In Situ and *In Vitro* Models to Investigate the Role of Oestrogen and Oestrogen Metabolism in Pulmonary Vascular Disease

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Abstract

Pulmonary arterial hypertension is an incurable vasculopathy, which affects significantly more women than men. Hence, female sex hormones may be intimately involved in the initiation and progression of disease pathogenesis. Indeed, current evidence suggests dysregulated oestrogen biosynthesis and metabolism, result in microenvironment favouring excessive proliferation of pulmonary artery smooth muscle cells, leading to significant vascular remodelling. Impairment of signalling via the bone morphogenetic protein receptor II (BMPR*II*) signalling pathway might also be partially accountable for increased cellular proliferation, and the sex dimorphism associated with the disease. Much of the currently available evidence regarding the pathophysiological mechanism of this vasculopathy was gained using experimental animal models of pulmonary hypertension, we aimed to develop and employ *in situ* and *in vitro* models to investigate the role of oestrogen and its metabolism in pulmonary arterial hypertension.

Signalling through aryl hydrocarbon receptor results in altered expression of *Phase I and II* metabolising enzymes, including oestrogen metabolising enzymes. Using an *in-silico* approach, we demonstrated that the importance of aryl hydrocarbon receptor in experimental animal model of pulmonary hypertension induced by exposure to Sugen 5416 and chronic hypoxia. Initial quantitative real-time polymerase chain reaction analysis revealed that expression of *CYP1A1* gene might be decreased in cultured pulmonary arterial smooth muscle cells from female patients, rats exposed to chronic hypoxia and Smad1 heterozygous mice. *CYP1A1* gene expression was, however, greatly increased in the Sugen 5416/hypoxic experimental animal model. We further demonstrated that in this specific model several genes under the transcriptional activation of the aryl hydrocarbon receptor, such as NAD(P)H quinone dehydrogenase 1 and aryl hydrocarbon receptor repressor, are increased. *In vitro* stimulation of human pulmonary arterial smooth muscle cells with Sugen 5416, which is a potent agonist of the aryl hydrocarbon receptor, resulted in increased expression of CYP1A1 and 1B1. We further demonstrated by employing R.E.A.P. fractionation protocol, that stimulation of smooth muscle cells with Sugen 5416 resulted in the translocation of the aryl hydrocarbon receptor from the cytoplasm to the nucleus, where it was able to exert its transcriptional activity. The blockade of the aryl hydrocarbon receptor pathway by CH223191, resulted in attenuated expression of these oestrogen metabolising enzymes only in smooth muscle cells derived from pulmonary arteries of control subjects. In human pulmonary microvascular endothelial cells, simulation with Sugen 5416 and aryl

hydrocarbon receptor inhibitor, FICZ, increased apoptosis of these cells as assessed via reduced cell number and increased cleavage of Caspase 3. Hence, Sugen 5416 may induce vascular injury, leading to initiation of pathophysiological mechanisms of experimental PH. Even though the aryl hydrocarbon receptor pathway interacts with hypoxia-inducible factor signalling, we showed that stimulation of pulmonary artery smooth muscle cells does not alter mediators involved in the latter pathway. Furthermore, we showed that sex dimorphism might exist in the basal expression of mediators involved in the hypoxia-inducible factors signalling pathway (HIFs). In smooth muscle cells derived from pulmonary arteries of female PAH patients, the expression of HIF1α was significantly increased, while the regulators of HIF1α proteasomal degradation were significantly decreased. Equally, in smooth muscle cells derived from pulmonary arteries of male PAH patients, protein expression regulators of HIF1α proteasomal degradation were significantly decreased. *In vitro*, we demonstrated that there a possible synergy exists between the aryl hydrocarbon receptor and hypoxia-inducible factor signalling pathways, where co-stimulation with Sugen 5416 and hypoxia resulted in increased proliferation of smooth muscle cells derived from pulmonary arteries of female PAH.

We also aimed to develop and employ an *in vitro* model to investigate the role of oestrogen and its metabolism in the pathophysiological mechanism of pulmonary arterial hypertension, and to quantitatively assess oestrogen metabolism in this model. We showed that stimulation with 2-methoxyoestrogens increased expression of prostacyclin synthase in pulmonary microvascular endothelial cells from female control subject. Using immunoblotting technique, we further demonstrated that in smooth muscle cells derived from pulmonary arteries of male control subjects 2-methoxyoestradiol increased protein expression of Id3 and had no significant effect in smooth muscle cells derived from pulmonary arteries of female control subjects. It appears that in female pulmonary artery smooth muscle cells 2-methoxyoestradiol increased the expression of p27/Kip1 in a concentration-dependent manner. In fact, *in vitro* stimulation with 2-methoxyoestradiol attenuated serum-induced proliferation in smooth muscle cells derived from pulmonary arteries of control subjects of both sexes. In smooth muscle cells derived from pulmonary arteries of PAH patients, 2-methoxyoestradiol only reduced cellular proliferation in female cells, while having no effect in male cells. We have also examined the effects of 2- and 4-hydroxyoestradiol on serum-induced proliferation in pulmonary artery smooth muscle cells from male control subjects. Here we found both metabolites mediated attenuation of cellular proliferation, with 2-hydroxyoestradiol mediating its effects through its biotransformation into 2-

methoxyoestradiol, as catechol-O-methyl transferase inhibition restored cellular proliferation. Inhibition of catechol-O-methyl transferase did not have an effect on 4-hydroxyoestradiol-mediated reduction of serum-induced proliferation.

Using the *in vitro* model of pulmonary arterial hypertension employing pulmonary artery smooth muscle cells, we have assessed the effects of treprostinil on oestrogen metabolism in these cells using high performance liquid chromatography/flux analysis. We demonstrated that pulmonary artery smooth muscle cells derived from female and male PAH patients might metabolise 17β-oestradiol differently than those derived from control subjects. Moreover, treprostinil might affect the metabolism of 17β-oestradiol to oestrone, probably by affecting the activity of 17β-hydroxysteroid dehydrogenase type 2. Basal protein expression level as assessed by immunoblotting technique indicated that the latter enzyme might be increased in pulmonary artery smooth muscle cells derived from male PAH patients. To overcome certain shortcomings of high performance liquid chromatography/flux analysis for investigation of oestrogen metabolism, we aimed to develop and optimise a liquid chromatography tandem mass spectrometry approach. We have established the technique, and optimised separation of methoxyoestrogens and wash steps. The novel approach was used to assess oestrogen metabolism in *in vitro* model of pulmonary arterial hypertension, showing that smooth muscle cells derived from female PAH patients produce significantly more oestrone and 17α-isomer of oestradiol. Moreover, smooth muscle cells derived from male PAH patients metabolised the least 17β-oestradiol in comparison with female patient cells. Although the technique is now established, further optimisation is required to achieve reliable quantification of oestrogen metabolites in reasonable concentration ranges, as currently the method appears unreliable at concentration ranges for some oestrogen metabolites.

Using a hypothesis-free metabolomic screen we also demonstrated that certain metabolic pathways associated with energy reactive oxygen species and L-tryptophan metabolism might be affected in pulmonary artery smooth muscle cells.

Using *in situ* model we showed that induction of the aryl hydrocarbon receptor by Sugen 5416 leads to alterations in the expression of oestrogen metabolising enzymes, such as CYP1B1 and CYP1A1. Thereby possibly affecting the pathogenic mechanisms by introducing an imbalance of oestrogen metabolism. By using *in vitro* model of pulmonary arterial hypertension, we also showed methoxyoestrogen the role of methoxyoestrogens, 2-

and 4-hydroxyoestrogens in the attenuation of cellular proliferation in pulmonary artery smooth muscle cells. Furthermore, we established oestrogen metabolic profile in pulmonary artery smooth muscle cells derived from controls and patients of both sexes and quantified these metabolites. We have therefore successfully applied *in situ* and *in vitro* approaches to investigate the role of oestrogen and oestrogen metabolism in the pathogenesis of pulmonary arterial hypertension.

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Nothing worth having comes easy.

Author's Declaration

I declare that this thesis has been written entirely by myself and is a record of the work performed by myself, except where acknowledgement has been made. This thesis has not been previously submitted for a higher degree at this or any other university.

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List of Abbreviations

20-HETE	20-hydroxyeicosatetraenoic acid
3βHSD	3β Hydroxysteroid dehydrogenase
3MC	3-methylcholanthrene
5-HT	5-hydroxytryptamine or serotonin
17βE2	17β-oestradiol
17αE2	17α-oestradiol
2OHE1	2-hydroxyoestrone
2OHE2	2-hydroxyoestradiol
4OHE1	4-hydroxyoestrone
4OHE2	4-hydroxyoestradiol
16αOHE1	16α-hydroxyoestrone
16αOHE2	16α-hydroxyoestradiol
2MeOE1	2-methoxyoestrone
2MeOE2	2-methoxyoestradiol
4MeOE1	4-methoxyoestrone
4MeOE2	4-methoxyoestradiol
17βHSD type 2	17β hydroxysteroid dehydrogenase type 2
17βHSD type 3	17β hydroxysteroid dehydrogenase type 3
%CV	coefficient of variation
%IS	ion suppression
%ME	matrix effects
%RE	recovery
AA	arachidonic acid / antibiotic anti-mycotic
AC	adenosine cyclase
AF-1	activation function 1
AF-2	activation function 2
AhRR	aryl hydrocarbon receptor repressor
AhR	aryl hydrocarbon receptor
ALK-1	activin A receptor type II-like kinase-1
AP1	Jun/Fos/activator protein 1
APAH	associated PAH
API (ARP9)	aryl hydrocarbon receptor associated 9
ARNT	AhR nuclear translocator
ATP	adenosine triphosphate

B[a]P	benzo[a]pyrene
BCA	bicinchoninic acid
bHLH	basic helix-loop-helix
BMP	bone morphogenetic protein
BMP4	bone morphogenetic protein
BMPRI	bone morphogenetic protein receptor type I
BMPRII	bone morphogenetic protein receptor II
BMPRII +/−	*BMPRII* allele
BrdU	5-bromo-2-deoxyuridine
BSS	buffered saline solution
Ca-CAM	calcium-calmodulin
CAD	coronary artery disease
cAMP	cyclic adenosine monophosphate
CBP	CREB-binding protein
CCB	calcium channel blocker
CDK	cyclin/cycle dependent kinase
cDNA	complementary DNA
CE-Q	catechol-oestrogens and quinones
cGMP	cyclic guanosine monophosphate
CHD	coronary heart disease
cHx	chronic hypoxia
CKI	cyclin-dependent kinase inhibitor
CoA	coenzyme A
COMT	catechol-O-methyl transferases
COPD	chronic obstructive pulmonary disease
COX	cyclooxygenase enzymes
CSS	charcoal-stripped serum
CTEPH	chronic thromboembolic pulmonary hypertension
CT	cycle threshold
CVD	cardiovascular disease
CYP	cytochrome P450 enzyme
CYP1A1	cytochrome P450, family 1, subfamily A, polypeptide 1
CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1
DAG	diacyl glycerol
DHEA	dehydroepiandrosterone
DHEA-S	DHEA-sulfate
DHT	dihydrotestosterone

DMEM	Dulbecco’s modified eagle medium
DMSO	dimethyl sulfoxide
dNTP	deoxynucleotide triphosphate
DPBS	Dulbecco's phosphate-buffered saline
DRE/XRE	dioxin- and/or xenobiotic-response elements
E1	oestrone
E2	oestradiol
E3	oestriol
EC	endothelial cell
EET	epoxyeicosatrienoic acid
EIS	electron ion spray voltage
eNOS	endothelial NO synthase
ER	oestrogen receptor
ERA	ET-1 receptor antagonist
ERE	oestrogen-response elements
ERα	oestrogen receptor α
ERK	extracellular signal-regulated kinase
ERK1/2	mitogen-activated protein kinases p42/p44
ESI	electrospray ionisation technique
ETRA	endothelin-1 receptor antagonists
ET-1	endothelin-1
FSH	follicle-stimulating hormone
FSH-β	β subunit of ovine follicle-stimulating hormone
GC-MS	gas chromatography-mass spectrometry
GPCR	G-protein coupled receptor
GPER1	G-protein coupled oestrogen receptor
GPR30	G-protein coupled oestrogen receptor
GFR	growth factor receptor
GSH	glutathione
GSTA1	glutathione S-transferase alpha 1
HIF	hypoxia-inducible factor
HIF1α	hypoxia-inducible factor 1α
HIF1β	hypoxia-inducible factor 1β
hPASMC	human pulmonary artery smooth muscle cell
HPLC	high-performance liquid chromatography
hPMEC	human pulmonary microvascular endothelial cell
HRP	horseradish peroxidase

Hsp90	heat shock protein 90
HPAH	hereditable PAH
Hsp90	heat shock protein 90
Hx	hypoxia/hypoxic
Id	inhibitor of the DNA-binding
Id1/3	DNA-binding protein inhibitor Id1/3
IGF	insulin-like growth factor
IGF-1	insulin growth factor 1
IL-6	interleukin 6
iNOS	inducible NO synthase
InsP3R	inositol-activated receptors
IP	relaxant prostacyclin IP receptor
IP3	inositol trisphosphate
IPAH	idiopathic PAH
I.S.	internal standards
JNK	c-Jun N-terminal kinase
Kv	voltage-gated potassium channels
LOD	limit of detection
LV	left ventricle
MAP	mitogen activated protein
MAPK	mitogen-activated protein kinase
M-COMT	membrane-bound COMT
MCX	mixed-mode, strong cation-exchange
MESA	multi-ethnic study of atherosclerosis
MLC-K	myosin light-chain kinase
mPAP	mean PAP
MRM	multiple reaction monitoring
MS	mass spectrometry
MCT	monocrotaline
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor kappa-B
NO	nitric oxide
NQO1	NAD(P)H dehydrogenase (quinone 1)
Nrf2	nuclear factor erythroid 2–related factor 2
NYHA	New York Heart Association
P	phosphorylation
P23	prostaglandin E synthase 3

PA	pulmonary arteries
PAB	pulmonary artery banding
PAH	pulmonary arterial hypertension
PAP	pulmonary arterial pressure
PAS	Per-Arnt-Sim
PCAH	polycyclic aromatic hydrocarbons
PCH	pulmonary capillary hemangiomatosis
PCL-β/δ	phospholipase C
PCWP	pulmonary capillary wedge pressure
PDE51	phosphodiesterase-5 inhibitors
pErk1/2	phosphorylation of the extracellular signal-regulated kinase1/2
PFP	pentafluorophenyl
PGI2	prostacyclin
PGIS	prostacyclin synthase
PH	pulmonary hypertension
PHD	prolyl hydroxylase enzymes
PI3K	phosphoinositide-3-kinase
PKA	protein kinase A
PKC	protein kinase C
PLC-β/δ	phospholipase C β/δ
PMEC	pulmonary microvascular EC
PPAR	peroxisome proliferator-activated receptor
PPHN	persistent pulmonary hypertension of the newborn
PPHTN	portal hypertension
p-Smad1/5/8	phospho-Smad1/5/8
PUFA	polyunsaturated fatty acid
PVDF	polyvinylidene difluoride
PVOD	pulmonary veno-occlusive disease
qRT-PCR	quantitative real-time PCR
ROS	reactive oxygen species
RQ	relative quantification
RT-PCR	reverse transcription polymerase chain reaction
RV	right ventricle
RVF	RV failure
RVH	RV hypertrophy
RVSP	right ventricular systolic pressure
RV/LV+S	right ventricular hypertrophy index

RXR	retinoid X receptor
S100A4/Mts1	S100Ca^{2+}-binding protein A4
S-COMT	soluble COMT
SEM	standard error of the mean
SERM	selective oestrogen receptor modulators
SERT	serotonin transporter
SERT+ mice	overexpression of the 5-HT transporter in mice
sGC	soluble guanylyl cyclase
SHBG	sex hormone-binding globulin
Shc	Src-homology and collagen-homology
Smad	decapentaplegic homologs
Smad-1/5/8	mothers against decapentaplegic homologs
SMC	smooth muscle cell
SP1	specificity protein 1
SPE	solid-phase extraction
Su5416	Sugen 5416
SULT	sulfotransferase
TCDD	2,3,7,8-tetrachlorodibenzodioxin
TEM	electron ion spray temperature
TF	transcription factor
TGF-β	transforming growth factor-β
TNFα	tumour necrosis factor α
TNS	trypsin neutralization solution
TPH	tryptophan hydroxylase
TXA2	thromboxane A2
UGT1A	UDP glucuronosyltransferase 1 family polypeptide A cluster
UV	ultra-violet
VEGF	vascular endothelial growth factor
VEGF-A	vascular endothelial growth factor-A
vHL	von Hippel-Lindau tumour suppressor protein
Vm	membrane potential
VSMC	vascular smooth muscle cell
Wise	Women's Ischemia Syndrome Evaluation

Chapter I

1 Introduction

Oxygen is one of the most fundamental requirements of life for most organisms, as the aerobic cellular respiration provides the energy required for the maintenance of elementary life functions.

1.1 Pulmonary Circulation

The cardiovascular system (CVS) is an intricate system of organs enabling normal circulation of the blood throughout the body. An important part of the CVS is the pulmonary circulation, which receives deoxygenated blood from the heart through the right atrium and ventricle, and pulmonary arteries leading to the lung. In the lung, the pulmonary arteries (PAs) divide into numerous small arterioles and capillaries forming capillary beds, which encapsulate the alveoli. Pulmonary respiration or gaseous exchange of carbon dioxide and oxygen takes place in small PAs and successive capillaries, which are often named functional pulmonary capillaries (Levitzky, 2013). The oxygenated blood then drains form the lung via pulmonary veins to the left atrium and is circulated from the left ventricle throughout the body.

1.1.1 *The Structural and Functional Organisation of the Pulmonary Circulation*

The structure and architecture of the pulmonary circulation greatly reflect its physiological function, which is to provide a high flow, high capacitance, low pressure and low resistance system of delivering deoxygenated blood from the heart to the lung's microcirculation for efficient gaseous exchange.

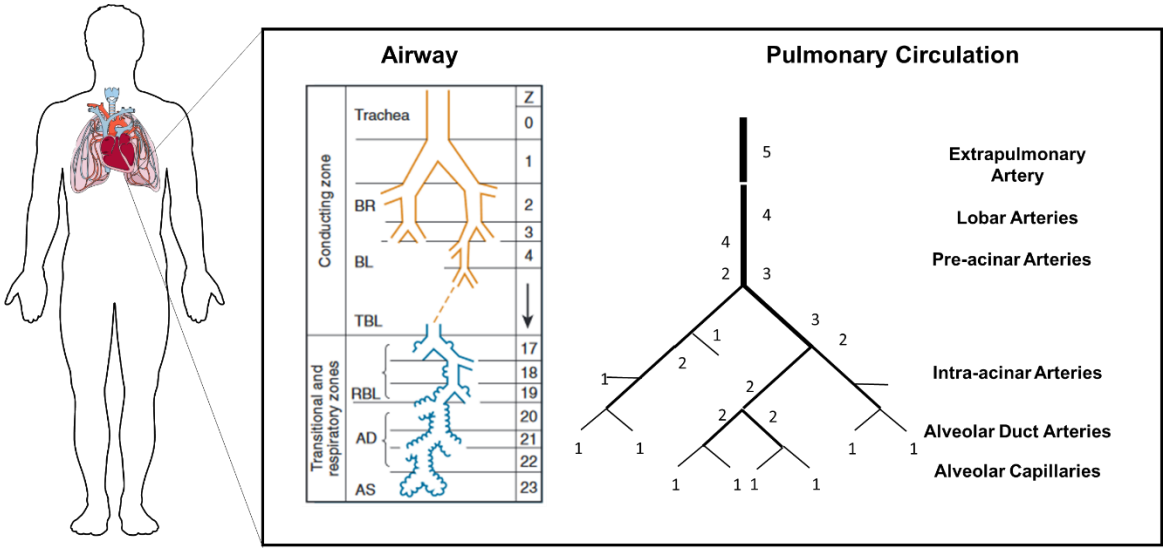


Figure 1–1: The branching of the airways and pulmonary circulation in a human body.

The pulmonary circulation is a closed system and is separated from the systemic circulation. Within the pulmonary circulation, the blood vessel organisation closely follows the organisation of airways, meaning the branching of pulmonary arteries, which deliver deoxygenated blood from the right heart to the lung, is branched in close association with the airways. The branching pattern of the main pulmonary artery extending from the heart to small arterioles reaching alveoli, where gaseous exchange occurs, equates to 15 orders of vessel branching. Adapted from (Powell et al., 2016). The numbers in the diagram indicate the order of airways and pulmonary circulation branching.

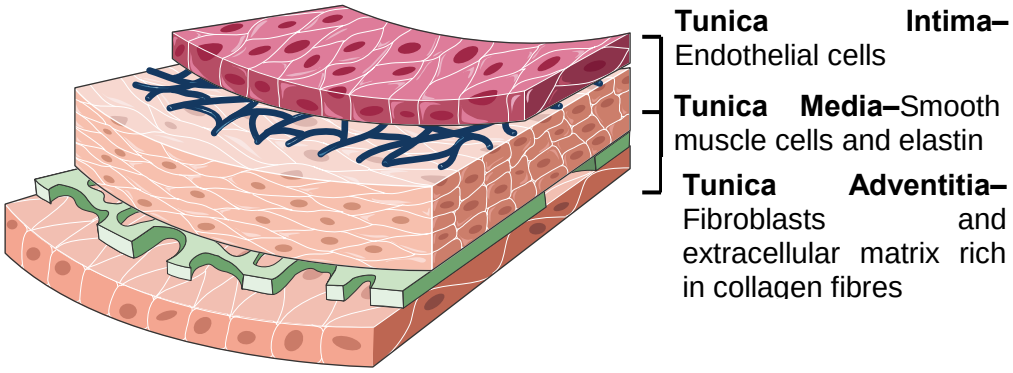


Figure 1–2: The structure of vessel wall.

The structure of the vascular wall is shared by systemic and pulmonary circulation. The innermost layer, termed tunica intima, is composed mainly from endothelial cells and sub-endothelial connective tissue. The function of the middle layer, tunica media, composed mainly from vascular smooth muscle cells and elastin, is to regulate the vascular tone and thereby associated vascular pressure. The outermost layer, tunica adventitia, is composed of fibroblasts and extracellular matrix, which is rich in collagen fibres, and conveys structural integrity of vessels exposed to high pressures.

All the vessels in the vascular system share the same composition of the vessel wall, which generally consists of three histologically distinct layers: i) intima, ii) media and iii) adventitia, which are separated with basement membrane, as shown in Figure 1–2. In arteries, the outermost layer, the tunica adventitia, is composed of myofibroblasts, which deposit extracellular matrix rich in collagen fibres, giving arteries their elastic character and maintain their structural integrity in high-pressure physiological environment. The tunica media, is composed of vascular smooth muscle cells (VSMCs), exhibiting contractile phenotype, and elastin. While this layer regulates the vascular tone, the innermost layer, tunica intima, consisting of a single layer of endothelial cells (ECs), is imperative for the maintenance of vascular homeostasis.

To accommodate the changing function of these vessel, the structure and size of the pulmonary arteries changes following the vessel branching within the lung (Elliott and Reid, 1965). The main PA which extends from the right ventricle and branches into PAs supplying deoxygenated blood to the left and right lung, is characterised by its utmost elastic properties, due to high content of elastic and collagen fibres forming the numerous elastic laminae contained within the medial layer. The elastic character of this large artery, due to the presence of 7 elastic laminae, allows it to act as a buffer, reducing the pulsating effects in the PAs further away from the heart. PA branching pattern is, as previously described, in close association with the branching pattern of the airways, with the character of the transitional arteries becoming less elastic further away from the heart, meaning the vessel wall contains fewer elastic laminae (Jones and Capen, 2011). The PAs in the lung exhibit the elastic and transitional character up to 9^{th} airway branching generation (Elliott and Reid, 1965;Jones and Capen, 2011). The character of PAs alters further in the very distal part of the lung, first becoming muscular, then transitioning to partially-muscular or non-muscular arteries, as shown in Figure 1–1. These pulmonary vessels are characterised by a medial layer consisting of smooth muscle cells (SMCs). The medial layer becomes progressively thinner and/or contains very few SMCs (De Mello and Reid, 1997;Jones and Capen, 2011). Similarly, with the branching of the PAs, the diameter of the vessel changes, becoming progressively smaller, with the diameter of large elastic PAs being greater than 3,200μm. In transitional PAs, the diameter reduces to 3,200–2,000μm, and becomes smaller in muscular and non-muscular PAs, which have the diameter of more than 150μm and less than 130μm, respectively (Jones and Capen, 2011). Most of the non-muscular PAs, leading to the alveoli, have the diameter of less than 50μm, with the average diameter of pulmonary capillaries, reaching the alveoli, being only approximately 6μm (Jones and Capen, 2011).

1.1.2 *The Development of the Pulmonary Circulation*

The development of the lung and pulmonary vasculature begins early during the embryonic development. The simultaneous development of both organ systems reflects the imperative for role of vascularisation for normal lung organogenesis (Joshi and Kotecha, 2007). The organogenesis of the lung and the development of pulmonary circulation are poorly understood, with limited understanding of the early stages of development and the regulation of the lung's vascularization (deMello et al., 1997;Steinhorn, 2010). Pereda and colleagues recently published novel findings on the morphogenesis of pulmonary circulation in humans (Pereda et al., 2013). Currently, three theories explaining the morphogenesis of pulmonary vasculature exist: i) vasculogenesis, ii) combination of central angiogenesis accompanied with peripheral vasculogenesis, and ii) distal angiogenesis accompanied with vascular remodelling (Figure 1–3), with the last two theories being most widely accepted.

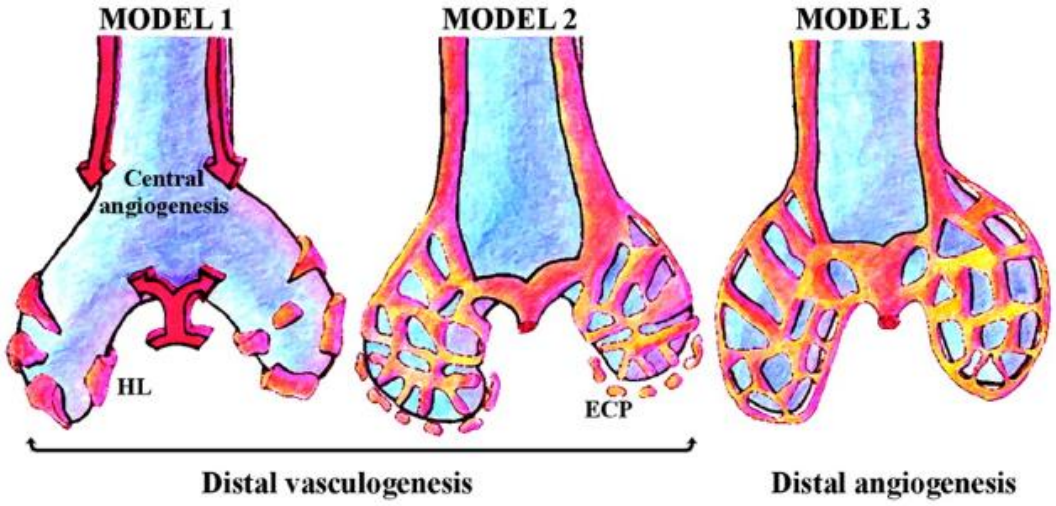


Figure 1–3: Schematic representation of theories explaining the morphogenesis of pulmonary vasculature.
Diagram adapted from (Parera et al., 2005).

Generally, lung morphogenesis can be characterised by four developmental stages: i) pseudoglandular, ii) canicular, iii) saccular and iv) alveolar. During the pseudoglandural stage, the lung appears as two primitive lung buds and primitive proximal forms of airways, whose development and budding are driven by growth signals from the mesenchyme (Rosenthal and Bush, 2002). According to the theory of pulmonary circulation forming through a combination of central angiogenesis accompanied by peripheral vasculogenesis, the development of the hierarchical pulmonary vasculature begins with the formation of the *truncus arteriosus*. The latter represents the origin from which the proximal macro-vessels,

the aorta and the main PA are formed (Gao and Raj, 2010). As PA and pulmonary veins are extending from aortic arches and vessel outgrowths from the heart, respectively (Rosenthal and Bush, 2002), the morphogenesis of pulmonary vasculature is also closely related to that of the heart (Peng et al., 2013b). Simultaneously, the PAs and veins up to the 7th branching generation are developing independently from the microvasculature, through process of central sprouting or angiogenesis (deMello et al., 1997). During canicular phase, the distal airways and pulmonary vessels are formed. Irregular vessel plexus in association with the airways are formed by vasculogenesis, during which capillary vessels are formed by the transformation of mesenchymal cells into vessel-forming endothelial cells (Pereda et al., 2013). After the irregular vascular beds are established, these vessels undergo extensive remodelling, whereby the character of these vessels is altered (Pereda et al., 2013). The branched structure of airways is formed during the process of branching morphogenesis with the last generation of the airways ending in saccules formed during the saccular phase (Gao and Raj, 2010;Pereda et al., 2013;Metzger et al., 2008). During this developmental stage, the distal vascular bed undergoes extensive modification, where the density of small vessels and capillaries is increased, and the capillaries are reorganised (Jones et al., 2014). The lung's segmentation organisation is finalised at the end of the embryonic period (Pereda et al., 2013). The theory of central angiogenesis with peripheral vasculogenesis speaks in favour of parallel, yet independent, development of the central and peripheral vascular systems within the lung. DeMello and colleagues have reported that in Swiss-Weber mouse foetuses few connections between the central and peripheral pulmonary vessels, forming a complete functional pulmonary vascular system, are established during pseudoglandural phase The extent of these connections becoming exceedingly more significant by the end of gestation period (deMello et al., 1997;deMello and Reid, 2000). The proposed primary mechanism of fusion of pulmonary macro- and micro-vessels is vasculogenesis (Hall and Haworth, 1987;Hall et al., 2000). The theory of distal angiogenesis proposes that the capillaries encapsulating the terminus of the airways in the distal lung are formed by the extension from pre-existing vessels, synchronous with the growth of lung buds (Parera et al., 2005). The distal capillary network expands by sprouting and nonsprouting angiogenesis, where the expansion of vessel network is supported by pre-existing capillaries or the mesenchyme, respectively (Risau, 1997;Risau and Flamme, 1995). Dynamic vascular remodelling is essential in distal angiogenesis, resulting in the gradual muscularisation of proximal vessels, the growth and fusion or degeneration of certain pulmonary vessels (Parera et al., 2005). During the embryonic development, the pulmonary vasculature is prepared to assume its role of blood oxygenation, which occurs immediately upon birth. During alveolarisation stage,

which begins in the late gestation period and lasts until adulthood in humans, mature alveoli are formed from saccules. Additionally, the number of alveoli is increased during this phase (Jones et al., 2014), the capillaries encapsulating the terminal airways buds begin to mature and the surface area for gas exchange of micro-vessels is significantly increased (Gao and Raj, 2010;Burri, 2006;Hislop, 2005). The maturation of pulmonary vasculature continues birth for several years after (Perl and Whitsett, 1999;Papamatheakis et al., 2013).

The growth and development of the pulmonary vessel bed is vigorously regulated at every developmental stage to achieve the appropriate branching pattern of the vessels with the correct size and vessel wall characteristics (Jones and Capen, 2011). The most important vascular growth factor in the formation of the vascular plexus within the lung is the vascular endothelial growth factor-A (VEGF-A), whose spatial and temporal isoform expression, stimulates and directs angiogenesis and vasculogeneis, resulting in vascular development and increased vascular density (Tirziu and Simons, 2009;Voelkel et al., 2006;Healy et al., 2000). Other signalling molecules and pathways, such as the bone morphogenetic protein (BMP) belonging to the transforming growth factor β superfamily, have also been implicated in the regulation of pulmonary vascular development (Chen et al., 2004).

1.1.3 *The Physiological Changes in the Pulmonary Circulation at Transition at Birth*

Prior to birth, the foetal lung is hyperexpanded and filled with liquid, which is produced within the lung. Therefore, the placenta is serving as the main organ for gaseous exchange during gestation. Most of the oxygenated blood from the placenta circulates from the right to the left atrium via the *foramen ovale* and bypasses the lung circulation completely via the *ductus arteriosus*. Hence, the proportion of the combined cardiac output to the lung is low *in utero* (Rasanen et al., 1996;Gao and Raj, 2017). During this period, the pulmonary vasculature is relatively insensitive to vasoactive stimuli (Lewis et al., 1976;Rasanen et al., 1998). Additionally, the pulmonary vascular resistance is high due to low number of pulmonary vessels and the thickness of the vessel wall in the developing lung (Lakshminrusimha, 2012). As gestation advances, the growth of the lung and pulmonary vasculature results in increased proportion of the blood flow to the lung and decreased pulmonary vascular resistance (Rasanen et al., 1996). At the end of gestation, the pulmonary vessels become more sensitive to vasoactive stimuli and the presence of oxygen, resulting in actively sustained vasoconstriction in hypoxic conditions, contributing to a significant

increase in vascular resistance (Lewis et al., 1976;Heymann et al., 1977). Within moments after birth, with the first breath, the lung assumes its primary role of gaseous exchange. The increased partial pressure of oxygen (pO_2) within the lungs, due to initiation of ventilation, results in stimulation of reflex vasodilation. This leads to significant decrease in pulmonary vascular resistance and an increase in pulmonary blood flow. The physical expansion of the lung with the first breath is associated with the release of vasoactive factors, resulting in increased pulmonary blood flow (Fineman et al., 1995). The mechanisms contributing to sudden changes within the pulmonary circulation also include: i) rapid reorganisation of SMCs within the pulmonary vessel wall, resulting in remodelled vessels having larger luminal cross-section (Haworth et al., 1987), ii) recruitment of intra-acinar arteries, iii) the release of vasodilatory mediators, and iv) gradual vascular remodelling (Haworth et al., 1987). Increased blood flow to the lung helps with the closing of foetal shunts, resulting in established normal circulation of blood from the right ventricle to PAs.

1.1.4 *The Regulation of Vascular Tone and Blood Flow in the Pulmonary Circulation*

Vascular tone is defined as the level of constriction of blood vessels, regulating the vascular resistance and blood flow through these vessels. The vascular tone and blood flow of the pulmonary circulation, which both partially depend on the structure of artery wall, are closely regulated to ensure efficient blood oxygenation in a high-flow, low pressure and low resistance system. Nonetheless, a certain degree of vascular tone is always maintained in the pulmonary vasculature in basal conditions (Bergofsky et al., 1963). Several mechanisms are employed to maintain the low pulmonary vascular resistance, with numerous factors affecting the vascular tone, ranging from passive (changes in cardiac output and left atrial pressure) to active (vasoactive factors and pO_2) (Carden et al., 2005).

The resting vascular tone is partially regulated by i) local production of nitric oxide (NO), produced in the endothelium (Cooper et al., 1996) and ii) regulation of the membrane potential of PASMCs, where local activity of potassium channels, leads to membrane depolarisation and activation of voltage-gated calcium channels (Nelson and Quayle, 1995), as shown in Figure 1–4. In VSMCs, there are at least five different types of potassium channels expressed (Mandegar and Yuan, 2002), with voltage-gated potassium channels (Kv) being associated with the maintenance of vascular tone in PASMCs in resting (Mandegar and Yuan, 2002;Evans et al., 1996). Sustained vasoconstriction and proliferation

of SMCs were associated with impaired function of Kv in PAH (Yuan et al., 1998). Although less than in the systemic vasculature, the sympathetic nervous system may also significantly contribute to the maintenance of basal vascular tone in the lung, mainly through inhibition of α2-adrenoreceptors according to (Duke and Stedeford, 1960).

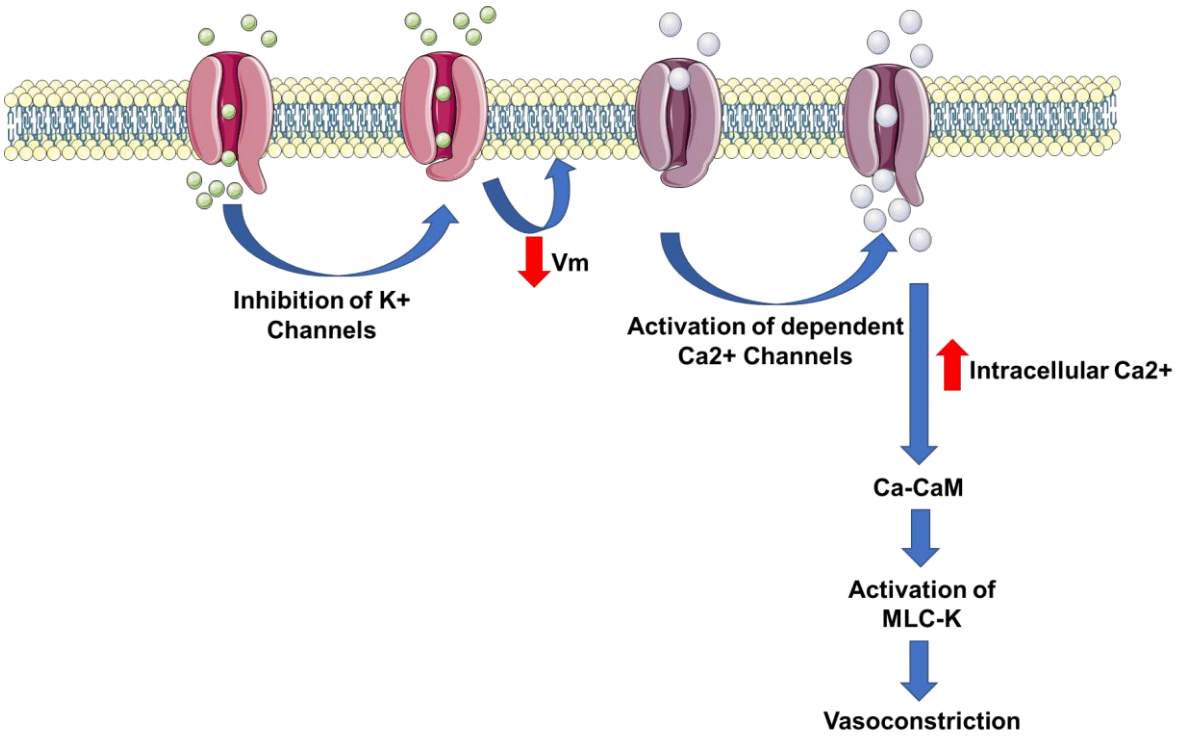


Figure 1–4: The maintenance of vascular tone in pulmonary arterial smooth muscle cells. In vascular smooth muscle cells, vasoconstriction (and vasodilation) is mediated through changes in the activity of voltage-dependent K^+channels, leading to changed activity of voltage-gated Ca^{2+} channels. The subsequent depolarisation of the membrane potential, and activation of voltage-dependent Ca^{2+} channels, result in increased intracellular levels of Ca^{2+}, thereby activating calcium-calmodulin, which in turn activates the myosin light-chain kinase. The latter phosphorylates the head of the myosin light chain, activating the contraction of smooth muscle, and resulting in vessel constriction.

Vm=Membrane potential, Ca-CAM=Calcium-calmodulin, MLC-K=Myosin light-chain kinase

After birth, the vascular tone in pulmonary circulation is mainly regulated by the ECs, which produce and respond to stimuli such as hormones, neurotransmitters and vasoactive factors (Sandoo et al., 2010). The vascular tone is regulated through the paracrine effects these vasoactive compounds exert on SMCs. The local balance of circulating prostaglandins (PGD_2, PGF_2, PGE_1, PGE_2 and prostacyclin), thromboxane A_2, serotonin (5-HT), and endothelin-1 (ET-1) modulates vascular tone (Gao and Raj, 2017), with the vasoactive compounds having varying effects on the vascular resistance, depending on the concentration, initial pulmonary vascular tone, absence of endothelium, etc. (Hughes and

Morrell, 2001). Interestingly, factors mediating vasodilation have only slight effect on pulmonary circulation when oxygen is abundant (Mazza Jr. and Taichman, 2006). 5-HT and ET-1 partially mediate their effects of increased vascular resistance through activation of protein kinase C (PKC), resulting in release of calcium ions from intracellular storage and inhibition of adenylate cyclase (Jackson, 2000). The mechanisms of vasoconstriction induced by 5-HT and ET-1 are shown in Figure 1–5.

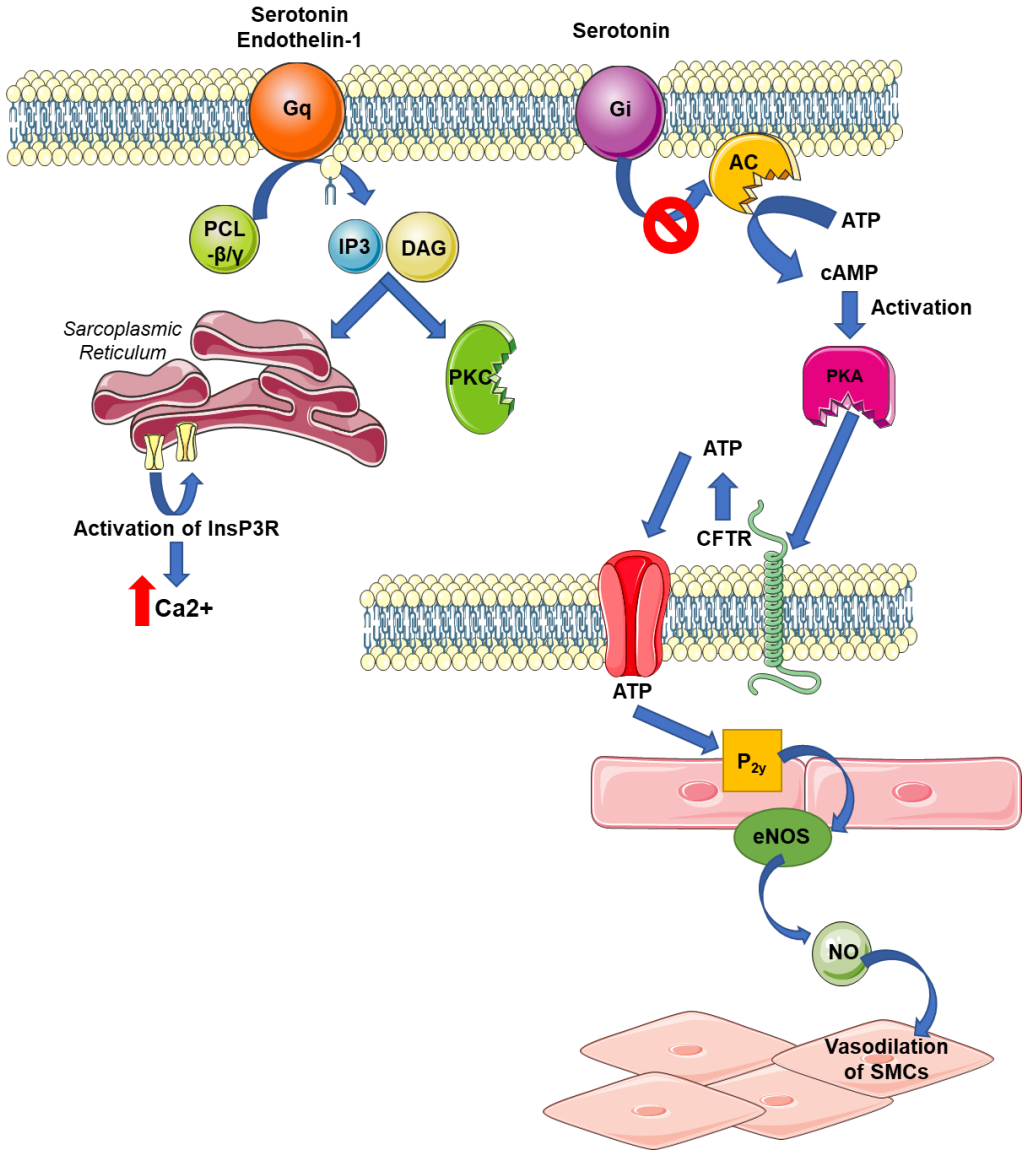


Figure 1–5: Mechanism of vasoconstriction mediated by endothelin-1 and serotonin in vascular smooth muscle cells.

The binding of endothelin-1 or serotonin to corresponding receptors, results in the activation of Gq and/or Gi subunit of G-protein coupled receptors, respectively. The activation of Gq subunit results in the activation of phospholipase C, which hydrolyses phosphatidylinositol 4,5-bisphosphate, forming cellular membrane, resulting in the formation of diacyl glycerol (Rich et al.) and inositol trisphosphate. The latter mediators act on inositol triphosphate receptors in the sarcoplasmic reticulum and protein kinase C, leading to increased intracellular levels of Ca^{2+} and vasoconstriction, respectively. The activation of Gi subunit leads to inhibition of membrane-bound adenosine cyclase, resulting in decreased levels of cyclic adenosine monophosphate, which mediates vasodilation.

Diagram adapted from (Hughes and Morrell, 2001). Vasodilation of smooth muscle cells is mediated by the production of cAMP by adenylate cyclase, which activates PKA, which is followed downstream by increased ATP release. ATP then binds to endothelial purinergic receptors on endothelial cells and activates endothelial NOS to stimulate NO-dependent vasodilation.

AC=Adenosine cyclase, ATP=Adenosine triphosphate, cAMP=Cyclic adenosine monophosphate, DAG=Diacyl glycerol, eNOS=Endothelial nitic oxide synthase, InsP3R=Inositol-activated receptors, IP3=Inositol trisphosphate, NO=Nitric oxide, PCL-β/δ=Phospholipase C, PKA= Protein kinase A, PKC=Protein kinase C, P_{2y}=Endothelial purinergic receptors 2y.

1.2 Pulmonary Hypertension

Sufficient blood oxygenation is essential for the execution of normal physiological functions, which closely depend on undisturbed energy metabolism. In pulmonary hypertension (PH), a class of severe and progressive vasculopathies, the pulmonary vascular network is altered due to narrowing, obstruction or loss of PAs, resulting in diminished capacity of gaseous exchange within the lung. The pathological changes within the PA network are a consequence of deregulated vasoconstrictive processes and progressive remodelling, forcing a naturally low-pressure, low-resistance system to become a high-pressure, high-resistance system. The persistently increased pulmonary arterial pressure (PAP) and resistance, resulting in prolonged excessive strain on the right heart, ultimately lead to right-sided heart hypertrophy and right heart failure.

1.2.1 *Clinical Definition*

As the pulmonary circulation is a low-pressure system, the PAP at rest is normally considerably lower than that measured in systemic arteries, and is in the range of 14±3mmgHg, with PAP of 20mmHg still considered as normal (Galiè et al., 2016). Clinically, the haemodynamic definition of PH is given as mean PAP (mPAP) of >25mmHg in rest, with pulmonary capillary wedge pressure (PCWP) ≤15mmHg (Hoeper, 2009;Galie et al., 2004b). Although additional criteria of mPAP >30mmHg during physical was also used in the past, it was recently shown in a comprehensive literature review by Kovacs and colleagues that mPAP during physical exertion is greatly dependent on the level of exercise and age, and should therefore not be used for clinical diagnosis (Kovacs et al., 2009).

1.2.2 *Classification of Pulmonary Hypertension*

Due to the complexity and diversity of PH aetiology, the therapeutic management and prediction of patient prognosis were historically difficult. The World Health Organisation introduced the Evian clinical classification system in 1998, which categorised vasculopathies according to exhibited common histological features, pathophysiological mechanisms, clinical presentation and available therapeutic options, in an effort to improve the management of the diseases (Simonneau et al., 2013). The classification was amended several times; with the latest updated clinical classification introduced in 2013 (Barst et al., 2006) shown in Table 1-1.

Table 1-1: Updated World Health Organisation Classification of Pulmonary Arterial Hypertension (2013).

1 Pulmonary arterial hypertension (PAH)
1.1 Idiopathic PAH (IPAH)
1.2 Hereditable PAH (HPAH)
1.2.1 BMPR-II
1.2.2 ALK-1, ENG, SMAD9, CAV1, KCNK3
1.2.3 Unknown
1.3 Drug and toxin-induced
1.4 Associated PAH (APAH)
1.4.1 Connective tissue disease
1.4.2 HIV infection
1.4.3 Portal hypertension (PPHTN)
1.4.4 Congenital heart disease
1.4.5 Schistosomiasis
1.4.6 Chronic haemolytic anaemia
1' Pulmonary veno-occlusive disease (PVOD) and/or pulmonary capillary hemangiomatosis (PCH)
1'' Persistent pulmonary hypertension of the newborn (PPHN)
2 Pulmonary hypertension associated with left heart disease
2.1 Left ventricular systolic dysfunction
2.2 Left ventricular diastolic dysfunction
2.3 Valvular disease
2.4 Congenital/acquired left heart inflow/outflow tract obstruction and congenital cardiomyopathies
3 Pulmonary hypertension associated with lung diseases and/or hypoxia
3.1 Chronic obstructive pulmonary disease (COPD)
3.2 Interstitial lung disease
3.3 Other pulmonary diseases with mixed restrictive and obstructive pattern
3.4 Sleep-disordered breathing
3.5 Alveolar hypoventilation disorders
3.6 Chronic exposure to high altitude
3.7 Developmental lung disease
4 Chronic thromboembolic pulmonary hypertension (CTEPH)
5 Pulmonary hypertension with unclear multifactorial mechanisms
5.1 Hematologic disorders: chronic haemolytic anaemia, myeloproliferative disorders, splenectomy
5.2 Systemic disorders: sarcoidosis, pulmonary histiocytosis, lymphangioleiomyomatosis
5.3 Metabolic disorders: glycogen storage disorder, Gaucher disease, thyroid disorders
5.4 Other: tumoral obstruction, fibrosing mediastinitis, chronic renal failure, segmental PH

In a clinical setting, however, the severity of symptoms in PH is often assessed using the New York Heart Association (NYHA) functional classification system. The system details the severity of clinical symptoms and the limitations of patient's physical ability (Table 1-2). Both classifications are used in clinical setting to assist medical professionals in the selection of the best available therapeutic options for the management of the disease and aid the accurate prediction of patient prognosis.

Table 1-2: New York Heart Association Classification of Functional Status of Patients with Pulmonary Hypertension.

Class I	Patients with pulmonary hypertension. No resulting limitation of physical activity. Ordinary physical activity does not result in undue dyspnoea or fatigue, chest pain or near syncope.
Class II	Patients with pulmonary hypertension with a slight limitation of physical activity. Comfortable at rest. Ordinary physical activity causes undue dyspnoea or fatigue, chest pain or near syncope.
Class III	Patients with pulmonary hypertension with marked limitation of physical activity. Comfortable at rest. Less than ordinary activity causes undue dyspnoea or fatigue, chest pain or near syncope.
Class IV	Patients with pulmonary hypertension with inability to carry out any physical activity without symptoms. Patients manifest signs of right heart failure. Dyspnoea and/or fatigue may even be present at rest. Discomfort is increased by any physical activity.

1.2.2.1 An Overview of Underlying Aetiologies of Pulmonary Arterial Hypertension

The first class of PAH according to the WHO classification, is represented by idiopathic PAH, previously also named primary PH, and describes the condition where no apparent cause for increased PAP and pulmonary arterial resistance is identified. The second class of PAH aetiology is termed heritable PAH, describing the vasculopathy with an underlying genetic cause, where the individuals with clinical presentation of the disease harbour a mutation associated with the disease phenotype or increased disease risk. The third class of PAH aetiology is described as drug or toxin-induced. Gurtner was the first to published a review on the possible association of the use of appetite depressing drug aminorex fumarate and the sudden epidemic of PAH in the late 1960s, which disappeared when this anorexic medication was withdrawn from the market (Gurtner, 1985). Almost three decades later, further association of appetite depressing drugs was observed in France, where the number of PAH diagnosis was increased in female population regularly taking dexfenfluamine

and/or fenfluramine (Brenot et al., 1993). A direct consequential relationship between increased risk of PAH and appetite-suppressant drugs, based on a case-control study, was reported in (Abenhaim et al., 1996), followed by an editorial suggesting these medications cause an increased in extracellular levels of 5-HT (Voelkel, 1997) resulting in pulmonary vasoconstriction (McGoon and Vanhoutte, 1984), and hyperplasia as well as hypertrophy of pulmonary artery SMCs (PASMCs) (Lee et al., 1994). Since, the catalogue of drugs and toxins related to PH greatly increased, with amphetamine derivatives, L-tryptophan, cocaine and serotonin reuptake inhibitors named as likely or possible risk factors (Price et al., 2012). The fourth and final class of PAH aetiology is considered as PH developed due to the presence of several primary underlying conditions, such as autoimmune diseases or diseases affecting the immune system, or cardiovascular conditions affecting the systemic circulation or the heart.

Several pulmonary diseases, leading to increased pressure within the lung circulation, clinically manifest as PH, although their aetiology is mainly associated with progressive narrowing of the small pulmonary veins, rather than arteries, the proliferation of alveolar capillaries or the failure of pulmonary circulation transition at birth, respectively.

1.2.3 *Diagnosis of Pulmonary Hypertension*

The difficulty with early diagnosis of PH is that at the onset of the disease patients do not experience significant symptoms. The first symptoms arise during progressed stages of PH and are associated with difficult breathing, such dyspnoea, during moderate everyday physical activity. With disease progression, other symptoms such as fatigue, weakness, angina, dizziness and syncope may also appear. These symptoms, however, can be mild in nature, and are non-specific (Galiè et al., 2016;ActelionPharmaceuticalsLtd). In more advanced stages, symptoms associated with insufficient oxygenation are triggered by minimal physical activity and are accompanied by symptoms associated with right-side heart failure. Due to initial non-specific symptoms, the time taken from disease onset to diagnosis was estimated to be on average of more than 2 years (Humbert et al., 2006;Badesch et al., 2010), meaning that at the time of diagnosis patients are usually exhibiting progressive stages of the disease (Gaine and Rubin, 1998;Humbert et al., 2006).

The diagnosis of PAH is demanding, usually requiring invasive procedures to confirm the clinical condition. The indication suggestive of PH in an individual is first evaluated using

non-invasive methods, as per clinical practice guidelines for the diagnosis of PAH in Europe. If the initial physical examination and electrocardiogram demonstrate elevated probability of PAH, further examinations, such as echocardiography, pulmonary function tests, arterial blood gas analysis, chest radiography and high-resolution computed tomography of the lungs are required to determine whether the symptoms are caused by a secondary left heart disease or lung disease (Schannwell et al., 2007). If no secondary diagnosis is made, then the patient must undergo a ventilation and perfusion lung scan to evaluate the circulation of air and blood within the lung. These procedures are used to determine whether the symptoms may be caused by chronic thromboembolic pulmonary hypertension. In case no changes in the lung's perfusion are detected, the diagnosis of PAH is considered (Gaine and Rubin, 1998;Humbert et al., 2006). Once the diagnosis of PAH is made, cardiac magnetic resonance imaging, blood tests and immunology tests, abdominal ultrasound and right heart catheterization are performed to confirm the diagnosis, determine the aetiology of the disease and assess the functional and haemodynamic impairment of the patient (Gaine and Rubin, 1998). Following PAH diagnosis, vasoreactivity assessment is used to determine patient's response to calcium channel blockers (CCBs), which can be used for treatment of certain forms of PAH.

Parameters such as functional class of the disease, right ventricular function, pulmonary haemodynamics, together with walking distance and peak oxygen uptake can be utilised to make a reliable prediction of patients prognosis (Schannwell et al., 2007).

1.2.4 *Epidemiology of Pulmonary Arterial Hypertension*

Longitudinal observational studies and national registries provided much of the information on the clinical and haemodynamic characteristics exhibited by the patients, as well as invaluable data on the PAH patient population demographics (Hoeper and Gibbs, 2014). Most recently gathered epidemiological data indicates there are between 1–3.2 newly diagnosed PAH cases annually, per million of population (Ling et al., 2012;Escribano-Subias et al., 2012). There is between 6.6–16 individuals per million of adult population currently affected by PAH (Ling et al., 2012;Escribano-Subias et al., 2012), with the overall population prevalence 52 cases per million population in Scotland (Peacock et al., 2007).

The demographics of PAH patients changed significantly over the years, since the clinical data from the fist compiled registry by the National Institutes of Health registry on PAH was

published (Rich et al., 1987), with current reported median age at the time of diagnosis for PAH being 50 years (Ling et al., 2012). According to Hoeper and colleagues, patients diagnosed with IPAH are even older at the time of diagnosis (Hoeper et al., 2013). Contrary, most recent registry demonstrated that patients with IPAH are younger at diagnosis compared to patients presenting with PAH of a different aetiology (Gall et al., 2017). Due to initial non-specific symptoms and late diagnosis, it was reported that approximately 70–80% of patients at the time of diagnosis had impairment associated with Class III and IV according to the NYHA classification (McGoon et al., 2008;Badesch et al., 2010;Ling et al., 2012). Impairment associated with Class III and VI according to the NYHA reported in the most recent Giessen Pulmonary Hypertension Registry was 82% (Gall et al., 2017).

Since the availability and approval of novel therapeutic strategies for PAH, the survival of appropriately treated PAH patients has greatly improved. Nonetheless, less than two thirds of patients survive 3 years once the PAH diagnosis was made (Humbert et al., 2010a;Benza et al., 2012), and the average survival time from diagnosis has been estimated to be 5–7 years (Gomberg-Maitland et al., 2011;Kane et al., 2011;Benza et al., 2012). The registries carried out in the UK and Spain, revealed that the 5-year mortality rate decreased significantly and was between 30–40% (Ling et al., 2012;Escribano-Subias et al., 2012). The 5-year survival of PAH patients with exhibiting functional classes of impairment according to NYHA Classes I/II, III and IV is approximately 80%, 60% and 40%, respectively (Gall et al., 2017). The survival of patients is closely associated with the underlying aetiology of the disease, with the poorest survival observed for PAH associated with underlying medical conditions (Gall et al., 2017). According to the latest registry, the elderly population of PAH patients exhibits significantly more impairment as per the NYHA functional classification compared to the Aspire registry (Hurdman et al., 2012), which is in accordance with (Ling et al., 2012), who proposed that patients with PAH aged 50 or less, exhibit better survival despite more severe haemodynamic impairment.

The NIH registry first reported a significantly higher proportion of women developed the clinical disease compared to men (Rich et al., 1987). Modern registries suggest the gender bias observed in PAH is even more pronounced, with women being between 2.3 and 4.3-times more likely to develop PAH than males (Escribano-Subias et al., 2012;Humbert et al., 2006;Badesch et al., 2010;Hoeper et al., 2013;Peacock et al., 2007;Walker et al., 2006). The latest registry on PH, published in 2017, reports an almost two-fold higher ratio of female patients presenting the clinical disease compared to male patients (Gall et al., 2017). The

proportion of female patients with clinical PAH in recent registries is shown in Figure 1–6A, and the percentage of females diagnosed with IPAH is presented in Figure 1–6B.

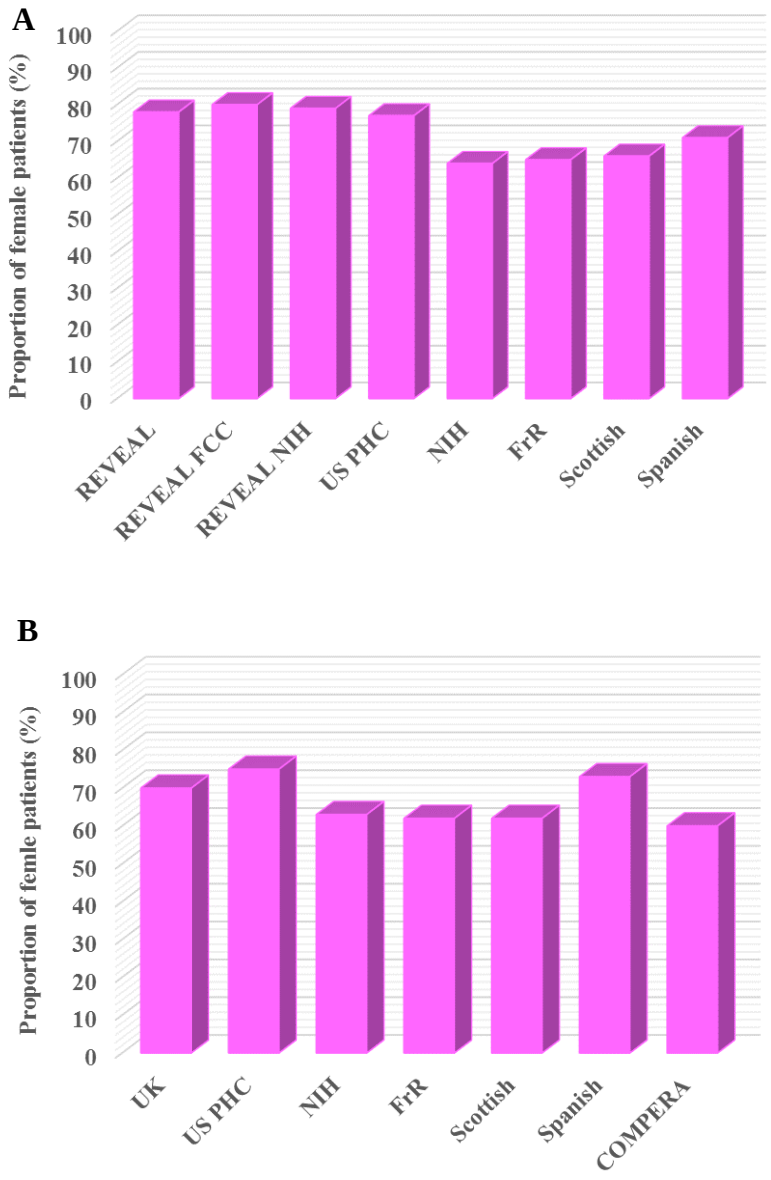


Figure 1–6: Proportion of females with clinically diagnosed pulmonary arterial hypertension in epidemiological studies and registries.
The diagram (A) depicts the proportion of female patients with pulmonary arterial hypertension, while the diagram (B) depicts the proportion of females diagnosed with idiopathic form of pulmonary arterial hypertension, as recorded by epidemiological registries carried out in Europe and United States. Adapted from (McGoon and Humbert, 2014).

Recent data suggests that certain classifications of PAH exhibit even stronger female to male predominance (Roberts et al., 2009;Kawut et al., 2008), probably due to the epidemiological features of the predisposing conditions (i.e. connective tissue disease, systemic sclerosis, certain congenital cardiac anomalies leading to congenital heart disease and systemic lupus

erythematosus), which affect more females (Manes et al., 2012). Nonetheless, it was suggested that correcting for the sex bias of the underlying condition still preserves sex bias in APAH (Kawut et al., 2008). The sex bias in PAH was recently shown to diminish with the onset of menopause in women, indicating oestrogen metabolic pathways might be vital in the onset and progression of PAH (Hoeper et al., 2013). Although initially the survival data suggested that post-menopausal female patients have a slightly better survival rate compared to male patients despite similar treatment (Shapiro et al., 2012), newer data indicates that female patients in general seem to have significantly better survival compared to males (Gall et al., 2017).

1.2.5 *Pathobiology of Pulmonary Arterial Hypertension*

The development and progression of PAH were shown to involve a complex interaction between genetic and endogenous/exogenous factors. While the presence of underlying genetic mutation might predispose individuals to PAH, other factors such as an underlying disease, exposure to drugs or toxins are usually required for the clinical disease to arise. The interaction of the pathophysiological processes contributing to the onset and progression of clinical PAH is depicted in Figure 1–7.

The vascular endothelial lining is the site of the production of vasoactive compounds (Figure 1–8), which regulate the vascular tone and resistance in systemic and pulmonary vessels through paracrine regulation of SMCs. Hypoxia (Hx) and inflammation were both shown to play a notable role in endothelial damage and/or dysfunction, which is essential in the pathobiology of PH (Budhiraja et al., 2004). Numerous studies reported that the removal of endothelium resulted in a rapid and spontaneous onset of vasoconstriction in PAs (Aaronson et al., 2002). Hence, in PAH, the initial pathological insult causing endothelial dysfunction, first results in an imbalance in the production and/or secretion of vasoactive mediators. The prolonged paracrine effects of vasoactive mediators lead to sustained vasoconstriction of small PAs and arterioles in otherwise low pressure, low resistance system (Filipe et al., 2008;Novella and Hermenegildo, 2011). Although in the systemic circulation, the physiological response to hypoxia results in vasorelaxation, in pulmonary vasculature vasoconstriction is observed to preserve ventilation/perfusion matching and the maximal possible oxygenation of the blood. The disruption of the vascular endothelium leads to increased vascular tone and vasoconstriction in small PAs. The resulting Hx conditions influence further disease development.

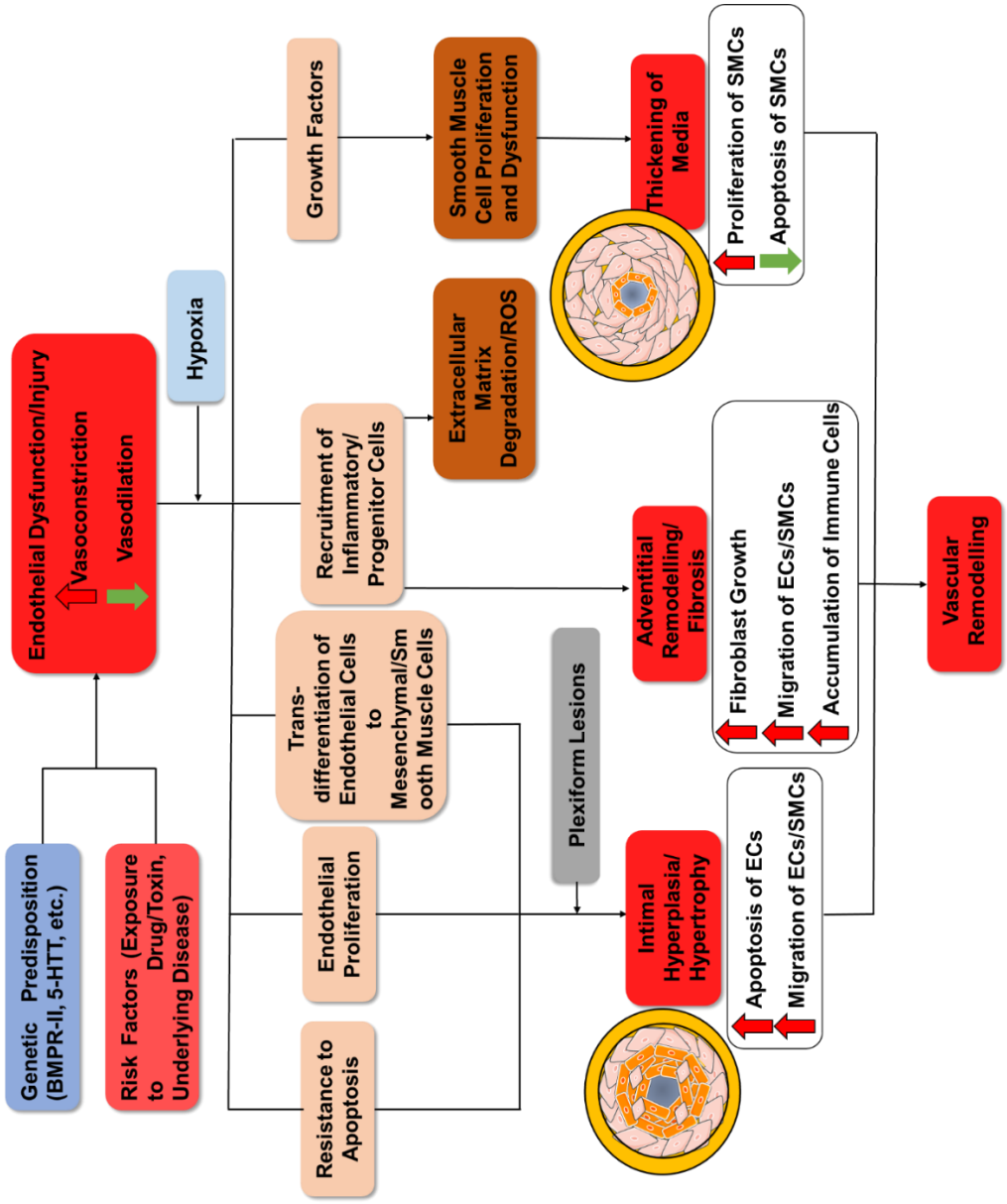


Figure 1–7: Pathophysiological processes contributing to the onset and progression of pulmonary arterial hypertension.

The onset of clinical pulmonary arterial hypertension is believed to be caused by the presence of at least two interrelating genetic and/or endogenous/exogenous factors. The pathophysiological disease process first entitles vascular injury, causing the apoptosis of endothelial cells. The disruption of the vascular endothelium leads to increased vascular tone and vasoconstriction in small pulmonary arteries. The resulting hypoxic conditions influence further disease development. Dysregulation in the production of vasoactive and growth factors leads to increased pulmonary artery smooth muscle cells proliferation and dysfunction, resulting in the thickening of the medial layer. The recruitment of inflammatory/progenitor cells causes degradation of extracellular matrix, which in turn enables migration of fibroblasts, smooth muscle, endothelial and progenitor cells to adventitial layer. Here, the abovementioned cell types contribute to development of fibrosis. Monoclonal endothelial cell proliferation is associated with the establishment of apoptosis-resistant cell population, following vascular injury, and leads to the formation of plexiform lesions. Trans-differentiation of endothelial cells to mesenchymal or smooth muscular cells, alongside transition of contractile smooth muscle cells phenotype to synthetic phenotype, leads to increased synthesis and degradation of extracellular

matrix, contributing to the development of neointima. Diagram adapted from (Mandegar et al., 2004) and (Montani et al., 2013).

BMPR-II= Bone morphogenetic protein receptor II, 5-HTT= Serotonin transporter, EC= Endothelial Cells, SMC= Smooth Muscle Cells, ROS= Reactive oxygen species.

While the circulating concentrations of endogenous vasoconstrictors thromboxane A2 (TXA_2) and ET-1 are elevated in PAH, the levels of prostacyclin (PGI_2), a potent endogenous vasodilator in systemic and pulmonary vasculature (Moncada and Vane, 1978;Badesch et al., 2004;Barst et al., 2009), are reduced in PAH patients, as shown by the reduced concentration of its stable metabolites in patients' urine samples (Christman et al., 1992). Sustained vasoconstriction of small PAs and arterioles converts the pulmonary circulation, which is normally a low pressure, low resistance system into a high pressure, high resistance system (Filipe et al., 2008; Novella and Hermenegildo, 2011). Gene expression of prostacyclin synthase (PGIS), the enzyme converting prostaglandin H_2 to PGI_2, was detected in endothelial and SMCs of proximal and distal PAs, with greater production in the latter (Badesch et al., 1989). Hypoxia was shown to have a direct effect on the PGI_2 signalling, where animals exposed to hypoxia exhibited significant pathological changes in the pulmonary vascular plexus, reminiscent to those observed in PAH (Hoshikawa et al., 2001b;Faller, 1999;Herget et al., 2000). Furthermore, the production of ET-1, 5-HT and other vasoconstrictors is also transcriptionally regulated by low oxygen tension (Kourembanas and Bernfield, 1994). Nonetheless, PGI_2 was shown to oppose the onset of hypoxia- and monocrotaline-induced PAH in animal models (Geraci et al., 1999;Tahara et al., 2004), indicating the importance of PGI_2 metabolic pathways in PAH pathogenesis. The bioavailability of another potent vasodilatory, NO, is also decreased in disease setting (Klinger et al., 2013), possibly due to decreased expression of endothelial NO synthase (eNOS) in the lungs of patients with PH (Giaid and Saleh, 1995).

The initial endothelial dysfunction additionally affects PA remodelling through the imbalance of vasoactive compounds, favouring extensive vascular remodelling through increased production of vasoconstrictors, such as ET-1 and 5-HT, exhibiting mitogenic/pro-proliferative properties (Clapp et al., 2002). Vascular injury affects the phenotype of vascular PASMCs, leading to the transition SMCs with contractile character to synthetic/proliferative character (Tajsic and Morrell, 2011). SMCs having synthetic and/or proliferative phenotype, exhibit increased deposition of extracellular matrix, primarily in the adventitial layer (Stenmark et al., 2006b;Durmowicz et al., 1994). Dysregulation of metabolic pathways regulating PASMCs proliferation, migration and apoptosis, e.g. RhoA/ROCK (Barst et al.,

2011;Chan and Loscalzo, 2008;Sheikh et al., 2014), results in extensive muscularisation and thickening of the non-muscular distal vessels (Barst et al., 2011), leading to a significant reduction of the luminal area. Sheikh and colleagues reported significant increase in muscularisation of non-muscularised distal vessels and proliferation of SMCs in response to hypoxia (Sheikh et al., 2014). The disequilibrium in the production of vasoactive compounds having pro- and anti-mitogenic properties, also significantly contributes the thickening of the medial layer.

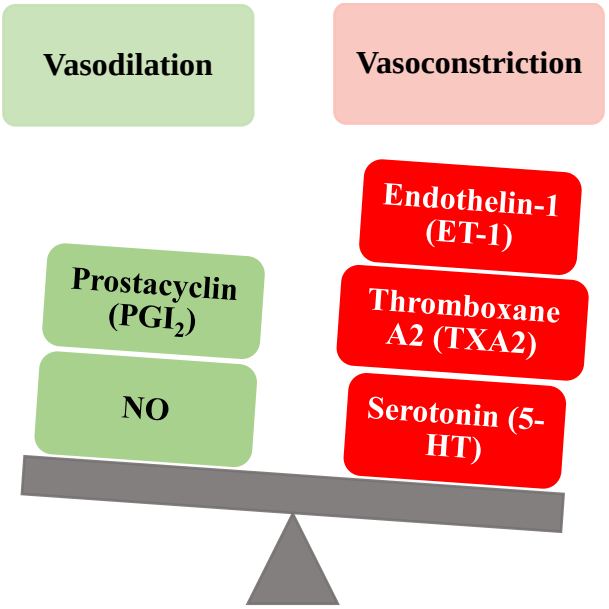


Figure 1–8: The equilibrium of vasoactive mediators in pulmonary arterial hypertension favours vasoconstriction.
Increased production of vasoconstrictors, and decreased production of vasodilators, results in sustained vasoconstriction, leading to increased mean pulmonary arterial pressure. Moreover, the mitogenic properties of these mediators have a significant impact on the remodelling of the pulmonary vasculature.

Histological abnormalities in the distal medium and small PAs observed in PAH progress from endothelial dysfunction and sustained vasoconstriction, to structural changes in the vascular wall. The ultimate morphological hallmarks of the disease include remodelling of pulmonary vasculature by extensive muscularisation of non-muscular vessels, neointima formation and obstruction of these vessels. Distinct remodelling of the adventitial layer was described to occur early in hypoxia, with significant proliferation of fibroblasts (Belknap et al., 1997). The initial recruitment of inflammatory/progenitor cells to vessel wall causes the degradation of extracellular matrix, which in turn enables migration of fibroblasts, SMCs, endothelial and progenitor cells to adventitial layer. Equally, trans-differentiation of ECs to

mesenchymal or SMCs, with transition of contractile SMCs phenotype to synthetic phenotype, leads to increased degradation and/or synthesis of extracellular matrix. The differentiation of fibroblasts into myofibroblast might also result in augmented production of extracellular matrix, and the remodelling of the adventitial layer (Stenmark et al., 2006a), which was also reported by (Durmowicz et al., 1994). The authors suggested that the expression of extracellular matrix proteins occurs in response to chronic hypoxia, therefore it appeared to be an adaptive mechanism to alterations in haemodynamic parameters and pO_2 (Durmowicz et al., 1994). Importantly, certain components of the extracellular matrix, shown to be increased in lung tissue form IPAH patients (Papakonstantinou et al., 2008), are associated with promoting the migration and proliferation of PASMCs, possibly contributing to the pathophysiological changes in the adventitial and medial layers, associated with muscularisation of otherwise non-muscularised vessels. The latter are mostly attributed to the differentiation of pericytes and/or other cell types, such as stem cells and fibroblasts, into SMCs and their subsequent proliferation and hypertrophy (Tuder et al., 2007;Rabinovitch, 2012). Importantly, migration of fibroblasts to the intimal and medial layers in hypoxic conditions and subsequent expansion was reported (Stenmark et al., 2002;Das et al., 2002). This resulted in increased production of extracellular matrix, and contributed to the reduction of vessel lumen through the formation of neointima (Tuder et al., 2007). The formation of neointima may be worsened by increased pressure within pulmonary vessels (Okada et al., 1997). A distinct absence of apoptosis described in SMCs and endothelial cells (ECs) was described in plexiform lesions in the lung samples of primary PH patients (Tuder et al., 2001b), with significant implication in complication of severe PAH with the formation of plexiform lesions. Specifically, following vascular injury, monoclonal EC proliferation was associated with the establishment of apoptosis-resistant cell population and formation of plexiform lesions. The latter are disorganised vascular formations composed of proliferating ECs, SMCs and infiltrated circulating immune and progenitor cells (Abe et al., 2010;Lee et al., 1998;Tuder et al., 1994), complicating the disease and leading to complete obliteration of distal PAs (Malenfant et al., 2013). The ECs in the plexiform lesions express markers of angiogenesis, therefore these lesions could be formed through a process of disorganised angiogenesis (Tuder et al., 2001a). The progression of vascular remodelling and the formation of plexiform lesions, albeit in experimental animal model, is shown in Figure 1–9. The distinct reduction of the vascular lumen in distal PAs and the obliteration of small vessels, result in significant reduction in the blood flow within the distal part of the lung.

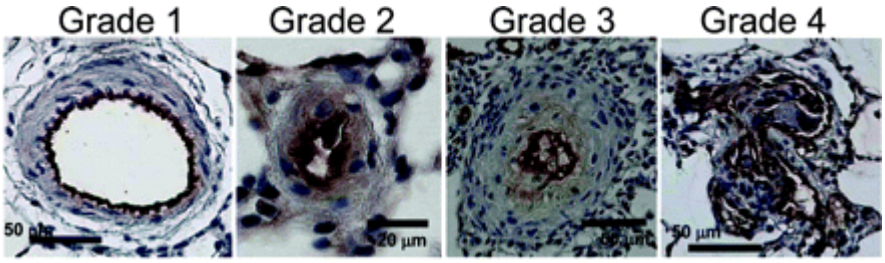


Figure 1–9: Gradual advancement of pulmonary arterial hypertension as per histopathological changes in the distal pulmonary arteries and lesions in Su5416/Hx rats. The vascular remodelling in Su5416/Hx animals closely resembles that observed in humans. Initially, only medial hypertrophy is observed, followed by proliferation and hypertrophy in the media intima. In the progressive stages of the disease, neointimal and plexiform lesions occur. Adapted from (Abe et al., 2010).

1.2.5.1 Serotonin in Pulmonary Arterial Hypertension

5-HT is derived from L-tryptophan by tryptophan hydroxylase (TPH), which converts L-tryptophan to 5-hydroxytryptophan in a rate-limiting reaction. The latter then serves as a precursor for L-aromatic acid decarboxylase, producing 5-hydroxytryptamine (5-HT) also known as serotonin. 5-HT serves as a neurotransmitter in central and peripheral tissues, with its production reported in the gastrointestinal tract and central nervous system. Moreover, in lung samples and pulmonary microvascular ECs (PMECs), the expression of *tph1* isoform is significantly increased and localised mainly in ECs (Eddahibi et al., 2006). The deletion of *tph1* gene in mice resulted in attenuation of pulmonary haemodynamics and vascular remodelling (Morecroft et al., 2007). Upon its synthesis, 5-HT is released into the bloodstream, from where it is actively taken up by platelets via the serotonin transporter (5-HTT or SERT). Activated platelets release 5-HT, which then, locally present in high concentrations, mediates vasoconstriction. Indeed, the exposure of isolated lungs from normoxic rats to lower concentrations of 5-HT induced a concentration-dependent vasodilation, while higher concentrations elicited vasoconstriction (Eddahibi et al., 1997). The especially potent vasoconstriction response to stimulation with 5-HT was reported in the resistance PAs, where vasoconstriction was mediated via 5-HT1B receptor (MacLean et al., 1996). The production of 5-HT in the peripheral arteries, specifically thoracic aorta and superior mesenteric artery, was proposed by (Ni et al., 2008). Conditioning of PASMCs with medium collected from PMECs, which were stimulated with tryptophan, induced hyperplasia of SMCs (Eddahibi et al., 2006). Interestingly, these effects were potentiated in cells derived from IPAH patients (Eddahibi et al., 2006). 5-HT induced cellular proliferation was further exacerbated in hypoxia (Eddahibi et al., 1999). The observed mitogenic effects

of 5-HT are associated with increased expression of SERT in hypoxia (Eddahibi et al., 1999). In HPAH, a set of polymorphism within the promoter of *SERT* gene was described, which are associated with earlier onset of the disease (Willers et al., 2006). Overexpression of the *SERT* gene in experimental model of PH was associated with the onset of PH phenotype in these animals and will be discussed in detail below (MacLean et al., 2004;White et al., 2011a).

1.2.5.2 Reactive Oxygen Species in Pulmonary Arterial Hypertension

The implication of reactive oxygen species (ROS) in the pathophysiology of PAH was proposed due to the ability of these reactive compounds to interfere with cellular processes via irreversible oxidative post-translational modifications and damage of proteins, enzymes and DNA. Indeed, there is increased generation of ROS in hypoxic models of PH (Hoshikawa et al., 2001a), and patients with clinical PAH (Reis et al., 2013), indicating that the imbalance in the production/removal of these compounds might result in pathophysiological changes in the pulmonary vasculature, including vascular remodelling. ROS are primarily produced by the nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase, Nox) family of enzymes, which are involved in the respiratory electron transport chain (Fulton et al., 2017). Seven isoforms of NADPH oxidases were described, including Nox1–4 and Duox1–2 (Bedard and Krause, 2007). The activation of Nox usually entails coordinated assembly of cytoplasmic proteins, which following post-translational modifications, form a functional unit (Bedard and Krause, 2007). Increased levels of *Nox4* mRNA were observed in murine hypoxic model of PH, monocrotaline-induced model of PH in rats, and IPAH patients (Mittal et al., 2007;Dorfmuller et al., 2011). Equally, increased levels of *Nox1* mRNA were reported in PASMCs derived from monocrotaline-treated rats (Veit et al., 2013). Most recently it was reported that the basal expression of Nox1/Nox4 is increased in PASMCs from PAH patients (Hood et al., 2016). Interestingly, stimulation of these cells with oestradiol and 16α-hydroxyoestrone (16αOHE1) resulted in increased ROS-dependent proliferation, sustained production of ROS and increased expression of Nox1, respectively (Hood et al., 2016). Generally, the introduction of small interfering RNA (siRNA) to silence the gene expression of *Nox1/Nox4* results in decreased proliferation of PASMCs, increased apoptosis, and attenuated PH phenotype (Mittal et al., 2007;Dorfmuller et al., 2011;Veit et al., 2013).

1.2.5.3Prostacyclin Synthase in Pulmonary Arterial Hypertension

PGI_2 is a lipid mediator with potent vasodilatory effects, primarily produced in the ECs. It is derived from arachidonic acid (AA) in a three-phase reaction, where cyclooxygenase enzymes (COX), produce the prostaglandin PGG_2, which is rapidly converted into PGH_2, and the latter then serves as a precursor for the PGIS, a specific prostanoid synthase. In vascular tissue, PGI_2 is the main metabolite formed from AA (Bunting et al., 1976). The enzyme is expressed in all vascular cells of proximal and distal PAs, with greater expression in the ECs (Badesch et al., 1989). While numerous human lung cancer cell line exhibit decreased expression of PGIS, the importance of this enzyme in lung cancer was shown by a transgenic murine model over-expressing this enzyme, which exhibited protection against lung tumour formation (Stearman et al., 2007). Equally, the expression of PGIS in pulmonary circulation of PAH patients is decreased compared to normal subjects (Tuder et al., 1999). A comprehensive set of PGIS promoter variants exists, resulting in heterogenous transcription of this gene (Stearman et al., 2014). Furthermore, in patients with clinical PAH the transcriptional activity of the PGIS gene is less compared to healthy individuals, which might underline the previously described low circulating levels of PGI_2 in PAH patients. Hx increased the expression of PGIS and release of PGI_2 in VSMC and ECs (Camacho et al., 2011). Alterations of PGIS expression and activity might be an adaptive response to hypoxic stress (Camacho et al., 2011).

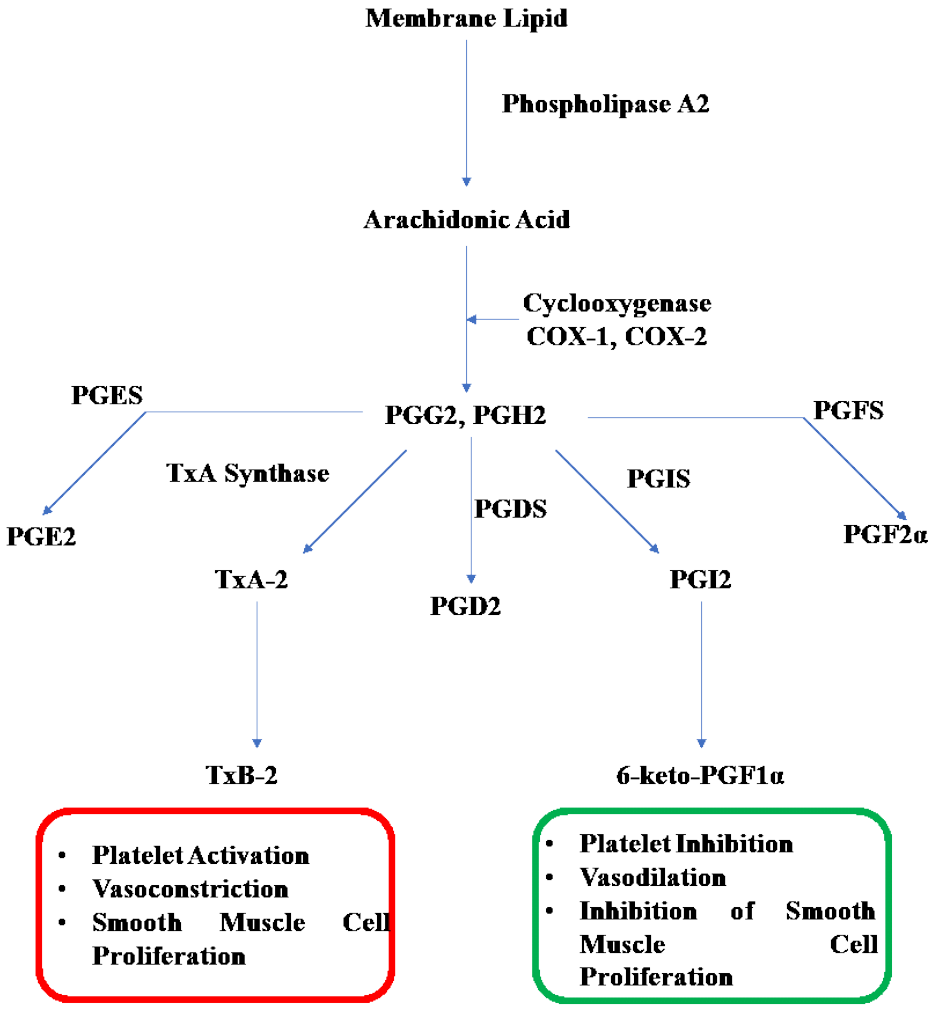


Figure 1–10: Prostaglandin biosynthesis.
Phospholipase A2 catalyses the hydrolysis of membrane lipids to liberate arachidonic acid, which is further metabolised by COX-1 and/or COX-2 to the unstable PGH2. Individual prostaglandin synthase enzyme then uses the precursor prostaglandin PGH2 to form distinct prostaglandins. TXAS, PGDS, PGES, PGIS and PGFS respectively generate, TxA2, PHD2, PGE2, PGI2 and PGF2α.

The beneficial effects of prostacyclin and its therapeutic analogues are attributed to the activation of the prostacyclin receptors (IP, DP, EP2 and EP4). These are located in the plasma membrane, leading to IP receptor coupling with Gs type of GPCR and resulting in production of cAMP, leading to vasodilation/vasorelaxation (Narumiya et al., 1999;Wise, 2003). Inhibitory prostacyclin receptors (EP3) and contractile receptors (EP1, FP and TP) couple Gi or Gq type of GPCR receptors, respectively. This results in i) inhibition of AC activity, and therewith associated restricted vasorelaxation, or ii) activation of PCL-β/γ, leading to increased production of DAG and IP3, which ultimately result in mobilisation of intracellular Ca^{2+} and PKC activation, associated with vasoconstriction.

The protective effects of PGI_2 in the vasculature far surpasses those mediated through vasodilation alone, suggesting anti-proliferative effect. In the heart, PGI_2 modulated the expression of collagen, an important ECM component (Yu et al., 1997), therefore it might also be associated with reduced fibrosis and remodelling. Genetic deletion of prostacyclin receptor resulted in aggravated intimal hyperplasia, associated with the absence of inhibitory effects on SMC proliferation and migration (Fetalvero et al., 2007). Vascular remodelling is associated with the de-differentiation of SMCs, whereby the contractile phenotype of these cells is substituted with synthetic, resulting in substantial increase in ECM component deposition and increase in proliferation and migration. The authors also observed that PGI_2 also opposes de-differentiation of SMCs by upregulating the expression of contractile proteins, thereby promoting enhanced contractile phenotype in human myometrial tissue (Fetalvero et al., 2008). The biosynthesis and PGI_2 signalling pathway in SMCs are depicted in Figure 1–11.

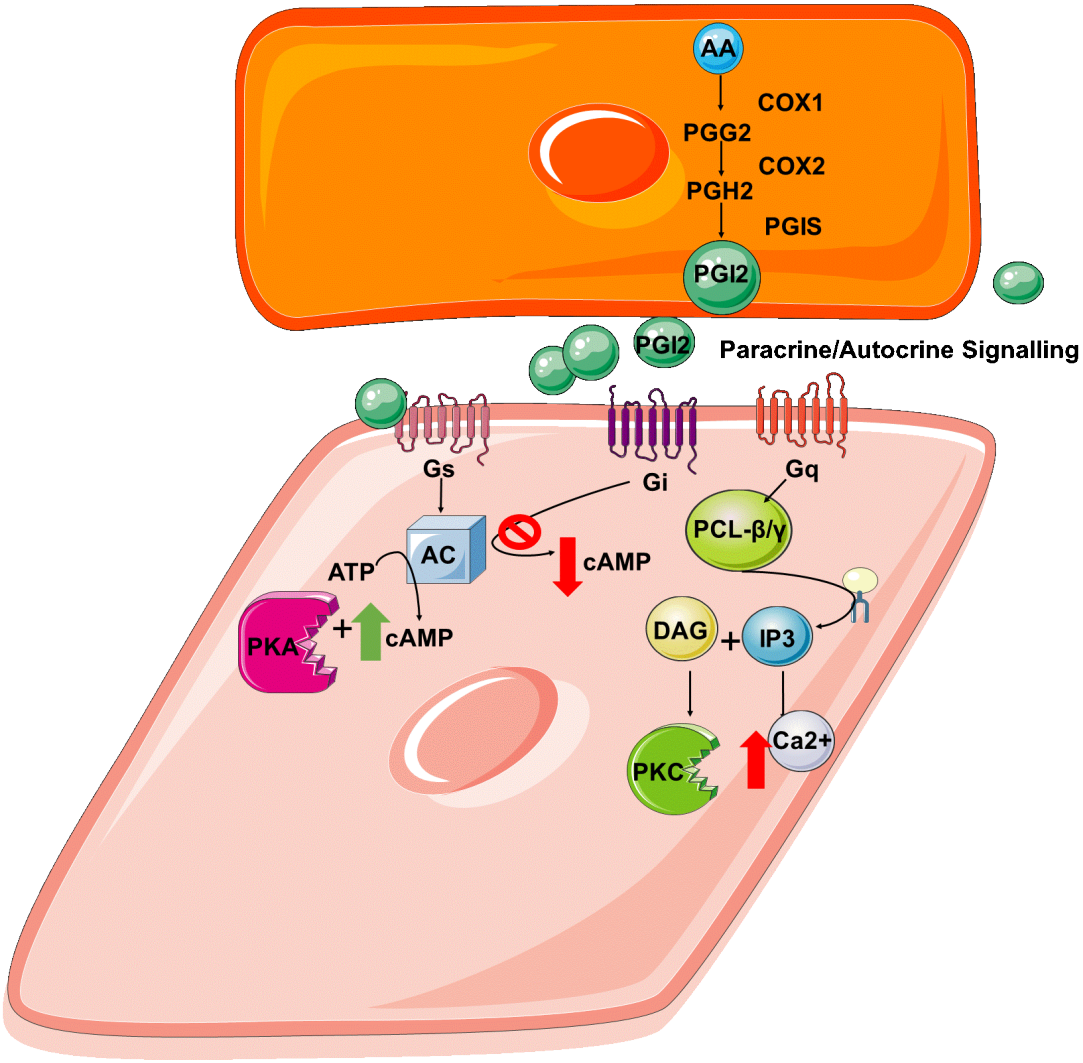


Figure 1–11: Prostacyclin biosynthesis and signalling pathway.
Prostacyclin is produced in endothelial cells from arachidonic acid, through a three-step reaction catalysed by cyclooxygenase and prostacyclin synthase enzymes, respectively. Prostacyclin then, in paracrine or autocrine manner, exerts its actions on smooth muscle cells, where it binds to prostacyclin receptors, resulting in activation of signalling pathways associated with the activation of G-protein coupled receptors (GPCR), leading to vasodilation or vasoconstriction, depending on the type of GPCR prostacyclin receptor is coupled with. Replicated from (Ivey et al., 2008).

EC= Endothelial cells, SMC= Smooth muscle cells, PGI2=Prostacyclin, IP=Relaxant prostacyclin IP receptor, AC=Adenylate cyclase, cAMP=Cyclin adenosine monophosphate; PKA=Protein kinase A, PLC-β/δ= Phospholipase C β/δ, DAG= Diacylglycerol, IP3= Inositol trisphosphate, PKC=Protein kinase C.

1.2.5.4 Interaction of Prostacyclin and the Peroxisome Proliferator-Activated Receptor Signalling

Many different ligands were shown to activate peroxisome proliferator-activated receptors (PPARs), which are ligand-inducible transcription factors, forming a subfamily of nuclear receptors comprising of PPARα, PPARγ and PPARβ/δ isoforms. The compounds that

activate PPARs include peroxisome proliferators, hypolipidaemic and anti-inflammatory drugs, and endogenous ligands such as fatty acids, eicosanoids and their derivatives (Berger and Moller, 2002). Several metabolites of AA were shown to activate PPARs (Forman et al., 1995;Yu et al., 1995). A screen for PPAR activators revealed that different PPARs isoforms are activated by prostaglandins to varying degrees (Yu et al., 1995), indicating the receptors possess differential affinities for these molecules. PGA_1, PGD_1 and PGD_2, and PGJ_2 increase the activation of PPARs for several ten-folds. Although, PGI_2 and its more stable metabolite $PGF_1\alpha$, were shown to only have a more marginal effect on PPAR activation (Yu et al., 1995), probably due to the very short half-life, PGI_2 was described as an endogenous ligand for PPARβ/δ and γ isoforms (Forman et al., 1995;Nemenoff et al., 2008). There is evidence that PGI_2 can activate PPARs directly (Forman et al., 1997) or indirectly, though the IP receptor (Falcetti et al., 2007), indicating it is PPAR endogenous ligand (Forman et al., 1997;Hertz et al., 1996). In addition to endogenous PGI_2, stable derivatives, such as iloprost and treprostinil, were also shown to activate PPAR isoforms (Forman et al., 1997;Ali et al., 2006). Therefore, it is plausible that some protective effects of these prostacyclin analogues are partly mediated through the PPAR signalling pathways. Ligand-activated PPARs form heterodimers with retinoid X receptors (RXR), and regulate gene expression by binding to the peroxisome proliferator response elements in promoter regions of target genes (Berger and Moller, 2002), mostly involved in glucose and lipid metabolism (Wang, 2010), inflammation (Becker et al., 2006), and cell survival (*KEGG PPAR signalling pathway*). Several functions were attributed to the isoforms activated by PGI_2, from modulating lipid and glucose metabolism, to inflammation, cell growth, and differentiation (Genini et al., 2012), as well as cardioprotective effects (Langenfeld et al., 2005). Specifically, in the CVS, prostacyclin receptor agonist beraprost, enhanced the expression of PPARγ and inducible NO synthase (iNOS), leading to diminished proliferation of aortic SMCs (Lin et al., 2008). Additionally, through the activation of PPARα/δ PGI_2 might also oppose the shift from contractile to synthetic phenotype, induced by increased pressure and/or shear stress, in vascular SMCs (Tsai et al., 2009).

Exposure of rats to certain peroxisome proliferator chemicals (e.g. phthalates) caused a marked reduction in oestrogen levels, leading to physiological irregularities in the animal reproductive systems (Davis et al., 1994). Therefore, it was postulated that PPARs could act to modulate oestrogen signalling pathways and metabolism. The RXRs were shown to be able to bind to ERE in the promoter region of oestrogen regulated genes and modulate their expression (Segars et al., 1993;Keller et al., 1995), indicating the existence of a cross-talk

between PPAR and oestrogen signalling pathways. By binding to ERE, PPARs act as competitive inhibitors (Keller et al., 1995), e.g. PPARγ mediates reduced transcription of aromatase gene and increased protein turnover (Yanase et al., 2001;Fan et al., 2005). Moreover, PPAR signalling led to increased degradation of ERα (Qin et al., 2003), resulting in altered oestrogen signalling. PPARα, which is constitutively expressed in the mouse lung (Becker et al., 2008b), increased the expression of 17βHSD type 1, leading to increased oestrone (E1) production (Corton et al., 1997;Davis et al., 1994). The treatment with peroxisome proliferator completely abolished *CYP2C11* gene expression, an enzyme which metabolises oestradiol to 2OHE1/2 and 16αOHE1/2 in males (Corton et al., 1997), indicating PPAR activity could modulate the expression of oestrogen metabolising enzymes. Interestingly, the expression of *CYP1B1* was shown to be induced through the activation of PPARα activation (Lovekamp-Swan et al., 2003;Murray et al., 2001). Equally, deletion of the *CYP1B1* gene in mice resulted in suppressed expression of PPARα, and the genes regulated by this receptor (Larsen et al., 2015).

1.2.6 *Right Ventricular Hypertrophy in Pulmonary Arterial Hypertension*

In the foetus, the thickness of the left and right ventricles is the same. Following the immense change in the pressure of the pulmonary vasculature upon the first breath and the maturation of pulmonary vessels during the first years of life, the anatomy of both ventricles' changes. Namely, the wall of the right ventricle (RV) remains thin (approximately 4mm) and the thickness of the left ventricle (LV) increases to be able to withstand greater afterload, closely related to the higher systemic pressure in the aorta compared to mPAP. In a healthy individual, the pulmonary circulation can, in times of increased oxygen requirements, accept several-fold higher stroke volume from RV with no change in pulmonary pressure (Matthews and McLaughlin, 2008). However, persistently increased pressure overload due to chronically increased mPAP results in increased wall thickness of RV (Bristow et al., 1998). The authors described additional changes in the RV geometrical character and chamber volume (Bristow et al., 1998). The compensatory RV hypertrophy with preserved cardiac output, in absence of proper therapeutic options due to late diagnosis and the focus of current treatments of PAH, progresses to maladaptive hypertrophy with contractile dysfunction, resulting in RV failure affecting the adequate perfusion of the pulmonary circulation (Ryan and Archer, 2014). Although most of the available therapeutic options for PAH are focused on vasoconstriction, the results of a dynamic cohort conducted by Sandoval

and colleagues, showed that the survival of PAH patients is significantly dependent on the RV function (Sandoval et al., 1994). Furthermore, the reduction in PVR does not necessarily result in improved RV function or has prognostic significance in treated patients (van de Veerdonk et al., 2011). The authors showed that the patients' survival is only significantly affected by the RV ejection fraction (van de Veerdonk et al., 2011).

Despite lower prevalence and incidence of PAH in men, they generally have worse survival compared to females (Humbert et al., 2010a;Ling et al., 2012;Escribano-Subias et al., 2012). The results of Multi-Ethnic Study of Atherosclerosis (MESA) indicated that the RV mass, volume and ejection fraction greatly depend on sex, age and race (Kawut et al., 2011). A study of cardiac volumetric measurements in a population of IPAH patients revealed that men have generally lower values of RV ejection fraction and stroke volume (Swift et al., 2015), possibly explaining the poor survival of men diagnosed with PAH.

1.2.7 *Current Treatment Options for Pulmonary Arterial Hypertension*

Currently, PAH is still considered as an incurable disease, even though, data from current PAH registries indicates that the 5-year survival of PAH patient post-diagnosis improved in comparison to the first published survival data by Rich and colleagues (Rich et al., 1987;Ling et al., 2012). Initially, it was reported that less than two thirds of patients survive 3 years once the PAH diagnosis was made (Humbert et al., 2010a;Benza et al., 2012). The average survival time from diagnosis was estimated to be 5–7 years (Gomberg-Maitland et al., 2011;Kane et al., 2011;Benza et al., 2012). The 5-year mortality rate reported in UK and Spanish registries was between 30–40% (Ling et al., 2012;Escribano-Subias et al., 2012). Recent data indicates that 5-year survival of PAH patients with exhibiting functional classes of impairment according to NYHA Classes I/II, III and IV is approximately 80%, 60% and 40%, respectively (Gall et al., 2017). Importantly, only estimated 19% of patients included in the NIH registry were treated for the disease long term (Medarov and Judson, 2015), while, according to the National Audit of Pulmonary Hypertension 2017, 99% of patients with PH diagnosis are on active therapy and referral (Digital, 2017).

The first therapeutic approaches were based on the use of vasodilators CCBs, providing alleviation of symptoms experienced by the patients, and non-specific therapies, such as anticoagulation therapy, diuretics and therapy with supplemental oxygen. Although there is

no direct evidence that the non-specific therapies have a beneficial effect in PAH, the use of diuretics is recommended to reduce the load on the right heart, reducing heart failure symptoms in these patients. Camerini and colleagues reported the beneficial effect of nifedipine, a CCB, on haemodynamic parameters within the lung of PAH patients. Moreover, prolonged administration of nifedipine resulted in improvement of symptoms and reduced signs of right heart failure (Camerini et al., 1980). Rich and colleagues reported the beneficial effects of therapy with high-dose CCBs in IPAH patients on heamodynamic parameters such as mPAP and PVR, right ventricular hypertrophy and the overall survival (Rich et al., 1992). The therapy using CCBs is particularly effective in approximately 12% of the patient population, who exhibit significant response to vasodilators, as per the vasoreactivity assessment carried out during the diagnostic procedures (Sitbon et al., 2005). Unfortunately, prolonged treatment with CCBs is effective in an extremely small proportion of PAH patients (Sitbon et al., 2005). Therefore, prolonged treatment with CCBs is not recommended by the WHO, especially for patients with more severe impairments according to the NYHA classification.

Currently, the recommendation for the treatment of PAH is to use one of the regulatory-approved treatments, including prostacyclin analogues, endothelin-1 receptor antagonists (ETRA) and phosphodiesterase-5 inhibitors (PDE5I). The approved therapeutic options are intended to improve the patient's symptoms and decrease the mortality rate. The choice of the most suitable therapeutic option is based on the disease severity, including haemodynamic parameters, functional limitations, and presence of right heart failure, route of administration, side effects and possible drug interactions with other concurrent therapies. Initially, the patients receive a monotherapy, and their response to the therapy is regularly monitored. Should the monotherapy not be sufficiently effective, then a combination therapy, using medications belonging to different treatment classes, is utilised (Galie et al., 2009;Badesch et al., 2007).

The first approved class of therapeutics for the treatment of PAH was prostacyclin and its analogues, which are derivatives of the endogenous molecule with prolonged half-life. The beneficial effects of prostacyclin analogues in SMCs were attributed to its effects through: i) the binding to the G-protein coupled receptors (GPCRs), leading to activation of PKA and increased production of cAMP, resulting in SMCs relaxation and vasodilation (Narumiya et al., 1999;Wise, 2003), and ii) the inhibition of SMC proliferation (Clapp et al., 2002). The anti-proliferative properties of prostacyclin analogues are not entirely understood, with

Clapp and colleagues having reported the anti-proliferative effects might be mediated in a cAMP-dependent manner (Clapp et al., 2002), while others have demonstrated prostacyclin analogues might activate the DNA-binding protein inhibitors Id1/Id3 through the bone morphogenetic protein receptor II (BMPRII) signalling pathway (Yang et al., 2010). Three prostacyclin analogues (epoprostenol, treprostinil and iloprost) are currently approved for the treatment of PAH and are used to manage moderate to severe PAH. Previously associated with intravenous administration, new generation prostacyclin exhibit improved pharmacokinetic properties and stability at room temperature, enabling improved routes of administration.

