DP. REVEAL: a contemporary US pulmonary arterial hypertension registry. *Eur Respir Rev* 2012;**21**:8–18.
11. Escribano-Subias P, Blanco I, López-Meseguer M, Lopez-Guarch CJ, Roman A, Morales P, Castillo-Palma MJ, Segovia J, Gómez-Sanchez MA, Barbera JA. Survival in pulmonary hypertension in Spain: insights from the Spanish registry. *Eur Respir J* 2012;**40**:596–603.
12. Hoeper MM, Huscher D, Ghofrani HA, Delcroix M, Distler O, Schweiger C, Grunig E, Staehler G, Rosenkranz S, Halank M, Held M, Grohé C, Lange TJ, Behr J, Klose H, Wilkens H, Filusch A, Germann M, Ewert R, Seyfarth HJ, Olsson KM, Opitz CF, Gaine SP, Vizza CD, Vonk-Noordegraaf A, Kaemmerer H, Gibbs JSR, Pittrow D. Elderly patients diagnosed with idiopathic pulmonary arterial hypertension: results from the COMPERA registry. *Int J Cardiol* 2013;**168**:871–880.
13. Humbert M, Sitbon O, Chaouat A, Bertocchi M, Habib G, Gressin V, Yaici A, Weitzenblum E, Cordier JF, Chabot F, Dromer C, Pison C, Reynaud-Gaubert M, Haloun A, Laurent M, Hachulla E, Simonneau G. Pulmonary arterial hypertension in France: results from a national registry. *Am J Respir Crit Care Med* 2006;**173**:1023–1030.
14. Skride A, Sablinskis K, Lejnieks A, Rudzitis A, Lang I. Characteristics and survival data from Latvian pulmonary hypertension registry: comparison of prospective pulmonary hypertension registries in Europe. *Pulm Circ* 2018;**8**:2045894018780521.

15. Ling Y, Johnson MK, Kiely DG, Condliffe R, Elliot CA, Gibbs JSR, Howard LS, Pepke-Zaba J, Sheares KKK, Corris PA, Fisher AJ, Lordan JL, Gaine S, Coghlan JG, Wort SJ, Gatzoulis MA, Peacock AJ. Changing demographics, epidemiology, and survival of incident pulmonary arterial hypertension: results from the pulmonary hypertension registry of the United Kingdom and Ireland. *Am J Respir Crit Care Med* 2012;**186**:790–796.
16. Hoeper MM, Huscher D, Pittrow D. Incidence and prevalence of pulmonary arterial hypertension in Germany. *Int J Cardiol* 2016;**203**:612–613.
17. Larkin EK, Newman JH, Austin ED, Hemnes AR, Wheeler L, Robbins IM, West JD, Phillips JA, Hamid R, Loyd JE. Longitudinal analysis casts doubt on the presence of genetic anticipation in heritable pulmonary arterial hypertension. *Am J Respir Crit Care Med* 2012;**186**:892–896.
18. Morris H, Denver N, Gaw R, Labazi H, Mair K, MacLean MR. Sex differences in pulmonary hypertension. *Clin Chest Med* 2021;**42**:217–228.
19. Theilmann AL, Hawke LG, Hilton LR, Whitford MKM, Cole DV, Mackeil JL, Dunham-snary KJ, Mewburn J, James PD, Maurice DH, Archer SL, Ormiston ML. Endothelial BMPR2 loss drives a proliferative response to BMP (bone morphogenetic protein) 9 via prolonged canonical signaling. *Arterioscler Thromb Vasc Biol* 2020;**40**:2605–2618.
20. Cirulis MM, Dodson MW, Brown LM, Brown SM, Lahm T, Elliott G. At the X-roads of sex and genetics in pulmonary arterial hypertension. *Genes (Basel)* 2020;**11**:1371.
21. Smith AM, Bennett RT, Jones TH, Cowen ME, Channer KS, Jones RD. Characterization of the vasodilatory action of testosterone in the human pulmonary circulation. *Vasc Health Risk Manag* 2008;**4**:1459–1466.
22. Xing D, Nozell S, Chen YF, Hage F, Oparil S. Estrogen and mechanisms of vascular protection. *Arterioscler Thromb Vasc Biol* 2009;**29**:289–295.
23. Ventetuolo CE, Mitra N, Wan F, Manichaikul A, Barr RG, Bluemke DA, Lima JAC, Tandri H, Ouyang P, Kawut M, Services H, Sciences I. Oestradiol metabolism and androgen receptor genotypes are associated with right ventricular function. *Eur Respir J* 2016;**47**:553–563.
24. Tello K, Richter MJ, Yogeswaran A, Ghofrani HA, Naeije R, Vanderpool R, Gall H, Tedford RJ, Seege W, Lahm T. Sex differences in right ventricular–pulmonary arterial coupling in pulmonary arterial hypertension. *Am J Respir Crit Care Med* 2020;**202**:1042–1046.
25. Umar S, Cunningham CM, Itoh Y, Moazeni S, Vaillancourt M, Sarji S, Centala A, Arnold AP, Eghbali and M. The Y chromosome plays a protective role in experimental hypoxic pulmonary hypertension. *Am J Respir Crit Care Med* 2018;**197**:952–955.
26. Upton PD, Morrell NW. TGF-β and BMPR-II pharmacology-implications for pulmonary vascular diseases. *Curr Opin Pharmacol* 2009;**9**:274–280.
27. Cunha SI, Magnusson PU, Dejana E, Lampugnani MG. Deregulated TGF-β/BMP signaling in vascular malformations. *Circ Res* 2017;**121**:981–999.
28. Goumans MJ, ten Dijke P. TGF-β signaling in control of cardiovascular function. *Cold Spring Harb Perspect Biol* 2018;**10**:a022210.
29. Morikawa M, Derynck R, Miyazono K. TGF-β and the TGF-β family: context-dependent roles in cell and tissue physiology. *Cold Spring Harb Perspect Biol* 2016;**8**:a021873.
30. Moses HL, Roberts AB, Derynck R. The discovery and early days of TGF-b: a historical perspective. *Cold Spring Harb Perspect Biol* 2016;**8**:a021865.
31. Kingsley DM. The TGF-β superfamily: new members, new receptors, and new genetic tests of function in different organisms. *Genes Dev* 1994;**8**:133–146.
32. Javelaud D, Mauviel A. Mammalian transforming growth factor-βs: Smad signaling and physio-pathological roles. *Int J Biochem Cell Biol* 2004;**36**:1161–1165.
33. Sanchez-Duffhues G, Williams E, Goumans MJ, Heldin CH, ten Dijke P. Bone morphogenetic protein receptors: structure, function and targeting by selective small molecule kinase inhibitors. *Bone* 2020;**138**:115472.
34. Shen MM. Nodal signaling: developmental roles and regulation. *Development* 2007;**1034**:1023–1034.
35. Namwanje M, Brown CW. Activins and inhibins: roles in development, physiology, and disease. *Cold Spring Harb Perspect Biol* 2016;**8**:a021881.
36. Chang H, Brown CW, Matzuk MM. Genetic analysis of the mammalian transforming growth factor-β superfamily. *Endocr Rev* 2002;**23**:787–823.
37. Visser JA. AMH signaling: from receptor to target gene. *Mol Cell Endocrinol* 2003;**211**:65–73.
38. Massagué J, Blain SW, Lo RS. TGFβ signaling in growth control, cancer, and heritable disorders. *Cell* 2000;**103**:295–309.