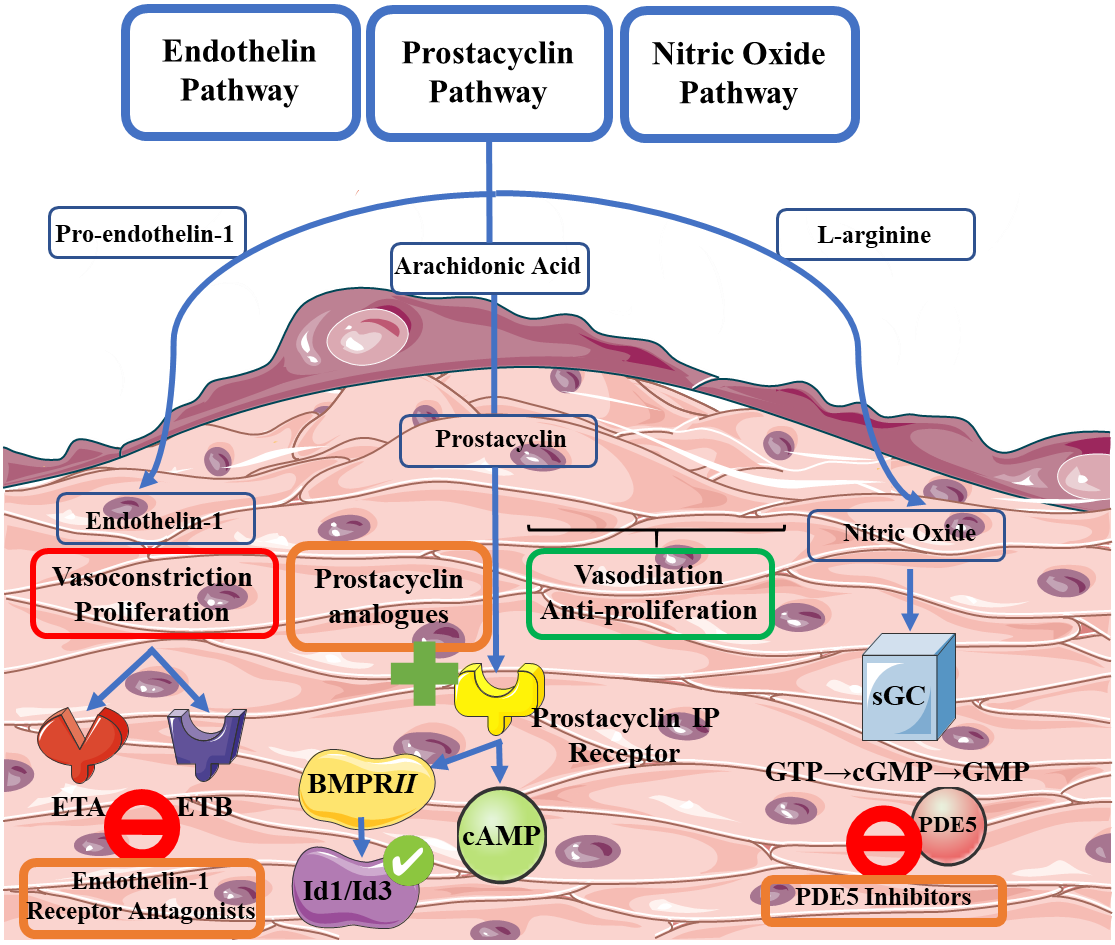


Figure 1–12: Diagram depicting signalling pathways targeted by current and emerging therapies in pulmonary arterial hypertension.
Currently approved therapeutic strategies in pulmonary arterial hypertension target three major signalling pathways involved in the regulation of vascular tone in pulmonary arteries: i) Endothelin, ii) Prostacyclin and iii) Nitric Oxide. Endothelial dysfunction and/or injury results in decreased production of endogenous vasodilatory mediators, i.e. prostacyclin and nitric oxide, while the expression of endothelin-1, promoting vasoconstriction and smooth muscle cell proliferation, is upregulated. Therefore, current therapeutic approaches focus on i) inhibiting the endothelin-1

signalling pathway through endothelin-1 receptor antagonists, or ii) administration of prostacyclin analogues to activate prostacyclin IP receptors, and iii) inhibition of phosphodiesterase 5, resulting in decreased decomposition of cyclic glucanosyl monophosphate, leading to vasodilation and arrest of proliferation. Adapted from (Humbert et al., 2014).

ETA= Endothelin A receptor, ETB= Endothelin B receptor, BMPRII= Bone-morphogenetic protein receptor II, Id1/Id3= DNA-binding protein inhibitor ID-1/3, cAMP= Cyclin adenosine monophosphate, sGC= Soluble guanylyl cyclase, GTP= Guanosine triphosphate, cGMP= Cyclic guanosine monophosphate, GMP= Guanosyl monophosphate, PDE5= Phosphodiesterase 5.

The vasoconstrictor properties of ET-1 and its importance in the maintenance of vascular tone were recognised, due to high amounts of ET-1 being produced by the lung. ET-1 is rapidly produced in direct response to the presence of specific stimuli, including hypoxia and low levels of oestrogens (Galie et al., 2004a). Increased levels of ET-1 in the plasma of PH patients were observed (Stewart et al., 1991), which might be a direct response to pulmonary vascular injury (Olave et al., 2012). The levels of ET-1 appear to be indicative of the disease severity, as assessed by PVR, mPAP and exercise capacity (Giaid et al., 1993;Rubens et al., 2001). Activation of ET-1 production was also reported in the rat monocrotaline and other models of PH (Miyauchi et al., 1993;Galie et al., 2004a). ET-1 acts through two endothelin-type receptors, ET_A and ET_B, where the former receptor is implicated in mediating vasoconstriction through G_i/G_q receptors and activation of protein kinase C, respectively (Vázquez-Prado et al., 1997;Warner et al., 1993), and the latter receptor is associated with the release of endogenous vasodilators (Hirata et al., 1993). While PASMCs express both receptor types constitutively, only ET_B is expressed in PAECs. Moreover, both types of receptors are differentially distributed in pulmonary vasculature, with greater expression of ET_A in proximal vessels, and greater expression of ET_B in distal vessels (Davie et al., 2002;Soma et al., 1999;MacLean et al., 1994). A marked up-regulation of ET receptors was reported in pre-clinical animal models (Li et al., 1994), and in PAs from PH patients (Galie et al., 2004a), with PAs also exhibiting increased ET-1 binding capacity (Davie et al., 2002), corroborating the importance of ET-1 signalling in the pathogenesis of the disease first proposed by MacLean and colleagues. Several studies to date indicated that the activity of ET_B receptor, involved in the clearance of ET-1, might be reduced (Langleben et al., 2006;Dupuis et al., 2000). A sex disparity was observed in the levels of ET-1, with male rats having higher levels of the endogenous molecule and more pronounced ET_A-mediated signalling in the kidney (Kittikulsuth et al., 2013), which could translate into increased vascular tone in this population compared to age-matched females (Stauffer et al., 2010). Hence, it is surprising that a meta study of patient data from six randomized placebo-controlled trials of ET-1 receptor antagonists (ERAs) revealed that, the differences in ET-1

expression led to greater response to ERAs in women compared to men (Gabler et al., 2012). A direct link between the levels of ET-1 and sex hormones was suggested, with oestrogens decreasing the gene expression of ET-1 in ECs (Bilsel et al., 2000). Currently available endothelin receptor antagonists can be divided into two classes: i) dual antagonists (ambrisentan), and ii) selective antagonists (bosentan, macitentan), where the dual antagonists target both ET-1 receptors, and the selective antagonists are selectively blocking ET_A receptor in SMCs. The selective antagonists have the advantage of decreasing ET-1-mediated vasoconstriction and proliferation, while the beneficial effects of ET_B remain unaltered.

The last class of approved therapeutics for PAH are phosphodiesterase 5 inhibitors (PDE5I), which target the cyclic guanosine monophosphate (cGMP)-specific PDE5 enzyme. The NO signalling pathway is involved in the regulation of vascular tone by mediating vasodilation of small vessels. Produced in ECs, the NO mediates relaxation of the underlying SMCs through the activation of soluble guanylyl cyclase (sGC) enzyme, resulting in the production of cGMP, a secondary messenger mediating vasodilation either by: i) affecting intracellular levels of Ca^{2+}, ii) activation of Ca^{2+}-sensitive K^+ channels thereby increasing K^+ influx (Archer et al., 1994), or iii) by stimulation of de-phosphorylation of myosine fibres (Nakamura et al., 2007). The PDE enzyme catalyses the degradation of cGMP, leading to termination of signal transduction. Several PDE isoforms (PDE1–6) exist, with PDE5 being abundantly expressed in the pulmonary vasculature (Corbin et al., 2005). Increased expression of PDE3 and PDE5 isoforms *in vitro* and *in vivo* were associated with PH phenotype (Murray et al., 2002), indicating that increased degradation of cGMP might be affecting vascular reactivity. Sildenafil and tadalafil are currently approved for the treatment of moderate to severe PAH. PDE5I were also shown to inhibit proliferation, although the exact mechanism is not yet fully understood (Li et al., 2007). Another class of therapeutics, associated with the NO pathway, directly stimulates sGC resulting in cGMP production, and stabilising the activation of this enzyme by NO (Stasch and Evgenov, 2013). Concurrent use of PDE5I and sGC stimulators is not recommended due to the risk of provoking systemic hypotension (Humbert and Ghofrani, 2016).

Patients who do not respond to available therapies or exhibit a significant decline in their clinical condition, and are deemed to be eligible for lung transplantation, are referred to a transplantation centre (George et al., 2011).

As available therapeutic approaches do not offer reversal of haemodynamic changes, the need for novel treatments, tackling the extensive vascular remodelling, is apparent, especially, as most of the described therapies demonstrate only marginal improvement in survival (Stenmark and Rabinovitch, 2010). Pullamsetti and colleagues produced a comprehensive review of the novel therapeutic concepts, currently still in experimental or clinical stages, including novel approaches to vasodilation using prostacyclin receptor agonists, and prospective i) anti-proliferative treatment strategies targeting tyrosine kinases, ii) transcription factor-based strategies targeting PPARγ, *Notch*, and *Wnt/β* signalling pathways, iii) inflammation-targeting strategies, iv) epigenetic modulation-based therapies utilising miRNA mimics, histone deacetylase inhibitors and DNA methyltransferase inhibitors, v) BMPR*II* modulation strategies focused on restoration of *BMPRII* expression and signalling, and vi) regenerative approaches aimed at targeting vascular pruning by induction of angiogenesis by early endothelial progenitor cells (Pullamsetti et al., 2014).

1.3 Underlying Genetic Causes of Pulmonary Arterial Hypertension

The occurrence of HPAH, previously termed primary PH, within families was first reported more than 65 years ago. Since, immense advances were made in terms of understanding the disease inheritance pattern, which is consistent with autosomal dominant mode (Loyd et al., 1984), and the genetic factors contributing to the disease onset. The transmission pattern indicated the possible X-chromosome linkage (Loyd et al., 1984). The hereditary form of the PAH only accounts for approximately 5% of entire PAH patient population (Thenappan et al., 2007). Nonetheless, the presence of mutations was linked with earlier onset of the disease, more severe haemodynamic impairment and poorer survival (Sztrymf et al., 2008;Girerd et al., 2010a). While the mutations in the *BMPRII* are the most characterised in PAH, numerous rare mutations in novel genes were connected with the onset of the hereditary form, including *ALK-1*, *KCNK3*, *ENG*, *ACVRL1*, *CAV1*, *SMAD9*, *EIF2AK4* and *BMPRIB*, causing 1–3% of HPAH cases (Austin et al., 2002).

1.3.1 *Mutations in BMPRII Gene*

The BMPR*II* is a constitutively active serine/threonine receptor kinase, belonging to the transforming growth factor-β (TGF-β) superfamily, found to be an important component of

PAH pathogenesis. The presence of mutations in the gene encoding various regions of the BMPR*II* are present in more than 70% of patients with HPAH (Lane et al., 2000;Deng et al., 2000;Trembath et al., 2001), and approximately 25% of IPAH patients (Thomson et al., 2000). Austin and colleagues reported that the risk of developing PAH in BMPR*II* mutation carriers is 14% and 42%, in males and females respectively (Austin et al., 2002). The mutations in the BMPR*II* gene seem to affect the expression levels, with BMPR*II* expression being decreased in patients with different forms of PAH (Atkinson et al., 2002), inferring that mutations occurring in the gene encoding the BMPR*II* receptor lead to diminished signalling though this pathway. Evidence indicates that the BMPR*II* signalling is significantly reduced in the PAECs derived from patients with IPAH and HPAH (Richter et al., 2004;Yang et al., 2005). The presence of a mutation in the BMPR*II* gene can generally be described as detrimental, as individuals with these mutations present clinical PAH more than 10 years earlier than patients that are not harbouring this defect (Girerd et al., 2010b), and exhibit higher mPAP and lower cardiac output (Evans et al., 2016). Moreover, studies showed that the presence of mutations in the BMPR*II* gene is closely associated with the severity of haemodynamic impairment of the pulmonary circulation, meaning these patients might require surgical intervention sooner than non-carriers (Sztrymf et al., 2008). Nonetheless, approximately 80% of individuals who are carriers of mutation in the BMPR*II* gene will never develop PAH (Hamid et al., 2009). Extremely low penetrance of the disease despite the presence of a mutation, strongly suggests more than one insult/injury is required for the disease to be presented clinically.

1.3.1.1 BMPRII Signalling Pathway

The binding of a BMP ligand or growth factor induces hetero-dimerization of type I and type II BMPR, results in the formation of an active receptor complex, which phosphorylates mothers against decapentaplegic homologs (Smad) 1/5/8. Phosphorylated Smad complex can then bind with Smad4, resulting in its translocation to the nucleus, and modification of gene expression through: i) direct interaction of Smad proteins with DNA or ii) indirect interaction through recruitment of co-activators, co-repressors and transcription factors (Massague et al., 2005). One of major downstream mediators of the BMPR*II* signalling pathway is the family of inhibitor of the DNA-binding (Id) proteins, which are involved in regulation of cellular differentiation and proliferation (Miyazono et al., 2010). The BMPR*II* signalling pathway can interact with other signalling pathways, such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 mitogen activated protein

(MAP) kinase pathways (Guo and Wang, 2009). Signalling through the BMPR*II* pathway was shown to promote survival in PAECs and inhibition of proliferation in PASMCs, while modulating the response of vascular cells to growth factors (Perez et al., 2011;Nakaoka et al., 1997;Teichert-Kuliszewska et al., 2006;Zhang et al., 2003).

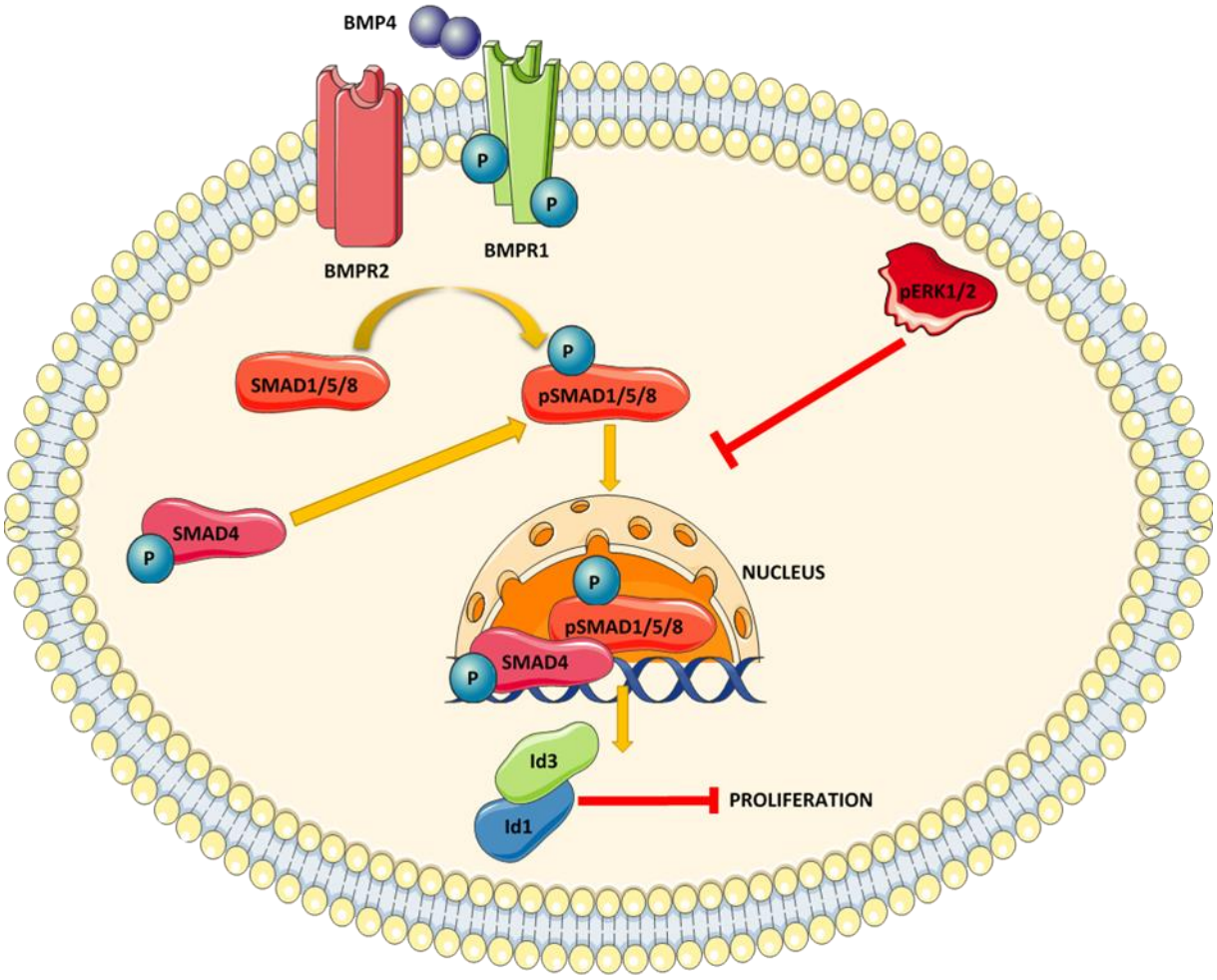


Figure 1–13: Bone morphogenetic protein receptor II (BMPRII) signalling pathway.
The binding of bone morphogenetic protein 4 or a suitable growth factors results in heterodimerisation of BMPRII with BMPRI, forming an active receptor complex, which leads to phosphorylation of Smad-1, -5 and -8. Phosphorylated Smad proteins then dimerise with Smad-4, and the complex is translocated into the nucleus, where it regulates the transcription of genes involved in cell growth, proliferation and apoptosis. The active extracellular signal–regulated kinases (ERK) and/or mitogen activated protein kinases inhibit the BMPRII signalling pathway, resulting in dysregulated cellular proliferation.

BMP4=Bone morphogenetic protein 4, BMPRII=Bone morphogenetic protein receptor type II, BMPRI=Bone morphogenetic protein receptor type I, Smad-1/5/8=Mothers against decapentaplegic homologs, P=Phosphorylation, ERK=Extracellular signal–regulated kinases, Id1/3=DNA-binding protein inhibitor Id1/3

In a healthy cell, the binding of a ligand to the BMPR*II* leads to the activation of PPARγ, which can block the Smad-independent signalling through the phosphorylation of the extracellular signal-regulated kinase1/2 (pErk1/2), leading the induction of genes p27 and p21, resulting in the senescence of the cell (Morrison and Farmer, 1999). However, when the BMPR*II* signalling is impaired by a mutation in the corresponding gene, then the binding of a growth factor to the receptors, causes the activation of Smad-independent signalling, resulting in VSMC proliferation *in vitro* (Sarjeant et al., 2003).

Stimulation of PAECs with oestrogen under hypoxic conditions resulted in decreased expression of proteins involved in the BMPR*II* signalling pathway (Ichimori et al., 2013). Under basal conditions, significantly less BMPR*II* receptor, phospho-Smad1/5/8 (p-Smad1/5/8), Id1 and Id3 proteins were expressed in the PASMCs derived from females compared to males (Mair et al., 2015), with similar observations made by Austin and colleagues in lymphocytes (Austin et al., 2009). *In vitro* experiments showed that oestrogen treatment leads to a reduction in the BMPR*II* signalling pathway through the activity of hypoxia-inducible factor 1α (HIF1α) and oestrogen receptor α (ERα) (Ichimori et al., 2013;Fessel et al., 2013), with the effects of oestrogen potentiated by five-fold in the presence of other pro-proliferative signals (Fessel et al., 2013). Hence, not surprisingly, disease penetrance in BMPR*II* mutation carriers is increased by altered oestrogen metabolism (Austin et al., 2009), with female gender reported as significantly affecting disease penetrance (Austin et al., 2009;Badesch et al., 2010). It was recently shown that the inhibition of the key enzyme in the local production of oestrogen in the pulmonary circulation, increases the BMPR*II* signalling pathway (Mair et al., 2014). Significantly, oestrogen regulates lysosomal activity through non-genomic signalling (Burlando et al., 2002), which has also regulating the BMPR*II* signalling pathway (Durrington et al., 2010).

1.3.2 *Mutations in ALK-1 Gene*

The activin A receptor type II-like kinase-1 (*ALK-1*) is a serine/threonine-protein kinase receptor in the TGF-β signalling pathway, which induces the phosphorylation of Smad1/5/8. Mutations in *ALK-1* gene was linked with the onset of HPAH and extremely poor patient prognosis in individuals harbouring this mutation compared to carriers of BMPR*II* mutation (Girerd et al., 2010a).

1.4 Animal Models of Pulmonary Arterial Hypertension

The survival of PAH patients' post-diagnosis remains poor (Humbert et al., 2010a;Benza et al., 2012), with the average survival time estimated to be 5–7 years (Benza et al., 2012;Gomberg-Maitland et al., 2011;Kane et al., 2011). Poor survival prognosis indicates that due to the complexity of PAH pathogenesis, most significant pathogenic signalling mechanisms are still unidentified, and consequently not therapeutically targeted. Animal models of the disease offer the opportunity to gain new insights into PAH pathogenesis and therewith associated effective pharmacological approaches. Numerous animal models were developed to assist the research of PAH. The features of some experimental animal models of PH are summarised in Table 1-3.

1.4.1 *Hypoxia-Induced Pulmonary Hypertension*

Most frequently, in research setting, PH in numerous animal species is induced by exposure to chronic hypoxia (cHx). The advantage of cHx models is the simplicity of model implementation and the range of species it can be used in. The exposure of rats to cHx induces remodelling of the pulmonary vascular plexus, including the muscularisation of small non-muscular PAs and increased muscularisation of pre-capillary arteries (Stenmark et al., 2009), associated with migration, proliferation and hypertrophy of SMCs (Stenmark et al., 2006b;Meyrick and Perkett, 1989). In cHx, thickening and fibrosis of large proximal PAs, leading to severe stiffening of these vessels, were documented (Drexler et al., 2008). The described progressive structural changes within the lung lead to an increase in the mean PAP. Although these animals developed increased mPAP, resulting in the onset of RV hypertrophy (RVH), this does not lead to RV failure (RVF), as seen in humans (Stenmark et al., 2009). In mice, exposure to cHx results in the muscularisation and thickening of distal and proximal PAs, although to a lesser extent compared to rats (Frank et al., 2008). The differences in response to cHx in mice are due to differential gene expression induced by Hx (Tada et al., 2008). Although this model was shown to exhibit highly reproducible and predictable response within a strain of animals, it is hindered by exceeding variability in the response to Hx between animal species, strain and animal's age (Stenmark et al., 2006b). The model is, however, additionally limited by relatively modest increases in mPAP and the

absence of plexiform lesions, which are characteristic for human PAH. Therefore, cHx animal models can only be regarded as models of less severe PH, and not PAH.

The cHx, although a classical animal model of PAH, does not completely recapitulate the human disease. Moreover, the extent of involvement of Hx-induced signalling pathways is not completely understood in human disease. In animals, prolonged exposure to cHx was associated with activation of hypoxia-induced signalling pathways, whereas intermittent cHx also resulted in altered oestrogen signalling through ERα (Wu et al., 2008). In this classical experimental model of PH, males develop more severe disease phenotype, and ovariectomy in female animals severely exacerbates the disease (Tofovic et al., 2006).

1.4.2 *Monocrotaline-Induced Pulmonary Hypertension*

In this classical animal model of PH, established in the late 1960s, the disease is induced by repetitive oral administration of a pyrrolizidine alkaloid found in the *Crotalaria Spectabilis* seeds, termed monocrotaline (MCT) (Kay et al., 1967). For MCT to be activated, the alkaloid is required to undergo transformation into a dehydrogenated pyrrole metabolite, through reactions catalysed by cytochrome P450 3A4 enzyme and oxidases (Lafranconi and Huxtable, 1984;Yao et al., 2014;Reid et al., 1998;Wilson et al., 1992). This results in an active bifunctional cross-linked MCT pyrrole. The MCT model is described as highly variable, as the establishment of the animal model heavily depended on liver metabolism, which exhibited great variance between animals, strains and species (Stenmark et al., 2009). MCT is metabolised more rapidly in livers from male rats compared to female rats (Lafranconi and Huxtable, 1984). Nowadays, the model is easily established in numerous species by a single injection of MCT or the active pyrrole. However, even the administration of the active compound does not result in reducing variability in this model, representing a significant limitation of this model.

The MCT-induced PH model is characterised by gradual alterations in the haemodynamic parameters, vascular remodelling and RV hypertrophy over a period of several weeks after initial induction, which is preceded by lung dysfunction (Lai et al., 1991;Gomez-Arroyo et al., 2012). Histological changes in the lungs of MCT-induced PH included intimal hyperplasia and medial hypertrophy, resulting in extensive thickening and remodelling of the vessel wall (Stenmark et al., 2009;Ryan et al., 2011) while distinctive plexiform pathophysiological lesions observed in other models of PH were not present (Gomez-Arroyo

et al., 2012). Exposure of MCT-treated animals to a second insult, resulted in development of plexiform lesions, vessel obliteration and severe RV hypertrophy (Okada et al., 1997). In addition to significant vasoconstrictor aspect of MCT-induced PH, the pathophysiological changes in this model are alleviated or reversed by most experimental treatments (Maarman et al., 2013), limiting its use to study severe PH in humans (Gomez-Arroyo et al., 2012). Proposed mechanisms of MCT-induced PH involve: i) direct induction of endothelial injury (Kay et al., 1967;Rosenberg and Rabinovitch, 1988), and ii) dysregulation of survival and proliferative pathways, and NO signalling in PAECs (Huang et al., 2010). MCT induces rapid sustained expression of phosphorylated Smad1, which accumulated in the nucleus, in cultured PAECs (Ramos et al., 2007). A decrease in BMPR*II* and increase in Smad6 were also observed (Ramos et al., 2007).

MCT model of PH, as one of the classical and well-established models, was extensively used in the research of PAH. More than two decades ago, Farhat and colleagues investigated the effects of oestrogens in male rats administered with MCT, demonstrating that treatment with 17β-oestradiol (17βE2) attenuated muscularisation of small PAs and RV hypertrophy (Farhat et al., 1993). As in cHx experimental model, also males treated with MCT develop more a severe disease phenotype compared to females. The protective effects of exogenous 17βE2 will be discussed later.

Early observations in so-called classic models of PH, such as cHx and MCT-induced PH, indicated that females exhibited less frequent and less severe PH than males (Rabinovitch et al., 1981;Rafikova et al., 2015). RVH and remodelling were observed in both of these animal models. However, significant dysfunction associated with RV maladaptive hypertrophy, and therewith associated higher mortality rate, is typically only seen in the MCT model (Gomez-Arroyo et al., 2012;Kato et al., 2003). To overcome these limitations of PAH animal models, novel models were developed more recently, that are thought to replicate the human phenotype more closely.

1.4.3 *Pulmonary Artery Banding*

The technique of pulmonary artery banding (PAB) is known since 1952, when the technique was developed to aid patients with congenital heart disease with secondary PAH due to increased blood flow to the lungs (Muller and Danimann, 1952). The surgical technique was adapted by Dias and colleagues to induce PH in animals, characterised by progressive

narrowing of PA and RVH (Maarman et al., 2013;Dias et al., 2002). PH is induced by physically constricting the PA by tightly tying a silk suture around an 18-gauge needle positioned alongside PA, which is then removed, and the animal is left to recover. The suture causes further constriction of the vessel in the growing animal, resulting in pressure-overload-induced RVH (Maarman et al., 2013;Dias et al., 2002). This model is mostly employed to investigate the molecular physiology and pathophysiological mechanisms involved in RVH and RV failure, which is the main cause of death in PAH. The PAB model is not employed frequently, as the initiation of this model requires highly technically skilled researchers and is also associated with significant mortality of animals.

1.4.4 *Serotonin Transporter Overexpressing Mice*

The MacLean research group reported the existence of a transgenic mouse model of PH, where the overexpression of the 5-HT transporter in mice (termed SERT+ mice) results in the development of PH in the absence of any other insults (MacLean et al., 2004). Instead, the group later showed that the disease phenotype is spontaneously developed in female SERT+ mice (White et al., 2012), characterised by PA remodelling, increased RV systolic pressure and RV hypertrophy, at 5–6 months of age. Male animals do not exhibit any PH phenotype at any age (White et al., 2011a). Moreover, in this model, exposure of transgenic female mice to hypoxia, results in the development of severe PH (White et al., 2011a)

1.4.5 *S100 Ca2+ Binding Protein A4 Overexpressing Mice*

Mice overexpressing the S100Ca^{2+}-binding protein A4 (S100A4/Mts1) represent another class of transgenic animal model of PH, established by (Ambartsumian et al., 1998). The overexpressed gene encoding the S100A4/Mts1 is associated with the regulation of cell proliferation, differentiation and apoptosis (Ambartsumian et al., 1998;Greenway et al., 2004). A small population of female mice with this transgenic modification developed PA remodelling with vascular lesions, with no apparent pathological changes in the right heart (Greenway et al., 2004;Dempsie et al., 2011). S100A4/Mts1 is upregulated in PAH (Greenway et al., 2004). In this model, animals develop histological changes in PAs, which resemble human neointimal lesions leading to occlusion of the vessel lumen (Greenway et al., 2004). The mechanism of action in PAH is associated with 5-HT, where the latter induces the release of S100A4/Mts1, which affected PASMC proliferation and migration (Lawrie et al., 2005).

1.4.6 *BMPRII Mouse Model of Pulmonary Arterial Hypertension*

The presence of mutations in the BMPR*II* coding gene in most HPAH population, also present in a significant proportion of IPAH patients, plays an important role in the pathophysiology of PAH. The impact of BMPR*II* mutations on the pathophysiology of the disease was extensively studied in mice heterozygous for the BMPR*II* allele (BMPR*II* $^{+/-}$). Beppu and colleagues were first to generate a mouse model carrying a mutant BMPR*II* allele (Beppu et al., 2000). Although authors later reported these mice had altered haemodynamic parameters and remodelled PAs compared to their wild-type littermates (Beppu et al., 2004;Song et al., 2005), pulmonary haemodynamics, PA wall thickness and index of muscularisation were not significantly different from wild-type littermates under normoxic (Nx) or Hx conditions (Long et al., 2006). Nonetheless, mice carrying the specific R899X mutation in the BMPR*II* gene, develop significant pulmonary vascular remodelling, characterised by increased right ventricular systolic pressure (RVSP), muscularisation of small pulmonary vessels and vaso-occlusive lesions, but only with a second insult, in this case with 16α-hydroxyoestrone (16 αOHE1) (West et al., 2008b). Specifically, these animals exhibit changes in angiogenesis, vasoreactivity, and injury response (West et al., 2008b). Chronic treatment of BMPR*II* deficient mice with 5-HT resulted in increased PA systolic pressure, RVH, and vascular remodelling (Long et al., 2006). The experimental PH phenotype in mice heterozygous for the BMPR*II* allele is exaggerated in cHx (Long et al., 2006). In the BMPR*II* model, however, no intimal and plexiform lesions are observed.

1.4.7 *Smad1 Conditional Knock-Out Mice Model of Pulmonary Arterial Hypertension*

Although the mutations in the gene encoding for the BMPR*II* receptor were described as the main genetic cause of HPAH, the role of the BMP signalling in the disease setting remained elusive. Smad1 protein is activated directly by the BMP receptors, as one of the down-stream signalling proteins of the BMP and the TGF-β signalling pathways. The conditional *Smad1* knock-out model was developed to observe by Huang and colleagues, who aimed to determine the role of *Smad1* in adult animals (Huang et al., 2002). *Smad1* conditional knock-out animals exhibit altered haemodynamic parameters and vascular remodelling of the pulmonary circulation similar to animals harbouring the specific R899X mutation in the

BMPR*II* gene (Long et al., 2011). Cross-breeding of both genetically engineered models results in animals with significantly increased RV systolic pressure compared to single heterozygous mice. Hence, it appears that the effect of both gene knock-outs is synergistic and increases susceptibility of animals for developing PAH. Female *Smad1* heterozygous mice develop PAH phenotype spontaneously at 5–6 months of age, characterised by haemodynamic alterations and pulmonary vascular remodelling, while male animals never developed the disease (Mair et al., 2015). In these female animals, ovariectomy results in decreased haemodynamic parameters and vascular remodelling, respectively (Mair et al., 2015). Nonetheless, in this model, despite established PAH phenotype, female animals do not develop RVH (Mair et al., 2015). Interestingly, the expression of phosphorylated Smad1 is decreased in the medial and intimal layers of the vessel wall compared to normal subjects, where Smad1 was mostly localised within the nucleus of the ECs (Yang et al., 2005).

Although transgenic models of PAH are useful to investigate the role of a specific pathway in the development and progression of the disease, they are possibly limited by not representing all the complex features of PAH pathogenies. Table 1-3 summarises the features of commonly used experimental models of PH.

Table 1-3: Summary of Features of Animal Models of Pulmonary Arterial Hypertension.

Model	Species	Pulmonary Hypertension	Medial Hypertrophy	Intimal Lesions	Plexiform Lesions
CH	R, M	Low	Yes	No	No
MCT	R, M	High	Yes	No	No
PAB	R,M	Low	No	No	No
5-HTT	M	Yes	Yes	No	No
S100A4/Mts4	M	Low	Yes	Yes	No
BMPR*II*	M	Low	Yes	No	No
Smad1	M	Low	Yes	No	No

CH= Chronic hypoxia, MCT= Monocrotaline, PAB= Pulmonary arterial binding, 5-HTT= Serotonin transporter, BMPR*II*= Bone morphogenetic protein receptor *II*, R= Rat, M= Mouse

None of the models described above develops pathophysiological changes in the pulmonary vasculature that exactly mimic all the hallmarks of the human disease. White and colleagues reported the development of plexiform-like lesions and severe vascular pruning in their animal model, where rats, which underwent left pneumonectomy and were additionally treated with MCT (White et al., 2007).

1.4.8 *Sugen 5416 and Hypoxia-Induced Pulmonary Arterial Hypertension*

A novel alternative model of PH was recently developed by Taraseviciene-Stewart and colleagues, where rats are given one dose of Sugen 5416 (Su5416), a VEGF receptor inhibitor (see Figure 1–14), and then exposed to cHx (Taraseviciene-Stewart et al., 2001). The protocol for model establishment differs in rats and mice, where the former is given a single dose of Su5416, and the latter receive several injections (Ciuclan et al., 2011). Although a single dose of Su5416 in Nx causes mild PH characterised by moderate PA remodelling in rats, only coupling of Su5416 with cHx results in the development of severe persistent PH (Taraseviciene-Stewart et al., 2001). Ciuclan and colleagues reported that upon return of the mice from hypobaric chambers, the disease phenotype was reversed, unlike with rats where the disease persisted and progressed even after the animals were removed from Hx (Ciuclan et al., 2011). These observations were somewhat similar to the disease onset and progression occurring in humans. For instance, in Su5416/Hx model of PH medial wall hypertrophy and neointimal thickening occur 3–5 weeks after model initiation (Abe, 2017). Additionally, complex plexiform lesions are formed approximately 13–14 weeks following model initiation (Abe et al., 2010). The mechanism by which the disease phenotype is induced in animals is not well understood. However, it is believed that exposure to Su5416 and Hx results in endothelial dysfunction, leading to vasoconstriction and vascular remodelling. Additionally, in diseased animals a population of apoptosis-resistant PAECs is believed to be detrimental in establishing the disease phenotype with angio-obliterative lesions (Stenmark et al., 2009;Jurasz et al., 2010). In terms of molecular mechanism of Su546/Hx model, a dysregulation of TGF-β and BMPR*II* signalling pathways was also observed, with considerable induction of HIF1α in these animals (Ciuclan et al., 2011).

Tofovic and colleagues were the first to report that female sex in Su5416/Hx model is associated with the onset of more severe PAH complicated by plexiform lesions, as observed in humans, while males only developed occlusion of vessels. However, plexiform lesions

are not always present (Tofovic et al., 2012;Dean et al., 2016), which would suggest an advantage of this model over other known PH models. Moreover, in this model oestrogens appear to be detrimental to the disease development, and even worsened the disease phenotype, featuring complex vascular lesions and exceedingly elevated mPAP (Tofovic et al., 2012). Ovariectomy and/or inhibition of endogenous oestrogen production in this model are associated with lessened and delayed disease phenotype (Bilan et al., 2013;Tofovic and Rafikova, 2009). Albeit female animals develop a more severe disease phenotype as characterised by vascular remodelling and the presence of complex vascular lesions in this model, they exhibit a lesser change in the RV mass and hypertrophy (Tofovic et al., 2012;Tofovic and Rafikova, 2009), which closely recapitulates the observations of epidemiological studies in humans. Su5416/Hx model is furthermore characterised by relative ineffectiveness of available therapeutic options in the reversal of disease phenotype. Due to extensive similarities of Su5416/Hx model with PAH observed in humans, this is the model of choice for numerous researchers. Notably, the murine Su5416/Hx model is not frequently used, as mice do not develop severe PAH with plexiform lesions (Abe, 2017).

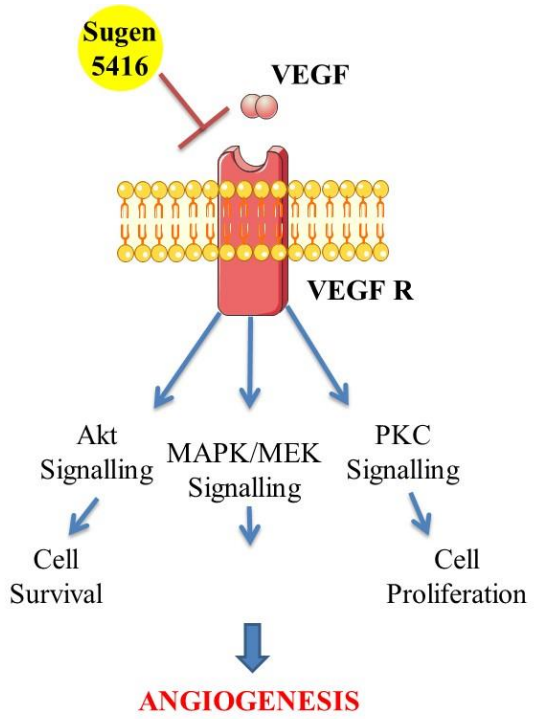


Figure 1–14: Effects of vascular endothelial growth factor receptor inhibition by Sugen 5416.
A schematic representation of possible pathways through which Sugen 5416 affects angiogenesis.

As discussed above, it appears that sex plays an exceedingly important role in the onset of clinical and experimental PAH phenotypes, in humans and animals, respectively. Therefore,

the understanding of biosynthesis of steroidal sex hormones and their metabolism is vital in the understanding of the mechanisms behind the observed sex disparity in PAH.

1.5 Steroidogenesis

The term steroid hormone is used to describe all hormones exhibiting steroidal character, with their functions throughout the body being mediated through nuclear receptors, regulating genomic mechanisms, and/or membrane-bound receptors, regulating rapid-response intracellular signalling pathways. Biologically active steroidal hormones are synthesised from cholesterol in a complex multi-step, multi-enzyme biological process termed steroidogenesis. Steroid hormones, depending on the class of the hormone being generated, are biosynthesised at different locations within the body, with most of sex hormones produced in the gonads and corpus luteum, adrenal gland, and placenta, with low levels also being produced in peripheral tissues such as liver, heart, skin, adipose tissue and brain (Cui et al., 2013). Although sex hormones are primarily responsible for the development and regulation of primary and secondary sex characteristics, they are also critical for the functional regulation of peripheral tissues (Cui et al., 2013).

In the first step of steroidogenesis (simplified scheme is provided in Figure 1–15), the cholesterol, transported to mitochondria, is metabolised by various cytochrome P450 enzyme to pregnenolone, which serves as an immediate precursor molecule for the synthesis of all steroid hormones. In the subsequent reactions, catalysed by several cytochrome P450 enzymes, pregnenolone is bio-transformed to dehydroepiandrosterone (DHEA), which is the most abundant steroid hormone found in humans. Even though, the main function of DHEA is to serve as an intermediate in the biosynthesis of oestrogen and androgen hormones, it was shown that DHEA possesses some biological activity. In the cHx and MCT models, DHEA prevented and reversed the experimental PH phenotype as assessed by haemodynamic and remodelling parameters (Bonnet et al., 2003;Meloche et al., 2010). The protective character of DHEA in PAH might be due to increased expression of sGC and vasodilation associated with improved responsiveness to NO (Oka et al., 2007). Additionally, DHEA was shown to increase the expression of BMPR*II*, leading to increased apoptosis in hPASMCs from PAH patients (Meloche et al., 2010). In Su5416/Hx model, chronic treatment with DHEA resulted in improved cardiac function due to: i) antioxidant properties, ii) decreased RV systolic pressure and iii) preserved contractile function of RV (Alzoubi et al., 2013). Indeed, decreased plasma levels of DHEA are associated with the onset of PAH in men (Ventetuolo

et al., 2016). Following successful completion of phase II clinical trials involving the use of DHEA for treatment of PAH, phase III clinical trials have now commenced (Meloche et al., 2010).

The DHEA can be bio-transformed to DHEA-sulphate (DHEA-S) by DHEA sulfotransferase, which circulates tightly bound to albumin, exhibiting prolonged half-life (White and Portfield, 2013). 3β Hydroxysteroid dehydrogenase (3βHSD) catalyses the reaction of biotransformation of DHEA to androstenedione, which serves as the precursor molecule for the synthesis of oestrogens and androgens. Two 3βHSD were identified in humans (Mason, 1993), while five different isoforms are known in mice (Payne et al., 1997). Androstenedione can be directly converted to testosterone by 17β hydroxysteroid dehydrogenase type 3 (17βHSD type 3), whereas the reverse reaction is catalysed by the 17β hydroxysteroid dehydrogenase type 2 (17βHSD type 2). Both precursors, androstenedione and testosterone, can be converted to 17βE2 and oestrone, respectively, in the reaction of aromatisation catalysed by aromatase. Aromatase is the key enzyme in the generation of oestrogens, also termed cytochrome P450 19A1, and is expressed in numerous tissues throughout the body, from the gonads, brain, adipose tissue, placenta, and blood vessels, including pulmonary arteries, meaning there is localised production of oestrogens in peripheral tissues.

1.5.1 *Female Steroid Sex Hormones – Oestrogens*

There are three main oestrogens in a human body, oestrone (E1), 17βE2, and oestriol (E3), where biological potency of oestrogens is E2>E1>E3. Oestradiol exists in two different isomers, 17β-oestradiol (17βE2) and 17α-oestradiol (17αE2), where the former possesses greater biological activity. The levels of oestrogens change during the life time of a female. During pregnancy, E3 is the dominating circulating oestrogen, with high levels of this hormone being produced by the placenta. In women of child-bearing age, E3 is formed from E1 and E2 through reaction of 16α-hydroxylation. Prior to menopause, the circulation concentration of 17βE2 depends greatly on the stage of the oestrous cycle, with normal range of 15–350pg/mL (0.06–1.3nM), and the levels increasing approximately 2-fold during ovulation. In peri-menopausal women, the levels of 17βE2 begin to fall, and the circulating levels of 17βE2 in post-menopausal women are usually less than 10pg/mL (<0.04nM) (Laboratories), with E1 assuming the role of the main circulating oestrogen in terms of concentration. Prior to menopause, 17βE2 is mostly produced in the ovaries and adrenal

gland, and acts in a paracrine way to regulate distal target tissues. In post-menopausal women and men, however, the oestrogens are primarily formed from androgens in the peripheral tissues, such as the adipose tissue, skin, etc. (Grodin et al., 1973;MacDonald et al., 1979), with 17βE2 acting in a paracrine or intracrine manner (Simpson, 2003). In post-menopausal women, E1 is synthesised mainly in the adipose tissue, with DHEA serving as the precursor molecule (Cui et al., 2013). Due to their steroidal character, most of circulating oestrogens in the vascular system are transported bound to the sex hormone-binding globulin (SHBG), and to lesser extent serum albumin. Only a small proportion of oestrogens exist in the circulation is unbound, and therefore biologically active.

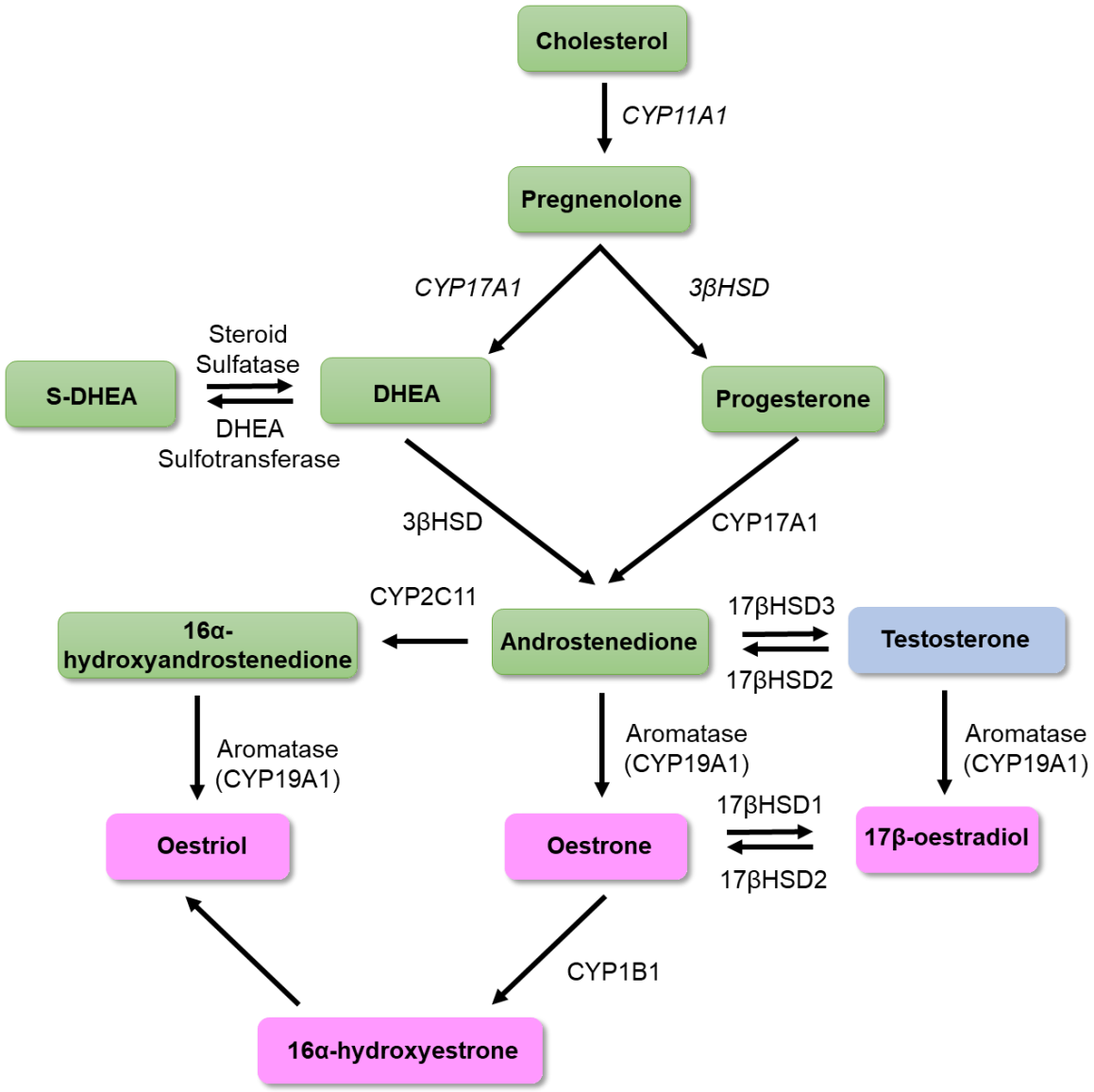


Figure 1–15: Biosynthesis of oestrogen steroid hormones form the precursor molecule, cholesterol.

Cholesterol is the precursor molecule for the biosynthesis of all major steroid hormone classes. It is converted into pregnenolone in a biotransformation reaction catalysed by CYP11A. Pregnenolone is then further converted by either CYP17A1 to DHEA or 3β-hydroxysteroid dehydrogenase to progesterone. Both intermediates are then bio-transformed into androstenedione, which can be either converted into testosterone in a biotransformation reaction catalysed by 17β-hydroxysteroid dehydrogenase type 3, or into 16α-hydroxy-androstenedione by the activity of CYP2C11. Testosterone, as the primary male sex hormone, can be converted into 17β-oestradiol by the activity of aromatase. 17β-oestradiol, as the primary female sex hormone, can be bio-transformed into oestrone or oestriol by the activity of 17β-hydroxysteroid dehydrogenase type 2 and CYP enzymes, respectively. Green colour indicates the intermediates involved in the production of both, androgens and oestrogens, while oestrogens are coloured pink and androgens are blue.

HSD3β=3β-Hydroxysteroid dehydrogenase, 17βHSD3=17β-Hydroxysteroid dehydrogenase type 3, DHEA=Dehydroepiandrosterone, 17βHSD2=17β-Hydroxysteroid dehydrogenase type 2, 17βHSD1=17β-Hydroxysteroid dehydrogenase type 1, CYP19A1=Aromatase, CYP=Cytochrome P450 enzyme.

1.5.2 *Aromatase*

Aromatase belongs to the cytochrome P450 enzyme (CYP) superfamily, catalysing the rate-limiting final-step reaction in the biosynthesis of 17βE2 (Simpson and Santen, 2015), consisting of three consecutive oxidation reactions. Therefore, for its normal function aromatase requires the availability of oxygen and the nicotinamide adenine dinucleotide phosphate (NADPH) cofactor. The functional form of aromatase is associated with NADPH cytochrome P450 reductase (Amarneh et al., 1993). The aromatase catalytic activity is specific to aromatisation of androgen hormones, specifically androstenedione and testosterone. Evidence indicated that the enzymes' activity in tissues is regulated through stringent tissue-specific expression of aromatase, depending on tissue-specific gene promoters (Simpson et al., 1994;Simpson, 2003). Ten different tissue-specific gene promoters regulating the expression of aromatase, were described so far by Bulun and colleagues, all activated by specific activators (hormones, cytokines, and signalling molecules) under physiological or pathophysiological conditions (Bulun et al., 2005). For example, the expression of aromatase in the ovaries is regulated by the follicle-stimulating hormone (FSH), while cytokines and tumour necrosis factor α (TNFα) regulate its expression in the adipose tissue (Cui et al., 2013). Prostaglandin PGE_2 was shown to stimulate aromatase expression in the adipose tissue, while aromatase expression was correlated with cyclooxygenase 1 and 2 expressions in breast cancer (Brueggemeier et al., 1999). The structure of the gene encoding aromatase enables that regardless whichever of the promoters is used for gene transcription, alternative splicing of the mature mRNA results in the formation of identical enzyme in all aromatase-expressing tissues (Zhao et al., 2016). Interestingly the expression level of aromatase in the adipose tissue of post-menopausal women is higher than that of pre-menopausal women (Misso et al., 2005). Authors therefore concluded that the capacity of the adipose tissue to produce oestrogens increases with age. Additionally, aromatase activity in humans is regulated by a rapid phosphorylation/dephosphorylation mechanism (Charlier et al., 2011).

The association of obesity, increased aromatase expression, and oestrogen production in PAH disease setting was long recognised (Grodin et al., 1973;Key et al., 2003). The local oestrogen biosynthesis describes the production of oestrogens in the extragonadal tissues, where the newly synthesised hormones act locally (Labrie et al., 1998). Local production of oestrogens, although small in comparison to circulating levels, results in high localised concentrations, exerting biological functions (Simpson and Davis, 2001). For example, the

local production of oestrogens is considerably higher in cancerous breast tissue than in plasma (de Jong et al., 1997). Indeed, Mair and colleagues demonstrated that aromatase is expressed in hPASMCs and vascular lesions from patients with PAH (Mair et al., 2014). In men and post-menopausal women, the levels of circulating oestrogens reflect the local production of these hormones in the peripheral tissue (Simpson, 2003). The use of aromatase inhibitors for treatment of oestrogen-dependent cancers has long been recognised. More recently, aromatase inhibitors have also been considered in PAH, where a recently published results of the phase 2 clinical trial to determine the safety and efficacy of anastrazole, showed there was a significant reduction in E2 levels with no change in RV ejection fraction (Kawut et al., 2017).

1.5.3 *Metabolism of Oestrogens*

Oestrogen activity in the body is regulated by deactivation of highly potent 17βE2, including its conversion to a less-active forms such as E1 or E3. Moreover, oestrogens can also be biotransformed into oestrogen derivatives, which cannot interact with oestrogen receptors (Kotov et al., 1999). Oestrogens are primarily metabolised by the CYPs, which are *Phase I* drug-metabolising enzymes, catalysing oxidative biotransformation of oestrogens and other steroids, most frequently at positions C2, C4, and C16 (Zhu and Conney, 1998), although numerous other hydroxylated metabolites were characterised (Lee et al., 2002;Lee et al., 2001). As the liver is the primary site of steroid metabolism, most of P450 enzymes are abundantly expressed here. Nonetheless, numerous inducible isoforms of CYPs were found to be expressed in the extrahepatic tissue, such as lung tissue (Hukkanen et al., 2001), indicating the lung is also a site of significant oestrogen metabolism (see Figure 1–16). The resulting oestrogen metabolites possess a more polar, and therefore hydrophilic character, and serve as precursor molecules for *Phase II* drug-metabolising enzymes, which are typically transferases catalysing biotransformation reactions of conjugation. Hydroxylated oestrogens serve as precursors to oestrogen sulfotransferase enzymes (SULTs), which catalyse reaction of sulphurylation, resulting in sulphate oestrogen metabolites. The resulting conjugated metabolites are generally i) more easily excretable due to enhanced hydrophilic character, ii) metabolically inactive, or iii) pharmacologically inactive, meaning they are unable to interact with their respective receptors (Jancova et al., 2010). The most abundant oestrogen conjugates are sulphates and glucuronides (Raftogianis et al., 2000), where the former exhibit prolonged half-life (Coughtrie et al., 1998) and might therefore serve as an oestrogen reservoir with concentrations of E1-sulfate approximately 10-fold higher than

unconjugated oestrogen (Pasqualini et al., 1989). Oestrogen glucuronides, instead, are primarily excreted from the body. Glutathione (GSH) and methyl oestrogen conjugates are also well documented.

In the process of oestrogen metabolism certain emerging compounds, such as metabolites containing hydroxyl groups at positions C2 or C4 is the steroidal backbone, can be further metabolised by CYP450 enzymes. The formation of catechol-oestrogens and quinones (CE-Qs) or semiquinones, resulting from further oxidation of 2- and/or 4-hydroxylated oestrogens, where the latter can covalently bind to DNA to form depurinating adducts and induce mutations (Cavalieri et al., 1997). Additionally, catechol-oestrogens might also enter redox cycling, resulting in enhanced generation of hydrogen peroxide, hydroxyl radicals and other ROS (Yager and Liehr, 1996). These reactive catechol-oestrogens serve as precursor molecules for catechol-O-methyl transferases (COMT), enzymes catalysing methylation reaction. Although the most active pathway towards catechol-oestrogen excretion is methylation, to a lesser extent, reactive catechol-oestrogens might also be metabolised to glucuronides and sulphate oestrogen conjugates (Zhu and Conney, 1998;Raftogianis et al., 2000). Soluble COMT (S-COMT) and membrane-bound COMT (M-COMT) enzymes exist, which differ in their relative affinity for their substrates, catalytic capacity and tissue expression (Raftogianis et al., 2000).

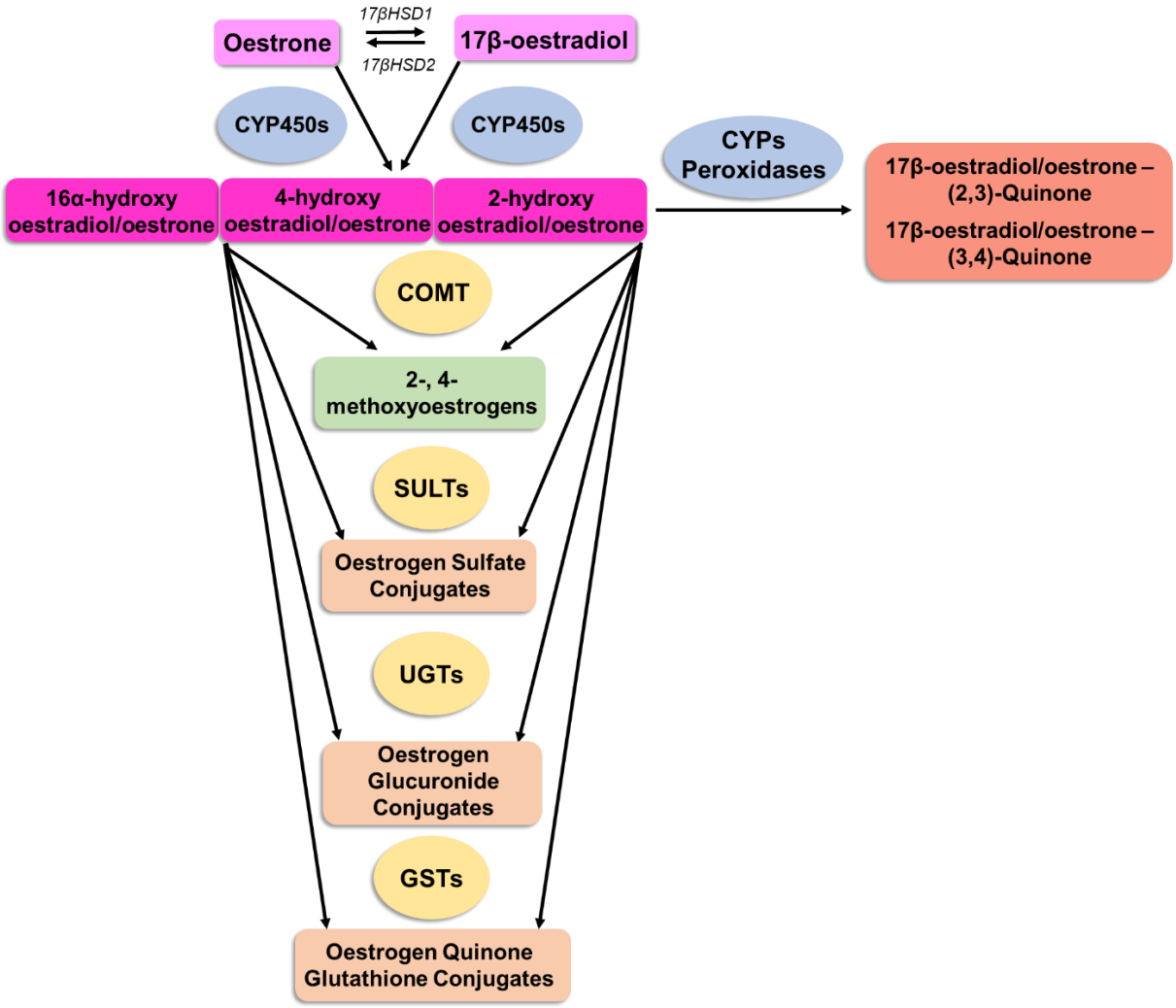


Figure 1–16: Metabolism of oestrogen hormones.
Oestrone and 17β-oestradiol can be converted into one another by the activity of 17β-hydroxysteroid dehydrogenase, and both oestrogen hormones can be metabolised by CYP450 enzymes to numerous hydroxylated oestrogens. The principal hydroxylated oestrogen metabolites are 2- and 4-hydroxyoestrogens, with only small proportion of 16α-hydroxy-oestrogens being formed. The 2- and 4-hydroxylated oestrogens can be further metabolised by COMT, producing 2- and 4-methoxyoestrogens, which have been shown to mediate protective effects in the cardiovascular system. Other reactions of biotransformation of hydroxylated oestrogens include the formation of oestrogen sulfate conjugates by the activity of UGT, which exhibit prolonged elimination half-lives and might therefore serve as an oestrogen reservoir, and the formation of oestrogen glucuronide conjugates, by the activity of UTG, which are primarily eliminated from the body. Oestrogens can also be further oxidised into catechol quinones and semi-quinones, which can form covalent bonds with DNA, resulting in depurinating adducts and therewith associated DNA damage.

17βHSD2=17β-hydroxysteroid dehydrogenase type 2, 17βHSD1=17β-Hydroxysteroid dehydrogenase type 1, CYP=Cytochrome P450 enzyme, COMT=Catechol-O-methyl transferase, SLUT=Oestrogen sulfotransferase enzymes, UGT= Glucuronosyltransferase, GST= Glutathione S-transferase

1.5.4 *Regulation of Oestrogen Signalling*

As discussed above, oestrogen signalling locally might be partly regulated by the biosynthesis of oestrogens via aromatase and transformation of oestrogens into sulphate conjugates, which are present in high concentrations but cannot bind to and activate oestrogen receptors. The oestrogen sulphates, however, serve as precursors for steroid sulfatase enzymes, hydrolysing E1/E2-sulfate to an active unconjugated from. Reportedly, the activity of E1 sulphatase in the breast tumour tissue was much higher than that of aromatase, indicating sulphate conjugates could also be the likely source of plasma oestrogens (Santen et al., 1986), specifically 17βE2 (MacIndoe, 1988), through interconversion of unconjugated E1 by 17βHSD type 1. Overexpression of 17βHSD isoforms 1 and 2 was associated with poorer prognosis and disease recurrence in breast cancer (Gunnarsson et al., 2001;Song et al., 2006). Steroid sulphatase is also capable of hydrolysing DHEA-S, which has little hormonal activity, but represents a large precursor reservoir for oestrogen biosynthesis. Nonetheless, biological effects in target tissues can only be mediated by hormonally active oestrogens, depending on their potency and receptor affinity through their interaction with oestrogen receptors (Kuiper et al., 1997). Oestrogen receptors (ER), belonging to the class I steroid hormone nuclear receptor superfamily, include receptor ERα and ERβ, with the latter receptor discovered approximately 20 years ago (Mosselman et al., 1996). ERs, as other nuclear receptors are comprised of six domains, where the N-terminal A/B domain is the regulatory domain also termed activation function 1 (AF-1), followed by C domain, which is the DNA-binding domain. Domain D represents the hinge region, which is responsible for nuclear localisation of the receptor, followed by the hormone binding domain E and the C-terminal F domain, which is involved in regulation of receptor activity and conformation.

The central DNA-binding domain and the N-terminal regulating domain represent the most and the least conserved regions of ERs, respectively. Both ER receptors have distinct roles in oestrogen signalling yet share a significant percentage of sequence homology. Due to their extensive homology, ERα and ERβ exhibit similar affinities for 17βE2. Additionally, E1 and E3, although possessing high receptor affinity, are weaker agonists of ERs compared to 17βE2 (Heldring et al., 2007). The inactive ERs are present in the nucleus, and bound with heat-shock and other proteins, which disassociate upon the binding of oestrogen or oestrogen-like compound. The binding of a suitable ligand results in the activation of the receptors, forming dimers and interacting with specific segments of DNA, oestrogen-

response elements (ERE), leading to transcription of target genes. Several splice variants were described for both receptors, and although it is not known whether all variants of ERα are translated and expressed as proteins, several variant isoforms of ERβ were characterised in tissues (Scobie et al., 2002;Palmieri et al., 2004), termed ERβ1–5 (Moore et al., 1998). Leung and colleagues reported that although the full-length ERβ, also termed ERβ1, is the only functional receptor isoform, the other variants can function as dimerising partners, thereby affecting the receptor activity (Leung et al., 2006). Conversely, there are functionally distinct ERβ isoform with greater transcriptional activity than ERβ1 (Wilkinson et al., 2002). Hence, it appears that the expression of variant ERβ isoforms may significantly modulate oestrogen signalling through affecting i) receptor activity, ii) target gene expression and iii) co-activator interaction, in a ligand-dependent manner (Ramsey et al., 2004).

Both ER receptors were shown to be expressed within the lung, with ERβ levels being approximately two-fold higher compared to ERα in the primary lung tumours (Kuiper et al., 1997). In tissues such as ovaries and the uterus both ER subtypes are present. However, there are significant differences in peripheral tissues, such as the liver, lung, etc. exist, with significantly more ERβ expressed within the lung compared to ERα (Kuiper et al., 1997).

ERs were also shown to induce target gene transcription through interaction with DNA-bound transcription factors, instead of ERE (Sukovich et al., 1994) and ligand-independent receptor activation through modulation of kinase/phosphatase activity in the absence of hormone (Weigel and Zhang, 1998). Alternatively, oestrogens were shown to mediate rapid non-genomic effects (Szego and Davis, 1967), possibly through cytoplasmic ER, which is tethered to the inner plasma membrane through protein interactions (Levin, 2005). Since, it was established that these effects are associated with activation of four signalling pathways: i) phospholipase C/protein kinase C (Morley et al., 1992;Bulotta et al., 2009), ii) Ras/Raf/MAPK (Klinge et al., 2005), iii) phosphoinositide-3-kinase (PI3K)/AKT (Lee et al., 2005), and iv) cAMP/PKA (Farhat et al., 1996;Chen et al., 1998). Filardo and colleagues observed rapid transient activation of the GPER by oestrogen via an inhibitory signal mediated by cAMP (Filardo et al., 2002).

1.5.4.1 Classical Ligand-Dependent Direct Activation of Oestrogen Receptor

The steroid properties of oestrogens and oestrogen-like compounds allow the molecules to freely diffuse through the cellular membrane into the cytoplasm and nucleus. Therefore, genomic oestrogenic effects are mediated through ligand-dependent direct activation of ERs, which bind to specific ERE sequences. Shortly, the binding of a suitable ligand to the hormone-dependent activation function 2 (AF-2) induces a transformational change in the receptor, leading to receptor activation and dissociation of the inhibitory multiprotein complex, which in absence of suitable ligands keeps ERs in their inactive form. The displacement of inhibitory complex also allows for i) the receptor to be translocated into the nucleus, ii) promotes dimerization of ERs, and iii) increases receptor affinity for ERE through induction of conformational changes exposing high affinity DNA binding site (Hall et al., 2001). DNA-bound homo-/hetero-dimers of ERs can interact with co-factors, which as co-regulators can either enhance or reduce the transcription of the target gene, thereby mediating ligand- and tissue-specific oestrogen signalling (Kase, 2003). The transcriptional activity of ERs is dependent on the AF-1 and AF-2, located in the C- and N-terminus, respectively. Out of the two transcriptionally active domains, the AF-2 possesses the strongest transactivation function. The maximal transcriptional activity of the receptors can only be achieved when both activation functions are engaged (Tzukerman et al., 1994;Maggi, 2011). The duration and magnitude of biological response to oestrogen stimulation is tightly regulated by i) the dissociation of the ligand from the receptor, and ii) the degradation of the ER receptor bound to the DNA (Kase, 2003). As some of the ligands exhibit very small dissociation constants, main degradation mechanism is the ubiquitin-proteasome pathway (Nawaz and O'Malley, 2004), where the degradation of the receptor is enhanced once it is bound to hormone. ERβ degradation is an oestrogen-dependent process, partially regulated by the conformational position of F domain, which in absence of a ligand protect the receptor from degradation (Tateishi et al., 2006).

1.5.4.2 Ligand-Independent Activation of Oestrogen Receptor

Many post-translational modifications, such as phosphorylation, acetylation, ubiquitination, methylation, sumoylation and palmitoylation have been identified, with approximately 22 identified modification sites in ER (Faus and Haendler, 2006;Le Romancer et al., 2011). Less is known regarding post-translational modifications of ERβ. The latter isoform can be

glycosylated, leading to increased receptor stability (Cheng and Hart, 2001). Aforementioned modifications were shown to modulate numerous aspects of ER activity, from receptor stability, subcellular localisation, and dimerization to receptor's ability to bind to DNA sequences and interact with co-regulators (Le Romancer et al., 2011). Phosphorylation at serine residues (S102, S104, S106 and S118) within ERα were associated with increased transcription activity, which may either be constitutive or induced by oestrogens (Medunjanin et al., 2005;Kato et al., 1995;Rogatsky et al., 1999). Phosphorylation at residue Y52 results in ligand-independent activation of transcription and enhanced receptor stability (He et al., 2010).

1.5.4.3 Non-Classical Ligand-Dependent Activation of Oestrogen Receptor

Ligand-bound ER can also interact with other transcription factors bound to their response elements, resulting in association of the ER with promoters, which may or may not include ERE-like sequences. The exact mechanism of this non-classical mechanism of oestrogen signalling is not well understood, however, it was shown that ERs can interact with transcription factors such as nuclear factor kappa-B (NF-κB), specificity protein 1 (SP1) and Jun/Fos/activator protein 1 (AP1) at corresponding DNA binding sites. The interaction of ERα with NF-κB and AP1, respectively, was shown to negatively affect the expression of several genes, including human interleukin 6 (IL-6) (Ray et al., 1997), TNFα (Srivastava et al., 1999), and quinone reductase (Montano and Katzenellenbogen, 1997). Agonists and antagonists of ERs, supress and stimulate gene transcription mediated through ER interaction with AP1, respectively (Jakacka et al., 2001) Although certain mutations might lead to abolishment of the classical oestrogen signalling, such ERs might still be able to their mediate effects through the non-classical pathways (Jakacka et al., 2001). Activation of both regulatory domains of ER is required for the activity of ERα, while only activation of the AP1 regulatory domain in the presence of selective oestrogen receptor modulators (SERMs) is required for ERβ activity (Webb et al., 1999). It was proposed that the binding of the Fos/Jun transcription factors at the AP1 binding site, leading to the recruitment of CPB/p300 and associated proteins, including p160 co-activators, allows for the interaction of ERs through the non-classical signalling at the AP1 binding site (Kushner et al., 2000). Stimulation with BSA-bound 17βE2 induced transcription of genes without the involvement of ER classical signalling (Watters et al., 1997).

1.5.4.4 Non-Genomic Oestrogen Signalling

Primarily, oestrogen signalling is characterised by a latency in the onset of effects, resulting in altered gene and protein expression, mediated by ERs localised within the nucleus (King and Greene, 1984). Multiple rapid actions of oestrogens have also been identified, indicating non-genomic signalling pathways may also be activated by oestrogens. Non-genomic oestrogen signalling pathways are activated by i) ERs tethered to the plasma membrane and/or ii) G-protein coupled receptor GPER1. A variant of the ERα is specifically targeted and localised to plasma membrane by post-translational modification of palmitoylation (Li et al., 2003). Moreover, some investigators reported that phosphorylation of ERs might also target these receptors to the inner plasma membrane (Balasenthil et al., 2004), while others observed that tethering of ERs to plasma membrane is dependent on the presence of specific adaptor proteins, such as Src-homology and collagen-homology (Shc) and insulin-like growth factor (IGF) protein (Evinger and Levin, 2005). Oestrogen stimulation increased the association of ERα with PI3K, leading to increased kinase activity, resulting in the activation of protein kinase B/Akt and cell survival pathways (Simoncini et al., 2000). Equally, stimulation of ECs with 17βE2 resulted in increased activity of eNOS, with MAPK and PI3K signalling pathways mediating these effects, depending on the concentration of the stimulant (Simoncini et al., 2000). Enhanced eNOS expression and activity are considered vaso-protective, mediating vasodilation through relaxation of SMCs.

The discovery of the G-protein coupled oestrogen receptor 1 (GPER1/gpr30) was prompted by reports of rapid activation of signalling pathways classically associated with growth factor and/or G protein-dependent signalling, leading to receptor cloning and characterisation in the late 1990s. Gpr30, previously described as an orphan receptor, is activated by 17βE2 in numerous breast cancer cell lines (Filardo et al., 2000). Gpr30 receptor is widely expressed throughout the body, and is also present the lung (Kvingedal and Smeland, 1997). Activation of gpr30 by 17βE2 results in the activation of Erk1/2 MAP kinases through EGFR transactivation (Filardo et al., 2000). Moreover, the same researchers later showed that gpr30 also activated adenylate cyclase (AC), leading to mobilisation of cAMP Activation of gpr30, localised at the endoplasmic reticulum, results in the mobilisation of intracellular Ca^{2+} storage through activation of PI3K, mediating rapid cellular response (Revanche et al., 2005). Interestingly, vascular SMCs of gpr30-deficient animals exhibited enhanced response to ET-1 mediated vasoconstriction, therefore the

authors concluded gpr30 might regulate vasoconstrictor activity of ECs, possibly leading to a reduction of blood pressure (Meyer et al., 2012).

1.5.5 *Oestrogens in Disease*

Numerous diseases exhibit sex dimorphism, including some cancers and cardiovascular disease. The evidence of the implications of female sex hormones are discussed below.

1.5.5.1 Oestrogens in Cancer

To illustrate the role of oestrogens in lung disease, the importance of lung cancer is considered here. Recent statistical data indicates that lung cancer is the second most common cancer following breast cancer amongst women, accounting for 12% of all diagnosed cases (Siegel et al., 2017), with a documented 22% increase in incidence in women within the past 30 years (Chakraborty et al., 2010). There is a direct association of lung cancer risk with hormone replacement therapy (Adami et al., 1989). Moreover, only a small proportion of lung cancers in females can be attributed directly to smoking (Chakraborty et al., 2010), indicating the majority of cases are directly related to female sex. Although, it was shown that females diagnosed with lung cancer have better survival than males (Cerfolio et al., 2006), no conclusive evidence exists on the effects oestrogens and female gender might have on survival in lung cancer (Schwartz et al., 2007). Additionally, the mortality of female patients receiving anti-oestrogen treatment for breast cancer significantly reduces the mortality associated with lung cancer (Bouchardy et al., 2011). Nonetheless, akin to PAH, female sex was recognised as a risk factor for development of lung cancer, indicating the lung might be exceedingly sensitive to oestrogen, which might be directly associated with the pro-proliferative environment resulting in disease pathogenesis and onset. Inhibition of aromatase in the cHx and Su5416/Hx models of PAH resulted in the attenuation of the models, however only in female animals (Mair et al., 2014).

1.5.5.2 Oestrogens in Cardiovascular Disease

Cardiovascular disease (CVD) is one of the leading causes of death in the developed countries according to WHO. Female sex is associated with later onset of CVD, as well as reduced mortality of females compared to males. For example, females present with CVD 7–10 years later than males (Mosca et al., 2011) and that coronary heart disease (CHD) in

pre-menopausal women is predominantly associated with smoking (Prescott et al., 1998). Interestingly, Women's Ischemia Syndrome Evaluation (Wise) revealed that oestrogen deficiency in pre-menopausal women is associated with a significantly increased risk of coronary artery disease (CAD) (Bairey Merz et al., 2003). Moreover, the onset of menopause significantly increases the risk of CHD in women, and is associated with exacerbation of the disease (Matthews et al., 1989). Hence, it appears that endogenous oestrogens might be mediating protective effects within the cardiovascular system.

Losordo and colleagues investigated the association of ER expression with significant CAD, showing reduced expression of receptors in samples derived from female individuals with CAD (Losordo et al., 1994). ERα and ERβ differentially regulate the expression of identical target genes within the same tissue (Lindberg et al., 2003). The relative expression of ER isoforms in cell types within the CVS might be an important aspect of oestrogen-mediated protective effect. Indeed, activation of ERβ in rat vascular SMCs led to enhanced expression of the iNOS, while activation of ERα resulted in negative regulation of the same gene (Tsutsumi et al., 2008). Interestingly, the expression of ERα in the vascular ECs varied in pre-menopausal women, depending on the stage of oestrous cycle and oestrogen levels. In post-menopausal women, the levels of ERα are significantly reduced compared to pre-menopausal women during late follicular stage (Gavin et al., 2009). Indeed, the duration of oestrogen stimulation affects the expression of both ER isoforms in vascular endothelium, with chronic exposure resulting in enhanced expression of ERα and decreased expression of ERβ (Ihionkhan et al., 2002). Moreover, cHx led to increased expression of ERβ in ECs of PAs (Lahm et al., 2012). A genome-wide expression profiling study to characterise genes specifically involved in the pathogenesis of PAH demonstrated that ERα is upregulated in PAH patients of both sexes (Rajkumar et al., 2010). Numerous genes regulated by ERα, involved in TGFβ and NO signalling, are altered in PAH. Recently the relative expression of ER in PASMCs derived from control subjects and PAH patients subjects of both sexes was reported (Wright et al., 2015). There is significantly more ERβ expressed in PASMCs derived from male PAH patients compared to cells derived from control subjects, while there is significantly more ERα expressed in cells derived from female PAH patients compared to control subjects. Hence, it appeared that the changes in the expression of ERs might be associated with disease setting, having significant consequences for oestrogen signalling. Treatment of ovariectomised female mice with 17βE2 attenuated cardiac hypertrophy following transaortic constriction compared to matched male animals, which were treated with vehicle (van Eickels et al., 2001). These observations might be dependent on ERβ

isoform (Skavdahl et al., 2005). Interestingly, the ERβ isoform might also be responsible for normal lung development and physiology (Patrone et al., 2003).

1.5.5.3 Androgens in Cardiovascular Disease

Testosterone is the main male sex hormone, synthesised from cholesterol through the formation of pregnenolone, progesterone and androstenedione, which is then transformed to testosterone by the activity of 17β-hydroxysteroid dehydrogenase type 3. As significantly more men are affected by CVD, it was long perceived that female sex hormones are protective. Nonetheless, recent epidemiological data indicated that increased prevalence of atherosclerosis, coronary heart disease (CHD), metabolic syndrome and hypertension in men are associated with decreased plasma levels of testosterone (Jones;Jones, 2010). Moreover, lower testosterone levels are also correlated with increased mortality in CVD and cancer (Khaw et al., 2007;Ponikowska et al.), while higher levels were shown to reduce the risk of cardiovascular events (Ohlsson et al., 2011). Testosterone mediated vasodilation in numerous vascular plexus, including pulmonary vasculature (Smith et al., 2008). Indeed, in men with chronic heart failure (CHF), the administration of exogenous testosterone improved the afterload of the LV, probably through decreasing systemic vascular resistance and consequently increased cardiac output (Pugh et al., 2003).

1.6 Cytochrome P450 Enzymes

In humans, there are approximately 57 genes encoding functional enzymes belonging to the CYP superfamily, while the existence of additional 58 pseudogenes was also described (Nelson et al., 2004a). The members of CYP450 superfamily, constituting the principal assembly of enzymes involved in oxidative metabolism of endogenous and exogenous compounds, are divided into families and sub-families, according to the extent of sequence identity, with members of CYP450 families exhibiting extensively overlapping substrate specificities (Nebert and Russell, 2002). As discussed above, CYP450 enzymes catalyse a variety of oxidative biotransformation reactions, including hydroxylation, S-oxidation, N- and O-dealkylation, and epoxidation of numerous compounds: i) endogenous molecules, including steroid hormones, sterols, eicosanoids and retinoids, and ii) exogenous compounds, including numerous drugs and environmental pollutants playing an important role in the biosynthesis and detoxification of reactive compounds (Nebert and Russell, 2002). The CYP450 enzymes were implicated in the bioactivation of inert toxins, possibly

leading to mutagenesis and carcinogenesis (Nebert and Russell, 2002). To illustrate the importance of CYP450 enzymes in the biosynthesis and metabolism of endogenous compounds, six enzymes participate (cholesterol desmolase, 11β-hydroxylase, aldosterone synthesizing enzyme, 17α-hydroxylase, aromatase and 21-hydroxylase) in the process of steroidogenesis, synthesis of oestrogens and interconversion of androgens into oestrogens (Nebert and Russell, 2002). Although generally, CYP450 enzymes are found in most tissues, due to their role in detoxification, it is believed that the majority of CYP450 enzymes are widely expressed in the liver. There is also significant, albeit lower, expression in other tissues which are regularly exposed to environmental pollutants, such as the lung, gastrointestinal tract and kidney (Nebert and Russell, 2002;Krishna and Klotz, 1994). According to a review by Lee and colleagues, numerous CYP450 enzymes are also present in target tissues for oestrogen (Lee et al., 2003). Most CYP450 enzymes exhibit tissue-specific expression pattern (Nishimura et al., 2003). Interestingly, not all CYP isoforms are expressed in the liver. Certain isoforms, e.g. CYP1A1, were found to be extensively expressed in adult liver, while their expression in foetal liver was only marginal, albeit the most abundant expression of CYP1A1 was detected in the lung (Nishimura et al., 2003). Additionally, isoforms exhibiting region-specific catalytic selectivity, such as CYP1B1, are essentially only expressed in extrahepatic oestrogen-responsive tissues (Lee et al., 2003).

CYP450 enzymes are haemoprotein monooxygenases, meaning the active site contains a protoporphyrin-IX haem with an iron(III) covalently linked to the protein by the sulphur atom of a proximal cysteine residue (Meunier et al., 2004). Moreover, the conjugated ring system of the haem is vital for enzyme's catalytic activity. Generally, the reaction catalyse by CYP450 enzymes can be written as:

$$NAD(P)H + H^+ + O_2 + RH \rightarrow NADP^+ + H_2O + ROH,$$

where the required electrons for the reaction are provided by the cofactor NADPH from the associated P450 reductase enzyme (Meunier et al., 2004). The catalytic cycle of CYP450s enzymes is initiated upon the binding of a suitable molecule in the active site, resulting in altered arrangement of iron (III) ion. The electron transfer from associated P450 reductase to the iron (III) centre, resulting in its transition to ferrous state, produces a highly active reduced form of CYP450 enzyme, which can bind an oxygen molecule. Following the binding of oxygen molecule to the ferrous cytochrome, the second reduction step, which is the rate-limiting, occurs. The CYP450 enzyme catalytic cycle is terminated with two

protonation steps, resulting in the formation of a peroxide complex, ultimately leading to the release of a water molecule and oxidised substrate, and the decoupling of the reaction (Meunier et al., 2004).

1.6.1 *NADPH-dependent Oxidative Metabolism of Oestrogens*

NADPH-dependent oxidative metabolism of oestrogens by CPY450 enzymes represents the first step in the body's attempt towards inactivation of these steroid hormones, containing their biological effects in target tissues, leading to their eventual excretion. Recent evidence indicates, oestrogen metabolism might not be as straightforward, as numerous metabolites have been shown to exert biological effects, which are independent of their parental hormone (Lee et al., 2003;Zhu and Conney, 1998). See Figure 1–17 for a summary of the biological effects of oestrogens in pulmonary circulation.

Generally, the expression CYP450 enzymes is induced by the presence of enzymes' substrate, especially for the many inducible extrahepatic CYP450 isoforms. The induction of CYP450 enzymes is a closely regulated process, allowing cells to appropriately respond to exposure of environmental pollutants (Williams et al., 2005). CYP450 enzymes exhibit certain substrate specificity, also resulting in their preferential site of oxidation reaction within the substrate molecule. Hence, the expression pattern of CYP450 enzymes might affect the local production of oestrogen metabolites. Lee and colleagues have recently characterised oxidative metabolism of 17βE2 and E1 of 15 different CYP450 enzymes (Lee et al., 2003).

Although numerous CYP450 isoforms metabolise 17βE2, some exhibit very little or no activity for oxidative metabolism of this hormone. The positions of hydroxylation for different cytochrome P450 enzymes are summarised in Table 1-4. Predominant hydroxylation positions in Table 1-4 are shown in bold. With 17βE2 as substrate, CYP1A1 and CYP1A2, isoforms exhibit high catalytic activity for hydroxylation at position C2. Most CYP isoforms also exhibit catalytic activity for hydroxylation at position C4. Interestingly, numerous isoforms have also shown a detectable activity for the conversion of 17βE2 to E1, with CYP2A6 isoform having most substantial activity for this reaction. The catalytic activity of CYP1A2 for E1 being almost two-fold than that for 17βE2. Although CYP1B1 catalyses hydroxylation at positions C2 and C4 with 17βE2 and E1 as substrates, with the

latter reaction being favoured. While CYP1A1 has only weak activity for hydroxylation of 17βE2 at position C16α, and no activity for hydroxylation of E1 at the same position, CYP3A4 exhibits high activity for these two reactions. Moreover, the ratio of 4OHE1/2OHE1 is almost two-fold compared to ratio 4OHE2/2OHE2 for this specific isoform. Out of all the isoforms investigated by Lee and colleagues, CYP1B1 possesses a predominant activity for hydroxylation at position C4, with the highest ratio of 4OHE2/2OHE2 (Lee et al., 2003). Nevertheless, the absolute amount of these metabolites formed by CYP1B1 is still lower than that of CYP1A1 and CYP1A2 (Lee et al., 2003).

Table 1-4: Positions of hydroxylation for different cytochrome P450 enzymes.

Cytochrome P450 Isoform	Substrate	Position of Hydroxylation
CYP1A1/2	17βE2	**C2**/**C4**/C6α/C7α/C15α/C16α/C6β/C12β/C16β
CYP1A1/2	E1	C2/**C4**/C6α/C7α/C15α/C16α/C6β/C12β/C16β
CYP1B1	17βE2	C2/**C4**/C6α/C7α/C15α/C16α/C6β/C12β/C16α/C16β
CYP1B1	E1	C2/**C4**/C6α/C7α/C15α/C16α/C6β/C12β/C16α/C16β
CYP3A7	17βE2	/
CYP3A7	E1	C2/**C4**
CYP2C8	17βE2	C2/C4/C6α/C7α/C15α/C16α/C6β/C12β//C16β
CYP2C8	E1	C2/C4/C6α/C7α/C15α/C16α/C6β/C12β/C16α/C16β
CYP3A4	17βE2	C2/C4/C6α/C7α/C15α/**C16α**/C6β/C12β/C16α/C16β
CYP3A4	E1	C2/C4/C6α/C7α/C15α/**C16α**/C6β/C12β/C16α/C16β

1.6.2 *Aryl Hydrocarbon Receptor Signalling Pathway*

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor, belonging to the superfamily of basic helix-loop-helix (bHLH)/Per-Arnt-Sim (PAS) transcription factors (Gu et al., 2000), and is associated with regulation of biological response to polycyclic aromatic hydrocarbons (PCAHs) and other environmental pollutants. AhR was shown to regulate the expression of xenobiotic-metabolising enzymes, including CYP450 enzymes. AhR has a role in development and physiological homeostasis. AhR knock-out mice demonstrated the is an association between AhR and the induction of target genes such as *CYP1A1*, *CYP1A2*, and *CYP1B1* upon stimulation with a potent, selective AhR agonist, 2,3,7,8-tetrachlorodibenzodioxin (TCDD) (Fernandez-Salguero et al., 1995). Moreover,

AhR knock-out animals exhibited numerous phenotypical abnormalities, including decreased number of immune cells in the periphery, observed in young animals, while mature animals also presented cardiac hypertrophy with fibrosis, abnormal blood vessel formation in the liver, and calcification of the uterus (Fernandez-Salguero et al., 1995). AhR-deficient mice did not develop tumours following injection with a known carcinogen benzo[a]pyrene (B[a]P), indicating the possible role of AhR signalling pathway in carcinogenesis and tumour progression (Shimizu et al., 2000). This may be through induction of enzymes involved in the activation of inactive pro-carcinogenic compounds to active carcinogens. Equally, previously, an association between *CYP1A1* induction and tumorigenesis in the lung was described (Shimada and Fujii-Kuriyama, 2004).

The AhR is ubiquitously expressed in most tissues and cell types, with significant levels of cDNA present in the placenta, lung, kidney, and liver (Dolwick et al., 1993), and lower expression in the heart (Carver et al., 1994). The expression of AhR was mainly immunolocalized to epithelial cells in these organs. Although it was shown that AhR is expressed in ECs derived from pulmonary microvasculature (Zhang and Walker, 2007), comprehensive literature on basal (and pathological) expression and localisation of AhR in pulmonary vasculature was lacking. Most recently, immunohistochemistry technique was employed to determine the localisation of AhR expression in human lung tissue, showing the receptor is mainly present in ECs and SMCs of pulmonary veins and arteries, while it was also detected in bronchial and alveolar sac epithelia (Li et al., 2017).

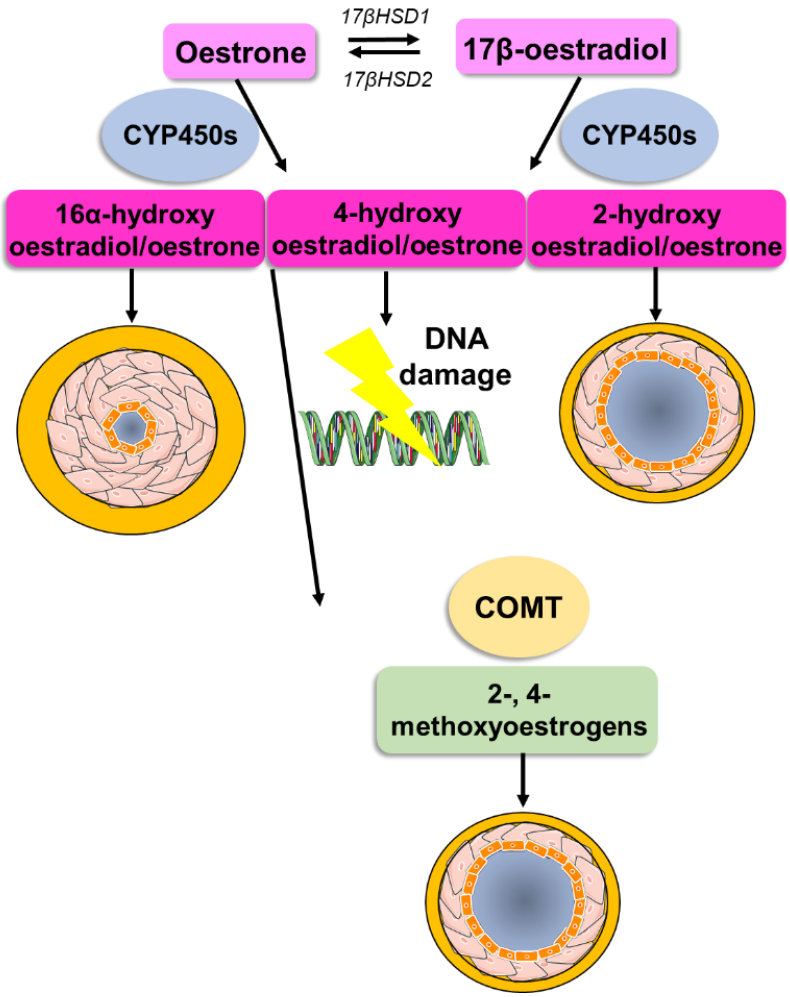


Figure 1–17: Schematic representation of the role of oestrogen metabolites in the onset and progression of pulmonary arterial hypertension.
Oestrogens are metabolised by cytochrome P450 enzymes, which catalyse the introduction of hydroxyl group at numerous positions within the steroidal backbone of these hormones, resulting in the formation of numerous oxidised oestrogen metabolites. Most commonly, 2- and 4-hyroxyoestrogens are formed, with small proportion of 16α-hydroxy-oestrogens. The latter have been shown to stimulate proliferation of smooth muscle cells, resulting in extensive vascular remodelling. The 2-hydroxyoestrogens exhibit anti-proliferative properties, while the 4-hydroxyoestrogens are mostly associated with the production of reactive oxygen species, associated with DNA damage. Moreover, 2- and 4-hyroxyoestrogens can be further metabolised to 2- and 4-methoxyoestrogens, which were shown to possess anti-proliferative properties, and were suggested to oppose the onset of pulmonary arterial hypertension in animal models.

1.6.3 *Activation of Aryl Hydrocarbon Receptor*

The AhR signalling pathway (see Figure 1–18 for summary) is initiated by endogenous/exogenous ligand diffusion through the plasma membrane into the cell, and its consecutive binding with the cytosolic AhR protein, which is associated with heat shock protein 90 (Hsp90), prostaglandin E synthase 3 (P23) and AhR associated 9 (AIP/ARA9) (Pongratz et al., 1992;Kazlauskas et al., 2000;Carver et al., 1998;Carver and Bradfield, 1997;Ma and Whitlock, 1997). The AhR-associated protein complex maintains the receptor in a conformation suitable for ligand binding and prevents the translocation of the receptor into the nucleus in the absence of a suitable ligand. The ligand binding activates AhR, leading to the dissociation of AIP and P23 proteins, while the ligand-receptor complex is then translocated into the nucleus, where the Hsp90 dissociates from AhR, enabling its heterodimerization with AhR nuclear translocator (ARNT or HIF1β) (Probst et al., 1993;Hankinson, 1995). AhR-ARNT complex, yielding a functional transcription factor, is then able to bind to dioxin- and/or xenobiotic-response elements (DRE/XRE) in the DNA sequence, resulting in altered transcription of target genes, amongst which *CYP1A1*, *CYP1A2*, and *CYP1B1* are the best characterised (Denison et al., 2011). The AhR target genes also include NAD(P)H dehydrogenase (quinone 1) (*NQO1*), UDP glucuronosyltransferase 1 family polypeptide A cluster (*UGT1A*), and glutathione S-transferase alpha 1 (*GSTA1*) (Brauze et al., 2017). CBP/p300 is a co-activator of the AhR-ARNT complex (Kobayashi et al., 1997). The AhR signalling is attenuated by: i) the binding of the ARNT with the aryl hydrocarbon receptor repressor (AhRR), which is under the transcriptional regulation of AhR, resulting in decreased availability of functionally active transcription factor (Mimura et al., 1999), or ii) ubiquitin/proteasome degradation of the ligand-bound AhR (Davarinos and Pollenz, 1999).

1.6.4 *The Function of Aryl Hydrocarbon Receptor in the Cardiovascular System*

The role of AhR in the CVS was first suggested by studies in AhR-deficient mice. With age these developed cardiac hypertrophy with fibrosis, suggesting AhR pathway may be imperative in cardiac development (Carreira et al., 2015;Vasquez et al., 2003). The absence of AhR resulted in increased expression of HIF1α and consequently vascular endothelial growth factor (VEGF) (Thackaberry et al., 2002), leading to compensatory hypertrophy of

cardiomyocytes (Vasquez et al., 2003). AhR-deficient mice developed systemic hypertension associated with significantly increased levels of circulating and tissue vasoactive molecules, such as ET-1 (Lund et al., 2003). AhR deficient mice have a potentiated response to changes in pO_2, leading to significant Hx-mediated increase in in ET-1 and systemic blood pressure (Lund et al., 2008). Activation of the AhR signalling pathway results in the induction of numerous *Phase I* drug metabolising enzymes, including CYP1A1, and leads to increased production of ROS, which in turn, may lead to endothelial dysfunction (Park et al., 1996). There is a correlation between endothelial dysfunction, characterised by disrupted integrity and increased permeability, and activation of AhR pathway (Toborek et al., 1995). Interestingly, there are neonatal vascular structures in AhR-deficient mice, indicating this signalling pathway might be associated with maturation of the vessels, during which the neonatal vasculature is replaced by matured vessel plexus (Lahvis et al., 2000). Due to the presence of abnormal vascular architectures in AhR-deficient mice, it can be inferred this signalling pathway may be also imperative in normal vascular development (Lahvis et al., 2000).

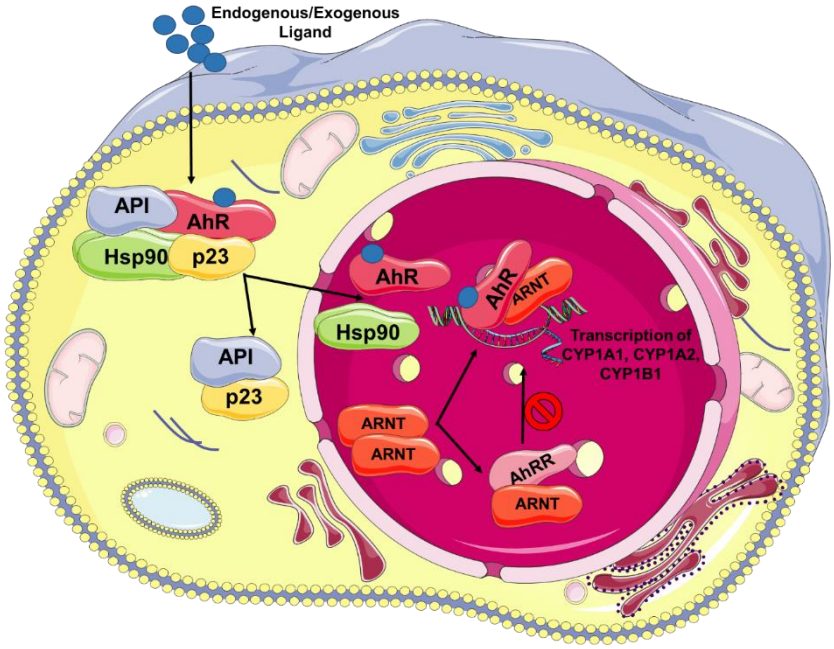


Figure 1–18: Aryl hydrocarbon receptor signalling pathway.

Binding of a suitable ligand to AhR in the cytoplasm, causes the dissociation of accessory proteins, resulting in the translocation of the activated receptor into the nucleus. In the nucleus, the receptor heterodimerises with ARNT, and then binds to dioxin or xenobiotic response elements, resulting in altered transcription of target genes.

AhR=Aryl hydrocarbon receptor, API (ARP9)= Aryl hydrocarbon receptor associated 9, Hsp90=Heat shock protein 90, p23=Prostaglandin E synthase 3, ARNT= AhR nuclear translocator, AhRR= AhR repressor.

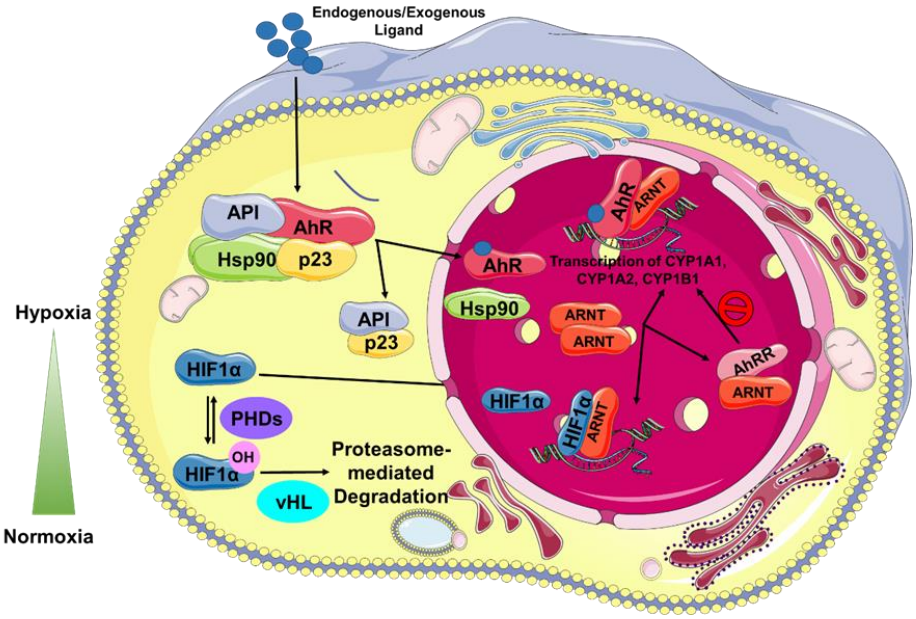


Figure 1–19: The interaction of hypoxia inducible factor signalling pathways and the aryl hydrocarbon receptor signalling pathway.

In hypoxia, the proteasomal degradation of HIF1α is diminished, as the activity of PHDs and vHL is reduced due to decreased partial pressure of oxygen. In such circumstances, HIF1α is translocated into the nucleus, where it competes with AhR for binding with ARNT, thereby reducing the availability of ARNT for signalling through the AhR pathway.

HIF1α=Hypoxia inducible factor 1α, PHDs= Prolyl hydroxylase enzymes, vHL= von Hippel-Lindau tumour suppressor protein

1.6.5 *Interactions between the AhR Signalling Pathway and Hypoxia-Inducible Factor Signalling*

Figure 1–19 provides a summary of the interactions between the AhR signalling pathway and HIF signalling. Hypoxia-inducible factors (HIFs) belong to the same subfamily of transcription factors as AhR, the bHLH/PAS family. Six different HIFs exist, with members HIF1–3α denoting hypoxia-inducible factors, and members HIF1–3β denoting AhR nuclear translocators. While all HIFs are constitutively expressed, only the nuclear translocators are stable in Nx conditions. Unlike with AhR, activation of HIFs does not require the binding of a suitable endogenous/exogenous ligand, as the transcription factors respond directly to changes in the partial pressure of oxygen in the cellular environment. The regulation of HIF1α activity occurs by hydroxylation-dependent proteasome-mediated degradation of these transcription factors. In Nx conditions, prolyl hydroxylase enzymes (PHDs) catalyse hydroxylation of two conserved proline residues of HIF1α, for which the enzymes require molecular oxygen as a co-factor. Three different PHDs exist, with PHD2 exhibiting the highest affinity for HIF1α (Berra et al., 2003). A complex of the von Hippel-Lindau (vHL) tumour suppressor protein and a ubiquitin ligase E3 then binds to the hydroxylated HIF1α (Kamura et al., 2000), leading to poly-ubiquitination of and proteasomal degradation of HIF1α. Mitogen-activated protein kinases p42/p44 (Erk1/2) mediates phosphorylation of HIF1α *in vitro*, leading to increased HIF1α-dependent transcriptional activity (Richard et al., 1999). In Hx, the activity of PHDs is greatly reduced or inactive due to the absence of a suitable co-factor. Therefore, the HIF1α is stabilised, as its proteasome-mediated degradation is greatly decreased. This leads to the translocation and accumulation of the transcription factor into the nucleus, where it heterodimerises with ARNT, also named HIF1β (Jiang et al., 1996). The heterodimer binds to Hx response enhancer elements within the DNA sequence (Liu et al.), resulting in transcription of target genes involved in angiogenesis, metabolism, apoptosis, etc. (Semenza, 2009). Equally, HIF2α, first characterised by Tian and colleagues, has approximately 50% of sequence homology with HIF1α (Tian et al., 1997), binds to HIF1β, resulting in the induction of specific target genes as shown by (Ema et al., 1997). HIF2α is abundantly expressed in the developing lung and might have an important role in the normal vascularisation of the lung (Ema et al., 1997).

Hence, it appears that HIF1β, involved in AhR- and HIF1α-mediated signalling, is an important modulator of signalling pathways through its association with several transcription factors, forming active transcription complexes. Moreover, competitive recruitment of

ARNT (HIF1β) might result in repression of genes targeted by these two signalling pathways (Nie et al., 2001). A reciprocal repression of the AhR and HIF1α pathways exists in certain cell lines, with the degree of repression depending on the sequence of introduced stimuli (Nie et al., 2001). Interestingly, HIF1α possesses a very high affinity for ARNT, resulting in significant competition with the AhR (Gradin et al., 1996). Nonetheless, these two distinct signalling pathways may also be interacting through other co-activators (Kobayashi et al., 1997;Ebert and Bunn, 1998). In some cell lines there is no reciprocal repression of the AhR and HIF1α pathways (Nie et al., 2001). Hence, the interaction between these signalling pathways may differ with respect to cell type, tissue and species (Chan et al., 1999).

1.6.6 *Interaction of the AhR Signalling Pathway with other Signalling Pathways*

There might be cross-talk between the AhR and ER signalling pathways (see Figure 1–20), inferring AhR agonists might modulate the activity of ERs (Umbreit and Gallo, 1988). The anti-oestrogenic effects of TCDD, a potent AhR agonist, resulting in disturbed oestrogenic cycle in female mice were documented (Umbreit and Gallo, 1988). Additionally, administration of a single dose of TCDD results in significantly reduced ER levels in the liver and uterus (Romkes et al., 1987). TCDD administration abolishes 17βE2-induced cell proliferation due to interference with expression of several cyclins required for advancement through cell cycle (Buchanan et al., 2002). In breast and endometrial cancer cell lines, TCDD-stimulated *CYP1A1* gene induction only occurred in ER-positive cell lines (Ricci et al., 1999). Moreover, TCDD treatment resulted in enhanced expression of TGFβ, was also implicated in the pathogenesis of PAH (Ricci et al., 1999). In rats the exposure to TCDD may lead to the onset of breast and ovarian cancer (Jenkins et al., 2007). In the absence of 17βE2, activation of AhR receptor by 3-methylcholanthrene (3MC) resulted in induction of ERE-mediated transcription (Ohtake et al., 2003). Ligand-activated AhR, upon its translocation into the nucleus and heterodimerisation with ARNT, associates with either of the unliganded ERs, further recruiting the p300 co-activator (Ohtake et al., 2003). Evidence further suggested that in the absence of 17βE2, ligand-bound AhR might recruit ERα through specific protein interaction, resulting also in the induction of ERα-target genes (Ohtake et al., 2003;Matthews et al., 2005). Nonetheless, oestrogen concentrations, depending on numerous aspects such as age and type of tissue, might define whether AhR ligands have oestrogenic or anti-oestrogenic effects (Ohtake et al., 2003). ARNT might function as a co-activator for both ER isoforms, through association with the ligand binding domain, in an

17βE2-dependent manner (Brunnberg et al., 2003). AhR-target genes are only induced in ER-positive breast cancer cell lines following treatment with TCDD, while no such response was noticeable in ER-negative cell lines (Vickers et al., 1989). Independently, several research groups observed the recruitment of ERα to XRE/DRE in the presence of ligand-activated AhR (Beischlag and Perdew, 2005;Matthews et al., 2005). Matthews and colleagues further reported that knock-down of ERα expression resulted in significantly diminished induction of *CYP1A1* gene in the presence of TCDD (Matthews et al., 2005). The interaction of ERα with the AhR signalling pathway may represent a negative regulation mechanism of oestrogen signalling (Matthews and Gustafsson, 2006).

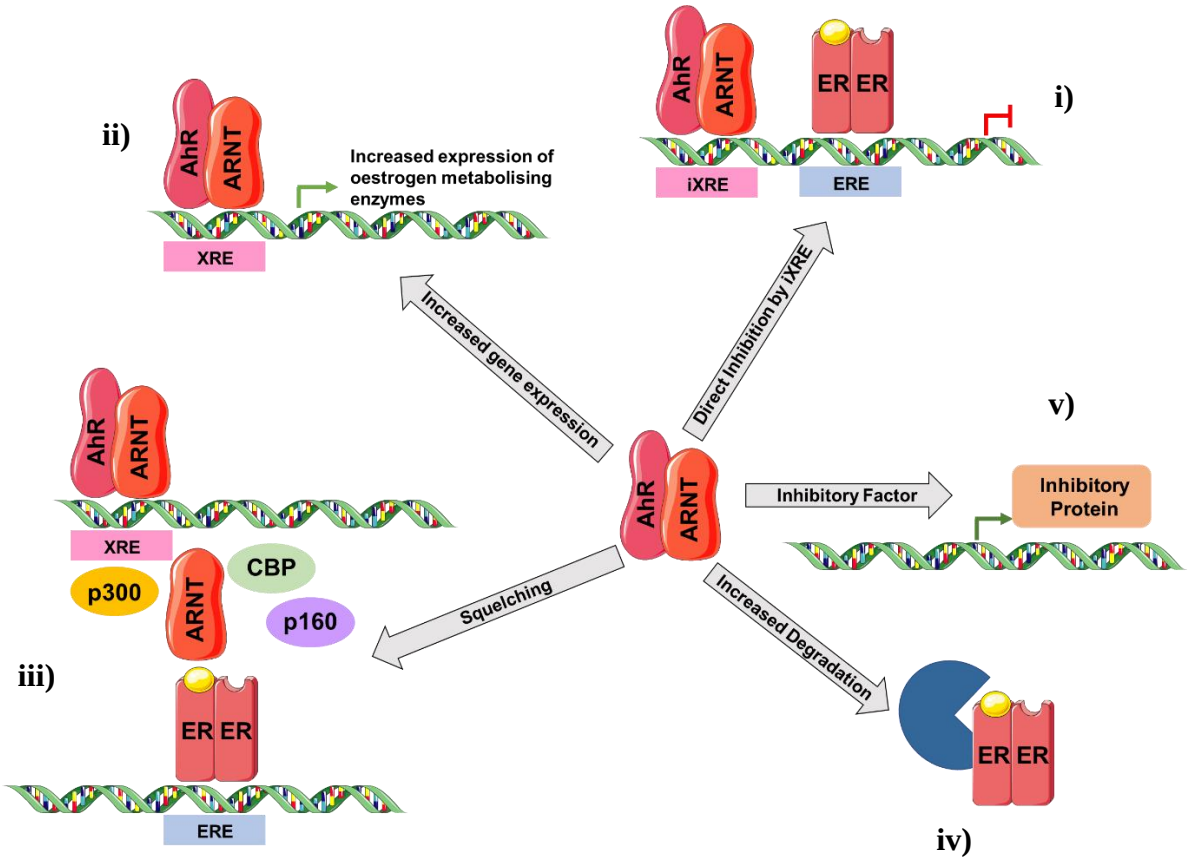


Figure 1–20: Schematic representation of interaction between oestrogen signalling pathway and aryl hydrocarbon receptor signalling pathway.
The proposed mechanism of interaction between oestrogen and AhR signalling pathways is by i) direct inhibition of oestrogen signalling through the binding of AhR/ARNT heterodimer to inhibitory response elements within the oestrogen target genes, ii) through induction of oestrogen metabolising enzymes by the AhR signalling pathway, resulting in increased production of metabolites with lower affinity for oestrogen receptors, iii) competing for and/or squelching of coactivator proteins shared by both signalling pathways, iv) increased proteasomal degradation of oestrogen receptors through activation of AhR signalling pathway, or v) induction of gene encoding for an inhibitory protein through activation of AhR signalling pathway. Adapted from (Matthews and Gustafsson, 2006).

XRE=Xenobiotic response element, ERE=Oestrogen response element, p300=Histone acetyltransferase p300, CBP= CREB-binding protein, p160= Nuclear Receptor Coactivator p160

1.6.7 ***CYP1B1***

Cytochrome P450, family 1, subfamily B, polypeptide 1 (CYP1B1) isoform is generally considered as an extrahepatic CYP450 isoform, exhibiting constitutive as well as inducible expression (Eltom et al., 1999). Post-translational mechanisms may also be involved in the regulation of CYP1B1 expression, by showing the activation of AhR by 3MC had no effect on the *CYP1B1* gene expression levels, while induction was still observed on protein level (McFadyen et al., 2003). This isoform is highly expressed in foetal brains and kidneys (Hakkola et al., 1997). The expression of CYP1B1 in the lung tissue was shown by Spivack and colleagues, who evaluated its role in lung carcinogenesis, showing significantly higher levels are displayed in tumours (Spivack et al., 2001). In the CVS, vascular SMCs display the highest expression of CYP1B1, while the expression in the ECs is much lower (Zhao et al., 1998a). Many single nucleotide polymorphisms were identified in the gene encoding *CYP1B1*, and numerous polymorphisms indeed were associated with carcinogenesis, including: i) cancers occurring in oestrogen-target tissues and lung cancer (De Vivo et al., 2002;Wang et al., 2011;Goodman et al., 2001;Murray et al., 1997;Xu et al., 2012), or ii) increased activation of pro-carcinogens (Shimada et al., 1996). Interestingly, females who present specific *CYP1B1* polymorphisms may have increased risk of lung cancer (Xu et al., 2012).

1.6.7.1 The Role of CYP1B1 in Cardiovascular Disease

Studies showed that CYP450 enzymes might be involved in the pathogenesis of CVD through metabolism of AA, producing either potent vasoconstriction mediators such as i) 20-hydroxyeicosatetraenoic acid (20-HETE) (Fleming, 2005), which exert their biological effects at relatively low concentrations in small arteries and arterioles (Harder et al., 1995), or ii) epoxyeicosatrienoic acids (EETs), which are potent vasodilators in small vessels (Harder et al., 1995). Therefore, it appears CYP450 enzymes can regulate the vascular tone, and these metabolites might also regulate pulmonary and cardiac functions by activation of ion channels (Ma et al., 1993;Harder et al., 1995). In addition to their vasoconstrictor properties, 20-HETE induce a significant concentration-dependent increase in cell proliferation of VSMCs, with the proliferation probably mediated through the MAPK signalling pathway (Uddin et al., 1998). Hence, CYP1B1 might play an important part in vascular remodelling of small resistance arteries (Ding et al., 2013). Inhibition of CYP1B1 activity in spontaneously hypertensive rats by TMS reduced cardiovascular hypertrophy,

vascular reactivity, endothelial dysfunction and systemic blood pressure (Jennings et al., 2014). Therefore, the research into the role of CYP1B1 in PAH pathogenesis has not been unmerited and has led to important findings regarding its role in the disease setting.

1.6.7.2 The role of CYP1B1 in Pulmonary Arterial Hypertension

Significant expression levels of CYP1B1, which primarily catalyses the hydroxylation of 17βE2 and E1 to 4OHE2/E1, in the lung (Spivack et al., 2001;Zhao et al., 1998b) indicated local oestrogen metabolite production might have important implications in the pathology of lung cancer, CVD and PAH. Preliminary gene expression experiments indicated the expression of *CYP1B1* gene may be sex-specific, with 10-fold decrease in expression levels in lymphocytes associated with PAH onset in female *BMPRII* mutation carriers, but not in males (West et al., 2008a). Recently, White and colleagues explored the differences in gene expression in SERT+ animal model of PAH, to identify genes which might be associated with the development and progression of PAH, showing that *CYP1B1* gene is significantly upregulated (White et al., 2011b). The basal expression of CYP1B1, as assessed by *in-situ* immunolocalization and protein blotting techniques, is significantly higher in IPAH patients compared to control subjects, while the expression is further enhanced in the presence of 17βE2 or 5-HT (White et al., 2012;White et al., 2011b). Additionally, in *BMPRII* mutation carriers, the presence of *CYP1B1* polymorphisms is associated with significantly higher penetrance of PAH (Austin et al., 2009). The absence or inhibition of CYP1B1 appeared to attenuate some of the pathophysiological characteristics of PAH to various degrees. Namely, CYP1B1 inhibition significantly reduced pulmonary vascular remodelling, RVH, RV systolic pressure and the appearance of occlusive lesions in small PAs in cHx/SU5416 model of PAH (White et al., 2012). 16αOHE1, a metabolite produced by CYP1B1, whose increased circulating levels are associated with increased risk of breast cancer in humans and mice (Bradlow et al., 1986;Bradlow et al., 1985), was shown to stimulate hyper-proliferative response in PASMCs derived from patients exhibiting PAH (White et al., 2012). This was attenuated by co-treatment with potent CYP1B1 inhibitor, indicating the direct role of this CYP450 isoform in the induction of proliferative response in PAH. To demonstrate the role of 16αOHE1 in the onset of PAH, White and colleagues administered the metabolite to mice, showing animals developed increased RV systolic pressure, pulmonary vascular remodelling and RVH (White et al., 2012), indicating CYP1B1 and its metabolites may be imperative modifiers associated with increased probability for the onset of PAH.

1.6.8 ***CYP1A1***

Although cytochrome P450, family 1, subfamily A, polypeptide 1 (CYP1A1) isoform is generally considered as an extrahepatic CYP450 isoform, it is substantially expressed in the foetal liver during embryonic development (Omiecinski et al., 1990;Kitada and Kamataki, 1994), with its levels reducing quickly, resulting in miniscule expression in the adult liver. CYP1A1 is not constitutively expressed in various tissues examined of the C57BL/6N mouse, however induction with 3MC caused significant increase in the lung, liver and ECs of blood vessels (Dey et al., 1999). *CYP1A1* is barely detectable in the mouse heart (Choudhary et al., 2003). Due to its inducible nature, hundred-fold differences in the expression of *CYP1A1* were reported human lung microsomes (Zanger and Schwab, 2013;Kim et al., 2004). More than ten different polymorphisms of the *CYP1A1* gene exist, with differing allele frequency, were characterised (Ingelman-Sundberg, 2004;Monostory et al., 2009;Sim, 2015). Although some research groups reported an association of *CYP1A1* polymorphisms with increased risk of lung, and oestrogen-related cancers, such as cervical and breast cancer (Wright et al., 2010;Sergentanis et al., 2012;Singh et al., 2007), no conclusive association was established (Masson et al., 2005). However, the *MspI* polymorphism of *CYP1A1* gene, is associated with increased risk of CVD, especially in smokers (Wang et al., 2002). *CPY1A1* genetic polymorphisms may exhibit racially distinct patterns (Cosma et al., 1993); leading to current insufficiency of conclusive evidence of whether *CYP1A1* polymorphisms are functionally significant in disease onset. Nevertheless, it needs to be considered that simultaneous presence of a polymorphic variant of *CYP1A1* exhibiting increased metabolic rate of oestrogen metabolism and a variant of *COMT* and/or *GST* exhibiting low catalytic activity, might result in the accumulation of hydroxylated oestrogen metabolites, leading to increased production of oestrogen quinones and semi-quinones, both having carcinogenic potential (Kisselev et al., 2005). The presence of *COMT* variant exhibiting low catalytic activity might be of special importance in disease, as reduced formation of methoxyoestrogens can affect the negative feedback regulation of oestrogen metabolism catalysed by i) CYP1A1, and ii) CYP1B1 (Dawling et al., 2003). Although the main metabolic pathway catalysed by CYP1A1 appears to be hydroxylation at position C2, according to Badawi and colleagues, this isoform exhibits higher catalytic activity for 16α-hydroxylation compared to CYP1B1, representing the principle contributor to formation of 16α-hydroxy-oestrogens (Badawi et al., 2001).

1.6.8.1 The Physiological Role of CYP1A1

CYP1A1 null mice, generated by Dalton and colleagues, are viable and demonstrate no specific phenotype (Dalton et al., 2000). Interestingly, in *AhR* null mice the expression of CYP1A1 could not be detected, even upon treatment with potent AhR activator TCDD (Buters et al., 1999), indicating the expression of CYP1A1 is under complete control of AhR signalling pathway. Considering, the basal expression levels of CYP1A1 is low, it can be inferred that CYP1A1 is not imperative for normal physiological development (Buters et al., 1999), rather the most significant physiological role of this CYP450 isoform is associated with oxidative metabolism of environmental pollutants, resulting in their removal and/or activation. Nonetheless, CYP1A1 was shown to catalyse a highly stereoselective formation of hydroxylated AA and epoxyeicosatrienoic acid (Schwarz et al., 2004), which are associated with vasodilation and anti-apoptotic properties (Carroll et al., 1996;Zhang et al., 2001).

1.6.8.2 The Role of CYP1A1 in Cardiovascular Disease and Cancer

The level of CYP1A1 expression in individuals with established CVD, is significantly increased in the RV (Thum and Borlak, 2000). Moreover, in individuals with dilated cardiomyopathy, *CYP1A1* expression was detected in the right atrium and associated aortas (Elbekai and El-Kadi, 2006). *CYP1A1* expression in cardiomyocyte culture in rats was only observed in culture older than 24 hours (Thum and Borlak, 2000). CYP1A1 might also mediate its role in CVD by catalysing the conversion of omega 3 (n-3) polyunsaturated fatty acids (PUFAs) into highly potent vasodilator mediators. Deletion of the *CYP1A1* gene in C57Bl/6J mice resulted in diminished NO-dependent regulation of blood pressure (Agbor et al., 2014). CYP1A1 might therefore contribute to eNOS activation, and therewith associated regulation of vasodilation and blood pressure.

1.7 Sex and Pulmonary Arterial Hypertension

The predominance of female sex in PAH was reported in numerous epidemiological studies and registries, as previously described (Rich et al., 1987;Badesch et al., 2010;Humbert et al., 2006;Ling et al., 2012). Although recently it was reported that female predominance in the incidence of PAH is greatly diminished in elderly population (Hoeper et al., 2013), women generally develop a more severe disease as per NYHA functional classification (Farber et

al., 2015). The direct association between female sex hormones and the disease pathogenesis was first described in a report of six young women developing the disease after prolonged period of oral contraceptive use (Kleiger et al., 1976). A significant proportion of women with prolonged exposure to exogenous oestrogens, either through use of oral contraceptives or hormone replacement therapy, were also diagnosed with PAH (Sweeney and Voelkel, 2009). Nonetheless, oral contraceptives are currently only contraindicated in women exhibiting Class III/IV PAH as per NYHA functional classification (Thorne et al., 2006), due to otherwise prohibitively high morbidity and mortality risk during pregnancy (Bedard et al., 2009). Generally, permanent contraception or progesterone-containing contraceptive are recommended instead of oestrogen-containing contraceptives (Hemnes et al., 2015). Despite the strong prevalence of females developing the disease, it was shown that men generally exhibit poorer prognosis and higher mortality rate (Escribano-Subias et al., 2012;Humbert et al., 2010b), which could be directly related with poorer RV function in men (Swift et al., 2015;Marra et al., 2016). Higher serum levels of 17βE2 were reported to have a beneficial effect on the structure and/or function of the RV (Kawut et al., 2011;Ventetuolo et al., 2011), while higher serum levels of testosterone and DHEA appear to be associated with increased RV mass and volume (Ventetuolo et al., 2011). Therefore, unsurprisingly, in a population of subjects without signs of clinical CVD, women have higher RV ejection fraction (Tandri et al., 2006). It also appears that in CVD, women exhibit preserved RV function (Cleland et al., 2003). Additionally, in PAH, men were shown to have less favourable pulmonary and cardiac haemodynamic parameters (Shapiro et al., 2012), and exhibit a more significant gradual deterioration in RV function following introduction of therapy compared to women (Jacobs et al., 2014). Therefore, the superior survival of women diagnosed with PAH might be a direct consequence of the protective effects that 17βE2 has on the RV function and structure. Recently in a population of PAH patients, it was shown that administration of anastrazole did not cause RV dysfunction in men (Kawut et al., 2017).

Despite the evidence acquired in clinical studies, indicating exogenous oestrogens might be promoting the pathogenesis of PAH, the evidence gathered in animal studies contradicts human studies by showing endogenous 17βE2 might exert protective effects in pulmonary vasculature and the RV (Frump et al., 2015;Wang et al., 2018).

1.7.1 *Evidence of oestrogen in Experimental Models of Pulmonary Hypertension*

The discrepancy between the evidence derived from clinical and animal studies, leading to the recognition of the 'oestrogen paradox', will be discussed below. It should be noted however, that generally first reports on sex differences in animal models showed male animals developed more severe phenotypes compared to female animals in terms of mPAP and RV hypertrophy (Rabinovitch et al., 1981), which is now know to greatly depend on the choice of the animal model employed.

1.7.1.1 Oestrogens in the Monocrotaline-Induced Pulmonary Hypertension

The MCT model of PAH, is one of the classical and well-established models, and was extensively used extensively in the research of PAH. More than two decades ago, Farhat and colleagues investigated the effects of oestrogens in male rats administered with MCT, demonstrating that treatment with 17βE2 attenuated muscularisation of small PAs and RV hypertrophy (Farhat et al., 1993). Equally, although female animals appeared to develop less severe phenotype when exposed to MCT, exacerbated disease phenotype and poorer survival caused by ovariectomy was rescued by administration of 17βE2 (Tofovic et al., 2006; Yuan et al., 2013). Furthermore, in male rats with established PH phenotype, induced by MCT, treatment with 17βE2 resulted in the reversal of disease progression, as assessed by the loss of pulmonary micro-vessels and RV hypertrophy, and resulted in the survival of all animals (Umar et al., 2011). Curiously, inhibition of CYP1B1 improved survival in this model (Johansen et al., 2016).

1.7.1.2Oestrogen in the Hypoxia-Induced Pulmonary Hypertension

The cHx model, although a classical animal model of PAH, does not completely recapitulate the human disease. Moreover, the extent of involvement of Hx-induced signalling pathways is not completely understood in human disease. In animals, prolonged exposure to Hx is associated with activation of Hx-induced signalling pathways, whereas intermittent Hx also results in altered oestrogen signalling through ERα (Wu et al., 2008). Similarly, as in the MCT model females develop a less severe disease phenotype, with ovariectomy severely

exacerbating the condition (Tofovic et al., 2006). In Hx models, the protective effects of exogenous 17βE2, mediated through both ER isoforms, were found to include attenuation of remodelling in pulmonary vasculature and RV (Lahm et al., 2012). Interestingly, the authors showed the protective effects of 17βE2 do not depend on its metabolism, as the use of specific inhibitor resulted in enhanced effects of 17βE2 on its haemodynamic targets. However, possibly more relevant to the disease, endogenous 17βE2 is pathogenic in this model, as anastrozole reverses experimental PH phenotype in female Hx animals (Mair et al., 2014).

1.7.1.3 Oestrogens in Sugen 5416 and Chronic Hypoxia-Induced Pulmonary Arterial Hypertension

The disease phenotype of PAH induced by combination of Sugen 5416 and Hx remarkably resembles that observed in humans, featuring complex vascular lesions and exceedingly elevated mPAP. Therefore, it might not be surprising that it is female animals who develop a more severe disease phenotype as characterised by vascular remodelling and the presence of complex vascular lesions in this model, yet exhibiting a lesser change in the RV mass and hypertrophy (Tofovic et al., 2012;Tofovic and Rafikova, 2009), which closely recapitulates the observations of epidemiological studies. Ovariectomy and/or inhibition of endogenous oestrogen production in this model is associated with lessened and delayed disease phenotype (Bilan et al., 2013;Tofovic and Rafikova, 2009). However, possibly more relevant to the disease, endogenous 17βE2 is pathogenic in this model as anastrozole and metformin (through aromatase inhibition) reverse PAH in female Su5416/Hx rats (Mair et al., 2014;Dean et al., 2016).

1.7.2 *Role of Oestrogens in Pulmonary Arterial Hypertension Onset and Progression*

The accumulating evidence of the involvement of oestrogens in the pathogenesis of PAH resulted in increased interest in the role of specific oestrogen metabolites in the underlying disease mechanisms.

1.7.2.1 The Role of 16α-Hydroxyoestrone in Pulmonary Arterial Hypertension

Oestrogens in extrahepatic tissues are mainly metabolised to 4-hydroxyestrogens, however, numerous extrahepatic CYP450 isoforms were shown to exhibit high catalytic activity at position C2 and moderate activity at position C16α, with the formation of 2- and 16α-hydroxylated oestrogens being mutually exclusive (Sepkovic and Bradlow, 2009). The ratio of circulating levels of 2OHE1/16αOHE1 was hypothesised to be a suitable indicator of breast cancer risk. The detrimental effects of 16αOHE1 were first shown in animal oestrogen-responsive cancer, where mice having elevated capacity of hydroxylation of oestrogens at position C16α spontaneously developed mammary tumours (Bradlow et al., 1985). 16αOHE1 induced significant increase in spontaneous DNA repair synthesis in mouse mammary epithelial cells (Telang et al., 1992). The potency of 16αOHE1 signalling through ERs, is attributed to its capacity to form covalent adduct formation with the receptor (Swaneck and Fishman, 1988), resulting in constitutively active receptor complexes (Lustig et al., 1989). In the context of PAH, the 16αOHEs are regarded as pathogenic as the circulating and urinary levels of 16αOHE1 were significantly increased in patients (Austin et al., 2009). The levels of this metabolite were also increased in animals exposed to cHx (White et al., 2012;Austin et al., 2009). The influence of 16αOHE1 on the pathogenesis of PAH was further demonstrated by the direct induction of disease phenotype in in female mice (White et al., 2012) and in *BMPRII* haplo-sufficient mice (Fessel et al., 2011) when animals were treated with this metabolite. Amongst the hydroxylated oestrogen metabolites, 16αOHE1 is the only metabolite to induce significant proliferation of hPASMCs (White et al., 2012).

1.7.2.2 The Effects of 2-Methoxyoestradiol

Numerous studies have reported protective effects of circulating oestrogens in the onset and progression of PAH in experimental animal models (Resta et al., 2001;Farhat et al., 1993;Umar et al., 2011). The parental oestrogens are mainly metabolised to 2OHE1/2 by CYP1A1 in extrahepatic tissues (Aoyama et al., 1990), which are then biotransformed into methoxyoestrogens by COMT. All enzymes involved in the formation of methoxyoestrogens are expressed in vascular SMCs and ECs (Zacharia et al., 2001), suggesting these metabolites are regularly formed in vasculature. As an anti-carcinogenic agent, 2MeOE2 was shown to exhibit anti-mitogenic and anti-angiogenic properties in

vascular ECs (Fotsis et al., 1994). This metabolite can be converted under physiological conditions by the activity of 17βHSD type 2 to 2MeOE1, which is largely devoid of any protective properties in cardiovascular cells. The opposite transformation, catalysed by 17βHSD type 1, was shown to occur in the vascular system and attenuates vascular remodelling in MCT-induced model of PH (Tofovic et al., 2008a;Tofovic et al., 2006). Co-treatment of hPASMCs and lung fibroblasts with 2MeOE1 and 17βHSD type 2 inhibitor, inhibited the growth of both cell types (Tofovic et al., 2008b). Moreover, the 2MeOEs opposes the PAH onset and progression in animal models (Fotsis et al., 1994;Tofovic et al., 2006), through i) the inhibition of VSMCs proliferation and migration, ii) enhancing production of vasodilators PGI_2 and NO (Barchiesi et al., 2006;Seeger et al., 1999;Tsukamoto et al., 1998), and iii) inhibiting ET-1 production (Dubey et al., 2001). The presence of *CYP1A1* and *COMT* variants exhibiting high catalytic activity might be considered as protective in disease (Kisselev et al., 2005), as the resulting methoxyoestrogens, especially 2MeOE2, act as antiangiogenic and anti-carcinogenic compounds (Zhu and Conney, 1998), possibly reducing the extent of vascular remodelling in CVD (Kisselev et al., 2005). The antiproliferative effects of 2MeOE2 were shown to be concentration dependent in vascular SMCs, cardiac fibroblasts and breast cancer cells. SMCs and cardiac fibroblasts are intimately involved in the pathophysiological processes leading to clinical PAH (Dubey et al., 2003;Nishigaki et al., 1995;Tofovic et al., 2009;Dubey et al., 1998;Vijayanathan et al., 2006). Nonetheless, several lines of evidence indicate the anti-proliferative effects of 2MeOE2 might be highly dependent on experimental conditions and type of cells used (Lippert et al., 2000;Lai and Law, 2004). *In vivo*, 2MeOE2 can supress the expression of HIF1α, thereby affecting all the downstream target genes of this transcription factor (Becker et al., 2008a). Through its effects on the proteins involved in cell cycle regulation, and Akt/MAPK signalling pathway, 2MeOE2 also opposes the formation of neointima in rat carotid arteries, possibly indicating that this metabolite might reduce vasoocclusion (Barchiesi et al., 2006). Interestingly, 2MeOE2 only induced apoptosis in ECs, but not in SMCs (Fotsis et al., 1994;Yue et al., 1997). Moreover, similar observations were made *in vitro*, where treatment of A549 cells with 2MeOE2 under Hx conditions resulted in significantly reduced expression of both, HIF1α and HIF2α (Aquino-Galvez et al., 2016). Additionally, low circulating 2OHE1/16αOHE1 levels might be reflecting not only an increase in the production of pathogenic 16αOHE1, but also decreased hydroxylation of oestrogens at position C2, especially considering these two reactions might be mutually exclusive (Sepkovic and Bradlow, 2009). Furthermore, evidence indicated that the formation of protective 2MeOE2 might be inhibited by the 4-OHEs (Roy et al., 1990;Zhu and Conney,

1998). In accordance, cHx was shown to reduce the expression of CYP450 enzymes catalysing this reaction in extrahepatic tissues, e.g. CYP1A1 (Fradette et al., 2007).

1.7.2.3 The Effects of 2- and 4-Hydroxyoestradiol

In the liver, the dominant metabolic transformation of oestrogens is hydroxylation at position C2, with only small proportion of the hydroxylation occurring at position C4 (Zhu and Conney, 1998). In numerous extrahepatic tissues, however, the reaction of hydroxylation of oestrogens at position C4 represents the dominant metabolic conversion pathway, as most of extrahepatic CYP450 isoforms catalyse this reaction, previously reviewed in this work. Amongst extrahepatic isoforms, CYP1B1 exhibits preferential catalytic activity for hydroxylation at position C4, leading to the formation of biologically active metabolite, which has been associated with incidence of oestrogen-dependent cancers (Liehr, 2001). Interestingly, further biotransformation to methoxyoestrogens occurs much more rapidly in 2-hydroxyoestrogens (Roy et al., 1990). 2- and 4OHEs exhibit similar affinity for both ER isoforms, which is 5–10-fold lower than that of the parental hormone 17βE2 (Schutze et al., 1994). 4OHE2 was shown to mediate biological effects through pathways independent of ER (Das et al., 1997), however, the 4OHEs were also shown to potentially activate ER (Martucci and Fishman, 1976), and possibly have prolonged oestrogen effects due to slower dissociation rates (Zhu and Conney, 1998;Barnea et al., 1983). The possible mechanism by which 4OHEs could promote PAH, might depend on the promotion of cell proliferation and oxidative stress as observed in cancers (Gao et al., 2004;Liehr and Ricci, 1996;Rogan et al., 2003). Interestingly, 4OHE2 decreases phospho-Smad1/5/8(9) and Id expression in female hPASMCs, while increasing these in males commensurate with a decreased proliferative effect in male hPASMCs (Mair et al., 2015). It therefore appears that distinct oestrogen metabolites might exert sex-dependent effects in hPASMCs, which is something that was not considered previously. Generally, catechol-oestrogens readily undergo oxidation, forming quinones and semiquinones, which can result, due to their highly reactive nature, in the formation of covalent adducts with proteins (Markides and Liehr, 2005) or DNA (Cavalieri et al., 2002;Cavalieri et al., 2000), leading to carcinogenicity and/or genotoxicity. During the formation of quinones and semiquinones, ROS are also formed, whose accumulation can result in DNA damage and cytotoxicity. Interestingly, 4OHE2, but not 2OHE2, was shown to induce HIF1α and VEGFA expression at protein level through the PI3K/Akt pathway (Gao et al., 2004), however not all studies support this line of evidence

(Seeger et al., 2006). Contrary, Bradlow and colleagues have indicated that 2OHE1 might also exhibit anti-carcinogenic properties (Bradlow et al., 1996).

1.8 Aims and Objectives

According to the Home Office statistics 3.87 million animals were used for research purposes and 3.93 million procedures were carried out in year 2016 alone (Office, 2013). As the necessity of animals in research of disease pathogenies is undeniable, considering the complexity of signalling pathways, in addition to the need for pre-clinical assessment of novel therapeutic approaches, it is the responsibility of the researchers to drive the progress of these models and substitute them where possible with similarly suitable alternative models. While numerous animal models have been developed for the research of pulmonary arterial hypertension, not all models resemble the human disease in the most important histopathological hallmarks, meaning not all aspects of disease pathogenesis can be investigated in every model. Therefore, more effort must be made to understand the underlying mechanisms leading to the establishment of disease phenotype, and to establish alternative models, which could replace the use of animals in the initial stages of research hypothesis testing. The objective of this research work was to develop a novel *in vitro* model, which could be used to investigate the role of oestrogens and their metabolites in pulmonary vascular disease.

This was addressed by the following aims:

1.) To investigate the mechanism through which the animal model of pulmonary arterial hypertension employing Sugen 5416 coupled with chronic hypoxia is induced, utilising *in vitro* methods.
2.) To develop a liquid chromatography tandem mass spectrometry method to quantitively evaluate oestrogen metabolism in an *in vitro* model of pulmonary arterial smooth muscle cells.
3.) To characterise the metabolic profile of oestrogens in pulmonary arterial smooth muscle cells derived from female and male patients and control subjects, respectively.
4.) To investigate the effects of oestrogens metabolites in an *in vitro* model of pulmonary arterial smooth muscle cells, derived from patients and control subjects of both sexes, respectively.

Chapter II

2 Methods

2.1 Chemical Reagents and Equipment

All chemical and reagents were purchased from Sigma-Aldrich (UK) or Fisher Scientific (Loughborough, UK), unless stated otherwise. Materials for RNA and protein analyses were obtained from Qiagen (UK) and Life Technologies (UK), respectively. For experimental protocols involving RNA, reagents and plastic-ware, which were certified to be nuclease-free, were supplied by Ambion, Life Technologies (UK). Plastic-ware used for cell culture was supplied by Corning, Fisher Scientific (UK).

2.2 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

2.2.1 *Protein Solubilisation and Preparation*

For *in vitro* experiments, hPASMCs, at the end time point of an experiment, were placed on ice. The media was aspirated off, and the monolayer of cells was then washed twice with DPBS. 120μL of 1% (v/v) lauryl maltoside (LM, Abcam, UK) buffer in DPBS was added to each well, when protein was extracted from 6-well plates. When protein was extracted from unstimulated hPASMCs growing in a T75cm^2 culture flask, 600μL of 1% LM, supplemented with protease inhibitors (0.1mmol/L PMSF, 1μg/mL soybean trypsin inhibitor and 1μg/mL benzamidine), was used. The cells were then collected using a sterile plastic scraper, collected in pre-chilled 1.5mL Eppendorf tubes and left on ice for 30 minutes to allow for improved disassociation of protein complexes and protein solubilisation. Cell lysates were then sonicated in iced water, before centrifuging at 10,000rpm (4°C) to remove any debris.

Alternatively, when extracting protein from tissue, a piece of tissue was placed in a 2mL Eppendorf tube, containing 300μL of 1% LM buffer. A 5mm stainless-steel ball (Qiagen, UK) was added, and the tissue homogenised using the Tissue Lyser II (Qiagen, UK). Homogenised tissue was then left on ice for 30 minutes to promote disassociation of protein complexes and improve protein solubilisation. Tissue samples were then centrifuged at 10,000rpm for 10 minutes (4°C).

Protein supernatant was collected into a new pre-chilled Eppendorf tube, and stored at -80°C until further.

2.2.2 *Bicinchoninic Protein Assay*

Protein concentration in supernatants was determined using the bicinchoninic acid (BCA) assay (Pierce, Thermo Fisher, USA). To quantify protein concentration in collected samples, a standard curve was constructed within the 0.0–2.0mg/mL concentration range, using the albumin standard ampules consisting the BCA Kit, diluted in 1% LM. Briefly, suitable volume of the albumin standards and unknown samples was pipetted into a 96-well plate. BCA assay working solution was prepared by combining 9.8mL of reagent A with 200µL of reagent B. 200µL of the working solution was quickly added to each well and incubated at room temperature with gentle agitation on micro-plate shaker for 30 minutes. Protein concentration was then evaluated against the standard curve, by measuring the absorbance at 652nm with POLARstar OPTIMA microplate reader (BMG Labtech, Germany).

2.2.3*Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis*

Samples, containing equal amounts of protein, were prepared by adding NuPAGE® LDS (lithium dodecyl sulphate) Sample Buffer (4X) and NuPAGE® Reducing Agent (10X) (10mmolL/L dithiothreitol), and heated at 70°C for 10 minutes. Samples were then separated per their molecular weight on Novex® Tris-Glycine gels (Invitrogen, Life Technologies, UK) using Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis, under constant voltage at 110–150V in NuPAGE MOPS SDS running buffer (20x), containing 1% (v/v) NuPAGE® Antioxidant.

2.2.4 *Protein Transfer*

Once adequate separation of protein according to their weight was achieved, the protein was blotted onto hydrophobic Immobilon polyvinylidene difluoride (PVDF) membrane (Millipore, USA) using a wet blotting system by Life Technologies. Due to its hydrophobic character, the membrane was immersed into 100% methanol prior to use in order to activate it and expose membrane's full protein binding capacity. The buffer for wet blotting contained 5% (v/v) NuPAGE Transfer Buffer (20x) and 20% (v/v) methanol. Proteins were transferred onto PVDF membranes at constant voltage (35V) for 2 hours and 15 minutes. Transfer efficiency was assessed by a short incubation of membranes in 0.1% (w/v) solution

of Ponceau S stain, prepared in 5% (v/v) acetic acid solution. The stain was washed off the membranes in 10% (v/v) TBS buffer containing 0.1% (v/v) Tween-20 (Fisher Scientific, UK) (TBST) washing buffer, before blocking in 5% (w/v) milk prepared in TBST, for 1 hour at room temperature in order to minimise non-specific binding of the antibody. The residual blocking solution was washed off the membrane by numerous short washes in TBST.

2.2.5 *Immunoblotting*

The detection of a specific protein was achieved by an overnight incubation of the membrane in primary antibody (the dilutions and concentrations are provided in Table 2-1 on a shaker at 4°C. The membranes were then washed for 3x10 minutes in TBST washing buffer to remove any unbound primary antibody, before incubating in secondary antibody conjugated with horseradish peroxidase (HRP) (Table 2-1) prepared in 5% (w/v) BSA (Sigma, UK) in TBST for 1 hour at room temperature. The membrane was again washed in TBST buffer for 3x15 minutes. The secondary antibodies used were coupled with HPR, catalysing the oxidation reaction of the luminal, which is present in the substrate Immobilon western chemiluminescence HRP substrate (Merck Millipore, USA) or Pierce ECL Western Blotting Substrate (Thermo Scientific, USA). The light emitted during the reaction catalysed by HPR, was captured on X-ray film (Kodak, UK), allowing protein visualisation after film processing using MIN-R Mammography Processor. When samples were probed for several proteins, membranes were washed in TBST for 5 minutes, following a 15-minute incubation in the Restore western blot stripping buffer (Thermo Scientific, UK) on a shaker, during which the bound antibodies were stripped. Equally, to reduce the number of times the PVDF membranes were stripped, the membranes were cut alongside suitable molecular-weight size markers, labelled and probed as described previously.

The membranes were re-probed for another protein of interest, or for loading control (β-actin, GAPDH). Protein expression was quantified by densitometry analysis using TotalLab TL100 v2006 programme (TotalLab, United Kingdom). In the following chapters the representative immunoblotting images are shown. Where images have been altered from full blots to remove redundant samples, it is stated in the legend.

Table 2-1: List of antibodies used for immunoblotting.

Primary Antibody	Dilution		Supplier	Molecular Weight (kDa)	Secondary Antibody	Concentration
AhRR	1:100	ab108518	Abcam	76	Rb	1:5000
PHD2	1:1000	D31E11	Cell Signalling	50	Rb	1:5000
HIF1α	1:500	NB100-105	Novus Biologicals	120/100	Ms	1:5000
ARNT (HI1β)	1:2000	ab2771	Abcam	87	Ms	1:5000
vHL	1:5000	NB100-41384	Novus Biologicals	17	Go	1:5000
Caspase 3	1:1000	9662	Cell Signalling	35/19/17	Rb	1:5000
VEGFR2	1:1000	ab39256	Abcam	151	Rb	1:5000
p27/Kip1	1:500	ab54563	Abcam	22	Ms	1:5000
Lamin A	1:1000	ab26300	Abcam	74	Rb	1:5000
Nucleoporin	1:2500	ab2460	Abcam	55	Ms	1:5000
β actin	1:1000	A5441	Sigma	42	Ms	1:10000
CYP1A1	1:1000	ab79819	Abcam	58/51	Rb	1:5000
CYP1A1 (A-9)	1:1000	sc-393979	Santa Cruz	56/51	Ms	1:5000
CYP1B1	1:1000	ab78044	Abcam	61	Rb	1:10000
CYP1B1	1:1000	ab33586	Abcam	61	Rb	1:5000
17βHSD2 (H-12)	1:1000	sc-373990	Santa Cruz	43	Ms	1:5000
AhR	1:500	ab84833	Abcam	95	Rb	1:5000
α tubulin		ab80779	Abcam	50	Ms	1:10000
BMPR*II*	1:500	612292	BD Biosystems	115	Ms	1:5000
Id1	1:1000	M085	Cal Bioreagents	20	Rb	1:5000

Id3	1:1000	M100	Cal Bioreagents	17	Rb	1:5000
pSmad1/5/9	1:1000	9511	Cell Signalling	55–60	Rb	1:5000
GAPDH	1:1000	ab9484	Abcam	40	Ms	1:10000

AhRR= AhR repressor, PHD2= Prolyl hydroxylase 2, HIF1α= Hypoxia-inducible factor 1α, ARNT (HI1β)= Aryl hydrocarbon receptor nuclear translocator, vHL= von Hippel-Lindau tumour suppressor , VEGFR2= Vascular endothelial growth factor receptor 2, p27/Kip1= Cyclin-dependent kinase inhibitor 1B (p27/Kip1), CYP1A1=, Cytochrome P450 1A1, CYP1B1=, Cytochrome P450 1B1, 17βHSD2= 17β-hydroxysteroid dehydrogenase type 2, AhR= Aryl hydrocarbon receptor, BMPR*II*= Bone morphogenetic protein receptor II, Id1= Inhibitor of DNA binding 1, Id3= Inhibitor of DNA binding 3, pSmad1/5/9= phosphorylated Smad 1/5/9

Table 2-2: List of positive controls.

Protein	**Positive Control**	**#**	**Supplier**
CYP1A1	HeLa whole cell lysate	ab29545	Abcam
CYP1B1	Human liver tissue lysate, total protein	ab29889	Abcam
CYP1B1	Rat liver lysate		N/A
AhR	Rat kidney lysate		N/A
HIF1α	$CoCl_2$-treated hPASMC lysate		N/A
Caspase 3	Resveratrol-treated EC lysate		N/A

HIF1α= Hypoxia-inducible factor 1α,), CYP1A1=, Cytochrome P450 1A1, CYP1B1=, Cytochrome P450 1B1, AhR= Aryl hydrocarbon receptor

2.3 RNA Analysis

2.3.1 *RNA Extraction*

To extract RNA from hPASMCs, 6-well plates or T75cm^2 culture flasks were placed on ice at the end time point of an experiment. The media was aspirated, and the cell monolayer was washed with ice-cold DPBS, before adding 700µL of QIAzol (Qiagen, UK) per well in 6-well plates or per culture flask. The cells were collected using a sterile plastic scraper and lysates placed in a pre-chilled 1.5mL Eppendorf tube. Whole tissue sample RNA was extracted by placing the lung tissue in a pre-chilled 2mL Eppendorf tubes containing 700µl of QIAzol. A 5mm stainless steel bead (Qiagen, UK) was added to each sample, and tissue was homogenised in Tissue Lyser II (Qiagen, UK) using 4x30 second intervals. The RNA samples were immediately stored at -80°C until RNA extraction.

RNA purification was carried out using miRNeasy mini kit (Qiagen, Germany) as per the manufacturer's instructions. Briefly, RNA samples were defrosted on ice and left at room temperature for 5 minutes to promote disassociation of nucleoprotein complexes. 140µL of chloroform was added to each tube containing the homogenate and capped securely. The tubes were then shaken vigorously for 15 seconds and left at room temperature for 3 minutes to improve the subsequent separation of phases. Samples were then centrifuged at 12,000g for 15 minutes (4°C). This step allowed for the separation of the sample in three distinct phases: aqueous (top, colourless), interphase (middle, white) and organic (bottom, red) phase. The aqueous phase, containing the RNA, was transferred to a new labelled RNase-free 1.5mL Eppendorf tube, to which a 1.5 volume of 100% ethanol was added to precipitate the RNA. The aqueous phase and ethanol were thoroughly mixed by pipetting, before loading onto a RNeasy Mini spin column, and centrifuging at 8,000g for 15 seconds at room temperature. The flow through was discarded, and the column in which RNA was captured, was washed with 350µL of RWT buffer. The flow through was again discarded. A DNase digestion was performed on column to remove any residual trances of DNA in the sample. The working solution for DNase digestion was prepared by diluting DNase I stock solution with RDD buffer in a 1:7 ratio. 80µL of the working solution was then pipetted directly onto each RNeasy Mini spin column membrane and left to incubate at room temperature for 30 minutes, to ensure complete DNA digestion. Following the DNase digestion, 350µL of RWT buffer was added to Mini spin column and centrifuged at 8,000g for 15 seconds. The flow through was discarded, before 500µL of RPE buffer was pipetted onto the column and

centrifuged at 8,000g for 15 seconds. The wash step with RPE buffer was immediately repeated, however, this time the samples were centrifuged at 8,000g for 2 minutes. To remove any residual RPE buffer, the Mini spin column was placed in a new 2mL collection tube, and centrifuged at maximal speed of 14,000rpm (Centrifuge 5417R, Eppendorf, UK) for 1 minute. The Mini spin column was then transferred to a new RNase-free 1.5mL Eppendorf tube, and 30µL of RNase-free H2O was pipetted directly onto each RNeasy Mini spin column membrane. To elute the RNA, the samples were then centrifuged at 10,000g for 1 minute at room temperature. This step was repeated with the eluate, to maximise the RNA elution. Immediately after elution, the samples were transferred to ice or stored at -80°C.

2.3.2 RNA Quantification

The quality and concentration of the extracted RNA was determined using Nanodrop 1000 spectrophotometer (Thermo Scientific, UK). The absorbance at 260nm and 280nm was measured, to assess the presence and concentration of RNA and protein, respectively. The quality of the RNA was assessed using the 260nm/280nm ratio. The ratio of approximately 2 indicated the sample contains pure RNA. Values lower than 2 indicated, the presence of a contaminant, such as protein, phenol or other compounds with absorbance at 280nm. Samples with 260nm/280nm ratio of approximately 2 were considered to contain RNA of good purity. The concentration of RNA was determined by the spectrophotometer by using a form of the Beer-Lambert equation:

$$Concentration\ of\ RNA\ \ (ng/\mu L) = \frac{(260nm\ Absorbance \times Extinction\ coefficent)}{Path\ length\ in\ cm},$$

Where the extinction coefficient was 40ng-cm/µL.

2.3.2 ***Reverse Transcription***

Samples considered to contain good purity RNA were subjected to a two-step reverse transcription polymerase chain reaction (RT-PCR) to assess the levels of mRNA expression. Firstly, the samples were reverse transcribed to complementary DNA (cDNA), using the TaqMan® Reverse Transcription Reagents Kit (Life Technologies, UK). Briefly, for one 20µL reverse transcription reaction, master-mix was prepared by mixing 10X RT buffer, 25mM magnesium chloride, 2.5mM deoxynucleotide triphosphates (dNTPs), 50 µM random hexamers, 20U/µL RNase Inhibitor and 50 U/µL Multiscribe. The master-mix was pipetted

into wells on a MicroAmp 96-Well Plates (Thermo Fisher Scientific, UK), and 500ng–1µg of RNA template was added. The MicroAmp 96-Well Plate was quickly vortexed and centrifuged at 1,000rpm for 2 minutes. The RNA template was reverse transcribed using the Veriti® Thermal Cycler (Life Technologies, UK), per the following thermal cycling parameters:

Table 2-3: Thermal cycling conditions for reverse transcription PCR.

Stage	Temperature (°C)	Time (minutes)	Action
1	10	25	Annealing
2	48	30	Reverse transcription
3	95	5	Inactivation of reverse transcription
4	4	∞	Short-term storage

2.3.3 *Quantitative Real Time Polymerase Chain Reaction*

To assess the levels of mRNA in the samples, the Applied Biosystems™ TaqMan™ Gene Expression Assays (Life Technologies, UK) quantitative real-time PCR (qRT-PCR) was used. Briefly, cDNA was added to a mixture of TaqMan® Universal Master Mix II and Taqman® probes (Life Technologies, UK) tagged with FAM fluorescent label, to measure the strength of the fluorescence, where a change in fluorescence after each amplification cycle is proportional to the level of the target gene expression. The ViiA7™ Real Time PCR System (Life Technologies, UK) was used to measure fluorescence of the qRT-PCR.

Each sample was performed in triplicate and expression was determined relative to housekeeping control primers as listed in Table 2-4–Table 2-6.

Table 2-4: List of mRNAs TaqMan primers used in human samples.

mRNA Name	Assay ID
HIF1α	Hs00153153_m1
vHL	Hs00184451_m1
P4HA2	Hs00990001_m1
AhR	Hs00907314_m1
ARNT (HIF1β)	Hs01121918_m1
CYP1B1	Hs00164383_m1
CYP1A1	Hs01054797_g1
NQO1	Hs02512143_s1
HSD17b2	Hs0015993_m1
PTGIR	Hs00168765_m1
β2m	Hs00187842_m1

Table 2-5: List of mRNAs TaqMan primers used in rat samples.

mRNA Name	Assay ID
HIF1α	Rn01472831_m1
vHL	Rn00583795_m1
P4HA2	Rn01495731_m1
AhR	Rn0565750_m1
ARNT	Rn00688999_m1
CYP1A1	Rn01418021_g1
CYP1B1	Rn00564055_m1
AhRR	Rn01537444_m1
NQO1	Rn00566528_m1
Kdr	Rn00564986_m1
β2m	Rn00560865_m1

Table 2-6: List of mRNAs TaqMan primers used in mouse samples.

mRNA Name	Assay ID
CYP1A1	Ms00487218_m1
β2m	Ms00437762_m1

Table 2-7: Thermal cycling conditions for quantitative real time PCR.

	Temperature (°C)	Time	Action	Stage
1	50	2 minutes		Hold
2	95	10 minutes		Hold
3	95	15 seconds	Denaturing	Cycle
4	60	1 minute	Polymerisation	Cycle

For quantitation analysis of gene expression, a comparative cycle threshold (CT) method was used, where CT is the number of cycles in the PCR reaction required for fluorescence to surpass a threshold level of fluorescence and is inversely proportional to the measured mRNA. The CT value is inversely proportional to the measured mRNA level. A ΔCT value was obtained from the difference between the CT value of the mRNA of interest and the CT value of the housekeeper CT. By calculating the difference of ΔCT values of the test sample and control sample, a ΔΔCT value was obtained. The relative quantitation (RQ) was calculated from the following equation:

$$RQ = 2^{-\Delta\Delta CT}.$$

This method of quantitative comparison allowed the test samples to be expressed as a fold change in contrast to the control samples.

2.4 Cell Culture

All cell culture experiments were performed under sterile conditions in a class II laminar flow cabinet. Cell culture was maintained in a humidified incubator at 37°C, with a supply of gaseous mixture of 5% CO_2 95% air.

2.4.1 *Human Pulmonary Arterial Smooth Muscle Cells*

Human pulmonary artery smooth muscle cells (hPASMCs) derived from the distal pulmonary arteries (with external diameter of 1mm) by explant protocols previously described in (Wharton et al., 2000;Johnson et al., 1994), from subjects with no reported presence of PAH and subjects diagnosed with either idiopathic or heritable form of PAH,

were generously provided by Prof Nicholas W. Morrell (University of Cambridge). Patient information for cell lines utilised in this work is shown in Table 2.3.

Table 2-8: Information on cell lines derived from patient and control subjects.

Cell line	Gender	Age	Disease status	Medications
9MP	M	72	Emphysema	
34MP	M	62	Adenocarcinoma (Metastatic disease)	
75MP	M	78	Squamous cell carcinoma	
79MP	M	60	Mild bronchiectasis/adenocarcinoma	
103MP	M	52		
110MP	M		APAH (Eisenmenger's Syndrome)	
23MP	M	43	HPAH (C347R)	
56MP	M		HPAH (W9X)	
67MP	M	22		
80MP	F			
82MP	F	71	Adenocarcinoma	
84MP	F	59		aspirin, simvastatin, bisoprolol, ramipril, metalazine, quinine sulphate, loperamide
105MP	F	57	COPD, Emphysema	
106MP	F	70	Left lower lobectomy	
35MP	F			
37MP	F	24	IPAH	
38MP	F			
73MP	F	30	R899X	IV prostanoids, warfarin, zopliclone, mebeverine, frusemide by transplantation
113MP	F		IPAH	
115MP	F		Associated PAH (Congenital Heart Disease)	

Primary cell line stocks were stored in the liquid nitrogen tank facility, in 10% (v/v) dimethyl sulfoxide (DMSO), 20% (v/v) FBS (Sera Laboratories International, UK) Dulbecco's modified eagle medium (DMEM, Life Technologies, UK). To initiate cell culture, hPASMCs were taken on dry ice from the storing facility, and rapidly defrosted in water bath maintained at 37°C. Immediately after thawing, the content of the cryo-vial was transferred into a T75cm^2 culture flask, and even dispersion was ensured by pipetting the cell suspension using stripette serological pipettes. HPASMCs were grown in DMEM supplemented with 10% (v/v) FBS and 1% (v/v) antibiotic anti-mycotic (AA) solution (10,000 units/mL penicillin G, 10 mg/mL streptomycin sulphate and 25µg/mL amphotericin B, Sigma Aldrich, UK). Cell culture media was replenished every 48 hours. Herein, donated cells derived from male and female individuals were used, as specified in each of the following sections.

2.4.1.1 Sub-culturing of Human Pulmonary Arterial Smooth Muscle Cells

Cells were grown until 85-90% confluency, and then sub-cultured for experiments between passage 3 and 8. Cell culture media was aspirated from the culture flask, and the cell monolayer was then washed twice with sterile Dulbecco's phosphate-buffered saline (DPBS, Life Technologies, UK). 2mL of 0.25% trypsin/0.1% (w/v) EDTA solution in PBS (Life Technologies, UK) was equally distributed over the entire culture flask and incubated at 37°C for between 2–3 minutes, or until the cells have started to detach. 8mL of 10% FBS DMEM was then immediately added to neutralise the trypsinisation process. Cells were seeded for experiments by diluting the cell stock suspension with an appropriate dilution factor. The medium was always replenished 24 hours after a newly generated sub-culture.

2.4.1.2 Charcoal-Stripped Foetal Bovine Serum

Oestrogens endogenously present in FBS were removed by charcoal stripping for all experiments. 0.1g of dextran-activated charcoal (Sigma-Aldrich, UK) was added per 100mL of FBS, and left under gentle agitation over night at 4°C. FBS was then centrifuged at 1811x*g* for 30 minutes at 4°C. The stripped serum was filtered using a sterile disposable filter unit with 0.22µm CN membrane filter (Thermo Fisher Scientific, UK). The process was repeated and CS-FBS was then aliquoted and frozen until use. In the results section this is referred to as Charcoal-stripped serum (CSS).

2.4.1.3 Pulmonary Artery Smooth Muscle Cell Proliferation Assay Using Haemocytometer

HPASMCs were counted manually using a haemocytometer to evaluate proliferation of the cells in culture. For proliferation assays, cells were seeded in 24-well plates, were 1mL of cell suspension during passaging was added to 24mL of 10% FBS DMEM and maintained until reaching 60% confluency. HPASMCs were then serum deprived using 0.2% CS-FBS PRF DMEM between 16 and 24 hours, to synchronise cell cycle before stimulation. HPASMCs were stimulated with appropriately diluted agonist for 24–72 hours in the presence of 2% (v/v) CS-FBS PRF DMEM. Stimulations of 0.2%, 2 % and 10% CS-FBS PRF DMEM were studied as negative and positive controls, respectively. Where necessary, hPASMCs were stimulated with appropriately diluted antagonist, 45 minutes prior to agonist stimulation. The media, agonist and if appropriate antagonists, were replaced every 48 hours. Each experimental condition was performed in triplicate. Upon completion of appropriate stimulation duration, media was aspirated, and the cell monolayer was washed twice with sterile DPBS. 150µL of trypsin/EDTA solution was added to each well, and cells were incubated at 37°C for 2 minutes to aid trypsinisation. Once the cells had detached, they were re-suspended using 0.5mL of 10% FBS DMEM per well, and gently pipetted to mix. The content of each well was then transferred into 1.5mL Eppendorf tubes, and spun in a pre-cooled centrifuge (4°C) at 4,000rpm for 10 minutes. The supernatant was then carefully aspirated, not to disturb the cell pellet, which was re-suspended in 200uL of 10% FBS DMEM and shortly vortexed to ensure even suspension. The haemocytometer is designed 10µL of prepared cell suspension was applied onto AC1000 Improved Neubauer counting chamber (Hawksley, Lancing, UK), guaranteeing distribution of the suspension in the chamber due to capillary action. Using a hand tally counter, the number of cells was counted in four counting fields, with the set volume of $0.1mm^3$ (Figure 2–1). From the number of counted cells, the average number of cells in 1mL of cell suspension was calculated according to the following formulae:

$$Number\ of\ cells\ in\ 1mL = \frac{N}{4} \times 10^4$$

The number of cells in the volume used to suspend the cell pellet was then calculated.

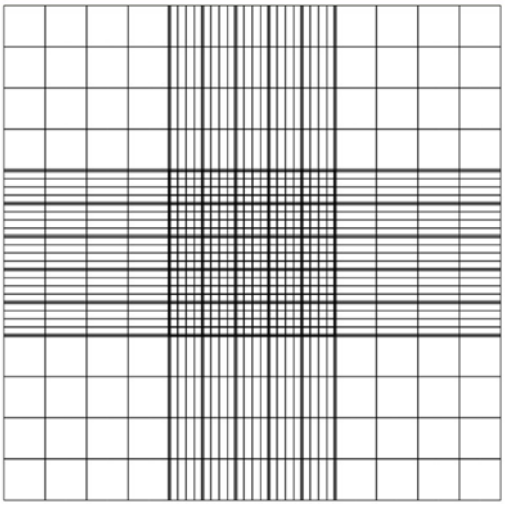


Figure 2–1: Haemocytometer counting chamber.

2.4.1.4 Pulmonary Artery Smooth Muscle Cell Proliferation Assay Using Haemocytometer in Hypoxia

HPASMCs were seeded as previously described in section 2.4.1.3, immediately after seeding, the 24-well plates were placed in a hypoxia incubator chamber (Bilrups Rothenburg, US), ensuring lowered atmospheric oxygen tension (1% O_2, 5% CO_2 (v/v)), where the cells were maintained between 24 and 72 hours. After every 24 hours, the hypoxia incubator chamber was opened in sterile environment and gassed to ensure that the cellular environment did not become overly acidic, causing cell death or altered cellular metabolism. The procedure was done as quickly as possible in order to reduce the exposure of cells to normal atmospheric oxygen tension.

2.4.1.5 Pulmonary Artery Smooth Muscle Cell Proliferation Assay Using 5-Bromo-2-deoxyuridine Incorporation

The proliferation of hPASMCs was also evaluated using the 5-bromo-2-deoxyuridine (BrdU) incorporation assay. When added to the cells, the reagent, which is a derivative of uridine, acts as the natural structural analogue of thymidine, incorporating into the newly synthesised DNA during the cell cycle. Protocol for adherent cell culture, provided by the Calbiochem® BrdU Cell Proliferation Assay manufacturer (Calbiochem, Merck Millipore, Germany), was followed. Briefly, hPASMCs were seeded in a 96-well plate, using 100µL of cell suspension per 10mL, and grown maintained in 10% FBS DMEM until reaching

approximately 60% confluence. HPASMCs were then serum-starved in 0.2% (v/v) FBS DMEM for 16-24 hours. Cells were stimulated with the appropriate agonist for a further 72 hours in the presence of 1% (v/v) CSS-PRF DMEM. Control groups consisting of 0.2% and 10% FBS DMEM stimulations were studied as a negative and positive control, respectively. Where necessary, hPASMCs were incubated with the appropriate antagonist 45 minutes prior to agonist stimulation. Each experimental condition was performed in quadruplicate. Two control conditions were utilised, blank control (1% CSS-PRF DMEM) and background control (no BrdU label). The BrdU label was prepared as follows, 1µL of the label was diluted in 2mL of 1% CSS-PRF DMEM, and 20µL of diluted stock was added to each well (except the background control group) 24 hours before assessment of proliferation. The contents of the wells were emptied, and the plate was carefully blotted dry, before adding 200µL of fixative/denaturing solution was added to each well, resulting in fixation and permeabilization of cells, denaturation of the DNA for the incorporated BrdU to be subsequently detected.

2.4.1.6 Fractionation

HPASMCs were grown in 100mm cell culture dishes until they reached approximately 80-90% confluence. Growth medium was then aspirated off, the cell monolayer washed with 10mL of DPBS, and 10mL of 1% CSS-PRF DMEM media was to each cell culture dish. HPASMCs were then stimulated with 1µM Sugen 5416 for 1, 15, 30, 60 and 90 minutes, starting with the longest stimulation time point. At experimental endpoint, the cell culture dishes were immediately placed on ice, and the media was aspirated off and hPASMCs were washed with ice-cold DPBS twice. For separation of nuclear and cytoplasmic protein fractions we have employed modified R.E.A.P protocol described in (Suzuki et al., 2010). Briefly, cell monolayer was collected into pre-chilled 1.5mL Eppendorf tube by adding 1mL of ice-cold DPBS to each cell culture dish and scrapped using a sterile plastic cell scraper. The samples were then centrifuged for 10 seconds at 10,000rpm in a centrifuge pre-chilled to 4°C. The supernatant was carefully removed, and 900µL of 0.1% (v/v) NP-40 (Sigma, UK) prepared in DPBS was added to each cell pellet and triturated 5 times using a p1000 pipette tip to ensure the cell pellet was equally dispersed. 300µL aliquot of the dispersed cell pellet was transferred into a new labelled 1.5mL Eppendorf tube. 100µL of the NuPAGE® LDS Sample Buffer (4X) was added to whole cell lysate and kept on ice. The remainder of the sample was again centrifuged as before. 330µL of the supernatant, forming the cytoplasmic fraction, were then carefully removed and transferred to a new labelled 1.5 mL

Eppendorf tube and kept on ice. 100µL of the NuPAGE® LDS Sample Buffer (4X) was added to the cytoplasmic fraction, heated at 70°C for 1 minute and stored on ice. The remainder of the supernatant was removed, and the remaining pellet was re-suspended in 1mL of ice-cold 0.1% NP40-DPBS by triturating once using a p1000 pipette tip, followed by a 10 second centrifugation at 10,000rpm in a centrifuge pre-chilled to 4°C. The supernatant was removed and discarded, and the pellet was re-suspended in 90µL of NuPAGE® LDS Sample Buffer (4X). Whole cell lysate and nuclear fraction were sonicated in icy water for 10 seconds before heating at 70°C for 1 minute. Prepared samples were used for western blot.

2.4.2 *Human Pulmonary Microvascular Endothelial Cells*

Human pulmonary microvascular endothelial cells (hPMECs) were purchased from Promocell (UK). Equally, media and products required for sub-culturing of HPMECs were supplied by Promocell.

2.4.2.1 Sub-culturing of Human Pulmonary Microvascular Endothelial Cells

Upon purchase, the vial of HPMECs was carefully defrosted in a water bath, set to 37°C, cleaned with 70% (v/v) ethanol, and then in sterile conditions immediately transferred to a T25cm^2 containing Endothelial cell growth medium MV supplemented with Endothelial cell growth medium MV SupplementMix. The cells were left to adhere for 24 hours before the media was replaced. The cells were fed every 24–48 hours until reaching approximately 80% confluence. To sub-culture HPMECs, the Promocell DetachKit was warmed at room temperature for at least 30 minutes before use. The growth media was aspirated, and the monolayer of hPMECs was washed with HEPES Buffered saline solution (HEPES/BSS) (100µL/cm^2 of vessel surface) by gentle agitation. The washing solution was then carefully aspirated, and 100µL/cm^2 of vessel surface of Typsin/EDTA solution (0.04 %/0.03 %) was added, and the cells were left to detach at room temperature for 2 minutes. During this time cells were carefully monitored under the microscope, and when cells started to detach, this process was aided by gently tapping the sides of the cell culture vessel. To neutralise the activity of Typsin/EDTA solution, 100µL of Trypsin neutralization solution (TNS) per cm^2 of cell culture flask surface was added and mixed with gentle agitation. Cell suspension was carefully transferred into a 15mL falcon tube and centrifuged at 220xg for 3 minutes at room

temperature. The supernatant was then aspirated off, and the cell pellet was gently re-suspended in supplemented Endothelial cell growth medium MV. The number of cells in the suspension was then assessed using a haemocytometer, and cells were seeded at 10,000–20,000cells/cm^2.

2.4.2.2 Apoptosis Assay in Pulmonary Microvascular Endothelial Cell

HPMECs were counted using the Countess II FL Automated Cell Counter (Thermo Fisher Scientific, UK), together with the disposable slides to evaluate the number of cells in culture. For apoptosis assays, cells were seeded in 24-well plates at 8,000–10,000 cells/cm^2 and maintained until reaching approximately 90% confluency. HPMECs were then serum deprived using phenol red-free Endothelial cell basal medium MV, supplemented with 0.2% CS-FBS (v/v) for 2 hours, to synchronise cell cycle before stimulation. HPMECs were then stimulated with appropriately diluted agonists for 24 hours in the presence of 2% (v/v) CS-FBS PRF Endothelial cell basal medium MV. Stimulations of 0.1%, 2 % and supplemented Endothelial cell growth medium MV were studied as negative and positive controls, respectively. Each experimental condition was performed in triplicate. Upon completion of appropriate stimulation duration, media was aspirated, and the cell monolayer was washed with sterile DPBS. 150µL of trypsin/EDTA (0.05%) solution was added to each well, and cells were incubated at room temperature for 2 minutes to allow the cells to begin trypsinisation. The cell culture plates were gently tapped to aid detaching. Once the cells had detached, they were re-suspended using 0.5mL of 10% FBS DMEM per well, and gently pipetted to mix. As represented the end time point of the experiment, we have used DMEM instead of Endothelial cell growth medium MV for resuspension, due to the cost of the Endothelial cell growth medium MV. The content of each well was then transferred into 1.5mL Eppendorf tubes, and spun in a pre-cooled centrifuge (4°C) at 220g for 3 minutes. The supernatant was then carefully aspirated, not to disturb the cell pellet, which was re-suspended in 200µL of 10% FBS DMEM and shortly vortexed to ensure even suspension. 10µL of prepared cell suspension was applied onto disposable Countess II FL Automated Cell Counter slides, guaranteeing distribution of the suspension in the chamber due to capillary action. Resveratrol (Sigma-Aldrich, UK) at a concentration of 100µM was used as a positive control of apoptosis in these cells.

2.4.2.3 Measuring Prostacyclin Release Using an Enzyme-Linked Immunosorbent Assay

PMECs were stimulated with 1nM oestrogen metabolites (Steraloids, USA) for 1 hour or 24 hours. 20µM arachidonic acid was used as (Sigma, UK). The cell plates were kept on ice, while the media was collected and stored at -20°C until subsequent analyses. Cells were then washed twice with ice-cold PBS, and the cell monolayer was lysed with 60µL of 1% LM solution. Protein concentration in collected samples was determined as described previously (Chapter 2.2.2).

The release of PGI_2 was measured using 6-keto $PGF_{1\alpha}$ ELISA Kit (Enzo Life Sciences, USA), as PGI_2 is quickly oxidised to form a stable metabolite, 6-keto $PGF_{1\alpha}$. Our preliminary results have shown that 6-keto $PGF_{1\alpha}$ levels were out of the detection limit of the ELISA Kit. To circumvent this, 6-keto $PGF_{1\alpha}$ was extracted from the medium using liquid-liquid extraction, as described in Cayman Chemical 6-keto Prostaglandin F1α EIA Kit, Item No. 515211. Briefly, the proteins present in 1mL of media were first precipitated with acetone (4x the sample volume). Samples were then thoroughly vortexed, and incubated at 4°C for 5 minutes, before centrifugation at 3,000g for 10 minutes. Supernatant was decanted into fresh test tubes, and the acetone was evaporated by vacuum centrifugation. Solid residue was then resuspended in 1mL of NaCl solution (~28%, w/v). 6-keto $PGF_{1\alpha}$ was extracted from hydrophilic NaCl solution phase by the addition of 5mL of ethyl acetate:acetone (75:25, v/v). The addition of organic phase was repeated twice, and the extracts were collected to a new test tube. Organic phases were collected and stored at -20°C. The organic solvent was evaporated using vacuum centrifugation immediately before carrying out the ELISA. The concentration of 6-keto $PGF_{1\alpha}$ was quantified as per manufacturer's instructions. Briefly, 6-keto $PGF_{1\alpha}$ was assayed with specific alkaline phosphatase-labelled conjugation, and quantification was determined by spectrophotometry analysis at 405nm with correction between 570 and 590nm (SpectraMax M2, Molecular Devices, US).

The release of PGI_2 was assayed in duplicate and is expressed as pg of PGI_2 per µg of protein.

2.5 High Performance Liquid Chromatography

2.5.1 *Flux/HPLC Analysis of Oestrogen Metabolism in Human Pulmonary Artery Smooth Muscle Cell In Vitro Model*

HPASMCs were plated in 100mm cell culture dishes and maintained in 10% FBS DMEM media until reaching approximately 70% confluence. The growth media was then replaced with 0.2% CSS-PRF DMEM for 16–24 hours, to ensure the cells were in the same stage of the cell cycle. When stimulating the cells, quiescing media was replaced with 1% CSS-PRF DMEM to maintain adequate cell growth, and hPASMCs were stimulated for 48 hours with 50nM (136.19ng) 17βE2 radioactively labelled with carbon (^{14}C) at position C4. ^{14}C-17βE2 with specific activity 55mCi/mmol and concentration, 0.1mCi/mL was purchased from American Radiolabelled Chemicals, Inc. (Missouri, USA). At the end time point of the experiment, cell culture dishes were placed on ice, and the spent media was collected into pre-chilled 15mL falcon tubes containing 0.1% (w/v) L-ascorbic acid (Sigma Aldrich, UK). The L-ascorbic acid was used to prevent the oxidation and degradation of catechol metabolites of 17βE2, which are extremely sensitive to oxidation. Immediately after collection, the media samples were stored at -20°C until further use. The cell monolayer was washed twice with ice-cold DPBS, and protein samples were collected by adding 600µL of Lysis and Extraction Buffer (ThermoFisher Scientific, UK), scraped carefully using a sterile plastic cell scraper and collected into pre-chilled 1.5mL Eppendorf tubes. Protein samples were left on ice for 30 minutes, before storing at -20°C until protein concentration was determined for normalisation of results, as described in section 2.2.2.

Metabolites formed form the radioactively labelled 17βE2 were extracted using solid-phase extraction (SPE), described in section 2.5.2. After SPE extraction, unlabelled metabolite standards prepared in 100% methanol were added to the extracted sample at a concentration of 10µg/mL for peak identification.

2.5.1.1 Analysis of Treprostinil Effects on Oestrogen Metabolism In Vitro

PASMCs were stimulated with treprostinil at two time points: i) 24 hours prior to addition of 50nM 14C-17βE2 (136.7pg), and ii) at the same time as 17βE2. After 48 hours, media samples were collected into tubes containing 10mg L-ascorbic acid (Sigma, UK) to prevent oxidation of oestrogen metabolites. The cell monolayer was washed twice with ice-cold PBS and lysed with 300µL of RIPA buffer (Thermo Scientific, USA). This experimental design enables to determine whether i) treprostinil affects oestrogen metabolism by directly altering enzyme catalytic activities, or ii) treprostinil affects oestrogen metabolism by altering enzyme expression.

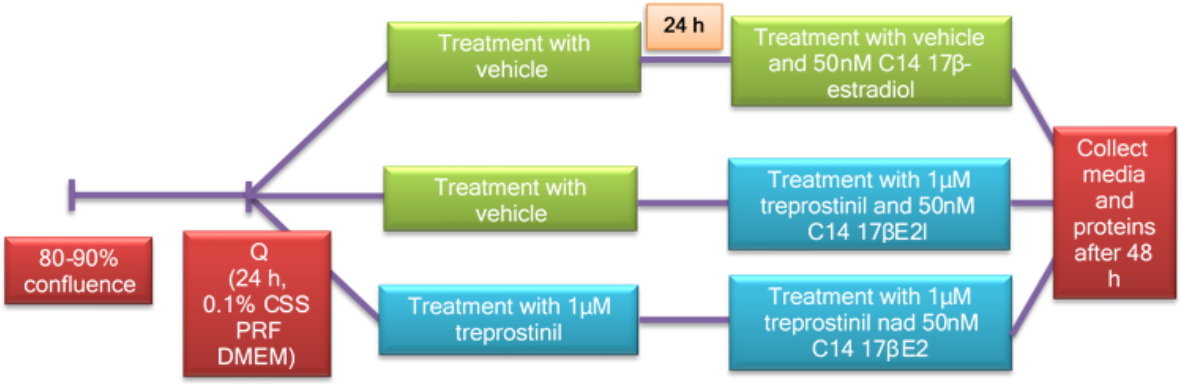


Figure 2–2: Experiment design to investigate the effects of treprostinil treatment on oestrogen metabolism in human pulmonary artery smooth muscle cells.

2.5.2 *Solid-Phase Oestrogen Metabolite Extraction*

The Oasis® HLB reverse-phase cartridges 3cc 60mg (Waters, UK) were used for solid-phase extraction of oestrogen metabolites from spent cell culture media. The cartridges were placed onto an extraction vacuum manifold (Waters, UK) to encourage the flow-through. The solid sorbent material in the cartridges was preconditioned first with 3mL of 100% methanol and then with 3mL of 100% water, prior to loading the biological sample or matrix containing known concentration of metabolite standards. The metabolites, having a steroid character, were retained in the sorbent material of the cartridge, which was subsequently re-equilibrated with 3mL of 100% water, followed by a wash of 3mL 5% (v/v) methanol in water. For flux/HPLC experiments, all solvents contained 0.1% (w/v) L-ascorbic acid. The oestrogen metabolites were eluted from cartridges with 3mL of 100% methanol. The eluents containing

concentrated and purified oestrogens were dried under a gentle stream of oxygen free nitrogen gas mixture at room temperature and reconstituted in the initial mobile phase containing water:acetonitrile:methanol (50:10:40, all HPLC Glass-distilled grade).

For HPLC analysis, samples were reconstituted in 250µL of mobile phase (water:methanol in the ratio 32:68). To certain samples 15µL of unlabelled oestrogen metabolite standard mixture was added, whilst 15µL of mobile phase was added to the rest of samples. Oestrogen metabolite standard mixture was prepared by mixing 200µL of each of the 10X standards (0.25mg/mL), resuspended in 300µL of mobile phase, after drying down under oxygen free nitrogen gas mixture at room temperature.

2.5.3 *Oestrogen Metabolism in Human Pulmonary Artery Smooth Muscle Cells*

A total of 40–70µL of each sample was injected onto the HPLC Sunfire C18 column (150mmx4.6mm, packed with 3.5µm C18 particles, 100Å, Waters, UK), maintained at 25°C, at a flow rate of 1mL/minute using the ASI-100 automated sample injector (Dionex, USA) and the P680 HPLC pump (Dionex, USA). Samples separated by HPLC were detected by UV and radio-labelled detection systems. To confirm peak identity, the samples were also separated with an Allure Biphenyl 5µm, 4.6x150mm column (Thames Restek, UK) maintained at 30°C, and using isocratic elution system with reverse phase chromatography with water and methanol in the mobile phase (water:methanol=32%:78%). %). Detection of radioactive metabolites was achieved by scintillation counting following mixing of column eluant with Ecoscint A scintillant (National Diagnostics, Georgia, USA; flow rate 1mL/min), using a LB509 β-scintillation counter (EG Berthold Technologies, Germany). Detection of unlabelled compounds was by ultra-violet (UV) absorption at wavelengths between 190nm to 220nm using a (PDA) Dionex PDA-100 detector (Dionex, USA).

All peak integrations for quantification were obtained from the sample run in the Sunfire C18 column. All HPLC data analysis was performed using Chromeleon 6.5 software (Dionex, USA).

2.6 Metabolomic Screen

2.6.1 *Cell Culture*

HPASMCs were plated in 100mm cell culture dishes, by adding 1mL of cell suspension to 9mL of 10% FBS DMEM and maintained until reaching approximately 70% confluence. Cells were then washed twice with warm DPBS, and the media was replaced with 1% CS-FBS PRF-DMEM. To assess the changes in the metabolome of hPASMCs, cells were stimulated with 50nM 17βE2 for 48 hours. Quenching of the metabolites was performed by putting the cell culture dishes on ice, and washing twice with DPBS, before 1 mL of methanol:acetonitrile:water=50:30:20 (v/v) was added to each dish. The extraction mixture was kept cold in a mixture of dry ice and methanol. Collected samples were then shaken for 12 minutes at 4°C, before being spun at 13,000rpm for 15 minutes (0°C). The supernatant was then transferred to labelled HPLC vials (Thermo Scientific, UK), while the protein pellet was used for determining protein concentration. All samples were stored at -80°C until analysis using pHILIC method.

2.6.2 *Metabolic Screen Using Liquid Chromatography Tandem Mass Spectrometry*

The samples were separated with a SeQuant® ZIC®-pHILIC Polymeric column (5µm, PEEK 150X4.6mm, Merck Millipore, UK) in gradient mode with 100% acetonitrile and 20mmol ammonia carbonate. Separated ions were analysed using LTQ Orbitrap mass spectrometer from Thermo Fisher Scientific (Hemel Hempstead, UK). Metabolomic screen data analysis was performed with open-source MZmine 2.12.4 data system.

2.7 Liquid Chromatography Tandem Mass Spectrometry

2.7.1 *Optimisation of Oestrogen Metabolite Extraction and Separation*

The purification of biological samples is generally required prior to sample analysis using the LC-MS/MS technique, to eliminate the major sources of interference and to concentrate the sample when low concentration of the analyte is present. This purification method must be optimised to permit efficient analyte recovery and to avoid presence of particulates, which might interfere with the LC-MS/MS method. To optimise the preparation of biological samples to be investigated using the Shimadzu QTRAP® 6500 LC-MS/MS System, the extraction of 1ng mixture of oestrogen metabolites was used to determine the nature of 1) sorbents in SPE cartridges, 2) wash steps and 3) elution solvents resulting in highest extraction yields and most reproducible LC-MS/MS results.

2.7.1.1 Optimisation of Extraction

The Oasis Hydrophilic-Lipophilic Balance (HLB) and Mixed-mode, strong Cation-eXchange (MCX) 3cc/60mg (both Waters, UK) SPE cartridges have been compared, where both cartridges are described as reverse-phase and contain water-wettable polymers. The HLB SPE cartridges are characterised as strongly hydrophilic, having high capacity and retention, resulting in their wide application range, while the MCX cartridges allow greater selectively of retention through manipulation of pH, as per Watters website. To simultaneously assess the effects of the sample matrix on the investigated variables, the mixture of oestrogen metabolites was prepared either in aqueous medium or cellular medium. A mixture of all oestrogen metabolites, with the final amount of 10ng, was added to 10 mL of either water or PRF-DMEM cellular media complemented with 1% CSS and spiked with 10µL of 1µg/mL of I.S. prior to extraction. The solid sorbent material in the cartridges was preconditioned first with 3mL of 100% methanol and then with 3mL of 100% water, prior to loading the biological sample or matrix containing known concentration of metabolite standards. The metabolites, having a steroid character, were retained in the sorbent material of the cartridge, which was subsequently re-equilibrated with 3mL of 100% water, followed by a wash of 3mL 5% (v/v) methanol in water. The oestrogen metabolites

were eluted from the extraction cartridge by either 3mL of 100% methanol or 100% acetonitrile (Sigma-Aldrich, UK). Eluents containing concentrated and purified oestrogens were dried at 40°C under a gentle stream of oxygen-free nitrogen gas mixture at room temperature, and reconstituted in 70µL of the initial mobile phase containing H_2O:ACN=90:10. Post-spiked samples were prepared by extracting 10mL of PRF-DMEM cellular media complemented with 1% CSS, as described above. The eluent was then spiked with 10µL of 1µg/mL oestrogen metabolite mixture and 10µL 1µg/mL I.S. The unextracted sample was prepared by pipetting 10µL of 1µg/mL oestrogen metabolite mixture and 10µL 1µg/mL I.S. into a clean vial and drying at 40°C down under oxygen-free nitrogen stream. Together these samples allow assessment of recovery and matrix suppression.

2.7.1.2 Optimisation of Wash Step

A mixture of all oestrogen metabolites, with the final amount of 10ng, was added to 10mL of PRF-DMEM cellular media complemented with 1% CSS and spiked with 10µL of 1µg/mL of I.S. prior to extraction. The solid sorbent material in the cartridges was preconditioned first with 3mL of 100% methanol and then with 3mL of 100% water, prior to loading the biological sample or matrix containing known concentration of metabolite standards. The metabolites, having a steroid character, were retained in the sorbent material of the cartridge, which was subsequently re-equilibrated with 3mL of 100% water, followed by a two wash steps with 1) 3mL 5% (v/v) methanol in water and 2mL 30% (v/v) methanol in water or 2) 3mL of 100% water, followed by 3mL 5% (v/v) methanol in water, respectively. The oestrogen metabolites were eluted from the extraction cartridge by either 3mL of 100% methanol or 3mL of 100% ACN. Eluents containing concentrated and purified oestrogens were dried under a gentle stream of oxygen-free nitrogen gas mixture at room temperature and reconstituted in 70µL of the initial mobile phase containing H_2O:ACN=90:10. The post-spiked and unextracted samples were prepared as described above.

2.7.2 *Liquid Chromatography Tandem Mass Spectrometry - Analysis of Oestrogen Metabolism in Human Pulmonary Artery Smooth Muscle Cells In Vitro Model*

HPASMCs were plated in 100mm cell culture dishes and maintained in 10% FBS DMEM media until reaching approximately 70% confluence. The growth media was then replaced with 0.2% CSS-PRF DMEM for 16–24 hours, to ensure all cells were in the same stage of the cell cycle. When stimulating the cells, quiescing media was replaced with 1% CSS-PRF DMEM to maintain adequate cell growth, and hPASMCs were stimulated for 48 hours with 50nM (136.19ng) 17βE2. At the end time point of the experiment, cell culture dishes were placed on ice, and the spent media was collected into pre-chilled 15mL falcon tubes. Immediately after collection, the media samples were stored at -80°C until further use. The cell monolayer was washed twice with ice-cold DPBS, and protein samples were collected by adding 600µL of 1% (v/v) LM buffer (Abcam, UK), scrapped carefully using a sterile plastic cell scraper and collected into pre-chilled 1.5mL Eppendorf tubes. Protein samples were left on ice for 30 minutes, before storing at -80°C until protein concentration was determined for normalisation of results, as described in section 2.2.2.

Metabolites formed form the 17βE2 were extracted using SPE, described in section 2.5.2, and stored at -80°C until derivatisation. To all samples, 10µL of 1µg/mL of I.S., $^{13}C_3$-E1 and $^{13}C_3$-17βE2 (Sigma-Aldrich, UK) was added prior to SPE extraction. For LC-MS/MS L-ascorbic acid was not added to any of the solvents used in the SPE.

2.7.3 *Derivatisation with PPZ in Methylation Conditions*

Extracted biological samples were dried under continuous stream of oxygen free nitrogen at 40°C. Then, 10µL of 1M $NaHCO_3$ (Sigma Aldrich, UK), 10µL 1mg/mL 1-(5-fluoro-2,4-dinitrophenyl)-4-methylpiperazine (1,PPZ, Tokyo Chemical Industry Ltd, UK) and 70µL 100% acetone were added to the dry residue, and shortly vortexed. Oestrogen metabolites and reagents were incubated at 60°C for 1 hour. The reaction was quenched by the addition of 500µL of H_2O:methanol=1:1 to each sample. The samples were again dried under continuous stream of oxygen free nitrogen, at 40°C, to complete dryness. To introduce a permanent positive charge into each molecule of oestrogen metabolite, the samples were further methylated by adding 100µL of methyl iodide (CH_3I, Sigma Aldrich, UK), and

incubating the mixture at 60°C for 30 minutes. The mixture was then dried down under a gentle stream of oxygen free nitrogen, and the dry residue was reconstituted in 70µL of initial mobile phase with composition H_2O:acetonitrile=90:10.

Derivatised samples were either analysed on an LC-MS/MS setting immediately, or they were stored at -80°C.

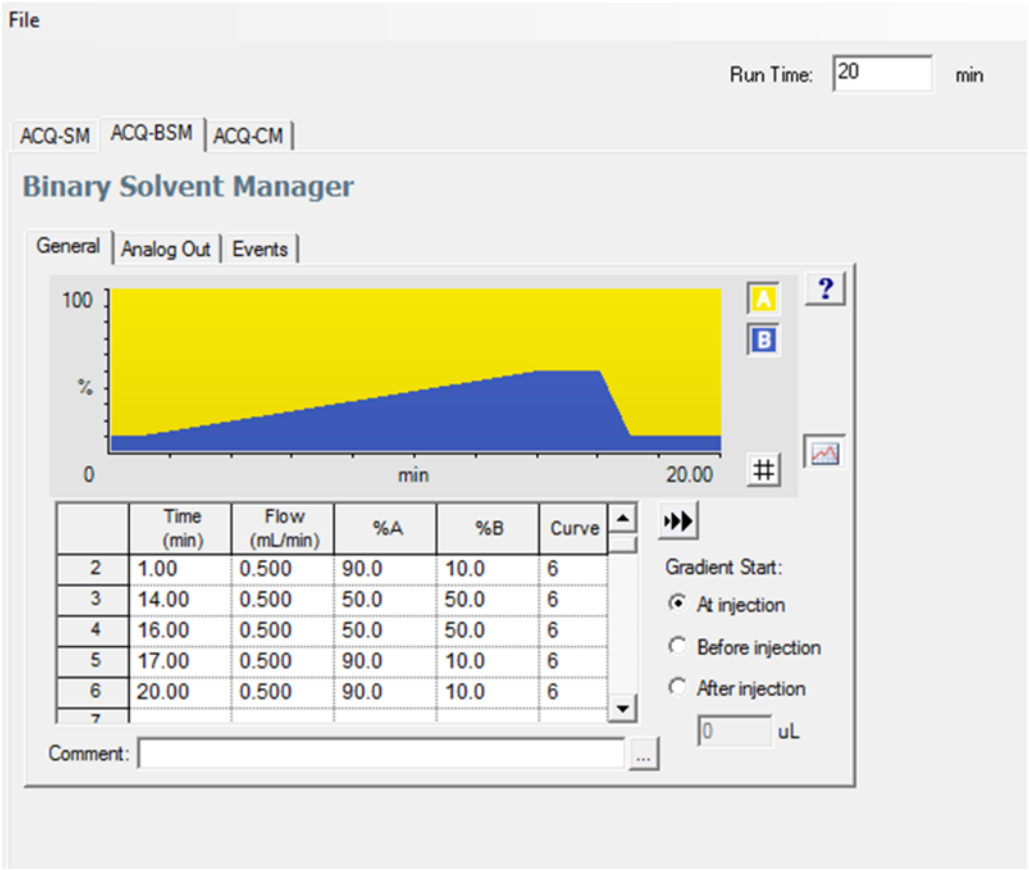


Figure 2–3: Gradient programme used to separate oestrogen metabolites within ACE Excel 2 C18-PFP (150x2.1mm) column, maintained at 25°C.

2.7.4 *Liquid Chromatography Tandem Mass Spectrometry*

Samples to be analysed on the Shimadzu QTRAP® 6500 LC-MS/MS System (AB SCIEX, UK), were maintained at 5°C. 30µL of each sample was injected onto the ACE Excel 2 C18-PFP (150x2.1mm) column (Hichrom Ltd, UK). Gradient separation method was employed, by an eluent composition initially set to 10% (B) 0.1% formic acid in acetonitrile (v/v) and 90% (A) 0.1% formic acid in water (v/v). The column was operated at 25°C with a flow rate of 0.5mL/min, with the following gradient program: 10% B to 50% B in 13 minutes, then maintained at 50% B for 2 minutes, linearly decreasing to 10% B in 3 minutes, finally the column was equilibrated with 10% B for 3 minutes (see Figure 2–3).

Turbo electron ion spray voltage (EIS) and temperature (TEM) were maintained at 5.5kV and 500°C, respectively. Multiple reaction monitoring (MRM), in positive polarity, with unit mass resolution for the precursor and product ions was used for quantitation of oestrogen metabolites.

The triple quadrupole mass spectrometer contains two mass filters (Q1 and Q3), while Q2 is used as collision cell to fragment molecule of interest. The Q1 is typically used to select the parental ions of interest generated in the ion source, hence the Q1 mass corresponds to molecular weight of the derivatised parental ion to charge ratio (m/z). The precursor ions are then fragmented in Q2, and the product ions are separated by m/z ratio in Q3. Therefore, Q3 mass is used to select specified fragments of precursor ion to the electron multiplier. The Q1 and Q3 masses of oestrogen metabolites analysed in the spent media samples are provided in Table 2-9.

Table 2-9: Characteristic Q1 and Q3 masses of oestrogen metabolites derivatised with PPZ/methylation conditions used for LC-MS/MS identification and analysis.

Identification	Q1 Mass (Da)	Q3 Mass (Da)	Time (ms)	DP (Volts)	CE (Volts)	CXP (Volts)
E1	549.1	502.3	30	141.0	59.0	20.0
17βE2	551.1	504.3	30	51.0	59.0	20.0
17αE2	551.1	504.3	30	51.0	59.0	20.0
2OHE1	565.0	58.0	30	130.0	129.0	10.0
4OHE1	565.0	58.0	30	130.0	129.0	10.0
16αOHE1	565.0	58.0	30	130.0	129.0	10.0
2OHE2	567.1	58.0	30	136.0	121.0	8.0
4OHE2	567.1	58.0	30	136.0	121.0	8.0
16αOHE2	567.1	58.0	30	136.0	121.0	8.0
2MeOE1	579.0	58.0	30	66.0	121.0	8.0
4MeOE1	579.0	58.0	30	66.0	121.0	8.0
2MeOE2	581.0	280.0	30	111.0	57.0	28.0
4MeOE2	581.0	250.0	30	61.0	63.0	24.0
^{13}C-E1	552.3	505.3	30	135.0	39.0	15.0
^{13}C-17βE2	554.3	507.3	30	135.0	35.0	15.0

DP= De-clustering potential, CE= Collision energy, CXP= Collision exit potential

Nineteen MRM transitions were monitored with dwell time set at 30ms. Collision energy was set for each metabolite separately. Data acquisition and processing were controlled by

the Analyst Software (version 1.6.3) of the instrument. All LC-MS/MS data analyses was performed using MultiQuant 3.0.2 software (AB SCIEX, UK).

2.7.5 Validation of Assay for Oestrogen Metabolites in Cell Media

The limits of detection (LODs) was determined for each of the oestrogen metabolites of interest as the lowest concentration at which the observed signal was clearly separable from the baseline noise. Calibration curves for oestrogen metabolites of interest were obtained using linear regression without weighting. The linearity of the assay was assessed daily on three consecutive days using six concentrations from 0.2 to 100ng/mL, while the concentration of I.S. ($^{13}C_3$-E1 and $^{13}C_3$-17βE2) was kept constant at 100ng/mL for both standards. The uncertainty of measurements for the same sample was expressed in coefficient of variation (%CV), calculated by dividing standard deviation of the sample measurements with average value, multiplied by 100. Recovery and matrix effects were determined at concentration of 200ng/mL (n=2). The recovery (%RE) for each oestrogen metabolite was estimated as the ratio of peak areas for each analyte to I.S. spiked before extraction, divided by the ratio of peak areas of each analyte analyte to IS spiked post-extraction, multiplied by 100:

$$\%RE = \frac{\left(\frac{Peak\ area\ (Analyte - Prespiked)}{Peak\ Area\ (IS - Prespiked)}\right)}{\left(\frac{Peak\ area\ (Analyte - Postspiked)}{Peak\ Area\ (IS - Postspiked)}\right)} \times 100$$

It is important to consider matrix effects (%ME) when developing a quantitative LC-MS/MS method, as the presence of unknown compounds, which react unpredictively, in the sample might affect the methods' accuracy and reproducibility. %ME was calculated as the ratio of peak areas of each post-spiked analyte with the peak area of unextracted analyte:

$$\%ME = \frac{Peak\ area\ (Analyte - Postspiked)}{Peak\ area\ (Unextrcted)}.$$

Ion suppression (%IS) is defined as reduced response of the detector due to competition of compounds present in the sample with the analyte of interest for the efficiency in the ionisation source. When looking at several analytes of interest within a complex sample, the

competition of these compounds for the efficiency in the ionisation source might also result in ion suppression. %IS was estimated as the ratio of each oestrogen metabolite to IS response ratio, divided by unextracted mixture of oestrogen metabolites, multiplied by 100:

$$\%IS = \frac{\left(\frac{Peak\ area\ (Analyte - prespiked)}{Peak\ Area\ (IS - prespiked)}\right)}{\left(\frac{Peak\ area\ (Unextracted\ Mixture)}{Peak\ Area\ (IS - Unextracted\ Mixture)}\right)} \times 100$$

2.8 Statistical Analysis and Data Handling

Data presented herein was processed and analysed using GraphPad Prism (California, USA), version 5 and 6. The values are expressed as the mean value ± the standard error of the mean (SEM). Herein, N denotes the number of cell lines used in an experiment, while the number of replicates indicates the number of times the experiment was repeated using a cell line. To evaluate the statistical significance of differences between groups, One-Way ANOVA followed by Tukey's or Bonferroni post-hoc tests, depending on whether all possible pairwise comparisons across all groups were examined or not. Where required, a Two-Way ANOVA followed by Sidak Post-hoc test was utilised. A probability level of $p<0.05$ was defined as being statistically significant.

Chapter III

3 *In Vitro* Investigation of Mechanisms Involved in the Establishment of Experimental Pulmonary Arterial Hypertension Phenotype in the Sugen 5416 and Chronic Hypoxia Model

3.1 Introduction

Numerous advances in medical research have been attributed to the use of animal models. According to Ericsson and colleagues, pre-clinical studies using animal models to appropriately and accurately replicate human diseases, offer rapid access to robust data, leading to imperative insight into the pathophysiological disease mechanisms and aid the development of novel treatment options (Ericsson et al., 2013). The research of complex diseases associated with numerous co-founding factors and aetiologies, such as PAH, might therefore rely heavily on animal models to elucidate underlying biological mechanism. This may require utilising more than one model to ensure robustness of gathered information. Although recent advances in genetic engineering have resulted in the generation of numerous novel models, these unfortunately do not generally recapitulate all the complex features of PAH pathophysiology. Moreover, larger number of genetically modified animals are required to maintain the genetically engineered line. Hence, it is not surprising that the number of animals in research has been increasing slowly since the beginning of 21st century, mostly due to increasing proportion of procedures using animals that have been genetically modified or harbour genetic mutations.

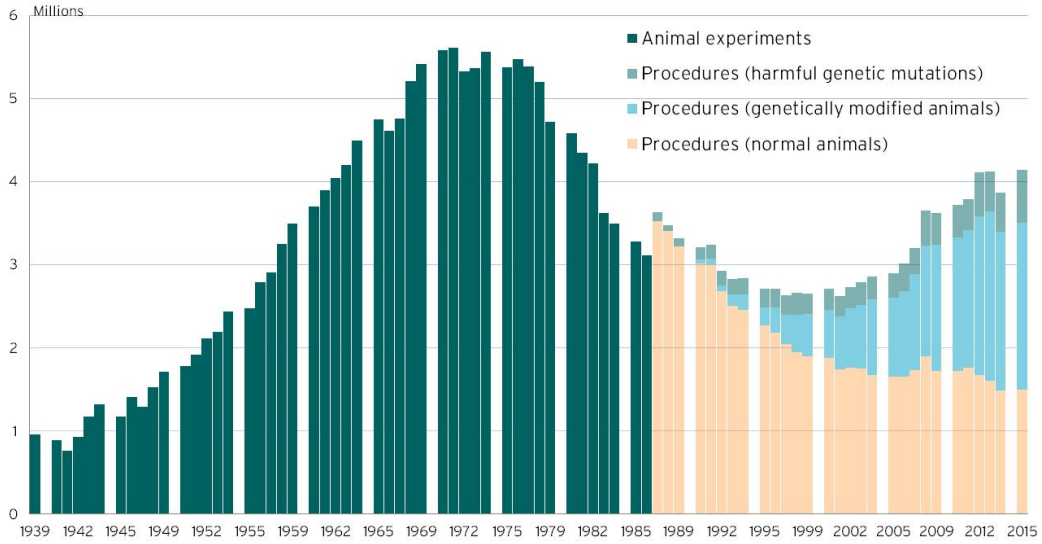


Figure 3–1: The trend of use of animals in research since 1930s to date.
The diagram showing the number of animals used in biomedical research per year was acquired from Understanding Animal Research.

In the UK, however, due to the efforts of British government to regulate more stringently the use of animals in research through establishment of the National Centre for the Replacement, Refinement and Reduction of Animals in Research, the proportion of percentage of experimental procedures on animals has not increased compared to the

increase in all bioscientific experiments. The main aims of the NC3Rs are to promote researchers to develop novel methods enabling i) minimisation of the number of animals used per experiment, ii) replacement or complete avoidance of the use of animals, and iii) improvement the welfare of animals and minimise their suffering (NC3Rs). It has been proposed that, the development of new research technologies and refinement of existing models could in the future lead to significant reduction, and possible absolute replacement, of the use of animals in research.

To accurately replicate human PAH in terms of the histopathological changes observed within the distal pulmonary vasculature, including the occurrence of angio-obliterative lesions generally requires multiple stimuli. The Su5416/Hx model in rats is established by administration of a single dose of 20mg/kg of Sugen 5416 to the animals, which are then exposed to hypoxia for 14 or 21 days. Animals are then retained in Nx conditions for 6 to 10 weeks so that the experimental PH phenotype develops fully. This model recapitulates the human disease in terms of the presence of occlusive lesions. Additionally, the animals developed higher mPAP than Hx alone. In this model female animals developed the disease more frequently compared to males, and oestrogens apparently mediate a detrimental role in this specific model (Tofovic et al., 2012), this model could be regarded as most appropriate model to investigate PAH pathophysiology and the effectiveness of novel therapeutic approaches. As Su5416 is considered to be a potent ligand of the AhR, expressing high affinity for the receptor, the role of the AhR has been considered in the mechanism driving the onset of PAH phenotype in this animal model. In terms of the AhR mediating CVD, the receptor absence was associated with the occurrence of abnormal hepatic vascular structures according to (Lahvis et al., 2000). The absence of the receptor in *AhR* null mice also resulted in increased expression of HIF1α and VEGF in the heart (Thackaberry et al., 2002), both of which have been associated with compensatory mechanisms resulting in cardiac hypertrophy (Vasquez et al., 2003). According to Sauzeau, absence of AhR in the vasculature was associated with increased thickness of arteria media vessel wall due to increased number of SMCs (Sauzeau et al., 2011). The activation of AhR in the cardiovascular system was also shown to increase the expression of *Phase I* metabolising enzymes, leading to subsequent increase in ROS (Park et al., 1996).

However, as the mechanism by which the disease phenotype is established in the Su5416/Hx model is largely unknown, it is important to determine which cellular pathways are involved to comprehend the suitability of the model.

Aims of this chapter:

1) To investigate the mechanism through which the animal model of pulmonary arterial hypertension employing Sugen 5416 coupled with chronic hypoxia is induced, utilising *in vitro* methods.
2) To establish whether proteins involved in the AhR/HIF1α signalling pathways are differentially expressed in the lungs of animals with established disease phenotype and their control littermates, and in hPASMCs, derived from patients and control subjects of both sexes.
3) To establish an *in vitro* cellular model using human pulmonary arterial and human pulmonary microvascular endothelial cells, recapitulating the molecular changes observed in animal model of Sugen 5416 coupled with chronic hypoxia.

3.2 Results

3.2.1 *Cells Derived from Female Patients with Pulmonary Arterial Hypertension Exhibit Decreased Gene Expression of Cytochrome P450 1A1*

White and colleagues conduced a microarray analysis to determine any sex differences in the genetically altered mouse that overexpresses the human gene for the serotonin transporter (SERT+ mouse), as only female SERT+ mice developed the PAH phenotype (White et al., 2011b). Thearray revealed the up-regulation of several genes in female SERT+ PAs, including *CYP1B1*, *CEBPβ* and *FOS* genes (White et al., 2011b). *CYP1A1* is induced by the AhR and is a good marker of AhR activity (Hu et al., 2007). To assess whether sex differences in *CYP1A1* gene expression levels exist and establish the importance in the disease onset and progression, we examined the expression of the gene encoding this enzyme in models of PAH.

Firstly, we examined hPASMCs derived from female PAH patients compared to female control cells. We observed that level of expression of the *CYP1A1* gene, determined using quantitative RT-PCR analysis, might be decreased in hPASMCs derived from female PAH patients compared to female control cells (Figure 3–2).

A slight variability in the Ct value of the housekeeper gene *β-2-microglobulin* was detected in the group of hPASMCs derived from female PAH patients. The normalisation of the Ct values of the gene of interest is important, as it enables the compensation of intra- and inter-kinetic RT-PCR variations, allowing to compare samples in the same experiment and between experiments. The quality of the data was examined by calculating the standard deviation of the Ct values. Generally, within our lab, the results for which the variability described by standard deviation of less than 1 amongst all groups were considered useable, and this criterion was also applied for this work. When the fold change in the expression of the gene of interest is high, there is a greater tolerance for the variation in the expression of the housekeeping gene, which has a lesser impact the interpretation of the results. Conversely, when looking at relatively small fold differences, the tolerance in the variation of housekeeping gene may be less. Therefore, optimisation of the assay may be required. The coefficient of variation for these experiments, calculated by dividing the standard

deviation with the mean Ct value, was less than 5%, which is still acceptable. We recognise that with borderline results, larger variability of the housekeeper gene) could artificially result in greater significance of results, however, the variability of the housekeeper gene Ct values was within the acceptable limits of variation. To improve the experiment in order to produce highly accurate and reliable results, other housekeeping genes, which generally exhibit less variability could be employed, as described in (de Jonge et al., 2007). According to de Jonge *et al.*, the housekeeping genes exhibiting the least variability are different ribosomal proteins and ornithine decarboxylase antizyme 1.

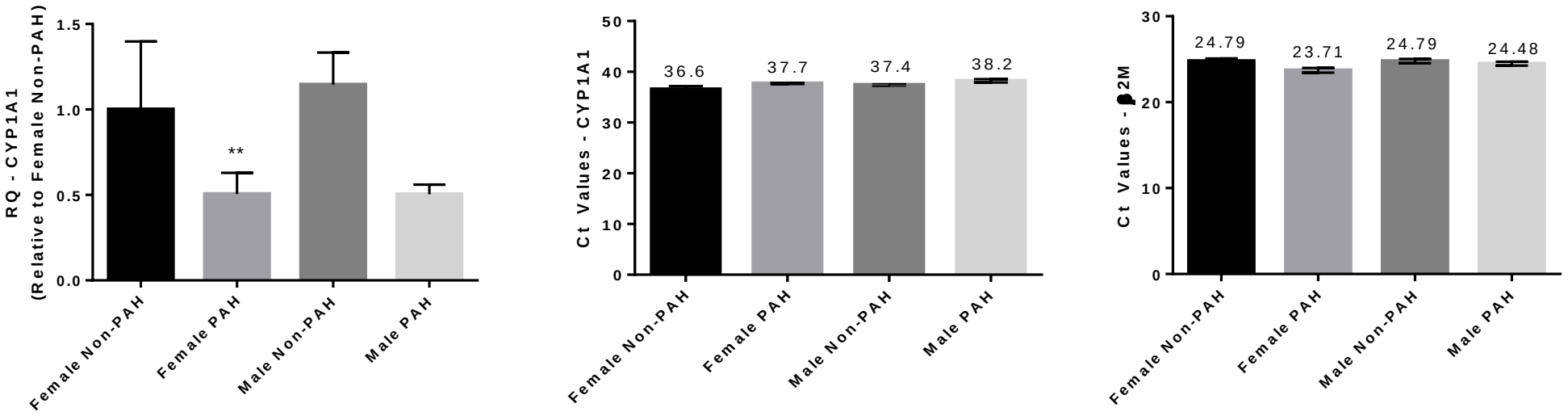


Figure 3–2:The relative expression of the gene encoding the CYP1A1 is decreased in human pulmonary artery smooth muscle cells from female PAH patients.

Pulmonary artery smooth muscle cells (PASMCs) were explanted from distal arteries of control subjects and patients suffering from pulmonary arterial hypertension (PAH) and cultured as described previously. The expression of *CYP1A1* was assessed by Taqman quantitative Real Time-PCR. Results were normalised to the *β-2-microglobulin*. N=3–4 per group in triplicate, **p≤0.01, One-way ANOVA followed by Tukey's post-hoc test. Data is expressed as $RQ \pm \frac{RQ_{max}}{RQ_{min}}$, where RQ=Relative quantification.

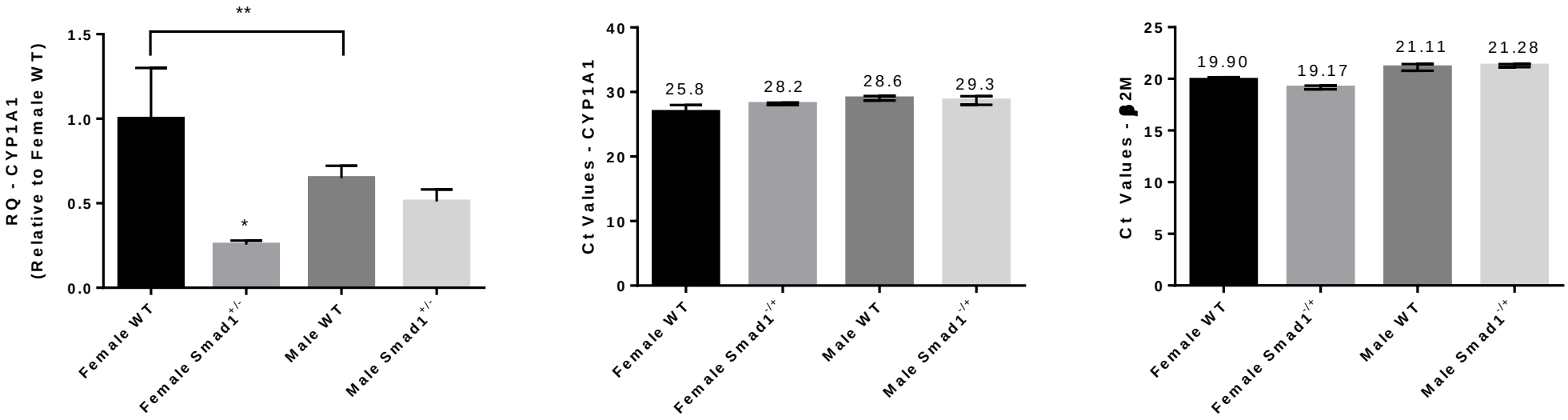


Figure 3–3:The relative expression of the gene encoding the CYP1A1 is decreased in whole lungs from female Smad1 heterozygous mice.