39. Bandyopadhyay A, Tsuji K, Cox K, Harfe BD, Rosen V, Tabin CJ. Genetic analysis of the roles of BMP2, BMP4, and BMP7 in limb patterning and skeletogenesis. *PLoS Genet* 2006;**2**:2116–2130.
40. Biernacka A, Dobaczewski M, Frangogiannis NG. TGF-β signaling in fibrosis. *Growth Factors* 2011;**29**:196–202.
41. Compton LA, Potash DA, Brown CB, Barnett JV. Coronary vessel development is dependent on the type III transforming growth factor β receptor. *Circ Res* 2007;**101**:784–791.
42. Atkinson C, Stewart S, Upton PD, Machado R, Thomson JR, Trembath RC, Morrell NW. Primary pulmonary hypertension is associated with reduced pulmonary vascular expression of type II bone morphogenetic protein receptor. *Circulation* 2002;**105**:1672–1678.
43. Ten Dijke P, Arthur HM. Extracellular control of TGFβ signalling in vascular development and disease. *Nat Rev Mol Cell Biol* 2007;**8**:857–869.
44. Jiang H, Salmon RM, Upton PD, Wei Z, Lawera A, Davenport AP, Morrell NW, Li W. The prodomain-bound form of bone morphogenetic protein 10 is biologically active on endothelial cells. *J Biol Chem* 2016;**291**:2954–2966.
45. Annes JP, Munger JS, Rifkin DB. Making sense of latent TGFβ activation. *J Cell Sci* 2003;**116**:217–224.
46. Constam DB. Regulation of TGFβ and related signals by precursor processing. *Semin Cell Dev Biol* 2014;**32**:85–97.
47. Derynck R. TGF-β-receptor-mediated signaling. *Trends Biochem Sci* 1994;**19**:548–553.
48. Heldin CH, Moustakas A. Signaling receptors for TGF-β family members. *Cold Spring Harb Perspect Biol* 2016;**8**:a022053.
49. Aykul S, Martinez-Hackert E. Transforming growth factor-β family ligands can function as antagonists by competing for type II receptor binding. *J Biol Chem* 2016;**291**:10792–10804.
50. Nickel J, Ten DP, Mueller TD. TGF-β family co-receptor function and signaling. *Acta Biochim Biophys Sin (Shanghai)* 2018;**50**:12–36.
51. Chang C. Agonists and antagonists of TGF-β family ligands. *Cold Spring Harb Perspect Biol* 2016;**8**:a021923.
52. Seki T, Yun J, Oh SP. Arterial endothelium-specific activin receptor-like kinase 1 expression suggests its role in arterialization and vascular remodeling. *Circ Res* 2003;**93**:682–689.
53. Desroches-Castan A, Tillet E, Bouvard C, Bailly S. BMP9 and BMP10: two close vascular quiescence partners that stand out. *Dev Dyn* 2022;**251**:178–197.
54. Lebrin F, Goumans MJ, Jonker L, Carvalho RLC, Valdimarsdottir G, Thorikay M, Mummery C, Arthur HM, Ten DP. Endoglin promotes endothelial cell proliferation and TGF-β/ALK1 signal transduction. *EMBO J* 2004;**23**:4018–4028.
55. Velasco S, Alvarez-Muñoz P, Pericacho M, ten Dijke P, Bernabéu C, López-Novoa JM, Rodríguez-Barbero A. L- and S-endoglin differentially modulate TGFβ1 signaling mediated by ALK1 and ALK5 in L6E9 myoblasts. *J Cell Sci* 2008;**121**:913–919.
56. Lee Y, Lee J, Nam SK, Hoon Jun Y. S-endoglin expression is induced in hyperoxia and contributes to altered pulmonary angiogenesis in bronchopulmonary dysplasia development. *Sci Rep* 2020;**10**:3043.
57. Massagué J, Seoane J, Wotton D. Smad transcription factors. *Genes Dev* 2005;**19**:2783–2810.
58. Hayashi H, Abdollah S, Qiu Y, Cai J, Xu YY, Grinnell BW, Richardson MA, Topper JN, Gimbrone MA, Wrana JL, Falb D. The MAD-related protein Smad7 associates with the TGFβ receptor and functions as an antagonist of TGFβ signaling. *Cell* 1997;**89**:1165–1173.
59. David CJ, Massagué J. Contextual determinants of TGF-β action in development, immunity and cancer. *Nat Rev Mol Cell Biol* 2018;**19**:419–435.
60. Itoh Y, Koinuma D, Omata C, Ogami T, Motizuki M, Ichi Yaguchi S, Itoh T, Miyake K, Tsutsumi S, Aburatani H, Saitoh M, Miyazono K, Miyazawa K. A comparative analysis of Smad-responsive motifs identifies multiple regulatory inputs for TGF-β transcriptional activation. *J Biol Chem* 2019;**294**:15466–15479.
61. Katagiri T, Imada M, Yanai T, Suda T, Takahashi N, Kamijo R. Identification of a BMP-responsive element in Id1, the gene for inhibition of myogenesis. *Genes Cells* 2002;**7**:949–960.
62. López-Rovira T, Chalaux E, Massagué J, Rosa JL, Ventura F. Direct binding of Smad1 and Smad4 to two distinct motifs mediates bone morphogenetic protein-specific transcriptional activation of Id1 gene. *J Biol Chem* 2002;**277**:3176–3185.
63. Miyazono K, Ten Dijke P, Heldin CH. TGF-β signaling by Smad proteins. *Adv Immunol* 2000;**75**:115–157.
64. Zhang YE. Non-Smad pathways in TGF-β signaling. *Cell Res* 2009;**19**:128–139.
65. Lee MK, Pardoux C, Hall MC, Lee PS, Warburton D, Qing J, Smith SM, Derynck R. TGF-β activates Erk MAP kinase signalling through direct phosphorylation of ShcA. *EMBO J* 2007;**26**:3957–3967.
66. Ma J, Sanchez-Duffhues G, Goumans MJ, ten Dijke P. TGF-β-induced endothelial to mesenchymal transition in disease and tissue engineering. *Front Cell Dev Biol* 2020;**8**:260.
67. Sánchez-Duffhues G, García de Vinuesa A, van de Pol V, Geerts ME, de Vries MR, Janson SGT, van Dam H, Lindeman JH, Goumans MJ, ten Dijke P. Inflammation induces endothelial-to-mesenchymal transition and promotes vascular calcification through downregulation of BMPR2. *J Pathol* 2019;**247**:333–346.
68. Seay U, Sedding D, Krick S, Hecker M, Seeger W, Eickelberg O. Transforming growth factor-β-dependent growth inhibition in primary vascular smooth muscle cells is p38-dependent. *J Pharmacol Exp Ther* 2005;**315**:1005–1012.
69. Nasim T, Ogo T, Ahmed M, Randall R, Chowdhury HM, Snape KM, Bradshaw TY, Southgate L, Lee GJ, Jackson I, Lord GM, Gibbs JSR, Wilkins MR, Ohta-ogo K, Nakamura K, Girerd B, Coulet F, Soubrier F, Humbert M, Morrell NW, Trembath RC, Machado RD. Molecular genetic characterization of SMAD signaling molecules in pulmonary arterial hypertension. *Hum Mutat* 2011;**32**:1385–1389.
70. Shintani M, Yagi H, Nakayama T, Saji T, Matsuoka R. A new nonsense mutation of SMAD8 associated with pulmonary arterial hypertension. *J Med Genet* 2009;**46**:331–337.