Whole lungs of *Smad1* heterozygous mice of both sexes, and their age-matched C57BL/6 litter mates, used as control animals, were prepared, as described previously, to assess the expression level of *CYP1A1* by Taqman quantitative Real Time-PCR. Results were normalised to the *β-2-microglobulin*. N=4–6 per group in triplicate, *p<0.05, **p≤0.01, One-way ANOVA followed by Dunnett's post-hoc test. Data is expressed as $RQ \pm \frac{RQ_{max}}{RQ_{min}}$, where RQ=Relative quantification.

3.2.2 *Female Smad1 Heterozygous Mice Exhibit Decreased Gene Expression Levels of Cytochrome P450 1A1*

The tissue of Smad1 heterozygous mice and their WT counterparts used in these experiments was kindly provided by Dr Mair. The haemodynamic and remodelling parameters observed in these animals are published in (Mair et al., 2015). The expression of the *CYP1A1* gene was also assessed in whole lung from female and male *Smad1* heterozygous mice, and their age-matched C57BL/6 litter mates, which served as controls. The relative quantitation of the gene showed that *CYP1A1* gene is significantly decreased in the lungs derived from *Smad1* heterozygous mice compared to female control animals (Figure 3–3). This observation indicates that in this Nx PAH generic model exhibiting the PAH disease phenotype (Han et al., 2013;Mair et al., 2015), the expression of *CYP1A1* is decreased compared to age-matched female litter mates. Interestingly, unlike the increased expression level of *CYP1B1*, reported previously in animals and hPASMCs (White et al., 2012) exhibiting PAH phenotype, the expression of *CYP1A1* was reduced.

3.2.3 *The expression level of Cytochrome P450 1A1 is Significantly Decreased in Experimental Pulmonary Hypertension Induced by Hypoxia in Sprague-Dawley Rats of Both Sexes*

To further investigate the expression of *CYP1A1* gene in models of PAH, the relative quantitation of the gene was assessed in lungs from the classical cHx model of PH. We studied this in Sprague-Dawley rats exposed to either Hx or Nx conditions for 28 days. The tissue used in these experiments was kindly provided by Dr Murphy. The haemodynamic and remodelling parameters observed in these animals after exposure to chronic hypoxia were published in (Murphy, 2017). We observed a significant decrease in the relative quantitation of the *CYP1A1* gene in female and male animals exposed to Hx compared to their Nx controls (Figure 3–4).

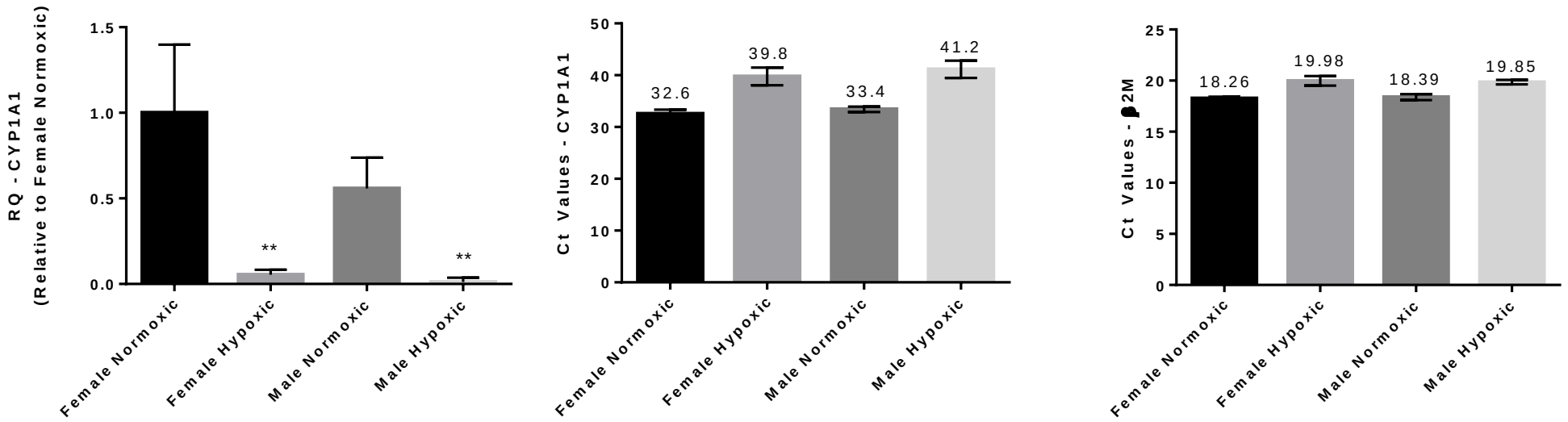


Figure 3–4: The relative expression of the gene encoding the CYP1A1 is decreased in female and male rats exposed to hypoxia for 28 days.

Whole lung of Sprague-Dawley rats of both sexes, exposed to hypoxia for 28 days, and their normoxic litter mates, used as control animals, were prepared, as described previously, to assess the expression level of *CYP1A1* by Taqman quantitative Real Time-PCR. Results were normalised to the *β-2-microglobulin*. N=4–6 per group in triplicate, *p<0.05, **p≤0.01, One-way ANOVA followed by Dunnett's post-hoc test. Data is expressed as $RQ \pm \frac{RQ_{max}}{RQ_{min}}$, where RQ=Relative quantification.

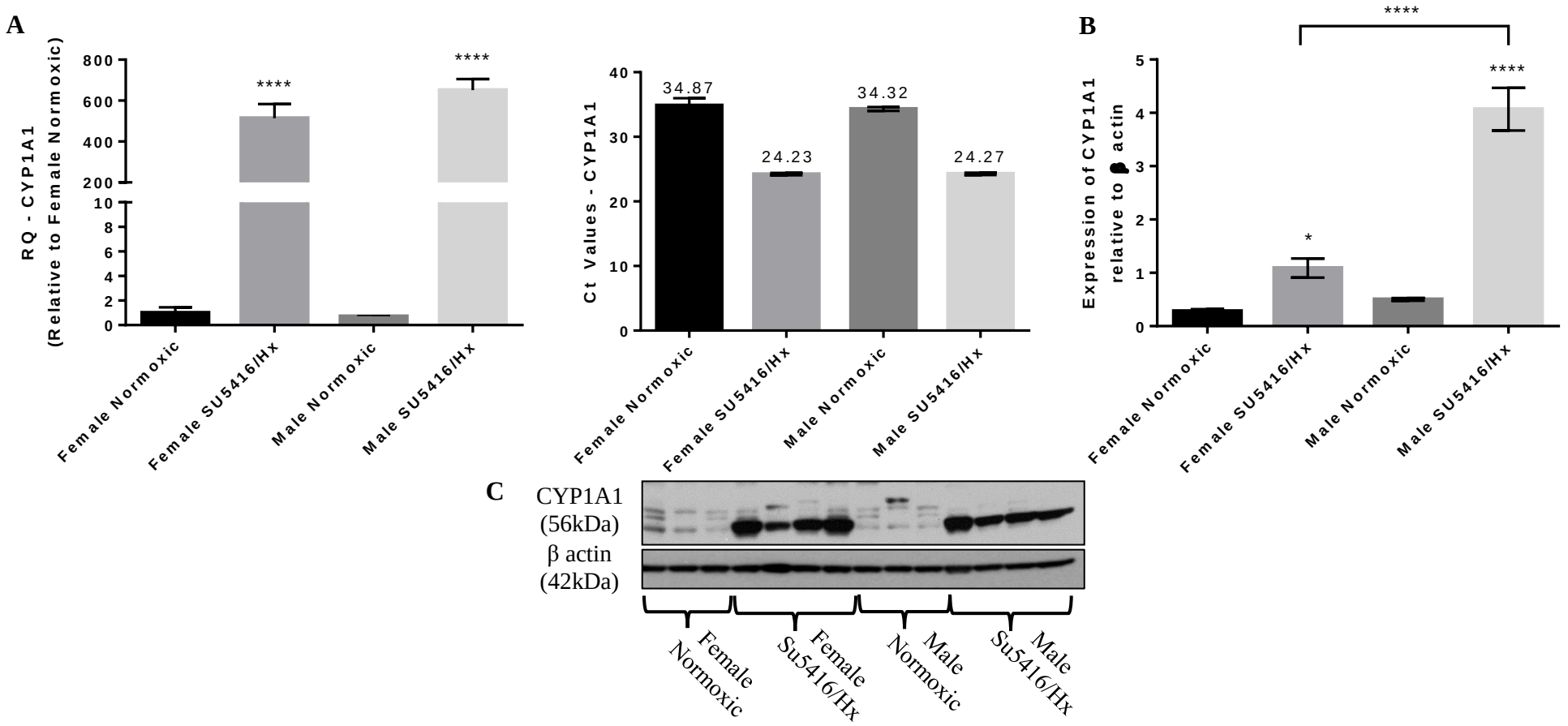


Figure 3–5: The expression of CYP1A1 is increased in whole lungs from female and male rats treated with Sugen 5416 and exposed to hypoxia.

Samples of whole lung of Wistar Kyoto rats, of both sexes, treated with single dose of Sugen 5416 and exposed to hypoxia and their normoxic controls, were prepared, as described previously, to assess the expression level of *CYP1A1* by Taqman quantitative Real Time-PCR (A). Results were normalised to *β-2-microglobulin*. N=4–6 per group in triplicate, ****p≤0.0001, One-way ANOVA followed by Dunnett's post-hoc test. Data is expressed as $RQ \pm \frac{RQ_{max}}{RQ_{min}}$, where RQ=Relative quantification. The level of CYP1A1 protein expression was assessed using immunoblotting (B). N=3–6 per group in duplicate, *p<0.05, ****p≤0.0001, One-way ANOVA followed by Dunnett's post-hoc test. Representative immunoblot is shown in (C).

3.2.4 *The Expression of Cytochrome P450 1A1 Gene is Increased in Experimental Pulmonary Hypertension Induced by Sugen 5416 and Chronic Hypoxia*

Lastly, the level of *CYP1A1* gene expression was addressed in the Su5416/Hx model, which is currently widely used to investigate the pathophysiological mechanisms of PAH onset and progression, due to histological, pathophysiological and epidemiological characteristics similar to those observed in humans. Here, the relative quantitation of the gene was assessed in the whole lungs from Wistar Kyoto rats treated with Su5416 and exposed to Hx or in age-matched litter mates kept in Nx conditions (Mair et al., 2014). The experimental PH phenotype in Su5416/Hx model was established by a single dose of 20mg/kg Su5416, followed by 14 days in Hx and further 14 days in Nx (Mair et al., 2014). Su5416/Hx-treated animals developed right ventricular systolic pressure of approximately 110mmgHg, with 10–15% remodelled and 20–30% occluded vessels, and right ventricular hypertrophy index (RV/LV+S) of 0.6 (Mair et al., 2014). The relative expression of the *CYP1A1* gene in female and male treated animals was significantly increased (Figure 3–5A), while no difference between the sexes was detected. Using immunoblotting technique to assess the level of protein expression we show that CYP1A1 protein expression was also increased in Su5416/Hx-treated animals of both sexes. The quantification of the protein expression also indicated that the CYP1A1 expression in male rats treated with Su5416/Hx is significantly higher than in age-matched female rats (Figure 3–5B).

The AhR signalling pathway is considered to be an organism's innate mechanisms to efficiently deal with exposure to environmental toxins Therefore, the activation results in induction of genes involved in metabolism of these substances, including the *CYP1B1* gene. We have therefore also examined the level of relative quantitation of *CYP1B1* in the whole lungs. We observed that in male and female animals exposed to Su5416 and cHx resulted in a significantly increased expression of CYP1B1 compared to their corresponding Nx controls, on both gene and protein level (Figure 3–6). The evaluation of the expression of CYP1B1 protein, however, was limited by the quality of the western blot. The diffuse nature of the bands and relatively high background limited the accuracy of determining the boarders of the protein bands and hence the measurement of optical density. The diffuse nature of the bands could be associated with the presence of post-translational modifications of CYP1B1

or the condition of the tissue used for this experiment. Additionally, the anti-CYP1B1 antibody used exhibited some off-target binding, leading to poorer results.

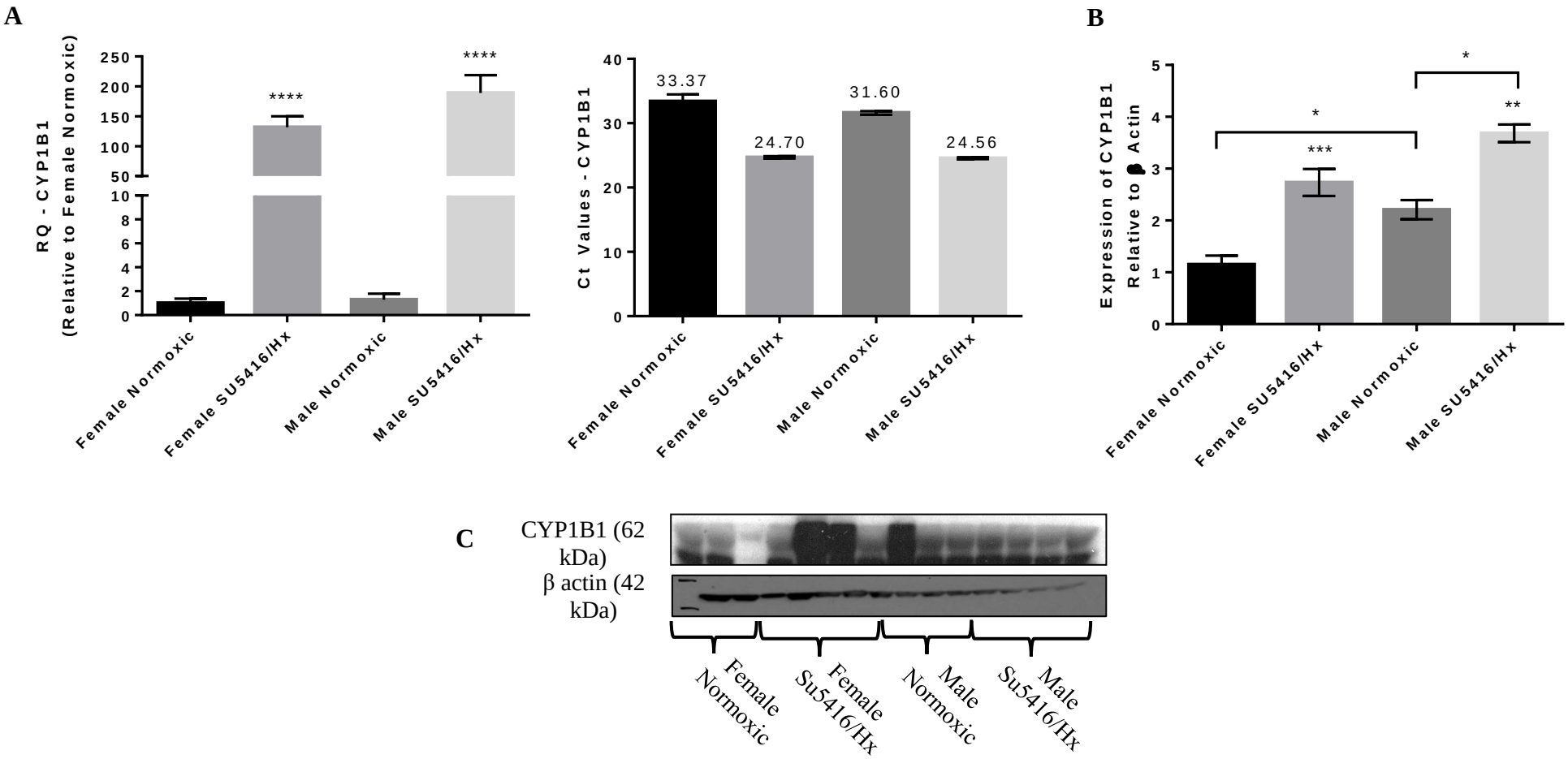


Figure 3–6: The expression of CYP1B1is increased in whole lung from female and male rats treated with Sugen 5416 and exposed to chronic hypoxia.

Samples of whole lung of Wistar Kyoto rats, of both sexes, treated with single dose of Sugen 5416 and exposed to hypoxia and their normoxic controls, were prepared, as described previously, to assess the expression level of *CYP1B1* by Taqman quantitative Real Time-PCR (A). Results were normalised to the *β-2-microglobulin*. N=4–6 per group in triplicate, *p<0.05, **p≤0.01, ***p≤0.001, One-way ANOVA followed by Dunnett's post-hoc test. Data is expressed as $RQ \pm \frac{RQ_{max}}{RQ_{min}}$, where RQ=Relative quantification. The level of CYP1B1 protein expression was assessed using immunoblotting (B). N=3–6 per group in duplicate, *p<0.05, ****p≤0.0001, One-way ANOVA followed by Dunnett's post-hoc test. Representative immunoblot is shown in (C).

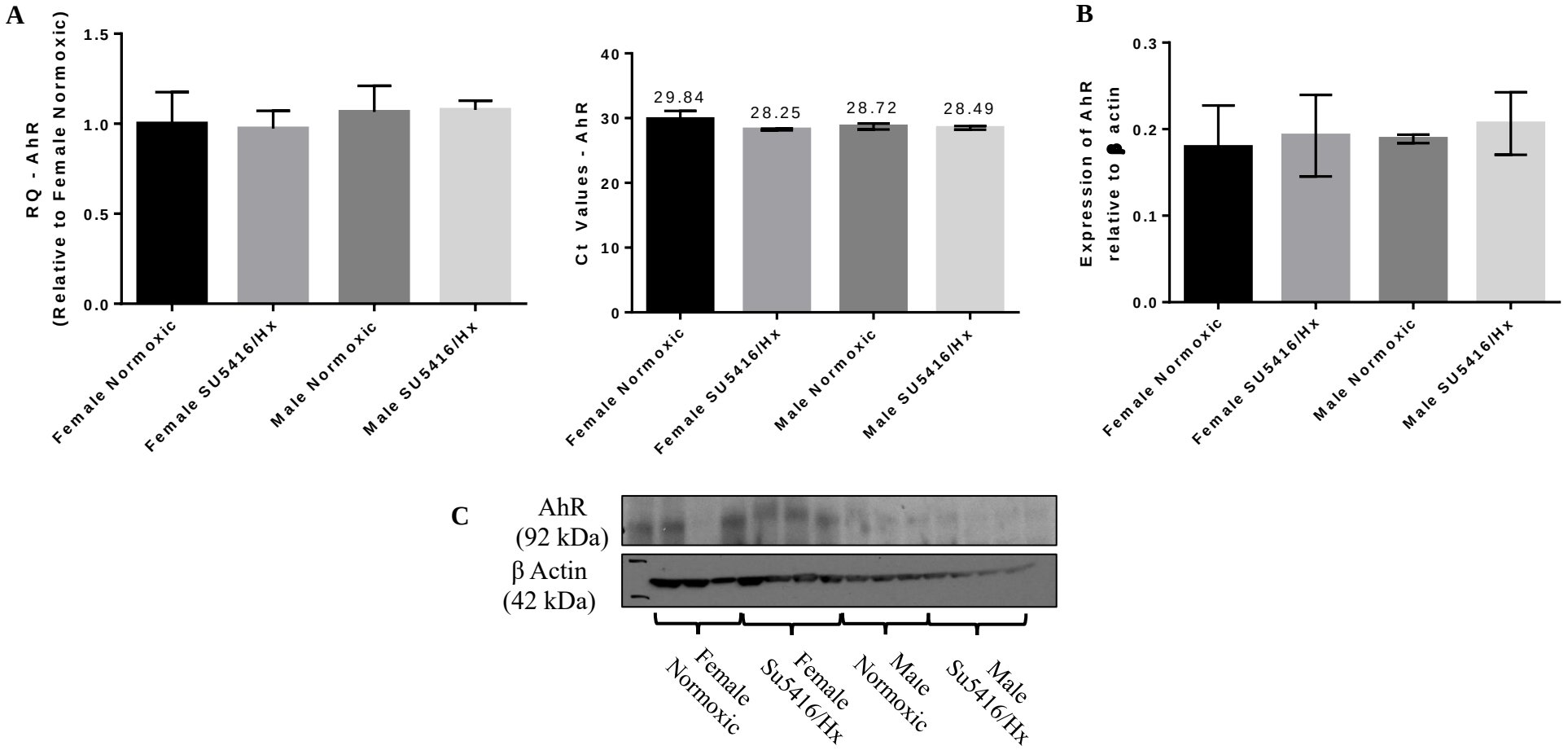


Figure 3–7: The expression of aryl hydrocarbon receptor is unchanged in whole lung samples from female and male rats treated with Sugen 5416 and exposed to chronic hypoxia.

Samples of whole lung of Wistar Kyoto rats, of both sexes, treated with single dose of Sugen 5416 and exposed to hypoxia and their normoxic controls, were prepared, as described previously, to assess the expression level of *AhR* by Taqman quantitative Real Time-PCR (A). Results were normalised to *β-2-microglobulin*. N=4–6 per group in triplicate, One-way ANOVA followed by Dunnett's post-hoc test. Data is expressed as $RQ \pm \frac{RQ_{max}}{RQ_{min}}$, where RQ=Relative quantification. The level of AhR protein expression was assessed using immunoblotting (B). N=3–6 per group in duplicate, One-way ANOVA followed by Dunnett's post-hoc test. Representative immunoblot is shown in (C).

When we assessed the relative quantitation of the *AhR* gene in lungs from male and female Su5416/hx rats and their controls, no significant differences were observed (Figure 3–7). The evaluation of the expression of AhR on protein level was limited by the level of expression and the diffuse nature of the bands, limiting the accuracy of determining the boarders of the protein bands and hence the measurement of optical density. *ARNT(HIF1β)* is the key binding partner of the AhR to produce an active receptor dimer. *ARNT(HIF1β)* gene levels in Su5416/Hx-treated females was significantly lower compared to female Nx controls (Figure 3–9). In male animals no such significant differences were observed (Figure 3–9).

The AhR signalling pathway is negatively regulated by the induction of the AhRR, which serves to bind to the AhR instead of the ARNT, and hence prevents the receptor's activity. Therefore, the relative expression level of the *AhRR* is another useful measure of the activity of the AhR signalling pathway. To confirm the AhR signalling pathways is greatly induced in the Su5416/Hx-treated animals, we have examined the relative quantitation of *AhRR* gene in whole lung samples. As previously with *CYP1A1* expression levels, we reported that the expression of *AhRR* gene was significantly higher in the Su5416/Hx-treated animals compared to their sex- and age-matched Nx controls (Figure 3–8). The magnitude of increase in the gene expression level of *AhRR* observed was comparable to that of *CYP1A1* gene. Moreover, we have also established that the expression of *AhRR* in Su5416/Hx male rats was significantly higher than in Su5416/Hx female rats. Equally, we observed that *AhRR* expression in Nx male rats was also significantly higher than in Nx female rats. Hence it appeared that there might be a sex specific difference in the level of *AhRR* expression, and therefore the AhR pathway might be differentially negatively regulated. The evaluation of the expression of AhRR protein, was limited by the quality of the western blot. The diffuse nature of the bands and relatively high background limited the accuracy of determining the boarders of the protein bands and hence the measurement of optical density. Additionally, the anti-AhRR antibody used exhibited some off-target binding, leading to poorer results.

As there is significant crosstalk between the AhR and the HIF1α signalling pathways, we also aimed to examine the levels of *HIF1α* gene expression in Su5416/Hx treated animals. As the HIF1α gene is normally expressed regardless of the partial pressure of O_2, and the latter only influences the stability and degradation of HIF1α on protein level, we investigated whether the basal level of gene expression is altered in treated animals compared to their Nx

controls. Herein, we report that the relative expression of *HIF1α* was significantly higher in Su5416/Hx treated males compared to treated females (Figure 3–10).

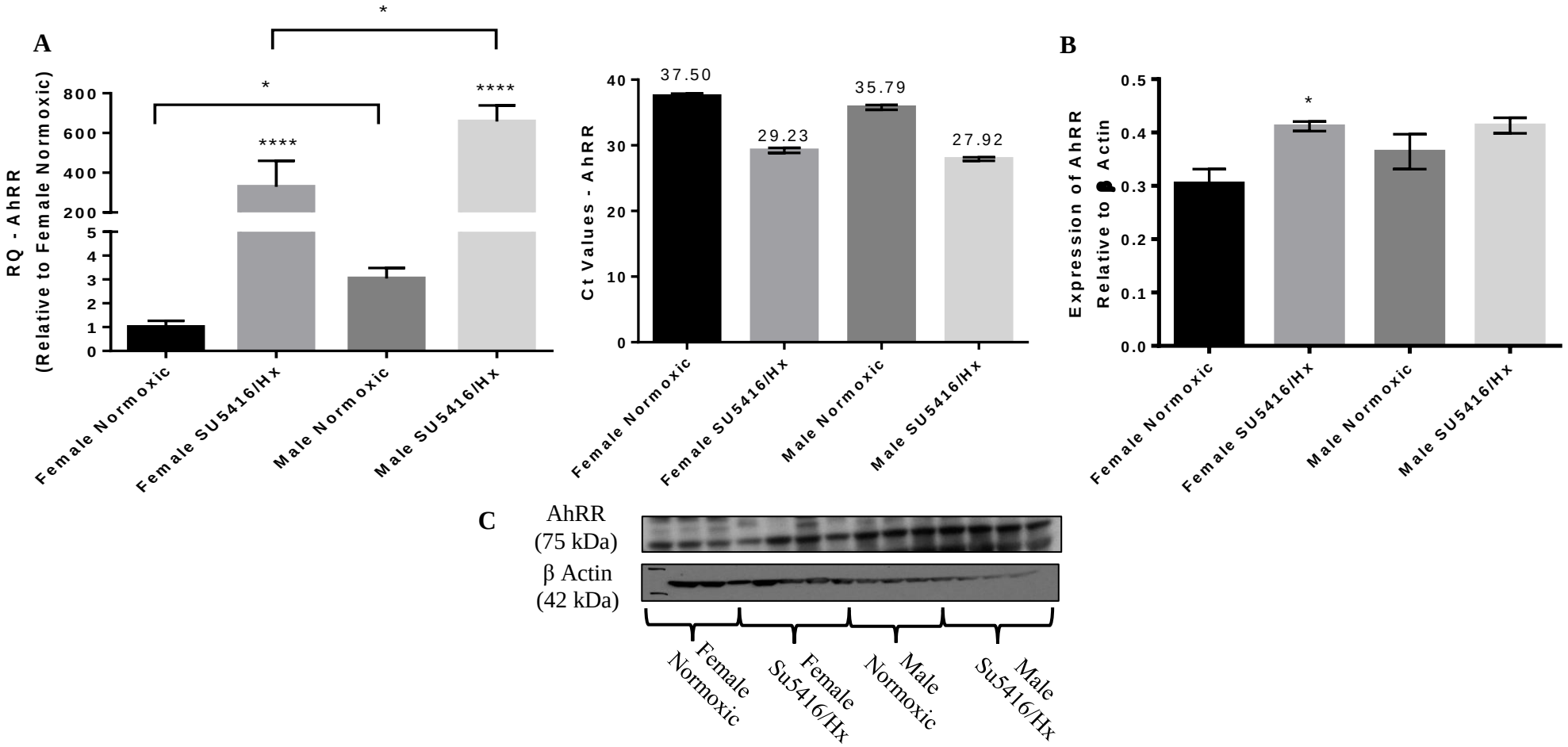


Figure 3–8: The expression of aryl hydrocarbon receptor repressor is increased in whole lungs derived from female and male rats treated with Sugen 5416 and exposed to chronic hypoxia.

Samples of whole lung of Wistar Kyoto rats, of both sexes, treated with single dose of Sugen 5416 and exposed to hypoxia and their normoxic controls, were prepared, as described previously, to assess the expression level of *AhRR* by Taqman quantitative Real Time-PCR (A). Results were normalised to *β-2-microglobulin*. N=3–6 per group in triplicate, ****p≤0.0001, One-way ANOVA followed by Dunnett's post-hoc test. Data is expressed as $RQ \pm \frac{RQ_{max}}{RQ_{min}}$, where RQ=Relative quantification. The level of AhRR protein expression was assessed using immunoblotting (B). N=3–6 per group in duplicate, *p<0.05, ****p≤0.0001, One-way ANOVA followed by Dunnett's post-hoc test. Representative immunoblot is shown in (C).

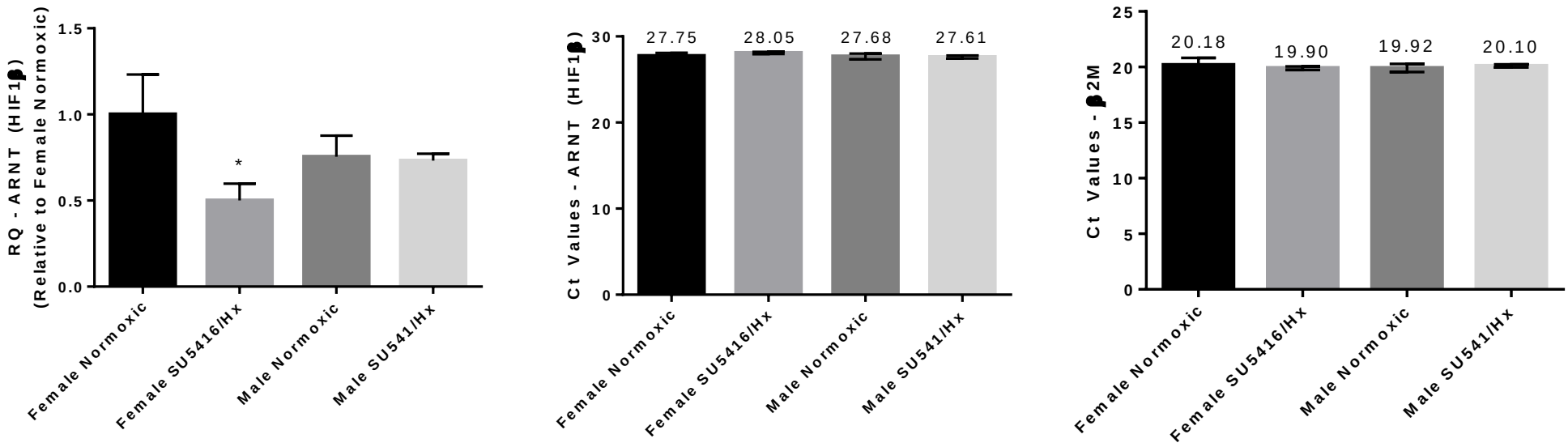


Figure 3–9: The relative expression of the gene encoding the ARNT (HIF1β) is decreased in whole lungs derived female rats treated with Sugen 5416 and exposed to chronic hypoxia.

Samples of whole lung of Wistar Kyoto rats, of both sexes, treated with a single dose of Sugen 5416 and subsequently exposed to chronic hypoxia, were prepared, as described previously, to assess the expression level of *ARNT (HIF1β)* by Taqman quantitative Real Time-PCR. Results were normalised to the *β-2-microglobulin*. N=4–6 per group in triplicate, *p<0.05, One-way ANOVA followed by Dunnett's post-hoc test. Data is expressed as $RQ \pm \frac{RQ_{max}}{RQ_{min}}$, where RQ=Relative quantification.

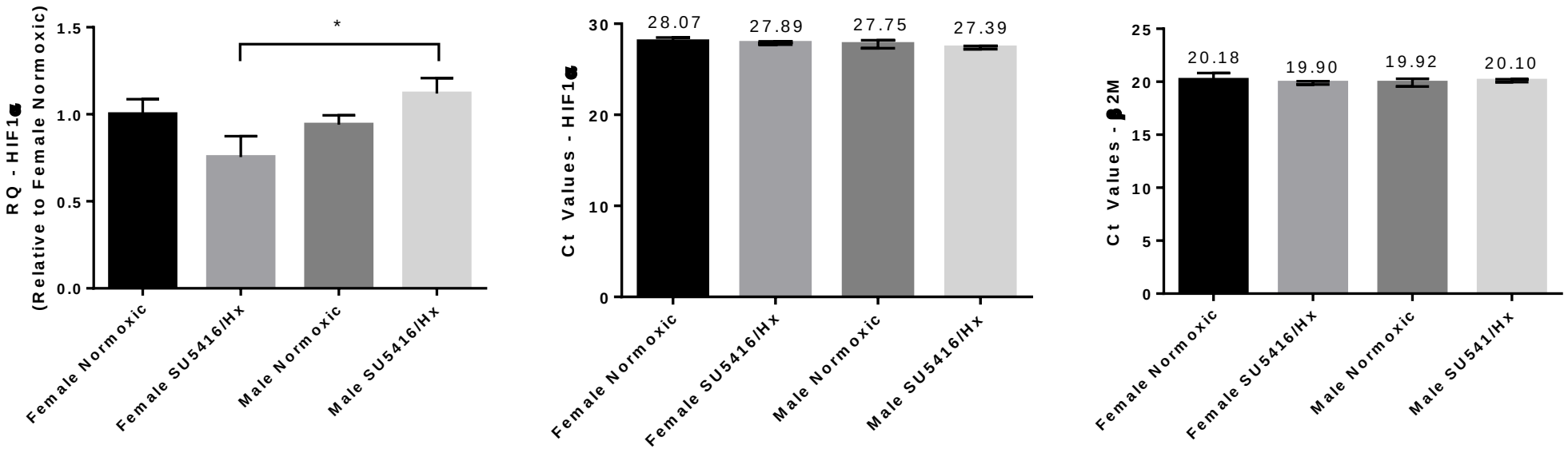


Figure 3–10: The relative expression of the gene encoding the hypoxia-inducible factor 1α is increased in whole lungs from male rats treated with Sugen 5416 and exposed to chronic hypoxia.

Samples of whole lung of Wistar Kyoto rats, of both sexes, treated with a single dose of Sugen 5416 and subsequently exposed to chronic hypoxia, were prepared, as described previously, to assess the expression level of *HIF1α* by Taqman quantitative Real Time-PCR. Results were normalised to *β-2-microglobulin*. N=4–6 per group in triplicate, *p<0.05, One-way ANOVA followed by Dunnett's post-hoc test. Data is expressed as $RQ \pm \frac{RQ_{max}}{RQ_{min}}$, where RQ=Relative quantification.

3.2.5 *The Expression of NAD(P)H Quinone Dehydrogenase 1 is Increased in the Lung of Sugen 5416-Hypoxic-Treated Animals, but not in Animals Exposed to Chronic Hypoxia*

The activation of AhR in mice results in the induction of a battery of genes through the nuclear factor erythroid 2–related factor 2 (Nrf2), which includes *CYP1A1* and *NQO1* (Yeager et al., 2009). The aim of assessing *NQO1* gene expression was to determine the animal model in which, the AhR signalling pathways is most likely activated. We also wished to assess the expression of another gene known to be regulated through AhR signalling pathway in Su5416/Hx treated animals. Prolonged vessel wall sheer stress has been associated with altered expression of the *NQO1* gene in the endothelial cells ECs (Chen et al., 2003), with the enzyme mediating the removal of genotoxic quinones to form hydroquinone, thereby conferring protective role against oxidative stress.

In the cHx model of PH, the only significant difference in *NQO1* observed was that females exhibited significantly higher expression level of the *NQO1* gene compared to males (Figure 3–11A). The *NQO1* gene was upregulated in Su5416/Hx treated animals of both sexes (Figure 3–11B).

A slight variability was observed in the Ct values of the housekeeper gene *β-2-microglobulin* for animal kept in Hx for 28 days and their Nx controls. The quality of the data was examined by calculating the standard deviation of the Ct values. Generally, we considered results with variability described by standard deviation of less than 1 to be useable, and this criterion was also applied for this work. Considering, the standard deviation of the housekeeper gene Ct values was within the acceptable limits, we believe the results are satisfactory. However, we recognise that with borderline results, larger variability of the housekeeper gene) could artificially result in greater significance of results, however, the variability of the housekeeper gene Ct values was within the acceptable limits of variation. The impact of the level of variability in the housekeeping gene expression is described in Chapter 3.2.1.

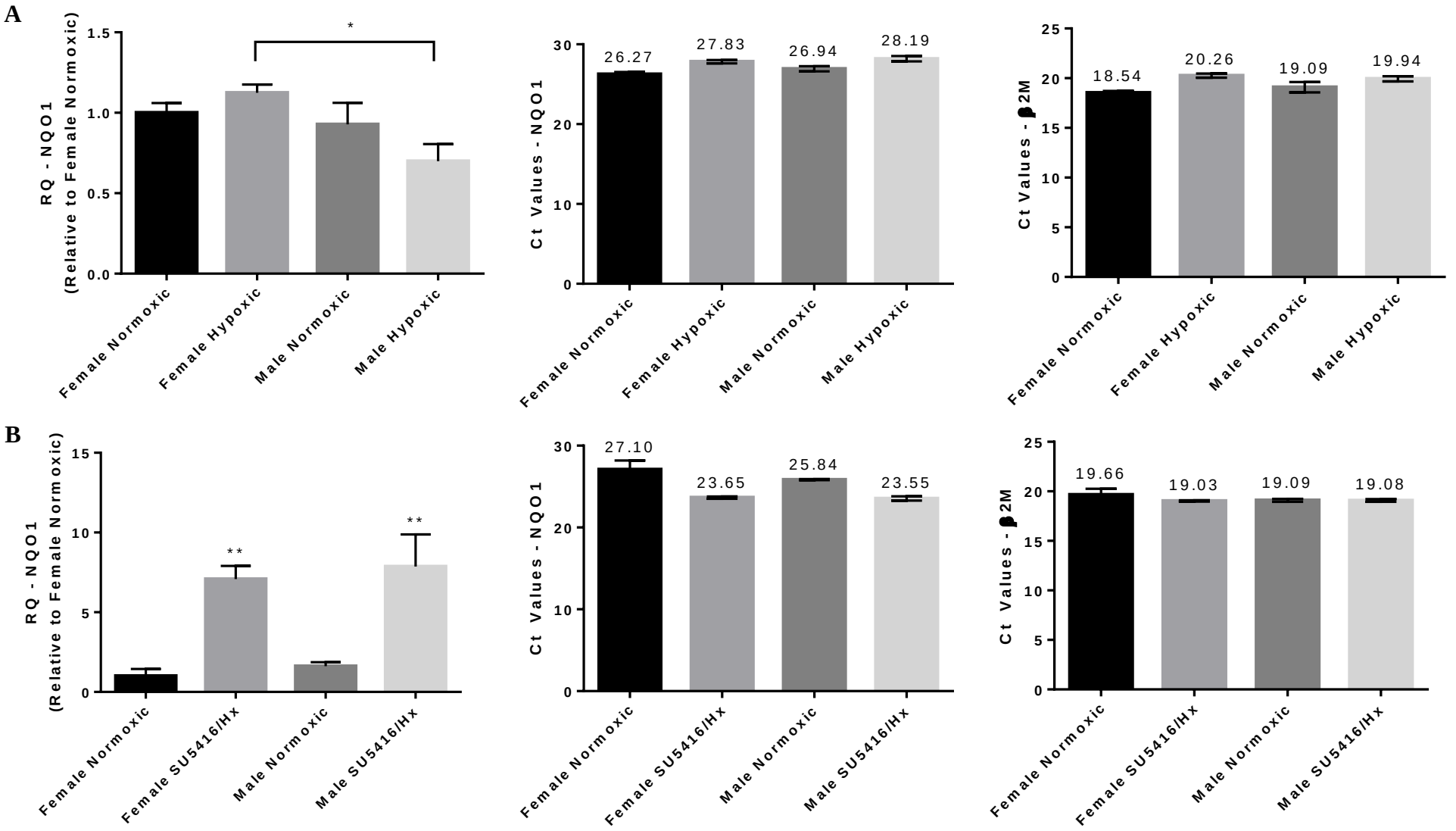


Figure 3–11: The relative quantitation NAD(P)H quinone dehydrogenase 1 is increased in the whole lungs from Sugen 5416 and hypoxia-treated animals.

Samples of whole lungs of two experimental PH rat models: cHx (A) and Su5416/Hx (B) of both sexes, were prepared, as described previously, to assess the expression level of *NQO1* by Taqman quantitative Real Time-PCR. Results were normalised to *β-2-microglobulin*. N=3–6 per group in triplicate, **p<0.01, One-way ANOVA followed by Tukey's post-hoc test. Data is expressed as $RQ \pm \frac{RQ_{max}}{RQ_{min}}$, where RQ=Relative quantification.

3.2.6 *Treatment of Human Pulmonary Artery Smooth Muscle Cells with Sugen 5416 Leads to Increased Expression of Cytochrome P450 1A1 and 1B1, but not Aryl Hydrocarbon Receptor or Hypoxia-Inducible Factor 1β*

As previously reported, AhR expression is increased in female PAH patient hPASMCs (Dean et al., 2016). Moreover, the AhR signalling pathway may be involved in the establishment of the disease-like phenotype in the Su5416/Hx animal model. We therefore aimed to assess the effect that stimulation of the AhR signalling pathway may have on the expression of CYP enzymes in female hPASMCs derived from control patients with no PAH. Both, CYP1A1 and CYP1B1, metabolise oestrogens and produce protective and/or detrimental oestrogen metabolites (Lee et al., 2003;White et al., 2012), which may be involved in PAH pathogenesis.

24 hour-long stimulation with Su5416 (1μM and 5μM) resulted in significant increase in the protein expression of CYP1A1 (Figure 3–12) and CYP1B1 (Figure 3–13) enzymes in female hPASMCs derived from control patients, while no such increase was observed for the AhR (Figure 3–15). We also showed that at gene expression levels, genes encoding for *CYP1A1* and *CYP1B1* were significantly increased with Su5416 stimulation, while no such increase was detected for *AhR* gene expression (Figure 3–14). Furthermore, in these cells, pre-treatment with AhR antagonist, CH223191 (100nM), antagonised the Su5416-induced decreased in CYP1A1 expression (Figure 3–12), but not CYP1B1 expression (Figure 3–13).

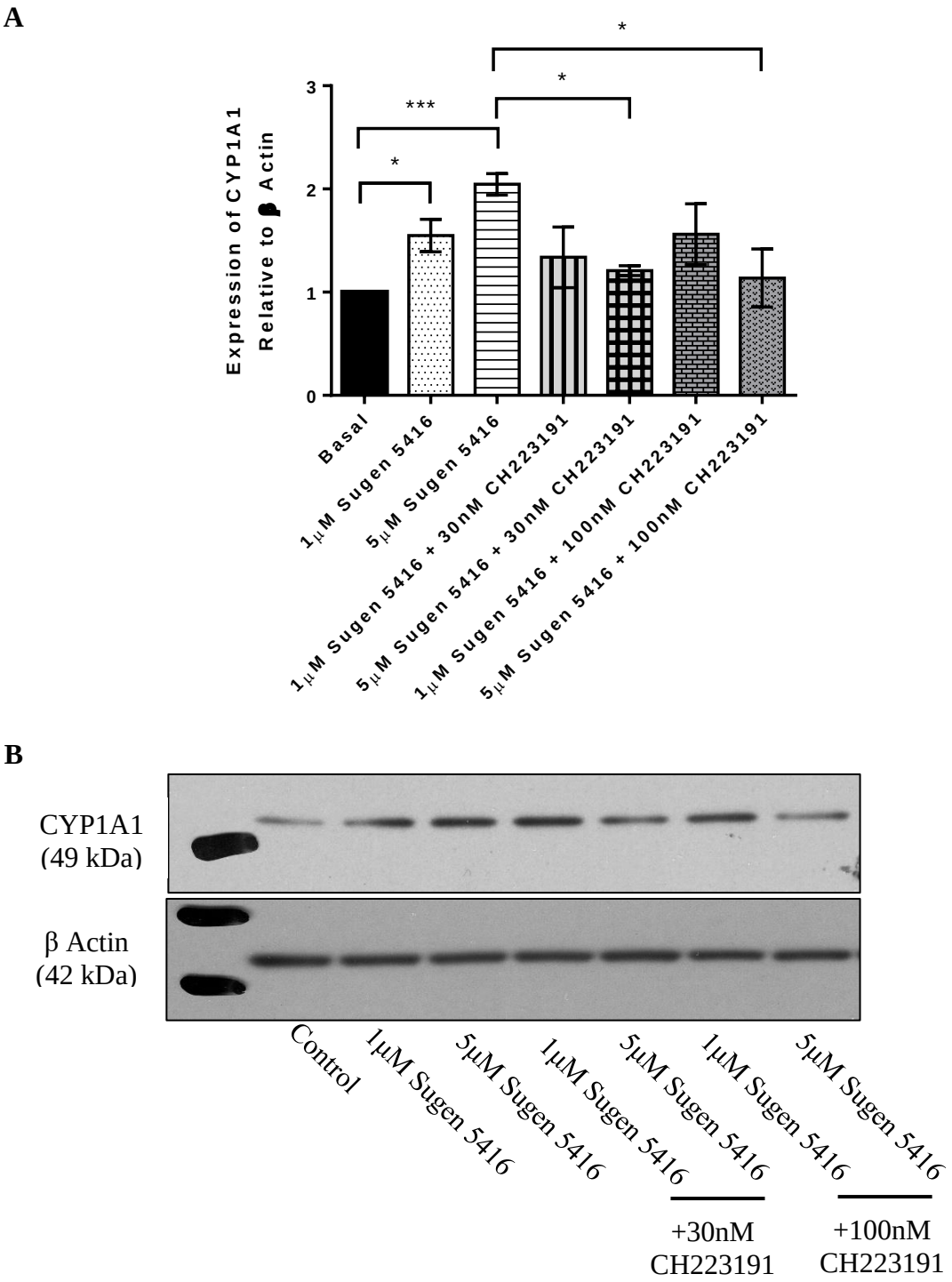


Figure 3–12: Expression of CYP1A1 is increased by treatment with Sugen 5416, and attenuated by AhR antagonist, CH223191, in human pulmonary artery smooth muscle cells from female controls.

HPASMCs were quiesced for 24 hours in 0.5% CSS PRF DMEM media. PASMCs were then treated with Sugen 5416 (1μM and 5μM) and CH223191 (30nM and 100nM), which was added 30 minutes before Sugen 5416, in the presence of 1% CSS PRF DMEM. (A) Protein expression of CYP1A1 was assessed as previously described, with representative immunoblotting image (B). N=3 per group, in duplicate, One-Way ANOVA with Bonferroni's post-hoc test. Data is expressed as fold-change of Control group±SEM.

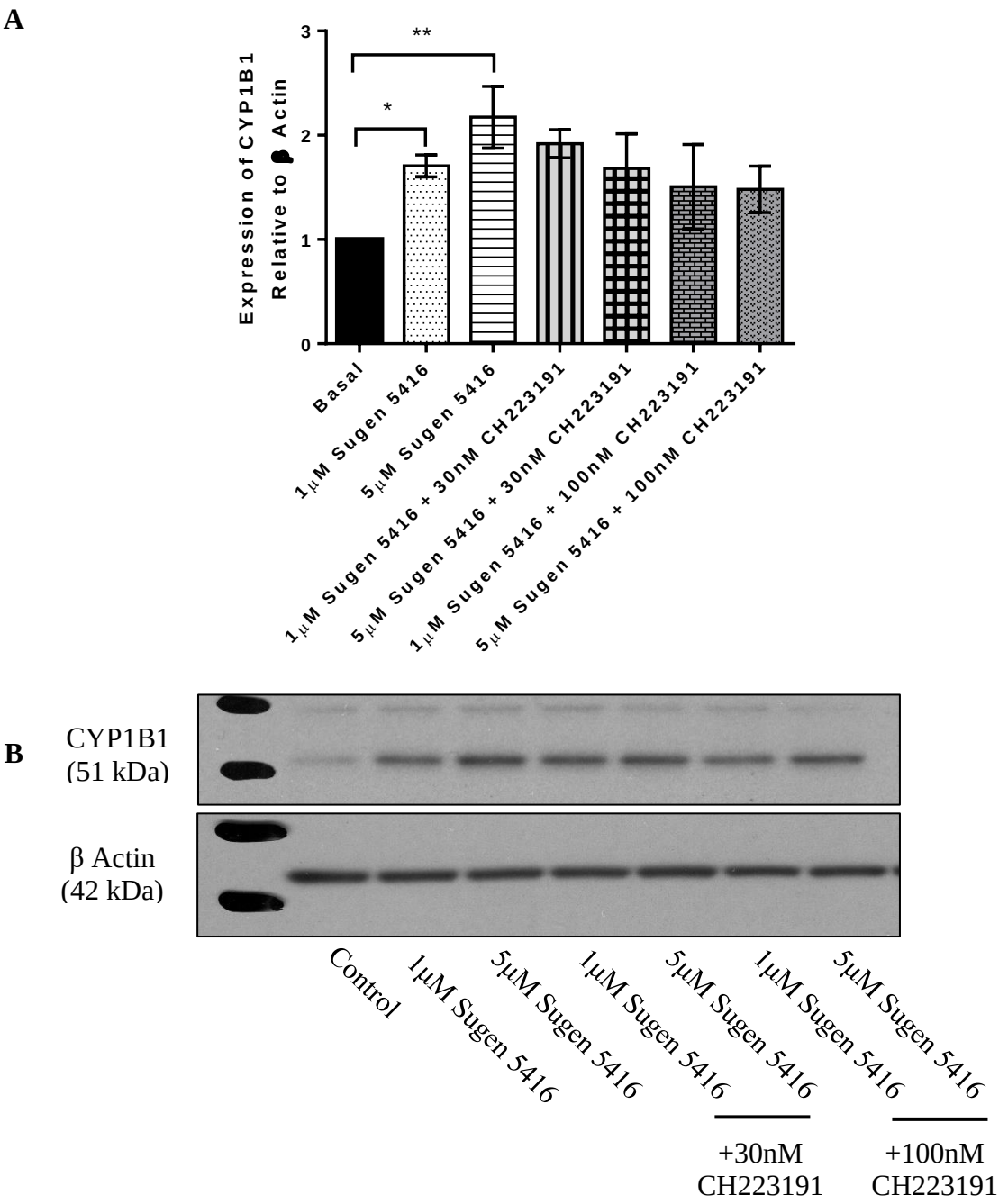


Figure 3–13: Expression of CYP1B1 is increased after treatment with Sugen 5416 in human pulmonary artery smooth muscle cells from female controls.

HPASMCs were quiesced for 24 hours in 0.5% CSS PRF DMEM media. PASMCs were then treated with Sugen 5416 (1μM and 5μM) and CH223191 (30nM and 100nM), which was added 30 minutes before Sugen 5416, in the presence of 1% CSS PRF DMEM. (A) Protein expression of AhR were assessed as described previously, with representative immunoblotting image (B). N=3 per group, in duplicate, One-Way ANOVA with Bonferroni's post-hoc test. Data is expressed as fold-change of Control group±SEM.

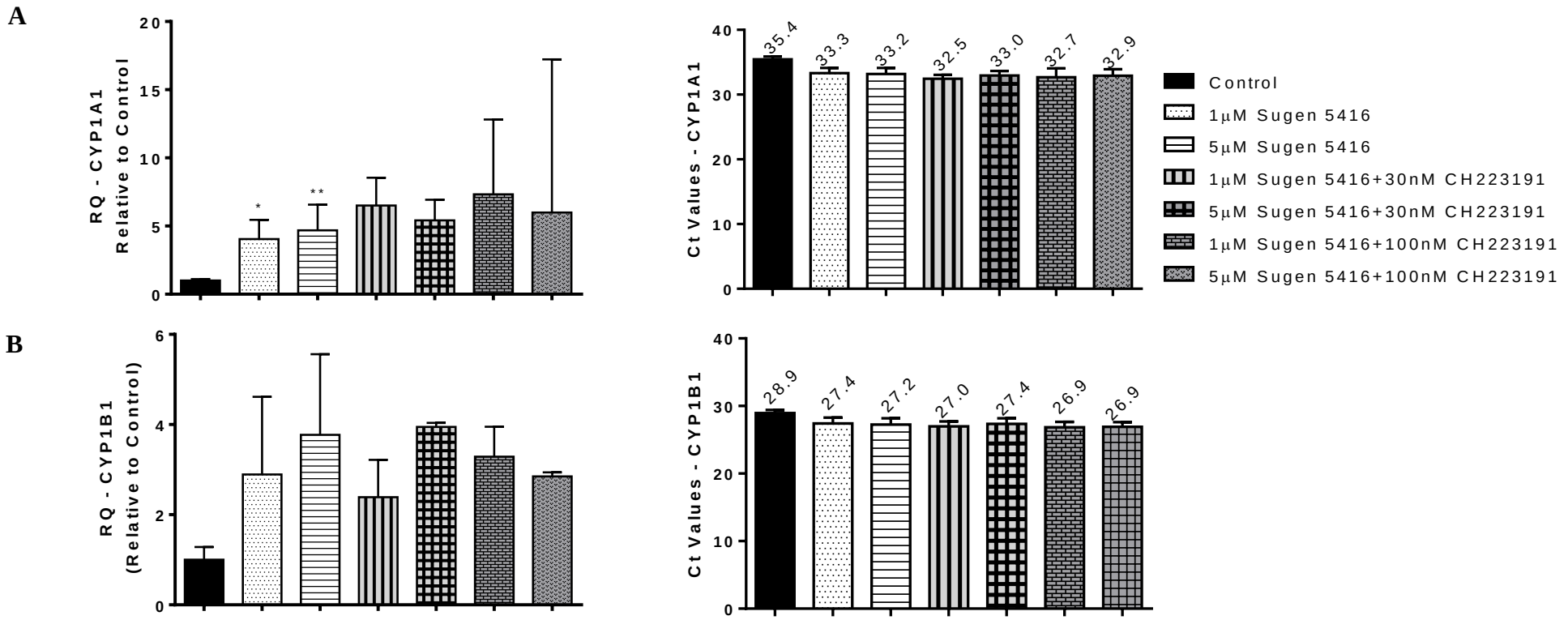


Figure 3–14: Expression of CYP1A1 and CYP1B1 at gene level is increased in human pulmonary artery smooth muscle cells from female controls after treatment with Sugen 5416 in the presence or absence of AhR antagonist, CH223191.

HPASMCs were quiesced for 24 hours in 0.5% CSS PRF DMEM media. PASMCs were then treated with Sugen 5416 (1μM and 5μM) and CH223191 (30nM and 100nM), which was added 30 minutes before Sugen 5416, in the presence of 1% CSS PRF DMEM. The expression level of *CYP1A1* and *CYP1B1* was assessed by Taqman quantitative Real Time-PCR. Results were normalised to *β-2-microglobulin*. N=3–4 per group, in duplicate, *p<0.05,**p≤0.01, One-Way ANOVA with Bonferroni's post-hoc test. Data is expressed as $RQ \pm \frac{RQ_{max}}{RQ_{min}}$, where RQ=Relative quantification.

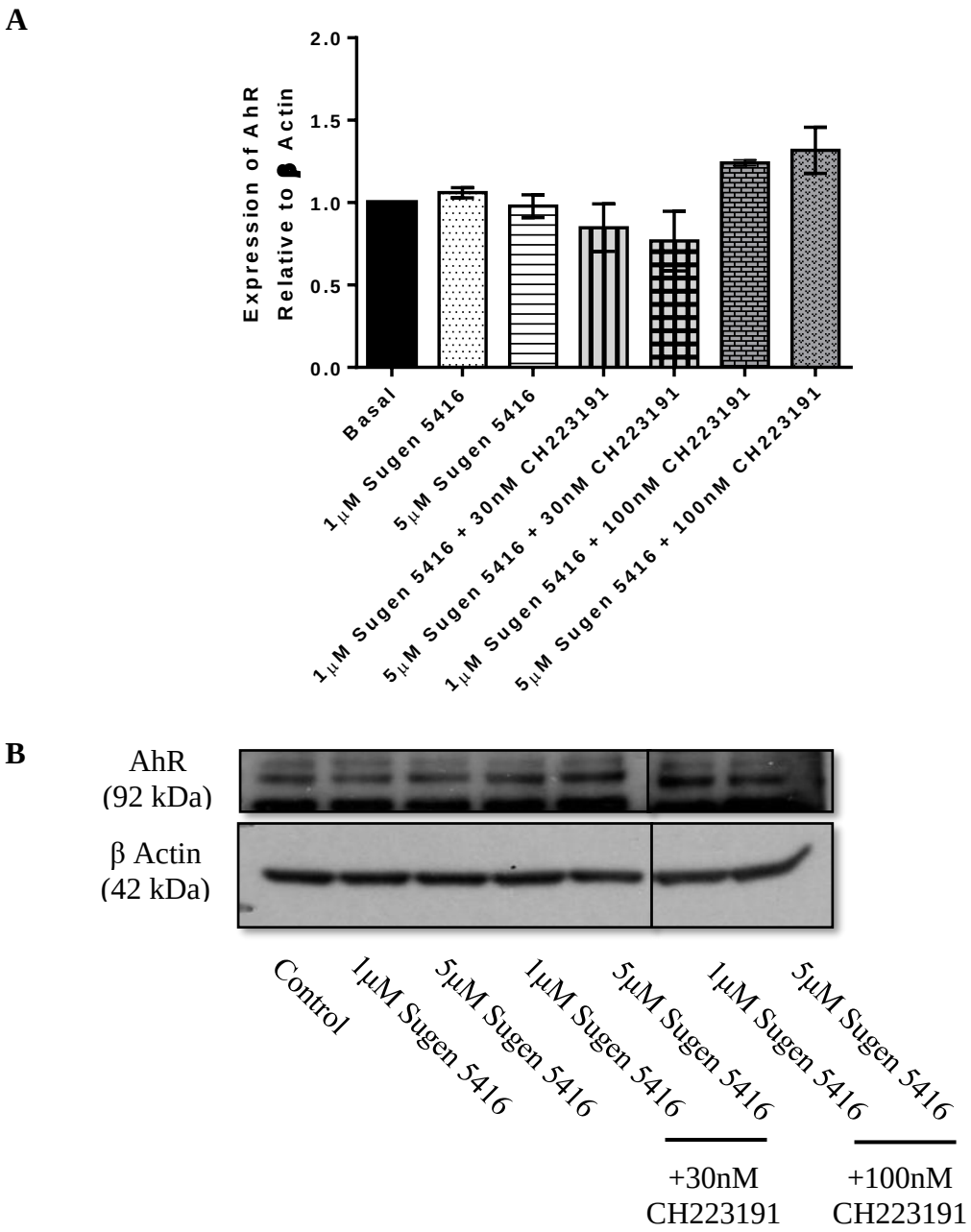


Figure 3–15: Protein expression of aryl hydrocarbon receptor remains unaltered by treatment with Sugen 5416 in the presence or absence of AhR antagonist, CH223191, in human pulmonary artery smooth muscle cells from female controls.

HPASMCs were quiesced for 24 hours in 0.5% CSS PRF DMEM media. PASMCs were then treated with Sugen 5416 (1μM and 5μM) and CH223191 (30nM and 100nM), which was added 30 minutes before Sugen 5416, in the presence of 1% CSS PRF DMEM. N=3 per group, in duplicate, One-Way ANOVA with Bonferroni's post-hoc test. Data is expressed as fold-change of Control group±SEM. The representative blot shown (B) was altered to remove Control, 1μM and 5μM Sugen 5416 treatment groups, otherwise appearing in the position now replaced by vertical line, on the same immunoblot.

3.2.7 *Treatment of Human Pulmonary Artery Smooth Muscle Cells with Sugen 5416 Leads to Increased Expression of Cytochrome P450 1A1 and 1B1, but not Aryl Hydrocarbon Receptor in Cells Derived from Female Patients*

As treatment of animals with Su5416 resulted in increased expression of target genes of the AhR signalling pathway, we aimed to investigate whether the use of Su5416 in the *in vitro* model of PAH might have a similar effect, considering the AhR is increased in PASMCs derived from female patients. The exposure of hPASMCs from female PAH patients to Su5416 yielded a significant increase in CYP1A1 and CYP1B1, at gene (Figure 3–16) and protein levels (Figure 3–17 and Figure 3–18), respectively. The difference in CYP1A1 expression compared to control treatment was more considerable as the change in CYP1B1 expression. Although the treatment with the AhR antagonist, CH223191 (30nM and 100nM) resulted in decreased expression of both CYP enzymes, this alteration was not statistically significant.

Although the results of immunoblotting indicate that there might be no change in the protein expression of AhR, the results are limited by the quality of the blot. The overexposed and diffuse nature of the bands, together with relatively high background limit the accuracy of determining the boarders of the protein bands and hence the measurement of optical density. The diffuse nature of the bands could be associated with the presence of post-translational modifications of AhR. Additionally, the anti-AhR antibody used might exhibited some off-target binding, leading to poorer results, or it should be considered that multiple isoforms of the protein exist. In the latter instance the role of these isoforms should be investigated.

3.2.8 *Treatment with Sugen 5416 does not Affect the Expression of Other Proteins Involved in the AhR and HIF1α Signalling Pathways*

We also examined the expression of AhR, the main receptor mediating the alterations in the expression of CYP1A1 and CYP1B1 enzymes, to determine whether the observed upregulation might be a direct consequence of an increased expression of the *AhR* gene.

Stimulation of female patient PASMCs with Su5416 did not result in significant changes in the expression of AhR, on gene or protein levels, respectively (Figure 3–19). Nor did we observe any significant changes in the expression of HIF1β and HIF1α (Figure 3–20 and Figure 3–21). Equally, treatment with Su5416 did not affect the protein expression levels of PHD2 and vHL, which are involved in the regulation of HIF1α oxygen-dependent degradation (Figure 3–22 and Figure 3–23). In Nx conditions, PHD2 catalyses hydroxylation of HIF1α, thereby enabling the binding of a complex formed of vHL and ubiquitin ligase E3 (Kamura et al., 2000). This then leads to poly-ubiquitination of and proteasomal degradation of HIF1α. In hypoxia, PHD2 is inactive, or its activity is greatly reduced, as the enzyme requires molecular oxygen as its co-factor, therefore, HIF1α is stabilised due to greatly decreased proteasome-mediated degradation, leading to its accumulation in the nucleus, (Jiang et al., 1996). The role of PHD2 and vHL in HIF1α signalling pathway were also described in Chapter 1.6.5.

The evaluation of protein expression of HIF1α and HIF1β were limited by the low expression levels of these proteins, leading to diffuse bands and higher background, rendering the determination of optical density difficult. Hence, it is difficult to draw solid conclusions regarding whether Su5416 might affect the expression of these two proteins.

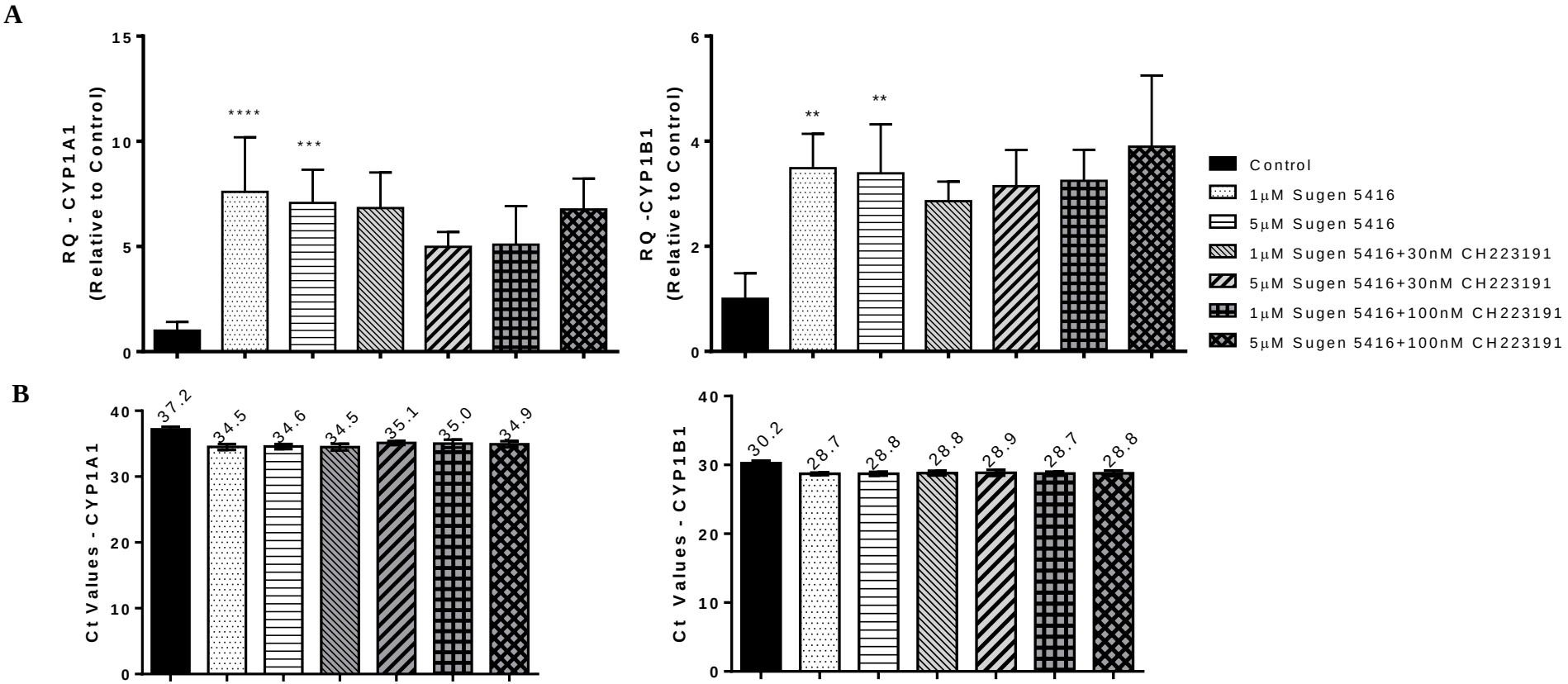


Figure 3–16: Expression of CYP1A1 and CYP1B1 at gene level is increased in human pulmonary artery smooth muscle cells from female PAH patients after treatment with Sugen 5416 in the presence or absence of AhR antagonist, CH223191.

HPASMCs were quiesced for 24 hours in 0.5% CSS PRF DMEM media. PASMCs were then treated with Sugen 5416 (1μM and 5μM) and CH223191 (30nM and 100nM), which was added 30 minutes before Sugen 5416, in the presence of 1% CSS PRF DMEM. The expression level of *CYP1A1* and *CYP1B1* was assessed by Taqman quantitative Real Time-PCR. Results were normalised to *β-2-microglobulin*. N=3–4 per group, in duplicate, **p≤0.01, ***p≤0.001, ****p≤0.0001, One-Way ANOVA with Bonferroni's post-hoc test. Data is expressed as $RQ \pm \frac{RQ_{max}}{RQ_{min}}$, where RQ=Relative quantification.

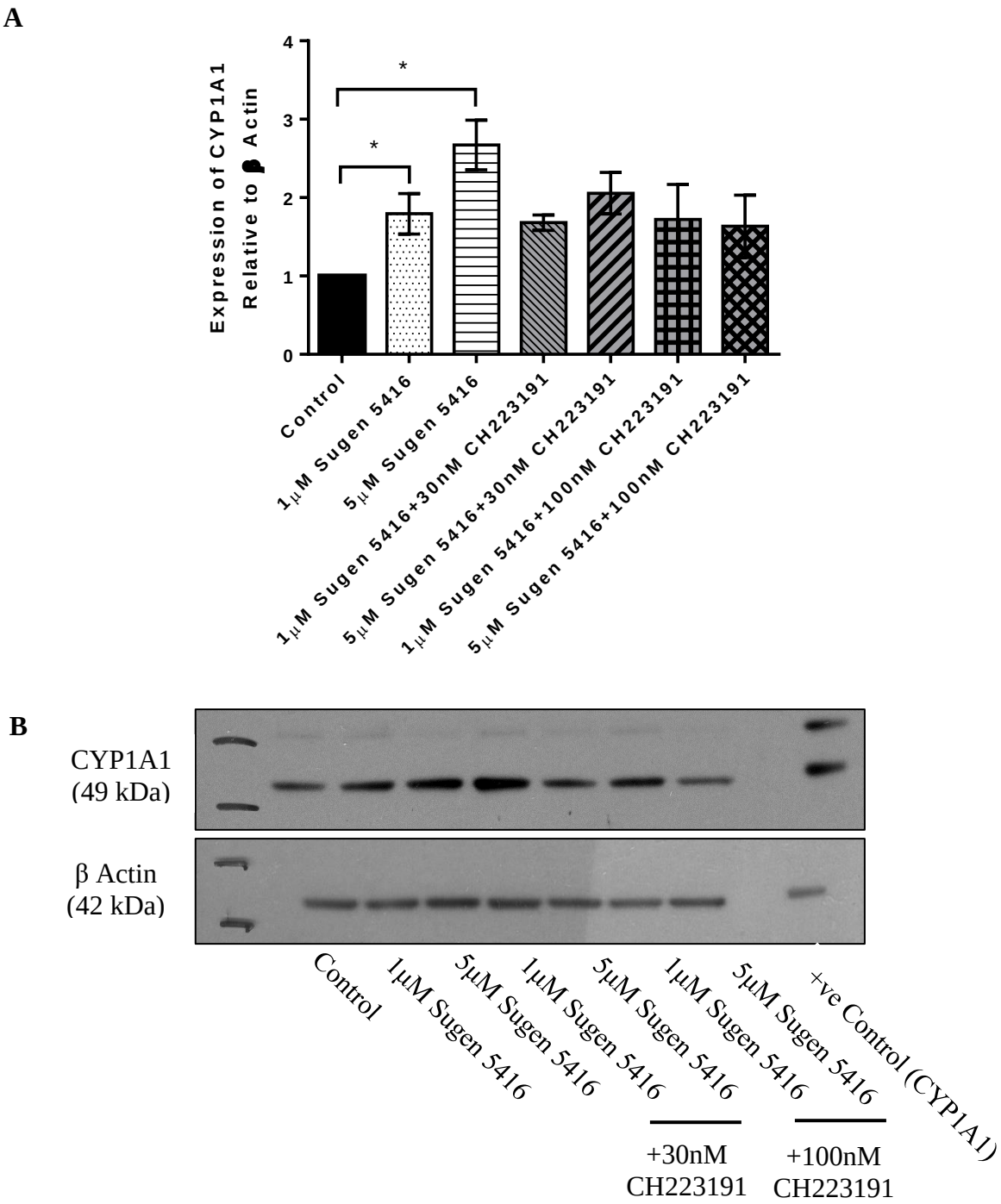
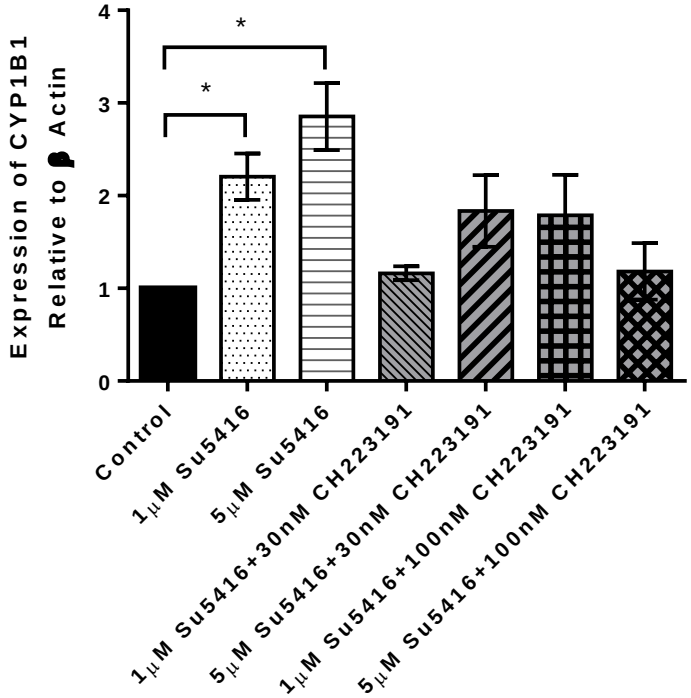


Figure 3–17: Protein expression of CYP1A1 is increased by stimulation with Sugen 5416 in the presence or absence of AhR antagonist, CH223191, in human pulmonary artery smooth muscle cells from female PAH patients.

HPASMCs were quiesced for 24 hours in 0.5% CSS PRF DMEM media. PASMCs were then treated with Sugen 5416 (1μM and 5μM) and CH223191 (30nM and 100nM), which was added 30 minutes before Sugen 5416, in the presence of 1% CSS PRF DMEM. (A) Protein expression of CYP1A1 were assessed as described previously, with representative immunoblotting image (B). N=3–4 per group, in duplicate, *$p<0.05$, **$p\leq0.01$, One-Way ANOVA with Bonferroni's post-hoc test. Data is expressed as fold-change of Control group±SEM.

A



B

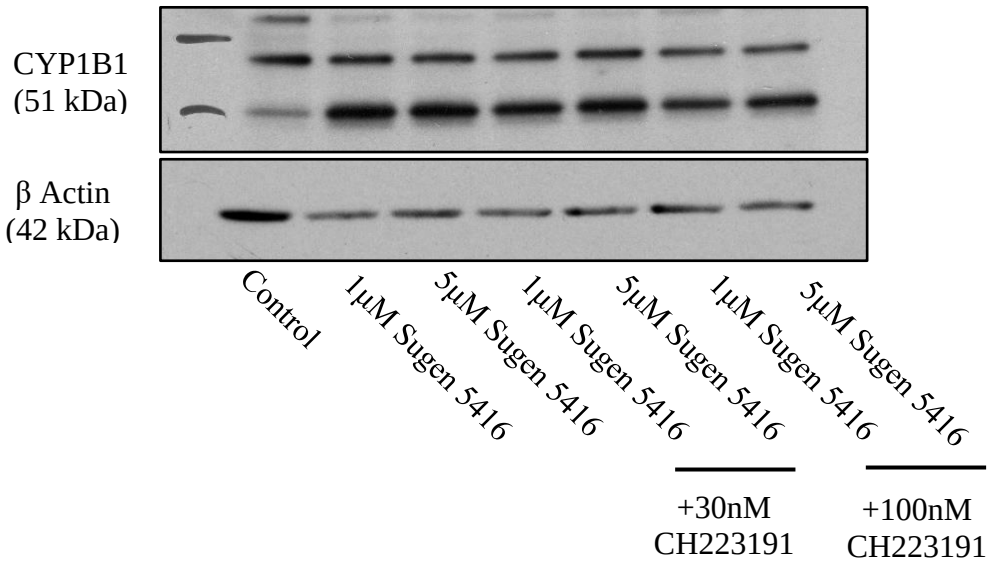


Figure 3–18: Protein expression of CYP1B1 is increased by stimulation with Sugen 5416 in the presence or absence of AhR antagonist, CH223191, in human pulmonary artery smooth muscle cells from female PAH patients.

HPASMCs were quiesced for 24 hours in 0.5% CSS PRF DMEM media. PASMCs were then treated with Sugen 5416 (1μM and 5μM) and CH223191 (30nM and 100nM), which was added 30 minutes before Sugen 5416, in the presence of 1% CSS PRF DMEM. (A) Protein expression of CYP1B1 were assessed as described previously, with representative immunoblotting image (B). N=4 per group, in duplicate, *p≤<0.01, One-Way ANOVA with Bonferroni's post-hoc test. Data is expressed as fold-change of Control group±SEM.

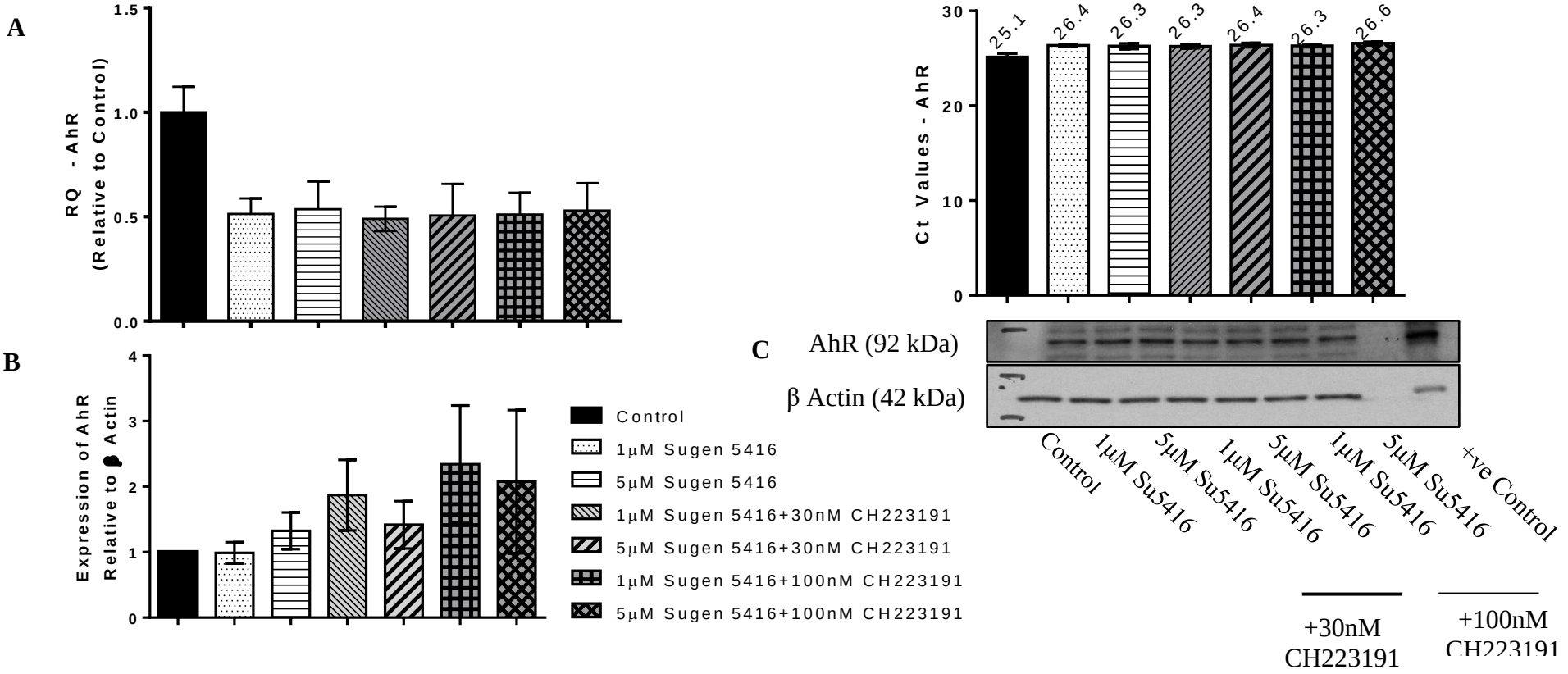
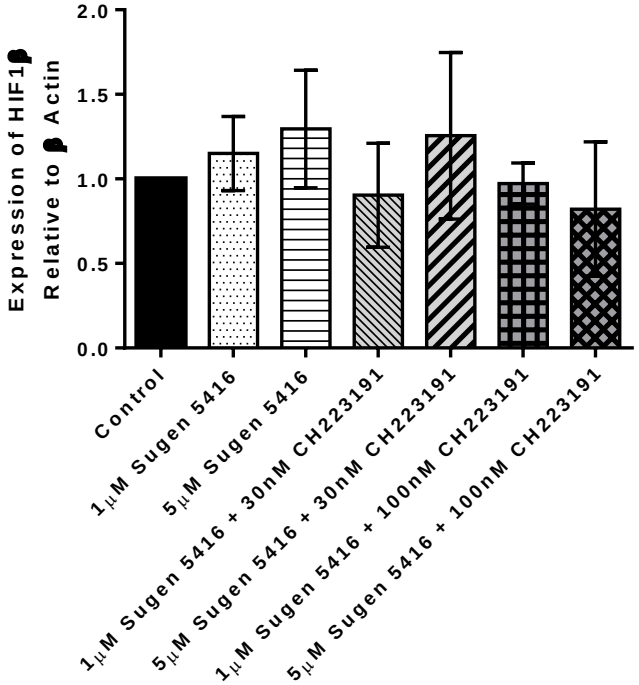


Figure 3–19: Expression of aryl hydrocarbon receptor remains unchanged by stimulation with Sugen 5416 in the presence or absence of AhR antagonist, CH223191, in human pulmonary artery smooth muscle cells from female PAH patients.

HPASMCs were quiesced for 24 hours in 0.5% CSS PRF DMEM media. PASMCs were then treated with Sugen 5416 (1μM and 5μM) and CH223191 (30nM and 100nM), which was added 30 minutes before Sugen 5416, in the presence of 1% CSS PRF DMEM. (A) When the expression level of the gene encoding for *AhR* was assessed by Taqman quantitative Real Time-PCR, the results were normalised to the *β-2-microglobulin*. N=4 per group, in duplicate, One-Way ANOVA with Bonferroni's post-hoc test. (B) Protein expression was assessed by immunoblotting. Data is expressed as fold-change of Control group±SEM or as $RQ \pm \frac{RQ_{max}}{RQ_{min}}$, where RQ=Relative quantification. Representative immunoblot is shown in (C).

A



B

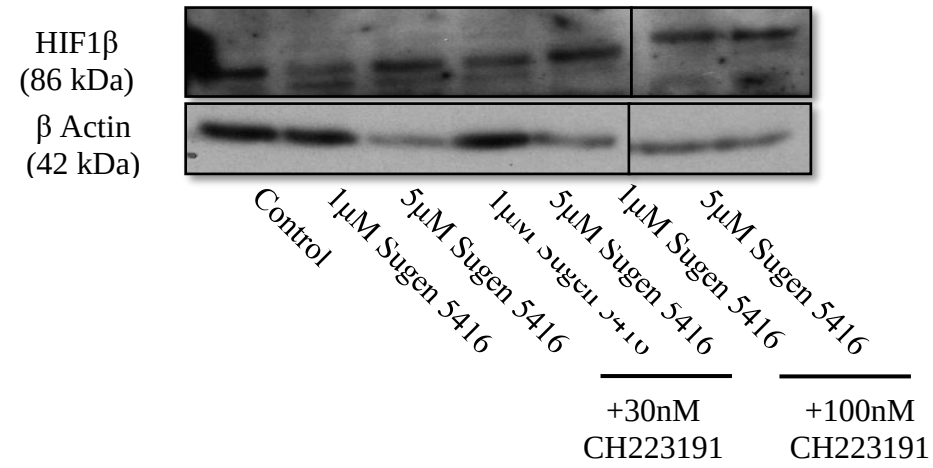


Figure 3–20: Protein expression of hypoxia-inducible factor 1β is unaltered by stimulation with Sugen 5416 in the presence or absence of AhR antagonist, CH223191, in human pulmonary artery smooth muscle cells from female PAH patients.

HPASMCs were quiesced for 24 hours in 0.5% CSS PRF DMEM media. PASMCs were then treated with Sugen 5416 (1μM and 5μM) and CH223191 (30nM and 100nM), which was added 30 minutes before Sugen 5416, in the presence of 1% CSS PRF DMEM. N=2–3 per group, in duplicate, One-Way ANOVA with Bonferroni's post-hoc test. Data is expressed as fold-change of Control group±SEM. The representative blot shown above was altered to remove Control, 1μM and 5μM Sugen 5416 treatment groups, otherwise appearing in the position now replaced by vertical line, on the same immunoblot.

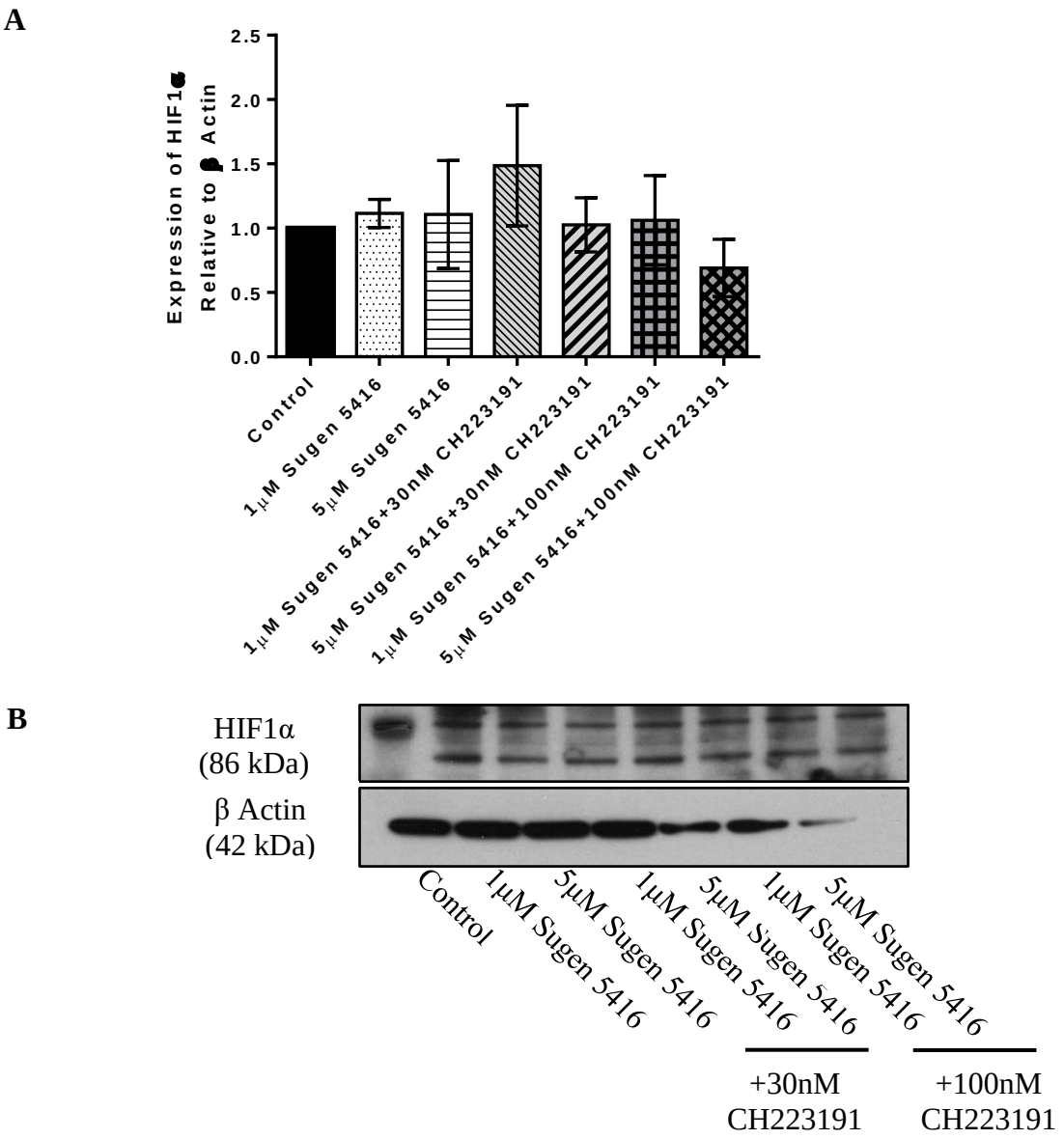


Figure 3–21: Expression of hypoxia-inducible factor 1α is unaltered by stimulation with Sugen 5416 in the presence of absence of AhR antagonist, CH223191, in human pulmonary artery smooth muscle cells from female PAH patients.

HPASMCs were quiesced for 24 hours in 0.5% CSS PRF DMEM media. PASMCs were then treated with Sugen 5416 (1μM and 5μM) and CH223191 (30nM and 100nM), which was added 30 minutes before Sugen 5416, in the presence of 1% CSS PRF DMEM. N=3 per group, in duplicate, One-Way ANOVA with Bonferroni's post-hoc test. Data is expressed as fold-change of Control group±SEM.

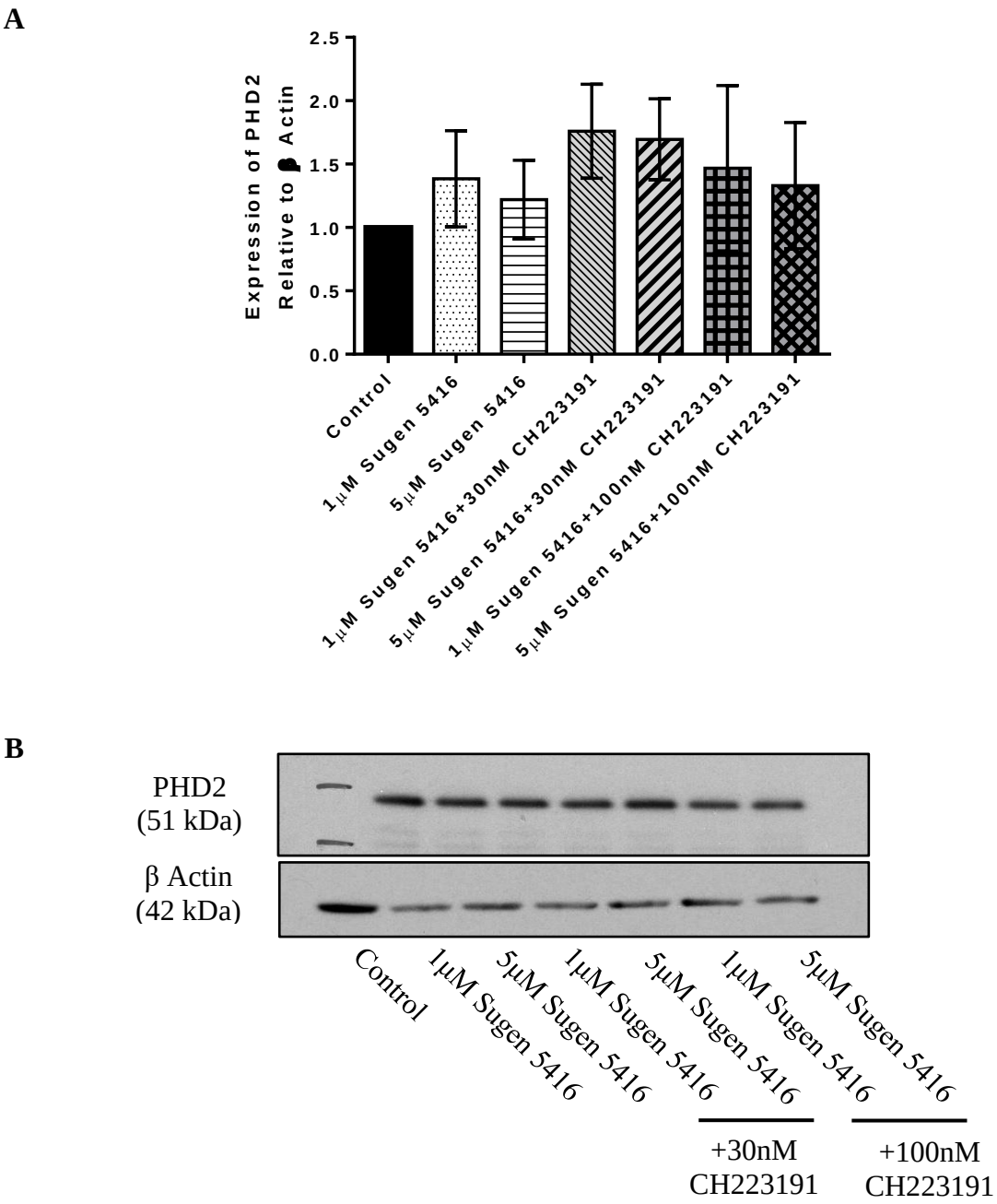
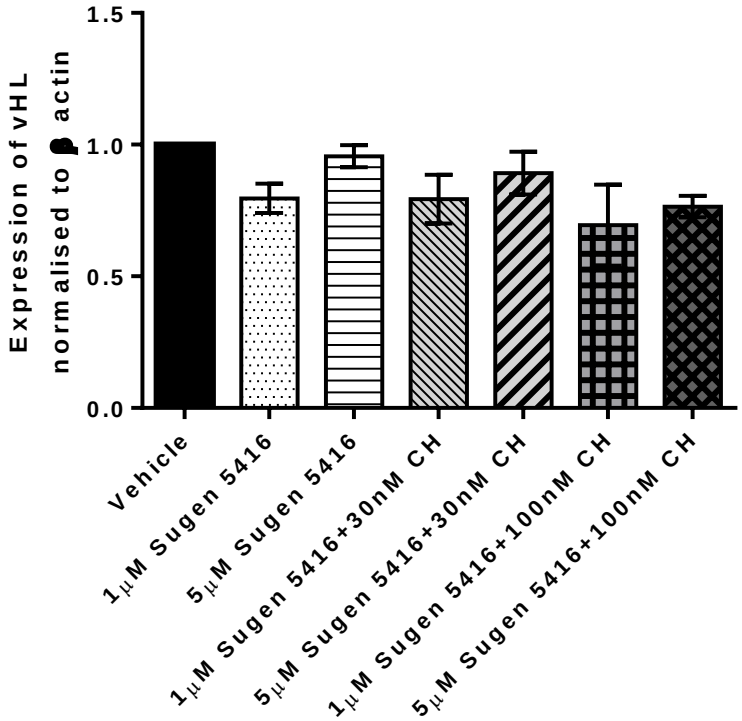


Figure 3–22: Expression of prolyl hydroxylase 2 is unchanged by stimulation with Sugen 5416 in the presence or absence of the AhR antagonist, CH223191, in human pulmonary artery smooth muscle cells from female PAH patients.

HPASMCs were quiesced for 24 hours in 0.5% CSS PRF DMEM media. PASMCs were then treated with Sugen 5416 (1μM and 5μM) and CH223191 (30nM and 100nM), which was added 30 minutes before Sugen 5416, in the presence of 1% CSS PRF DMEM. N=4 per group, in duplicate, One-Way ANOVA with Bonferroni's post-hoc test. Data is expressed as fold-change of Control group±SEM.

A



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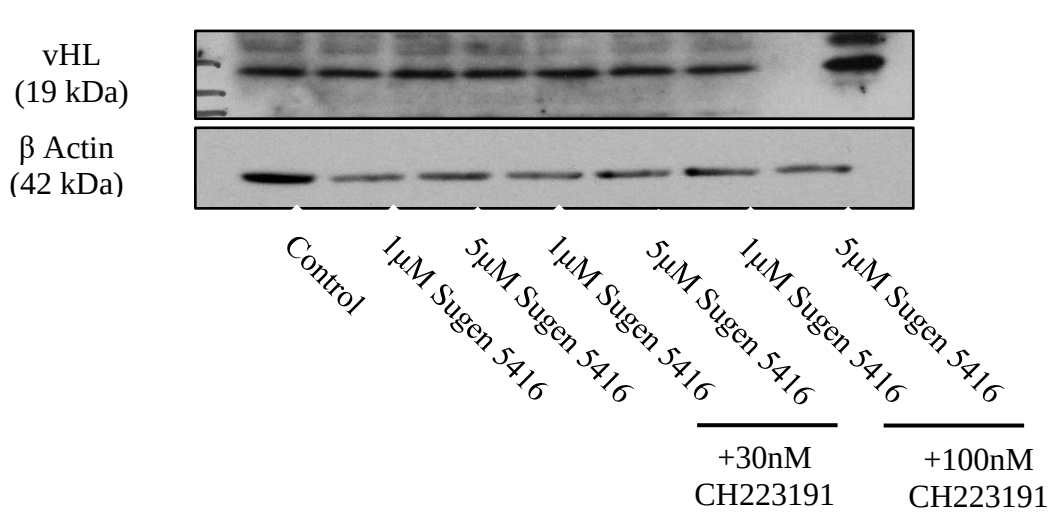


Figure 3–23: Expression of von Hippel-Lindau tumour factor is unchanged by stimulation with Sugen 5416 in the presence or absence of the AhR antagonist, CH223191, in human pulmonary artery smooth muscle cells from female PAH patients.

HPASMCs were quiesced for 24 hours in 0.5% CSS PRF DMEM media. PASMCs were then treated with Sugen 5416 (1μM and 5μM) and CH223191 (30nM and 100nM), which was added 30 minutes before Sugen 5416, in the presence of 1% CSS PRF DMEM. N=3 per group, in duplicate, One-Way ANOVA with Bonferroni's post-hoc test. Data is expressed as fold-change of Control group±SEM.

3.2.9 *The Expression of Cytochrome P450 1A1 Gene is Significantly Increased Only in Male Hyper-Responsive Animals Treated with Sugen 5416*

Recently, it was observed that a specific colony of Sprague Dawley (SD) rats develop severe PAH-like phenotype with a single dose of Su5416 and without subsequent exposure to cHx (Jiang, 2016). The SD-hyper-responsive rats exhibited enhanced mortality at 8 weeks compared to rats of other rat strains and colonies (Jiang, 2016). Interestingly, unlike the Su5416/Hx model in more common rat colonies, the proportion of male rats exhibiting severe phenotype of the disease exceeds that of female rats in SD-hyper-responsive colony (Jiang, 2016). In the following text we have termed SD-typical colony rats as non-responders, as they did not develop severe PAH phenotype after administration of a single dose of Su5416. While we have termed SD-hyper-responsive rats, which have developed severe PAH phenotype with Su5416 alone, as responders.

We have investigated the expression of gene encoding for the *CYP1A1* to assess the level of expression in hyper-responsive animals. The relative quantitation of *CYP1A1* gene was significantly increased in male hyper-responsive group compared to the male animals characterised by normal response to treatment with Su5416 (Figure 3–24). Exposure of female animals to a single dose of Su5416 did not result in significant difference in the relative expression of *CYP1A1* gene. Moreover, no difference between relative quantitation of *CYP1A1* gene between female and male normally responsive animals was observed.

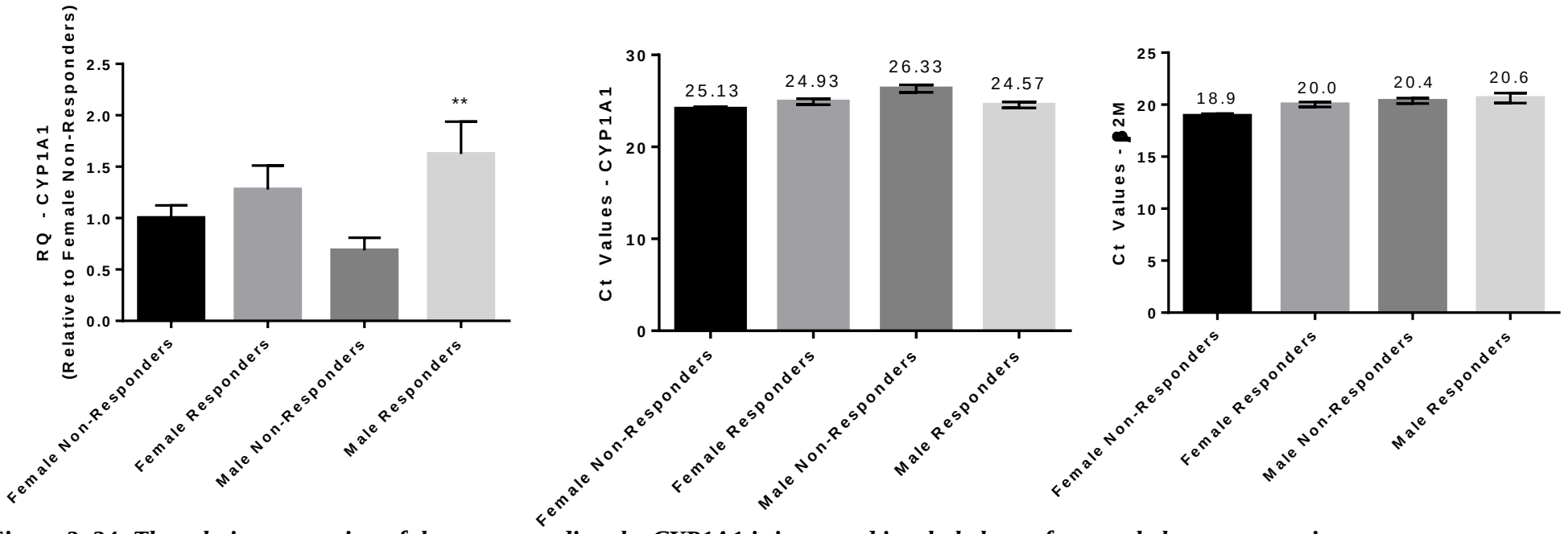


Figure 3–24: The relative expression of the gene encoding the CYP1A1 is increased in whole lungs from male hyper-responsive rats.

Whole lungs from hyper-responsive and typical Sprague-Dawley rat colonies, of both sexes, treated with a single dose of Su5416, were prepared, as described previously, to assess the expression level of *CYP1A1* by Taqman quantitative Real Time-PCR. Results were normalised to *β-2-microglobulin*. N=4–6 per group in triplicate, **p<0.01, One-way ANOVA followed by Dunnett's post-hoc test. Data is expressed as $RQ \pm \frac{RQ_{max}}{RQ_{min}}$, where RQ=Relative quantification.

3.2.10 *The Relative Expression of Cytochrome P450 1B1 and Aryl Hydrocarbon Receptor Repressor is Increased in Male Hyper-Responsive Rats, but not in Female*

Unlike the Su5416/Hx animal model of PAH, the treatment of male animals of hyper-responsive SD rat colony with a single dose of Su5416 results in the onset of a severe disease phenotype, without requiring exposure to cHx. As the expression of *CYP1B1* was previously reported to be increased in cHx and Su5416/Hx animal models, and CYP1B1 is considered as an important modulator of PAH development and progression, we wished to determine whether the expression of this gene is altered in animals who exhibit enhanced sensitivity to treatment with Su5416. We observed that the *CYP1B1* mRNA expression was significantly increased in whole lungs from male animals exhibiting hyper-responsive character, while the increase in female hyper-responsive animals was not significant (Figure 3–25). No difference between normally responsive animals to Su5416 of both sexes was observed, while hyper-responsive males exhibited significantly higher gene expression level as hyper-responsive females (Figure 3–25).

To determine whether the AhR signalling pathway may be ubiquitously activated in animals who exhibit hyper-responsive character for Su5416 treatment, we also aimed to assess the *AhRR* gene expression levels, as this gene is not normally expressed. The levels of *AhRR* gene in whole lung samples from male animals that develop a severe PAH-like phenotype in the absence of cHx was significantly higher than in male animals, that are characterised as normally responsive to Su5416 (Figure 3–26).

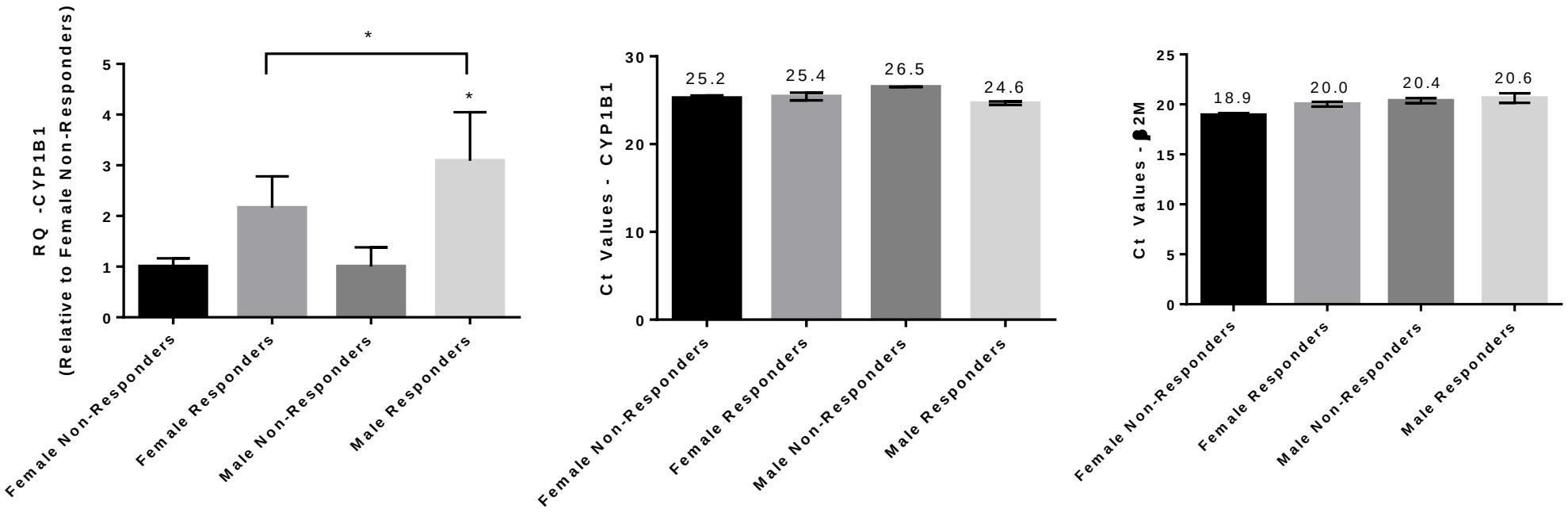


Figure 3–25: The relative expression of the gene encoding the CYP1B1 is increased in whole lungs from male hyper-responsive rats.

Whole lungs from hyper-responsive and typical Sprague-Dawley rat colonies, of both sexes, treated with a single dose of Su5416, were prepared, as described previously, to assess the expression level of *CYP1B1* by Taqman quantitative Real Time-PCR. Results were normalised to *β-2-microglobulin*. N=3–6 per group in triplicate, *p<0.05, One-way ANOVA followed by Dunnett's post-hoc test. Data is expressed as RQ $\pm \frac{RQ_{max}}{RQ_{min}}$, where RQ=Relative quantification.

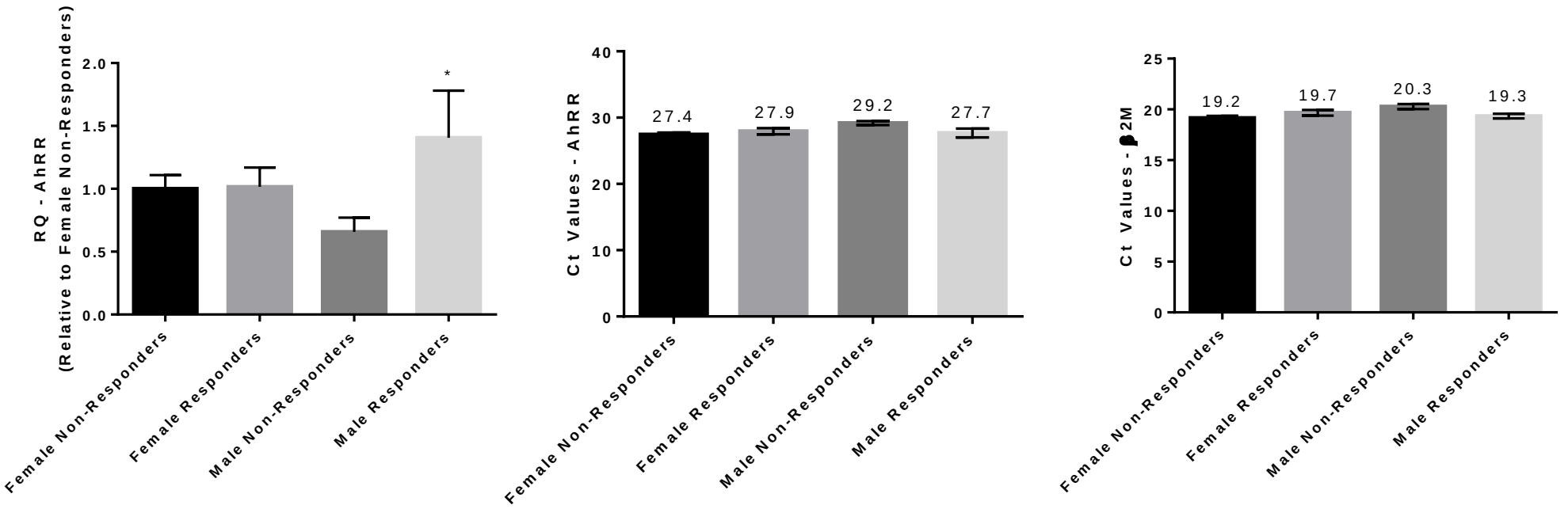


Figure 3–26: The relative expression of the gene encoding the aryl hydrocarbon receptor repressor is increased in whole lungs from male hyper-responsive rats.

Whole lungs from hyper-responsive and typical Sprague-Dawley rat colonies, of both sexes, treated with a single dose of Su5416, were prepared, as described previously, to assess the expression level of *AhRR* by Taqman quantitative Real Time-PCR. Results were normalised to the *β-2-microglobulin*. N=3–6 per group in triplicate, *p<0.05, One-way ANOVA followed by Dunnett's post-hoc test. Data is expressed as RQ $\pm \frac{RQ_{max}}{RQ_{min}}$, where RQ=Relative quantification.

3.2.11 *The Expression of the Aryl Hydrocarbon Receptor is Higher in Female Rats Exhibiting Hyper-Responsive Character to Sugen 5416 Treatment Compared to their Male Counterparts*

Considering, the expression of *CYP1A1* and *CYP1B1* were increased in male rats exhibiting hyper-responsive phenotype to treatment with Su5416, we determined the relative expression of the *AhR* gene in these samples to show whether male animals, which developed severe disease-like phenotype also exhibited increased expression of the *AhR* gene, responsible for the induction of these genes. The relative expression of the *AhR* as per Real-Time Quantitative PCR was significantly higher in female animals, which were characterised as hyper-responsive to Su5416 compared to hyper-responsive male animals (Figure 3–27).

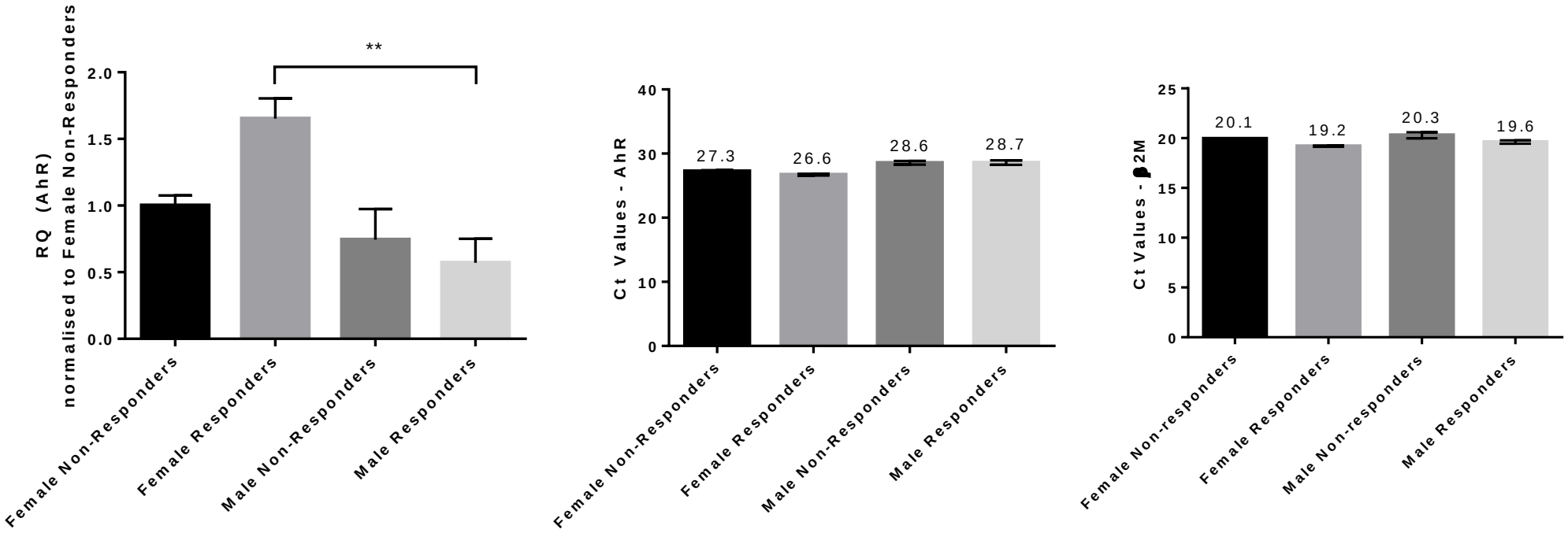


Figure 3–27: The relative expression of the gene encoding the aryl hydrocarbon receptor is increased in whole lungs from female hyper-responsive rats.

Whole lungs from hyper-responsive and typical Sprague-Dawley rat colonies, of both sexes, treated with a single dose of Su5416, were prepared, as described previously, to assess the expression level of *AhR* by Taqman quantitative Real Time-PCR. Results were normalised to *β-2-microglobulin*. N=4–6 per group in triplicate, *p<0.05, ***p≤0.001, One-way ANOVA followed by Dunnett's post-hoc test. Data is expressed as $RQ \pm \frac{RQ_{max}}{RQ_{min}}$, where RQ=Relative quantification.

3.2.12 *The Expression of ARNT (HIF1β) is Increased Only in Female Rats Exhibiting Hyper-Responsive Character to Sugen 5416*

The AhR is only capable of mediating the alterations in the gene expression of its target genes when coupled with the ARNT (HIF1β). Moreover, a crosstalk between the AhR and HIF1α signalling pathways exists, where the ARNT (HIF1β) also serves as binding partner for HIF1α. A diagram of the cross talk between the mentioned pathways is shown in Chapter 1.6.5. Importantly, the latter exhibits higher binding affinity for the ARNT (HIF1β) compared to AhR. Therefore, we examined the relative gene expression of the *ARNT (HIF1β)* in normally responsive animals and animals exhibiting a hyper-responsive character, of both sexes. The relative gene expression of *ARNT (HIF1β)* was significantly increased in female hyper-responsive animals compared to their normally responsive female controls (Figure 3–28). In male hyper-responsive animals, the relative expression of *ARNT (HIF1β)* was not altered when compared to males, which have normal response to Su5416 treatment. Moreover, the relative expression of *ARNT (HIF1β)* in normally responsive female animals was significantly lower than in corresponding male animals (Figure 3–28). The increased expression of *ARNT (HIF1β)* could be associated with increased number of male animals form the SD colony, which exhibit hyper-responsive character to Su5416 stimulation.

No change was observed in the expression of HIF1α (Figure 3–29) and NQO1 (Figure 3–30) between hyper-responsive and normally responsive.

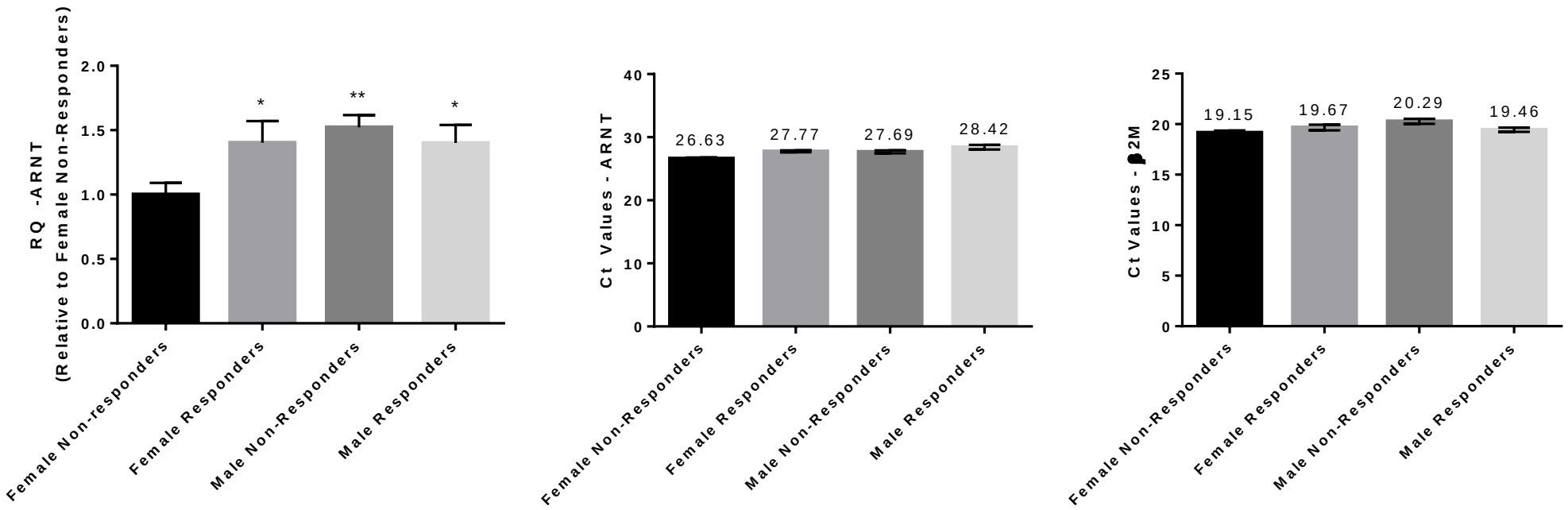


Figure 3–28: The relative expression of the gene encoding the aryl hydrocarbon receptor nuclear translocator protein (HIF1β) is increased in whole lungs from female hyper-responsive rats.

Whole lungs from hyper-responsive and typical Sprague-Dawley rat colonies, of both sexes, treated with a single dose of Su5416, were prepared, as described previously, to assess the expression level of *ARNT (HIF1β)* by Taqman quantitative Real Time-PCR. Results were normalised to *β-2-microglobulin*. N=4–6 per group in triplicate, *p<0.05, One-way ANOVA followed by Tukey's post-hoc test. Data is expressed as $RQ \pm \frac{RQ_{max}}{RQ_{min}}$, where RQ=Relative quantification.

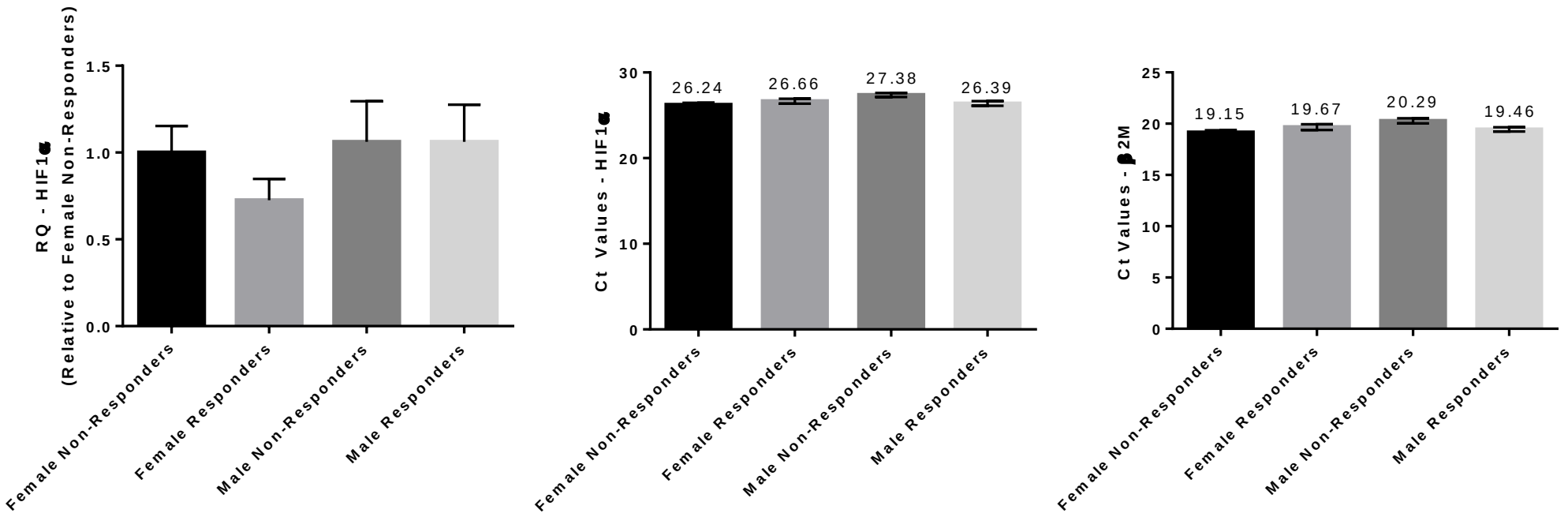


Figure 3–29: The relative expression of the gene encoding the hypoxia-inducible factor 1α in whole lungs derived from hyper-responsive rats of both sexes is unaltered.

Whole lungs from hyper-responsive and typical Sprague-Dawley rat colonies, of both sexes, treated with a single dose of Su5416, were prepared, as described previously, to assess the expression level of *HIF1α* by Taqman quantitative Real Time-PCR. Results were normalised to *β-2-microglobulin*. N=4–6 per group in triplicate, One-way ANOVA followed by Tukey's post-hoc test. Data is expressed as $RQ \pm \frac{RQ_{max}}{RQ_{min}}$, where RQ=Relative quantification.

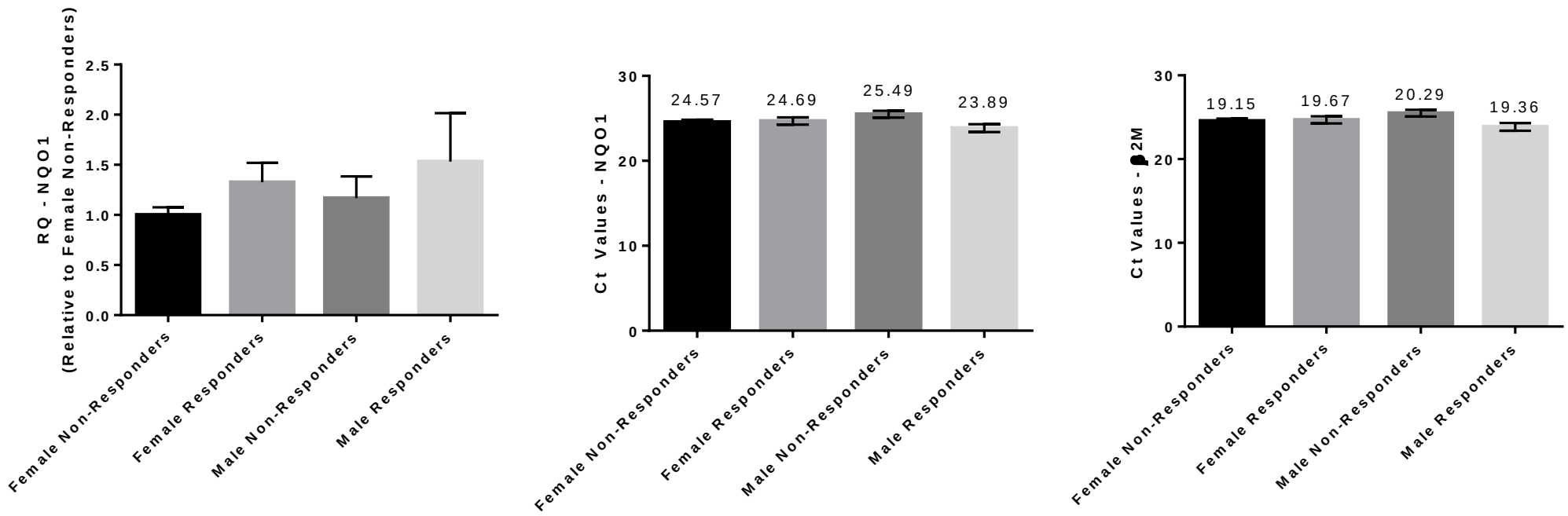


Figure 3–30: The relative expression of NAD(P)H quinone dehydrogenase 1 in the whole lungs from hyper-responsive rats of both sexes is unchanged.
Samples of whole lungs from hyper-responsive and typical Sprague Dawley rat colonies of both sexes, were prepared, as described previously, to assess the expression level of *NQO1* by Taqman quantitative Real Time-PCR. Results were normalised to *β-2-microglobulin*. N=3–6 per group in triplicate, One-way ANOVA followed by Dunnett's post-hoc test. Data is expressed as $RQ \pm \frac{RQ_{max}}{RQ_{min}}$, where RQ=Relative quantification.

3.2.13 *Male Rats Treated with Sugen 5416 and Chronic Hypoxia Exhibit Higher Levels of the Regulators of Hydroxylation-Dependent Proteasome-Mediated Degradation of HIF1α*

We previously discussed that we observed increased whole lung CYP1A1 expression at gene and protein level in Su5416/Hx-treated rats. Additionally, we looked at the expression of AhR and CYP1B1 in these animals. We have found no difference in the expression of AhR, however CYP1B1 expression was increased in treated animals of both sexes, with significantly higher levels in male treated animals compared to females.

Moreover, we investigated the levels of proteins involved in HIF1α signalling pathway, due to the crosstalk between both signalling pathways (diagram is shown in Chapter 1.6.5). We found that HIF1α was significantly increased in female Su5416/Hx-treated animals compared to Nx females, probably due to exposure to Hx. Interestingly, the levels of HIF1α in male Nx animals were significantly higher than in their female counterparts (Figure 3–31A). Nonetheless, no difference exists between Su5416/Hx-treated males and their Nx controls. When examining the expression of PHD2 and vHL, which are both involved in the regulation of hydroxylation-dependent proteasome-mediated degradation of HIF1α (see Figure 1–19), we discovered that Nx male animals exhibited higher levels of vHL compared to female rats kept in Nx conditions (Figure 3–31C). The levels of vHL in male treated animals are also significantly higher than those in treated females. The only significant difference in the expression of PHD2 was observed in male Su5416/Hx-treated animals compared to their female counterparts, where the expression in males was significantly higher than in females (Figure 3–31B).

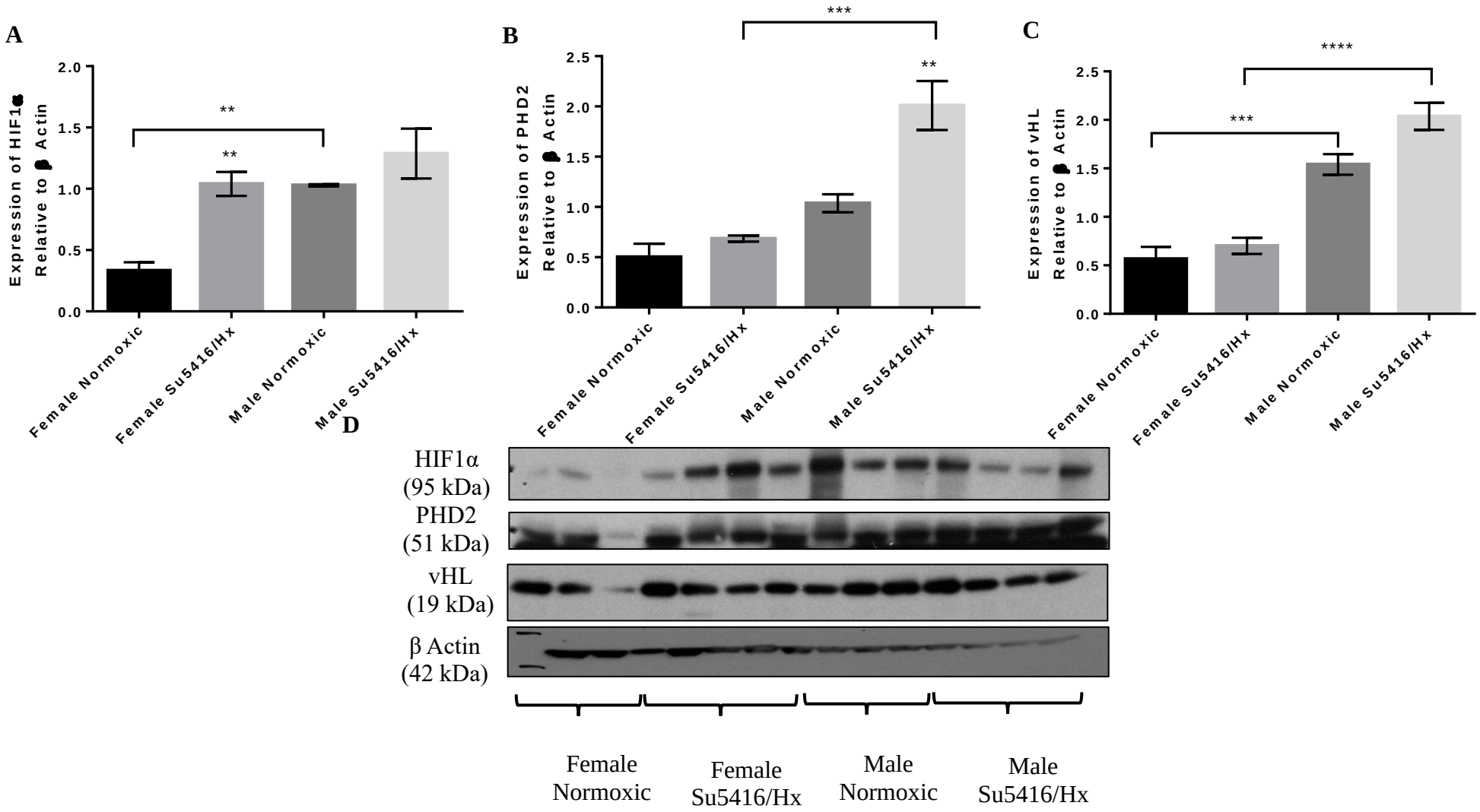


Figure 3–31: Prolyl hydroxylase 2 and von Hippel-Lindau tumour suppressor expression is increased in whole lungs from male rats treated with Sugen 5416 and chronic hypoxia.

Samples of whole lungs from Wistar Kyoto rats, of both sexes, treated with single dose of Sugen 5416 and exposed to hypoxia and their normoxic controls, were prepared, as described previously, to assess the expression level of *HIF1α*, *PHD2* and *vHL* by immunoblotting (A, B, C) with representative images shown in (D). Results were normalised to the β Actin. N=3–4 per group in duplicate, **p≤0.01, ***p≤0.001, ****p≤0.0001, One-way ANOVA followed by Bonferroni's post-hoc test. Data is expressed as mean±SEM.

3.2.14 *Relative Expression of Hypoxia-Inducible Factor β in Pulmonary Arterial Smooth Muscle Cells Derived from Female Patients*

The level of expression of HIF1α and β have not yet been examined in hPASMCs derived from control and PAH patients of both sexes. We therefore assessed the expression in unstimulated protein samples. The results indicate the expression of HIF1β could be significantly higher in female PAH patients compared to controls (Figure 3–32). However, due to high background and apparent low expression levels of HIF1β, it is only warranted to report an observational trend based on accumulative results of repeating the experiment on different occasions. As due to poorer quality of the HIF1β blot, the unequal loading could result in artificially increased expression.

The quantitation of expression level of the gene encoding HIF1α showed no difference between hPASMCs derived from control and patient subjects of both sexes (Figure 3–33A). However, when we examined the expression at protein level, we observed significant increase in HIF1α in hPASMCs derived from female PAH patients compared to controls (Figure 3–33B, C).

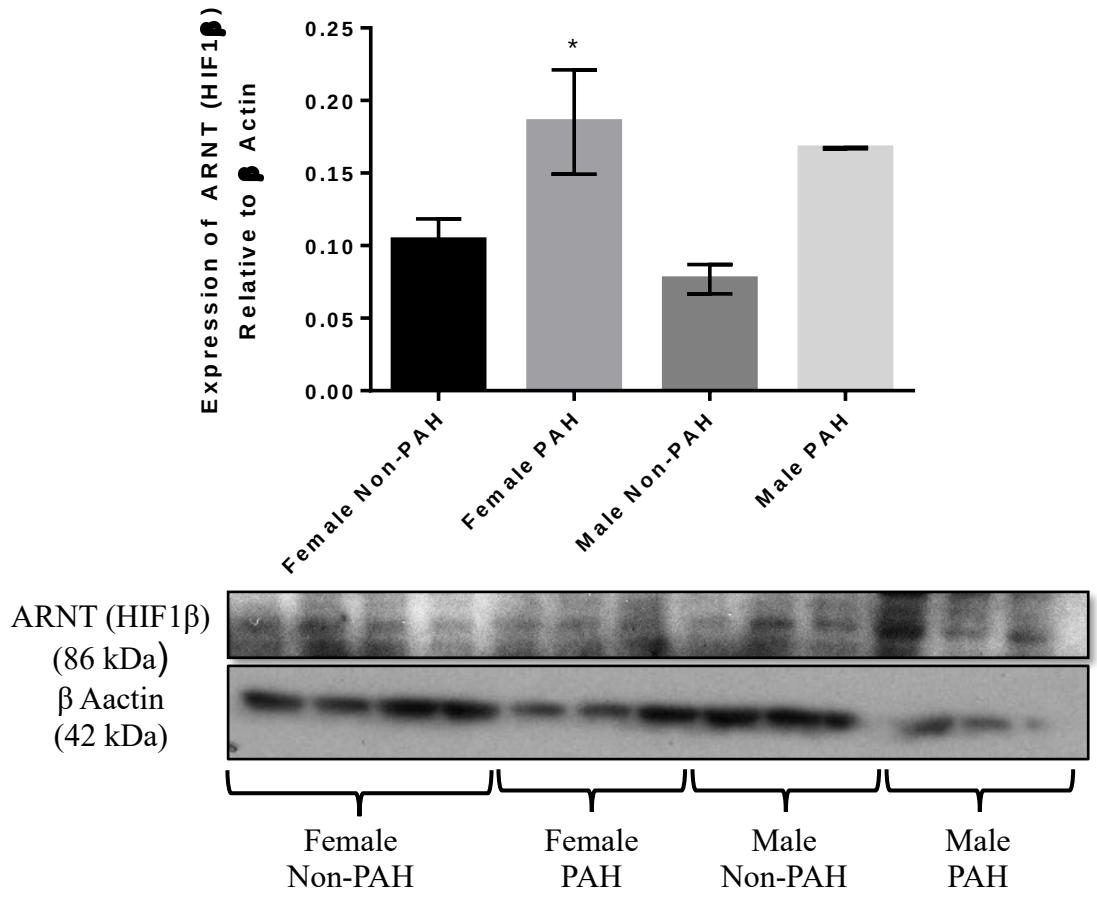


Figure 3–32: Expression of ARNT (HIF1β) is increased in unstimulated human pulmonary artery smooth muscle cells derived from female PAH patients.

HPASMCs were grown to reach approximately 80% confluence in 10% FBS DMEM media. PASMCs were then washed with ice-cold PBS and protein samples were collected and prepared as previously described. N=3–4 per group, in duplicate, *$p<0.05$, **$p\leq0.01$, One-Way ANOVA followed by Bonferroni's post-hoc test. Data is expressed as mean±SEM.

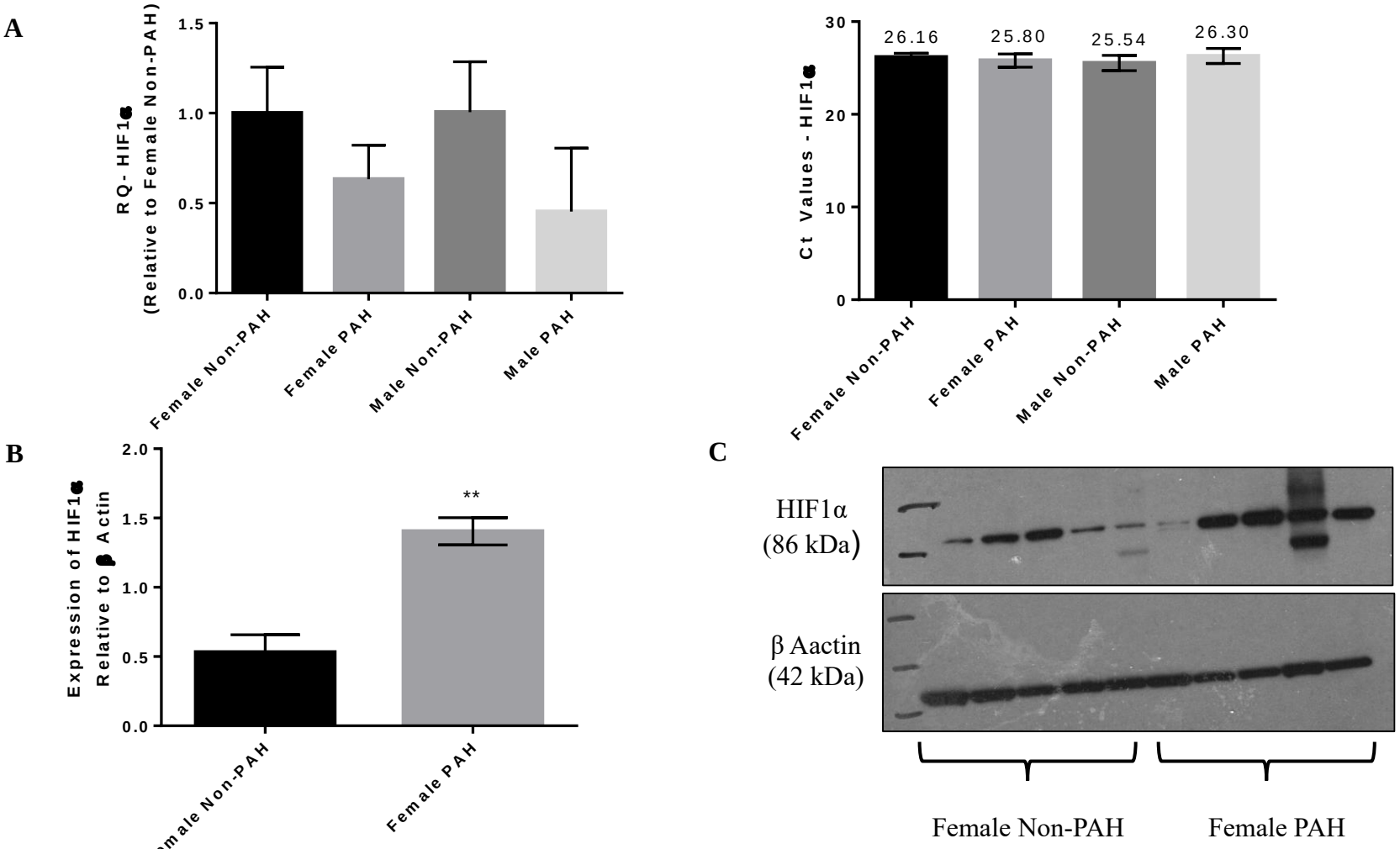


Figure 3–33: The basal expression of HIF1α at protein level is increased in unstimulated human pulmonary artery smooth muscle cells derived from female PAH patients.

HPASMCs were grown to reach approximately 80% confluence in 10% FBS DMEM media. PASMCs were then washed with ice-cold PBS and samples were collected and prepared as previously described to assess gene (A) and protein (B) expression levels. For assessment of gene *HIF1α* expression by Taqman quantitative Real Time-PCR, results were normalised to *β-2-microglobulin* expression level. N=3–5 per group, in duplicate, *p<0.05, **p≤0.01, One-Way ANOVA followed by Bonferroni's post-hoc test. Data is expressed as $RQ \pm \frac{RQ_{max}}{RQ_{min}}$, where RQ=Relative quantification. HIF1α expression at protein level was assessed using immunoblotting (B). N=3–5 per group, in duplicate, *p<0.05, unpaired t-test. Data is expressed as mean±SEM. Representative image of immunoblotting is provided in (C).

3.2.15 *Chronic Hypoxia Elicits Significant Changes in Relative Quantitation of Genes Involved in Aryl Hydrocarbon Receptor and Hypoxia-Inducible Factor 1α Signalling Pathways in Female, But Not in Male Rats*

In whole lungs from male SD rats exposed to cHx alone, the relative expression of *AhR* was significantly reduced compared to their Nx controls. When female rats exposed to cHx alone were compared to their Nx controls, no difference in the level of *AhR* expression was observed (Figure 3–34). However, the relative quantitation of *HIF1β (ARNT)* expression was significantly increased in female animals exposed to cHx compared to Nx controls (Figure 3–35). And while no significant difference was observed in male animals, we demonstrated that relative expression of *HIF1β (ARNT)* in male rats exposed to cHx alone was significantly less than in equally treated female animals (Figure 3–35).

Due to the possible crosstalk between the AhR and HIF1α signalling pathways, we also examined the relative quantitation of *HIF1α* gene expression in the whole lungs. We determined that only in female rats exposed to cHx alone, the relative quantitation of *HIF1α* gene expression was significantly increased compared to Nx controls (Figure 3–36). Interestingly, exposure to cHx did not elicit a significant change in *HIF1α* gene expression in males.

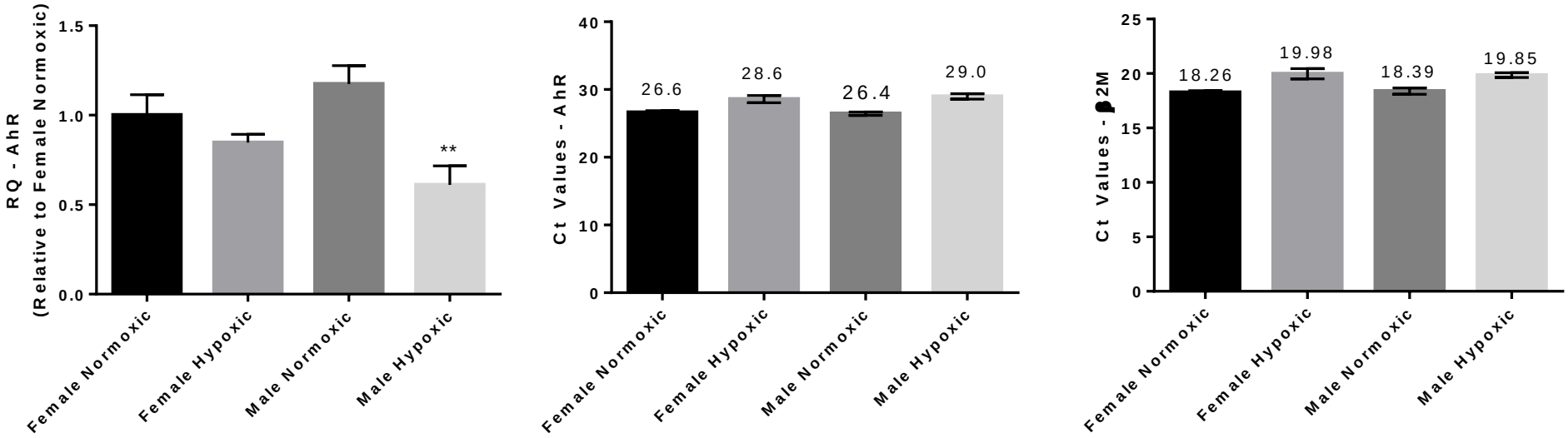


Figure 3–34: The relative expression of the gene encoding the aryl hydrocarbon receptor is decreased in whole lungs from male rats exposed to chronic hypoxia for 28 days

Samples of whole lung of Sprague-Dawley rats, of both sexes, exposed to chronic hypoxia or normoxia for 28 days, were prepared, as described previously, to assess the expression level of *AhR* by Taqman quantitative Real Time-PCR. Results were normalised to *β-2-microglobulin*. N=4–6 per group in triplicate, **p≤0.01, One-way ANOVA followed by Dunnett's post-hoc test. Data is expressed as $RQ \pm \frac{RQ_{max}}{RQ_{min}}$, where RQ=Relative quantification.

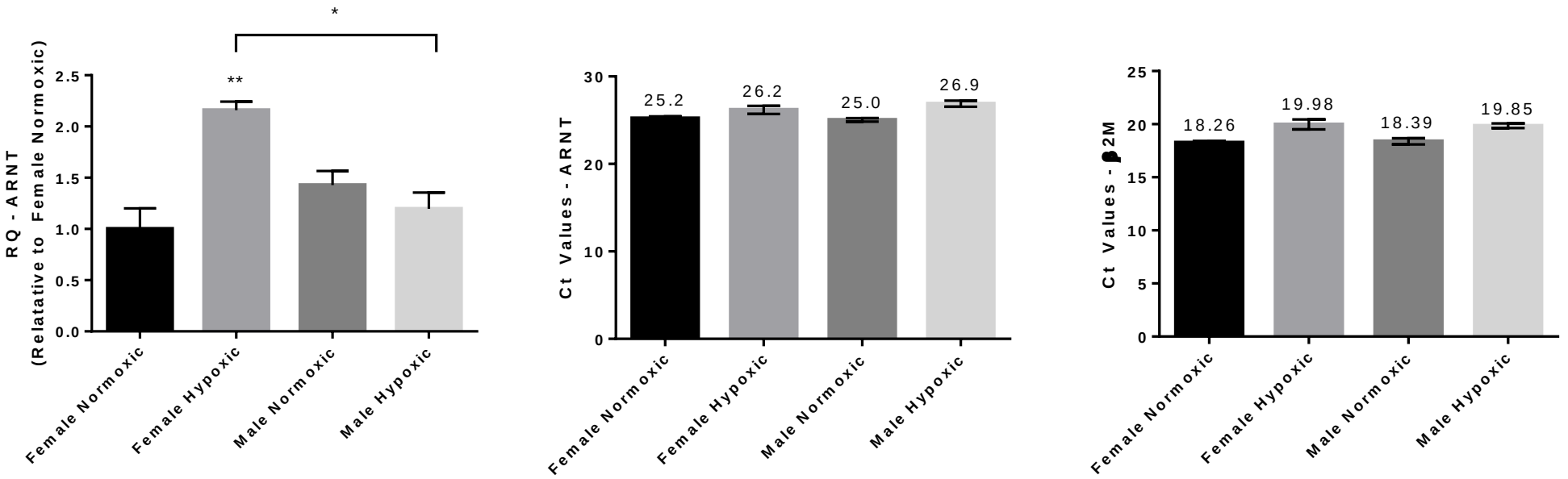


Figure 3–35: The relative expression of the gene encoding the HIF1β (ARNT)was only increased in whole lungs from female rats exposed to chronic hypoxia for 28 days.

Samples of whole lung of Sprague-Dawley rats, of both sexes, exposed to chronic hypoxia or normoxia for 28 days, were prepared, as described previously, to assess the expression level of *HIF1β (ARNT)* by Taqman quantitative Real Time-PCR. Results were normalised to *β-2-microglobulin*. N=4–6 per group in triplicate, **p≤0.01, One-way ANOVA followed by Dunnett's post-hoc test. Data is expressed as $RQ \pm \frac{RQ_{max}}{RQ_{min}}$, where RQ=Relative quantification.

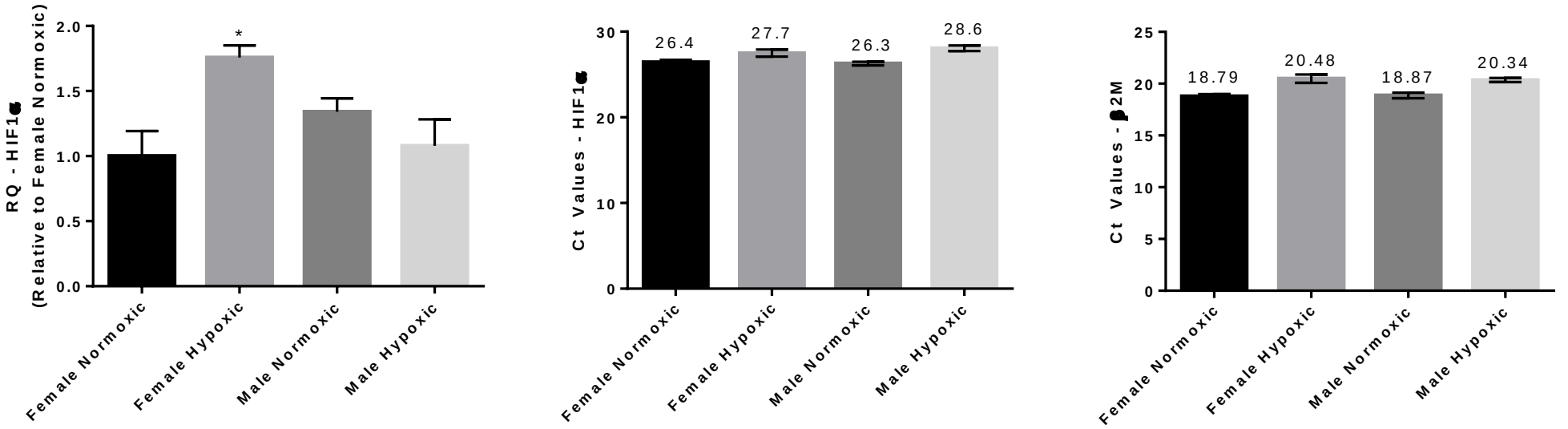


Figure 3–36: The relative expression of the gene encoding the HIF1α was only increased in whole lungs from female rats exposed to chronic hypoxia for 28 days.

Samples of whole lung of Sprague-Dawley rats, of both sexes, exposed to chronic hypoxia or normoxia for 28 days, were prepared, as described previously, to assess the expression level of *HIF1α* by Taqman quantitative Real Time-PCR. Results were normalised to *β-2-microglobulin*. N=4–6 per group in triplicate, **p≤0.01, One-way ANOVA followed by Tukey's post-hoc test. Data is expressed as $RQ \pm \frac{RQ_{max}}{RQ_{min}}$, where RQ=Relative quantification.

3.2.16 *Male Pulmonary Artery Smooth Muscle Cells Derived from PAH Patients Exhibit Higher Expression Levels of Proteins Regulating the Hydroxylation-Dependent Proteasome-Mediated Degradation of Hypoxia-Inducible Factor 1α Signalling Pathway, Aryl Hydrocarbon Receptor and its Repressor*

To determine whether the expression of proteins involved in the AhR and HIF1α signalling pathways are altered in diseased condition, we compared the basal levels of expression in protein samples derived from control and patient hPASMCs, of both sexes. We determined that in male hPASMCs the expression of AhR is increased in PAH patients compared to controls (Figure 3–37). Equally, basal expression of AhRR at protein level was also increased in male PAH patients compared to male controls (Figure 3–38). While, the basal expression of CY1B1, an important modulator of PAH onset and development, was found to be increased in female and male patient hPASMCs compared to suitable controls (Figure 3–40), no significant difference was observed for CYP1A1 (Figure 3–39).

When examining the basal level of expression of regulators of hydroxylation-dependent proteasome-mediated degradation of HIF1α (PHD2 and vHL), we observed that observed that PHD2 and vHL expression was increased in male patient PASMCs but decreased in female patient PASMCs (Figure 3–41 and Figure 3–42).

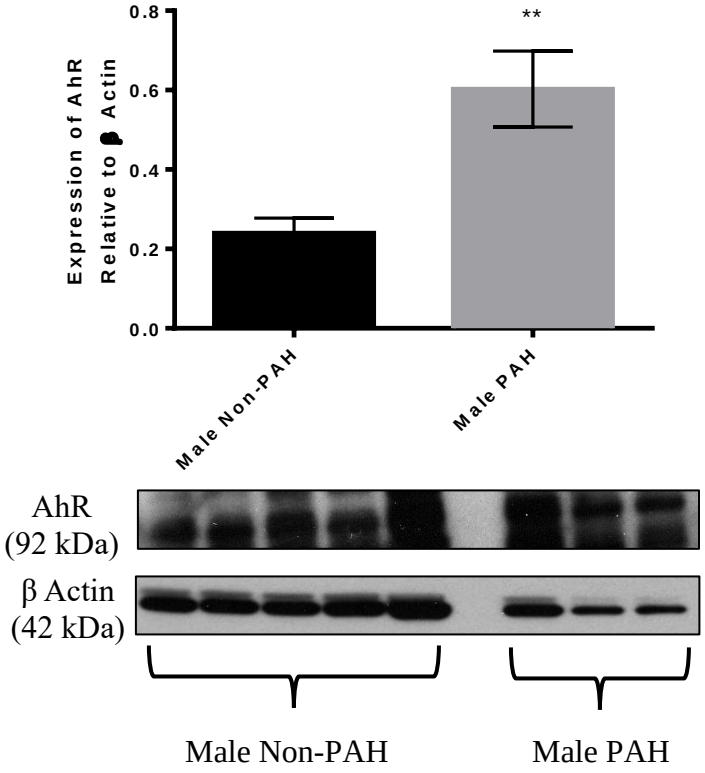


Figure 3–37: In human pulmonary artery smooth muscle cells from male PAH patients, the basal expression of aryl hydrocarbon receptor is increased at protein level.

HPASMCs were grown to reach approximately 80% confluence in 10% FBS DMEM media. PASMCs were then washed with ice-cold PBS, and protein samples were collected and prepared as previously described. N=3–5 per group, in duplicate. **p≤0.01, unpaired t-test. Data is expressed as mean±SEM.

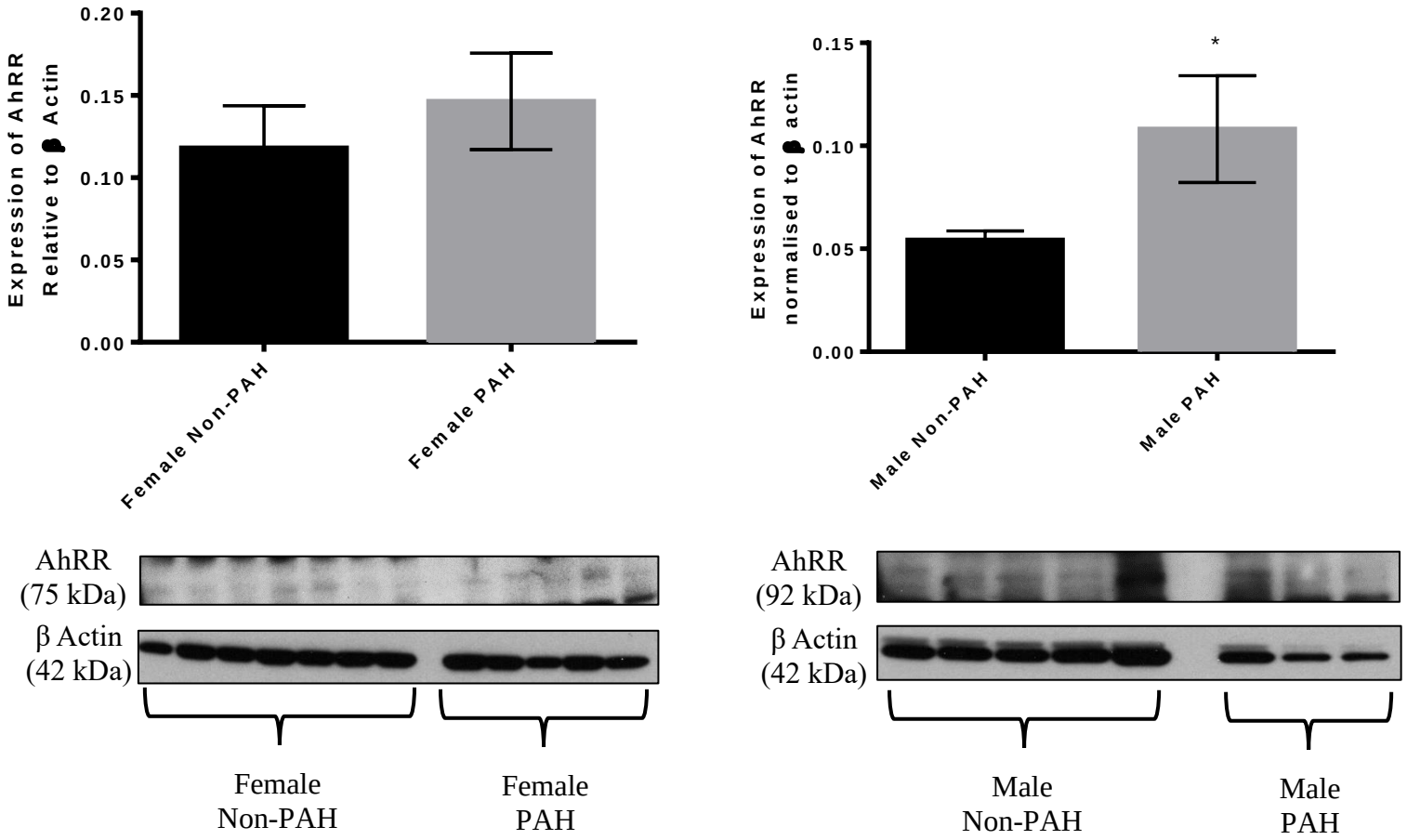


Figure 3–38: The basal expression of aryl hydrocarbon receptor repressor in human pulmonary artery smooth muscle cells from male PAH patients, is increased at protein level.

HPASMCs were grown to reach approximately 80% confluence in 10% FBS DMEM media. PASMCs were then washed with ice-cold PBS, and protein samples were collected and prepared as previously described. N=3–6 per group, in duplicate. *p<0.05, unpaired t-test. Data is expressed as mean±SEM.

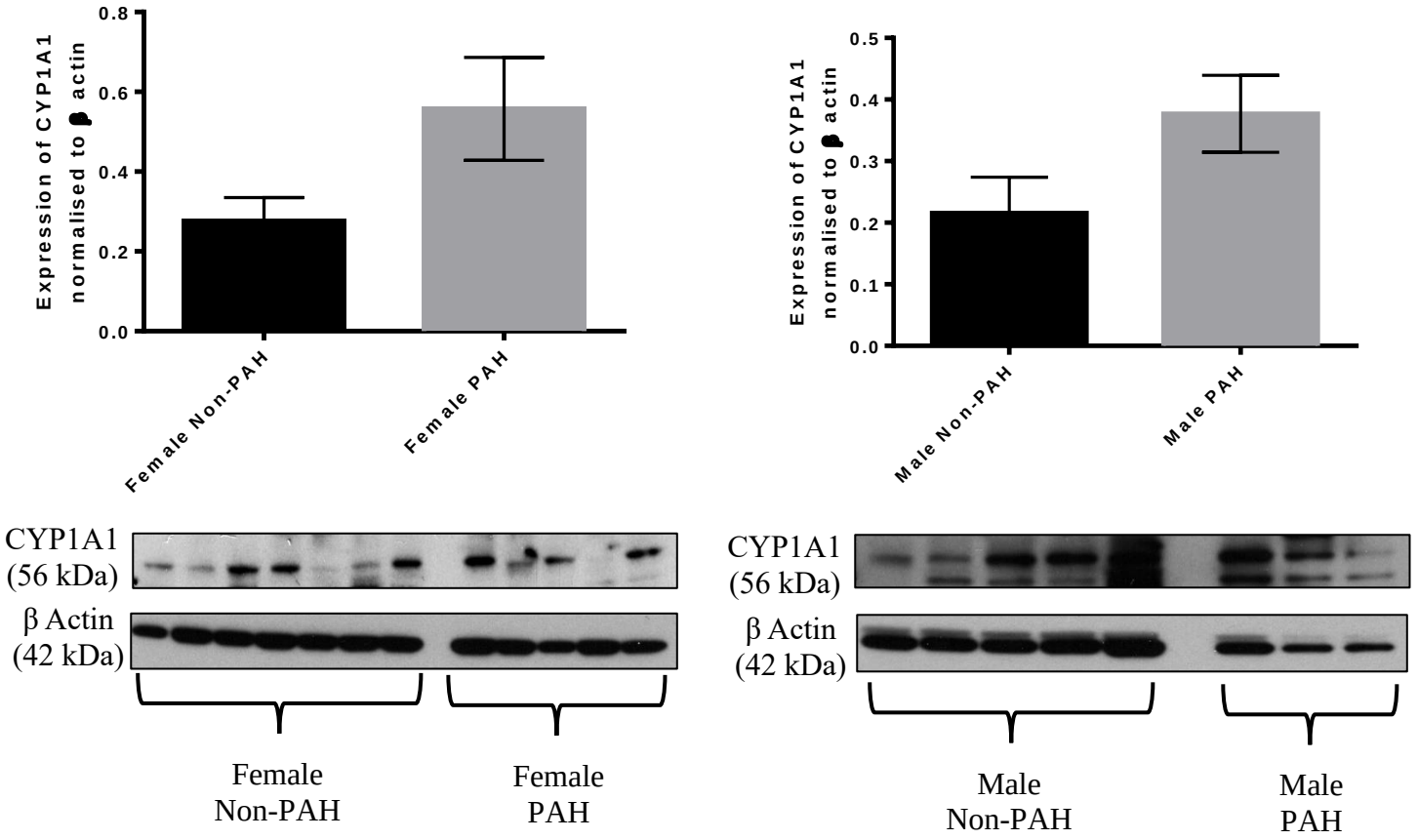


Figure 3–39: The basal expression of cytochrome P450 1B1 level in human pulmonary artery smooth muscle cells from male and female PAH patients, is not altered at protein level.

HPASMCs were grown to reach approximately 80% confluence in 10% FBS DMEM media. PASMCs were then washed with ice-cold PBS, and protein samples were collected and prepared as previously described. N=3–6 per group, in duplicate. unpaired t-test. Data is expressed as mean±SEM.

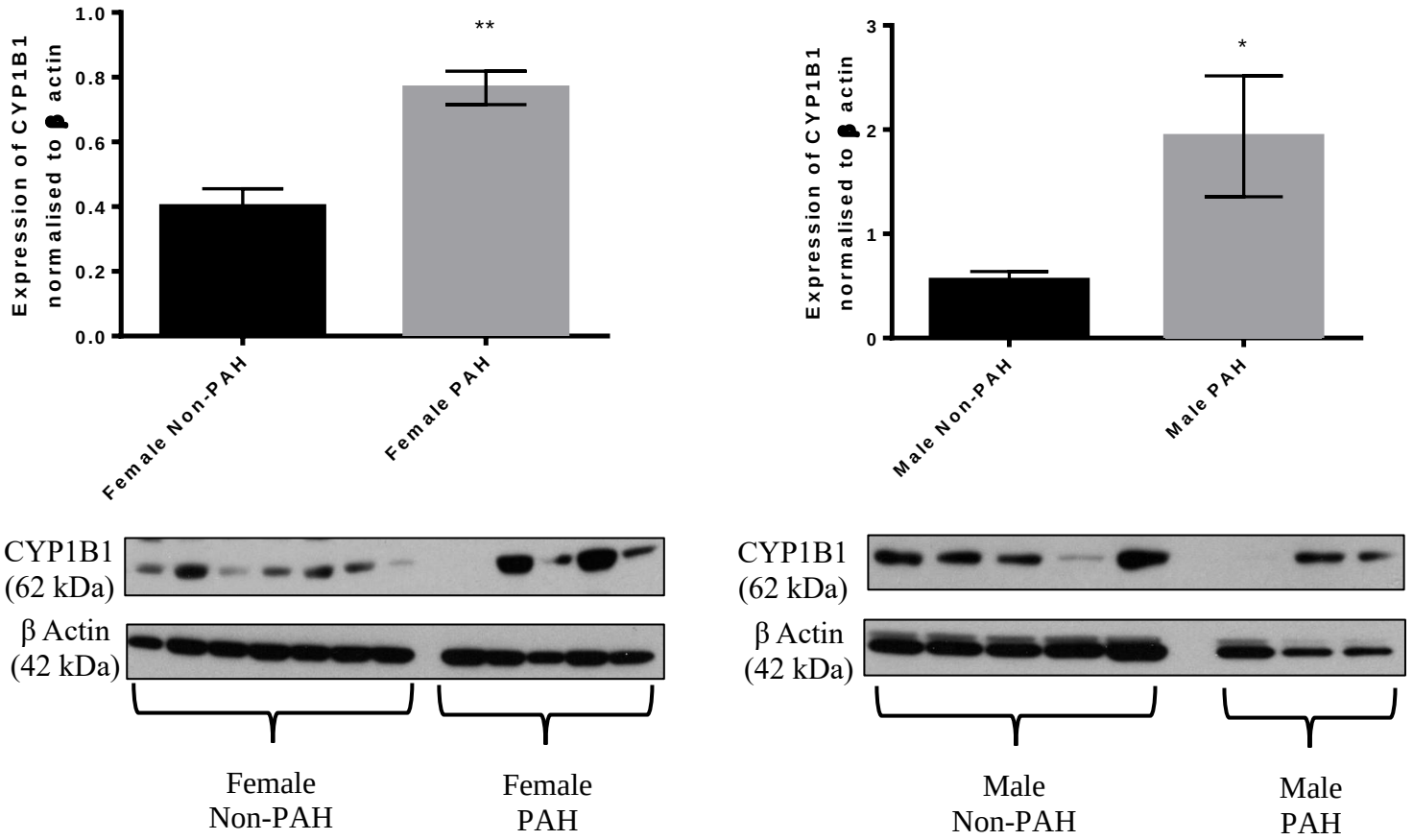


Figure 3–40: The basal expression of cytochrome P450 1B1 in human pulmonary artery smooth muscle cells from male and female PAH patients, is increased at protein level.

HPASMCs were grown to reach approximately 80% confluence in 10% FBS DMEM media. PASMCs were then washed with ice-cold PBS, and protein samples were collected and prepared as previously described. N=3–6 per group, in duplicate. *p<0.05, **p≤0.01, unpaired t-test. Data is expressed as mean±SEM.

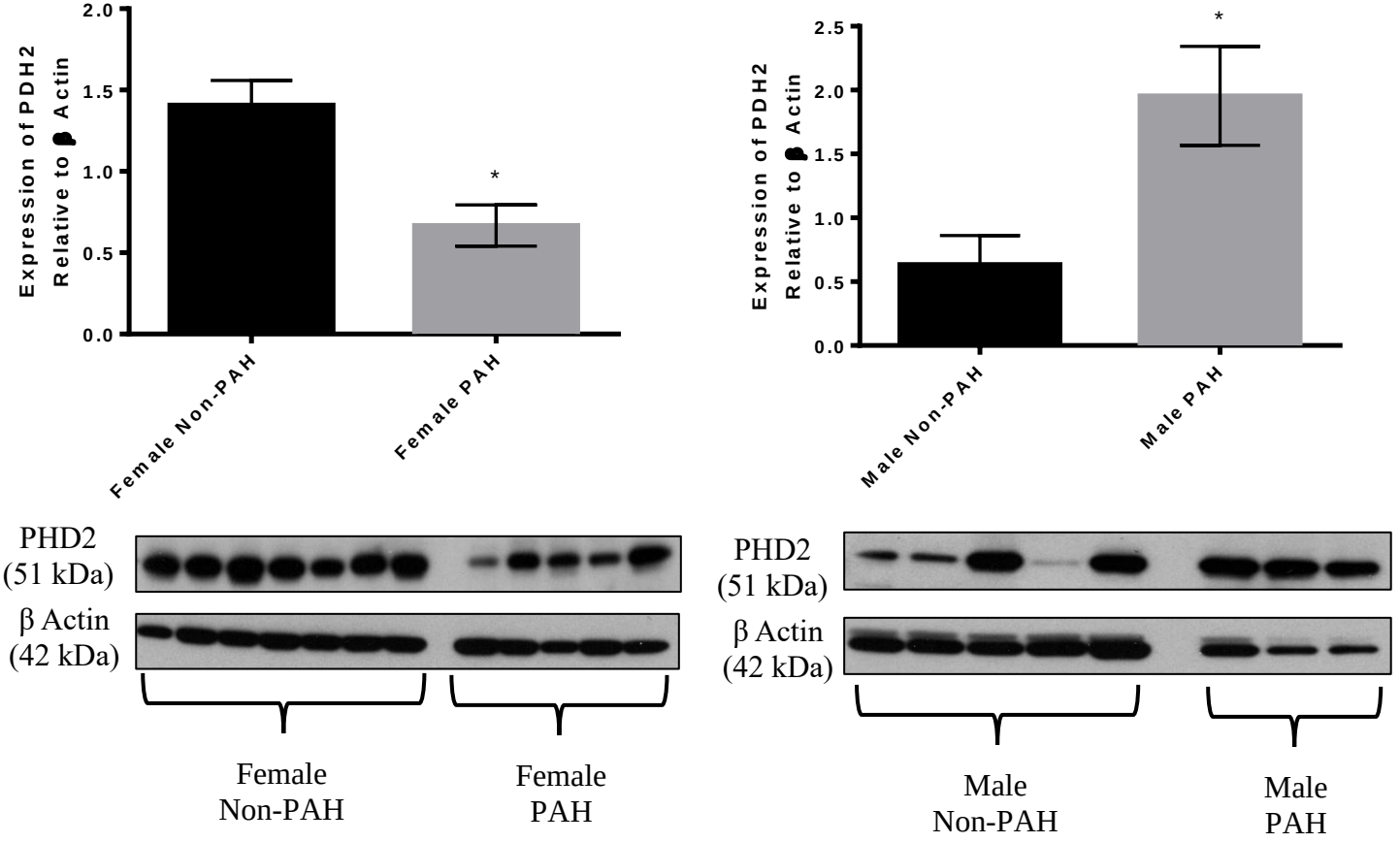


Figure 3–41: The basal expression of prolyl hydroxylase 2 in human pulmonary artery smooth muscle cells from male PAH patients, is increased at protein level.

HPASMCs were grown to reach approximately 80% confluence in 10% FBS DMEM media. PASMCs were then washed with ice-cold PBS, and protein samples were collected and prepared as previously described. N=3–6 per group, in duplicate. *p<0.05, unpaired t-test. Data is expressed as mean±SEM.

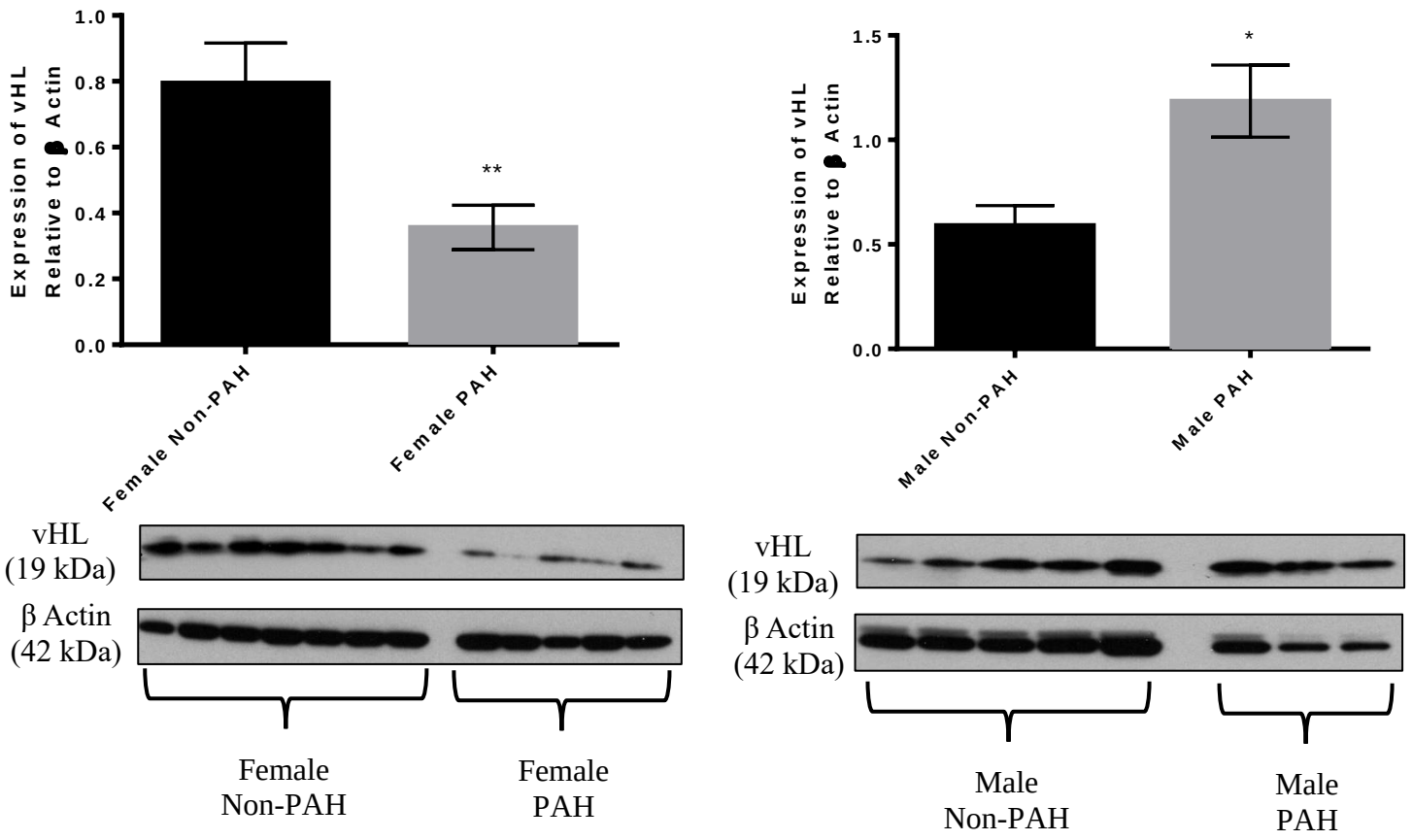


Figure 3–42: The basal expression of von Hippel-Lindau tumour suppressor in human pulmonary artery smooth muscle cells from male PAH patients, is increased at protein level.

HPASMCs were grown to reach approximately 80% confluence in 10% FBS DMEM media. PASMCs were then washed with ice-cold PBS, and protein samples were collected and prepared as previously described. N=3–6 per group, in duplicate. *p<0.05, **p≤0.01, unpaired t-test. Data is expressed as mean±SEM.

3.2.17 *Male Pulmonary Artery Smooth Muscle Cells Derived from Control Patients Exhibit Lower Levels of Hypoxia-Inducible Factor 1β and Cytochrome P450 1B1 than Female Cell Lines*

The AhR and HIF1α signalling pathways both appear to be involved in the onset of disease phenotype in PAH (Dean et al., 2017;Ray et al., 2008). Although, we have observed no difference in the expression of AhR (Figure 3–43 and Figure 3–44) and HIF1α in hPASMCs, there was a significant difference in the expression of ARNT (HIF1β). The expression of CYP1A1 did not differ between female and male control hPASMCs, possibly due to its inducible nature. Significantly less CYP1B1 was expressed in male control hPASMCs compared to female counterparts (Figure 3–43 and Figure 3–44). No significant difference in the expression of PHD2 and vHL was observed (Figure 3–43 and Figure 3–44).

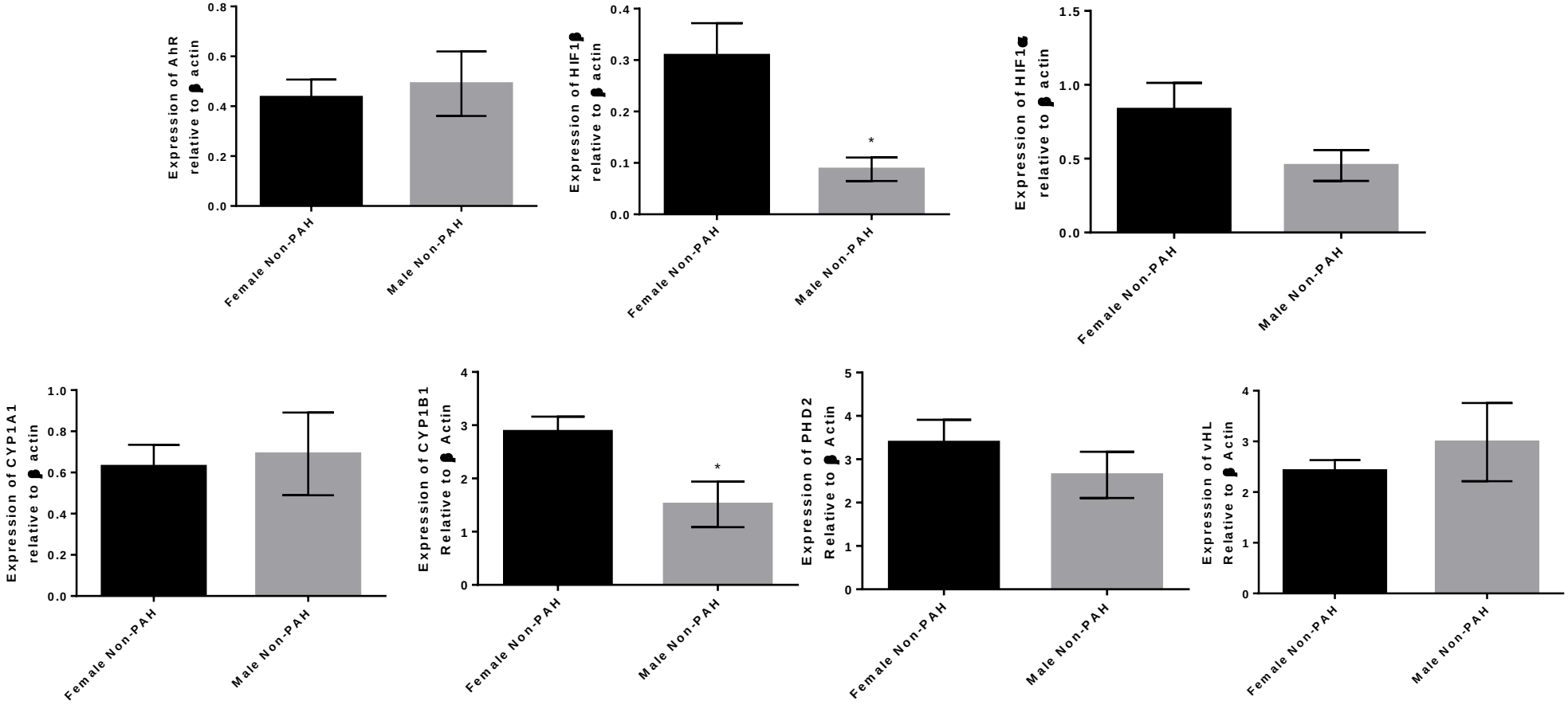


Figure 3–43: In human pulmonary artery smooth muscle cells from male control patients, the basal expression of cytochrome P450 1B1 and hypoxia-inducible factor 1β is decreased.

HPASMCs were grown to reach approximately 80% confluence in 10% FBS DMEM media. PASMCs were then washed with ice-cold PBS and protein samples were collected and prepared as previously described. N=3–6 per group, in duplicate, *p<0.05, unpaired t-test. Data is expressed as mean±SEM.

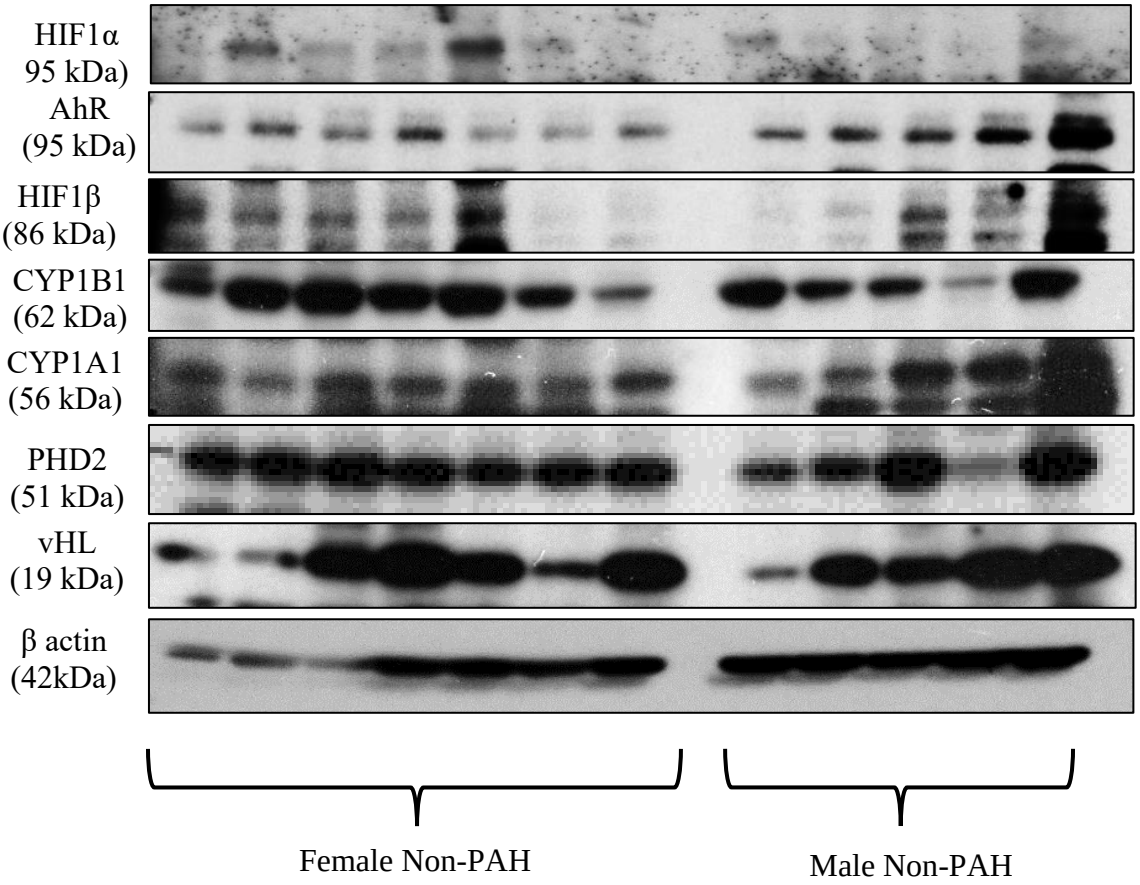


Figure 3–44: Representative immunoblot images of basal expression levels of proteins involved in AhR and HIF1α signalling pathways in pulmonary artery smooth muscle cells derived from controls.

HPASMCs were grown to reach approximately 80% confluence in 10% FBS DMEM media. PASMCs were then washed with ice-cold PBS, and protein samples were collected and prepared as previously described. N=3–6 per group, in duplicate.

3.2.18 *Male Pulmonary Artery Smooth Muscle Cells Derived from Clinical Patients Exhibit Higher Levels of Aryl Hydrocarbon receptor, and Proteins Involved in the Regulation of Oxygen-Dependent Degradation of Hypoxia-Inducible Factor 1α*

The expression of the mediators of AhR and HIF1α signalling pathways have not been assessed in terms of protein expression in female and male patient hPASMCs to determine whether a sex-related difference exists. A possible sex dimorphism might contribute to the higher penetrance of the disease in female population (Ling et al., 2012;Escribano-Subias et al., 2012).

No significant difference in the expression of HIF1α and 1β was observed between samples derived from female and male hPASMCs derived from PAH patients (Figure 3–45 and Figure 3–46). Moreover, the expression of CYP1A1 and CYP1B1 did not differ between male and female PAH patients (Figure 3–45 and Figure 3–46). However, significant differences were observed in the protein expression of AhR, vHL and PHD2, with significantly more protein expressed in male PAH patient hPASMCs compared to female counterparts (Figure 3–45 and Figure 3–46).

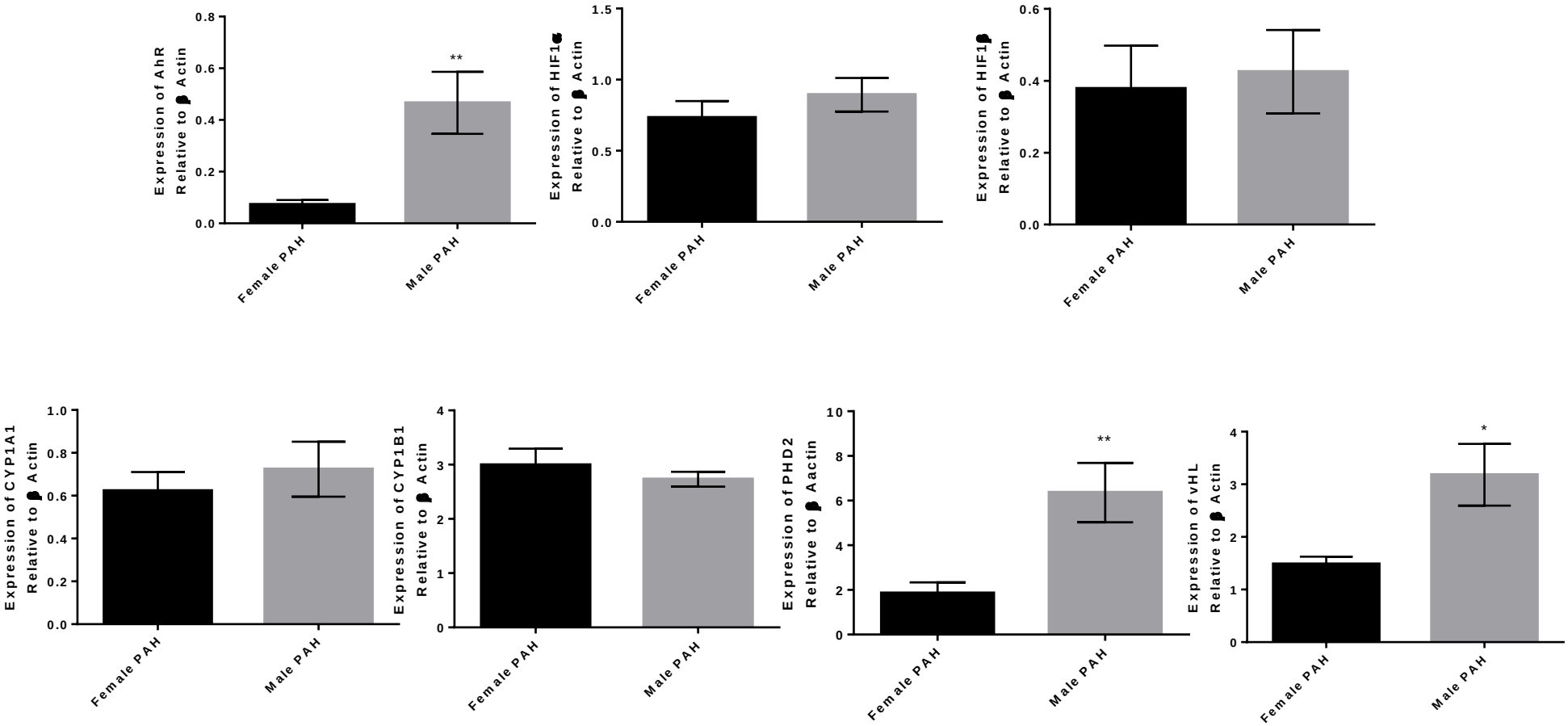


Figure 3–45: In human pulmonary artery smooth muscle cells derived from male PAH patients, the basal expression of aryl hydrocarbon receptor, prolyl hydroxylase 2 and von Hippel-Lindau tumour suppressor is increased.

HPASMCs were grown to reach approximately 80% confluence in 10% FBS DMEM media. PASMCs were then washed with ice-cold PBS and protein samples were collected and prepared as previously described. N=3–5 per group, in duplicate, *p<0.05, **p≤0.01, unpaired t-test. Data is expressed as mean±SEM.

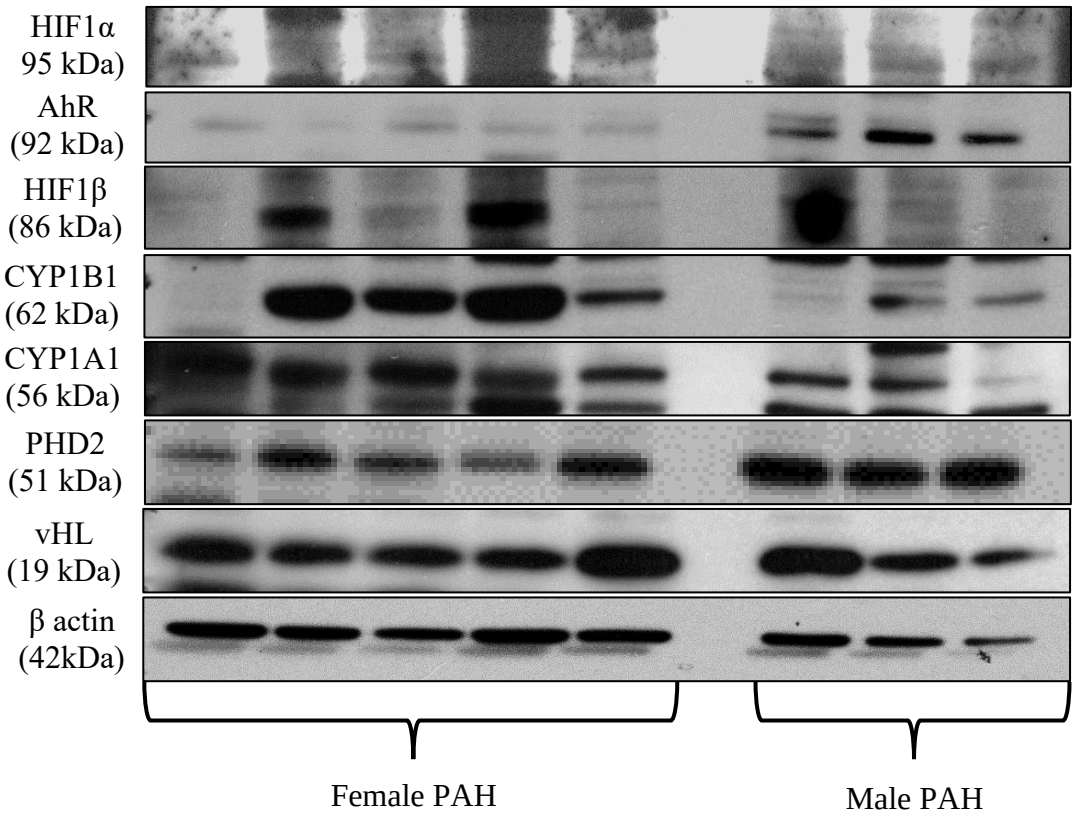


Figure 3–46: Representative immunoblot images of basal expression levels of proteins involved in AhR and HIF1α signalling pathways in pulmonary artery smooth muscle cells from patients.

HPASMCs were grown to reach approximately 80% confluence in 10% FBS DMEM media. PASMCs were then washed with ice-cold PBS, and protein samples were collected and prepared as previously described. N=3–5 per group, in duplicate.

3.2.19 *The Expression of Vascular Endothelial Growth Factor Receptor 2 is Significantly Decreased in Sugen 5416/Hypoxia-Treated Female Rats*

As Su5416 is a known selective inhibitor of the VEGF receptor 1/2. We investigated VEGFR2, for which Su5416 exhibits the greatest affinity, is expressed in whole lungs of Su5416/Hx model. For clarity, VEGFR isoforms 1 and 2 were previously known as Flk-1 and Kdr.

We observed that the relative expression level of Kdr gene is decreased in female rats, treated with a single dose of Su5416 and exposed to cHx, compared to Nx controls (Figure 3–47A), while in male animals no significant changes were observed. Equally, using immunoblotting technique we showed that at protein level, the expression of VEGFR2 was decreased in Su5416/Hx-treated female animals compared to their Nx controls (Figure 3–47B, C).

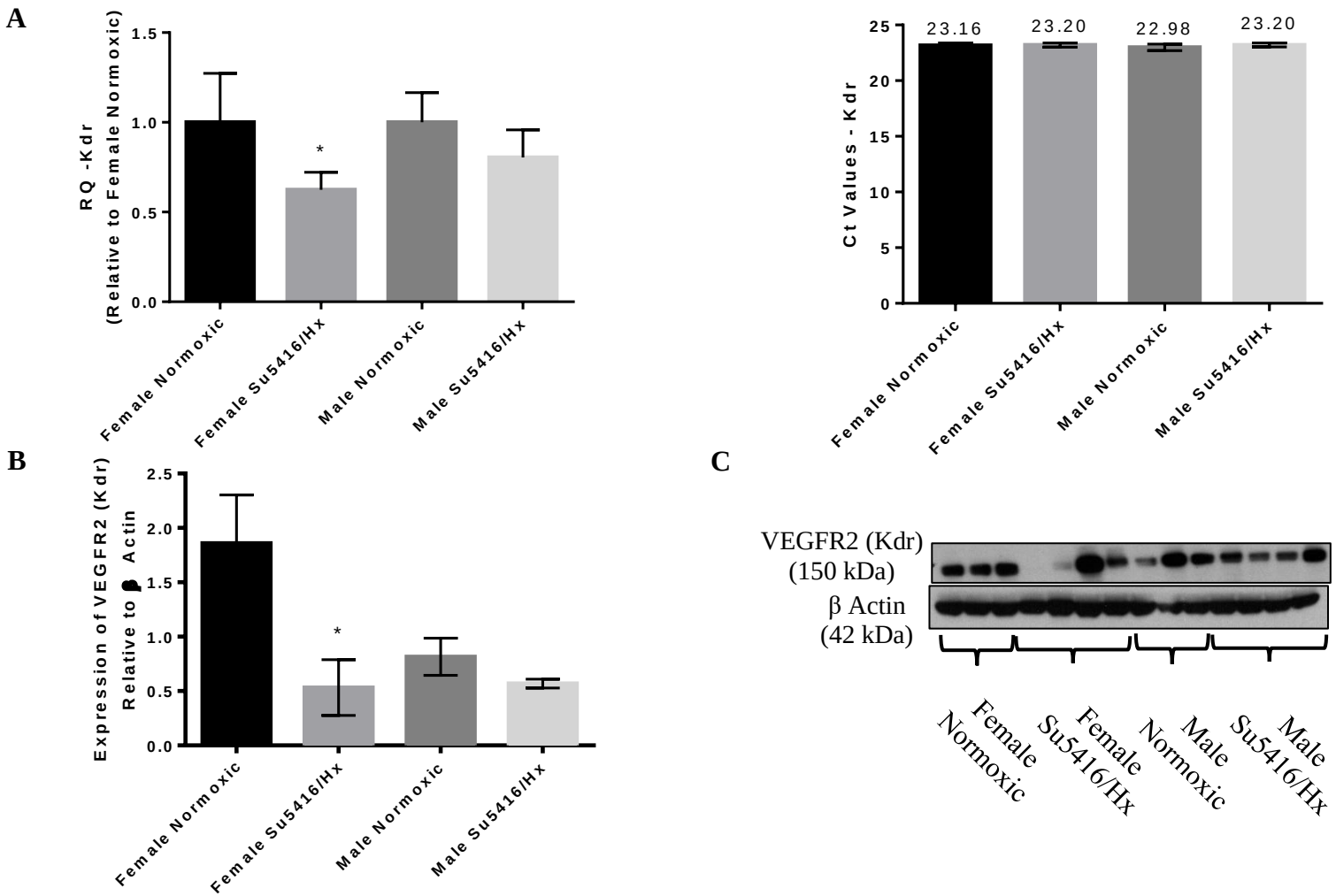


Figure 3–47: Expression of vascular endothelial growth factor receptor 2 in whole lung from Sugen 5416 model.

Samples of whole lung of Sprague-Dawley rats, of both sexes, treated with single dose of Sugen 5416 and exposed to hypoxia and their normoxic controls, were prepared, as described previously, to assess the expression level of *VEGFR2* by Taqman quantitative Real Time-PCR (A). Results were normalised to *β-2-microglobulin*. N=4–6 per group in triplicate, *p<0.05, One-way ANOVA followed by Dunnett's post-hoc test. Data is expressed as $RQ \pm \frac{RQ_{max}}{RQ_{min}}$, where RQ=Relative quantification. The level of VEGFR2 (Kdr) protein expression was assessed using immunoblotting (B). N=3–6 per group in duplicate, *p<0.05, One-way ANOVA followed by Dunnett's post-hoc test. Representative immunoblotting image is shown in (C).

3.2.20 *The Effects of Sugen 5416 in Control Human Microvascular Endothelial Cells*

The disruption of vascular endothelium, resulting in endothelial dysregulation, leads to increased vascular tone due to imbalance in the production of vasoactive compounds favouring vasoconstriction, as well as dysregulated proliferation and growth of SMCs due to alterations in molecules influencing proliferative pathways in these cells (Peiro et al., 1995). Several research groups have reported the imperative role of endothelium in the onset of PAH-like phenotype in animal models (Taraseviciene-Stewart et al., 2001;Ciuclan et al., 2011). In the Su5416/Hx model of PAH, early transient apoptosis of ECs is closely associated with the onset of experimental phenotype (Ciuclan et al., 2011) and expansion of apoptosis-resistant sub-population of ECs (Jurasz et al., 2010).

As previous studies reported the effects of Su5416 on ECs in whole lungs derived from animals exposed to Su5416/Hx, we aimed to investigate its effects in control human microvascular ECs. We assessed apoptosis of ECs by cell number and immunoblotting quantification of active Caspase 3. We have observed no significant difference between treatment with 2% CSS and 2% CSS with DMSO, indicating the percentage of DMSO added to deliver treatments did not affect the overall cell number. The number of cells was significantly reduced in 0.1% CSS and 100μM resveratrol treated cells, serving as positive control groups for apoptosis compared to 2% CSS (Figure 3–48A, B). In the negative control group, where ECs were exposed to endothelial cell growth medium MV SupplementMix, the number of cells was significantly increased compared to control group. The decrease in cell number in Su5416 treatment groups appeared to be dose related, with significant effect observed only in 1μM Su5416 treatment group (Figure 3–48A, B). FICZ, a potent AhR pathway activator, induced a significant decrease in cell number only at the concentration of 50nM (Figure 3–48A).

Treatment of ECs with positive control for apoptosis, 100μM resveratrol, resulted in significantly increased cleaved Caspase 3, while there was no significant difference in the growth medium treatment group, serving as negative control, compared to 2% CSS treated cells. Moreover, the treatment of ECs with FICZ also resulted in significantly increased proportion of cleaved Caspase 3 (Figure 3–48B). Stimulation of ECs with 1μM and 5μM Su5416 resulted in significantly increased cleavage of Caspase 3 compared to the 2% CSS control group (Figure 3–48B). Administration of 16αOHE1 *in vivo* in mice has been

previously shown to inflict a PH phenotype only in female animals (White et al., 2012;Johansen, 2014). We therefore wanted to determine whether the observed changes in the haemodynamic and remodelling parameters could be preceded by endothelial injury. Pro-apoptotic signalling as per increased active Caspase 3 was also observed upon exposure of ECs to 1nM 16αOHE1, but not 100nM 16αOHE1 (Figure 3–48B).

3.2.21 *The Proliferation of Human Pulmonary Arterial Smooth Muscle Cells in Normoxic Conditions is Not Affected by Sugen 5416*

During the onset and progression of PAH, the vascular wall of the distal non-muscular vessels of the pulmonary circulation gradually becomes thickened and muscularised (Barst et al., 2011) either through i) differentiation of pericytes and/or and fibroblasts, into SMCs, followed by their subsequent proliferation and hypertrophy (Tuder et al., 2007; Rabinovitch, 2012) or ii) the transition of SMCs phenotype from contractile to synthetic and/or proliferative phenotype, where the metabolic pathways regulating SMCs proliferation, migration and apoptosis, e.g RhoA/ROCK (Barst et al., 2011; Chan and Loscalzo, 2008; Sheikh et al., 2014) are dysregulated. There was extensive vascular muscularisation and significantly increased percentage of fully muscularised vessels in the lungs of their murine Su5416/Hx model compared to control group, or groups of animals exposed to Hx or Su5416 alone(Ciuclan et al., 2011).

To determine whether 48-hour stimulation with Su5416 affects the proliferation of SMCs, we have assessed the effects of stimulation with (100nM, 500nM, 1μM and 5μM) Su5416 in SMCs in Nx conditions by cell counting. We have compared the cell number in treatment groups to 1% CSS with 0.5% (v/v) DMSO. The culturing of cells in the presence of 10% CSS resulted in significantly increased number of cells, indicating increased proliferation. However, over a wide range of Su5416 concentration, we did not observe a statistically significant change in the cell number of SMCs compared to control group (Figure 3–49).

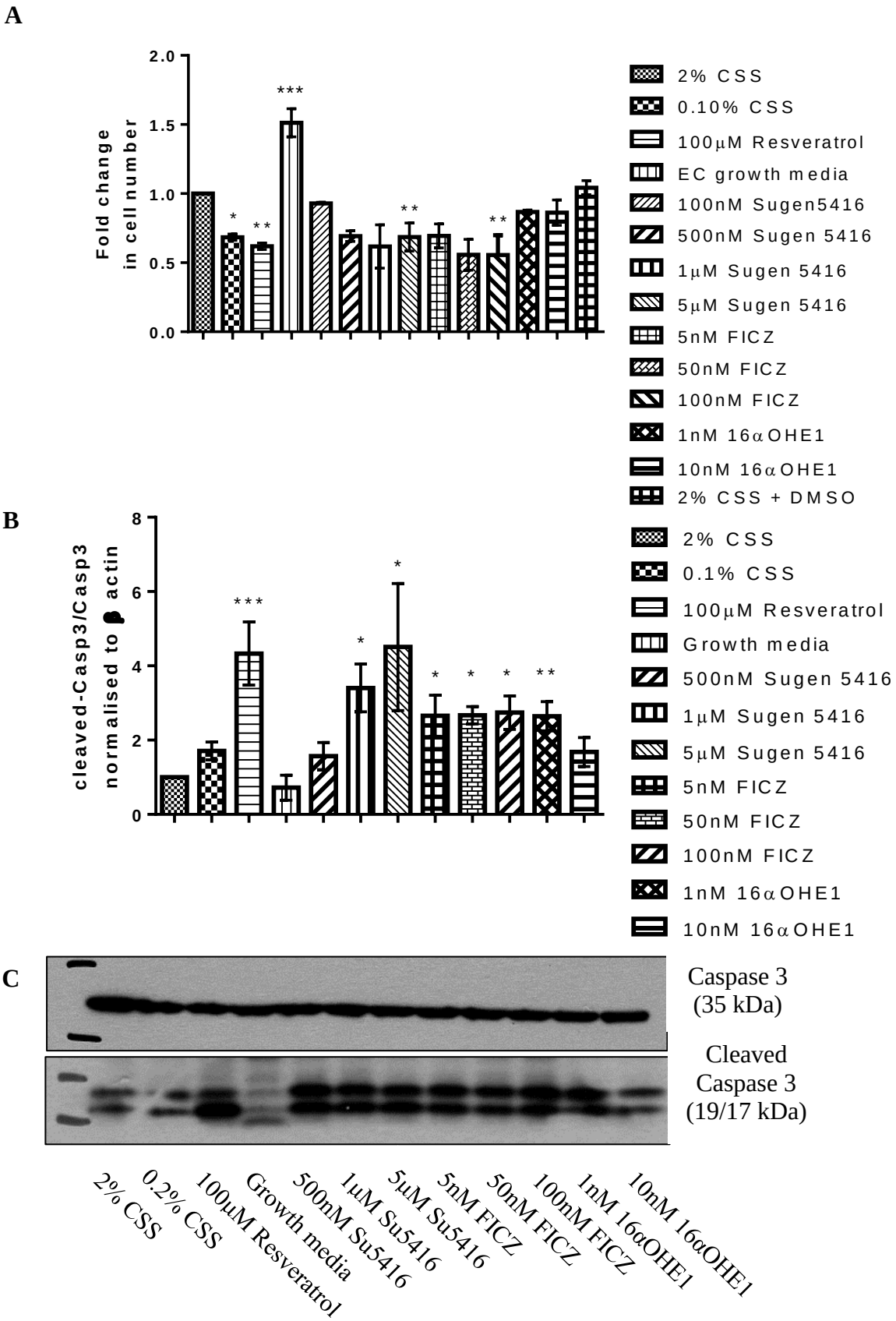


Figure 3–48: The Effects of Sugen5416 stimulation in human microvascular endothelial cells.

Samples of human pulmonary microvascular ECs derived from a female control subject, treated with Su5416(500nM–5μM), FICZ (5–50nM) and 16αOHE1 (1nM and 10nM), were prepared as described previously, to assess the cell number using automatic cell counter (A) and the level of protein expression of activated cleaved Caspase 3 (B) with representative visualisation shown in (C). Results of protein expression were normalised to the total Caspase 3. N=4 per group in duplicate, ***p<0.001, **p<0.01, *p≤0.05, One-way ANOVA followed by Dunnett's post-hoc test. Data is expressed as mean±SEM.

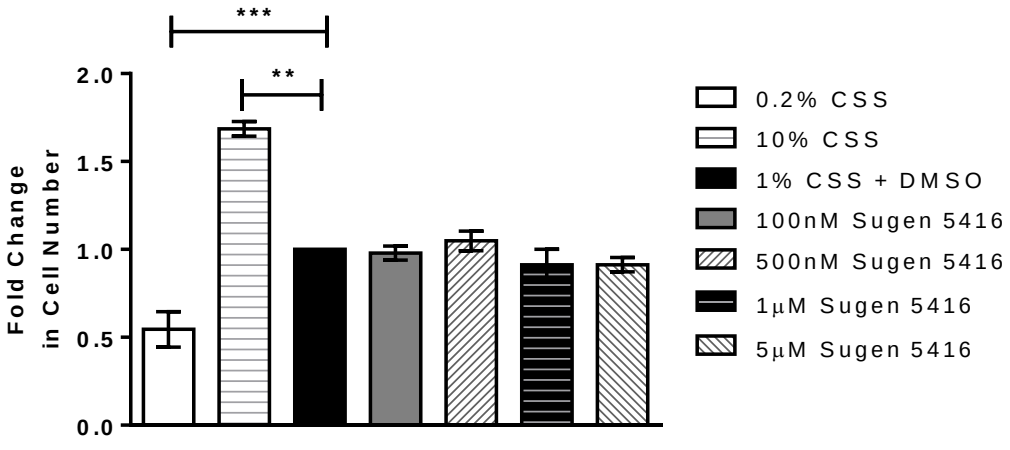


Figure 3–49: In normoxic conditions, Sugen 5416 does not increase proliferation pulmonary artery smooth muscle cells derived from females diagnosed with pulmonary arterial hypertension.

HPASMCs from female patients were quiesced for 24 hours in 0.5% CSS PRF DMEM media. Cells were incubated with a range of concentrations of Su5416. Experiments were conducted in the presence of 1% CSS PRF DMEM media. Cell number was assessed after 48 hours by haemocytometer. N=4 per group in triplicate, ***$p \leq 0.001$, **$p \leq 0.01$. One-way ANOVA followed by Bonferroni's post-hoc test. Data is expressed as percentage of 1% CSS +DMSO control ± SEM.

3.2.22 *The Proliferation of Human Pulmonary Arterial Smooth Muscle Cells in Hypoxic Conditions is Increased by Sugen 5416*

Animals treated with Su5416 only develop severe phenotype of PAH when exposed to cHx, while only a moderate phenotype is observed in animals in Nx conditions.

We have carried out preliminary experiments assessing the suitability of time points to determine the effects of Su5416 stimulation in ECs in Hx. To summarise, upon stimulation with Su5416, cells were placed in a Hx incubator chamber in sterile conditions. The chamber was then flushed with gas mixture containing lowered atmospheric oxygen tension (1% O2, 5% CO2 (v/v)) and placed in the incubator. This procedure was repeated every 24 hours. Out of the three time-points considered, at 24 (Figure 3–50A) and 72 hours (Figure 3–50B), stimulation with Su5416 either did not result in significant alterations in cell number, or resulted in drastically reduced cell number, indicating cellular death due to prolonged exposure to Hx. Therefore, all further experiments were considered at 48-hour time-point.

As in Nx conditions, culturing SMCs in the presence of 10% CSS prompted a significant increase in the cell number, indicating increased proliferation of these cells compared to the control group. In cHx, treatment with Su5416 elicited a concentration-dependent increase in cell number, with 1μM resulting in statistically significant changes in cellular proliferation (Figure 3–51).

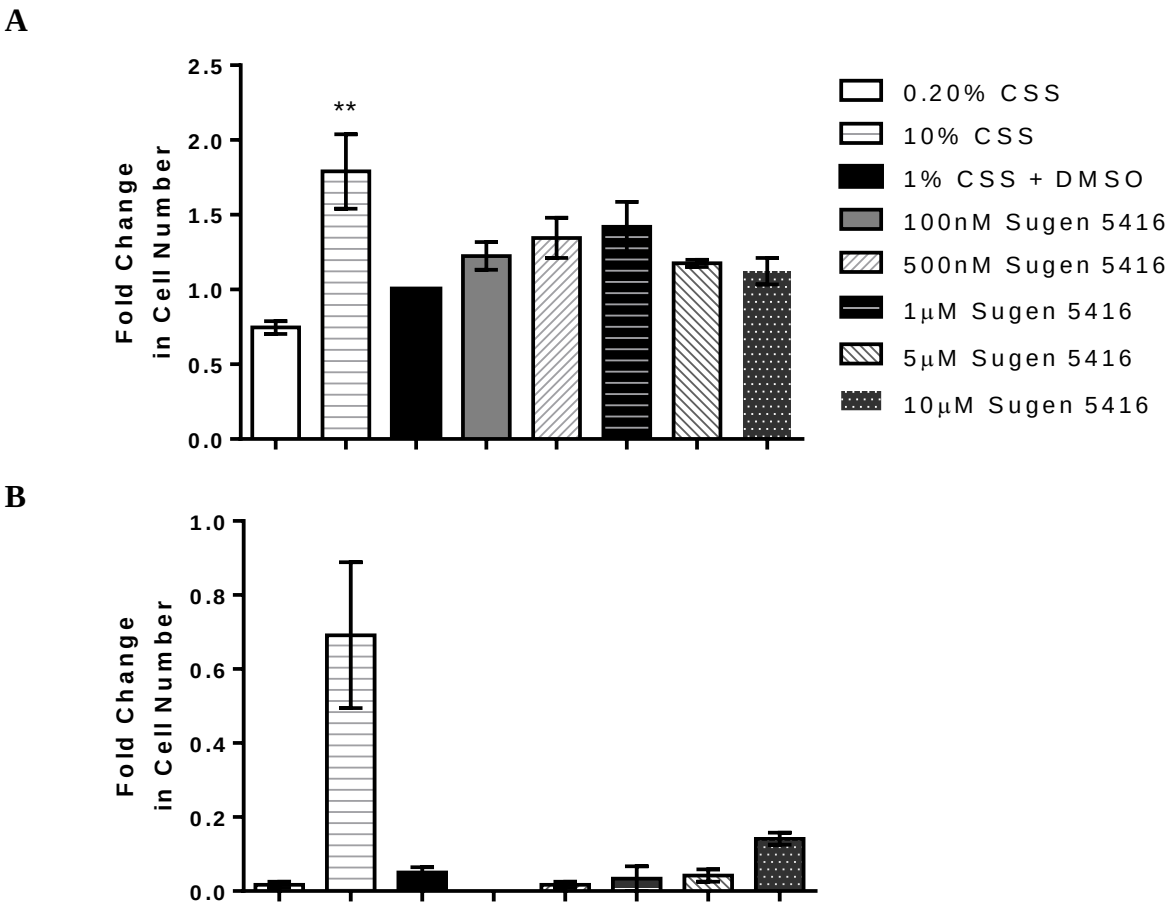


Figure 3–50: Time course experiment showed 24 hour and 72 hour time points were not suitable for further experiments in female human pulmonary artery smooth muscle cells.