71. Gräf S, Haimel M, Bleda M, Hadinnapola C, Southgate L, Li W, Hodgson J, Liu B, Salmon RM, Southwood M, Machado RD, Martin JM, Treacy CM, Yates K, Daugherty LC, Shamardina O, Whitehorn D, Holden S, Aldred M, Bogaard HJ, Church C, Coghlan G, Condliffe R, Corris PA, Danesino C, Eyries M, Gall H, Ghio S, Ghofrani HA, Gibbs JSR, Girerd B, Houweling AC, Howard L, Humbert M, Kiely DG, Kovacs G, MacKenzie Ross RV, Moledina S, Montani D, Newnham M, Olschewski A, Olschewski H, Peacock AJ, Pepke-Zaba J, Prokopenko I, Rhodes CJ, Scelsi L, Seeger W, Soubrier F, Stein DF, Suntharalingam J, Swietlik EM, Toshner MR, Van Heel DA, Noordegraaf AV, Waisfisz Q, Wharton J, Wort SJ, Ouwehand WH, Soranzo N, Lawrie A, Upton PD, Wilkins MR, Trembath RC, Morrell NW. Identification of rare sequence variation underlying heritable pulmonary arterial hypertension. *Nat Commun* 2018;**9**:1416.
72. Evans JDW, Girerd B, Montani D, Wang XJ, Galiè N, Austin ED, Elliott G, Asano K, Grünig E, Yan Y, Jing ZC, Manes A, Palazzini M, Wheeler LA, Nakayama I, Satoh T, Eichstaedt C, Hinderhofer K, Wolf M, Rosenzweig EB, Chung WK, Soubrier F, Simonneau G, Sitbon O, Gräf S, Kaptoge S, Di Angelantonio E, Humbert M, Morrell NW. BMPR2 mutations and

survival in pulmonary arterial hypertension: an individual participant data meta-analysis. *Lancet Respir Med* 2016;**4**:129–137.
73. Austin ED, Ma L, LeDuc C, Rosenzweig EB, Borczuk A, Phillips JA, Palomero T, Sumazin P, Kim HR, Talati MH, West J, Loyd JE, Chung WK. Whole exome sequencing to identify a novel gene (caveolin-1) associated with human pulmonary arterial hypertension. *Circ Cardiovasc Genet* 2012;**5**:336–343.
74. Kerstjens-Frederikse WS, Bongers EMHF, Roofthooft MTR, Leter EM, Douwes JM, Van Dijk A, Vonk-noordegraaf A, Dijk-bos KK, Hoefsloot LH, Hoendermis ES, Gille JJP, Sikkema-raddatz B, Hofstra RMW, Berger RMF. TBX4 mutations (small patella syndrome) are associated with childhood-onset pulmonary arterial hypertension. *J Med Genet* 2013; **50**:500–506.
75. Best DH, Sumner KL, Smith BP. EIF2AK4 mutations in patients diagnosed with pulmonary arterial hypertension. *Chest* 2016;**151**:821–828.
76. Ma L, Roman-Campos D, Austin ED, Eyries M, Sampson KS, Soubrier F, Germain M, Trégouët DA, Borczuk A, Rosenzweig EB, Girerd B, Montani D, Humbert M, Loyd JE, Kass RS, Chung WK. A novel channelopathy in pulmonary arterial hypertension. *N Engl J Med* 2013;**369**:351–361.
77. Machado RD, Southgate L, Eichstaedt CA, Aldred MA, Austin ED, Best DH, Chung WK, Benjamin N, Elliott CG, Eyries M, Fischer C, Gräf S, Hinderhofer K, Humbert M, Keiles SB, Loyd JE, Morrell NW, Newman JH, Soubrier F, Trembath RC, Viales RR, Grünig E. Pulmonary arterial hypertension: a current perspective on established and emerging molecular genetic defects. *Hum Mol Genet* 2015;**36**:1113–1127.
78. Machado RD, Eickelberg O, Elliott G, Geraci MW, Hanaoka M, Loyd JE, Newman JH, Soubrier F, Trembath RC, Chung WK. Genetics and genomics of pulmonary arterial hypertension. *J Am Coll Cardiol* 2009;**54**:32–42.
79. Hara H, Takeda N, Morita H, Hatano M, Amiya E, Maki H, Minatsuki S, Taki M, Shiraishi Y, Fujiwara T, Maemura S, Komuro I. Three novel BMPR2 mutations associated with advanced pulmonary arterial hypertension. *Hum Genome Var* 2017;**4**:17010.
80. Machado RD, Aldred MA, James V, Harrison RE, Patel B, Schwalbe EC, Gruenig E, Janssen B, Koehler R, Seeger W, Eickelberg O, Olschewski H, Elliott CG, Glissmeyer E, Carlquist J, Kim M, Torbicki A, Fijalkowska A, Szewczyk G, Parma J, Abramowicz MJ, Galie N, Morisaki H, Kyotani S, Nakanishi N, Morisaki T, Humbert M, Simonneau G, Sitbon O, Soubrier F, Coulet F, Morrell NW, Trembath RC. Mutations of the TGF-β type II receptor BMPR2 in pulmonary arterial hypertension. *Hum Mutat* 2006;**27**:121–132.
81. Hamid R, Cogan JD, Hedges LK, Austin E, Phillips JA, Newman JH, Loyd JE. Penetrance of pulmonary arterial hypertension is modulated by the expression of normal BMPR2 allele. *Hum Mutat* 2009;**30**:649–654.
82. Happé C, Kurakula K, Sun XQ, da Silva Goncalves Bos D, Rol N, Guignabert C, Tu L, Schalij I, Wiesmeijer KC, Tura-Ceide O, Vonk Noordegraaf A, de Man FS, Bogaard HJ, Goumans MJ. The BMP receptor 2 in pulmonary arterial hypertension: when and where the animal model matches the patient. *Cells* 2020;**9**:1422.
83. Dewachter L, Adnot S, Guignabert C, Tu L, Marcos E, Fadel E, Humbert M, Dartevelle P, Simonneau G, Naeije R, Eddahibi S. Bone morphogenetic protein signalling in heritable versus idiopathic pulmonary hypertension. *Eur Respir J* 2009;**34**:1100–1110.
84. Yang J, Li X, Li Y, Southwood M, Ye L, Long L, Al-Lamki RS, Morrell NW. Id proteins are critical downstream effectors of BMP signaling in human pulmonary arterial smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2013;**305**:312–321.
85. Hurst LA, Dunmore BJ, Long L, Crosby A, Al-Lamki R, Deighton J, Southwood M, Yang X, Nikolic MZ, Herrera B, Inman GJ, Bradley JR, Rana AA, Upton PD, Morrell NW. TNFα drives pulmonary arterial hypertension by suppressing the BMP type-II receptor and altering NOTCH signalling. *Nat Commun* 2017;**8**:14079.
86. Gore B, Izikki M, Mercier O, Dewachter L, Fadel E, Humbert M, Dartevelle P, Simonneau G, Naeije R, Lebrin F, Eddahibi S. Key role of the endothelial TGF-β/ALK1/endoglin signaling pathway in humans and rodents pulmonary hypertension. *PLoS One* 2014;**9**:e100310.
87. Selimovic N, Bergh CH, Andersson B, Sakiniene E, Carlsten H, Rundqvist B. Growth factors and interleukin-6 across the lung circulation in pulmonary hypertension. *Eur Respir J* 2009; **34**:662–668.