HPASMCs from female patients were quiesced for 24 hours in 0.50% CSS PRF DMEM media. Cells were incubated with a range of concentrations of Su5416 and exposed to hypoxia for 24 (A) and 72 (B) hours, respectively. Experiments were conducted in the presence of 1% CSS PRF DMEM media. Cell number was assessed after 48 hours by haemocytometer. N=4 per group in triplicate, ***p≤0.001, **p≤0.01, *p<0.05. One-way ANOVA followed by Bonferroni's post-hoc test. Data is expressed as percentage of 1% CSS +DMSO control ± SEM.

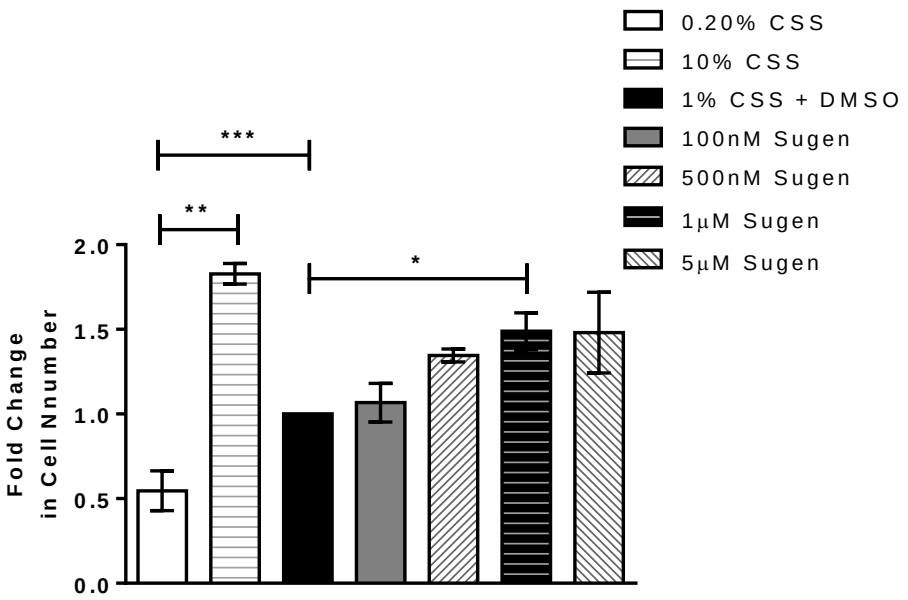


Figure 3–51: Stimulation with Sugen 5416 in hypoxia (0.1% O_2, 5% CO_2) increased proliferation of pulmonary artery smooth muscle cells derived from females diagnosed with pulmonary arterial hypertension.

HPASMCs from female patients were quiesced for 24 hours in 0.50% CSS PRF DMEM media. Cells were incubated with a range of concentrations of Su5416. Experiments were conducted in the presence of 1% CSS PRF DMEM media. Cell number was assessed after 48 hours by haemocytometer. N=4 per group in triplicate, ***p≤0.001, **p≤0.01, *p<0.05. One-way ANOVA followed by Bonferroni's post-hoc test. Data is expressed as percentage of 1% CSS +DMSO control ± SEM.

3.2.23 *Sugen 5416 Increased Localisation of the Aryl Hydrocarbon Receptor in the Nucleus in Female Patient Pulmonary Artery Smooth Muscle Cells*

In the absence of a suitable ligand, the inactive AhR is localised in the cytoplasm, where it is bound to accessory proteins, which keep the receptor in a conformation capable of binding a suitable ligand (see Figure 1–18). Upon receptor's activation, the ligand-bound receptor is translocated into the nucleus, where it encounters and binds to the ARNT (HIF1β), thereby forming the transcriptionally active complex, resulting in the induction of target genes. To determine whether stimulation of hPASMCs with Su5416 might result in the activation of the AhR, we have examined the expression of AhR in nuclear and cytoplasmic fraction utilising the REAP protocol.

We have observed that exposure of hPASMCs derived from female patients to Su5416 elicited increased expression of AhR in the nuclear fraction (Figure 3–52A). A significant difference in AhR expression between cytoplasmic and nuclear fractions was recorded at 90 minutes (Figure 3–52A). Our results indicate that at short time-points, stimulation with Su5416 directly resulted in the translocation of the AhR from cytoplasm to the nucleus.

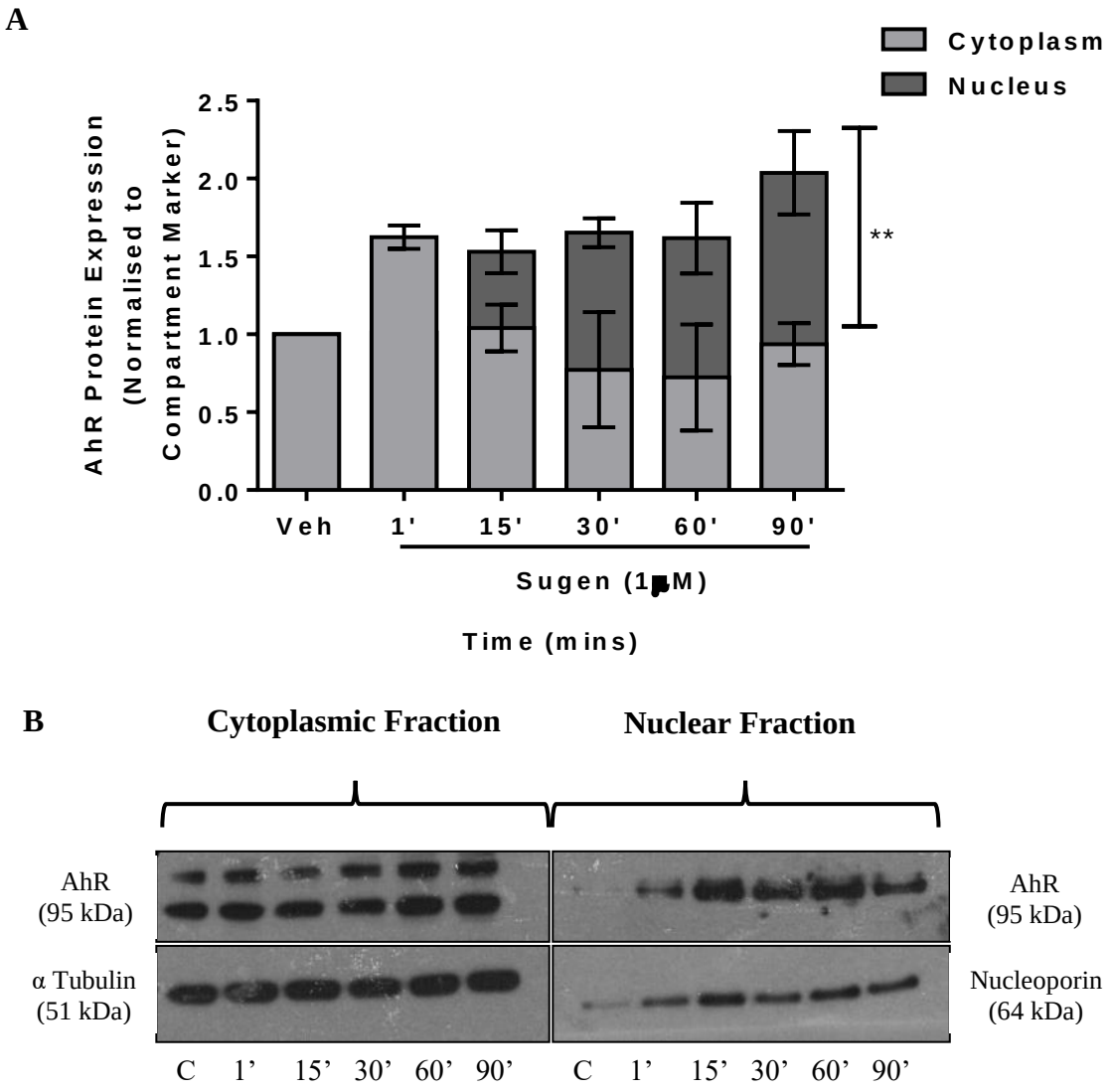


Figure 3–52: Nuclear translocation of aryl hydrocarbon receptor in pulmonary artery smooth muscle cells following time-dependent stimulation with 1μM Sugen 5416.

HPASMCs from female patients were washed with ice-cold PBS solution and incubated with 1μM Sugen5416 as indicated. Experiments were conducted in the presence of 1% CSS PRF DMEM media. Protein samples were prepared according to the REAP fractionation protocol. N=3 in duplicate, *p<0.05. Two-way ANOVA followed by Dunnett's post-hoc test. Data is expressed as fold-change to Veh±SEM.

3.3 Discussion

The evidence for the underlying reasons for the prevailing occurrence of PAH in females is still limited. However, recently emerging literature is supportive of a causative role of oestrogens and their altered metabolism in females (Rajkumar et al., 2010;White et al., 2011a;White et al., 2012;Mair et al., 2014). The pathogenesis of PAH in humans is characterised by numerous cofounding molecular processes, resulting in a disease, whose complex mechanism and novel therapeutic approaches are difficult to investigate. Thus, numerous animal models were developed to explore the individual contributions of these processes. Experimental models which recapitulate the important hallmarks of PAH, such as the Su5416/Hx model, are becoming the gold standard in the field of PAH research. The mechanism responsible for the development of Su5416/Hx experimental phenotype are still largely unknown. This work provides an understanding of the mechanisms driving the experimental PH in the Su5416/Hx animal model.

3.3.1 *CYP1A1 in Animal Models of Pulmonary Arterial Hypertension*

A significant upregulation of the gene encoding for the inducible oestrogen-metabolising enzyme *CYP1A1* was observed and considering that altered oestrogen metabolism was associated with the development of experimental and clinical PAH, we aimed to determine whether its expression might exhibit sex- or disease-related expression patterns. The relative expression of *CYP1A1* in clinical samples of *Smad1* heterozygous female mice and cHx animals of both sexes was significantly reduced. In samples derived from hPASMCs, however, the determination of *CYP1A1* gene expression was less reliable, probably due to more marginal expression of the enzyme in these cells and larger variability in the stability of housekeeper gene. Interestingly, the expression of *CPY1A1* in whole lungs from Su5416/Hx model was significantly increased in male and female animals. Our observations were consistent with previous reports that prior exposure to Hx greatly hindered AhR signalling induced by receptor activator 3,3',4,4',5-pentachlorobiphenyl (PCB 126) in human hepatocytes (Vorrink et al., 2014). Albeit the reduction in *CYP1A1* transcription was reported to be independent of HIF1α signalling in these cells (Allen et al., 2005). Nonetheless, microarray analysis in breast cancer cell line MCF7 indicated that the

expression of *CYP1A1* in these cells might be increased in Hx with unknown underlying mechanism (Elvidge et al., 2006).

We therefore wished to determine why *CYP1A1* expression was highly increased in the Su5416/Hx model, considering it was largely decreased in other experimental PH models. Recent report on the reversal of experimental PAH by metformin revealed that the hypoglycaemic drug mediates a decrease in the circulating levels of oestrogen and its metabolites by decreasing the expression of aromatase, CYP1B1 and the transcription factor affecting the expression of the latter enzyme, the AhR. *CYP1A1* gene expression was considerably more than that of *CYP1B1*, in female and male animals, respectively. This is likely to be due to the fact that CYP1B1 is constitutively expressed whilst CYP1A1 is an inducible enzyme and not normally expressed at significant levels. Therefore, the expression of *CYP1A1* is the most sensitive marker for AhR activation (Taraseviciene-Stewart et al., 2001). Although an increase in AhR expression was previously reported (Dean et al., 2016), we did not note such changes in the expression of AhR in animals with established Su5416/Hx-induced PAH phenotype. We hypothesised that the mechanism of Su5416/Hx-induced experimental PH might involve the activation of the AhR signalling pathway, as Su5416 was shown to activate AhR, and the corresponding murine receptor isoforms, with a potency comparable to that of TCDD (Mezrich et al., 2012). The regulation of the localisation of a ligand-activated transcription factor is a highly dynamic process, involving a mechanism associated with stabilisation of the receptors and retention in the cytoplasm, as well as its transport to the nucleus. The transportation of AhR between the cytoplasm and the nucleus was shown to occur also in the absence of an exogenous ligand (Richter et al., 2001). However, in the presence of an exogenous ligand, the import of AhR to the nucleus is significantly increased, without stopping nuclear export of the receptor (Richter et al., 2001). Hence, the binding of a suitable ligand, leading to enhanced receptor translocation to nucleus, might be important, and the increase in expression of the receptors might not be required for its enhanced transcriptional activity.

To determine whether the AhR signalling pathway may be activated in animals with established experimental disease phenotype, we have assessed the expression of several downstream target genes, such as the *AhRR* and *NQO1*, both of which have been found to be significantly increased in the lungs from Su5416/Hx animals. While the increment in the expression of *AhRR* gene was similar in magnitude of *CYP1A1*, the magnitude of change in the expression of *NQO1* was significantly lower. In the cHx animal model, we observed that

the *NQO1* gene was significantly reduced in male animals exposed to cHx alone when compared to their female counterparts. This might indicate that Hx might interfere with the induction of AhR signalling, as previously reported (Vorrink et al., 2014).

We demonstrated that several of the transcription target genes of the AhR signalling pathway are induced in the Su5416/Hx model, therefore we were interested to determine the effects of Su5416 compound in the *in vitro* model of hPASMCs. The importance of the use of *in vitro* models in research became increasingly significant, since the care for welfare of animals in research has become stringently regulated by the government and criticised by the public. We reduced the number of animals studied by carrying out all expression experiments in the lungs of animals used for previous studies. As an innovative approach to understanding the mechanism behind the Su5416/Hx-induced experimental PAH, we employed our established *in vitro* model, where we have stimulated hPASMCs derived from female control and clinical patients and assessed the expression of AhR target genes involved in oestrogen metabolism. We showed that in both cases, stimulation with Su5416 resulted in significant increase in protein and gene expression of CYP1A1. The observed increase in *CYP1A1* expression, however, was significantly larger than that observed with *CYP1B1*. Moreover, the assessment of CYP1B1 expression was hindered by the selection of available antibodies, which did not give reliable and precise results. Where possible, monoclonal antibodies were utilised, however, due to existence of different CYP isoforms, several bands were shown, despite taking great care to optimise the protocol. We have analysed the bands, which corresponded closely to the molecular weight specified by the provider. Nonetheless, the observed difference might be attributed to the inducible nature of CYP1A1, while CYP1B1 an inducible and constitutively expressed forms of exist (Eltom et al., 1999). Interestingly, changes in the expression of CYP1B1 due to gene induction, through activation of AhR by 3MC, was only observed at protein level (McFadyen et al., 2003), which corresponds with our observations in female control hPASMCs. Our published findings indicate that in Nx rats CPY1A1 was expressed mainly in the PA endothelium, while in Su5416/Hx-treated animals the expression was also detected in the medial layer of small PAs (Dean et al., 2017). It appeared that Su5416 administration influenced CYP1A1 expression in the Su5416/Hx model. When hPASMCs were pre-treated with an AhR antagonist, CH223191, prior to stimulation with Su5416, to determine whether the observed changes in the expression of oestrogen metabolising enzymes occurs through activation of the AhR, the effects in protein expression were reversed, but only in female hPASMCs derived from control subjects. This observation could be associated with reported increased

expression of AhR in female cell lines derived from clinical patients (Dean et al., 2016). We found that AhR expression was not changed by Su5416, specifically in hPASMCs. However, as previously discussed, it is possible that an increase in AhR expression is not required for the reported increase in the induction of *CYP1A1* and *CYP1B1* (Dean et al., 2017), as receptors' activation might be sufficient to confer the changes in transcription of target genes. We were interested in the effects Su5416 of on expression of ARNT (HIF1β), and mediators involved in Hx-inducible signalling pathway (HIF1α, PHD2 and vHL). This is of interest as there is possible crosstalk between the two pathways. Overexpression of ARNT (HIF1β) can alleviate Hx-induced decrease in *CYP1A1* expression (Vorrink et al., 2014). Therefore, we wanted to determine whether activation of the AhR pathway by Su5416 might affect its expression in hPASMCs. We found that Su5416 did not change the expression of ARNT (HIF1β) in hPASMCs. Equally, we assessed the expression of PHD2 and vHL, which are vital in the regulation of proteasome-mediated degradation of HIF1α in Nx. If Su5416 increased the expression of PHD2 and vHL in Nx, this could also affect the degradation of HIF1α in Hx, thereby enhancing the induction of AhR-regulated gene expression. No change in the expression of PHD2, vHL and HIF1α by Su5416 in Nx was observed. In Hx conditions, however, we previously reported that Su5416 reduced the levels of HIF1α in cytoplasmic and nuclear fractions (Dean et al., 2017), showing also AhR signalling pathway might interfere with the induction of Hx-inducible genes.

3.3.2 *AhR Signalling in Rat Colony Exhibiting Hyper-Responsiveness to Sugen 5416 Exposure*

The establishment of experimental PH in Su5416/Hx model was closely related to exposure of animals to cHx, as otherwise animals would only develop a moderate disease phenotype. However, a specific SD rat colony discovered in Canada developed a severe PAH phenotype when treated only with a single dose of Su5416, without the exposure to cHx (Jiang, 2016). The genetic background of rat strain played an important role in the response of animals to Su5416 treatment. Hyper-responsive animals exhibited increased haemodynamic and vascular remodelling parameters compared to typical SD strain and other commonly available rat strains (such as Lewis and Fischer rats), as assessed by RVSP and RV/(LV+S) parameters (Jiang, 2016). Additionally, the presence of angio-proliferative plexiform-like lesions in Nx was only reported in hyper-responsive animals (Jiang, 2016). These animals also exhibited increased apoptosis of ECs in small arterioles shortly after administration of Su5416 (Jiang, 2016). The genetic background differences of rat strains used to establish

experimental PAH models influenced the severity of experimental phenotype in Su5416/Hx model (Jiang, 2016). Unpublished data indicated that the SD strain with enhanced response to Su5416 treatment exhibits sex dimorphism, favouring the development of the disease in male animals (*Stewart et al., unpublished*). As exogenous 17βE2 was shown to exacerbate the Su5416/Hx phenotype in typical rat strains (Tofovic et al., 2012), it was our intention to employ an *in situ* approach to determine whether hyper-responsive male SD rats exhibit differential expression of mediators involved in AhR and HIF1α signalling pathways. Any observed differences might point towards important modifiers of PAH-like phenotype in these animals and link-in with our observations in Su5416/Hx model in typical rat strains. We therefore aimed to assess the expression of CYP enzymes associated with oestrogen metabolism and other proteins involved in the AhR and HIF1α signalling pathways in the lungs from normally responsive and hyper-responsive rats. The expression of *CYP1A1* and *CYP1B1* in this PH experimental model was only increased in males, which were confirmed to develop severe experimental PH without exposure to cHx, hence characterised as 'hyper-responsive'. Moreover, the expression of the *AhRR*, which is induced by the activation of the AhR to serve as negative regulator of this signalling pathway, was also increased in hyper-responsive male animals. Surprisingly, the expression of *AhR* in hyper-responsive male animals was significantly lower than in their female counterparts. Further investigations showed that *ARNT (HIF1β)*, which binds to AhR and thereby forms an active transcriptional heterodimer, was significantly increased in female hyper-responsive animals. In typical SD strain, males exhibited reduced expression of *ARNT (HIF1β)*. The overexpression of ARNT (HIF1β) was shown to rescue the inhibitory effects of Hx enforced upon AhR signalling pathway (Vorrink et al., 2014). The basal levels of expression of this particular protein might therefore be of utmost importance in the establishment of the experimental PH in female animals. Interestingly, in the SD colony exhibiting enhanced sensitivity to Su5416, the hyper-responsive female rats increased expression of *ARNT (HIF1β)*, while the expression of *HIF1α* remained unchanged at gene level in all the groups. We therefore examined whether the expression of HIF1α, and the regulator proteins involved in the oxygen-dependent ubiquitination and degradation, PHD2 and vHL in whole lung samples of Su5416/Hx model. The increase in the expression of HIF1α was observed at protein level in Su5416/Hx-treated female animals compared to their Nx controls, which may be due to the exposure of animals to Hx. Some caution is required with the interpretation of this data, as the level of HIFs was shown to be rather low in these tissues. Furthermore, we also noted that Nx males had significantly higher levels of HIF1α compared to Nx females. Unpublished results from our research group indicated that hyper-responsive

female SD rats had significantly more HIF1α at protein levels (*Docherty, unpublished*). Therefore, the possible increased expression of AhR, ARNT (HIF1β) and HIF1α in hyper-responsive SD female rats might result in higher coupling of these signalling pathways, resulting in the onset of experimental PAH. Whereas, in male hyper-responsive rats enhanced signalling through AhR might be important. Increased expression of AhR target genes in male hyper-responsive SD rats pointed toward a constitutively active receptor in these animals, warranting the investigation of such possibility. The investigation of the genetic background of hyper-responsive SD colony indicated that enhanced expression of genes under the transcriptional activation of AhR, such as *CYP1A1* and *CYP1B1* might be associated with increased probability of PAH phenotype establishment in these animals. It appears that the mechanisms driving the experimental PAH phenotype depended on the sex of the animal.

3.3.3 *Hypoxia-Inducible Factor 1α in Human Pulmonary Artery Smooth Muscle Cells*

A difference in gene induction in response to reduced O_2 exists between males and females in the right and left heart ventricles (Bohuslavova et al., 2010). Although the initial induction of genes in response to O_2 deprivation is higher in females, it is males who exhibit significantly higher levels of target genes regulated by Hx (Bohuslavova et al., 2010). To determine the basal expression of receptors and their binding partners involved in AhR and HIF1α signalling pathways, we prepared unstimulated hPASMCs derived from control and IPAH patients of both sexes. A trend of increased expression of HIF1β in female PAH patient hPASMCs was observed. However, due to lower expression level in hPASMCs and poor response of the employed antibody, the quantitation of this particular protein is not completely reliable. Nonetheless, the expression of HIF1α in these cells compared to female controls was significantly higher. Considering the affinity of the HIF1α for its obligatory binding partner for heterodimerisation is significantly higher than that of AhR, increased expression of the HIF1α might interfere with AhR signalling. Even though the documented increased binding of HIF1α to ARNT may be a direct consequence of decreased degradation of HIF1α during O_2 deprivation, resulting in increased proportion of the receptor in the receptive form to bind to ARNT, compared to AhR (Chan et al., 1999). Interestingly, in male rats exposed to cHx alone, we observed a significant decrease in the gene expression of *AhR*, which was not observed in females.

ARNT (HIF1β), the obligate partner of the AhR, is ubiquitously and constitutively expressed in all cells, as it is involved in pathways other than HIF1 signalling. Recently, conflicting evidence on the effects of Hx on the expression of ARNT (HIF1β) were reported. Mandl and Depping observed that Hx does not affect the expression levels (Mandl and Depping, 2014). Whilst others reported that decreased partial pressure of O_2 increases the expression of HIF1α and ARNT (HIF1β) (Wang et al., 1995). It appears that the induction of ARNT (HIF1β) by Hx greatly depends on the cell and tissue type, with induction being more common in tumour tissues. Moreover, ARNT (HIF1β) might also have a more restricted pattern of expression compared to HIF1α subunit. Our results indicate that in the whole lung samples from rats exposed to cHx alone, the expression of HIF1β is only increased in female animals, while the expression in Hx males is significantly reduced. Equally, in these animals only female Hx rats showed an increase in HIF1α. Hx therefore appeared to increase the expression of both HIFs in female rats, corroborating the findings of (Wang et al., 1995).

A comparison of basal levels of proteins involved in the AhR and HIF1α signalling pathways was not yet reported, therefore we aimed to examine the existence of possible basal differences in hPASMCs. When comparing the basal protein expression between control and patient PASMCs for each sex, we observed that in male patient hPASMCs the expression of AhR, AhRR, CYP1B1, PHD2 and vHL are significantly increased compared to controls, whilst in female cell lines, patient cells exhibited more CYP1B1, and less PHD2 and vHL. It appears that similarly, as in hPASMCs derived from male clinical patients, the expression of vHL and PHD2 was also increased in whole lungs of male rats with Su5416/Hx-induced experimental PAH. Some of these results should be considered with caution, as the protein expression is low, bands obtained for quantification do not have sharp edges despite considerable optimisation procedures, and on occasion contain multiple bands, indicating the presence of multiple isoforms. Nonetheless, the observations in the whole lung samples from female Su5416/Hx animals are in accordance with the observed increase in HIF1α expression in female animals. Moreover, the decreased expression of PHD2 and vHL might affect the basal expression of HIF1α and/or its expression in Hx. It may also be associated with less severe disease phenotype in male animals due to the absence of a synergy between AhR and HIF1α signalling pathways. We believe that this aspect of PAH pathogenesis must be further investigated. Since Hx was shown to induce the proliferation of SMCs derived from systemic arteries (Ray et al., 2008), increased expression of HIF1α observed in hPASMCs from female PAH patients might have implications in establishment of more pro-proliferative phenotype of these cells. Equally, in male patient hPASMCs increased

expression of protein regulating HIF1α degradation in basal levels might affect proliferation of these cells in Hx.

3.3.4 *Effects of Sugen 5416 in Pulmonary Microvascular Endothelial Cells*

In the Su5416/Hx animal model, establishment of the experimental disease phenotype involves vascular injury and hyper-proliferation of vascular cells (Taraseviciene-Stewart et al., 2001). As EC apoptosis may initiate vascular remodelling, and Su5416 induced apoptosis of ECs in animal models (Taraseviciene-Stewart et al., 2001;Jiang, 2016), we wished to examine this in hPMECs, which we considered as control ECs. Increased apoptosis of ECs might result in the selection of apoptosis-resistant ECs that contribute to the formation of angio-proliferative plexiform lesions in clinical and experimental PAH (Jurasz et al., 2010). Here we established that stimulation with Su5416, as well as with FICZ, which is a known AhR agonist, caused apoptosis of ECs as assessed by a reduction in cell number and immunoblotting for active Caspase 3. Recently we reported that stimulation of BOECs from PAH patients with Su5416 resulted in increased proliferation of these cells (Dean et al., 2017). Indeed, Su5416 might contribute to the development of plexiform-like lesions in some animals through imbalance in EC turnover, resulting in selection of apoptosis-resistant cells and increased proliferation of these apoptosis-resistant cells (Sakao et al., 2009;Dean et al., 2017). We also wished to assess whether Su5416 alone, or in the combination with cHx might affect the proliferation of hPASMCs. Our results indicated that in Nx stimulation of hPASMCs from female clinical patients with Su5416 had no effect on the proliferation of these cells. However, when cells were subsequently exposed to cHx, a significant increase in proliferation was recorded (Dean et al., 2017). There was increased proliferation of SMCs in animals after stimulation with Su5416 (Taraseviciene-Stewart et al., 2001). Contrary, the number of PCNA positive vessels and the percentage of fully muscularised vessels was only significantly increased when mice were treated with Su5416 and cHx (Ciuclan et al., 2011).

Finally, to investigate further the effects of Su4516 in the activation of the AhR in hPASMCs, we carried out fractionation analysis, thereby assessing the change in the localisation of this receptor from the cytoplasm to the nuclear fraction. We determined that stimulation of female hPASMCs derived from patients with Su5416 directly resulted in significantly increased localisation of AhR in the nucleus at 90-minute time-point. This is the first report of AhR translocation by SU5416 treatment in hPASMCs. Interestingly, in

BOECs stimulation with Su5416 reduced cytoplasmic expression of AhR, whilst nuclear expression remained constant (Dean et al., 2017). Therefore, it appeared that the expression of AhR in BOECs from patients was decreased, with unaltered activity of AhR within the nucleus.

In summary, the data presented herein, and published in (Dean et al., 2017), provides a new insight into the potential mechanisms behind the Su5416/Hx experimental PH model. In Nx, the AhR is expressed at low levels in whole lungs. Exposure to Su5416/Hx, results in increased expression of AhR (Dean et al., 2016). The in the animals showing PH phenotype, the RVSP, RVH and pulmonary vascular remodelling are all markedly increased by Su5416/Hx. The mechanism behind the induction of experimental PH is thought to affect the cell turn-over of ECs, resulting in Su5416-induced. Increased apoptosis of ECs may initiate vascular remodelling in experimental PAH. This could cause degeneration of pre-capillary arterioles or select apoptosis-resistant ECs that contribute to "angio-proliferative" plexiform lesions (Jurasz et al., 2010) As ECs are subject to stricter contact inhibition in intact arteries than hPASMCs, they do not normally proliferate. However, it was shown that BOECs from patients with PAH can exhibit increased proliferation (Toshner et al., 2009). Indeed, the dysregulated proliferation of ECs is considered a fundamental process in the development of clinical PAH (Tuder et al., 1994). Su5416 induced the proliferation of BOECs from PAH patients, which could perhaps precede and contribute to the selection of apoptosis-resistant ECs, leading to vascular remodelling and the development of occlusive lesions in some animals. The exposure of hPASMCs to Su5416 leads to increased activation of AhR, which is a complex and dynamic process involving the mechanism of receptor retention and stabilisation in the cytosol, and continuous nuclear export. Increased activation results in increased rate of nuclear AhR import without affecting nuclear AhR export (Richter et al., 2001). Firstly, increased activation of AhR induces the expression of aromatase, leading to increased local production of 17βE2 within the lung. 17βE2 can be converted to 2OHE2 by CYP1A1/2, CYP1B1 and CYP3A4. Moreover, CYP1A1 also metabolises E1 and 17βE2 to 2OHE2 and 16αOHE1, which are mitogenic in hPASMCs and may contribute to the development of PAH (White et al., 2012;Hood et al., 2016). Indeed, increased activation of AhR leads to increased expression of CYP1A1, and possibly CYP1B1, resulting in altered oestrogen metabolism. In Hx, exposure of hPASMCs to Su5416 results in increased proliferation, perhaps through the synergy of Su5416 and Hx signalling pathways via ARNT (HIF1β).

The effects of AhR on 17βE2 synthesis and metabolism are of interest as major PAH registries report a greater incidence of PAH among women than men (Shapiro et al., 2012) and 17βE2 metabolism is implicated in the increased penetrance of heritable PAH (HPAH) among female patients harbouring a mutation in the gene encoding (Austin et al., 2009). The resulting increase in EC apoptosis, as well as 17βE2 synthesis and metabolism, may contribute to experimental PH. This is summarized in Figure 3–54.Therefore, inhibition of AhR may be a potential novel approach to the treatment of PAH, should these new findings translate to the human situation.

To summarise, the AhR and HIF1α signalling pathways appear to have an important role in the establishment of experimental as well as clinical PAH and might possibly offer another attractive target for the development of novel treatment approaches for PAH.

3.3.5*Limitations of the Study*

Genotypic and phenotypic variation amongst animals used in research might limit scientific validity by enhancing experimental variability. Genetic background of animals is defined as an assortment of interactions of the complete genotype of the animal with the particular gene of interest, potentially resulting in the appearance of a specific phenotype. Therefore, the understanding of the effects of genetic background on phenotype is of utmost importance in research. Herein, a well-established SD rats from Canada exhibited hyper-responsive phenotype to treatment with Su5416. With the determination of complete genotypes of numerous animals used in research, the understanding of genetic background was improved. Most animals used research are namely derived from established laboratory strains, hence the genetic variation is expected to be rather uniform, due to prolonged in-breeding process. Although in-breeding should theoretically result in animal populations that are relatively genetically unvarying, mutations can occur and accumulate naturally, resulting in phenotypic variance (Yoshiki and Moriwaki, 2006). This could be the reason behind the apparent hyper-responsiveness to Su5416 reported in a colony of SD rats in Canada. Another issue arises when using specific transgenic strains, such as *Smad1* heterozygous mice. Transgenic animals usually have mixed genetic backgrounds, as the main effort is to create animals with specific genotype in order to investigate the role of that particular gene. Hence, the varying genetic background of such animals might have even more apparent influence on animals' phenotype. The natural presence/occurrence of such significant phenotypic

variance exposes the question whether it is appropriate to compare the effects of a treatment across different animal strains, and even species.

The age at which the animal was subjected to experiments might be another limiting factor. As shown by several transgenic models, described in Chapter 1.4, the phenotype might greatly depend on the age (and sex) of the animal, with specific phenotype only being present in older animals. The age-dependent changes in the phenotype might be attributable to significant effect ageing might have on transcription. Indeed, changes in the level of circulating oestrogens might affect the pattern of gene expression in the genes under oestrogen regulation. However, with age mutations might naturally accumulate, resulting in phenotypic variance. Hence for quality and comparable data, the age of animals for experiments should be determined on the basis of development or cellular ageing.

The choice of the housekeeping gene could also pose a further limitation of the study, as described above. The characteristic for the selection of an appropriate housekeeping gene for normalisation of RT-qPCR results is stable expression, which can be described by: i) small coefficient of variation and ii) maximal fold change. De Jonge and colleagues described the experiments undertaken to determine the most appropriate housekeeping genes (de Jonge et al., 2007). The variation of traditionally used housekeeping genes, i.e. β–2–macroglobulin (*B2M*), glyceraldehyde-3 phosphate dehydrogenase (*GAPDH*) and β actin (*ACTB*) was found to fluctuate considerable. Therefore, other genes were proposed to be used as housekeeping genes of choice. Equally, the low protein expression levels and the presence of multiple isoforms, with possible off-target binding, have limited some aspects of this study. Further optimisation should be considered in terms of using more specific protein detection systems with the exclusive used of monoclonal antibodies for detection and localisation.

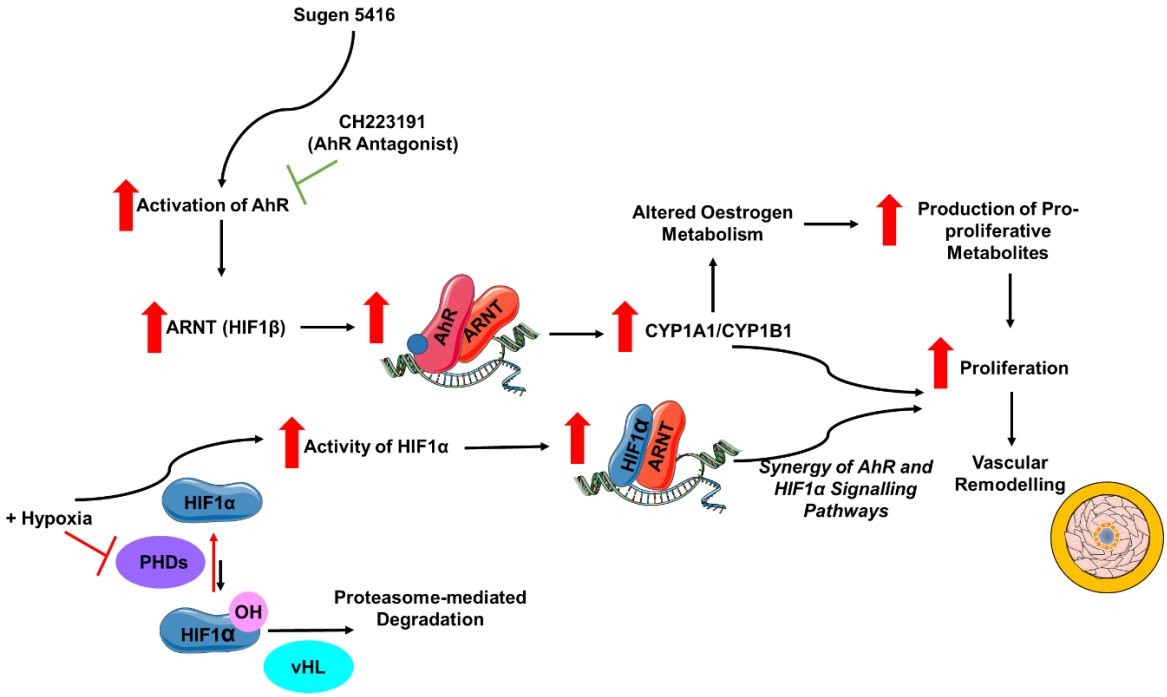


Figure 3–53: Proposed synergy of aryl hydrocarbon receptor and hypoxia-inducible factor 1α signalling pathways.

Herein we demonstrated the existence of a possible synergy of both insults in the establishment of the Su5416/Hx model. First, we provided data, that stimulation with Su5416 in female hPASMCs from PAH patients, results in increased localisation of AhR in the nucleus, indicating increased receptor activation. *In vivo* studies showed that the ARNT (HIF1β) protein levels are increased in diseased animals, and the levels of the protein are returned to normal by AhR antagonist CH223191. Second, we showed that exposure of hPASMCs derived from female PAH patients results in increased proliferation of these cells, while in Nx conditions proliferation was not significant. Hx may synergise with AhR activation through the ARNT (HIF1β). A simplified diagram of the proposed synergy is shown in Figure 3–53. Third, we provided evidence that in female hPASMCs stimulation with Su5416 results in increased expression of CYP1A1 and CYP1B1. And while exposure to AhR antagonist CH223191 only resulted in decreased expression of CYP1A1 in hPASMCs derived from female control subjects, it is anticipated that increased expression of these CYP enzymes might result in increased production of oestrogen metabolites promoting proliferation.

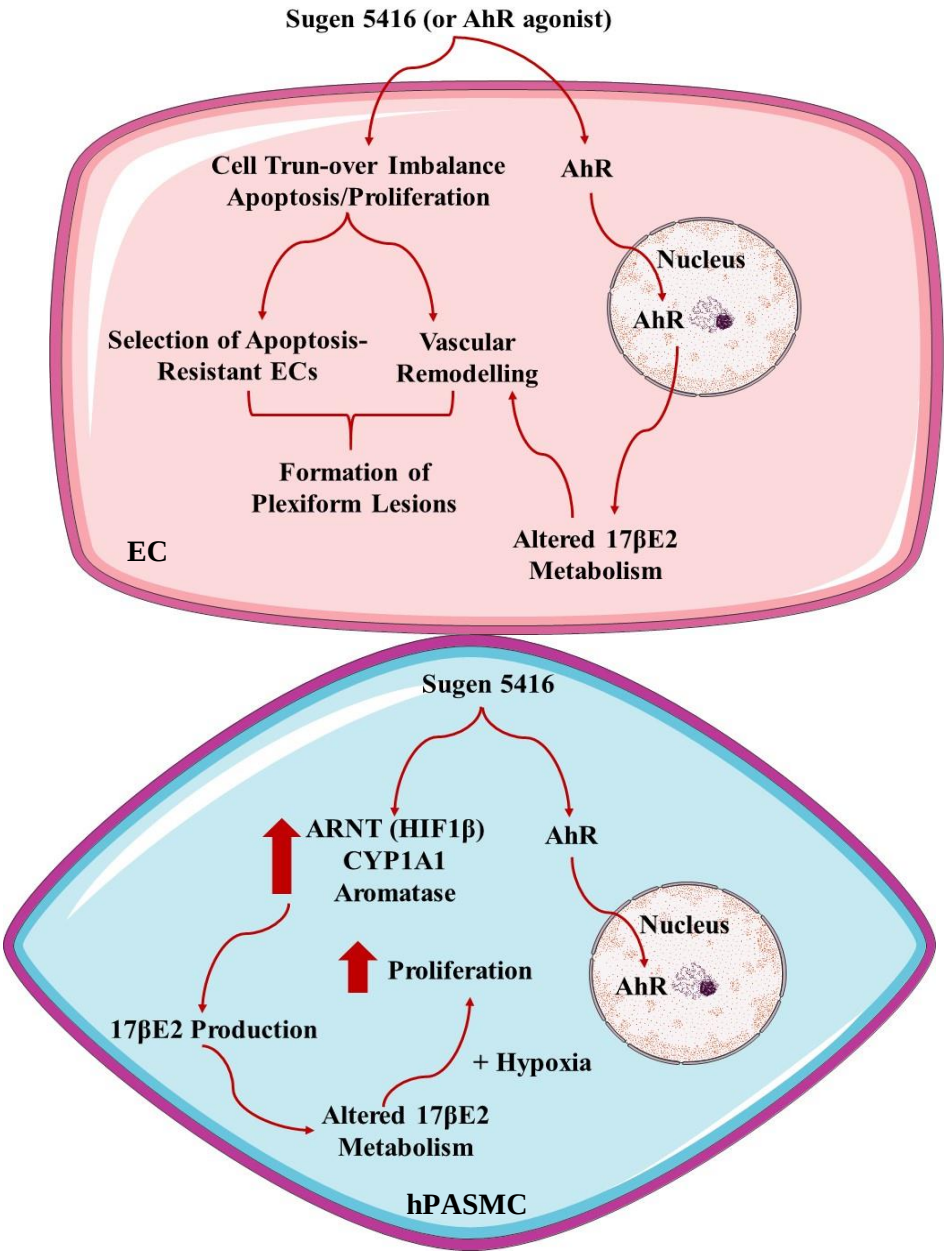


Figure 3–54: Proposed mechanism of Sugen 5416 and hypoxia-induced experimental phenotype of pulmonary arterial hypertension mediated through activation of the aryl hydrocarbon receptor.

In ECs, Su5416 causes an imbalance in cell turnover, favouring apoptosis of these cells. The insult result in vascular injury, leading to vascular remodelling and selection of apoptosis-resistant ECs. Furthermore, Su5416 activates AhR, leading to increased expression of oestrogen-metabolising enzymes, resulting in altered oestrogen metabolism. In SMCs, Su5416 leads to increased translocation of AhR from the cytoplasm to the nucleus, resulting in induced expression of ARNT (HIF1β), CYP1A1 and aromatase. Increased production of 17βE2 and altered oestrogen metabolism, through the synergy of AhR and HIF1α signalling pathways lead to increased proliferation of SMCs and vascular remodelling.

ECs= Endothelial cells, SMCs= Smooth muscle cells, AhR= Aryl hydrocarbon receptor, 17βE2= 17β-oestradiol, CYP1A1= Cytochrome P450 1A1

Chapter IV

4 An *In Vitro* Model to Investigate the Role of Oestrogen and Oestrogen Metabolism

4.1 Introduction

The importance of sex hormones in the pathogenesis of PAH was exposed when numerous large national registries and surveys repeatedly demonstrated that PAH affects predominantly women (Rich et al., 1987;Badesch et al., 2009;Escribano-Subias et al., 2012;Ling et al., 2012;Galiè et al., 2015). Therefore, much research was focused on discovering the causative mechanism by which oestrogen might possibly contribute to disease development in animal models and, most recently, in clinical setting. In animal models, disease susceptibility and severity were associated with abnormal synthesis of 17βE2, as shown in (Mair et al., 2014). It appeared that stimulation of vascular cells derived from female control subjects with 17βE2, suppresses the expression of final effectors of BMPR*II* signalling pathway (Mair et al., 2015). While Austin and colleagues reported that 17βE2 suppressed the expression of BMPR*II* receptor (Austin et al., 2012). Hence, increased levels of circulating hormone might contribute to establishment of conditions favouring SMC proliferation, leading to muscularisation and remodelling of distal PAs. The probability of clinical PAH in men, but not in women, was also shown to correlate with higher levels of endogenous 17βE2 (Ventetuolo et al., 2016). Increased level of endogenous 17βE2 and decreased levels of circulating sex hormone precursor DHEA-S in men were associated with more severe haemodynamic impairment and patient's lower tolerance of physical activity (Ventetuolo et al., 2016).

17βE2 is metabolised to numerous metabolites through oxidative biotransformation reactions, with most oestrogen-metabolising enzymes exhibiting high activity for hydroxylation at positions C2 and C4, and only moderate activity at position C16α. Evidence suggested that amongst most commonly produced hydroxylated oestrogens, only 16αOHE1 induced significant proliferation of PASMCs (White et al., 2012). White and colleagues provided evidence of dysregulation of oestrogen metabolism in PAH, reporting that the expression of CYP1B1 oestrogen-metabolising enzyme was increased in clinical PAH as well as in experimental models of PH (White et al., 2012). The anomalous metabolism of oestrogens, arising from pathogenic expression of oestrogen-metabolising enzymes, might therefore result in the accumulation of metabolites, capable of driving the pathological changes in the distal pulmonary vasculature due to their pro-proliferative properties. The hydroxylated oestrogens serve as precursors for conjugation reactions, resulting in metabolites that are easily excretable due to enhanced hydrophilic character and/or metabolically/pharmacologically inactive (Jancova et al., 2010). 2-Methoxyoestrogens have

been shown to mediate protective effects in terms of PAH pathogenesis, with demonstrated anti-mitotic and anti-angiogenic properties (Fotsis et al., 1994), further affecting the production of vasoactive compounds to favour vasodilation (Barchiesi et al., 2006;Seeger et al., 1999;Tsukamoto et al., 1998;Dubey et al., 2001). Administration of these metabolites resulted in protection against or reversal of haemodynamic impairment and vascular remodelling in animals with established PH phenotype (Tofovic et al., 2006;Tofovic et al., 2008b;Tofovic and Rafikova, 2009;Fotsis et al., 1994). It was previously suggested that the dysregulated oestrogen metabolism in disease setting might also include reduced formation of protective metabolites, due to the presence of enzyme isoform exhibiting decreased activity of MeOE formation and the presence of oestrogen-metabolising enzymes with increased activity (Kisselev et al., 2005). In such circumstances, the accumulation of hydroxylated oestrogens, which are normally rapidly metabolised to form methoxyoestrogens, could lead to formation of quinones and semiquinones, which due to their reactive character might result in increased production of ROS (Yager and Liehr, 1996) leading to protein as well as DNA damage (Cavalieri et al., 1997).

As discussed previously (in Chapter 3.1), each year millions of animals are used in research. According to the viewpoint of the Committee for Proprietary Medicinal Products, published in 1997, the ethical consideration requires the use of animals in research to be limited as much as possible. Approximately 30 years ago, Pearson has produced a perspective article on the possibility of *in vitro* models replacing the use of animals, especially in the assessment of drug toxicity and safety. Published work stated that *in vitro* studies might offer the advantage of producing relevant and precise results much more rapidly than animal studies (Pearson, 1986). Albeit, the use of animals in research might not be completely avoidable, the use of animal models to investigate the contribution of specific molecular processes to disease development is hindered by i) the lack of recapitulating the human condition, ii) the un-feasibleness of varying individual molecular processes to identify their contribution to the disease pathogenesis, and iii) incapability of measuring the responses to stimulation in real time (Benam et al., 2015). The *in vitro* model using hPASMCs was employed in the research of PAH pathology previously, however the role oestrogen metabolism in this model has not yet been fully investigated.

Although the primary biological functions of oestrogens are mediated in endocrine manner, the paracrine effects of oestrogens, due to high concentrations of locally produced hormones, might be importantly associated with pathological processes in numerous diseases (Simpson

and Davis, 2001;de Jong et al., 1997). Considering that emerging evidence points to the importance of individual oestrogen metabolites in the pathogenesis of PAH, the qualitative and quantitative assessment of the levels of oestrogen metabolites produced locally by the vascular cells to ascertain actual metabolomic profile of oestrogens in normal and diseased conditions is required. Currently, the available techniques to quantitatively measure oestrogen metabolites encompass various immunoassays and chromatographic techniques. Unfortunately, the use of these well-established techniques to determine the normal and diseased metabolic profile of oestrogens is hindered by i) poor reproducibility of results of immunoassays due to assay interferences (Tate and Ward, 2004), ii) the extremely low physiological concentration range of oestrogens, and iii) structural similarity of these compounds. Previously, significant efforts were made to establish a precise and sensitive measuring technique, using the high-performance liquid chromatography approach (HPLC) (Johansen, 2014), which has been successfully used to investigate oestrogen metabolism by several research groups (Rogan et al., 2003;Johansen et al., 2014;Delvoux et al., 2007). Nonetheless, the use of this technique to ascertain the metabolomic profile of oestrogens is hindered by i) difficult quantitation of oestrogen metabolites, ii) method's relatively high detection limit and low concentrations range of certain oestrogen metabolites, and iii) difficulty of peak identification, introducing the possible error of subjective analysis. According to Gaikwad, gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry have been extensively used to date for quantification of oestrogen metabolism (Gaikwad, 2013), with reported simultaneous detection and quantitation of oestrogen metabolites in tissue, urine and serum samples (Flores-Valverde and Hill, 2008;Dai et al., 2012;Carvalho et al., 2008). However the elaborate sample preparation methods and the requirement of analyte derivatisation, resulting in significant matrix effects and hence less robust assays (Ke et al., 2014), render these methods rather unsuitable.

For this study, the general hypothesis was that oestrogen metabolism is dysregulated in pulmonary arterial hypertension, and that the disparity observed in the incidence of this vasculopathy in women might be associated with increased production of oestrogen metabolites having pathogenic properties.

Aims of this chapter:

1) To assess the effects of oestrogen metabolites on the expression and activity of prostacyclin synthase in microvascular endothelial cells derived from control female subject.
2) To assess the effects of oestrogen metabolites on the expression of BMPR*II* signalling pathway in human pulmonary artery smooth muscle cells.
3) To assess the extent of anti-proliferative effects of 2-methoxyoestradiol in *in vitro* model of pulmonary hypertension consisting of pulmonary artery smooth muscle cells derived from control and patient subjects of both sexes.
4) To develop and validate a precise and sensitive liquid chromatography tandem mass spectrometry method to quantitatively assess the concentration of oestrogen metabolites in *in vitro* model of pulmonary hypertension consisting of pulmonary artery smooth muscle cells derived from control subjects and individuals with clinical pulmonary arterial hypertension.
5) Establishment of the oestrogen metabolic profile in *in vitro* model of pulmonary hypertension consisting of pulmonary artery smooth muscle cells derived from control and patient subjects of both sexes, using the novel quantitative technique.

4.2 Results

4.2.1 *2-Methoxyoestradiol and 2-Methoxyoestrone Significantly Increase Expression of Prostacyclin Synthase in Pulmonary Microvascular Endothelial Cells*

Sustained vasoconstriction is an important hallmark of PAH pathogenesis and is associated with dysregulated synthesis of vasoactive compounds. The circulating levels of endogenous vasodilator PGI_2 were reduced in patients (Moncada and Vane, 1978;Badesch et al., 2004;Barst et al., 2009); (Christman et al., 1992). PGIS is expressed in all vascular cells of proximal and distal PAs, with greater expression in the ECs (Badesch et al., 1989). Hx increased the expression of PGIS and release of PGI_2 in VSMC and ECs (Camacho et al., 2011). The effects of oestrogen metabolites on the release of PGI_2 were recently examined in human umbilical ECs (Jobe et al., 2013). Hydroxy and methoxy metabolites of oestradiol significantly increased the production of PGI_2 in pregnant and non-pregnant women, at physiological concentrations (Jobe et al., 2013). As the lung, is highly sensitive to oestrogens, we aimed to determine the effects of stimulation of ECs derived from pulmonary micro-vessels. Considering, the reported changes were rapid, we treated the hPMECs with 1nM oestrogen metabolites for 24 hours to assess whether these metabolites might affect the expression of PGIS. Herein, we showed that stimulation with oestrogen metabolites did not affect the expression of *PGIS* at gene level (Figure 4–1A). Nonetheless, significant increase in PGIS protein expression was caused by stimulation with 2-methoxyoestradiol and 2-methoxyoestrone (Figure 4–1B, C). 17βE2 and 4-methoxy metabolites caused an insignificant increase in PGIS expression at protein level. Equally, 2- and 4OHE2 had no effect on the expression of PGIS at protein level.

1nM concentration of oestrogen metabolites was selected for these experiments, as this was approximately close to the average reported physiological concentrations of oestrone and oestradiol. Furthermore, as currently the physiological plasma concentrations of other prominent oestrogen metabolites are not known, we concluded the estimate of oestrone, and oestradiol concentrations would be a good approximation.

We further wished to assess the release of PGI_2 in these cells to determine whether increased expression of PGIS might be associated with increased release of its product. We used 6-keto PGF1α immunoassay to determine the level of stable PGI_2 metabolite. Acute stimulation with oestrogen metabolites led to significantly decreased expression of 6-keto PGF1α when cells were treated with 16αOHE2 and 2MeOE1 (Figure 4–2A), indicating decreased release of its parental molecule PGI_2. The release of 6-keto PGF1α in positive control group, where cells were treated with 20μM arachidonic acid, was like that observed in treatment groups 16αOHE2, 2MeOE1 and 2MeOE2. Prolonged treatment with oestrogen metabolites though did not result in significant changes in the release of PGI_2 as assessed per 6-keto PGF1α levels (Figure 4–2B). Prolonged stimulation with 20μM arachidonic acid resulted in significantly increased production of 6-keto PGF1α, indicating the production of PGI_2 was also increased in this instance (Figure 4–2B).

4.2.2 *2-Methoxyoestradiol Increases Expression of Prostacyclin Synthase in Pulmonary Artery Smooth Muscle Cells from Male Control Subjects, but not in Female Cells*

Although the PGI_2 production has only been documented in ECs, the expression of PGIS has been also reported in vascular SMCs (Nakayama, 2005). As we have previously shown that certain oestrogen metabolites affect the expression of PGIS in hPMECs (Chapter 4.2.1), we wanted to investigate whether oestrogens also affect the expression of PGIS in hPASMCs. Herein, we show that the expression of PGIS is only significantly increased by 100nM 2MeOE2 in hPASMCs derived from male control subjects (Figure 4–3), while no change was observed in female cells (Figure 4–4). It appears that PGIS expression in PASMCs is only affected at higher pharmacological concentrations of oestrogens compared to PMECs, as significant changes were observed with 100nM and 1nM 2MeOE2, respectively. Moreover, oestrogen-induced changes in the expression of PGIS in PASMCs seem to be sex-dependent, with female hPASMCs not responding to oestrogen treatment.

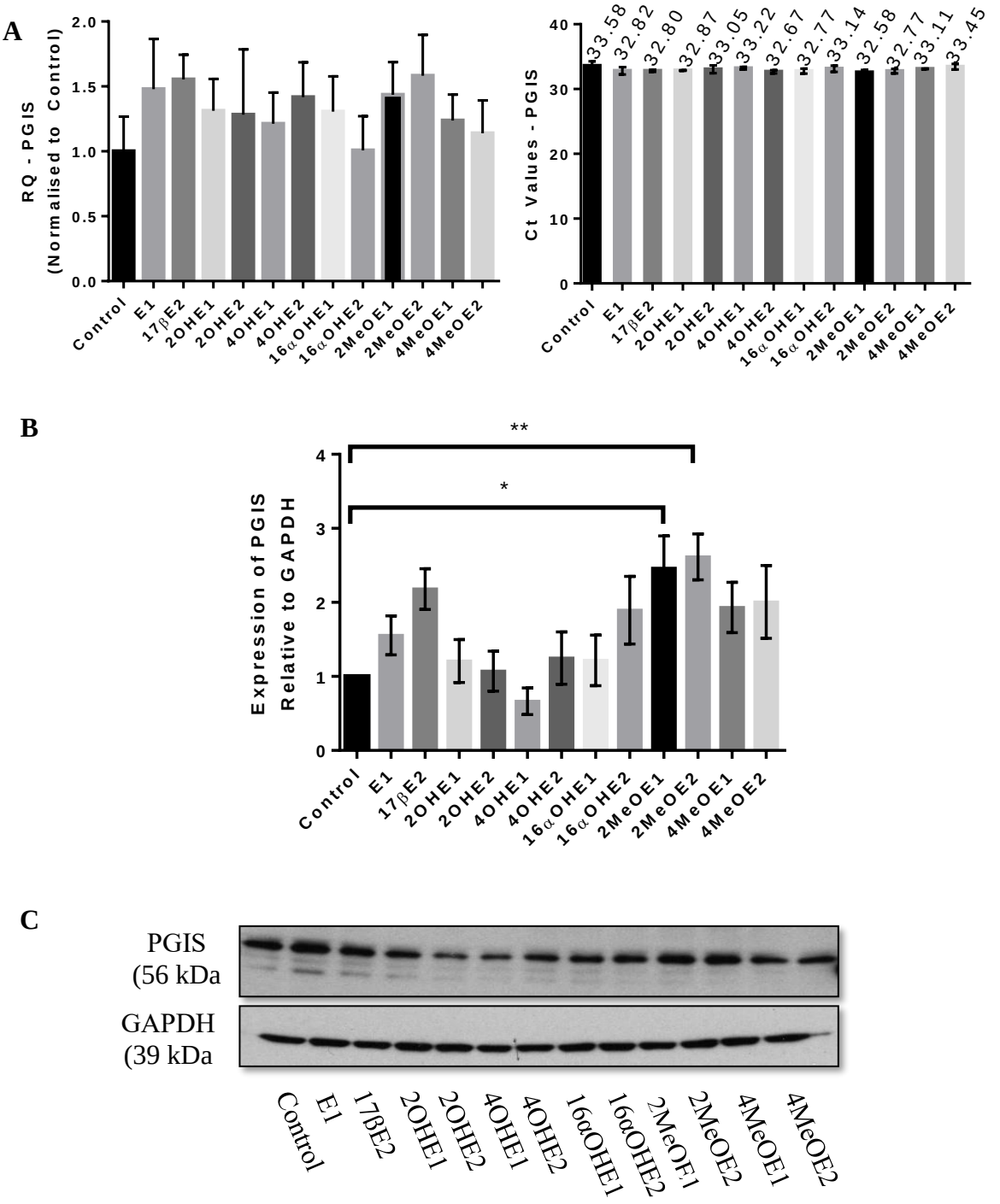
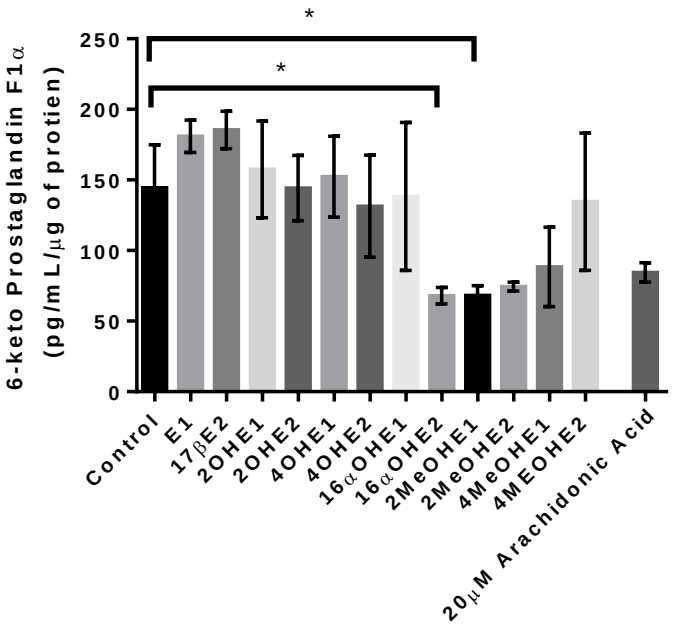


Figure 4–1: The protein expression of prostacyclin synthase in human microvascular endothelial cells derived from female control subjects is significantly increased by 1nM methoxyoestrogens.

Human pulmonary microvascular endothelial cells (hPMECs) derived from female control, purchased from Qiagen, were grown as previously described to approximately 80% confluence. Before stimulation with oestrogen metabolites (1nM) for 24 hours, cells were washed twice with ice-cold DPBS. (A) The expression of *PGIS* was assessed by Taqman quantitative Real Time-PCR. Results were normalised to *β-2-microglobulin*. N=3 per group in triplicate, One-way ANOVA followed by Tukey's post-hoc test. Data is expressed as $RQ \pm \frac{RQ_{max}}{RQ_{min}}$, where RQ=Relative quantification. (B) The level of PGIS protein expression was addressed using immunoblotting. N=3–6 per group in duplicate, *p<0.05, **p≤0.01, One-way ANOVA followed by Tukey's post-hoc test. Data is expressed as fold change to Control group±SEM. The representative immunoblotting image is shown in (C).

A



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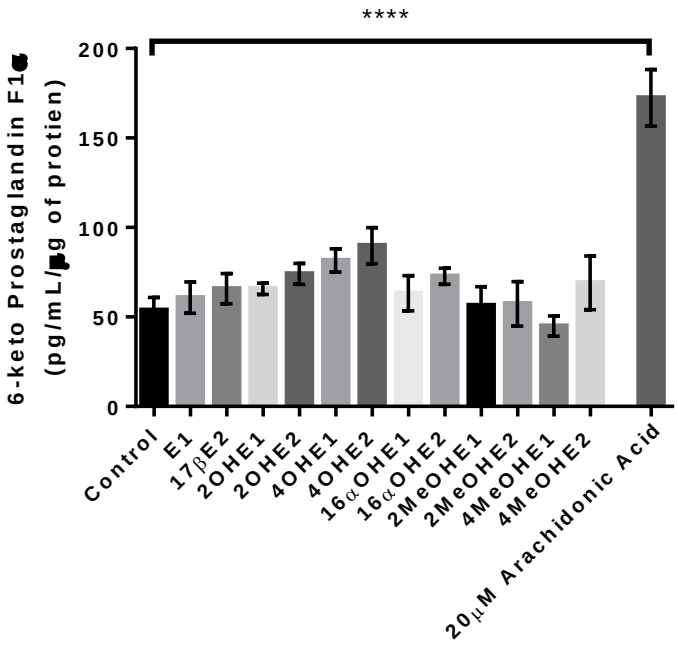


Figure 4–2: Acute stimulation with 1nM methoxyoestrogens decreases the release of prostacyclin from human microvascular endothelial cells.

Human pulmonary microvascular endothelial cells (hPMECs) derived from female control subjects, purchased from Qiagen, were grown as previously described to approximately 80–90% confluence. Before stimulation with oestrogen metabolites (1nM) for 1 hour (A) or 24 hours (B), cells were washed twice with ice-cold DPBS. The release of PGI_2 was assessed using 6-keto PGF1α ELISA Kit (Enzo Life Sciences, USA). Results were normalised to protein amount. N=3 per group in duplicate, ****p≤0.0001One-way ANOVA followed by Tukey's post-hoc test. Data is expressed as mean±SEM.

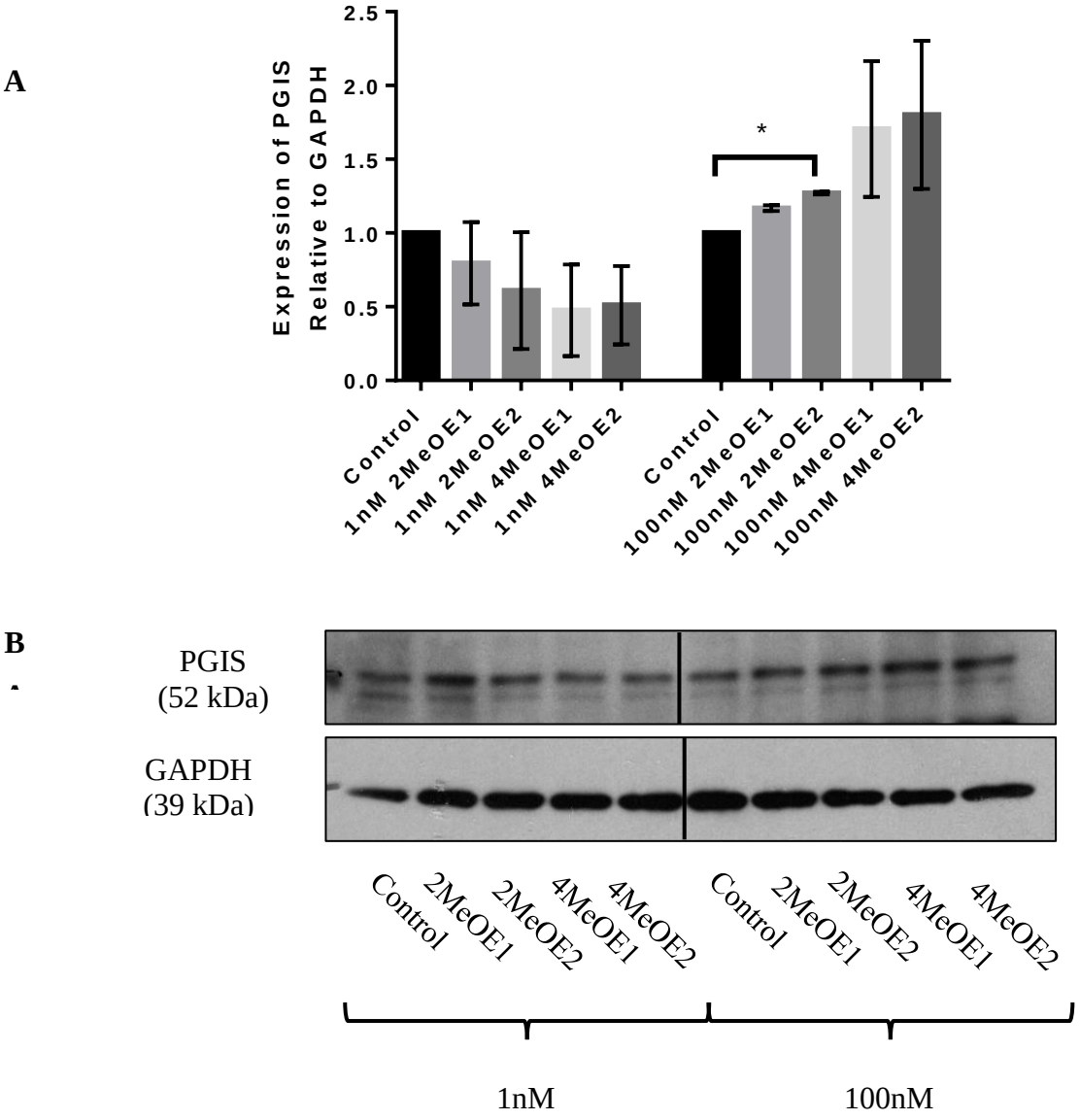


Figure 4–3: The protein expression of prostacyclin synthase is increased by 100nM 2-methoxyoestradiol in human pulmonary artery smooth muscle cells derived from male controls.

HPASMCs were grown to reach approximately 70% confluence in 10% FBS DMEM media. PASMCs were then serum starved for 24 hours, before stimulation with oestrogen metabolites (1nM, 100nM). Protein samples were collected and prepared as previously described. N=3 per group, in duplicate, *p<0.05, One-Way ANOVA, followed by Tukey's post-hoc test. Data is expressed as fold change of Control group±SEM Representative immunoblotting image is shown in (B).

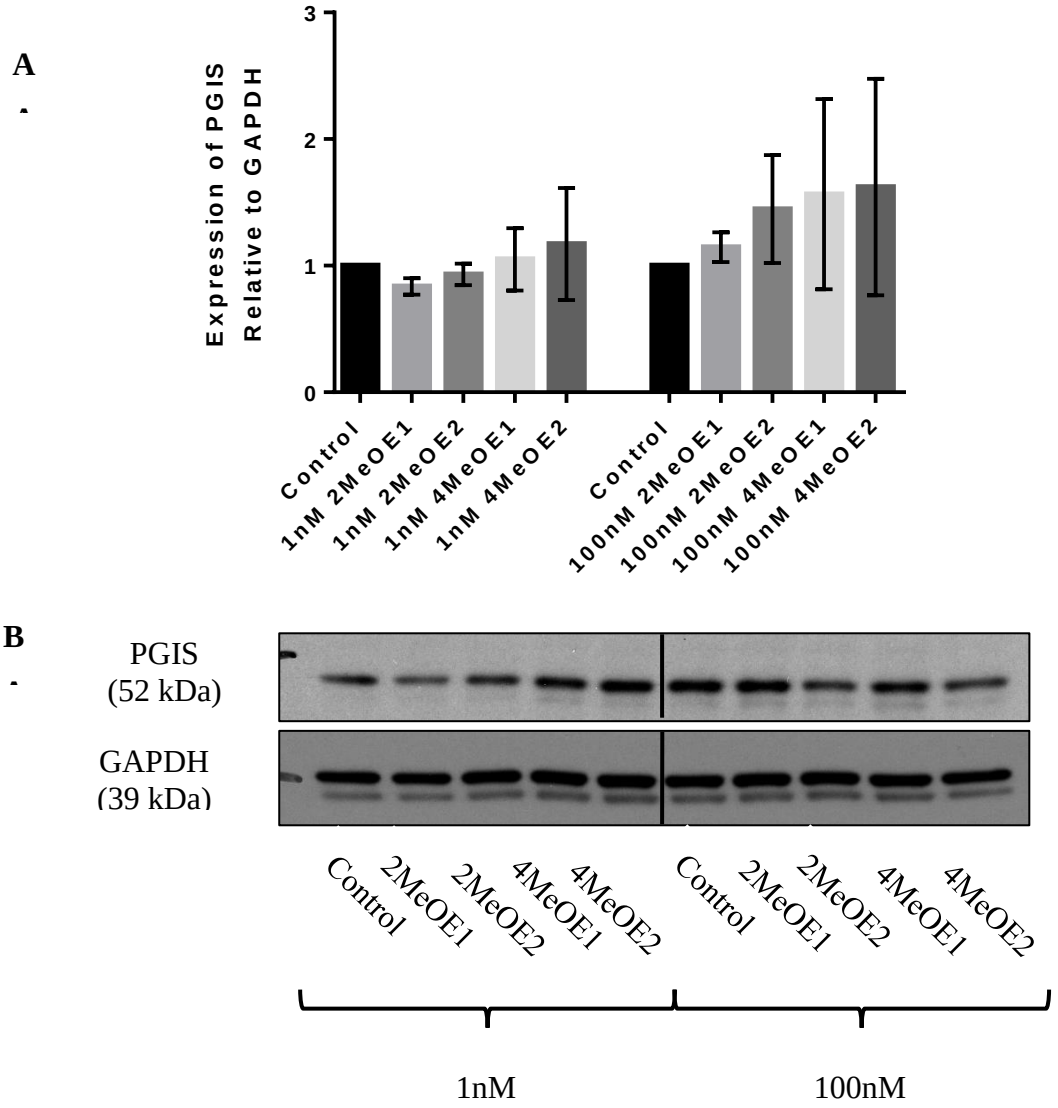


Figure 4–4: Methoxyoestrogens do not affect the protein expression of prostacyclin synthase in human pulmonary artery smooth muscle cells derived from female controls. HPASMCs were grown to reach approximately 70% confluence in 10% FBS DMEM media. PASMCs were then serum starved for 24 hours, before stimulation with oestrogen metabolites (1nM, 100nM). Protein samples were collected and prepared as previously described. N=3 per group, in duplicate, One-Way ANOVA, followed by Tukey's post-hoc test. Data is expressed as fold change of Control group±SEM. Representative immunoblotting image is shown in (B).

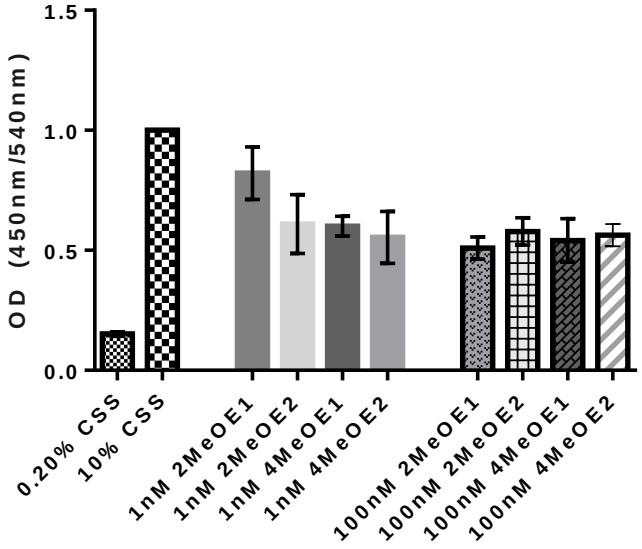
4.2.3 *Exposure to 2-Methoxyoestradiol Reduces Proliferation in Human Pulmonary Artery Smooth Muscle Cells of Both Sexes*

2-MeOE2 was shown to prevent and/or rescue the experimental PH phenotype in animal models (Fotsis et al., 1994;Tofovic et al., 2006;Tofovic et al., 2008b). As the protective properties of methoxy-oestradiol are partially mediated by their anti-proliferative effects on vascular cells (Barchiesi et al., 2006), we wished to assess these in *in vitro* models using cell lines derived from control subjects, which were subjected to pro-proliferative conditions.

Here we showed that exposure to physiological concentration of 2MeOE2 results in a trend of reduced proliferation in male hPASMCs derived from control subjects, as assessed by BrDU assay (Figure 4–5A). Equally, exposure to MeOEs reduced serum-induced proliferation in female hPASMCs from controls (Figure 4–6). At higher pharmacological concentrations all MeOEs seemed to diminish cell proliferation induced in the presence of 10% CSS. When hPASMCs were treated with inhibitors of ERα, ERβ and gpr30 receptors through which oestrogens mediate their effects, inhibition of all three receptors appeared to restore proliferation compared to 2MeOE2 only treated cells (Figure 4–7). Whilst inhibition of gpr30 had no effect on diminished proliferation when cells were treated with 4MeOE2, inhibition of both classical oestrogen receptors resulted in increased proliferation of cells despite treatment with 4MeOE2 (Figure 4–7). It therefore appeared that the anti-proliferative effects of 2MeOE2 and 4MeOE2 are mediated through ERα and β, while only 2MeOE2 might also affect proliferation through non-classical oestrogen signalling. These trends were also observed with BrDU assay (Figure 4–8), although more cell lines would need to be tested.

In female control cell lines, treatment with 2MeOE2 and 4MeOE2 resulted in significantly decreased serum-induced proliferation (Figure 4–9). Pre-exposure to ERα and ERβ antagonists resulted in observable trend of apparently increased proliferation, while the antagonism of gpr30 had no effect. Moreover, in these cell lines exposure to 2MeOE2 appeared to induce a concentration-dependent increase in the p27/Kip1 protein expression (Figure 4–10), showing this could be a potential mechanism through which 2MeOE2 protective properties are mediated.

A



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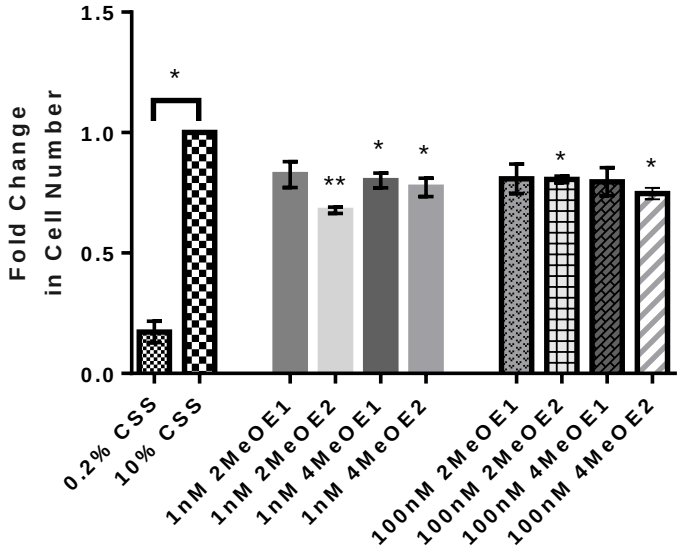


Figure 4–5: The anti-proliferative effects of methoxyoestrogens in male pulmonary artery smooth muscle cell lines derived from control subjects.

HPASMCs from male control subjects were serum starved for 24 hours. PASMCs were then stimulated by methoxyoestrogens (2MeOE1, 2MeOE2, 4MeOE1 and 4MeOE2) at physiological and pharmacological concentrations (1nM, 100nM). Experiments were carried out in the presence of 10% CSS PRF DMEM media. Cell counts were assessed after 72 hours by BrDU assay (A) or haemocytometer (B). (A) N=2 per group, repeated 8 times, One-Way ANOVA with Tukey's post-hoc test. Data expressed as mean±SEM. (B) N=3 per group, in triplicate, *$p<0.05$, **$p\leq0.01$, One-Way ANOVA with Tukey's post-hoc test. Data expressed as fold change of 10% control group±SEM.

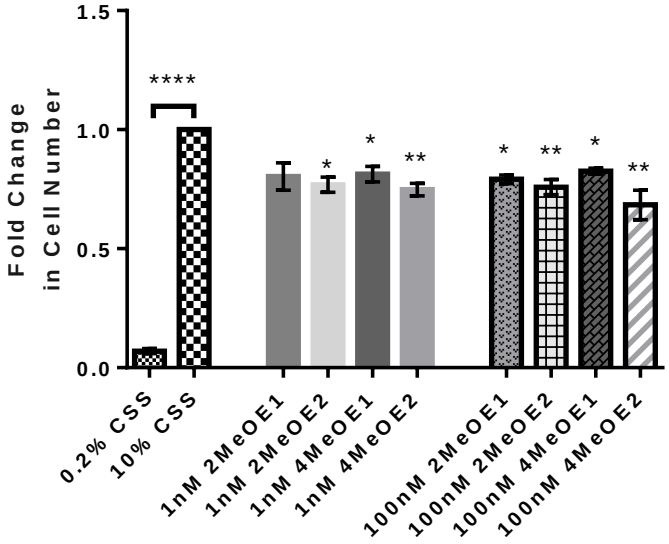


Figure 4–6: The anti-proliferative effects of methoxyoestrogens in female pulmonary artery smooth muscle cell lines derived from control subjects.

HPASMCs from male control subjects were serum starved for 24 hours. PASMCs were then stimulated by methoxyoestrogens (2MeOE1, 2MeOE2, 4MeOE1 and 4MeOE2) at physiological and pharmacological concentrations (1nM, 100nM). Experiments were carried out in the presence of 1% CSS PRF DMEM media. Cell counts were assessed after 72 hours by haemocytometer. N=4 per group, in triplicate, *$p<0.05$, **$p\leq0.01$, ****$p<0.0001$, One-Way ANOVA with Tukey's post-hoc test. Data expressed as fold change of 10% control group±SEM.

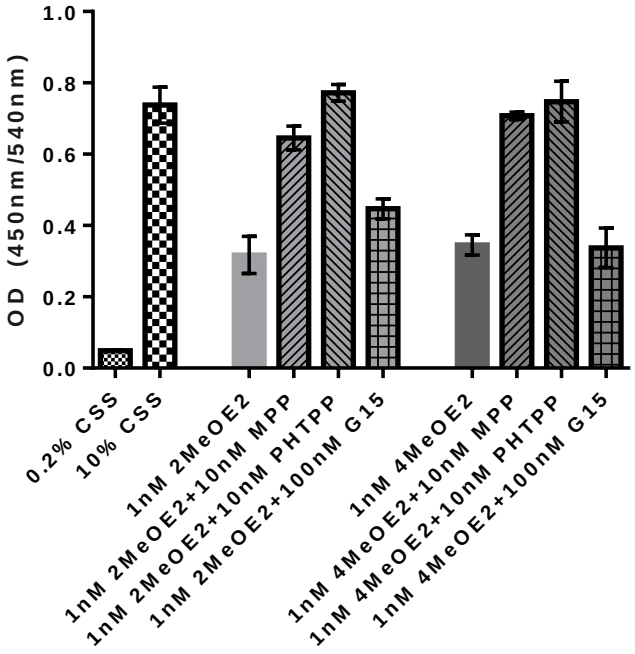


Figure 4–7: Inhibition of ERα and ERβ appears to restore proliferation of male control human pulmonary artery smooth muscle cells treated with 2- and 4-methoxyoestradiol.

HPASMCs from male control subjects were serum starved for 24 hours. PASMCs were then stimulated by ERα inhibitor (MPP, 10nM), ERβ inhibitor (PHTPP, 10nM), and gpr30 receptor (G15, 100nM) 30 minutes prior to stimulation with methoxyoestrogens (2MeOE2 and 4MeOE2) at physiological concentrations (1nM). Experiments were carried out in the presence of 10% CSS PRF DMEM media. Cell proliferation were assessed after 72 hours by BrDU assay. N=1 per group, repeated 4 times. Data expressed as mean±SEM.

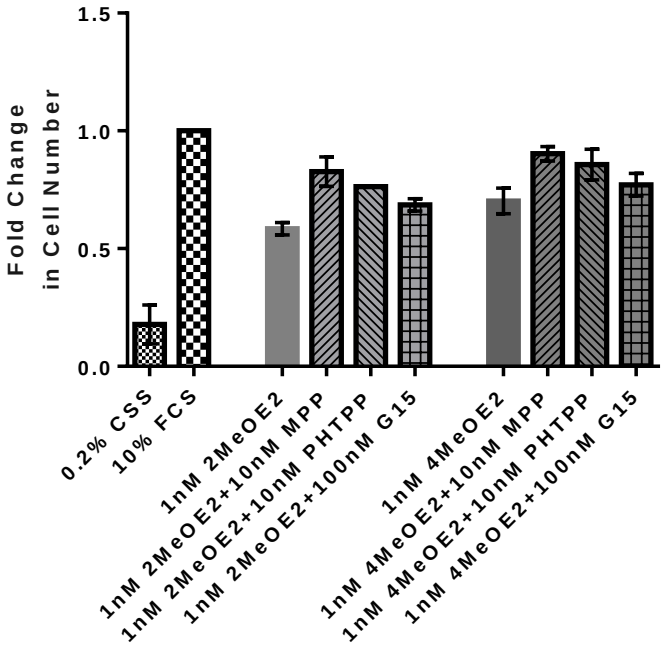


Figure 4–8: The mechanism of anti-proliferative properties of 2-methoxyoestradiol and 4-methoxyoestradiol in male human pulmonary artery smooth muscle cells derived from male control subject.

HPASMCs from male control subjects were serum starved for 24 hours. PASMCs were then stimulated by ERα inhibitor (MPP, 10nM), ERβ inhibitor (PHTPP, 10nM), and gpr30 receptor (G15, 100nM) 30 minutes prior to stimulation with methoxyoestrogens (2MeOE2 and 4MeOE2) at physiological concentrations (1nM). Experiments were carried out in the presence of 10% CSS PRF DMEM media. Cell proliferation were assessed after 72 hours by haemocytometer. N=2 per group, in triplicate, One-Way ANOVA with Dunnett's post-hoc test. Data expressed as fold change of 10% CSS group±SEM.

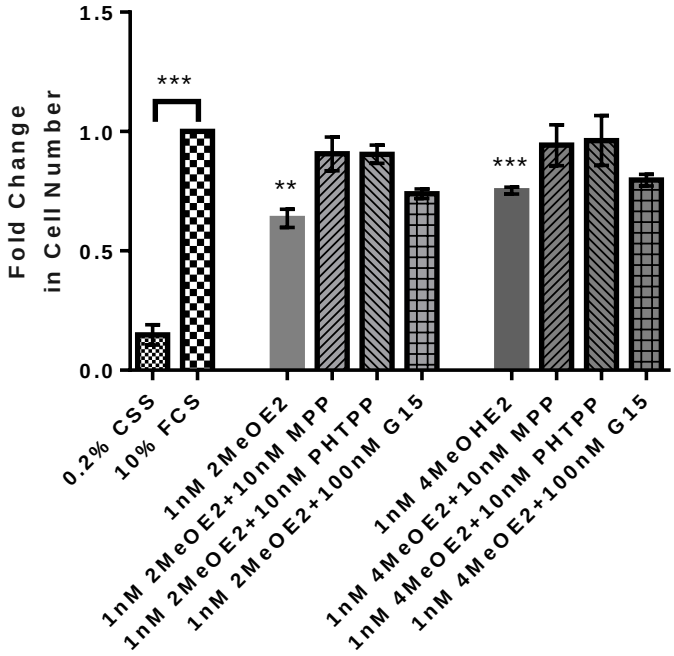


Figure 4–9: 2- and 4-methoxyoestradiol reduce serum-induced proliferation in female control pulmonary artery smooth muscle cells.

HPASMCs from male control subjects were serum starved for 24 hours. PASMCs were then stimulated by ERα inhibitor (MPP, 10nM), ERβ inhibitor (PHTPP, 10nM), and gpr30 receptor (G15, 100nM) 30 minutes prior to stimulation with methoxyoestrogens (2MeOE2 and 4MeOE2) at physiological concentrations (1nM). Experiments were carried out in the presence of 10% CSS PRF DMEM media. Cell proliferation were assessed after 72 hours by haemocytometer. N=3–4 per group, in triplicate, One-Way ANOVA with Dunnett's post-hoc test. Data expressed as fold change of 10% CSS group±SEM.

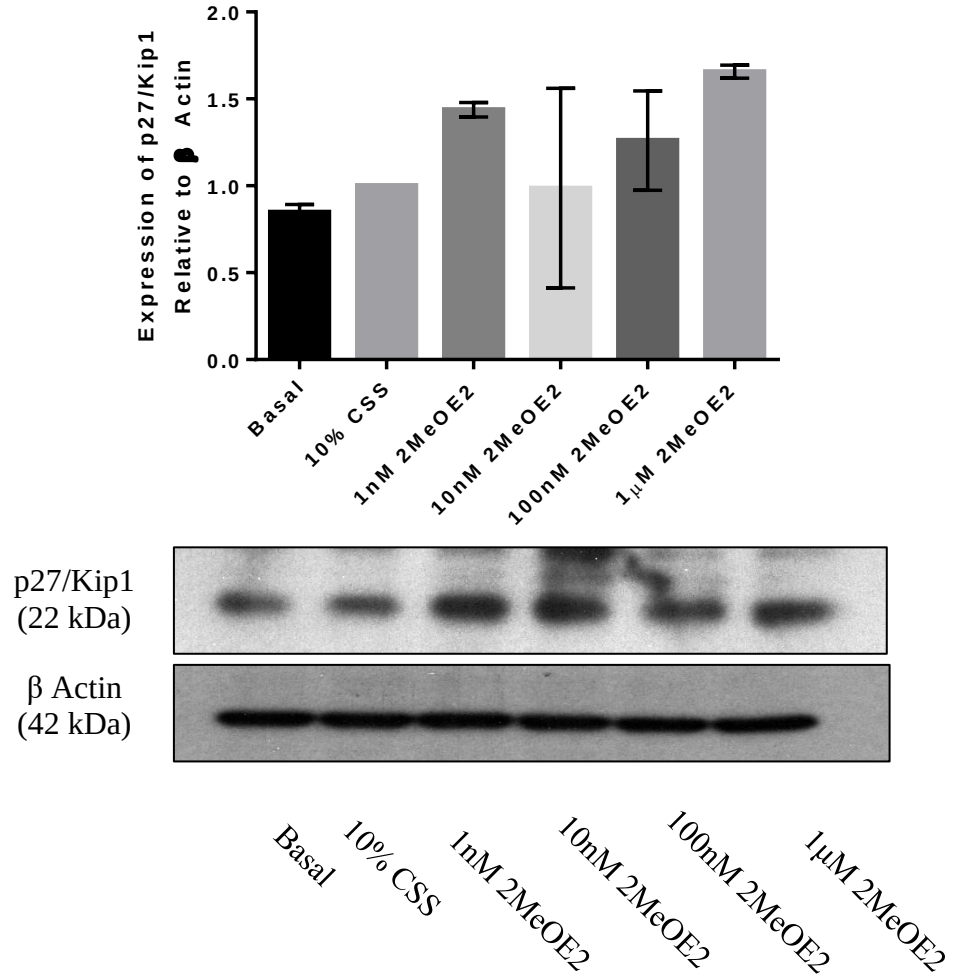


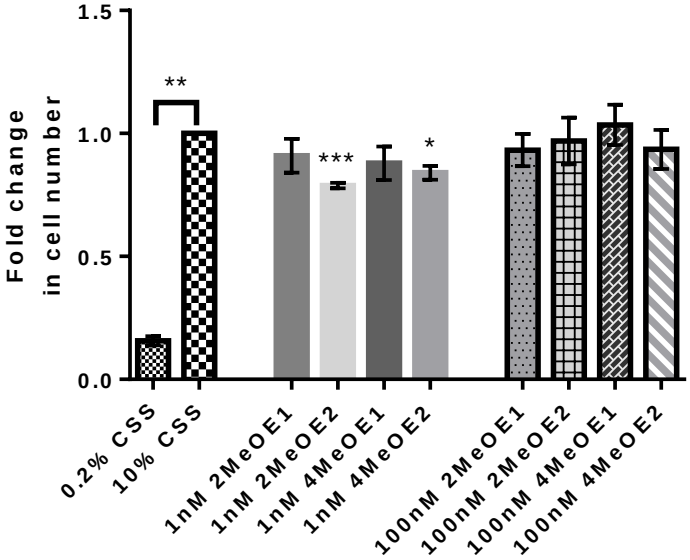
Figure 4–10: In human pulmonary artery smooth muscle cells from female control subjects, 2-mtehodyoestradiol induces protein expression of the cell cycle inhibitory protein p27/Kip1.

HPASMCs were quiesced for 24 hours in 0.5% CSS PRF DMEM media. PASMCs were then stimulated by 2MeOE2 (1nM, 10nM, 100nM and 1μM) in the presence of 10% CSS PRF DMEM. N=2 per group, in duplicate, One-Way ANOVA with Bonferroni's post-hoc test. Data is expressed as fold-change of 10% CSS group±SEM. The 10% CSS was set to 1, and other treatment groups are shown as fold change.

4.2.4 *2-Methoxyoestradiol Reduces Proliferation in Female, but not in Male Patient Pulmonary Artery Smooth Muscle Cells*

Evidence up to date, indicates that in animal models, administration of 2MeOE2 opposed the onset of experimental PH phenotype, while in diseased animals the metabolite improves the haemodynamic parameters and improved pulmonary vascular remodelling (Tofovic et al., 2008b). Therefore, we aimed to assess the documented anti-proliferative properties of 2MeOE2 in PAH *in vitro* model using cell lines derived from subjects with clinical PAH. Stimulation with 1nM 2MeOE2 and 4MeOE2 resulted in significant reduction of proliferation in female cell lines derived from patients (Figure 4–11A), while we observed no changes in male cell lines (Figure 4–11B). Moreover, it appeared that the metabolites had significant effects only at lower physiological concentrations.

A



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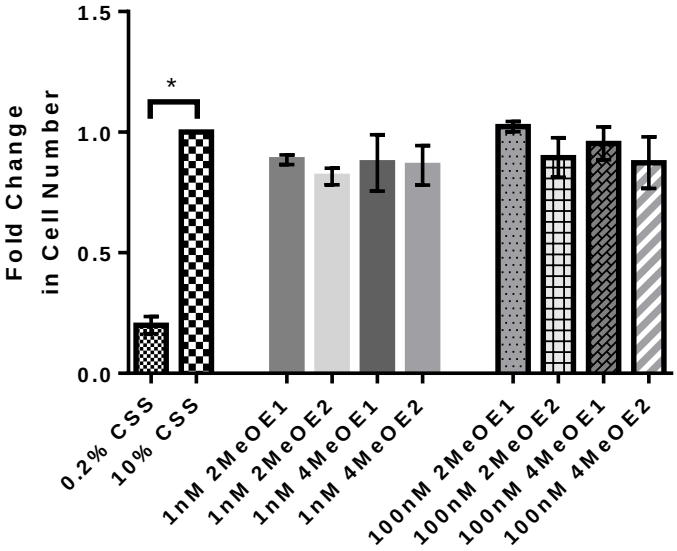


Figure 4–11: Methoxyoestrogens do not attenuate serum-induced proliferation in male patient pulmonary artery smooth muscle cells.

HPASMCs from female (A) and male (B) clinical patient subjects were serum starved for 24 hours. PASMCs were then stimulated by 2MeOE1, 2MeOE2, 4MeOE1 and 4MeOE2 (1nM, 100nM). Experiments were carried out in the presence of 10% CSS PRF DMEM media. Cell counts were assessed after 72 hours by haemocytometer. N=3–4 per group, in triplicate, *p≤0.05, **p<0.01, ***p<0.001, One-Way ANOVA with Dunnett's post-hoc test. Data expressed as fold change of 10% control group±SEM.

4.2.5 *In Female Control Pulmonary Artery Smooth Muscle Cells Only Methoxyoestrogens Appear to Increase the Expression of the BMPRII Signalling Pathway*

The protective effects of oestradiol in PAH could partially be attributed to the formation of protective MeOEs, which were shown to oppose the onset of experimental PH in animal models (Tofovic et al., 2006;Tofovic and Rafikova, 2009). 2MeOE2 decreased proliferation of vascular cells (Dubey et al., 1998;Tofovic and Rafikova, 2009;Vijayanathan et al., 2006), albeit experimental conditions and cellular type appear to be of noteworthy importance (Lippert et al., 2000;Lai and Law, 2004). As the arrest of proliferation of vascular cells seems to be one of the most important protective effects of 2MeOE2 in PAH, we aimed to assess the effects of 15 oestrogen metabolites, including methoxy-oestradiols, on the expression of mediators of the BMPR*II* signalling pathway in female hPASMCs derived from control subjects.

A trend of increased expression of BMPR*II*, pSmad1/5/(8)9 and Id3 at protein level was observed when cells were treated with MeOEs, however this increase was not statistically significant due to large variations in the response of the cell lines used (Figure 4–12). Therefore, we decided to further evaluate the effects of methoxyoestrogens at physiological and pharmacological concentrations, in hPASMCs of both sexes.

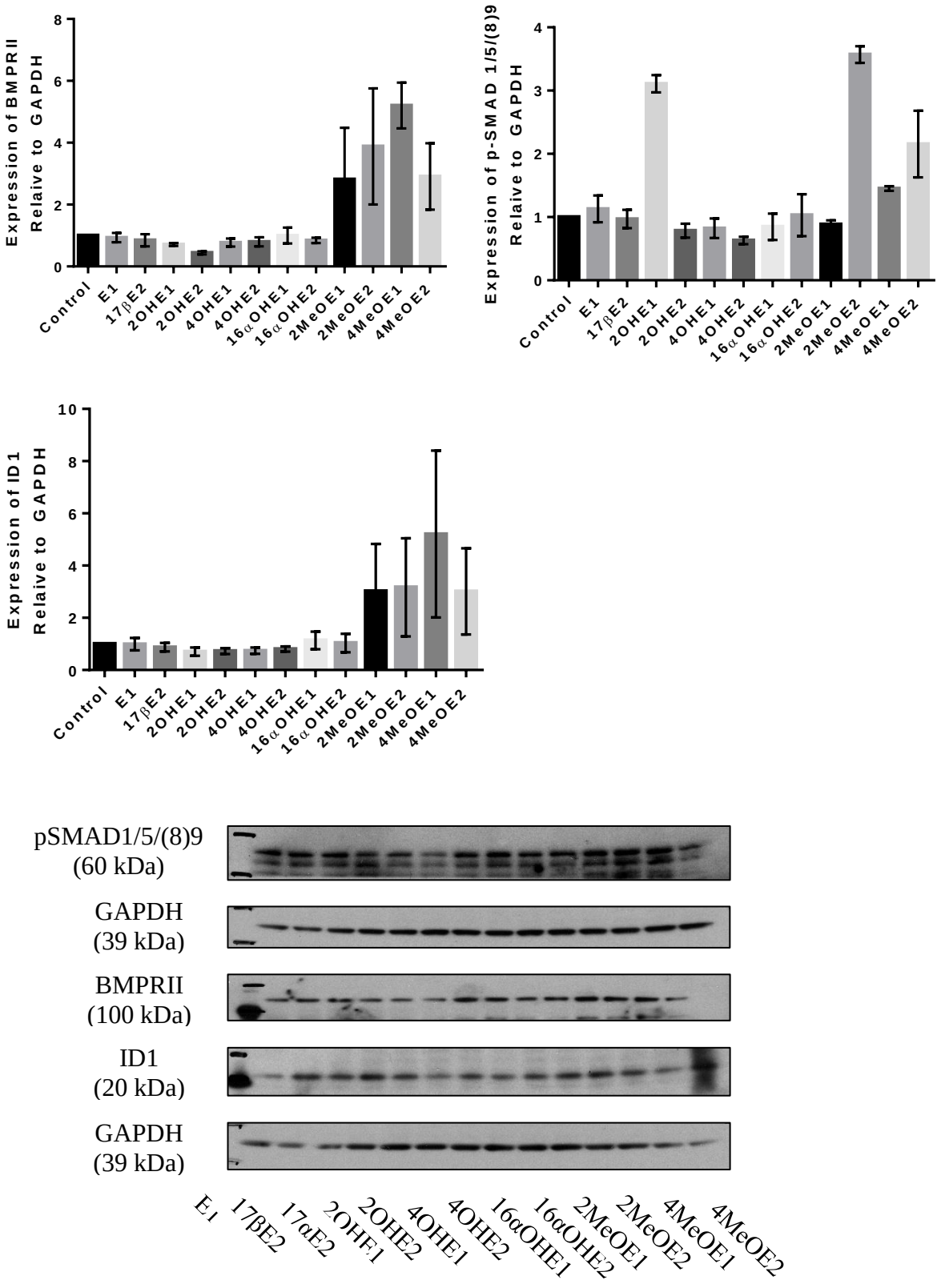


Figure 4–12: Methoxyoestrogens appear to increase the protein expression of effectors involved in the BMPRII signalling pathway in female control pulmonary artery smooth muscle cells.

HPASMCs were quiesced for 24 hours in 0.5% CSS PRF DMEM media. PASMCs were then stimulated by oestrogen metabolites (1nM) in the presence of 1% CSS PRF DMEM. The expression level of BMPRII, pSmad1/5/(8)9 was assessed using immunoblotting. Results were normalised to the GAPDH. N=3 per group, in duplicate, One-Way ANOVA with Bonferroni's post-hoc test. Data is expressed as fold change of Control group±SEM.

4.2.6 *2-Methoxyoestradiol Significantly Increased Expression of DNA-binding protein inhibitor Id-1 in Male but not in Female Control Pulmonary Artery Smooth Muscle Cells*

The mutations in the gene encoding the *BMPRII* receptor usually result in reduced receptor expression and/or activity (Atkinson et al., 2002). Although these mutations are present in most patients with HPAH, and in approximately 20–25% of patients with IPAH (Atkinson et al., 2002;Machado et al., 2009;Hamid et al., 2009), the low penetrance of the disease in these individuals indicates a second factor is required for disease onset. The basal expression of proteins involved in the BMPR*II* signalling pathway was significantly reduced in hPASMCs derived from female control subjects compared to males (Mair et al., 2015). Additionally, 17βE2 decreased the expression of DNA-binding protein inhibitor *Id1* and *Id3* in male control cells (Mair et al., 2015). The suppressive effects that 17βE2 exerted on the expression of BMPR*II* receptor were mediated through ERα receptor (Austin et al., 2012).

As the anti-proliferative properties of 2MeOE2 are well documented, and the metabolite was shown to oppose the onset of experimental PH in animal models, we wished to assess the effects of methoxy metabolites on expression of proteins involved in BMPR*II* signalling pathway, at a physiological and pharmacological concentrations.

Treatment with MeOEs did not affect the protein expression levels of BMPR*II* and pSmad/5/(8)9 in hPASMCs from control subjects of both sexes (Figure 4–13 and Figure 4–14). Exposure of male hPASMCs from control subjects to pharmacological concentration of 2MeOE2 resulted in increased expression of Id1, while Id3 expression was unaffected (Figure 4–13). In female cell lines, treatment with MeOEs did not result in significantly altered expression of Id1 and Id3 (Figure 4–14).

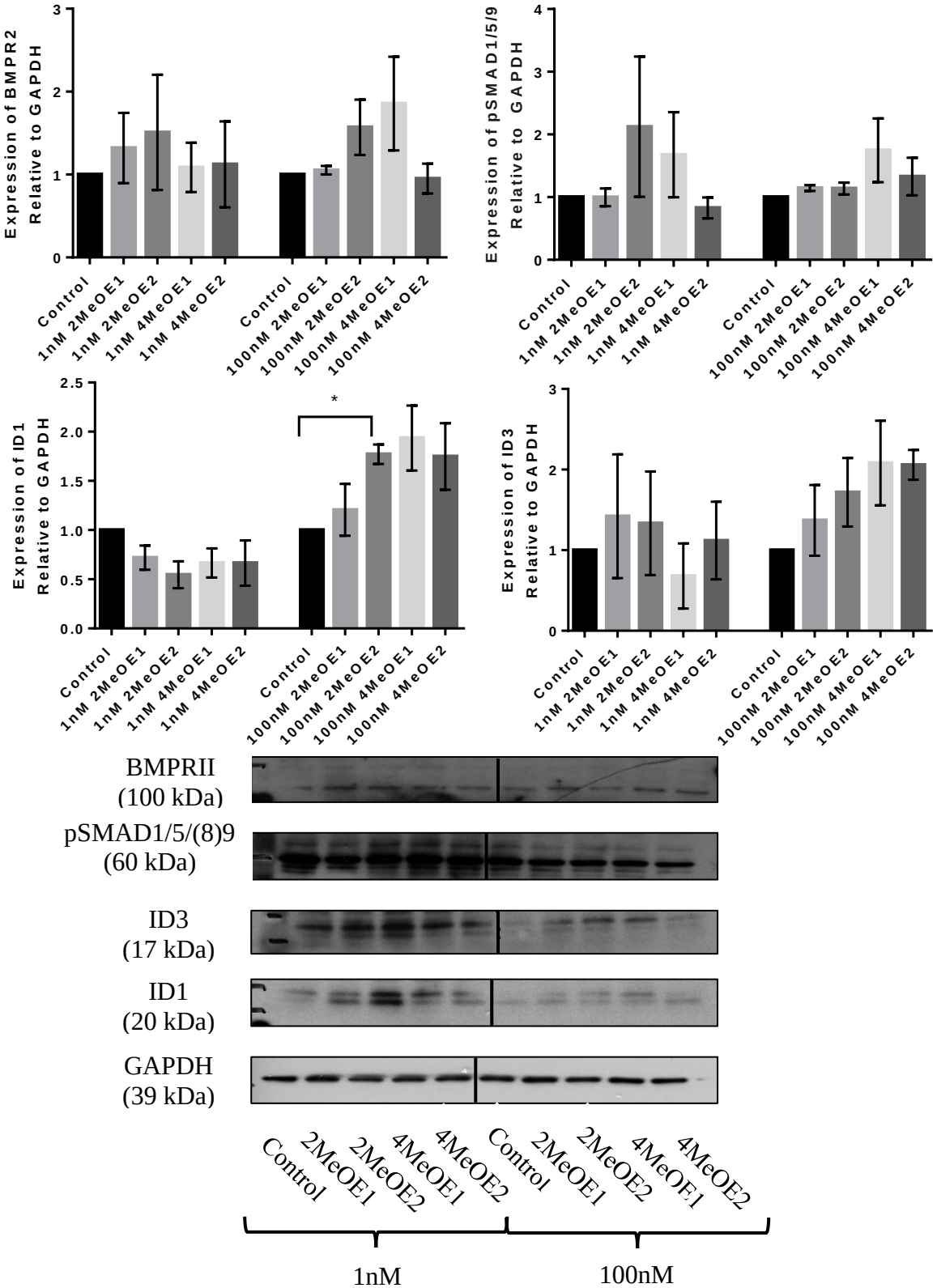


Figure 4–13: 2-Methoxyoestradil increases the protein expression of DNA-binding protein 1 in male control pulmonary artery smooth muscle.

HPASMCs were quiesced for 24 hours in 0.5% CSS PRF DMEM media. PASMCs were then stimulated by oestrogen metabolites (1nM, 100nM) in the presence of 1% CSS PRF DMEM. The expression level of BMPRII, pSmad1/5/(8)9, ID1 and ID3 was assessed using immunoblotting. Results were normalised to the GAPDH. N=3 per group, in duplicate, *p<0.05, One-Way ANOVA with Bonferroni's post-hoc test. Data is expressed as fold change of Control group±SEM.

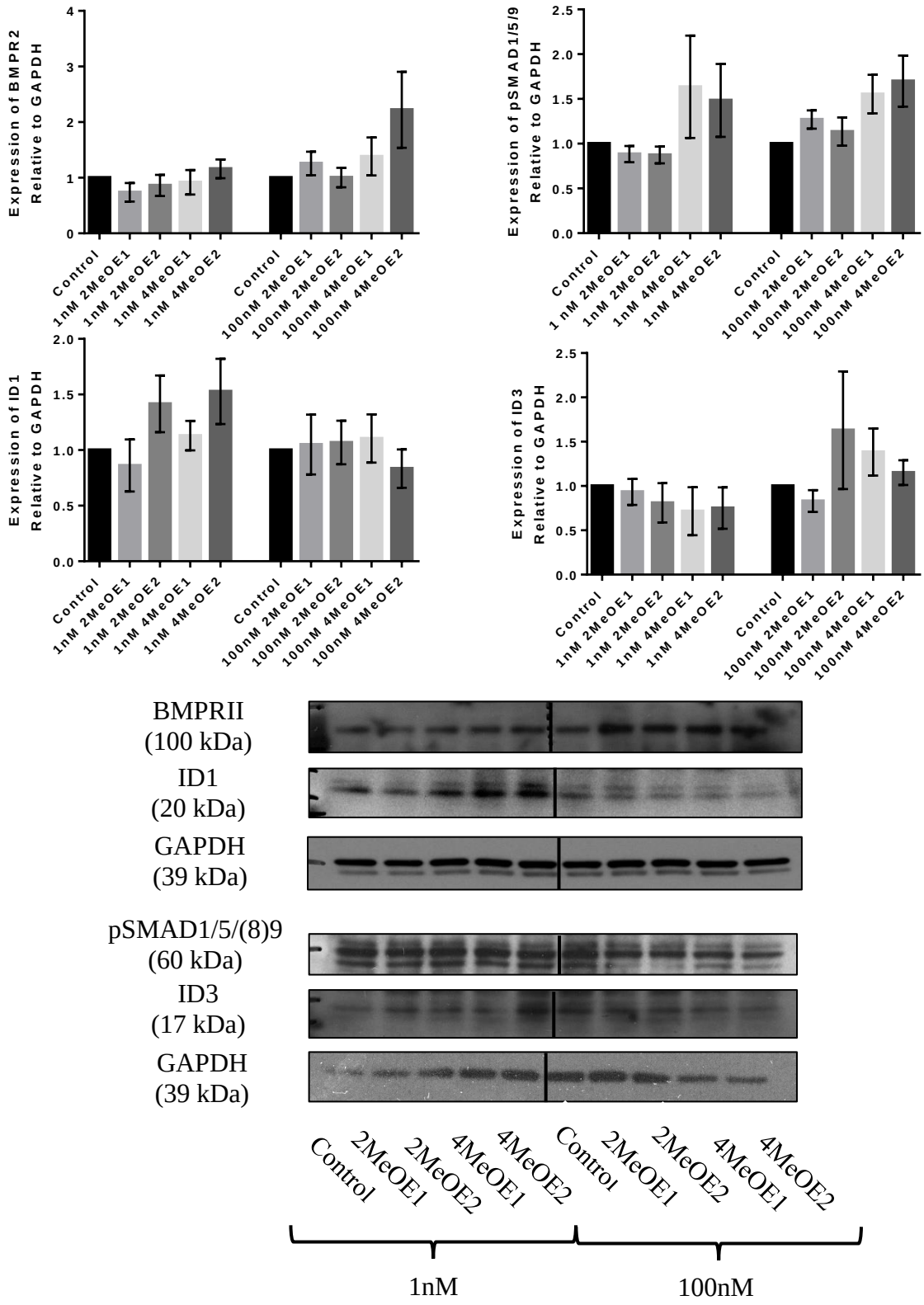


Figure 4–14: Methoxyoestrogens do not alter protein expression of effectors involved in the BMPRII signalling pathway in female control pulmonary artery smooth muscle cells.

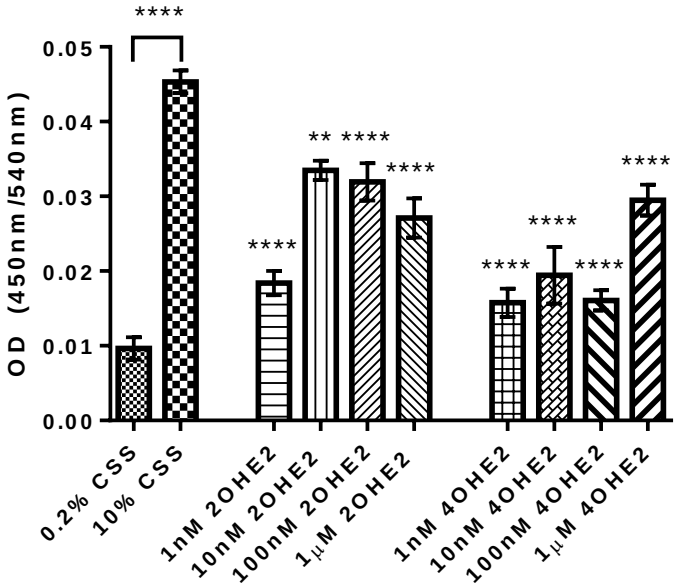
HPASMCs were quiesced for 24 hours in 0.5% CSS PRF DMEM media. PASMCs were then stimulated by oestrogen metabolites (1nM, 100nM) in the presence of 1% CSS PRF DMEM. The expression level of BMPRII, pSmad1/5/(8)9 , ID1 and ID3 was assessed using immunoblotting. Results were normalised to the GAPDH. N=3 per group, in duplicate, One-Way ANOVA with Bonferroni's post-hoc test. Data is expressed as fold change of Control group±SEM.

4.2.7 *2- and 4-Hydroxyoestradiol Oppose Proliferation in Male Control Pulmonary Artery Smooth Muscle Cells*

Albeit the presence of oestrogen-metabolising enzymes with increased activity has been linked to the pathology of numerous diseases, due to high production of hydroxylated oestrogens which may be metabolised to form highly reactive quinones and semiquinones, mutagenic activity has only been attributed to 4OHE2 (Liehr et al., 1986). Nonetheless, administration of 4OHE2 did not result in the onset of experimental PH in animals (Johansen, 2014). In male hPASMCs 4OHE2 increased expression of Id1, Id3 and pSmad1/5/8 (Mair et al., 2015). Firstly, we showed using the BrDU assay that treatment of male control PASMCs with 2OHE2 and 4OHE2 significantly decreased number of cells (Figure 4–15A). Considering both catechol oestrogens serve as precursor molecules for the COMT, forming protective oestrogen metabolites, we wished to ascertain whether the mechanism for observed decreased proliferation of hPASMCs might be due to their conversion to methoxyoestrogens. We therefore treated male control cell lines with COMT inhibitors, entacapone and OR-486, prior to stimulation with both oestrogen metabolites. Pre-treatment with entacapone opposed the anti-proliferative properties of 2OHE2, while the inhibition of COMT had no effect on anti-proliferative properties of 4OHE2 (Figure 4–15B).

When male control cell lines were stimulated with inhibitors of oestrogen receptors prior to stimulation with 2OHE2, inhibition of ERα and ERβ resulted in increased proliferation compared to 2OHE2 only treated cells (Figure 4–16). This indicates that in addition to the protective properties of 2MeOE2, protective properties of 2OHE2 might also be mediated through ER signalling. Additionally, inhibition of ERα also opposed the anti-proliferative properties of 4OHE2, indicating the decrease in the cell number might be also be mediated through ER signalling as opposed to the metabolite being biotransformed to 4MeOE2 (Figure 4–16).

A



B

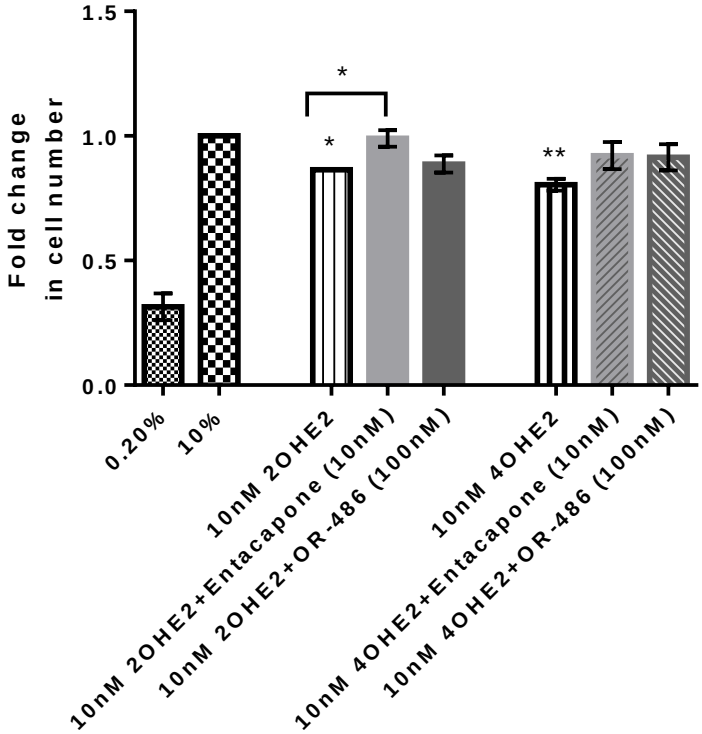


Figure 4–15: Treatment of male control cell lines with 2- and 4-hydroxyoestradiol results in decreased proliferation.

HPASMCs from male control subjects were serum starved for 24 hours. PASMCs were then stimulated 2OHE2 or 4OHE2 (1nM, 10nM, 100nM, 1μM). Otherwise, cells were pre-treated with COMT inhibitor entacapone (10nM) OR or-486 (100nM). Experiments were carried out in the presence of 10% CSS PRF DMEM media. Cellular proliferation was assessed after 72 hours either by BrDU assay (A) or haemocytometer (B). N=3 per group, in triplicate, One-Way ANOVA with Tukey's post-hoc test. Data expressed as fold change of mean±SEM (A) or fold change of 10% control group±SEM (B).

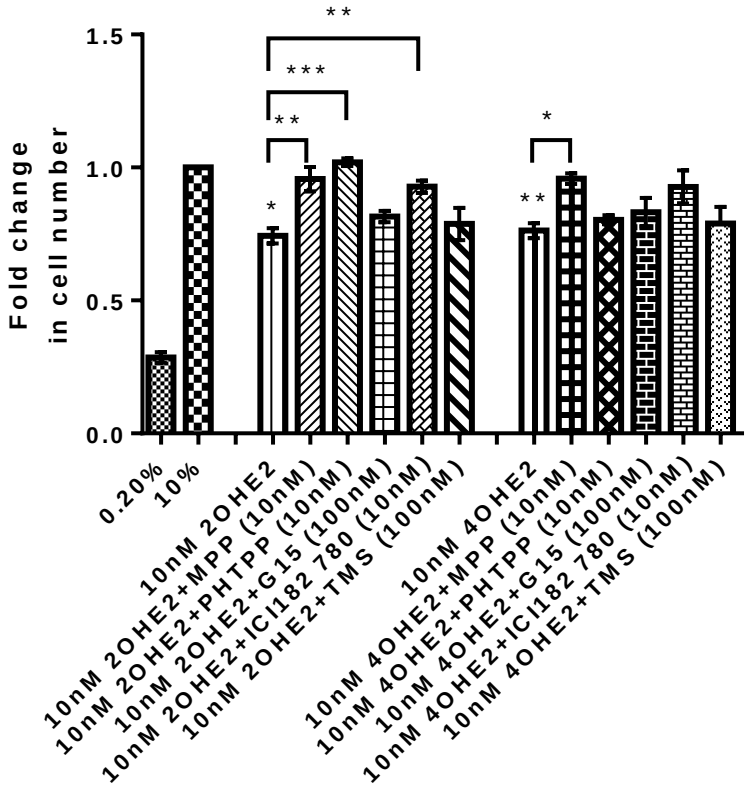


Figure 4–16: The anti-proliferative effects of 2- and 4-hydroxyoestradiol in male control pulmonary artery smooth muscle cells are mediated through classical oestrogen receptors.

HPASMCs from male control subjects were serum starved for 24 hours. PASMCs were then stimulated by ERα inhibitor (MPP, 10nM), ERβ inhibitor (PHTPP, 10nM), gpr30 receptor (G15, 100nM) or an ERα/β inhibitor (ICI182780, 10nM) 30 minutes prior to stimulation with 2OHE2 or 4OHE2 (10nM). Experiments were carried out in the presence of 10% CSS PRF DMEM media. Cell counts were assessed after 72 hours by haemocytometer. N=3 per group, in triplicate, One-Way ANOVA with Tukey's post-hoc test. Data expressed as fold change of 10% control group±SEM.

4.2.8 *High-Performance Liquid Chromatography Method to Investigate Oestrogen Metabolism In Vitro*

4.2.8.1 Treatment of Human Pulmonary Arteries with Treprostinil Significantly Alters the Production of Oestrone in Male Patient Pulmonary Artery Smooth Muscle Cells

Albeit prostacyclin analogues were the first approved class of therapeutics for the treatment of PAH, they are still frequently prescribed as part of combinational therapy to manage persistent symptoms. The beneficial effects of prostacyclin analogues include i) vasorelaxation and vasodilation (Narumiya et al., 1999; Wise, 2003), and ii) the inhibition of SMC proliferation (Clapp et al., 2002; Yang et al., 2010). Approved prostacyclin analogues, such as treprostinil and iloprost, are currently used to manage moderate to severe PAH.

Gathered evidence indicates that RXR can modulate the expression of oestrogen-regulated genes due to their interaction with promotor regions of these genes (Segars et al., 1993;Keller et al., 1995). PPARs affect oestrogen signalling through i) competitive inhibition of ERs (Keller et al., 1995), and ii) increased degradation of ERα (Qin et al., 2003), resulting in altered oestrogen signalling. Moreover, Becker and colleagues reported that PPARα, which is constitutively expressed in the mouse lung (Becker et al., 2008b), altered oestrogen metabolism through an increase of 17βHSD type 1 expression (Corton et al., 1997;Davis et al., 1994). Inhibition of PPARs was shown to modulate the expression of certain oestrogen metabolising enzymes according to (Corton et al., 1997). As evidence indicated that CYP1B1 may be induced through the activation of PPARα (Lovekamp-Swan et al., 2003;Murray et al., 2001), we were interested in the assessment of oestrogen metabolism in cell lines treated with a prostacyclin analogue, as the endogenous molecule and its metabolites were shown to activate certain PPAR isoforms (Forman et al., 1995;Nemenoff et al., 2008), directly (Forman et al., 1997) or indirectly, though the IP receptor (Falcetti et al., 2007). Prostacyclin and its analogues only have a marginal effect on PPAR activation due to their rapid elimination and degradation (Yu et al., 1995). Recently, iloprost and treprostinil, were shown to activate PPAR isoforms (Forman et al., 1997;Ali et al., 2006),

further stimulating our interest in the effects of prostacyclin analogue on oestrogen metabolism.

We employed high performance liquid chromatography (HPLC) to assess the effects of stimulation of treprostinil on oestrogen metabolism in in vitro model of PAH. Cells were treated with $^{14}C_4$-17βE2, which enabled the tracking and flux analysis of oestrogen metabolism in these cells. All cells were exposed to 50nM $^{14}C_4$-17βE2. Treatment groups T24B and TE48 referred to the time points when cells were stimulated with treprostinil (1μM): i) 24 hours prior to addition of 50nM $^{14}C_4$-17βE2 (T24B), and ii) at the same time as 17βE2 (TE48). Cells were then incubated with 50nM $^{14}C_4$-17βE2 for 48 hours, and the differences in cell growth during the duration of the experiment, was eliminated by normalisation of metabolite amount per amount of protein. It appears that the treatment of female cell lines derived from control subjects with treprostinil resulted in increased metabolism of 17βE2, as the amount of the parental oestrogen was decreased in both groups treated with treprostinil compared to control treatment (Figure 4–17A). Moreover, the metabolism of 17βE2 in other cell lines appeared to be faster, as the HPLC/flux experiments show that the amount of labelled 17βE2 was lower compared to female control cell lines, indicating the hormone was metabolised to other metabolites. Radioactively labelled 17βE2 was mainly metabolised to E1 in cell lines derived from female patient, male control and male patient subjects, respectively. The formation of E1 was considerably higher in female patient cell line compared to female and male control cell lines (Figure 4–17B). Treatment with treprostinil had no effect on the formation of E1 in female control and patient cell line. Preliminary results indicate that in male patient cell line, pre-treatment with treprostinil might resulted in increased production of E1 compared to control treatment, while co-treatment of cells with treprostinil and 17βE2 might result in decreased in E1 production compared to pre-treatment group (Figure 4–17B). In assessed samples we also detected 16αOHE1, 17αE2 and an unknown oestrogen metabolite, albeit the levels of these metabolites were considerably lower than both parental hormones. The amount of 17βE2 metabolised to 16αOHE1 was higher in the cell line derived from female patient compared to female control cell line (Figure 4–18A). Treatment with treprostinil did not affect the formation of 16αOHE1 compared to control treatment group across the cell lines used in these experiments. Interestingly, the formation of 16αOHE1 was higher in female patient cell line treated with 17βE2 compared to male patient cell line (Figure 4–18A). Conversion of 17βE2 to 17αE2 was only observed in cell lines derived from female patient and male control subjects, specifically in treprostinil treatment groups (Figure 4–18B).

Interestingly we also observed the formation of an unknown oestrogen metabolite (Figure 4–19), which could not be identified based on the retention time of the 15 unlabelled oestrogen metabolites used to determine the retention time using UV detection system. Nonetheless, due to the position of the ^{14}C within the 17βE2, we were satisfied labelled oestrogen was metabolised to this uncharacterised metabolite, whose formation was significantly enhanced when cells were co-treated with treprostinil and 17βE2 for 48 hours (TE48) compared to control treatment in female patient cell line. Representative chromatograms are shown in Figure 4–20 to Figure 4–23.

The effects of treprostinil treatment on oestrogen metabolic profile are shown in Figure 4–24. In female control cell line, the oestrogen metabolites present in the analysed media samples, indicated the enzymatic activity of 17βHSD type 2 and cytochrome P450 oestrogen metabolising enzymes, while there was no evidence of COMT activity (Figure 4–17 and Figure 4–18). In female patient, male control and patient cells, however, the present metabolites indicated that 17βHSD type 2 was more active compared to control cell line (Figure 4–24). Pre-treatment with treprostinil in male patient cells may result in increased activity of 17βHSD type 2, indicating the used prostacyclin analogue might affect the expression of this enzyme, while it does not affect the enzymes' activity (Figure 4–17B).

The presence of 16αOHE1 further indicated the presence and enzymatic activity of cytochrome P450 oestrogen metabolising enzymes, especially CYP1B1, whilst the formation of 17αE2 also showed that hPASMCs can convert the original substrate to this isomer. It is not clear, though, whether this was a spontaneous reaction, or a specific isomerase enzyme was required. The presence of an unknown metabolite implied that 17βE2 is the substrate of an oestrogen metabolising enzyme, whose end products we have not yet included in our research of oestrogen metabolism in *in vitro* model. In order to draw sound conclusions regarding the effect of treprostinil on oestrogen metabolism, these experiments would require to be repeated in more cell lines in order to increase the N number and allow statistical evaluation of the results. However, some of the preliminary results corroborate previous observations that prostacyclin analogues might affect oestrogen metabolism, specifically the 17βHSD expression (Corton et al., 1997;Davis et al., 1994), leading to altered production of enzymes' metabolites. Whether this occurs trough activation of PPARs still remains to be investigated.

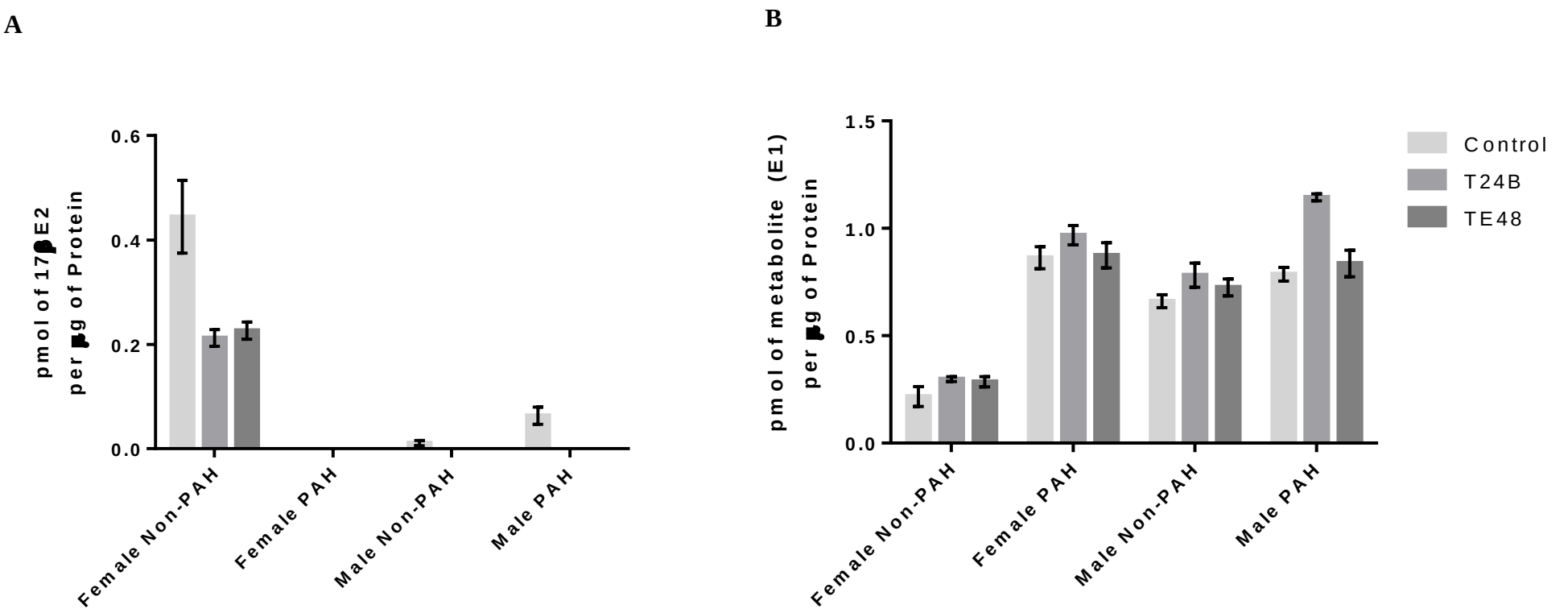


Figure 4–17: Treprostinil increases metabolism of 17β-oestradiol in human pulmonary arterial smooth muscle cells derived from female controls.
HPASMCs were grown to 60-70% confluence, as previously described, and serum starved for 16–24 hours. Cells were then stimulated with either treprostinil (1μM) l or vehicle (0.005% ethanol (v/v)) for 24 hours, prior to addition of 50nM $^{14}C_4$-17βE2, and treprostinil (1μM) or vehicle (0.005% ethanol) for further 48 hours. The spend media were collected and analysed using HPLC with scintillation counter. N=1 per group, repeated 3 times over three different passages. Data is expressed as mean±SEM.

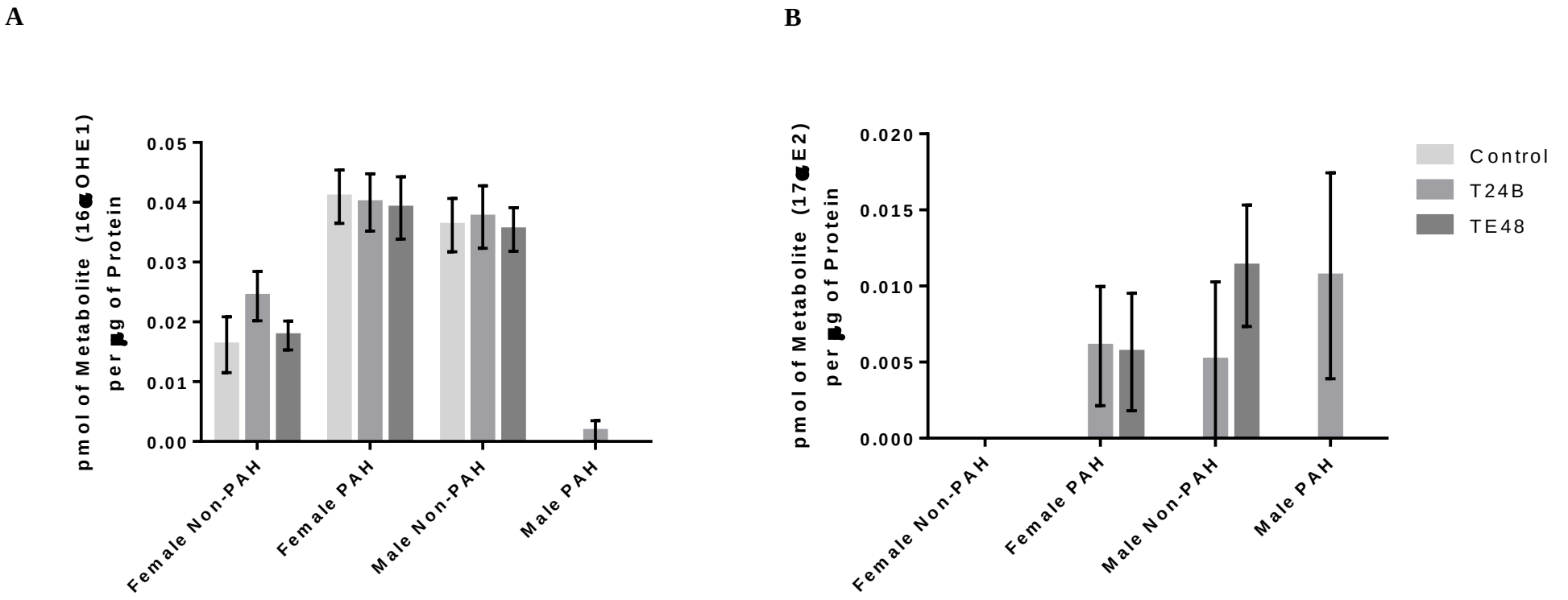


Figure 4–18: In human pulmonary artery smooth muscle cell line derived from male control subjects, treprostinil increases the formation of 17αE2 isoform.

HPASMCs were grown to 60-70% confluence, as previously described, and serum starved for 16–24 hours. Cells were then stimulated with either treprostinil (1μM) l or vehicle (0.005% ethanol (v/v)) for 24 hours, prior to addition of 50nM $^{14}C_4$-17βE2, and treprostinil (1μM) or vehicle (0.005% ethanol) for further 48 hours. The spend media were collected and analysed using HPLC with scintillation counter. N=1 per group, repeated 3 times over three different passages. Data is expressed as mean±SEM.

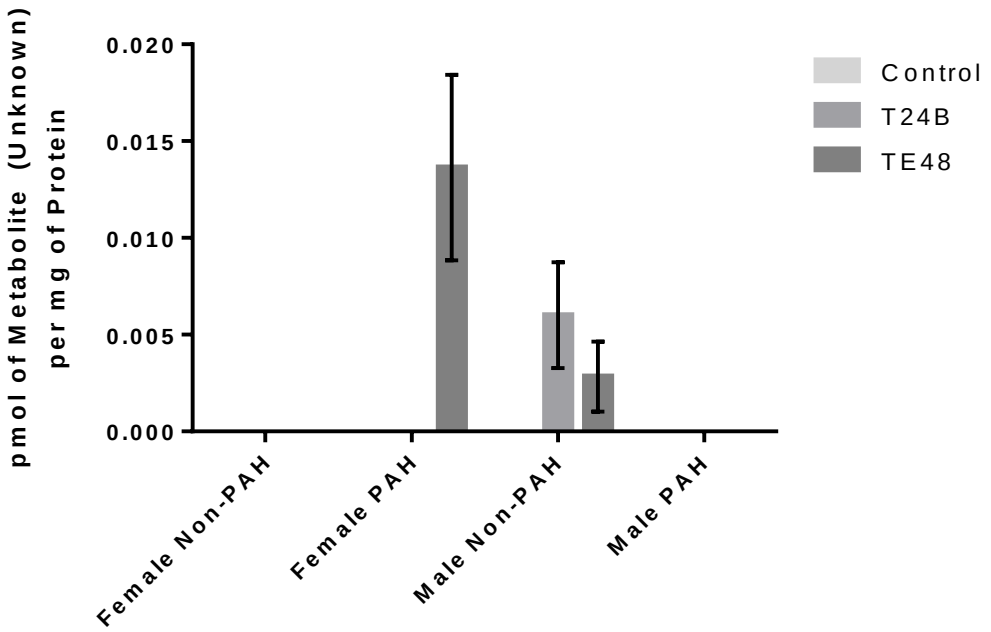


Figure 4–19: In human pulmonary artery smooth muscle cells from female patient, treprostinil increases the formation of an unknown oestrogen metabolite.

HPASMCs were grown to 60-70% confluence, as previously described, and serum starved for 16–24 hours. Cells were then stimulated with either treprostinil (1μM) l or vehicle (0.005% ethanol (v/v)) for 24 hours, prior to addition of 50nM $^{14}C_4$-17βE2, and treprostinil (1μM) or vehicle (0.005% ethanol) for further 48 hours. The spend media were collected and analysed using HPLC with scintillation counter. N=1 per group, repeated 3 times. Data is expressed as mean±SEM.

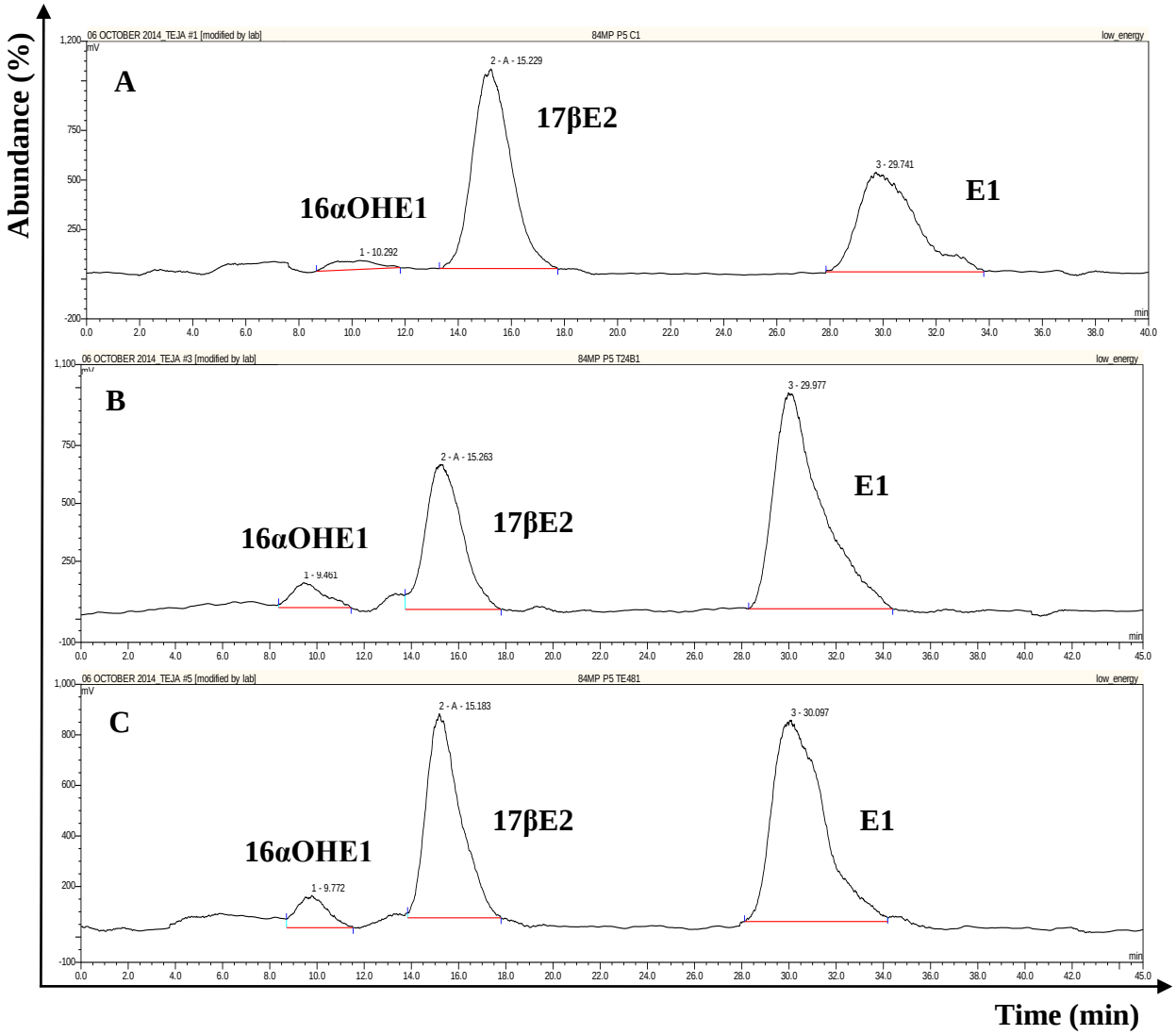


Figure 4–20: Representative high-performance liquid chromatography chromatograms for female control pulmonary artery smooth muscle cells (84MP).

A) Chromatogram of oestrogen metabolites formed when female control hPASMCs were stimulated with $^{14}C_4$-17βE2 for 48 hours. B) Chromatogram of oestrogen metabolites formed when female control cell lines were pre-treated with 1μM treprostinil 24 hours prior to stimulation with 50nM $^{14}C_4$-17βE2 and 1μM treprostinil. C) Chromatogram of oestrogen metabolism in female control cell lines co-stimulated with 50nM $^{14}C_4$-17βE2 and 1μM treprostinil for 48 hours.

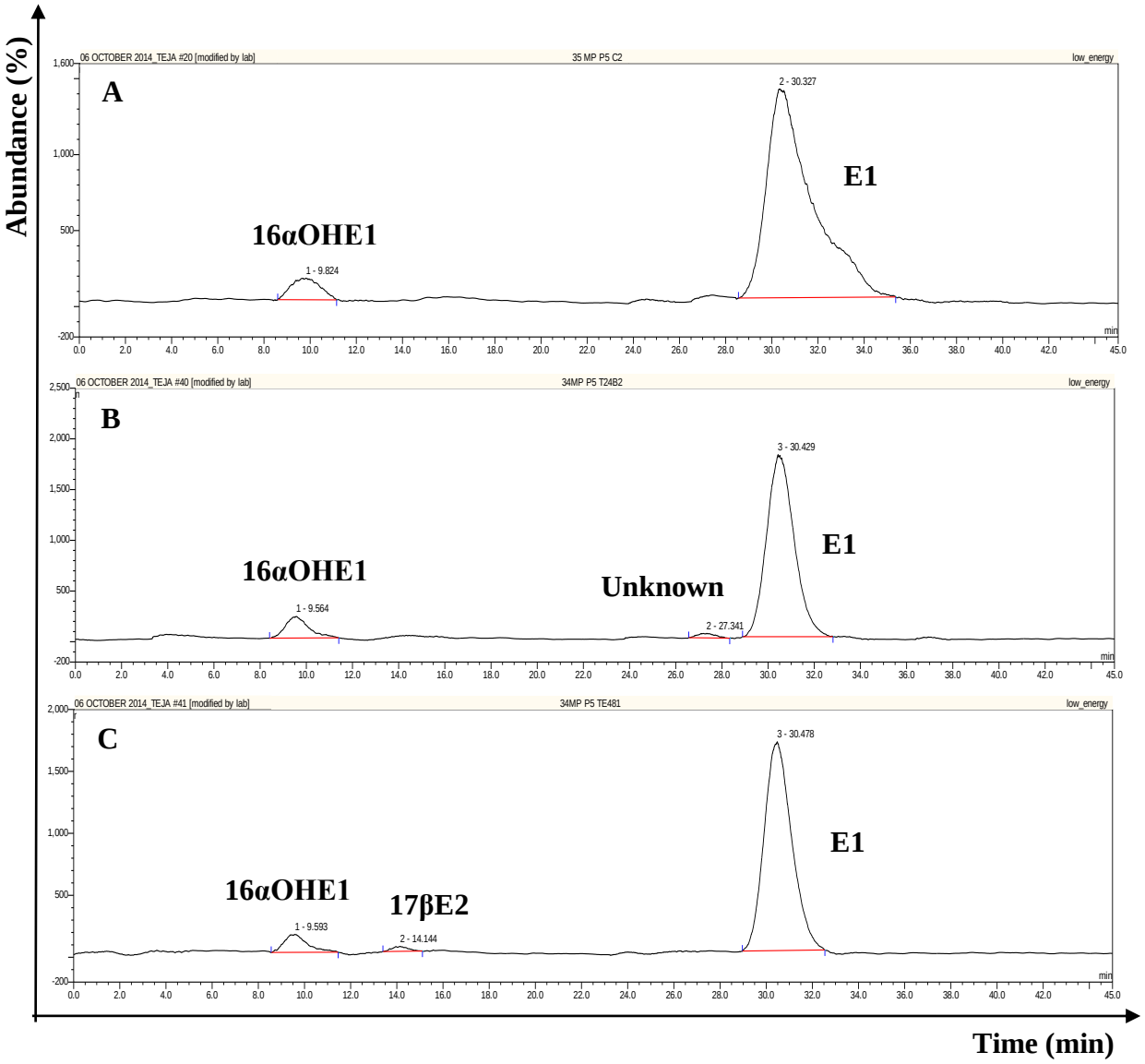


Figure 4–21: Representative high-performance liquid chromatography chromatograms for female patient pulmonary artery smooth muscle cells (35MP).

A) Chromatogram of oestrogen metabolites formed when female PAH patient hPASMCs were stimulated with $^{14}C_4$-17βE2 for 48 hours. B) Chromatogram of oestrogen metabolites formed when female control cell lines were pre-treated with 1μM treprostinil 24 hours prior to stimulation with 50nM $^{14}C_4$-17βE2 and 1μM treprostinil. C) Chromatogram of oestrogen metabolism in female control cell lines co-stimulated with 50nM $^{14}C_4$-17βE2 and 1μM treprostinil for 48 hours.

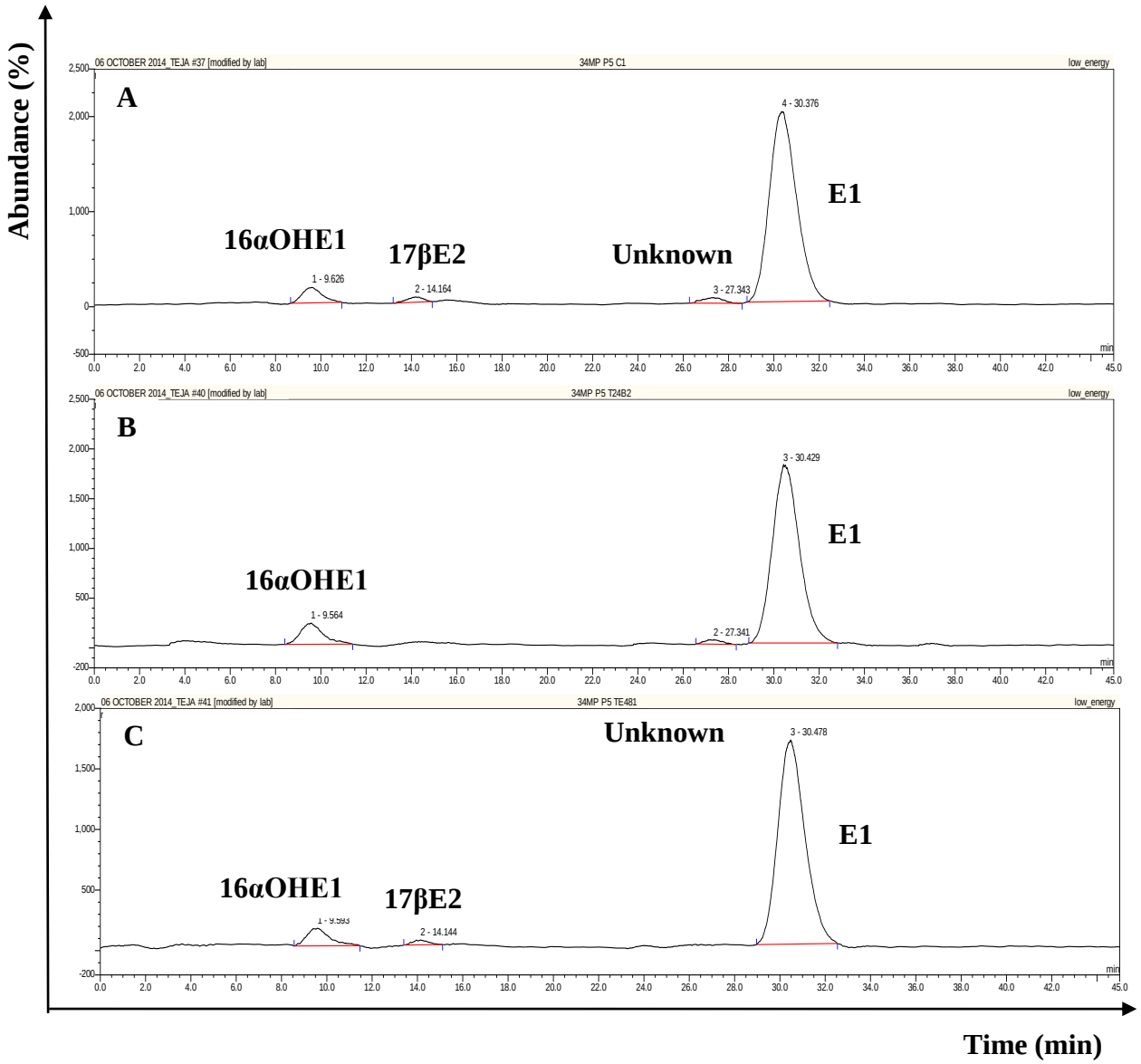


Figure 4–22: Representative high-performance liquid chromatography chromatograms for male control pulmonary artery smooth muscle cells (34MP).

A) Chromatogram of oestrogen metabolites formed when male control hPASMCs were stimulated with $^{14}C_4$-17βE2 for 48 hours. B) Chromatogram of oestrogen metabolites formed when female control cell lines were pre-treated with 1µM treprostinil 24 hours prior to stimulation with 50nM $^{14}C_4$-17βE2 and 1µM treprostinil. C) Chromatogram of oestrogen metabolism in female control cell lines co-stimulated with 50nM $^{14}C_4$-17βE2 and 1µM treprostinil for 48 hours.

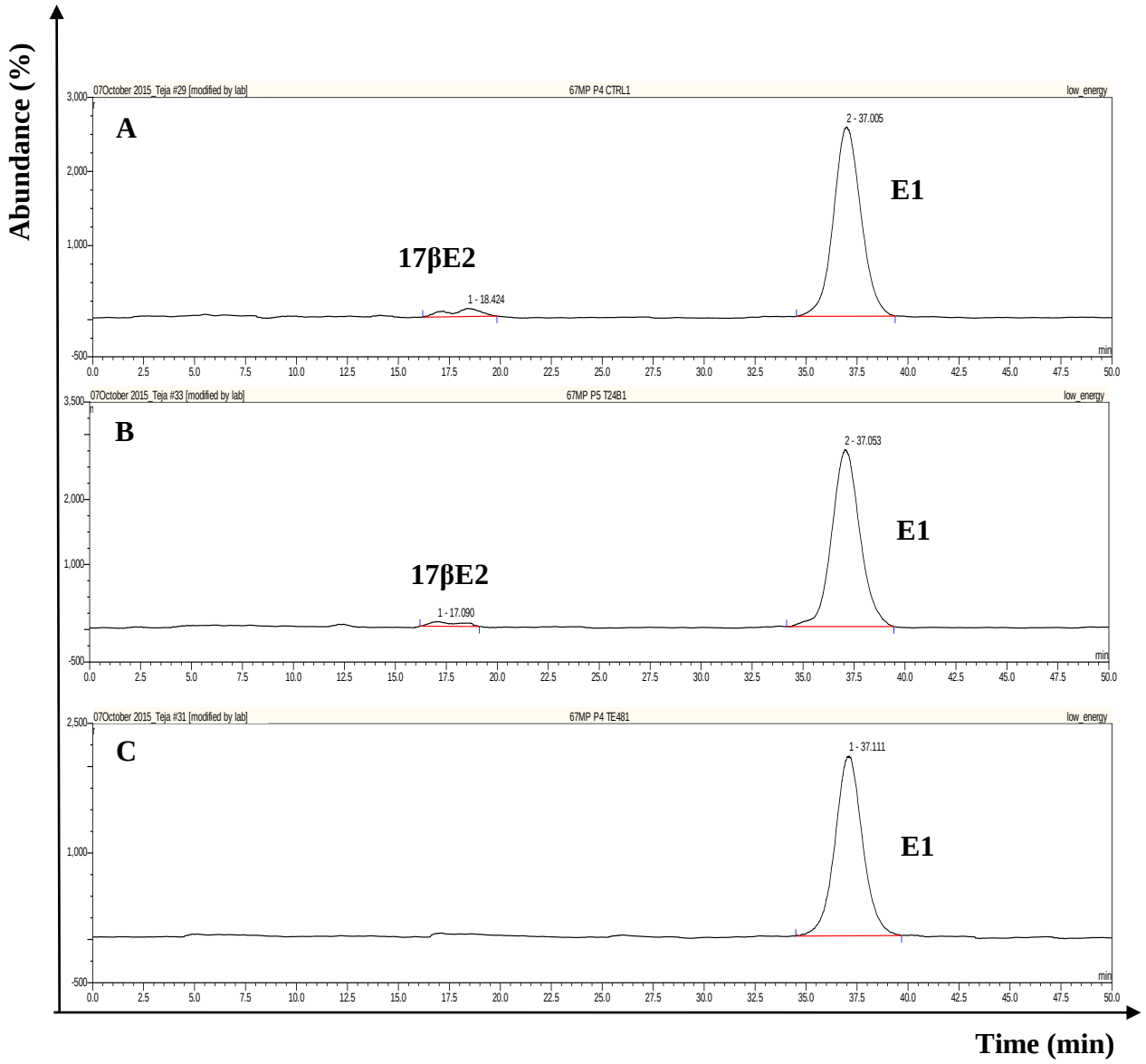


Figure 4–23: Representative high-performance liquid chromatography chromatograms for male patient pulmonary artery smooth muscle cells (67MP).

A) Chromatogram of oestrogen metabolites formed when male PAH patient hPASMCs were stimulated with $^{14}C_4$-17βE2 for 48 hours. B) Chromatogram of oestrogen metabolites formed when female control cell lines were pre-treated with 1μM treprostinil 24 hours prior to stimulation with 50nM $^{14}C_4$-17βE2 and 1μM treprostinil. C) Chromatogram of oestrogen metabolism in female control cell lines co-stimulated with 50nM $^{14}C_4$-17βE2 and 1μM treprostinil for 48 hours.

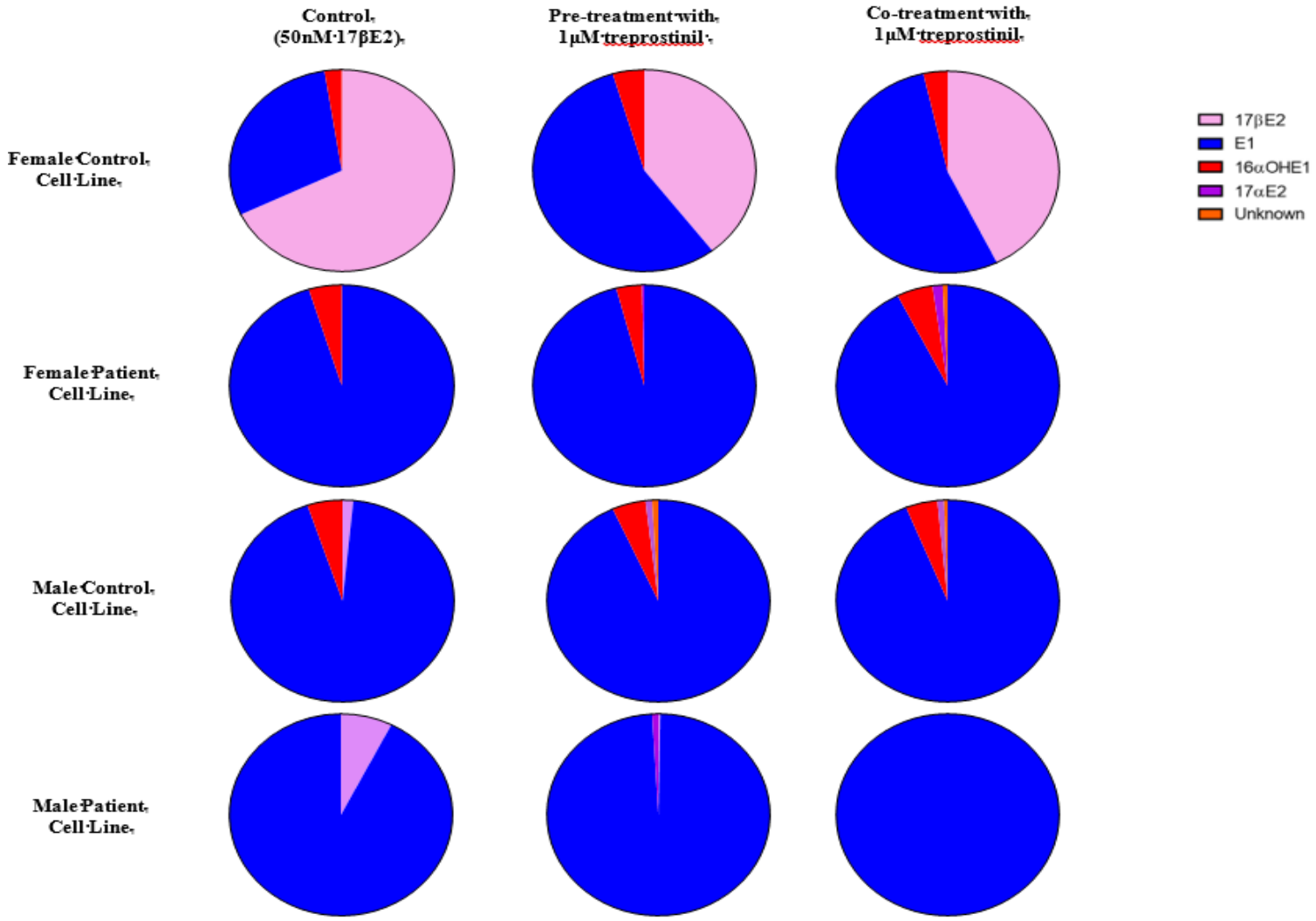


Figure 4–24: Oestrogen metabolic profile in pulmonary artery smooth muscle cells stimulated with treprostinil.

Female and male, control and patient PASMCs were incubated with $^{14}C_4$-17βE2 (50nM) for 48 hours in 1% (oestrogen-free) CSS PRF DMEM. Samples were extracted by solid phase extraction using Oasis® HLB cartridges and analysed by HPLC/flux method with UV and radiolabelled detection. N=1 cell line per group, repeated two or three times.

4.2.8.2 2,4,3',5'-Tetramethoxystilbene did not Alter Oestrogen Metabolism in Female Human Pulmonary Artery Smooth Muscle Cells

TMS, a potent inhibitor of the CYP1B1 oestrogen-metabolising enzyme, has been shown to attenuate the development of experimental PH phenotype in female SERT+ mice and mice exposed to cHx (Johansen, 2014). It also improved the overall survival in male rats exhibiting monocrotaline-induced PH, although it did not improve the haemodynamic parameters and vascular remodelling (Johansen, 2014). The beneficial effects of CYP1B1 inhibition may be mediated through anti-proliferative effects observed *in vitro*, where TMS reduced 17βE2-induced proliferation in hPASMCs (White et al., 2012). High local concentrations of oestrogens due to local synthesis (Mair et al., 2014) and metabolism (Johansen, 2014) was shown to potentially contribute to potent pathogenic environment, stimulating proliferation of VSMCs, and leading to muscularisation of small PAs. Considering the robust evidence of the detrimental role of CYP1B1 in the pathogenesis of PAH (Austin et al., 2009; White et al., 2012), we wished to assess the effects of its inhibition in vitro on oestrogen metabolism in female cell lines.

We showed that female control and patient cell lines metabolise 17βE2 differently (Figure 4–25A), with larger quantities of E1 produced in female patient cells compared to control cells (Figure 4–25B). Moreover, in accordance with previous studies, 16αOHE1 was only detected in female patient cells (Figure 4–27A). Its production in female patient cells stimulated with 17βE2 was higher compared to control cell line. We also observed the formation of 16αOHE2 and 2OHE1 in female control and patient cell lines, respectively (Figure 4–26A and Figure 4–27B). Treatment with TMS did not affect the overall oestrogen metabolism in control and patient cell lines, respectively, although a small decrease in the production of 16αOHE1 was observed (Figure 4–27A). Similarly, the production of 16αOHE2 in female control cells was decreased as well, albeit this was not significant (Figure 4–27B). Stimulation of female patient cell line with TMS also resulted in the formation of two unknown oestrogen metabolites (Figure 4–26 B, C).

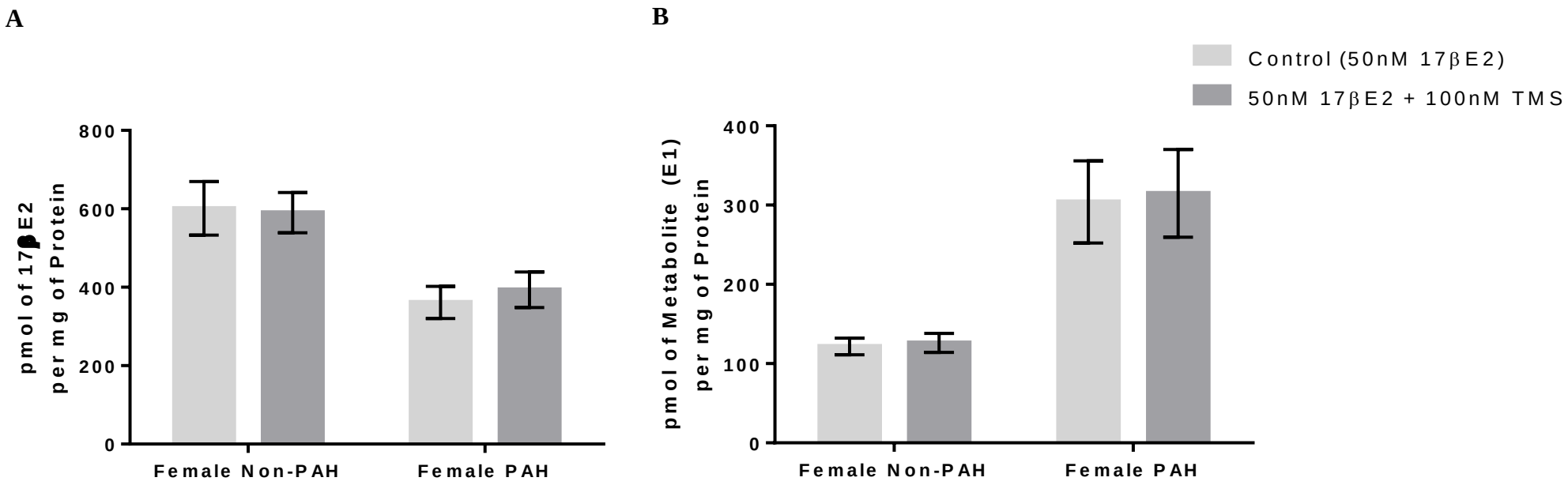


Figure 4–25: Treatment of human pulmonary arteries from female control subjects and clinical patients with 2,4,3',5'-tetramethoxystilbene (TMS) does not alter oestrogen metabolism.

HPASMCs were grown to 60-70% confluence, as previously described, and serum starved for 16–24 hours. Cells were then stimulated with 2,4,3',5'-tetramethoxystilbene (TMS, 100nM) 30 minutes prior to addition of 50nM $^{14}C_4$-17βE2. The spent media samples were collected after 48 hours and analysed using HPLC with scintillation counter. N=1 per group, repeated 3 times. Data is expressed as mean±SEM.

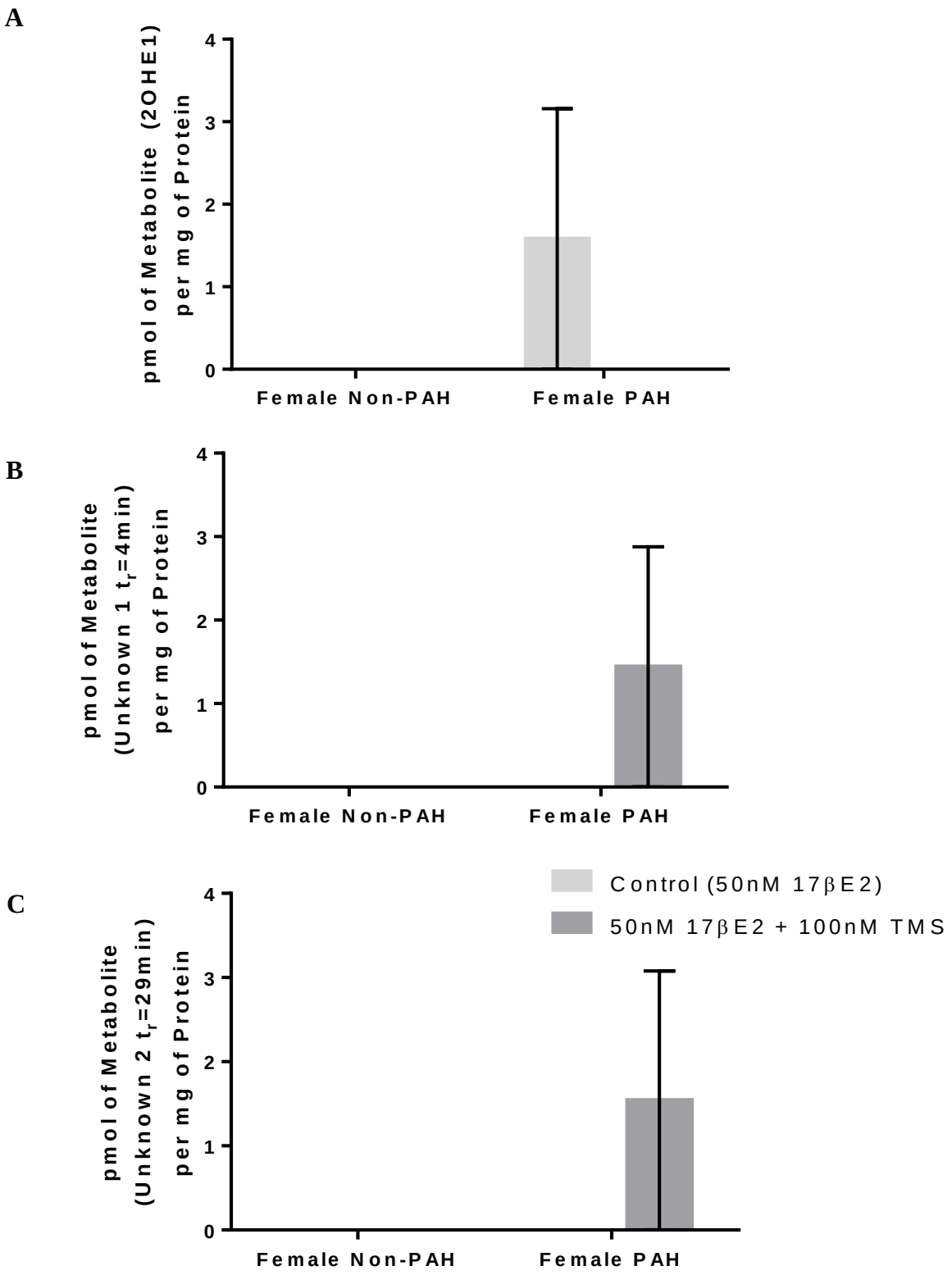


Figure 4–26: In human pulmonary artery smooth muscle cell line derived from female patient subject, treatment with 2,4,3',5'-tetramethoxystilbene (TMS) results in the formation of two unknown metabolites.

HPASMCs were grown to 60-70% confluence, as previously described, and serum starved for 16–24 hours. Cells were then stimulated with 2,4,3',5'-tetramethoxystilbene (TMS, 100nM) 30 minutes prior to addition of 50nM $^{14}C_4$-17βE2. The spent media samples were collected after 48 hours and analysed using HPLC with scintillation counter. N=1 per group, repeated 3 times. Data is expressed as mean±SEM.

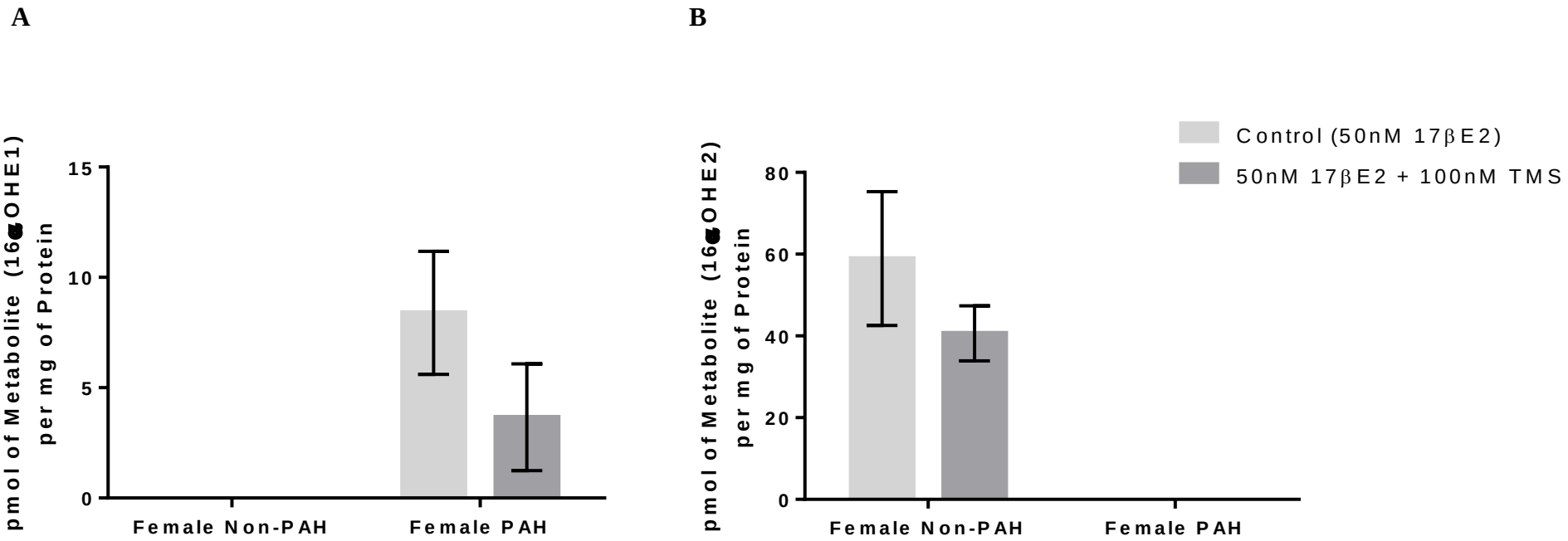


Figure 4–27: 2,4,3',5'-Tetramethoxystilbene (TMS) does not affect the metabolism of 17β-oestradiol to 16α-hydroxyoestrogens.

HPASMCs were grown to 60-70% confluence, as previously described, and serum starved for 16–24 hours. Cells were then stimulated with 2,4,3',5'-tetramethoxystilbene (TMS, 100nM) 30 minutes prior to addition of 50nM $^{14}C_4$-17βE2. The spent media samples were collected after 48 hours and analysed using HPLC with scintillation counter. N=1 per group, repeated 3 times, Two-Way ANOVA, followed by Tukey's multiple comparison post-hoc test. Data is expressed as mean±SEM.

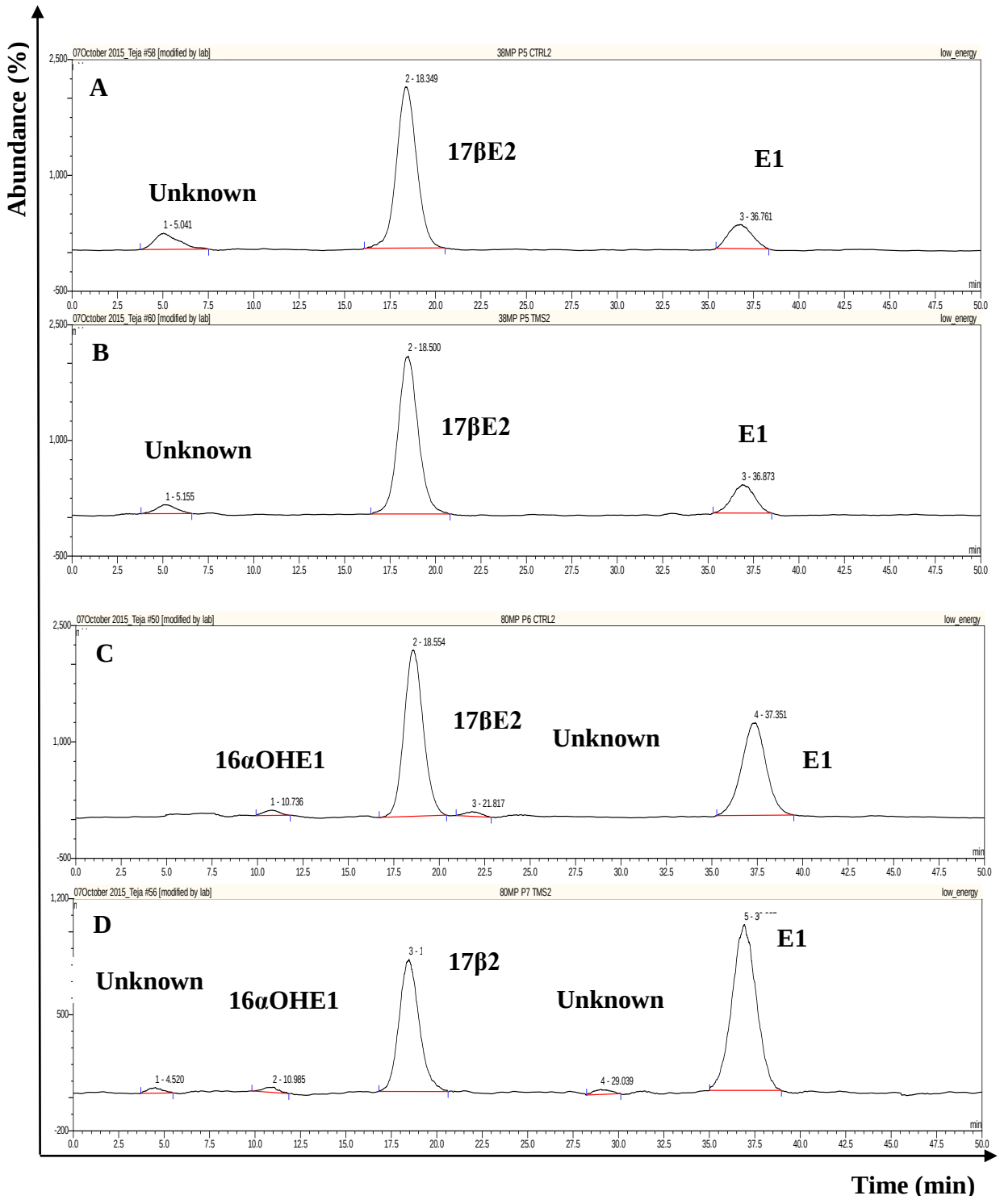


Figure 4–28: Representative high-performance liquid chromatography chromatograms for female control and patient pulmonary artery smooth muscle cells (80MP, 38MP). Chromatograms shown above depict oestrogen metabolites in female control and patient cell lines treated with 50nM $^{14}C_4$-17βE2 (A, C) and 2,4,3',5'-tetramethoxystilbene (TMS, 100nM), a potent cytochrome CYP1B1 inhibitor, in the presence of $^{14}C_4$-17βE2 (B, D).

4.2.9 *Expression of 17β-Hydroxysteroid Dehydrogenase Type 2 is Increased in Unstimulated Male Patient Pulmonary Arterial Smooth Muscle Cells*

17βHSD type 2 is the oxidoreductase catalysing the interconversion of 17βE2 to E1, with equally high catalytic activity for the interconversion of testosterone to androstenedione (Labrie et al., 1997), and is therefore generally considered to mediate anti-oestrogenic and anti-androgenic effects. 17βHSD type 2 is widely expressed in the peripheral tissues, including the lung and liver (Miettinen et al., 1996), as well as in oestrogen-target tissues, indicating its main metabolic role might be associated with the conversion of sex hormones to biologically less potent hormones. Previously, it was reported that exposure to cHx results in increased expression of 17βHSD type 1 enzyme, which catalyses the interconversion of the E1 to 17βE2 (Johansen, 2014), indicating that increased conversion of less biologically potent oestrogens to more biologically potent hormones, might be associated with dysregulated oestrogen metabolism observed in PAH. We assessed the expression levels of 17βHSD type 2 in PASMCs of both sexes, derived from control and PAH patient subjects to determine whether there is a difference in the expression associated with sex. At gene expression level, we observed no difference in the expression of 17βHSD type 2 between the groups (Figure 4–29A), indicating the expression level of this enzyme might not be affected by sex or disease.

Furthermore, at protein level, a trend of increased expression of 17βHSD type 2 in unstimulated human PASMCs derived from male subjects exhibiting clinical PAH phenotype compared to cells derived from control male subjects and female subjects exhibiting PAH phenotype, respectively was observed (Figure 4–29B). Although the results from immunoblotting should be considered with caution due to the quality of the blot, the results indicate that the protein expression might be affected on the level of microRNA or its degradation pathway, considering the expression at gene level was not altered across groups, our observations.

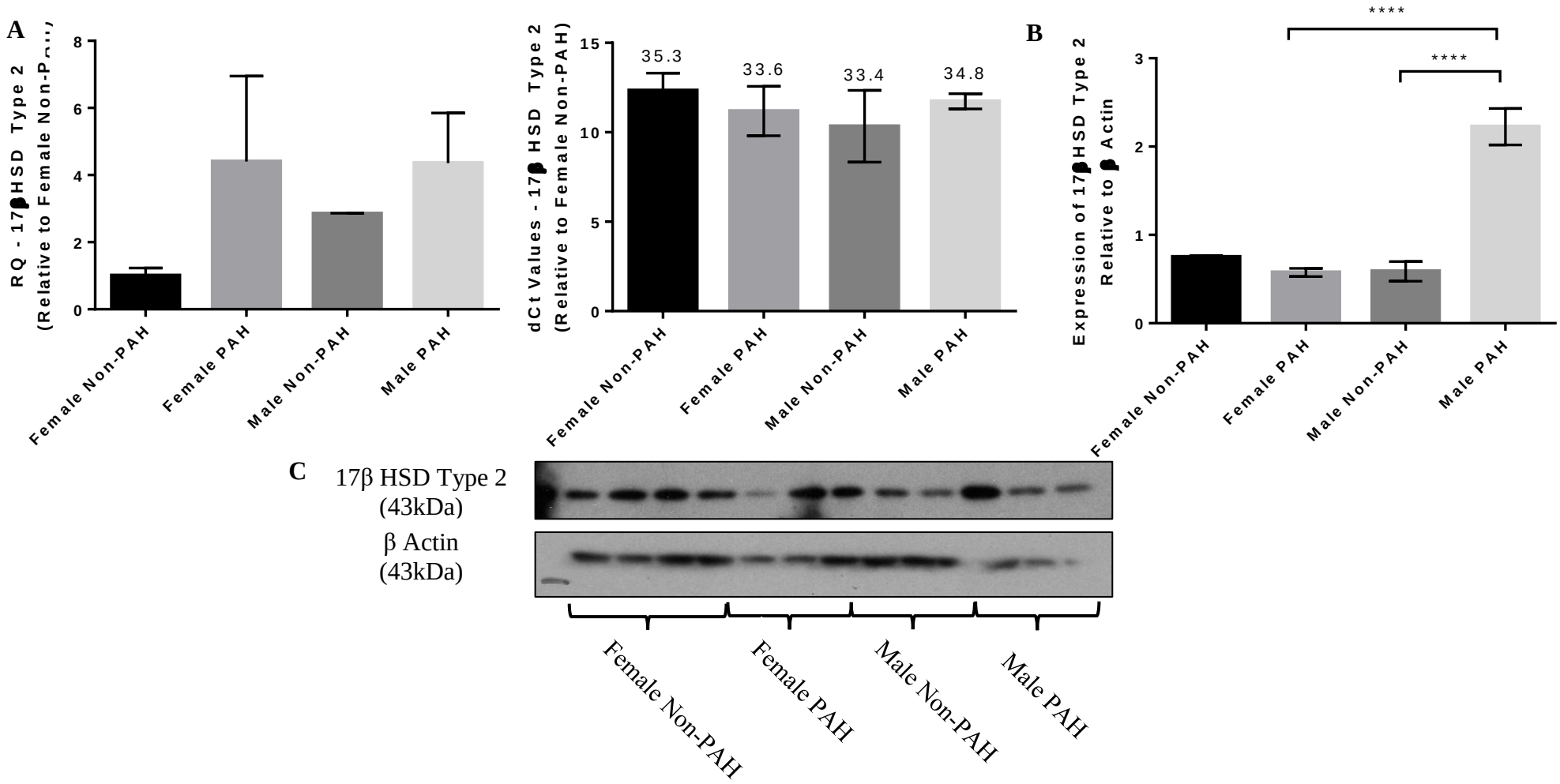


Figure 4–29: Expression of 17β-hydroxysteroid dehydrogenase type 2 is increased in unstimulated male human pulmonary artery smooth muscle cells derive from patients with pulmonary arterial hypertension.

HPASMCs were grown to reach approximately 80% confluence in 10% FBS DMEM media. PASMCs were then washed with ice-cold PBS and protein samples were collected and prepared as previously described. (A) The expression of *17βHSD type 1* was assessed by Taqman quantitative Real Time-PCR. Results were normalised to *β-2-microglobulin*. N=3–6 per group in triplicate, One-way ANOVA followed by Tukey's post-hoc test. Data is expressed as $RQ \pm \frac{RQ_{max}}{RQ_{min}}$, where RQ=Relative quantification. (B) The level of 17βHSD type 1 protein expression was addressed using immunoblotting. N=3–4 per group in duplicate, ****p<0.0001, One-way ANOVA followed by Tukey's post-hoc test. Data is expressed as fold change to Control group±SEM. The representative immunoblotting image is shown in (C).

4.2.10 *Hypothesis-Free Metabolomic Screen in Female Pulmonary Artery Smooth Muscle Cells Stimulated with 17β-Oestradiol*

When conducting HPLC/flux analyses of oestrogen metabolism in vitro we have been confronted with the presence of oestrogen metabolites that were previously not identified. Due to the position of radioactive label within the parental hormone, however, we were convinced the observed compounds were oestrogen metabolites, although we were unable to correlate them with the retention times of 15 most frequent metabolites.

To the best of author's knowledge, a metabolomic screen using an *in vitro* model of hPASMCs has not yet been carried out. Our aim was, therefore, to conduct a hypothesis-free metabolomic screen in female control and patient cell lines, treated with 17βE2 or vehicle, as described in Chapter 2.6.1. Sample preparation and analysis were performed at Strathclyde University, Glasgow with the support of Dr David Watson and Muhammad Abbas. Our aim was to identify any metabolites that are either altered with clinical disease onset or with 17βE2 stimulation, an insult known to contribute to PAH pathogenesis. Any identified changes in the metabolome could namely potentially point towards molecular pathways that might be imperative in disease pathogenesis but have not yet been investigated in the context of PAH. The library used to carry out the metabolomic screen was constructed at Strathclyde University and was kindly provided by Dr Watson.

The analysis was carried out in two modes, positive and negative, hence the results herein are presented in such manner as well. In positive mode, we observed that stimulation with 17βE2 did not lead to many changes in the metabolome of female control cell lines. A statistically significant increase in (S)-ATPA and an unknown complex was observed, with more of the molecule present when cells were stimulated with 17βE2. Similar observations were made in negative mode, where the only significant difference in the metabolome between vehicle- and 17βE2-treated cells was a fatty acid 4,7-dioxo-octanoic acid (*data not shown*). Interestingly, when comparing female patient cells stimulated with vehicle and 17βE2, more metabolites were shown to be significantly changed (Figure 4–30). A variety of peptides and chemical complexes were found to be altered in female patient cells stimulated with 50nM unlabelled 17βE2 compared to vehicle treatment. However due to their general character, it is difficult to establish their potential association with metabolic

pathways which might be affected in hPASMCs by 17βE2 stimulation (Figure 4–31). Interestingly, creatine, whose main physiological function is to recycle adenosine triphosphate (ATP), was significantly increased in female patient cells stimulated with 50nM unlabelled 17βE2. Moreover, the levels of glutathione were also affected by 17βE2 treatment in female patient cells. It therefore appears that energy metabolism and the formation of ROS are affected by oestrogens in female patient hPASMCs. The level of L-kynurenine, a catabolite of tryptophan which is the precursor of 5-HT, was also altered in female patient cells treated with 17βE2 (Figure 4–31), where treatment resulted in catabolite diminished concentration. While further catabolite products of L-kynurenine metabolism have been shown to possess vasoactive properties (Wang et al., 2010), the parental molecule is a known AhR ligand, shown to be increased in certain cancers, where it promoted tumour growth (Opitz et al., 2011). In negative mode, we did not observe significant changes in metabolites present in female patient cells stimulated with 17βE2 compared to vehicle treatment group.

When comparing the metabolome of vehicle-treated female controls to female patients, we observed significantly increased levels of glutathione and decreased levels of nicotinamide in female patient cell lines, indicating the formation of ROS might be dysregulated (Figure 4–32). The levels of L-carnitine and pantothenate, which are both involved in the synthesis and stabilisation of coenzyme A (CoA) and acetyl-CoA, respectively, were decreased with treatment in female control cell lines. In negative mode, we show that besides numerous complexes, the levels of pantothenate are slightly reduced in female patient cell lines (Figure 4–34). Equally, using positive mode, we have established that in female patient cells treatment with 17βE2, results in significantly altered levels of numerous chemical complexes, and levels of L-carnitine compared to female patient cell lines (Figure 4–33). The ratio of levels indicated that L-carnitine was decreased in female patient cell lines. Whilst in negative mode, treatment with 17βE2 resulted in increased levels of 2-oxoglutarate, which has an important role in oxidation reactions involving molecular oxygen, in female patient cell lines compared to female control cell lines (Figure 4–34).

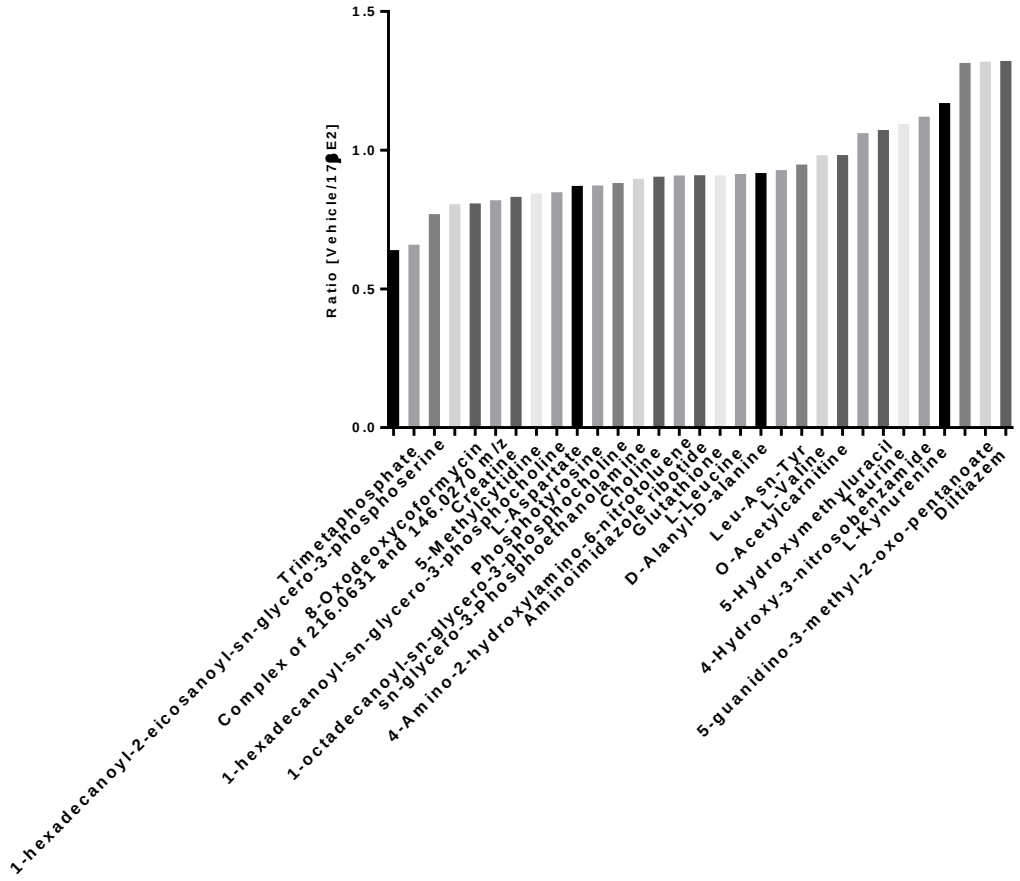


Figure 4–30: Metabolomic screen of pulmonary artery smooth muscle cells from female patient subjects comparing vehicle-treated cells with those treated with 17β-oestradiol using positive mode.

Female patient hPASMC were grown to 60-70% confluence, as previously described, and serum starved for 16–24 hours. Cells were then stimulated with vehicle (0.005% (v/v) EtOH in 1% CSS PRF DMEM) for 48 hours. The spend media were collected as previously described and analysed using LC-MS. N=3 per group, repeated 2–3 times, *$p \leq 0.05$, ****$p < 0.0001$, unpaired t-test.

The graph presents only the selection of metabolites, which were significantly altered by treatment with 17β-oestradiol.

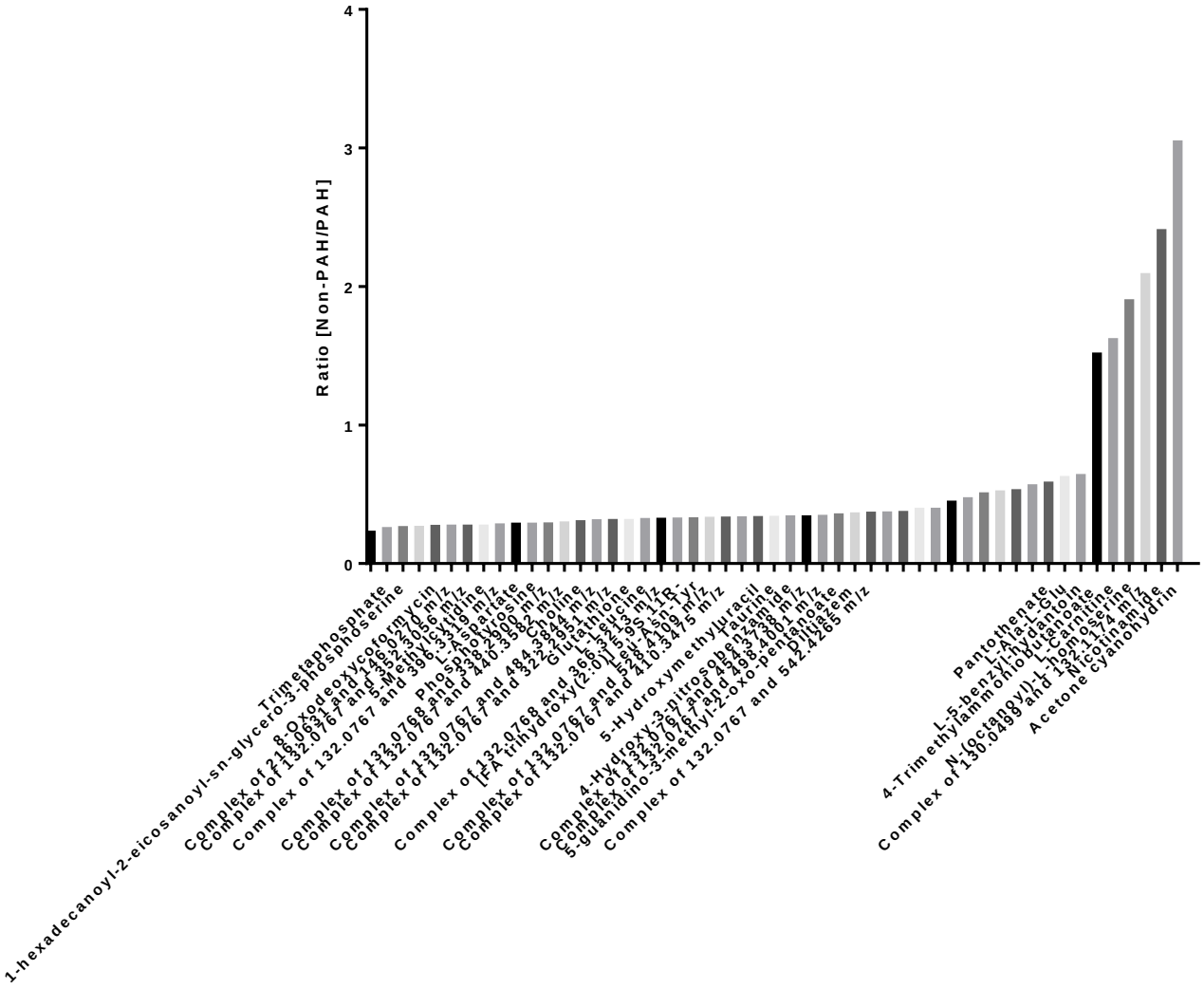


Figure 4–31: Metabolomic screen comparing vehicle-treated pulmonary artery smooth muscle cells from female control subjects to female patient subjects using positive mode. Female control hPASMC were grown to 60-70% confluence, as previously described, and serum starved for 16–24 hours. Cells were then stimulated with vehicle (0.005% (v/v) EtOH in 1% CSS PRF DMEM) for 48 hours. The spend media were collected as previously described and analysed using LC-MS. N=3 per group, repeated 2–3 times, *p≤0.05, ****p<0.0001, unpaired t-test.

The graph presents only the selection of metabolites, which were significantly different between vehicle-treated female cell lines derived from control and patient subjects.

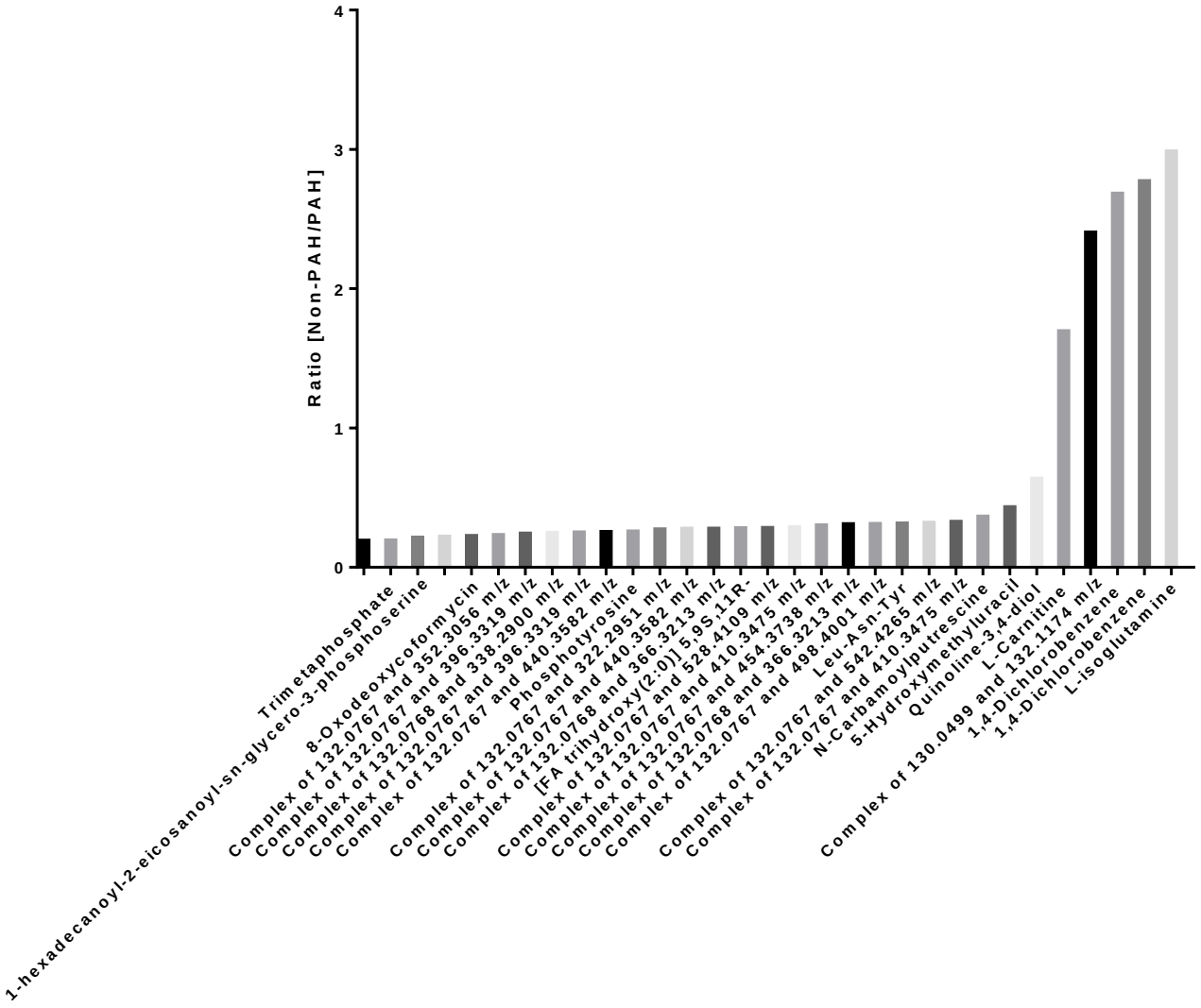


Figure 4–32: Metabolomic screen comparing vehicle-treated pulmonary artery smooth muscle cells from female control subjects to female patient subjects using positive mode
Female control hPASMC were grown to 60-70% confluence, as previously described, and serum starved for 16–24 hours. Cells were then stimulated with vehicle (0.005% (v/v) EtOH in 1% CSS PRF DMEM) for 48 hours. The spend media were collected as previously described and analysed using LC-MS. N=3 per group, repeated 2–3 times, *$p \leq 0.05$, ****$p < 0.0001$, unpaired t-test.

The graph presents only the selection of metabolites, which were significantly different between vehicle-treated female cell lines derived from control and patient subjects.

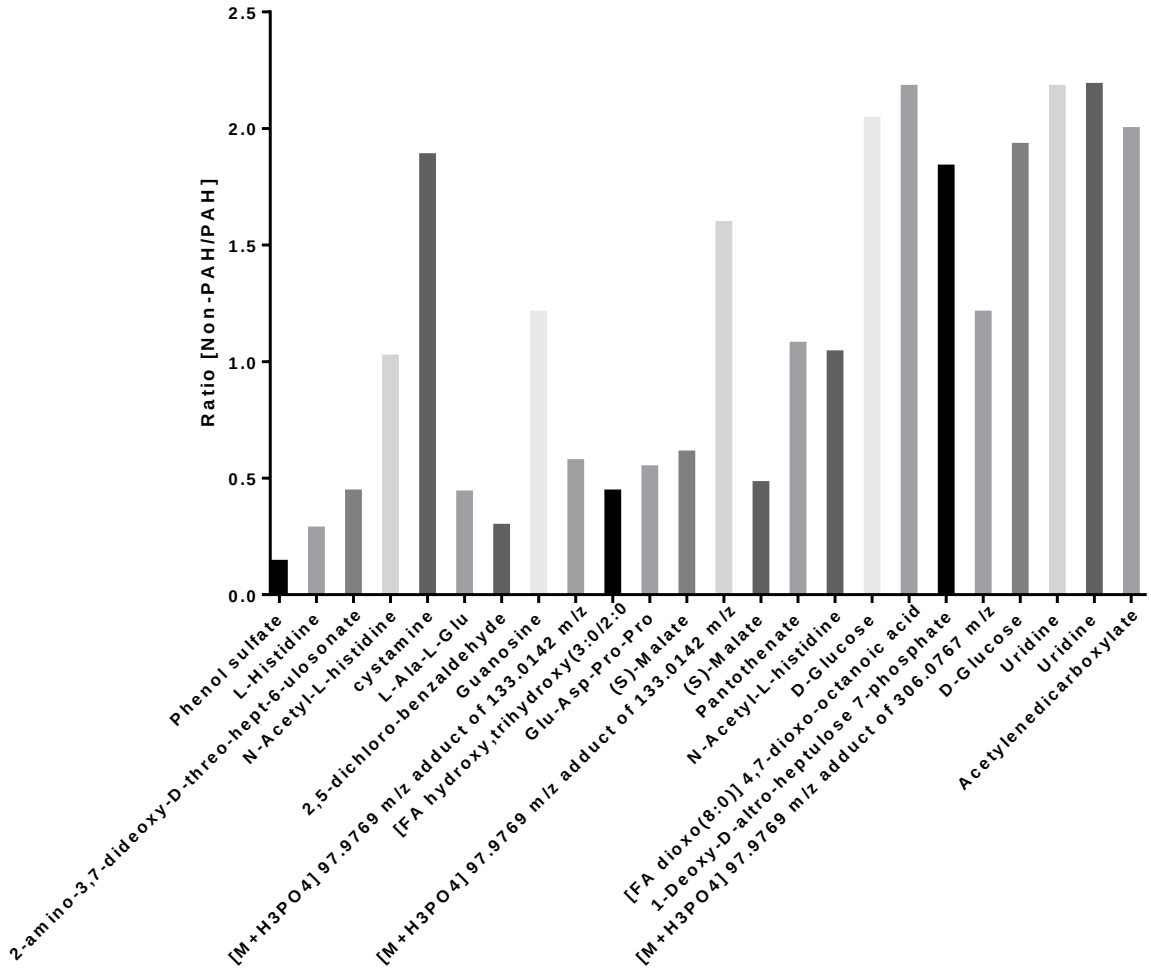


Figure 4–33: Metabolomic screen comparing vehicle-treated pulmonary artery smooth muscle cells from female control subjects to female patient subjects using negative mode.
Female control and patient hPASMC were grown to 60-70% confluence, as previously described, and serum starved for 16–24 hours. Cells were then stimulated with vehicle 17βE2 (50nM) in 1% CSS PRF DMEM for 48 hours. The spend media were collected as previously described and analysed using LC-MS. N=3 per group, repeated 2–3 times, *p≤0.05, ****p<0.0001, unpaired t-test.

The graph presents only the selection of metabolites, which were significantly different between vehicle-treated female cell lines derived from control and patient subjects.

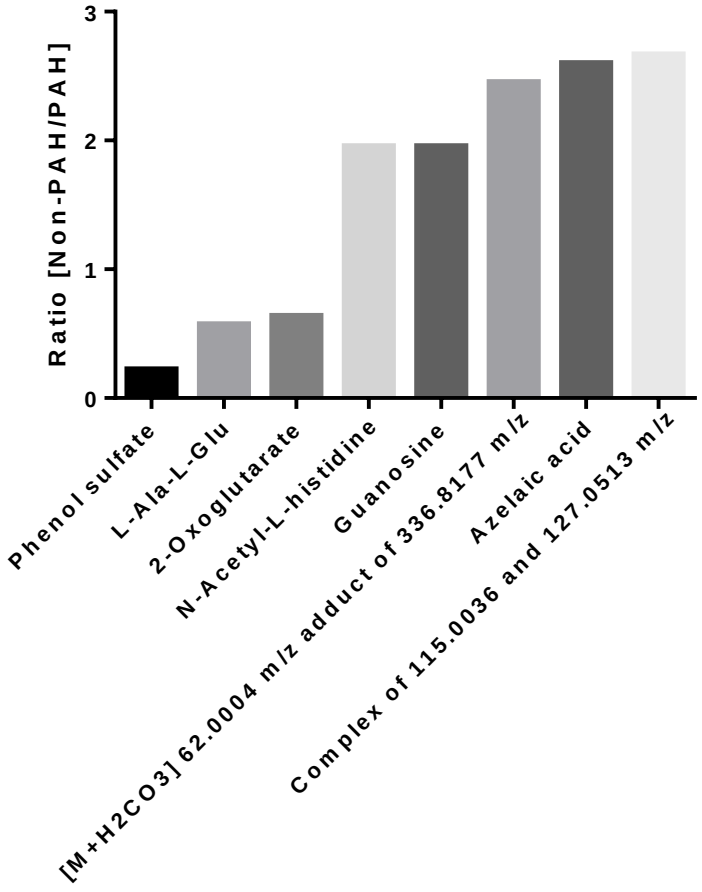


Figure 4–34: Metabolomic screen comparing 17β-oestradiol-treated pulmonary artery smooth muscle cells from female control subjects to female patient subjects using negative mode.

Female control and patient hPASMC were grown to 60-70% confluence, as previously described, and serum starved for 16–24 hours. Cells were then stimulated with vehicle 17βE2 (50nM) in 1% CSS PRF DMEM for 48 hours. The spend media were collected as previously described and analysed using LC-MS. N=3 per group, repeated 2–3 times, *p≤0.05, ****p<0.0001, unpaired t-test.

The graph presents only the selection of metabolites in female cell lines derived from controls compared to patient cell lines, which were significantly altered by 17β-oestradiol.

4.2.11 *Initial Development of Liquid Chromatography Tandem Mass Spectrometry Method for the Detection and Quantitation of Oestrogens in Spent Cell Media Samples*

Liquid chromatography tandem mass spectrometry (LC/MS/MS) is an analytical technique, providing information on structure, molecular mass and concentration of analytes present within a complex sample. The technique utilises separation of analytes by liquid chromatography or HPLC, and detection of these analytes by mass spectrometry (MS). Briefly, the compounds in samples are first separated by UHPLC, where the sample together with mobile phase is pumped through the separation column, resulting in differential elution of compounds, based on their interaction with stationary phase packed inside the column. The separated sample compounds are then immediately introduced into the mass spectrometer. In mass spectrometer, the sample components are firstly ionised, most often using the electrospray ionisation technique (ESI). Here, the sample introduce into the MS is dispersed by electrospray tip into a mist of highly charged droplets, having the same polarity as the capillary voltage, due to strong electric field applied at the tip. The charged droplets travel down a pressure gradient and potential gradient toward the analyser of the mass spectrometer. The temperature of the ESI source together with a stream of drying gas enable the de-solvation of charged droplets, leading to reduction of droplet size, to a critical point, when ions can be ejected in an electric field, resulting in the formation of analyte ions. Resulting emitted ions are then accelerated into the mass analyser, where the charged parental molecules or charged fragments of the analyte are separated in vacuum according to their mass-to-charge ratio by their behaviour within quadrupole analysers. The value of indicator quantity is then recorded by the detector, and signal is transformed into a mass spectrum. In tandem mass spectrometry, a precursor ion is selected based on its mass by a mass analyser. The selected ion then undergoes a gas-phase chemical reaction, resulting in the fragmentation of the ion to product ions of different masses, which are then passed to another mass analyser. The masses of the product ions are detected in the latter mass analyser, generating the mass ion spectrum. Due to the method's numerous advantages, such as small sample volumes, improved specificity and sensitivity, and short analysis times, LC-MS/MS is rapidly replacing other methods for steroid hormone analysis. Importantly, LC-MS/MS additionally offers the identification of unknown analytes present in a sample, based on their molecular weight and structure, determined by fragmentation pattern of the analyte.

As dysregulation of oestrogen metabolism has been associated with the onset of PAH, it is our belief that a technique is required, which could be employed to investigate the changes and/or differences in the oestrogen metabolism in patients and healthy individuals. Albeit numerous techniques are currently available to assess the levels of individual oestrogen metabolites, the considerable number of oestrogen metabolites, having similar structure and exhibiting high tendency for oxidation, severely hinder the separation and identification of these compounds. UHPLC was previously successfully used to investigate oestrogen metabolism by several groups (Rogan et al., 2003;Johansen et al., 2014;Delvoux et al., 2007). Nonetheless, this technique did not readily guarantee quantification of oestrogen metabolites, as it is partially hindered by high detection limits. Moreover, the identification of the metabolites when analysing complex samples might be impeded by the lack of means of metabolite identification. We therefore opted to develop an LC-MS/MS method to assess the profile of oestrogens in *in vitro* model of hPASMCs, which offers numerous advantages over other methods: i) the separation of oestrogen metabolites is not only based on liquid chromatography, but also the differences in metabolite molecular masses and chemical structures, ii) enhanced sensitivity of LC-MS/MS, achieved through the introduction of derivatising chemical groups, enables the detection of oestrogen metabolites on pico-molar level, and iii) the redundancy of radioactively labelled oestrogens introduces cost- and environment/health-benefits. Most often this technique has been used to investigate the levels of parental oestrogens (Nelson et al., 2004b), with Guo and colleagues recently reporting the quantification of 16αOHE1 in human sera samples (Guo et al., 2008).

However, the LC-MS/MS approach to oestrogen metabolism analysis requires the compounds to ionise for them to be separated in the electrical field according to their molecular mass, and oestrogens, as all other steroid-based compounds, do not ionise readily. Although chemical derivatisation, which enhances the sensitivity of the assay, is required, it could potentially introduce errors into the system.

The work presented in this chapter was done in close collaboration with Prof Ruth Andrew and Dr Natalie Homer at University of Edinburgh. The method development was a joint effort by the author and Ioannis Stasinopoulos, who has dedicated his time to establishing and validating the initial technique as a part of his internship research project.

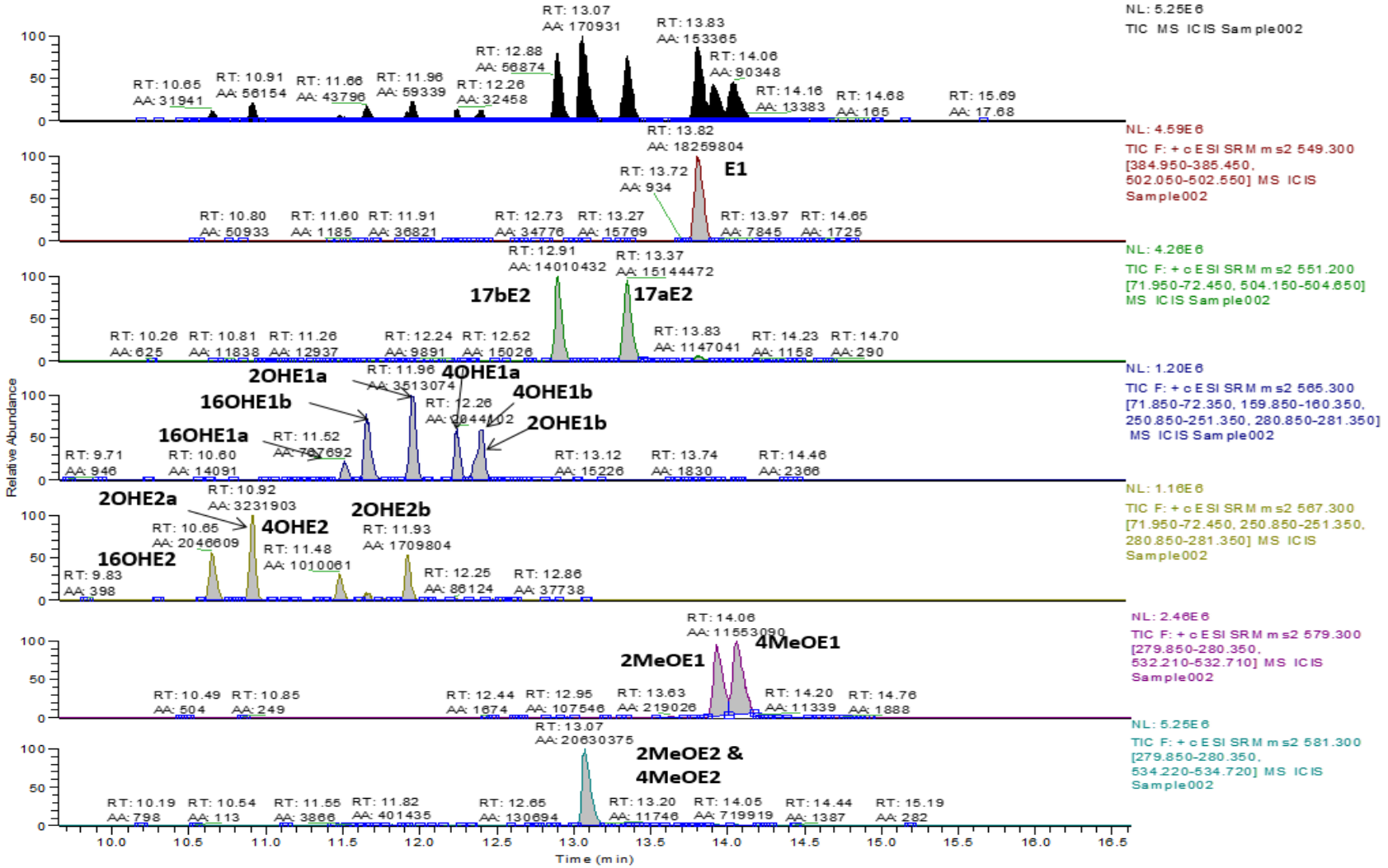


Figure 4–35: Initial liquid-chromatography tandem mass spectrometry method for the detection and quantitation of oestrogen metabolites in in vitro model.

4.2.11.1 Establishing a Method by Initial Liquid Chromatography Tandem Mass Spectrometry

The initial method for quantitation of oestrogen metabolites in aqueous matrix was established by Ioannis Stasinopoulos, who compared available ACE LC columns, by Hichrom Ltd, UK, which were suitable for separation of analytes with steroid character. The most suitable column was selected based on the peak shape and size, and the maximal resolving power achieved for E1 and 17βE2, as assessed by the difference in the retention time for these two parental oestrogens. For the separation of oestrogens, gradient elution was selected, as generally longer gradient times result in larger differences in the retention times of metabolites, and therefore better chromatographic separation.

The ACE Excel C18-AR column was selected for the separation of complex mixture of oestrogen metabolites, with gradient length (t_g) of 660 seconds at 25°C, where the gradient was applied from to increase the organic phase (CH_3CN+0.1% FA (v/v), B) from 10% to 80%, before the system was re-equilibrated to initial 10% of organic phase. Excellent separation of E1, 17αE2, 17βE2, 2OHE1, 2OHE2, 4OHE1, 4OHE2, 16αOHE1 and 16αOHE2 was achieved (Figure 4–35). The separation of methoxy metabolites, however, was not obtained. Considering the important impact of these metabolites in the treatment and prevention of the experimental PH in animal models, we deemed it necessary to alter the method to also be able to detect and quantify methoxyoestrogens.