88. Welsh CH, Hassell KL, Badesch DB, Kressin DC, Marlar RA. Coagulation and fibrinolytic profiles in coagulation patients with severe pulmonary hypertension. *Chest* 1996;**110**: 710–717.
89. Yndestad A, Larsen KO, Øie E, Ueland T, Smith C, Halvorsen B, Sjaastad I, Skjønsberg OH, Pedersen TM, Anfinsen OG, Damås JK, Christensen G, Aukrust P, Andreassen AK. Elevated levels of activin A in clinical and experimental pulmonary hypertension. *J Appl Physiol* 2009;**106**:1356–1364.
90. Guignabert C, Savale L, Boucly A, Thuillet R, Tu L, Ottaviani M, Rhodes CJ, Prévot G, Bergot E, Bourdin A, Howard LS, Fadel E, Beurnier A, Roche A, Jevnikar M, Jaïs X, Montani D, Wilkins MR, Sitbon O, Humbert M. Serum and pulmonary expression profiles of the activin signaling system in pulmonary arterial hypertension. *Circulation* 2023;**147**:1809–1822.
91. Ryanto GRT, Ikeda K, Miyagawa K, Tu L, Guignabert C, Humbert M, Fujiyama T, Yanagisawa M, Hirata K, Emoto N. An endothelial activin A-bone morphogenetic protein receptor type 2 link is overdriven in pulmonary hypertension. *Nat Commun* 2021;**12**:1720.
92. Newman JH, Phillips JA, Loyd JE. Review narrative review: the enigma of pulmonary arterial hypertension. *Physiol Med* 2008;**148**:278–283.
93. Hiepen C, Jatzlau J, Hildebrandt S, Kampfrath B, Goktas M, Murgai A, Cuellar Camacho JL, Haag R, Ruppert C, Sengle G, Cavalcanti-Adam EA, Blank KG, Knaus P. BMPR2 acts as a gatekeeper to protect endothelial cells from increased TGFβ responses and altered cell mechanics. *PLoS Biol* 2019;**17**:e3000557.
94. Goumans MJ, Valdimarsdottir G, Itoh S, Lebrin F, Larsson J, Mummery C, Karlsson S, Ten Dijke P. Activin receptor-like kinase (ALK)1 is an antagonistic mediator of lateral TGFβ/ALK5 signaling. *Mol Cell* 2003;**12**:817–828.
95. Ramachandran A, Vizán P, Das D, Chakravarty P, Vogt J, Rogers KW, Müller P, Hinck AP, Sapkota GP, Hill CS. TGF-β uses a novel mode of receptor activation to phosphorylate SMAD1/5 and induce epithelial-to-mesenchymal transition. *Elife* 2018;**7**:e31756.
96. Olsen OE, Sankar M, Elsaadi S, Hella H, Buene G, Darvekar SR, Misund K, Katagiri T, Knaus P, Holien T. BMPR2 inhibits activin and BMP signaling via wild-type ALK2. *J Cell Sci* 2018; **131**:2–11.
97. Ramachandran A, Mehić M, Wasim L, Malinova D, Gori I, Blaszczyk BK, Carvalho DM, Shore EM, Jones C, Hyvönen M, Tolar P, Hill CS. Pathogenic ACVR1 R206H activation by activin A-induced receptor clustering and autophosphorylation. *EMBO J* 2021;**40**: e106317.
98. McAllister KA, Grogg KM, Johnson DW, Gallione CJ, Baldwin MA, Jackson CE, Helmbold EA, Markel DS, McKinnon WC, Murrel J, McCormick MK, Pericak-Vance MA, Heutink P, Oostra BA, Haitjema T, Westerman CJJ, Porteous ME, Guttmacher AE, Letarte M, Marchuk DA. Endoglin, a TGF-β binding protein of endothelial cells, is the gene for hereditary haemorrhagic telangiectasia type 1. *Nat Genet* 1994;**8**:345–351.
99. Núnez-Gómez E, Pericacho M, Ollauri-Ibánez C, Bernabéu C, López-Novoa JM. The role of endoglin in post-ischemic revascularization. *Angiogenesis* 2016;**20**:1–24.
100. Koleva RI, Conley BA, Romero D, Riley KS, Marto JA, Lux A, Vary CPH. Endoglin structure and function. *J Biol Chem* 2006;**281**:25110–25123.
101. Yung LM, Yang P, Joshi S, Augur ZM, Kim SSJ, Bocobo GA, Dinter T, Troncone L, Chen PS, McNeil ME, Southwood M, de Frias SP, Knopf J, Rosas IO, Sako D, Scott Pearsall R, Quisel JD, Li G, Kumar R, Yu PB. ACTRIIA-Fc rebalances activin/GDF versus BMP signaling in pulmonary hypertension. *Sci Transl Med* 2020;**12**:eaaz5660.
102. Upton PD, Dunmore BJ, Li W, Morrell NW. An emerging class of new therapeutics targeting TGF, activin, and BMP ligands in pulmonary arterial hypertension. *Dev Dyn* 2022;**252**: 327–342.
103. Klinge CM. Estrogen receptor interaction with estrogen response elements. *Nucleic Acids Res* 2001;**29**:2905–2919.
104. Wilson S, Qi J, Filipp FV. Refinement of the androgen response element based on ChIP-Seq in androgen-insensitive and androgen-responsive prostate cancer cell lines. *Nat Publ Gr* 2016;**6**:32611.
105. Jacobsen BM, Horwitz KB. Progesterone receptors, their isoforms and progesterone regulated transcription. *Mol Cell Endocrinol* 2013;**357**:18–29.
106. Xu S, Yu S, Dong D, Tsz L, Lee O. G protein-coupled estrogen receptor: a potential therapeutic target in cancer. *Front Endocrinol (Lausanne)* 2019;**10**:725.
107. Arnal JF, Fontaine C, Billon-Galés A, Favre J, Laurell H, Lenfant F, Gourdy P. Estrogen receptors and endothelium. *Arterioscler Thromb Vasc Biol* 2010;**30**:1506–1512.
108. Fontaine C, Morfoisse F, Tatin F, Zamora A, Zahreddine R, Henrion D, Arnal JF, Lenfant F, Garmy-Susini B. The impact of estrogen receptor in arterial and lymphatic vascular diseases. *Int J Mol Sci* 2020;**21**:3244.
109. Frump AL, Goss KN, Vayl A, Albrecht M, Fisher A, Tursunova R, Fierst J, Whitson J, Cucci AR, Beth Brown M, Lahm T. Estradiol improves right ventricular function in rats with severe angioproliferative pulmonary hypertension: effects of endogenous and exogenous sex hormones. *Am J Physiol Lung Cell Mol Physiol* 2015;**308**:L873–L890.
110. Frump AL, Albrecht M, Yakubov B, Breuils-bonnet S, Nadeau V, Tremblay E, Potus F, Omura J, Cook T, Fisher A, Rodriguez B, Brown RD, Stenmark KR, Rubinstein CD, Krentz K, Tabima DM, Li R, Sun X, Chesler NC, Provencher S, Bonnet S, Lahm T. 17 β-Estradiol and estrogen receptor α protect right ventricular function in pulmonary hypertension via BMPR2 and apelin. *J Clin Invest* 2021;**131**:e129433.
111. Mannella P, Simoncini T. Progestogen effects at vascular level: the endothelial cells. *Horm Mol Biol Clin Investig* 2010;**3**:449–451.
112. Vazquez F, Rodriguez-Manzaneque JC, Lydon JP, Edwards DP, Luisa I-A M, Bert WO. Progesterone regulates proliferation of endothelial cells. *J Biol Chem* 1999;**274**:2185–2192.
113. Torres-Estay V, Carreño D V, San Francisco IF, Sotomayor P, Godoy AS, Smith GJ. Androgen receptor in human endothelial cells. *J Endocrinol* 2015;**224**:R131–R137.
114. English KM, Jones RD, Jones TH, Morice AH, Channer KS. Gender differences in the vasomotor effects of different steroid hormones in rat pulmonary and coronary arteries. *Horm Metab Res* 2001;**33**:645–652.
115. Tofovic SP, Jackson EK. Estradiol metabolism: crossroads in pulmonary arterial hypertension. *Int J Mol Sci* 2019;**21**:116
116. Smith A, Jones R, Channer K. The influence of sex hormones on pulmonary vascular reactivity: possible vasodilator therapies for the treatment of pulmonary hypertension. *Curr Vasc Pharmacol* 2005;**4**:9–15.
117. Jameson JJ, Cave DR. Hormonal and antihormonal therapy for epistaxis in hereditary hemorrhagic telangiectasia. *Laryngoscope* 2004;**114**:705–709.
118. Albiñana V, Bernabeu-Herrero ME, Zarrabeitia R, Bernabeu C, Botella LM. Estrogen therapy for hereditary haemorrhagic telangiectasia (HHT): effects of raloxifene, on endoglin and ALK1 expression in endothelial cells. *Thromb Haemost* 2010;**103**:525–534.
119. Sobrino A, Mata M, Laguna-Fernandez A, Novella S, Oviedo PJ, García-Pérez MA, Tarín JJ, Cano A, Hermenegildo C. Estradiol stimulates vasodilatory and metabolic pathways in cultured human endothelial cells. *PLoS One* 2009;**4**:e8242.
120. McCarthy TL, Chang WZ, Liu Y, Centrella M. Runx2 integrates estrogen activity in osteoblasts. *J Biol Chem* 2003;**278**:43121–43129.

121. Zhou S, Turgeman G, Harris SE, Leitman DC, Komm BS, Bodine PVN, Gazit D. Estrogens activate bone morphogenetic protein-2 gene transcription in mouse mesenchymal stem cells. *Mol Endocrinol* 2003;**17**:56–66.
122. Ong DB, Colley SM, Norman MR, Kitazawa S, Tobias JH. Transcriptional regulation of a BMP-6 promoter by estrogen receptor α. *J Bone Miner Res* 2004;**19**:447–454.
123. Yang NN, Bryant HU, Hardikar S, Sato M, Galvin RJ, Glasebrook AL, Termine JD. Estrogen and raloxifene stimulate transforming growth factor-beta 3 gene expression in rat bone: a potential mechanism for estrogen- or raloxifene-mediated bone maintenance. *Gene Expr* 1996;**137**:8.
124. Austin ED, Hamid R, Hemnes AR, Loyd JE, Blackwell T, Yu C, Phillips JA III, Gaddipati R, Gladson S, Gu E, West J, Lane KB. BMPR2 Expression is suppressed by signaling through the estrogen receptor. *Biol Sex Differ* 2012;**3**:6.
125. Mair KM, Yang XD, Long L, White K, Wallace E, Ewart M, Docherty CK, Morrell NW, Maclean MR. Sex affects bone morphogenetic protein type II receptor signaling in pulmonary artery smooth muscle cells. *Am J Respir Crit Care Med* 2015;**191**:693–703.
126. Mair KM, Wright AF, Duggan N, Rowlands DJ, Hussey MJ, Roberts S, Fullerton J, Nilsen M, Loughlin L, Thomas M, MacLean MR. Sex-dependent influence of endogenous estrogen in pulmonary hypertension. *Am J Respir Crit Care Med* 2014;**190**:456–467.
127. Kunzmann S, Ottensmeier B, Speer CP, Fehrholz M. Effect of progesterone on Smad signaling and TGF-β/Smad-regulated genes in lung epithelial cells. *PLoS One* 2018;**13**: e0200661.
128. Paulin R, Meloche J, Jacob MH, Bisserier M, Courboulin A, Bonnet S. Dehydroepiandrosterone inhibits the Src/STAT3 constitutive activation in pulmonary arterial hypertension. *Am J Physiol Hear Circ Physiol* 2011;**301**:1798–1809.
129. Braga M, Bhasin S, Jasuja R, Pervin S, Singh R. Testosterone inhibits transforming growth factor-β signaling during myogenic differentiation and proliferation of mouse satellite cells: potential role of follistatin in mediating testosterone action. *Mol Cell Endocrinol* 2012;**350**: 39–52.
130. Zhang G, Kang Y, Zhou C, Cui R, Jia M, Hu S, Ji X, Yuan J, Cui H, Shi G. Amelioratory effects of testosterone propionate on age-related renal fibrosis via suppression of TGF-β1/Smad signaling and activation of Nrf2-ARE signaling. *Sci Rep* 2018;**8**:10726.
131. Beck TN, Korobeynikov VA, Kudinov AE, Georgopoulos R, Solanki NR, Andrews-Hoke M, Kistner TM, Pépin D, Donahoe PK, Nicolas E, Einarson MB, Zhou Y, Boumber Y, Proia DA, Serebriiskii IG, Golemis EA. Anti-Müllerian hormone signaling regulates epithelial plasticity and chemoresistance in lung cancer. *Cell Rep* 2016;**16**:657–671.
132. Xie L, Vo-Ransdell C, Abel B, Willoughby C, Jang S, Sowa G. Caveolin-2 is a negative regulator of anti-proliferative function and signaling of transforming growth factor-β in endothelial cells. *Am J Physiol Cell Physiol* 2011;**301**:C1161–C1174.
133. Martinkovich S, Shah D, Planey SL, Arnott JA. Selective estrogen receptor modulators: tissue specificity and clinical utility. *Clin Interv Aging* 2014;**9**:1437–1452.
134. Ghaffari S, Nabi FN, Sugiyama MG, Lee WL. Estrogen inhibits LDL (low-density lipoprotein) transcytosis by human coronary artery endothelial cells via GPER (G-protein—coupled estrogen receptor) and SR-BI (scavenger receptor class B type 1). *Arter Thromb Vasc Biol* 2018;**38**:2283–2294.
135. Meyer MR, Prossnitz ER, Barton M. GPER/GPR30 and regulation of vascular tone and blood pressure. *Immunol Endocr Metab Agents Med Chem* 2014;**11**:255–261.
136. Unterleutner E, Rigassi L, Barchiesi F, Imthurn B, Dubey RK. Abstract P098: G-protein coupled estrogen receptor stimulates capillary formation by human umbilical vein endothelial cells via ALK1-SMAD 1/5/8 pathway activation. *Hypertension* 2015;**66**:AP098.
137. Filardo EJ, Quinn JA, Bland KI, Frackelton AR, Surgery EJF. Estrogen-induced activation of Erk- 1 and Erk-2 requires the G protein- coupled receptor homolog, GPR30, and occurs via trans—activation of the epidermal growth factor receptor through release of HB-EGF. *Mol Endocrinol* 2000;**14**:1649–1660.
138. Pupo M, Pisano A, Abonante S, Maggiolini M, Maria A. GPER activates notch signaling in breast cancer cells and cancer-associated fibroblasts (CAFs). *Int J Biochem Cell Biol* 2014; **46**:56–67.
139. Babicheva A, Yuan JXJ. Endothelial Notch1 in pulmonary hypertension: friend or foe? *Circ Res* 2020;**124**:176–179.
140. Berghausen EM, Janssen W, Vantler M, Gnatzy-Feik LL, Krause M, Behringer A, Joseph C, Zierden M, Ten Freyhaus H, Klinke A, Baldus S, Alcazar MA, Savai R, Pullamsetti SS, Wong DWL, Boor P, Zhao JJ, Schermuly RT, Rosenkranz S. Disrupted PI3K subunit p110α signaling protects against pulmonary hypertension and reverses established disease in rodents. *J Clin Invest* 2021;**131**:e136939.
141. Shafiq M, Jagavelu K, Iqbal H, Yadav P, Chanda D, Verma NK, Ghosh JK, Gaestel M, Hanif K. Inhibition of mitogen-activated protein kinase (MAPK)-activated protein kinase 2 (MK2) is protective in pulmonary hypertension. *Hypertension* 2021;**2**:1248–1259.
142. Zhou W, Liu K, Zeng L, He J, Gao X, Gu X, Chen X. Targeting VEGF-A/VEGFR2 Y949 signaling-mediated vascular permeability alleviates hypoxic pulmonary hypertension. *Circulation* 2022;**146**:1855–1881.
143. West J, Cogan J, Geraci M, Robinson L, Newman J, Phillips JA, Lane K, Meyrick B, Loyd J. Gene expression in BMPR2 mutation carriers with and without evidence of pulmonary arterial hypertension suggests pathways relevant to disease penetrance. *BMC Med Genomics* 2008;**1**:45.
144. Austin ED, Cogan JD, West JD, Hedges LK, Hamid R, Dawson EP, Wheeler LA, Parl FF, Loyd JE, Phillips JA. Alterations in oestrogen metabolism: implications for higher penetrance of familial pulmonary arterial hypertension in females. *Eur Respir J* 2009;**34**: 1093–1099.
145. Cherlet T, Murphy LC. Estrogen receptors inhibit Smad3 transcriptional activity through Ap-1 transcription factors. *Mol Cell Biochem* 2007;**306**:33–42.
146. Ito I, Hanyu A, Wayama M, Goto N, Katsuno Y, Kawasaki S, Nakajima Y, Kajiro M, Komatsu Y, Fujimura A, Hirota R, Murayama A, Kimura K, Imamura T, Yanagisawa J. Estrogen inhibits transforming growth factor β signaling by promoting Smad2/3 degradation. *J Biol Chem* 2010;**285**:14747–14755.
147. Malek D, Gust R, Kleuser B. 17-β-Estradiol Inhibits transforming-growth-factor-β-induced MCF-7 cell migration by Smad3-repression. *Eur J Pharmacol* 2006;**534**:39–47.
148. Matsuda T, Yamamoto T, Muraguchi A, Saatcioglu F. Cross-talk between transforming growth factor-β and estrogen receptor signaling through Smad3. *J Biol Chem* 2001;**276**: 42908–42914.
149. Yamamoto T, Saatcioglu F, Matsuda T. Cross-talk between bone morphogenic proteins and estrogen receptor signaling. *Endocrinology* 2002;**143**:2635–2642.
150. Kang HY, Huang KE, Chang SY, Ma WL, Lin WJ, Chang C. Differential modulation of androgen receptor-mediated transactivation by Smad3 and tumor suppressor smad4. *J Biol Chem* 2002;**277**:43749–43756.
151. Band AM, Laiho M. Crosstalk of TGF-β and estrogen receptor signaling in breast cancer. *J Mammary Gland Biol Neoplasia* 2011;**16**:109–115.
152. Thomas P, Pang Y. Protective actions of progesterone in the cardiovascular system: potential role of membrane progesterone receptors (mPRs) in mediating rapid effects. *Steroids* 2013;**78**:583–588.
153. Morey AK, Pedram ALI, Razandi M, Prins BA, Hu R, Biesiada E, Levin ER. Estrogen and progesterone inhibit vascular smooth muscle proliferation. *Endocrinology* 1997;**138**: 3330–3339.
154. Lee W-S, Harder JA, Yoshizumi M, Lee M-E, Haber E. Progesterone inhibits arterial smooth muscle cell proliferation. *Nat Med* 1997;**3**:1005–1008.
155. Wu W, Yuan P, Zhang S, Jiang X, Wu C, Li Y, Liu S. Impact of pituitary—gonadal axis hormones on pumonary arterial hypertension in men. *Hypertension* 2018;**72**:151–158.
156. Iwata N, Hasegawa T, Fujita S, Nagao S, Nakano Y. Effect of the interaction of metformin and bone morphogenetic proteins on ovarian steroidogenesis by human granulosa cells. *Biochem Biophys Res Commun* 2018;**503**:1422–1427.
157. Chang H, Cheng J, Huang H, Shi F, Leung PCK. Activin A, B and AB decrease progesterone production by down-regulating StAR in human granulosa cells. *Mol Cell Endocrinol* 2015; **412**:290–301.
158. Chang H, Cheng J, Klausen C, Taylor EL, Leung PCK. Effects of recombinant activins on steroidogenesis in human granulosa-lutein cells. *J Clin Endocrinol Metab* 2014;**99**: 1922–1932.
159. Zhang H, Klausen C, Zhu H, Chang H. BMP4 And BMP7 suppress StAR and progesterone production via ALK3 and SMAD1/5/8-SMAD4 in human granulosa-lutein cells. *Endocrinology* 2015;**156**:4269–4280.
160. Alzoubi A, Toba M, Abe K, Neill KDO, Rocic P, Fagan KA, Mcmurtry IF, Oka M. Dehydroepiandrosterone restores right ventricular structure and function in rats with severe pulmonary arterial hypertension. *Am J Physiol Hear Circ Physiol* 2013;**304**:1708–1718.
161. Walsh TP, Baird GL, Atalay MK, Agarwal S, Arcuri D, Klinger JR, Mullin CJ, Morreo H, Normandin B, Shiva S, Whittenhall M, Ventetuolo CE. Experimental design of the effects of dehydroepiandrosterone in pulmonary hypertension (EDIPHY) trial. *Pulm Circ* 2021; **11**:2045894021989554.
162. Dubey RK, Oparil S, Imthurn B, Jackson EK. Sex hormones and hypertension. *Cardiovasc Res* 2002;**53**:688–708.
163. Mikkonen L, Pihlajamaa P, Sahu B, Zhang FP, Jänne OA. Androgen receptor and androgen-dependent gene expression in lung. *Mol Cell Endocrinol* 2010;**317**:14–24.
164. Ventetuolo CE, Baird GL, Barr RG, Bluemke DA, Fritz JS, Hill NS, Klinger JR, Lima JA, Ouyang P, Palevsky HI, Palmisciano AJ, Krishnan I, Pinder D, Preston IR, Roberts KE, Kawut SM. Higher estradiol and lower dehydroepiandrosterone-sulfate levels are associated with pulmonary arterial hypertension in men. *Am J Respir Crit Care Med* 2016 ;**193**:1168–1175.
165. Baird GL, Archer-Chicko C, Barr RG, Bluemke DA, Foderaro AE, Fritz JS, Hill NS, Kawut SM, Klinger JR, Lima JAC, Mullin CJ, Ouyang P, Palevsk HI, Ventetuolo CE. Lower DHEA-S levels predict disease and worse outcomes in post-menopausal women with idiopathic, connective tissue disease- and congenital heart disease-associated pulmonary arterial hypertension. *Eur Respir J* 2018;**51**:1800467.
166. van Wezenbeek J, Groeneveldt JA, Llucià-Valldeperas A, van der Bruggen CE, Jansen SMA, Smits AJ, Smal R, van Leeuwen JW, dos Remedios C, Keogh A, Humbert M, Dorfmüller P, Mercier O, Guignabert C, Niessen HWM, Handoko ML, Marcus JT, Meijboom LJ, Oosterveer FPT, Westerhof BE, Heijboer AC, Bogaard HJ, Noordegraaf AV, Goumans MJ, de Man FS. Interplay of sex hormones and long-term right ventricular adaptation in a Dutch PAH-cohort. *J Hear Lung Transplant* 2022;**41**:445–457.
167. Hayes SA, Zarnegar M, Sharma M, Yang F, Peehl DM, Ten DP, Sun Z. SMAD3 Represses androgen receptor-mediated transcription. *Cancer Res* 2001;**61**:2112–2118.
168. Yu X, Li S, Xu Y, Zhang Y, Ma W, Liang C, Lu H, Ji Y, Liu C, Chen D, Li J. Androgen maintains intestinal homeostasis by inhibiting BMP signaling via intestinal stromal cells. *Stem Cell Reports* 2020;**15**:912–925.
169. Qiu T, Grizzle WE, Oelschlager DK, Shen X, Cao X. Control of prostate cell growth: BMP antagonizes androgen mitogenic activity with incorporation of MAPK signals in smad1. *EMBO J* 2007;**26**:346–357.
170. Bjørnerem Å, Straume B, Midtby M, Fønnebø V, Sundsfjord J, Svartberg J, Acharya G, Øian P, Berntsen GKR. Endogenous sex hormones in relation to age, sex, lifestyle factors, and

chronic diseases in a general population: the Tromsø study. *J Clin Endocrinol Metab* 2004;**89**: 6039–6047.
171. Humbert M, McLaughlin V, Gibbs JSR, Gomberg-Maitland M, Hoeper MM, Preston IR, Souza R, Waxman A, Escribano Subias P, Feldman J, Meyer G, Montani D, Olsson KM, Manimaran S, Barnes J, Linde PG, de Oliveira Pena J, Badesch DB. Sotatercept for the treatment of pulmonary arterial hypertension. *N Engl J Med* 2021;**384**:1204–1215.
172. Hart KN, Stocker WA, Nagykery NG, Walton KL, Harrison CA, Donahoe PK, Pépin D, Thompson TB. Structure of AMH bound to AMHR2 provides insight into a unique signaling pair in the TGF-β family. *Proc Natl Acad Sci USA* 2021;**118**:e2104809118.
173. Ricci M, Mohapatra B, Urbiztondo A, Birusingh RJ, Morgado M, Rodriguez MM, Lincoln J, Vatta M. Differential changes in TGF-β/BMP signaling pathway in the right ventricular myocardium of newborns with hypoplastic left heart syndrome. *J Card Fail* 2010;**16**:628–634.
174. Picard JY, Cate RL, Racine C, Josso N. The persistent Müllerian duct syndrome: an update based upon a personal experience of 157 cases. *Sex Dev* 2017;**11**:109–125.
175. Chong YH, Dennis NA, Connolly MJ, Teh R, Jones GT, van Rij AM, Farrand S, Campbell AJ, McLennan IS. Elderly men have low levels of anti-Müllerian hormone and inhibin B, but with high interpersonal variation: a cross-sectional study of the sertoli cell hormones in 615 community-dwelling men. *PLoS One* 2013;**8**:e70967.
176. Appt SE, Chen H, Clarkson TB, Kaplan JR. Premenopausal antiMüllerian hormone concentration is associated with subsequent atherosclerosis. *Menopause* 2012;**19**:1353–1359.
177. Dennis NA, Jones GT, Chong YH, van Rij AM, McLennan IS. Serum anti-Müllerian hormone (AMH) levels correlate with infrarenal aortic diameter in healthy older men: is AMH a cardiovascular hormone? *J Endocrinol* 2013;**219**:13–20.
178. De Kat AC, Monique Verschuren W, Eijkemans MJC, Broekmans FJM, Van Der Schouw YT. Anti-Müllerian hormone trajectories are associated with cardiovascular disease in women: results from the Doetinchem cohort study. *Circulation* 2017;**135**:556–565.
179. Choi SH, Jung YK, Jang JA, Han S. Idiopathic pulmonary arterial hypertension associated with a novel frameshift mutation in the bone morphogenetic protein receptor II gene and enhanced bone morphogenetic protein signaling: a case report. *Medicine (Baltimore)* 2019;**98**:e17594.
180. Kadariya D, Kurbanova N, Qayyum R. Association of anti-Mullerian hormone with C-reactive protein in men. *Sci Rep* 2019;**9**:13081.
181. Rabinovitch M, Guignabert C, Humbert M, Nicolls MR. Inflammation and immunity in the pathogenesis of pulmonary arterial hypertension. *Circ Res* 2015;**115**:165–175.
182. Chen X, Austin ED, Talati M, Fessel JP, Farber-Eger EH, Brittain EL, Hemnes AR, Loyd JE, West J. Oestrogen inhibition reverses pulmonary arterial hypertension and associated metabolic defects. *Eur Respir J* 2017;**50**:1602337.
183. Kawut SM, Pinder D, Al-Naamani N, McCormick A, Palevsky HI, Fritz J, Smith A, Mazurek JA, Doyle MF, MacLean MR, DeMichele A, Mankoff DA. Fulvestrant for the treatment of pulmonary arterial hypertension. *Ann Am Thorac Soc* 2019;**16**:1456–1459.
184. Kawut SM, Archer-chicko CL, Demichele A, Fritz JS, Klinger JR, Ky B, Palevsky HI, Palmisciano AJ, Patel M, Pinder D, Propert KJ, Smith KA, Stanczyk F, Tracy R, Vaidya A, Whittenhall ME, Ventetuolo CE. Anastrozole in pulmonary arterial hypertension. *Am J Respir Crit Care Med* 2017;**195**:360–368.
185. Kawut SM, Feng R, Zamanian RT, Ellenberg S, Bull TM, Chakinala MM, Hemnes A, Mathai SC, Lin G. Pulmonary hypertension and anastrozole (phantom): a randomized clinical trial. *Am J Respir Crit Care Med* 2023;**207**:A6727.
186. Limoges M, Langleben D, Fox BD, Shear R, Wieczorek P, Rudski LG, Hirsch AM, Schlesinger RD, Lesenko L. Pregnancy as a possible trigger for heritable pulmonary arterial hypertension. *Pulm Circ* 2016;**6**:381–383.
187. Olsson KM, Channick R. Pregnancy in pulmonary arterial hypertension. *Eur Respir Rev* 2016; **25**:431–437.
188. Maan AA, Eales J, Akbarov A, Rowland J, Xu X, Jobling MA, Charchar FJ, Tomaszewski M. The y chromosome: a blueprint for men's health? *Eur J Hum Genet* 2017;**25**:1181–1188.
189. Eggers S, Ohnesorg T, Sinclair A. Genetic regulation of mammalian gonad development. *Nat Rev Endocrinol* 2014;**10**:673–683.
190. Turner ME, Ely D, Prokop J, Milsted A. Sry, more than testis determination? *Am J Physiol Regul Integr Comp Physiol* 2011;**301**:R561–R571.
191. Yan L, Cogan JD, Hedges LK, Nunley B, Hamid R, Austin ED. The Y chromosome regulates BMPR2 expression via SRY: a possible reason 'why' fewer males develop pulmonary arterial hypertension. *Am J Respir Crit Care Med* 2018;**198**:1581–1583.
192. Yuan X, Lu ML, Li T, Balk SP. SRY Interacts with and negatively regulates androgen receptor transcriptional activity. *J Biol Chem* 2001;**276**:46647–46654.
193. Lee KH, Song GJ, Kang IS, Kim SW, Paick J-S, Chung CH, Rhee K. Ubiquitin-specific protease activity of USP9Y, a male infertility gene on the Y chromosome. *Reprod Fertil Dev* 2003; **15**:129–133.
194. Brown KA, Ham AL, Clark CN, Meller N, Law BK, Chytil A, Cheng N, Pietenpol JA, Moses HL. Identification of novel Smad2 and Smad3 associated proteins in response to TGF-b1. *J Cell Biochem* 2008;**611**:596–611.
195. Panning B. X-chromosome inactivation: the molecular basis of silencing. *J Biol* 2008;**7**:30.
196. Sado T, Hoki Y, Sasaki H. Tsix silences Xist through modification of chromatin structure. *Dev Cell* 2005;**9**:159–165.
197. Vallot C, Patrat C, Collier AJ, Huret C, Casanova M, Liyakat Ali TM, Tosolini M, Frydman N, Heard E, Rugg-Gunn PJ, Rougeulle C. XACT noncoding RNA competes with XIST in the control of X chromosome activity during human early development. *Cell Stem Cell* 2017; **20**:102–111.
198. Yasukochi Y, Maruyama O, Mahajan MC, Padden C, Euskirchen GM, Schulz V, Hirakawa H, Kuhara S, Pan XH, Newburger PE, Snyder M, Weissman SM. X chromosome-wide analyses of genomic DNA methylation states and gene expression in male and female neutrophils. *Proc Natl Acad Sci USA* 2010;**107**:3704–3709.
199. Credendino SC, Neumayer C, Cantone I. Genetics and epigenetics of sex bias: insights from human cancer and autoimmunity. *Trends Genet* 2020;**36**:650–663.
200. Qin S, Predescu D, Carman B, Patel P, Chen J, Kim M, Lahm T, Geraci M, Predescu SA. Up-regulation of the long noncoding RNA X-inactive–specific transcript and the sex bias in pulmonary arterial hypertension. *Am J Pathol* 2021;**191**:1135–1150.
201. Sripathy S, Leko V, Adrianse RL, Loe T, Foss EJ, Dalrymple E, Lao U, Gatbonton-Schwager T, Carter KT, Payer B, Paddison PJ, Grady WM, Lee JT, Bartolomei MS, Bedalov A. Screen for reactivation of MeCP2 on the inactive X chromosome identifies the BMP/TGF-β superfamily as a regulator of XIST expression. *Proc Natl Acad Sci USA* 2017;**114**:1619–1624.
202. Wang Z, Jinnin M, Nakamura K, Harada M, Kudo H, Nakayama W, Inoue K, Nakashima T, Honda N, Fukushima S, Ihn H. Long non-coding RNA TSIX is upregulated in scleroderma dermal fibroblasts and controls collagen mRNA stabilization. *Exp Dermatol* 2016;**25**: 131–136.
203. Trembath RC, Thomson JR, Machado RD, Morgan NV, Atkinson C, Winship I, Simonneau G, Galie N, Loyd JE, Humbert M, Nichols WC, Berg J, Manes A, McGaughran J, Pauciulo M, Wheeler L, Morrell NW. Clinical and molecular genetic features of pulmonary hypertension in patients with hereditary hemorrhagic telangiectasia. *N Engl J Med* 2001;**345**: 325–334.
204. Govani FS, Shovlin CL. Hereditary haemorrhagic telangiectasia: a clinical and scientific review. *Eur J Hum Genet* 2009;**17**:860–871.
205. Berg JN, Gallione CJ, Stenzel TT, Johnson DW, Allen WP, Schwartz CE, Jackson CE, Porteous MEM, Marchuk DA. The activin receptor-like kinase 1 gene: genomic structure and mutations in hereditary hemorrhagic telangiectasia type 2. *Am J Hum Genet* 1997;**61**: 60–67.
206. Kim YH, Vu PN, Choe SW, Jeon CJ, Arthur HM, Vary CPH, Lee YJ, Paul Oh S. Overexpression of activin receptor-like kinase 1 in endothelial cells suppresses development of arteriovenous malformations in mouse models of hereditary hemorrhagic telangiectasia. *Circ Res* 2020;**127**:1122–1137.
207. Mallet C, Lamribet K, Giraud S, Dupuis-Girod S, Feige JJ, Bailly S, Tillet E. Functional analysis of endoglin mutations from hereditary hemorrhagic telangiectasia type 1 patients reveals different mechanisms for endoglin loss of function. *Hum Mol Genet* 2015;**24**:1142–1154.
208. Mora-Luján JM, Iriarte A, Alba E, Sánchez-Corral MA, Cerdà P, Cruellas F, Ordi Q, Corbella X, Ribas J, Castellote J, Riera-Mestre A. Gender differences in hereditary hemorrhagic telangiectasia severity. *Orphanet J Rare Dis* 2020;**15**:63.
209. Letteboer TGW, Mager JJ, Snijder RJ, Koeleman BPC, Lindhout D, Ploos van Amstel HK, Zanen P, Westermann CJJ. Genotype-phenotype relationship in hereditary haemorrhagic telangiectasia. *J Med Genet* 2006;**43**:371–377.
210. Chowdhury FN, Chandrarathne GS, Masilamani KD, Labranche JTN, Malo S, Svenson LW, Jeerakathil T, Vethanayagam DP. Links between strokes and hereditary hemorrhagic telangiectasia: a population-based study. *Can J Neurol Sci* 2019;**46**:44–50.
211. Donaldson JW, McKeever TM, Hall IP, Hubbard RB, Fogarty AW. Complications and mortality in hereditary hemorrhagic telangiectasia. *Neurology* 2015;**84**:1886–1893.
212. Donaldson JW, McKeever TM, Hall IP, Hubbard RB, Fogarty AW. The UK prevalence of hereditary haemorrhagic telangiectasia and its association with sex, socioeconomic status and region of residence: a population-based study. *Thorax* 2014;**69**:161–167.
213. Albiñana V, Cuesta AM, De R-PI, Gallardo-Vara E, Recio-Poveda L, Bernabéu C, María Botella L. Review of pharmacological strategies with repurposed drugs for hereditary hemorrhagic telangiectasia related bleeding. *J Clin Med* 2020;**9**:1766.
214. Yaniv E, Preis M, Shvero J, Nageris B, Hadar T. Anti-estrogen therapy for hereditary hemorrhagic telangiectasia—a long-term clinical trial. *Rhinology* 2011;**49**:214–216.
215. Chi J, Chang HY, Haraldsen G, Jahnsen FL, Troyanskaya OG, Chang DS, Wang Z, Rockson SG, van de Rijn M, Botstein D, Brown PO. Endothelial cell diversity revealed by global expression profiling. *Proc Natl Acad Sci USA* 2003;**100**:10623–10628.
216. Gherezghiher TB, Michalsen B, Chandrasena REP, Qin Z, Sohn J, Thatcher GRJ, Bolton JL. The naphthol selective estrogen receptor modulator (SERM), LY2066948, is oxidized to an o-quinone analogous to the naphthol equine estrogen, equilenin. *Chem Biol Interact* 2012; **196**:1–10.