4.2.11.2 Separation of Methoxyoestrogens was Achieved by Replacement of High-Performance Liquid Chromatography Column

Separation of methoxyoestrogens was attempted by the author by i) decreasing temperature (T=10°C and 15°C), ii) prolonging the gradient time to t_g=23 minutes, and iii) reducing flow rate (0.5mL/min). None of the introduced changes in the LC-MS/MS method resulted in improved chromatographic separation of these metabolites.

Separation of methoxyoestrogens was achieved only by exchange of the ACE Excel C18-AR column for the ACE Excel C18-PFP column (Figure 4–36). Improved selectivity was achieved due to the presence of pentafluorophenyl (PFP) group, due to multiple mechanism of analyte separation. With ACE Excel C18-PFP column, the retention times of

methoxyoestrogens have changed. Specifically, with ACE Excel C18-AR column, the 4MeOE1 eluted from the column after 2MeOE1, while the order of metabolite elution was reversed with ACE Excel C18-PFP column. Importantly, the peaks presenting 2MeOE1 and 4MeOE1 were completely separated. Equally, the replacement of ACE Excel C18-AR column resulted in complete separation of the peaks corresponding to 2MeOE2 and 4MeOE2, which previously eluted from the column simultaneously.

4.2.11.3 Sample Matrix Affects Calibration Curves of Oestrogen Metabolites

As the initial method development was done using aqueous sample matrix, we aimed to determine whether the relationship between the ratio of peak area of an oestrogen metabolite to the peak area of corresponding internal standard with concentration of that metabolite was linear when recovered from cell medium. The ratio of peak areas is used instead of metabolite peak area to account for any losses during solid-phase extraction. We prepared two calibration curves for each of oestrogen metabolites in either aqueous or media matrices in the range of 5 to 2000ng/mL, and extracted these samples as previously described (Chapter 2.5.2). Extracted samples were then derivatised and methylated as described in Chapter 2.7.3.

While the relationship between the concentration and the ratio of oestrogen metabolites to the peak area of corresponding inert standard was linear in aqueous samples, the relationship was not linear in the media sample matrix for all steroids, with particular problems at lower concentrations (Figure 4–37 to Figure 4–41). Nonetheless, in the range between 500 and 2000ng/mL, linearity was obtained for most of oestrogen metabolites in both sample matrices. Therefore, the quantitation of oestrogen metabolites using the LC-MS/MS method might be hindered, as the expected concentrations of oestrogen metabolites are expected in physiological concentrations, i.e. the lower part of the calibration curves.

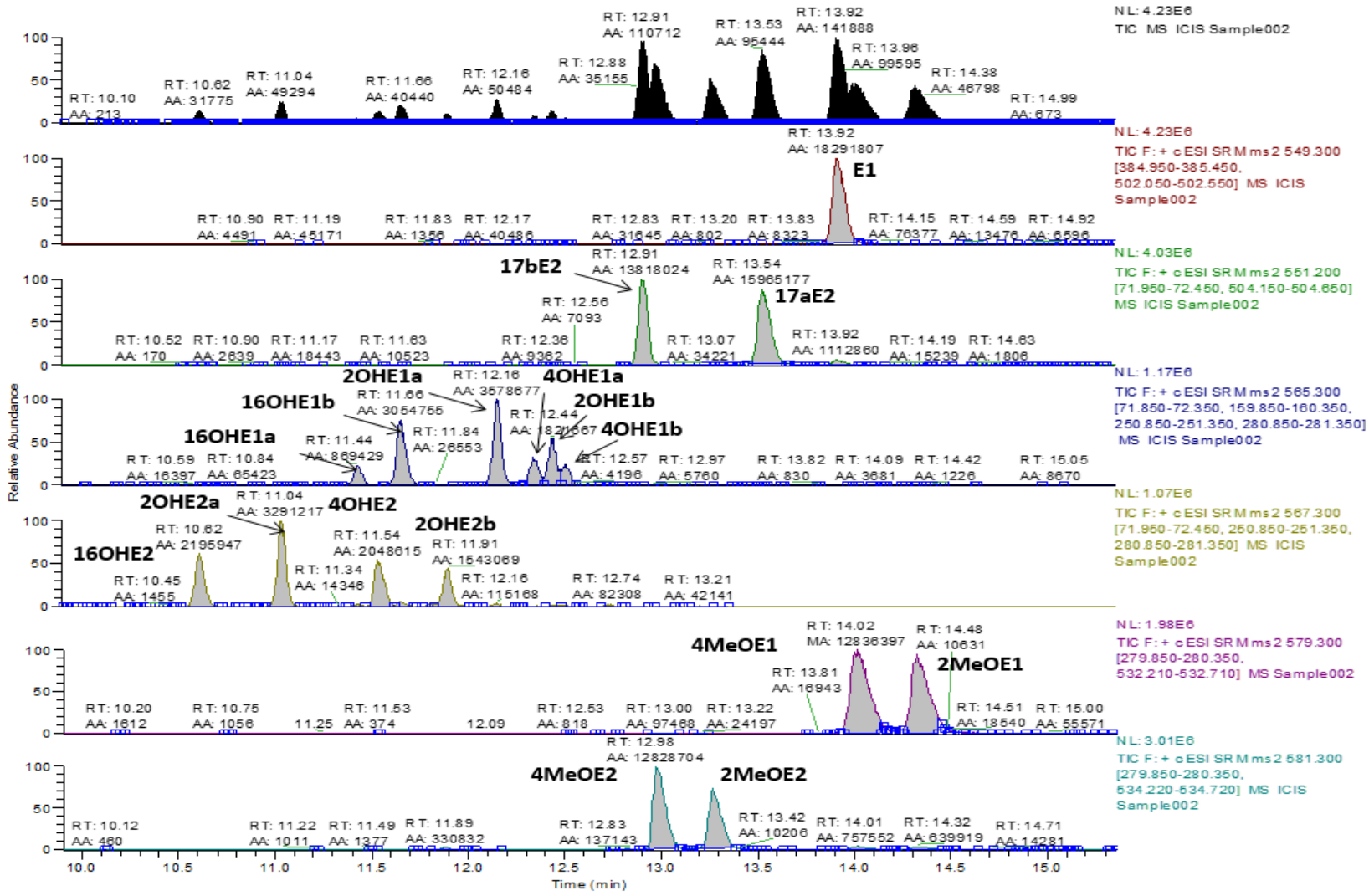


Figure 4–36:Liquid-chromatography tandem mass spectrometry method for the detection and quantitation of oestrogen metabolites in in vitro model, using ACE Excel C18-PFP column.

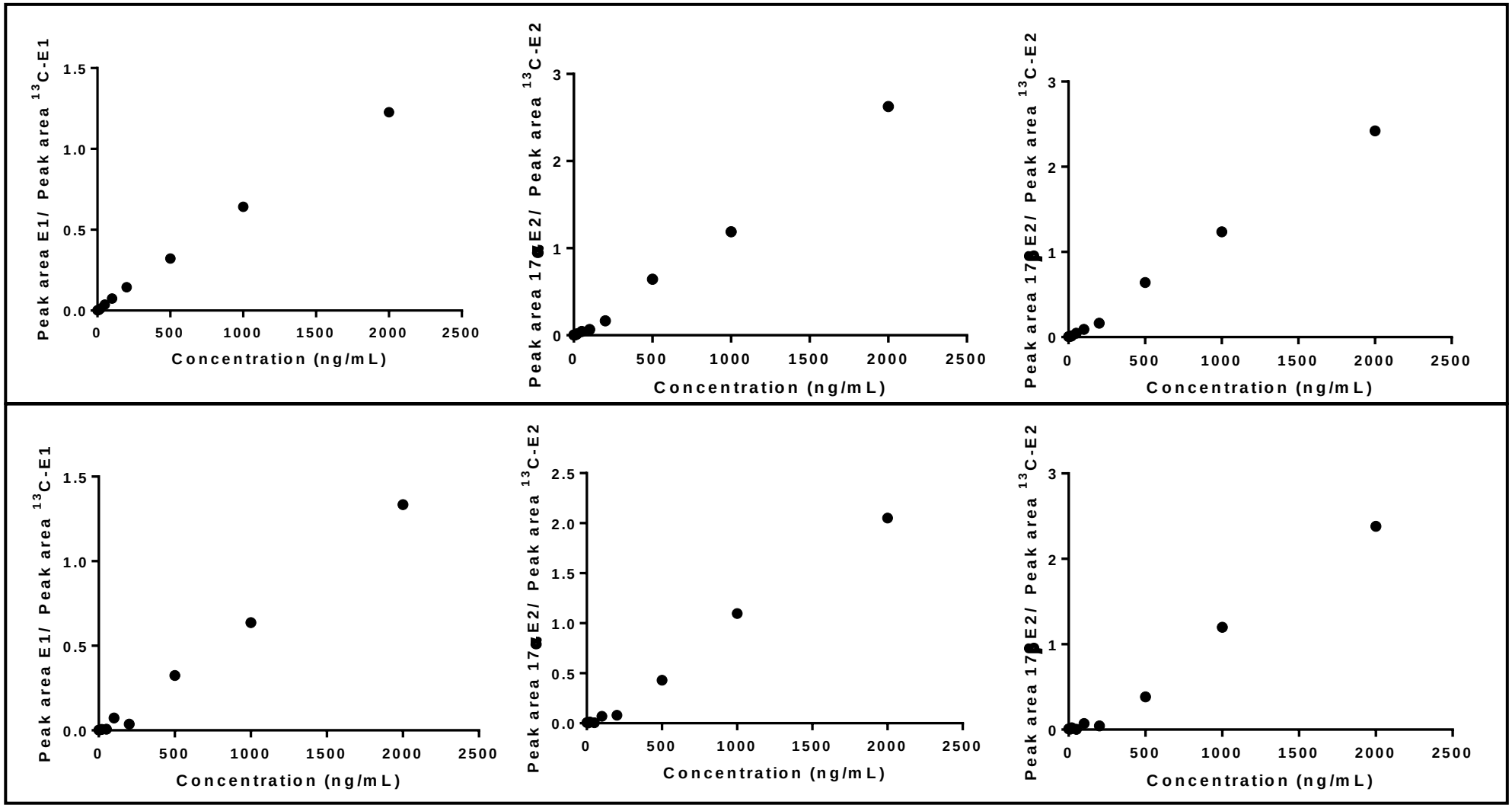


Figure 4–37: Calibration curves for oestrone, 17α-oestradiol and 17β-oestradiol prepared in two different sample matrices (aqueous – above and media – below).

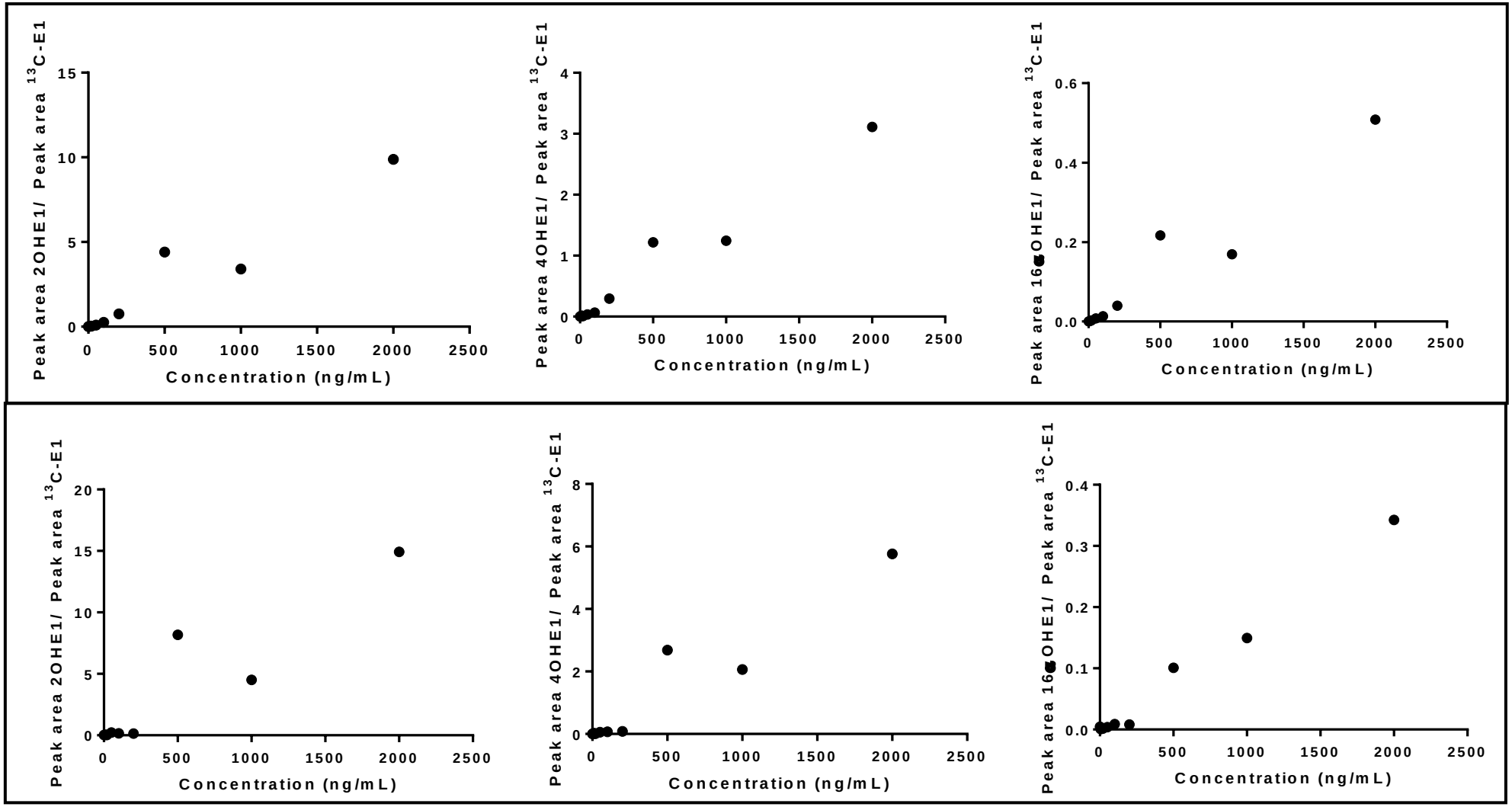


Figure 4–38: Calibration curves for 2- 4- and 16α-hydroxyoestrone prepared in two different sample matrices (aqueous – above and media – below).

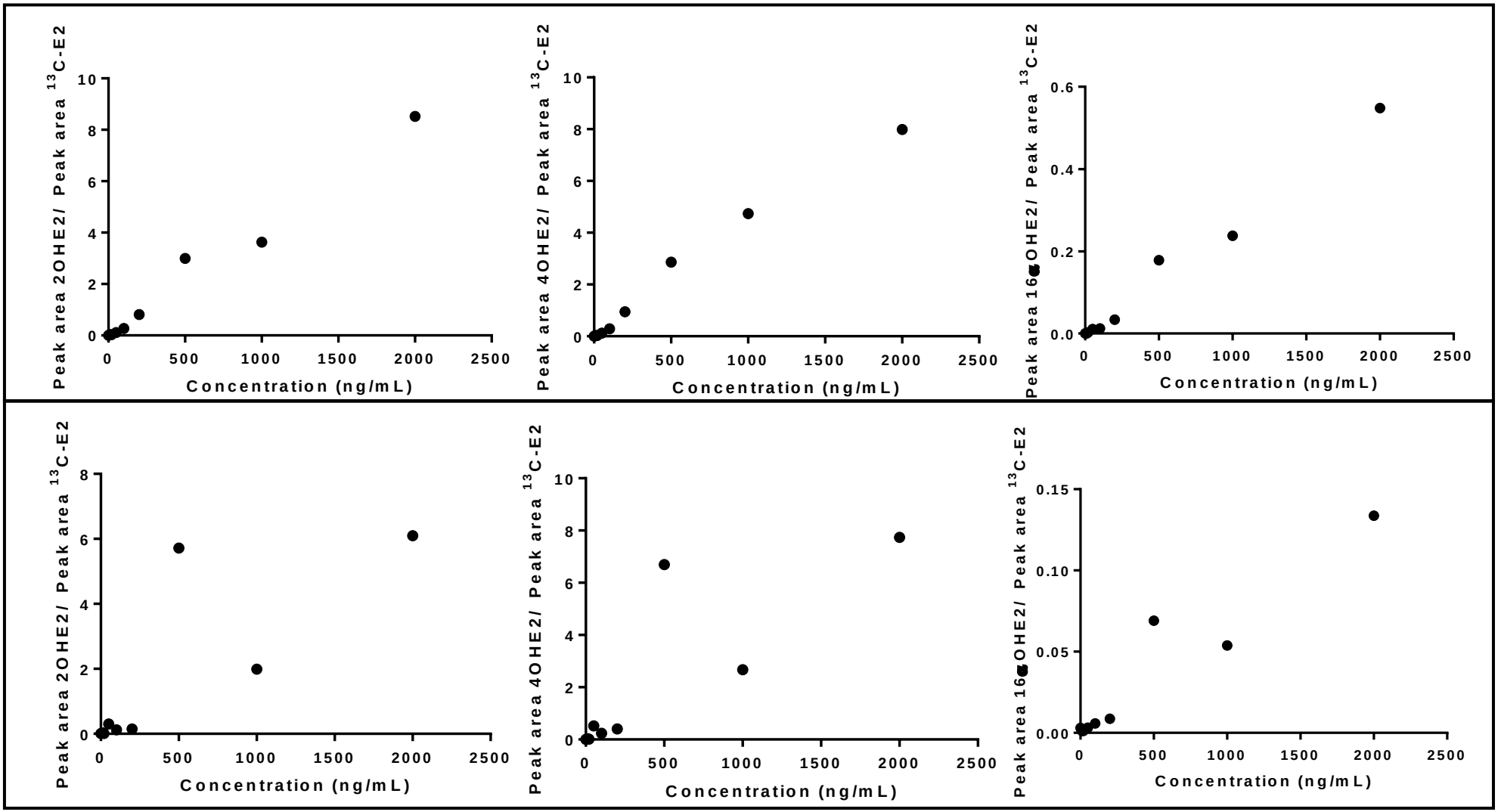


Figure 4–39: Calibration curves for 2- 4- and 16α-hydroxyoestradiol prepared in two different sample matrices (aqueous – above and media – below).

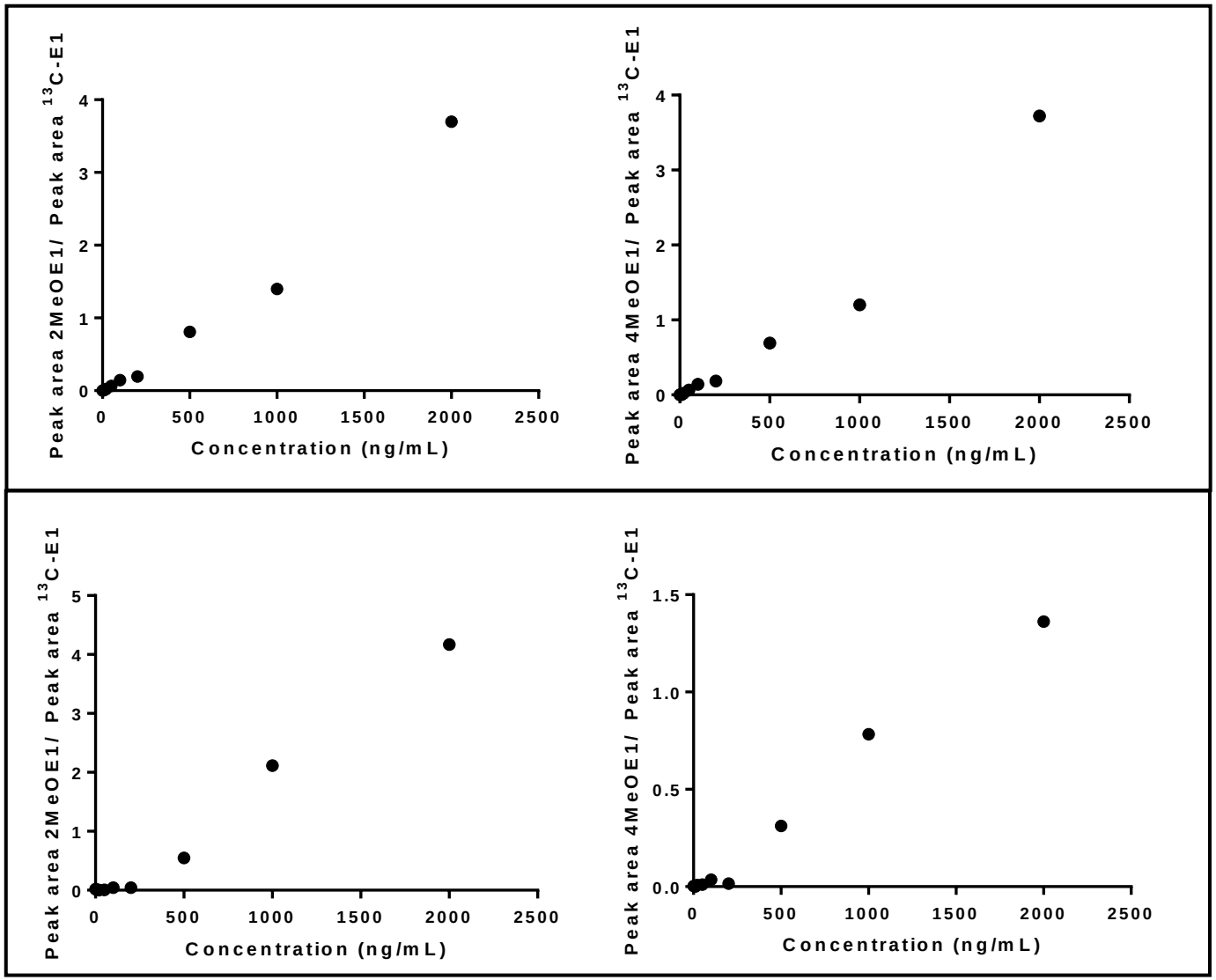


Figure 4–40: Calibration curves for 2- and 4-methoxyoestrone prepared in two different sample matrices (aqueous – above and media – below).

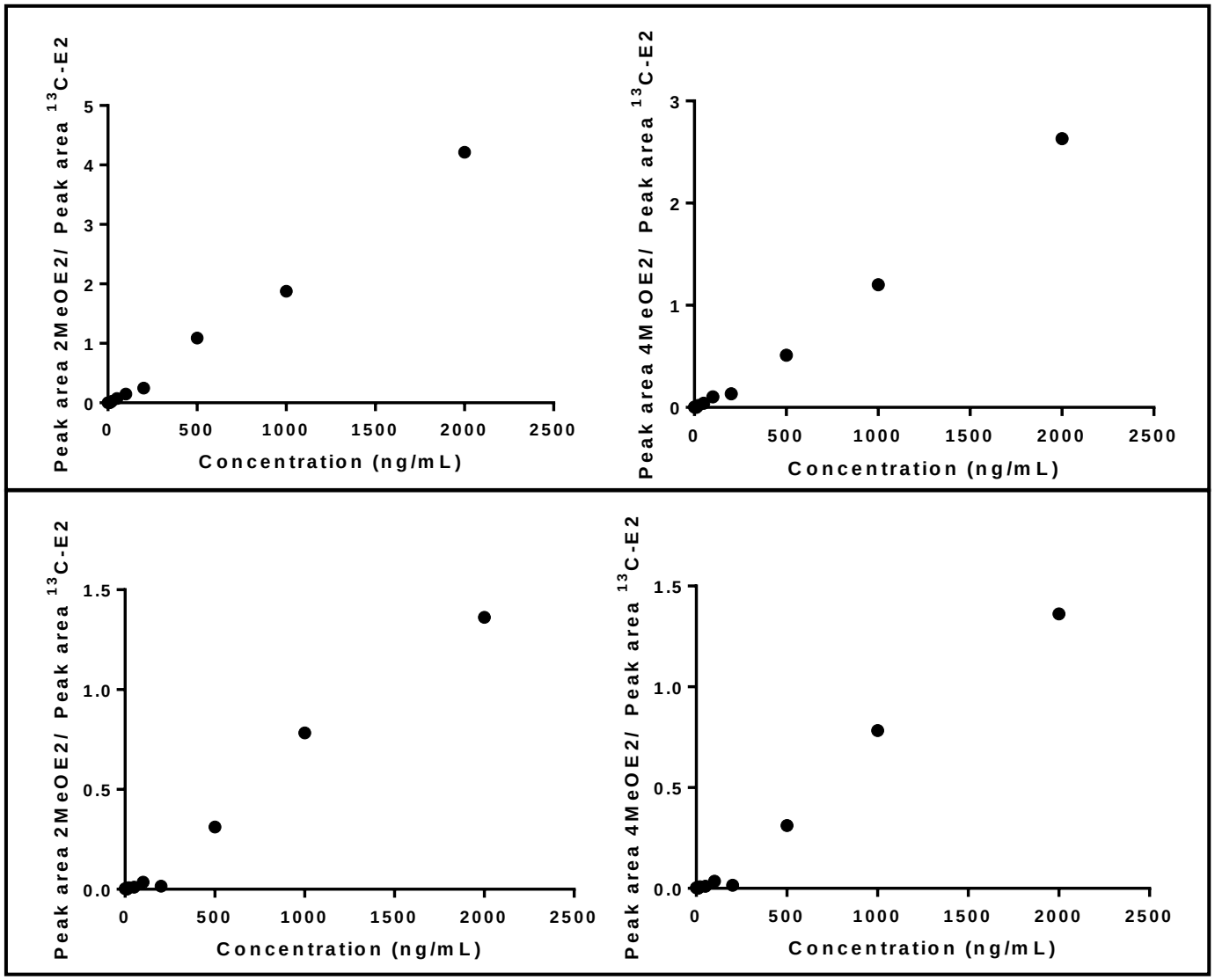


Figure 4–41: Calibration curves for 2- and 4-methoxyoestradiol prepared in two different sample matrices (aqueous – above and media – below).

4.2.11.4 Assessment of Wash Step for Solid-Phase Extraction of Oestrogen Metabolites from Media Sample Matrix

The solid-phase extraction protocol was optimised as previously described in (Johansen, 2014) for use with radio-HPLC. Due to the observed matrix effects of media, we wished to assess whether the extraction protocol may be further optimised. First, we assessed alternative types of solid-phase cartridges. Here we compared the HLB and MCX cartridges where the first cartridge is universal, exhibiting strongly hydrophilic character and can maintain high retention and capacity even if it dries, and the latter is a mixed-mode, strong cation-exchange cartridge, allowing for greater selectively of retention through manipulation of pH. The suitability of cartridges was assessed based on recoveries of oestrogen metabolites and internal standards. To recapitulate, recovery was calculated as the percentage of a given extracted oestrogen metabolite to unextracted metabolite. It follows from the equation, that the best cartridge for SPE is the one offering recoveries close to 100%. Here we showed that although MCX cartridges might offer better recovery of some metabolites, the HLB cartridges exhibit better recovery of internal standards labelled with stable ^{13}C isotope and most of oestrogen metabolites, when MeOH is used for elution (Figure 4–42 and Figure 4–44). Although ACN as eluent resulted in seemingly better recoveries, we observed large variability with the latter eluent. The difference in the recovery of hydroxylated oestrogens is shown in Figure 4–43, where the recovery of these metabolites in HLB extraction cartridges was poorer compared to the MCX cartridges. To circumvent this, samples of spent media could be divided, and applied to both cartridges, although this would inevitably lead to lower metabolite amount per each sample.

We then also assessed the wash steps used to wash out undesired matrix components, which are not bound to the cartridge. We show that the combination of a wash step with 100% H_2O followed by a wash with 5% (v/v) MeOH in water might wash out some of the oestrogen metabolites present in the samples, as the recoveries, especially for more hydrophilic metabolites, were lower in this instance (Figure 4–44B).

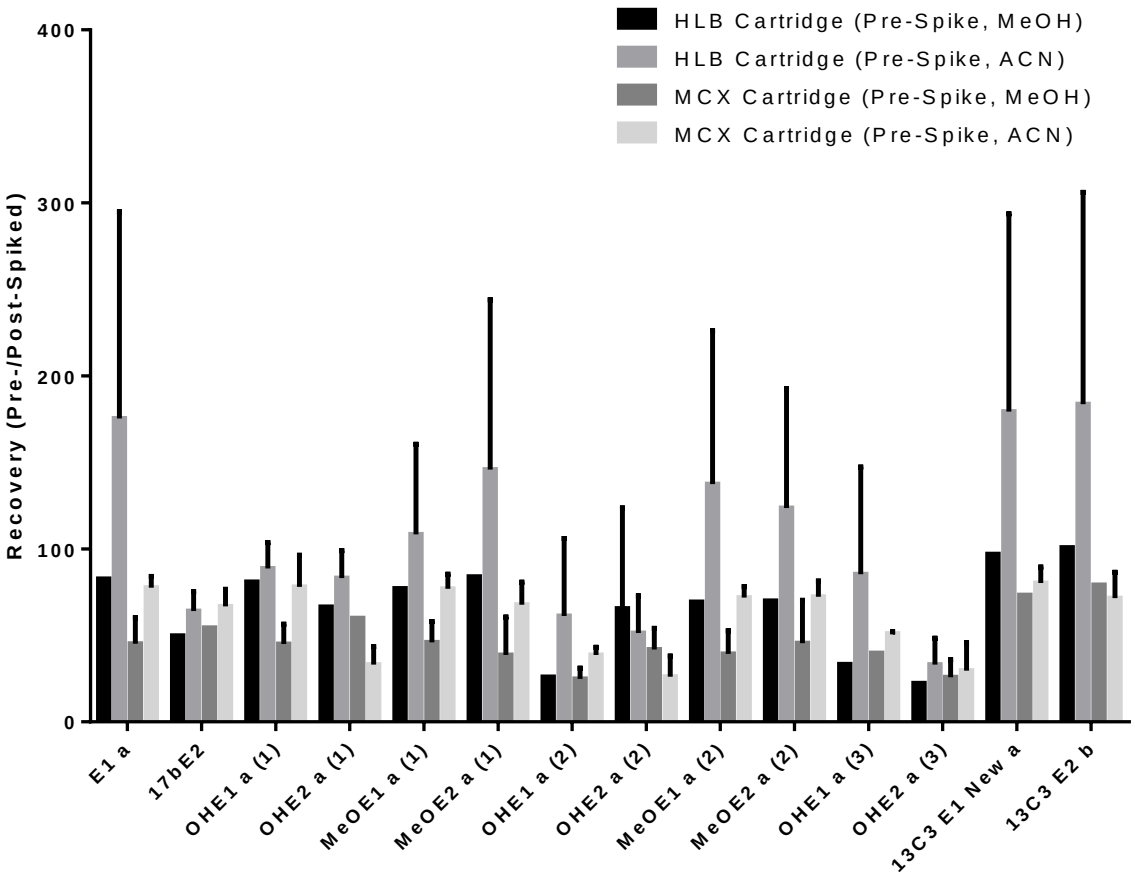
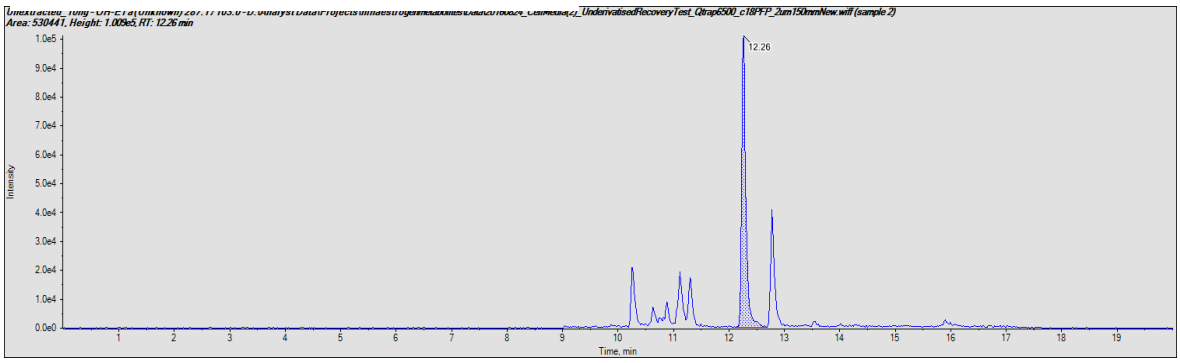


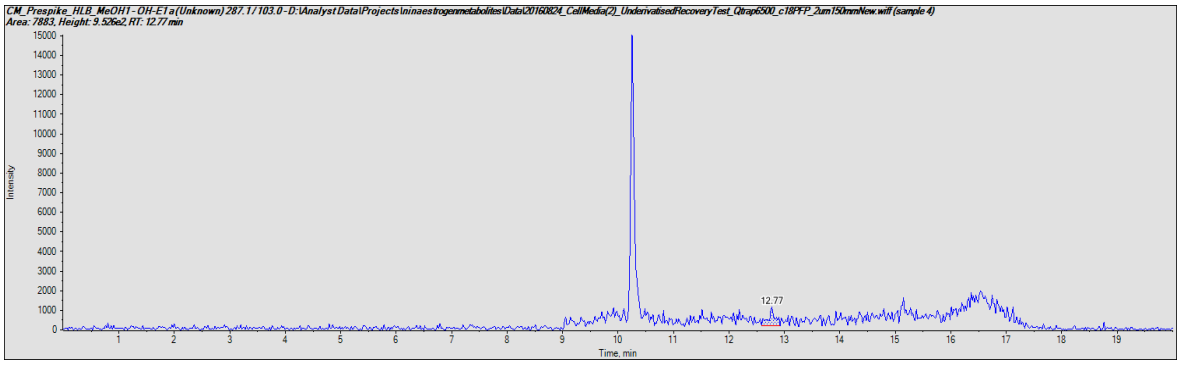
Figure 4–42: The combination of HLB cartridges for solid-phase extraction and methanol as eluent results in less variable recovery of oestrogen metabolites from cell media.

10ng of each oestrogen metabolite was added to 10mL of 1% CSS PRF DMEM media before extraction. MCX and HLB cartridges were used for solid-phase extraction, and either 3mL of methanol (MeOH) or acetonitrile (ACN) was used to elute the oestrogens. The recovery of oestrogen metabolite using solid-phase extraction cartridges Oasis® HLB was calculated by determining the ratio of pre- to post-spiked samples. The recovery of oestrogen metabolites was calculated by determining the ratio of pre- to post-spiked samples. Each extraction was performed in duplicate.

Unextracted Hydroxyoestrones (10ng)



Hydroxyoestrones (10ng) extracted using HLB cartridge (MeOH)



Hydroxyoestrones (10ng) extracted using MCX cartridge (MeOH)

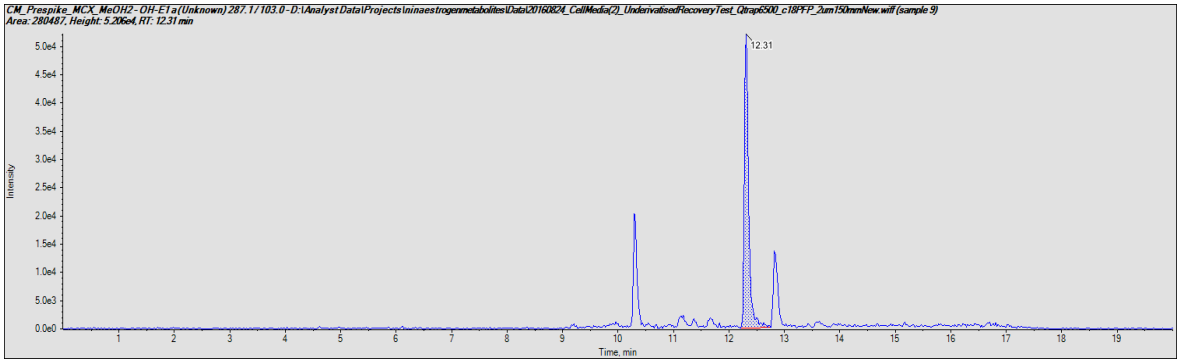
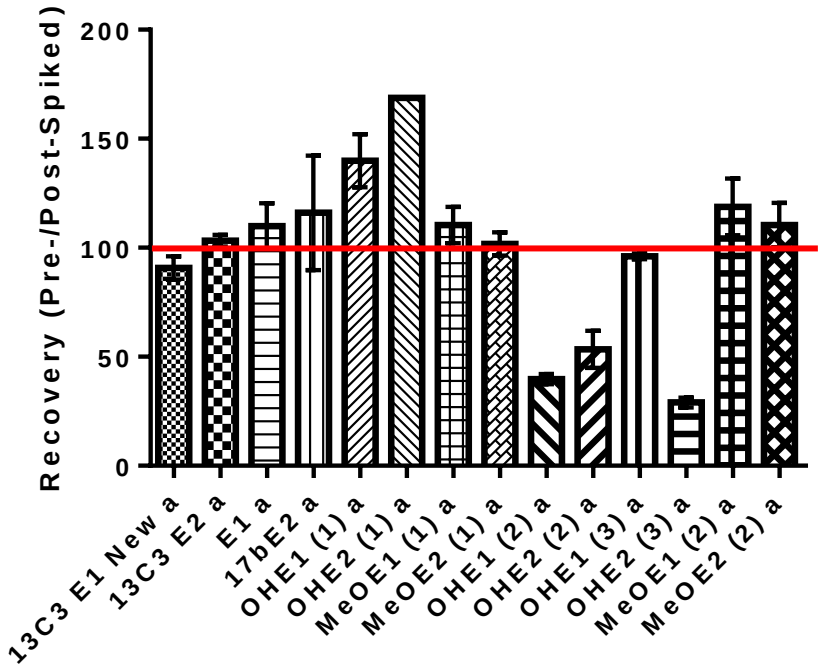


Figure 4–43: Liquid chromatography tandem mass spectrometry of unextracted hydroxyoestrones compared to those extracted using either HLB or MCX solid-phase extraction cartridges.

A



B

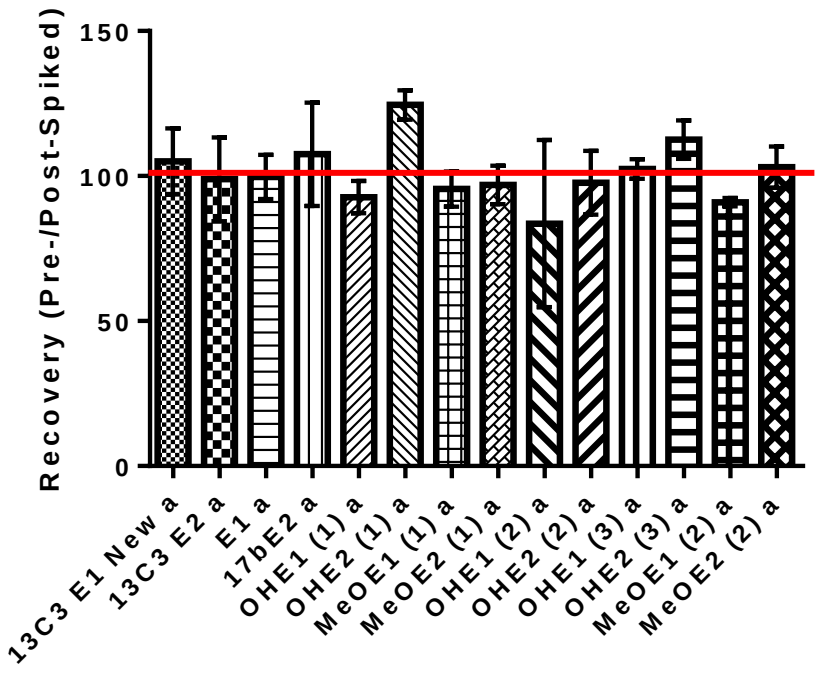


Figure 4–44: Recovery of hydroxy oestrogens is reduced by additional wash step with 100% water.

100ng of each oestrogen metabolite was added to 10mL of 1% CSS PRF DMEM media, either before (pre-spiked) or after extraction (post-spiked). The recovery of oestrogen metabolite using solid-phase extraction cartridges Oasis® HLB was calculated by determining the ratio of pre- to post-spiked samples. A) Solid-phase extraction with 5% (v/v) MeOH wash. B) Solid-phase extraction with 100% (v/v) H_20 and 5% (v/v) MeOH wash steps. Each extraction was performed in triplicate.

4.2.12 *Oestrogen Metabolomic Profile and Quantitation in In Vitro Model of Pulmonary Arterial Hypertension*

4.2.12.1 Calibration Curves for Oestrogen Metabolites of Interest in Pulmonary Arterial Hypertension in Media Sample Matrix

As we showed previously that the media sample matrix might affect the reaction of derivatisation and therefore hinder quantitation of oestrogen metabolites in spent media samples of naïve hPASMCs derived from control and patient subjects of both sexes, we have dedicated special care to construction of calibration curves. Here we show that over a wide concentration range of oestrogen metabolites included in these experiments, we have achieved satisfactory calibration curves as assessed per R-square values, which assesses the closeness of the data to the fitted regression curve (Figure 4–45 to Figure 4–48). Poorer fit of data to the linear regression curve was observed for hydroxylated oestrogens, as seen in Figure 4–46 and Figure 4–47.

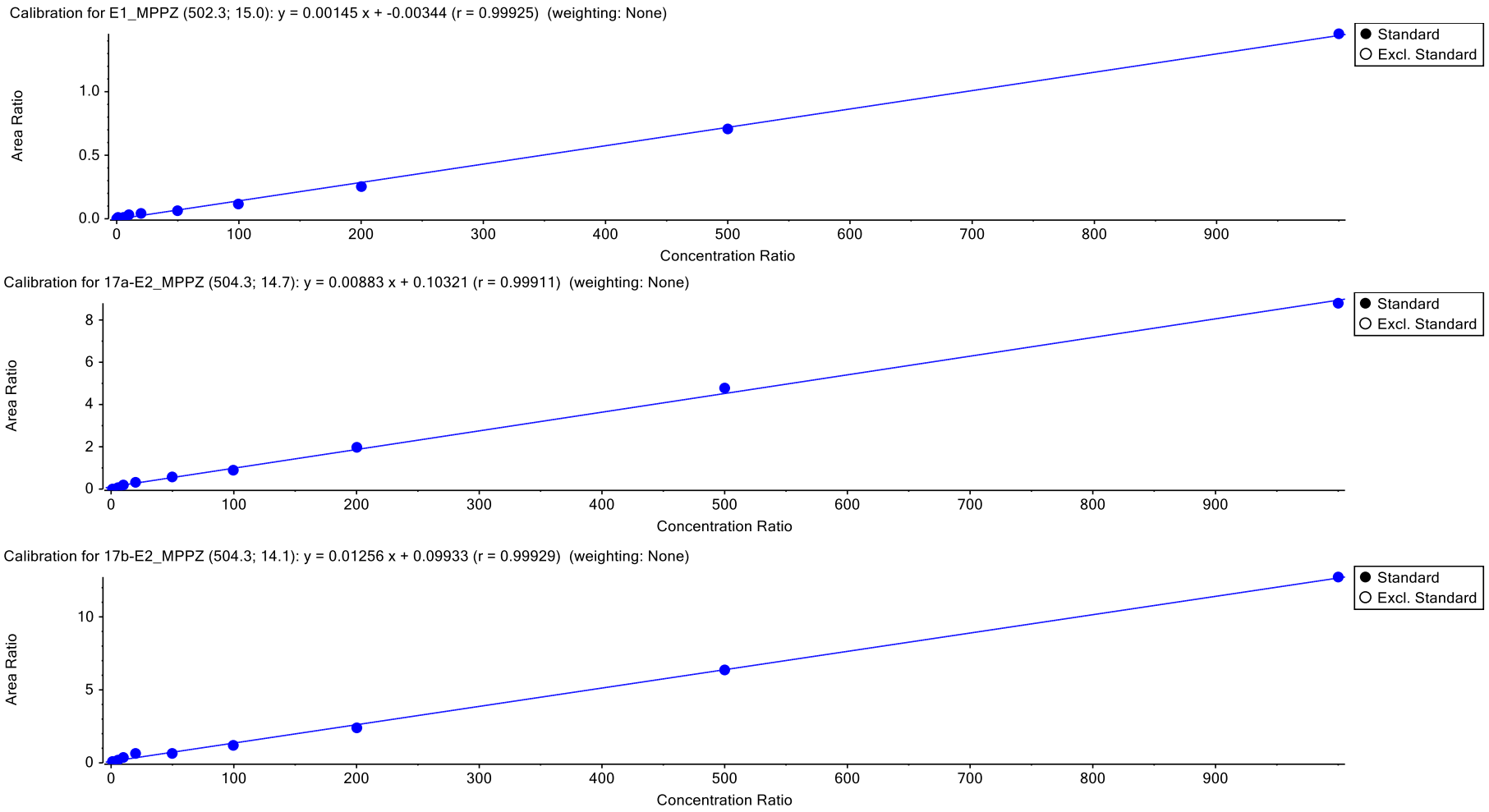


Figure 4–45: Calibration curves for oestrone, 17α-oestradiol and 17β-oestradiol prepared in media sample matrix, and used to quantify oestrogen metabolites in spent media samples of naïve cell cultures of human pulmonary arterial smooth muscle cells derived from control and patient subjects of both sexes.

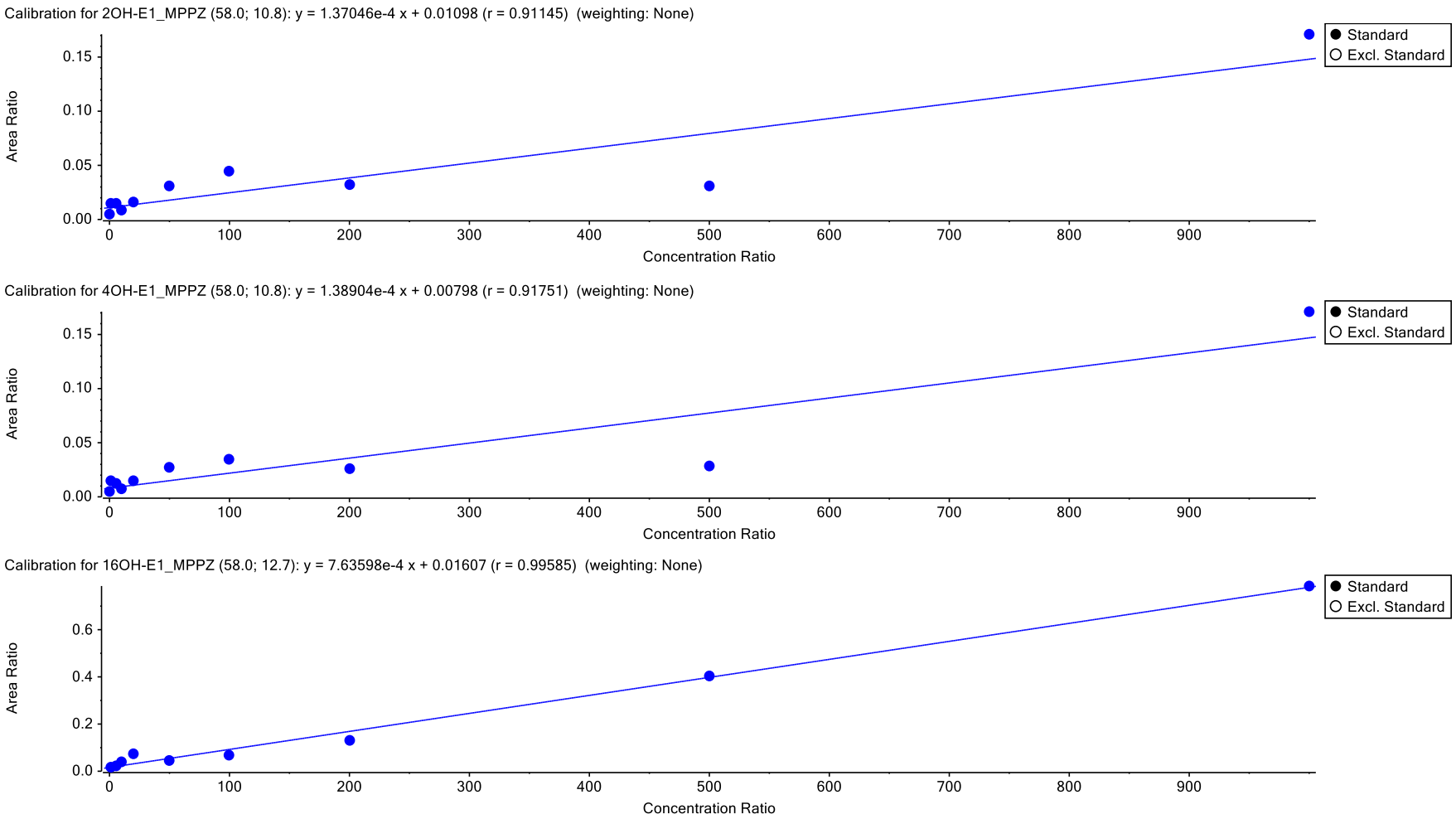


Figure 4–46: Calibration curves for 2-, 4- and 16α-hydroxyoestrone prepared in media sample matrix, and used to quantify oestrogen metabolites in spent media samples of naïve cell cultures of human pulmonary arterial smooth muscle cells derived from control and patient subjects of both sexes.

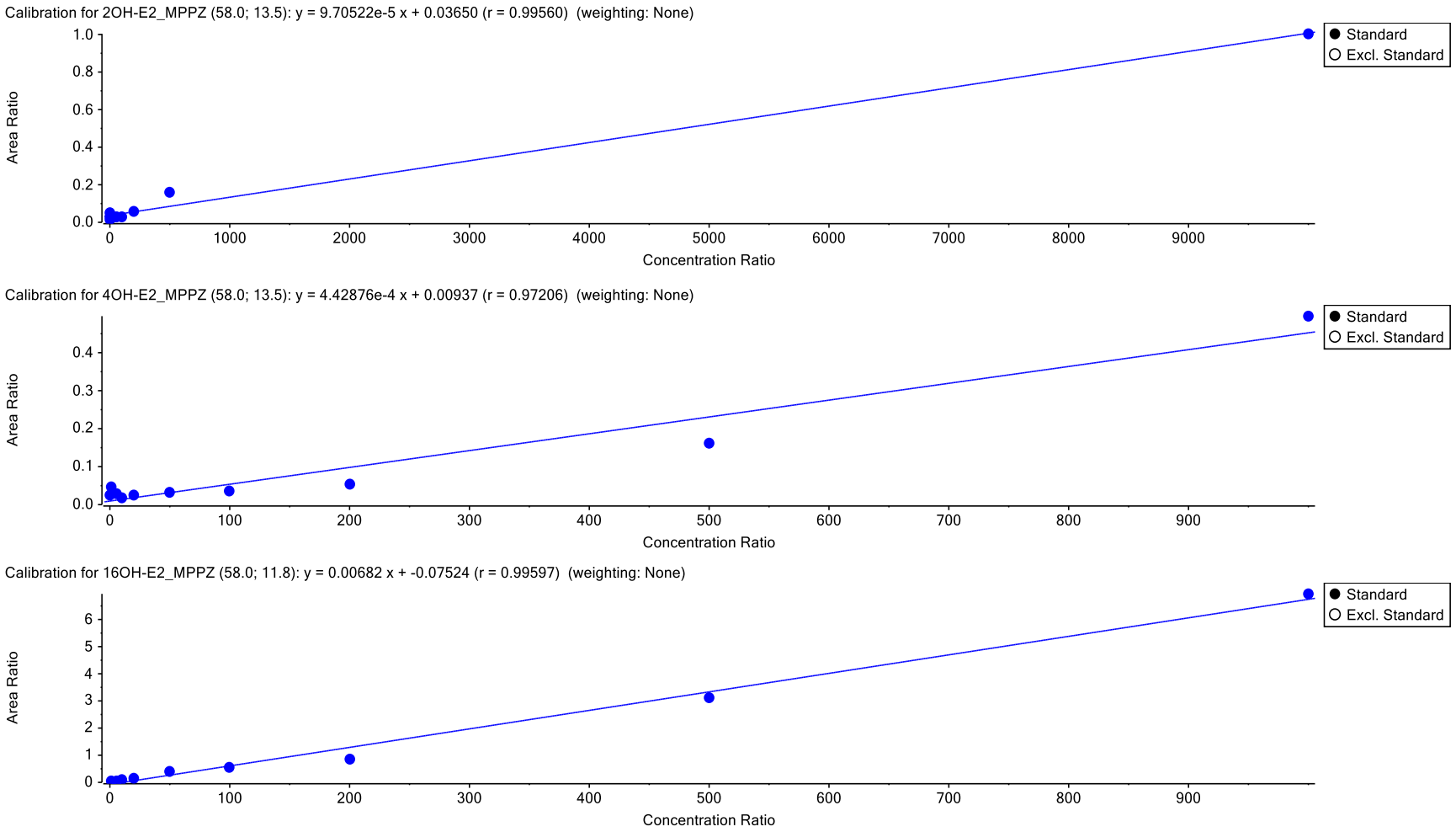


Figure 4–47: Calibration curves for 2-, 4- and 16α-hydroxyoestradiol prepared in media sample matrix, and used to quantify oestrogen metabolites in spent media samples of naïve cell cultures of human pulmonary arterial smooth muscle cells derived from control and patient subjects of both sexes.

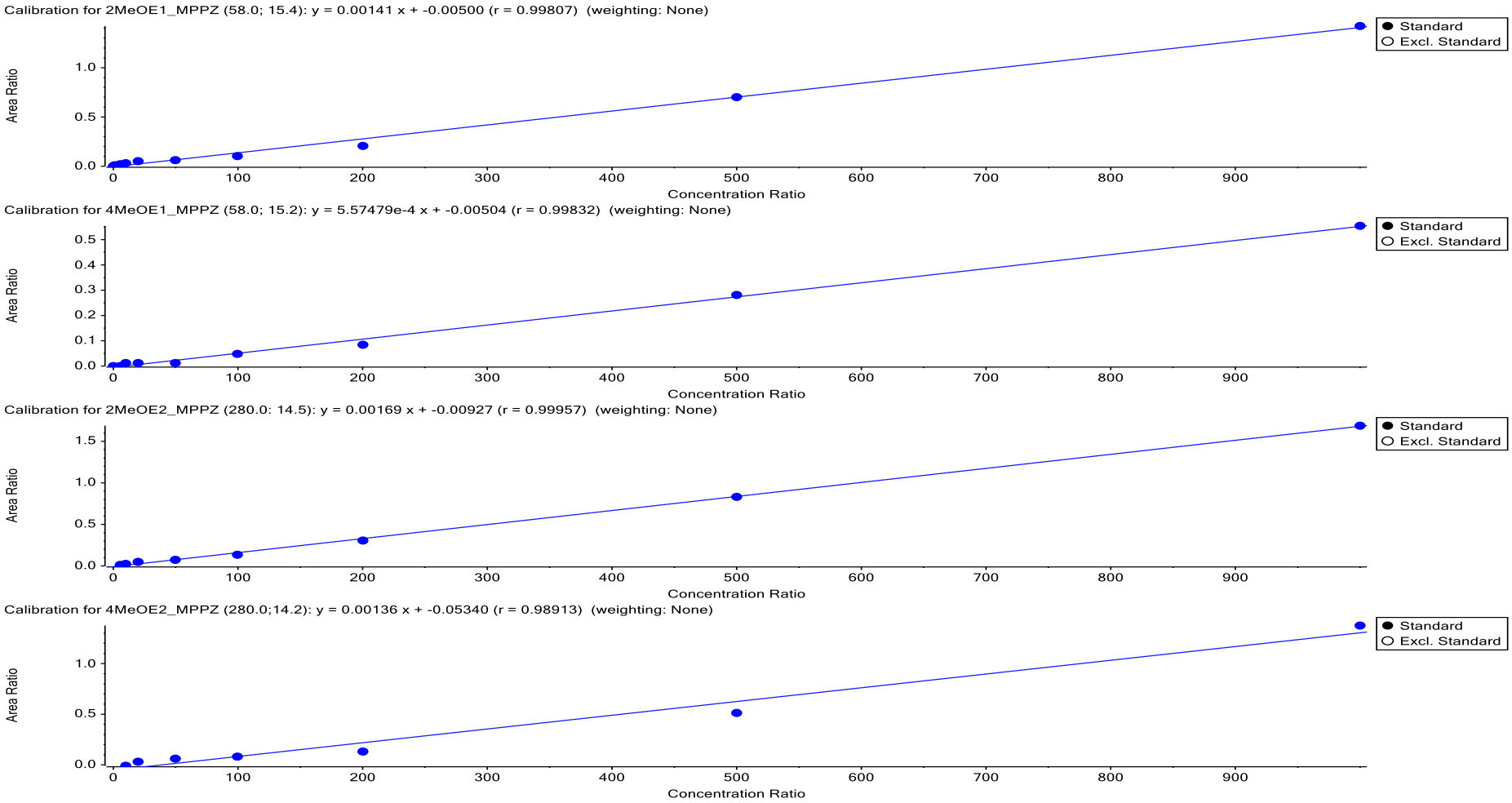


Figure 4–48: Calibration curves for 2- and 4- methoxyoestrogens prepared in media sample matrix, and used to quantify oestrogen metabolites in spent media samples of naïve cell cultures of human pulmonary arterial smooth muscle cells derived from control and patient subjects of both sexes.

4.2.12.2 Oestrogen Metabolic Profile in In Vitro Model of Pulmonary Arterial Hypertension

As the lung is a site of oestrogen synthesis and metabolism, and individual oestrogen metabolites have been shown to convey differential biological effects in PASMCs, we wished to establish a metabolic profile of oestrogens using the LC-MS/MS methodology. The data within this chapter is presented in two ways. Firstly, we showed the complete oestrogen metabolic profile in *in vitro* model of PAH. Next, we compared the levels of individual oestrogen metabolites for hPASMCs derived from control and PAH patient hPASMCs of both sexes, to determine whether there were significant differences in the amounts of these metabolites.

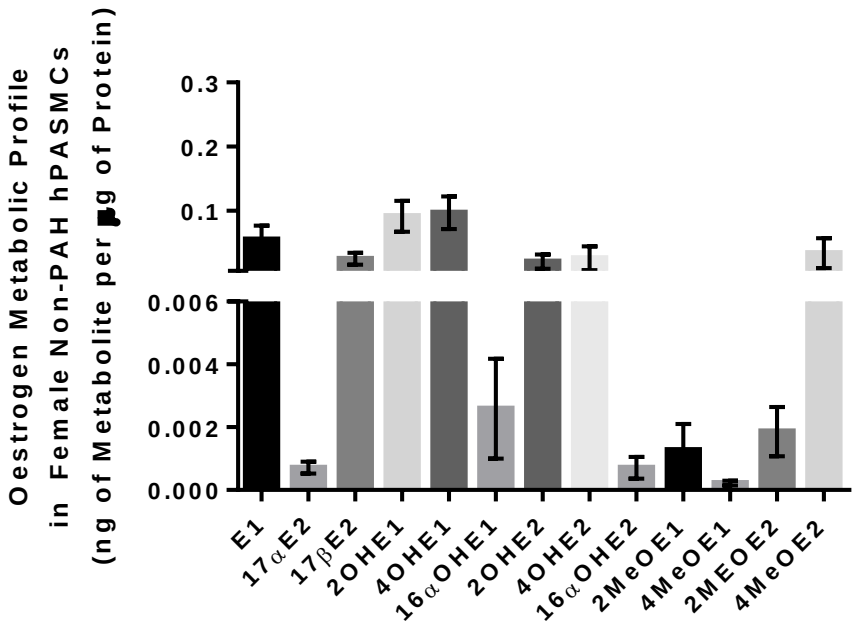
According to the profile, in cells derived from female control subjects, the metabolites present in highest concentrations were E1, 17βE2, 17αE2, 2OHE1, 4OHE1, 2OHE2, 4OHE2, and 4MeOE2 (Figure 4–49A). Although other metabolites are formed in these cells from 17βE2, their amounts were low. The results suggested in these cells oestrogen metabolising enzymes with high affinity for hydroxylation at positions C2 and C4 were present, alongside 17βHSD type 2, which converts highly active 17βE2 to less biologically active E1. Moreover, a significant proportion of 17βE2 was also converted to the geometrical isoform 17αE2, which has been in the past considered to be less biologically active. Whether this is a spontaneous isomerisation, or an enzyme-catalysed reaction is not clear. Considering the numerous extrahepatic CYP enzymes exhibits moderate or high affinity for hydroxylation at position C2, it is perhaps not surprising that we observed relatively high levels of 2OHE1. The presence of 16αOHE1 was also detected, while less of 16αOHE2 was present. In Chapter 1.6.1 we have summarised the catalytic affinities of CYP1A1 and CYP1B1 enzymes. CYP enzymes exhibit specific substrate preference, resulting in differential hydroxylation of E1 and 17βE2. The comparable levels of hydroxylated E1 and 17βE2 indicated that the present CYP enzymes did not exhibit significant preference for their substrates. Among the protective methoxyoestrogens, 4MeOE2 was present in the highest amount of metabolite per μg of protein in female control hPASMCs. The observed amount of 4MeOE2 also indicated relatively high activity of COMT in these cells. While 2OHE1, 4OHE1 and 2OHE2 were shown to be present, we have only measured low amounts of these metabolites. It is unclear why they have not been metabolised by COMT to form corresponding methoxyoestrogens. HPASMCs derived from female PAH patients metabolised 17βE2 mainly to E1, 2OHE1 and 4OHE1, with other metabolites present only

in moderate amounts (Figure 4–49B). Therefore, high enzymatic activity or increased expression of the 17βHSD type 2 was indicated. High amounts of 2OHE1 and 4OHE1 compared to those of 2OHE2 and 4OHE2, indicated there is a shift in the expression of CYP enzymes, favouring E1 as their substrate. In samples from female PAH patient hPASMCs, the production of 16α-hydroxy metabolites was smaller than in female control cell lines. We have observed no significant production of protective methoxyoestrogens in samples from female PAH patient hPASMCs.

In cell lines derived from male control subjects we observed the formation of E1, 2OHE1, 4OHE1, 2OHE2 and 4OHE2. 16αOHE1 and MeOEs were also detected, while other metabolites were present only in minor amounts. The metabolic profile of oestrogens indicated high activity of 17βHSD type 2, CYP enzymes exhibiting high and moderate affinity for hydroxylation at position C2 and C4, with only moderate activity at position C16α. Moreover, considering less of 16αOHE2 was produced, the enzymes appear to favour hydroxylation of E1 at position C16α. The detection of all methoxyoestrogens indicated the activity of COMT. The oestrogen metabolism in male PAH patient hPASMCs appeared to be especially influenced by the enzymatic activity of 17βHSD type 2, which has also been confirmed in female PAH patient hPASMCs (Figure 4–50B). Interestingly, like in female patient hPASMCs, the isomerisation of 17βE2 to 17αE2 appeared to be a significant part of oestrogen metabolism in these cells (Figure 4–50B). Although, the literature on the effects of 17αE2 in humans is very scarce, there are reports of its pro-proliferative effects in human neoplasms (Papendorp et al., 1985;Hajek et al., 1997). Moreover, 17αE2 was also shown to increase aromatase activity in a concentration- and time-dependent manner (Hoffmann et al., 2002). In these cells much of the 17βE2 introduced as the source for oestrogen metabolism has remained unmetabolized.

For easier interpretation of oestrogen metabolite profiles in hPASMCs measured using the LC-MS/MS methodology for *in vitro* investigation of oestrogen metabolism, pie charts are shown in Figure 4–51.

A



B

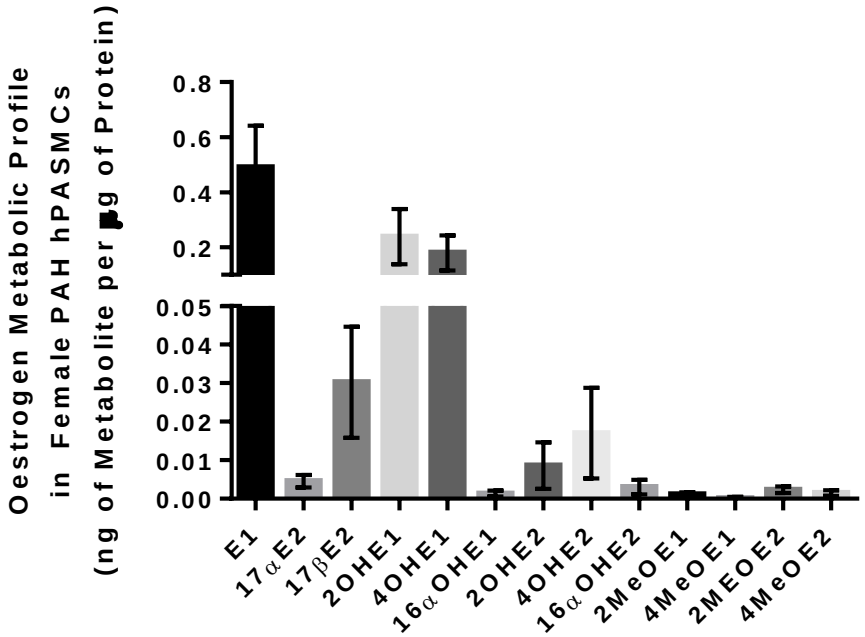
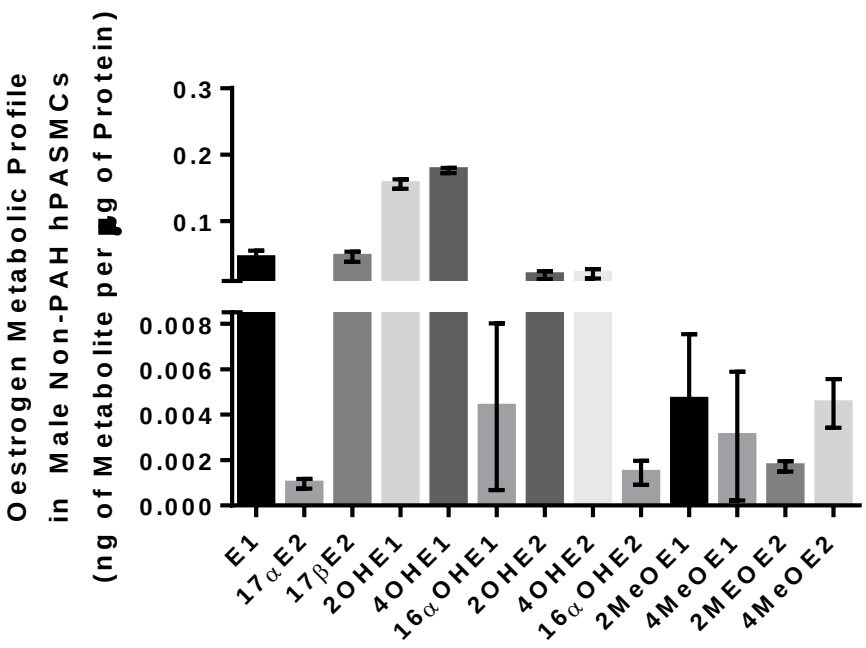


Figure 4–49: Oestrogen metabolic profile in human pulmonary arterial smooth muscle cells derived from females.

Female control and patient hPASMCs were grown to 60-70% confluence, as previously described, and serum starved for 16–24 hours. Cells were then stimulated with 17βE2 (50nM) for 48 hours. The spent media were collected and analysed using LC-MS/MS, as previously described. N=3–5 per group, repeated 1–2 times. Oestrogen metabolic profile was constructed.

A



B

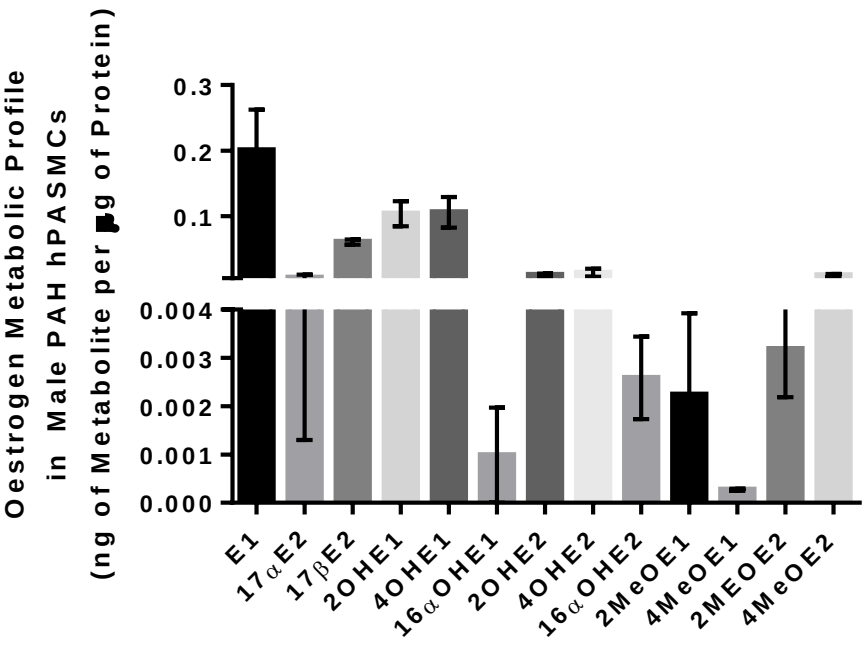


Figure 4–50: Oestrogen metabolic profile in human pulmonary arterial smooth muscle cells derived from males.

Male control and patient hPASMCs were grown to 60-70% confluence, as previously described, and serum starved for 16–24 hours. Cells were then stimulated with 17βE2 (50nM) for 48 hours. The spent media were collected and analysed using LC-MS/MS, as previously described. N=3–5 per group, repeated 1–2 times. Oestrogen metabolic profile was constructed.

To compare the levels of oestrogen metabolites formed between different hPASMCs derived from control and patient subjects of both sexes, we further presented the quantified levels for each of the metabolites. For OHEs, currently achievable limit of detection was relatively high, therefore the measured amounts of these metabolites could not be deemed reliable. We observed that the levels of conversion of 17βE2 into E1, was increased in female and male patient hPASMCs, however only in female cells the increase was significant compared to female controls (Figure 4–52A). Furthermore, the isomerisation of 17βE2 appeared to be increased in patient hPASMCs of both sexes, although the increase was only statistically significant in female patient hPASMCs (Figure 4–52B). It is not clear whether a specific isomerase enzyme is required for this reaction. Interestingly, it appeared that in male patient hPASMCs more of the 17βE2 remained unmetabolized compared to female patient hPASMCs (Figure 4–52C). No statistically significant changes in the formation of OHEs (Figure 4–53 and Figure 4–54) and MeOEs metabolites (Figure 4–55) were detected.

As all the results are expressed in ng of metabolite per μg of protein, we also calculated the amount of 17βE2 introduced as the source of oestrogen metabolism in these experiments, per μg of protein at 48-hour end time-point. The amount of 17βE2 used varied between approximately 0.11 ng/μg protein to approximately 1.1 ng/μg protein. These figures indicated that in certain instances significant loss of either 17βE2 or the metabolites formed from this source had occurred.

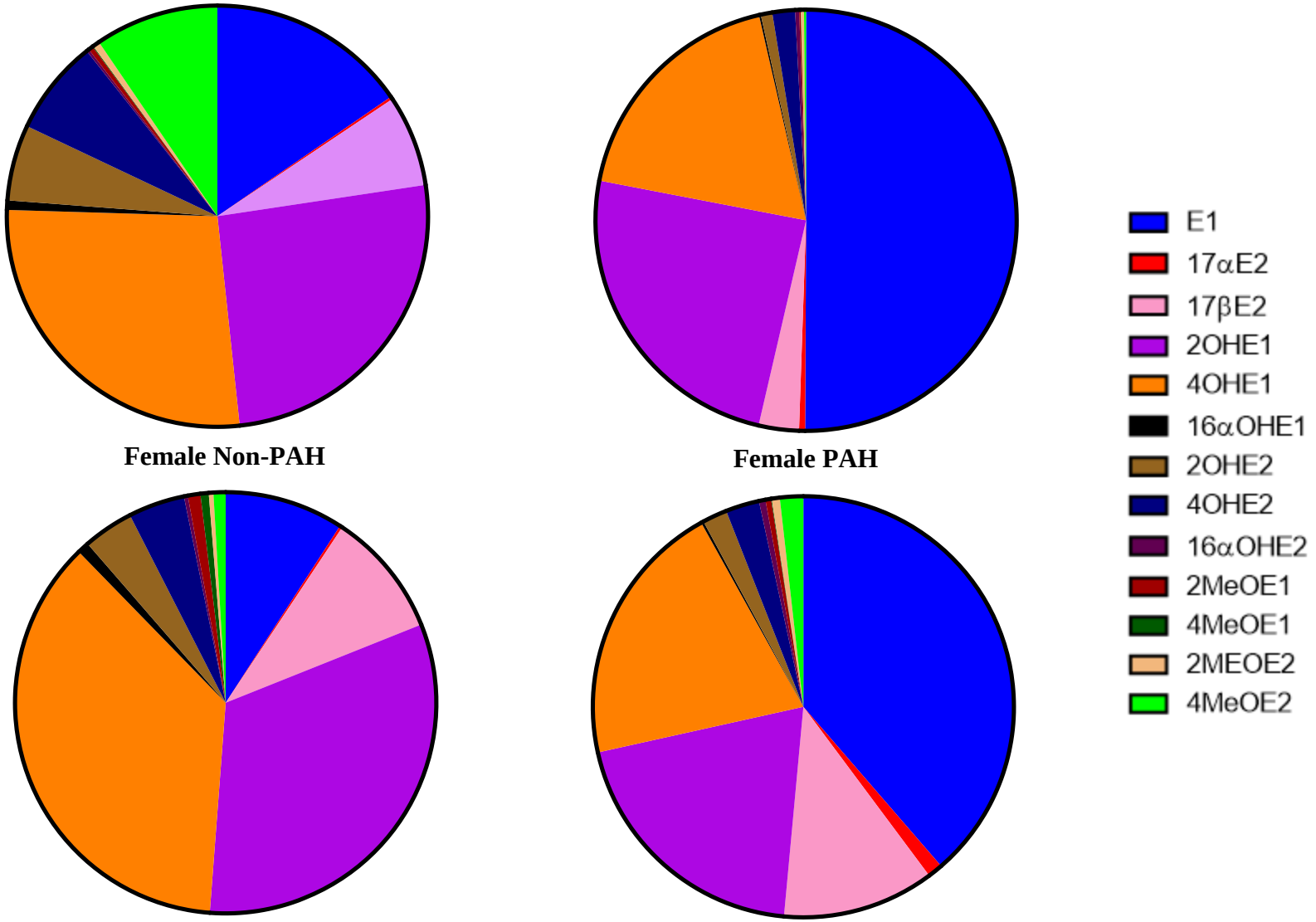


Figure 4–51: Oestrogen metabolite profile of pulmonary artery smooth muscle cells from controls and PAH patients of both sexes, determined using novel LC-MS/MS method to investigate oestrogen metabolism in vitro.

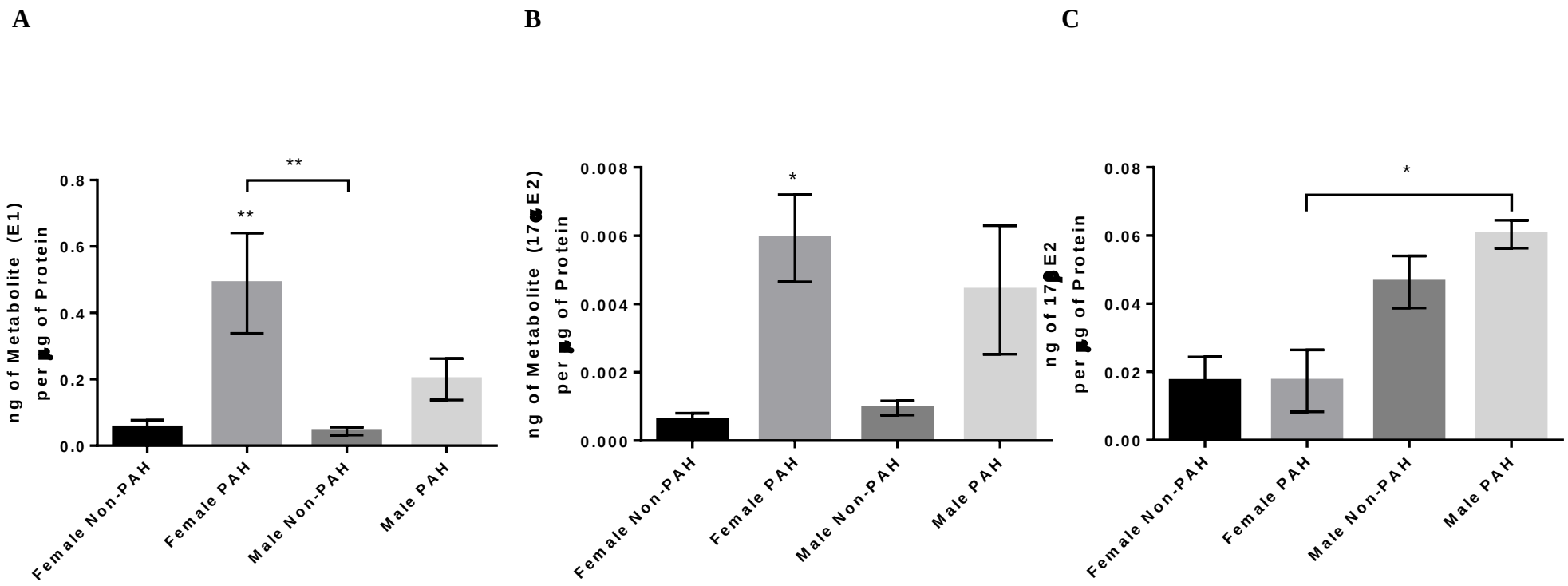


Figure 4–52: Comparison of oestrone, 17β-oestradiol and its 17α geometric isomer quantities in human pulmonary arterial smooth muscle cells determined using the novel liquid chromatography tandem mass spectrometry method.

HPASMCs were grown to 60-70% confluence, as previously described, and serum starved for 16–24 hours. Cells were then stimulated with 17βE2 (50nM) for 48 hours. The spent media were collected and analysed using LC-MS/MS, as previously described. N=3–5 per group, repeated 1–2 times, *p≤0.05, **p<0.01, One-Way ANOVA, followed by Tukey's multiple comparison post-hoc test. Data is expressed as mean±SEM.

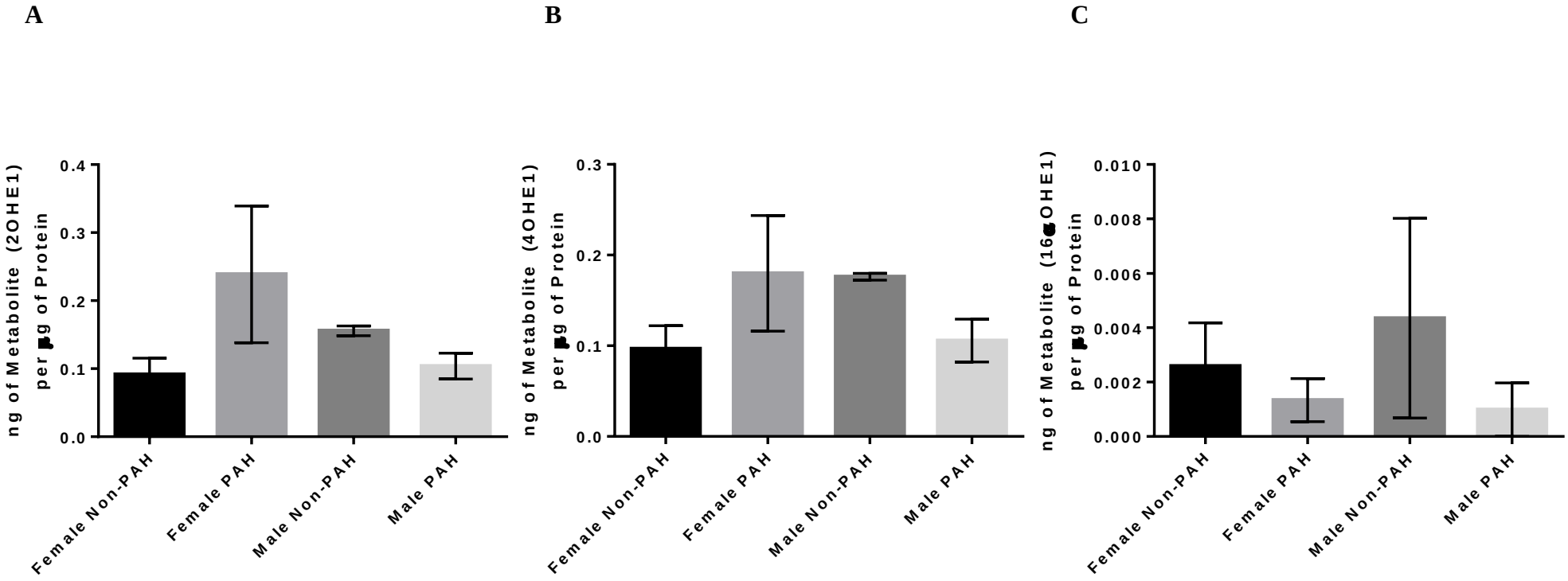


Figure 4–53: Comparison of 2-, 4- and 16α-hydroxyoestrone quantities in human pulmonary arterial smooth muscle cells determined using the novel liquid chromatography tandem mass spectrometry method.

HPASMCs were grown to 60-70% confluence, as previously described, and serum starved for 16–24 hours. Cells were then stimulated with 17βE2 (50nM) for 48 hours. The spent media were collected and analysed using LC-MS/MS, as previously described. N=3–5 per group, repeated 1–2 times, One-Way ANOVA, followed by Tukey's multiple comparison post-hoc test. Data is expressed as mean±SEM.

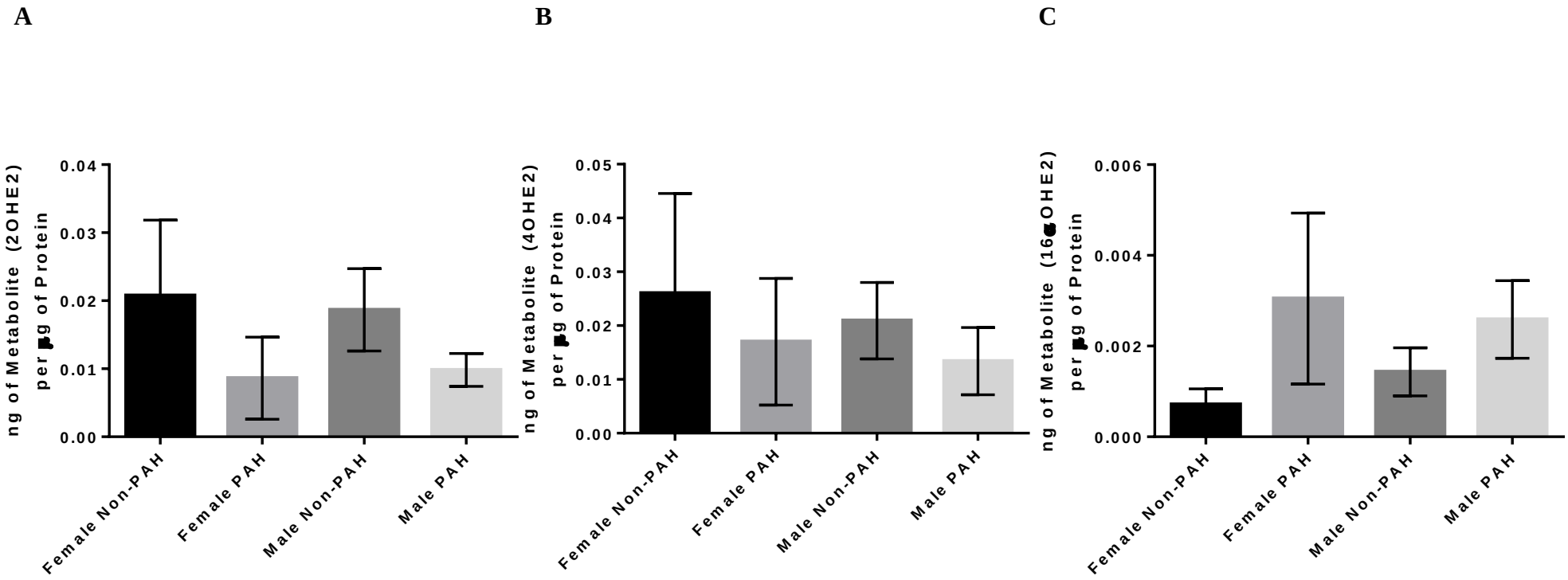


Figure 4–54: Comparison of 2-, 4- and 16α-hydroxyoestradiol quantities in human pulmonary arterial smooth muscle cells determined using the novel liquid chromatography tandem mass spectrometry method.

HPASMCs were grown to 60-70% confluence, as previously described, and serum starved for 16–24 hours. Cells were then stimulated with 17βE2 (50nM) for 48 hours. The spent media were collected and analysed using LC-MS/MS, as previously described. N=3–5 per group, repeated 1–2 times, One -Way ANOVA, followed by Tukey's multiple comparison post-hoc test. Data is expressed as mean±SEM.

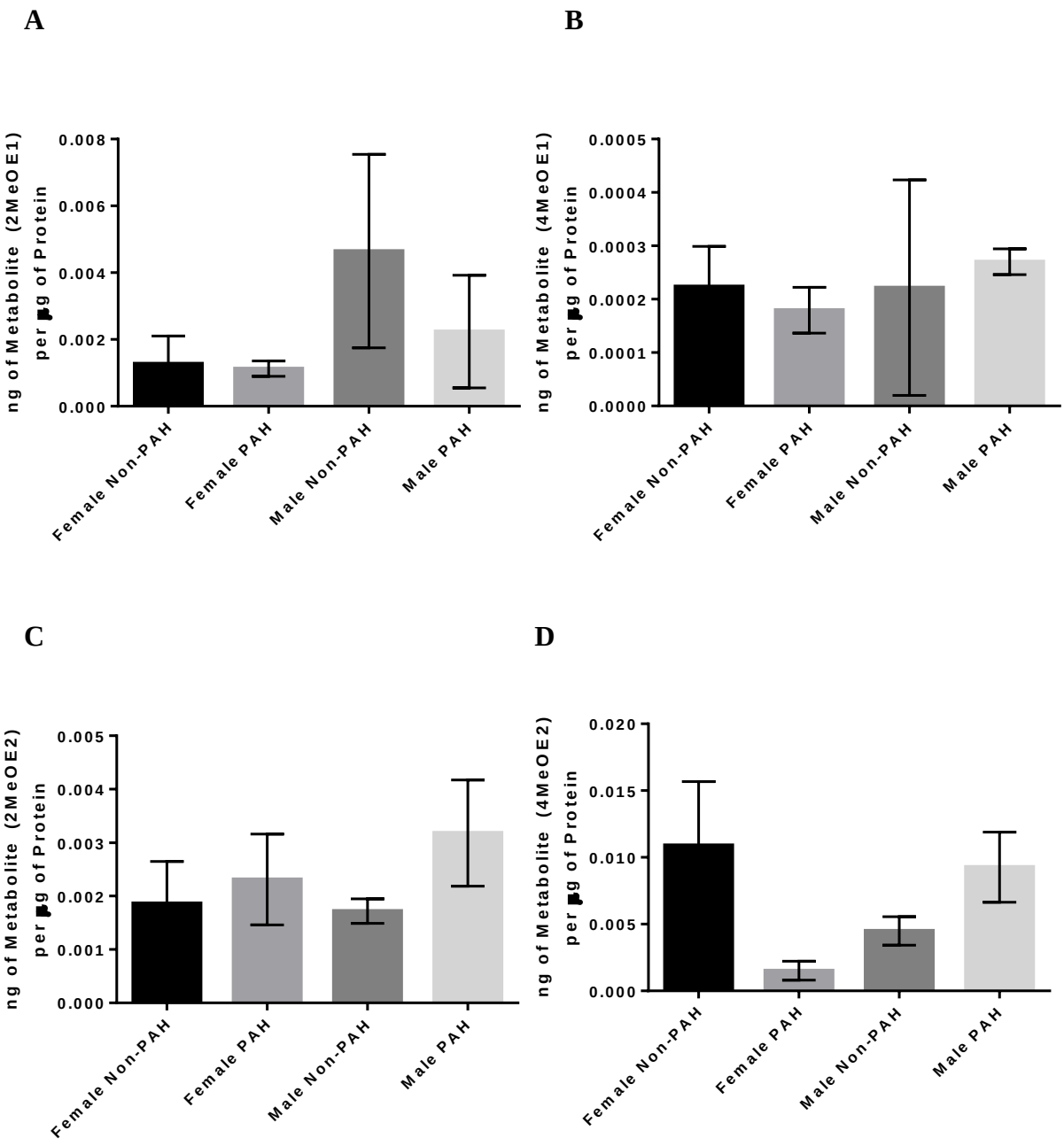


Figure 4–55: Comparison of 2-methoxy and 4-methoxy metabolites of oestrone and 17β-oestradiol quantities in human pulmonary arterial smooth muscle cells determined using the novel liquid chromatography tandem mass spectrometry method.

HPASMCs were grown to 60-70% confluence, as previously described, and serum starved for 16–24 hours. Cells were then stimulated with 17βE2 (50nM) for 48 hours. The spent media were collected and analysed using LC-MS/MS, as previously described. N=3–5 per group, repeated 1–2 times, *p≤0.05, **p<0.01, Two-Way ANOVA, followed by Tukey's multiple comparison post-hoc test. Data is expressed as mean±SEM.

4.3 Discussion

The molecular mechanisms associated with the role of sex in the development of PAH remain largely poorly investigated and understood. It appears that the effects of sex are two-fold. Firstly, the development of the clinical form of PAH is influenced by sex, with significantly higher proportion of women presenting the clinical form of the disease compared to men (Humbert et al., 2010a;Ling et al., 2012;Escribano-Subias et al., 2012). Secondly, according to survival data, women, albeit developing the disease more often, exhibit improved survival compared to men (Escribano-Subias et al., 2012;Humbert et al., 2010b). Women have improved cardiac function as assessed by higher ejection fraction and RV function (Swift et al., 2015;Marra et al., 2016). Moreover, in CVD, the RV function appeared to be better preserved in women compared to men (Cleland et al., 2003), with documented detrimental effects of testosterone and DHEA on RV mass and volume (Ventetuolo et al., 2011). In PAH, the initiation of appropriate therapy does not significantly reduce the deterioration in RV function in men compared to matched women patients (Jacobs et al., 2014).

Within the lung, oestrogen is produced and metabolised locally (Mair et al., 2014;Bieche et al., 2007). As numerous extrahepatic oestrogen metabolising enzymes were shown to be expressed within the lung, the array of metabolites that exert could be exerting their biological effects is considerable. Specifically, 16αOHE1 was implicated in the promotion of proliferation of VSMCs in PAH, while 2MeOE2 was shown to attenuate the proliferation of several vascular cell types (Fotsis et al., 1994;Barchiesi et al., 2006;Tsukamoto et al., 1998;Tofovic et al., 2009;Nishigaki et al., 1995). We hypothesised that in PAH, the local oestrogen metabolism is altered and favours the accumulation of metabolites driving the disease pathogenesis, whilst less of the protective metabolites is formed. We therefore wished to establish oestrogen metabolic profile of VSMCs and quantify the production of oestrogen metabolites. Furthermore, we aimed to assess the effects of oestrogen metabolites on specific aspects of PAH pathogenesis.

In human uterine artery ECs, 2OHE2, 4OHE2, 2MeOE2 and 4MeOE2 induced the expression of PGIS (Jobe et al., 2013). As PGIS is also widely expressed within the lung, we wished to assess whether oestrogen metabolites might be affecting the expression of this enzyme in the lung, and therefore possibly convey protective or pathogenic properties. We showed that in hPMECs, only 2MeOE1 and 2MeOE2 increase the expression of PGIS,

contrary to observations by (Jobe et al., 2013). Considering we report no changes in the gene expression encoding for *PGIS*, it is possible that MeOEs might stabilise the enzyme and protect it from degradation compared to other oestrogens, instead of inducing synthesis *de novo*. When assessing the effects of oestrogen metabolites on the release of PGI_2, we showed that acute stimulation led to significantly decreased expression of 6-keto PGF1α when cells were treated with 16αOHE2 and 2MeOE1, indicating decreased release of its parental molecule PGI_2. Interestingly, the release of 6-keto PGF1α in the positive control group for PGI_2 release (20μM arachidonic acid), was like the one observed in treatment groups 16αOHE2, 2MeOE1 and 2MeOE2. One might argue that it appears that the mentioned metabolites do not affect the release of PGI_2 from intracellular storage. However, as all prostaglandins also PGI_2 is produced as required in response to appropriate stimuli. Therefore, the observed decreased synthesis of PGI_2 in response to acute treatment with 16αOHE2, 2MeOE1 and 2MeOE2 should be further investigated. Prolonged exposure to oestrogen metabolites though did not result in significant changes in the release of PGI_2 levels, probably due to the selected time-points for these experiments. Furthermore, as PGIS is expressed not only in vascular ECs, but also in VSMCs, we assessed the effects that oestrogen metabolites might have on the expression of PGIS in PAs. Treatment of hPASMCs derived from male control subjects with pharmacological levels of 2MeOE2 resulted in increased expression of PGIS, whilst no effect was observed in cell lines derived from females. Hence, it appeared that 2MeOE2 effects on the expression of PGIS might be sex-dependent in VSMCs.

The protective properties of 2MeOE2 were first described in cancer research, where treatment with this metabolite opposed tumour progression by having antiproliferative and antiangiogenic activity (Sutherland et al., 2005) in numerous types of tumours, including lung (Huober et al., 2000). The protective effects in the lung were also described in PAH, where 2MeOE2 was found to prevent and attenuate the development of experimental PH phenotype in MCT, belomycine-induced and Su5416/Hx model (Tofovic et al., 2009;Tofovic et al., 2008b;Tofovic and Rafikova, 2009). We therefore wanted to assess the antiproliferative properties of methoxyoestrogens in *in vitro* model of PAH and determine whether the protective effects are sex-dependent. We treated PASMC cell lines derived from control subjects of both sexes with 1nM and 100nM MeOEs. We demonstrated that 2MeOE1, 2MeOE2 and 4MeOE2 reduce proliferation in control PASMCs of both sexes, at both concentrations. According to radioligand binding assays, the affinity of MeOEs for ER at physiological concentrations are not significant, compared to the parental hormone and

catechol oestrogens (Merriam et al., 1980). It is however noteworthy, that normal physiological concentration of 2MeOE2 is supposed to be <0.1nM. We therefore wished to assess whether some of the antiproliferative properties might be mediated through classical oestrogen signalling. Our results indicated that in male control hPASMCs, inhibition of ERα, ERβ and gpr30 restored proliferation, in the presence of 2MeOE2. Moreover, the antiproliferative properties of 4MeOE2 were only opposed by inhibition of both classical ER. Similar response was documented in female control PASMCs. Interestingly, 2MeOE2 only attenuated cellular proliferation in female patient PASMCs, while it had no effect on male patient PASMCs. The research showing the protective activity of 2MeOE2 as determined by prevention and/or attenuation of experimental PH phenotype was done in female animals only, therefore no description of *in vivo* effects of 2MeOE2 in male exist. Treatment of hPASMCs with 2OHE2 and 4OHE2, precursors in the formation of antiproliferative MeOEs, led to attenuated proliferation of these cells. Herein we showed that both catechol oestrogens decrease serum-induced proliferation of hPASMCs. To establish whether the observed attenuation of proliferation is due to the metabolism of catechol oestrogens to methoxy metabolites, we have pre-stimulated hPASMCs with COMT inhibitors. Indeed, our results indicate that inhibition of the conversion of 2OHE2 into corresponding methoxyoestrogen, at least restores cellular proliferation. Interestingly, inhibition of COMT only partially restored proliferation in the presence of 4OHE2, indicating that 4OHE2 might confer its anti-proliferative properties through other mechanisms. Due to reported high binding affinity of catechol oestrogens to classical ER, we also explored whether these metabolites might mediate some of their effects through ER signalling. 2OHE2 and 4OE2 have similar affinity for both ER isoforms, which is 5–10-fold lower than that of the parental hormone 17β-E2 (Schutze et al., 1994). The presence of ERα and ERβ antagonist, or the dual ER antagonist ICI 182 780, resulted in restored proliferation of hPASMCs in the presence of 2OHE2, while in the presence of 4OHE2 the proliferation was only restored by ERα antagonist. It appears that 4OHE2 may convey its effects through the ERα receptors. ERα is increased in hPASMCs derived from female PAH patients, therefore the anti-proliferative properties of 4OHE2 might not be associated with the amount of the present receptor but affect cellular proliferation through a different mechanism. The 4-OHEs were also reported to potentially activate ER (Martucci and Fishman, 1976), and possibly convey prolonged biological effects due to slower dissociation rates from receptors (Zhu and Conney, 1998;Barnea et al., 1983).

The BMPR*II* signalling pathway is intimately involved in the pathogenesis of PAH, and its dysregulation resulted in proliferation of PASMCs (Perez et al., 2011;Nakaoka et al., 1997) (Zhang et al., 2003) and apoptosis of PAECs (Teichert-Kuliszewska et al., 2006). 17βE2 reduced BMPR*II* signalling, in Hx through HIF1α (Ichimori et al., 2013) and ERα signalling (Fessel et al., 2013). Additionally, sex appeared to be associated with differential expression of proteins participating in the BMPR*II* signalling pathway, with PASMCs from female control subjects exhibiting lower levels of mRNA and protein expression of BMPR*II*, Smad1, Id1 and Id3 (Mair et al., 2015). We therefore wished to assess whether different oestrogen metabolites might affect the expression of components of the BMPR*II* pathway. We first conducted a preliminary assessment of 13 oestrogen metabolites, showing that methoxyoestrogens might increase the expression of BMPR*II*, pSmad1/5/8(9) and Id1. We further showed that in male PASMCs from control subject, 2MeOE2 significantly increased the expression of Id1, while the increase in female PASMCs was not statistically significant. The alterations in the expression of other components of the BMPR*II* signalling pathway were not statistically significant due to large variation in the response between cell lines. Interestingly, increased expression in Id1 was observed at higher concentration of 2MeOE2, while we previously observed antiproliferative effects of this metabolite at lower concentrations. The p27/Kip1 protein is a tumour suppressor, belonging to cyclin-dependent kinase inhibitors (CKIs), which bind to various cyclin/cycle dependent kinase (CDK) complexes throughout the cell cycle and negatively regulate cells' progression through cell cycle. p27/Kip1 primarily regulates CDK activity in response to extracellular signals, such as DNA damage, TGFβ, resulting in the formation of inhibition of cyclin E-CDK2 complexes and phase G_1 cell cycle arrest (Polyak et al., 1994;Koff et al., 1992;Kato et al., 1994). The expression of p27/Kip1 was significantly reduced in high serum environment, indicating VSMCs were progressing through cell cycle and proliferating, while overexpression of this tumour suppressor reduced cell proliferation (Fouty et al., 2001). Considering vascular remodelling is essential in the pathology of PAH, the role of p27/Kip1 in disease development might be important due to its role in the regulation of PASMCs proliferation. Previously, treatment of rats exposed to cHx with 17βE2 resulted in increased expression of p27/Kip1 animal lung tissue and cultured PASMCs (Xu et al., 2010). The authors summarised the mechanism by which 17βE2 might rescue the experimental PH phenotype in these animals might at least partially depend on increased expression of p27/Kip1. Moreover, the ability of 2MeOE2 to oppose the neointima formation appeared to at least partially depend on upregulation of p27/Kip1 expression in VSMCs (Barchiesi et al., 2006). Here we showed that stimulation of hPASMCs derived from female control subjects

with 2MeOE2 elicited a concentration-dependent increase in p27/Kip1 expression, which might be another mechanism by which 2MeOE2 might modulate hPASMC proliferation. This offers the possibility that 2MeOE2 conveys its anti-proliferative properties through various mechanisms, which could be concentration-dependent.

Previously, an HPLC/flux method was developed within our research group with the aim to trace oestrogen metabolism in *in vitro* model of PAH. Due to accumulating evidence indicating prostacyclin analogues, such as iloprost and treprostinil, used for treatment of PAH possess the ability to activate PPARs (Forman et al., 1997;Ali et al., 2006). Therefore, the protective effects of these analogues might be partly mediated through the PPAR signalling pathways. PPAR signalling was shown to modulate oestrogen signalling by i) altering the expression of ERs (Segars et al., 1993;Keller et al., 1995) or ii) binding to ERE and acting as competitive inhibitors (Keller et al., 1995). Activation of PPARγ results in reduced transcription of aromatase gene and increased protein turnover (Yanase et al., 2001;Fan et al., 2005), while PPARα, which is constitutively expressed in the mouse lung (Becker et al., 2008b), increases the expression of 17βHSD type 1, leading to increased E1 production (Corton et al., 1997;Davis et al., 1994). Interestingly, the expression of CYP1B1 was shown to be induced through the activation of PPARα activation (Lovekamp-Swan et al., 2003;Murray et al., 2001). We therefore treated hPASMCs derived from control and patient subjects of both sexes and examined how treatment with treprostinil affects oestrogen metabolism in these cells. All cells were exposed to 50nM $^{14}C_4$-17βE2, and cells were then stimulated with treprostinil (1μM): i) 24 hours prior to addition of 50nM $^{14}C_4$-17βE2 (*T24B*), and ii) at the same time as 17βE2 (*TE48*). The experiment was designed to ascertain whether treprostinil affected the expression of enzymes involved in the metabolism of oestrogen or merely affected the enzyme's activity. In female control cell line, preliminary results indicate that treprostinil increases the metabolism of oestrogen as shown by decreased amount of radioactively labelled 17βE still present in the samples. Moreover, in male patient cell line, pre-treatment with treprostinil resulted in increased production of E1, whereas co-treatment with treprostinil did not affect the conversion of 17βE2 to E1. Hence, we conclude that treprostinil affects the expression of 17βHSD type 2 expression level, which is in line with previous reports (Corton et al., 1997;Davis et al., 1994). Interestingly, treprostinil may only affect the expression of 17βHSD type 2 in male patient hPASMCs, indicting the mechanism behind the observed effect might be sex-dependent. A line of evidence indicates that in unstimulated hPASMCs, the basal expression of 17βHSD type 2 might be higher in male patient hPASMCs compared to female patient hPASMCs. Further investigation is merited

here, to establish whether the observed effects were mediated through PPAR activation and to ascertain whether PPAR signalling might be influenced by sex.

CYP1B1 is overexpressed PASMCs, in experimental and clinical PAH (Johansen, 2014). Its role in the pathogenesis of PAH ranges from production of 16αOHE1 and 4OHE1, exhibiting proliferative (White et al., 2012) and genotoxic (Cavalieri et al., 2000;Cavalieri et al., 1997) properties, respectively, to increased formation of ROS. CYP1B1 inhibition by TMS *in vivo* reversed pulmonary haemodynamic parameters as well as vascular remodelling (White et al., 2012). We therefore wished to assess the effects of CYP1B1 inhibition on oestrogen metabolism in PASMCs derived from female control and patient subjects. Although we observed significant differences in the metabolism of oestrogen between control and patient cell lines, treatment with TMS did not significantly affect the conversion of 17βE2 into other metabolites. The experiments were conducted using a single time-point; however, it may be a warranted notion to repeat these experiments using several experimental end-points.

During the HPLC/flux experiments used to trace the metabolism of 17βE2, we encountered the formation of unknown oestrogen metabolites. To possibly identify any metabolites that were not included in our research interests, and to correlate exposure to 17βE2 with dysregulation of other signalling pathways that might not yet be considered in PAH, we conducted a hypothesis-free metabolomic screen in female cell lines. Here we compared female control and patient cell lines, which were stimulated either by vehicle or 17βE2. Exposure of female control cell lined to 17βE2 did not affect the levels of numerous metabolites, whilst more metabolites were affected by 17βE2 in female patient cell lines, indicating energy and fatty acid metabolism might be affected alongside redox cycling. Indeed, exposure of hPASMCs to serotonin resulted in increased production of ROS, which according to the authors might cause DNA damage, and redox-sensitive proliferation (Hood et al., 2017). We further observed that the level of L-kynurenine, a catabolite of tryptophan which is the precursor of 5-HT, was also altered in female patient cells treated with 17βE2, where treatment resulted in catabolite diminished concentration. L-kynurenine is a known AhR ligand, shown to be increased in certain cancers, where it promotes tumour growth (Opitz et al., 2011). Here we showed that pathogenesis of PAH might be affected by 5HT in two-fold: i) through the formation of 5HT from its precursor tryptophan, and by ii) the degradation of this precursor to L- kynurenine, activating the AhR and resulting in altered oestrogen metabolism. The decrease in L-kynurenine levels in the 17βE2 female patient cell

lines might be associated with decreased turnover of tryptophan due to its increased conversion into 5HT.

Investigation of oestrogen metabolism is complicated by the sheer number of metabolites, which are similar in chemical structure, and are therefore difficult to separate chromatographically. We therefore wanted to develop an LC-MS/MS methodology, with which we could identify and quantify each of oestrogen metabolites. Herein we provide qualitative oestrogen metabolic profile in control and patient hPASMC cell lines of both sexes, and quantitation of these metabolites. We observed statistically significant differences in the production of E1 and 17αE2 in female patient hPASMCs compared to female control hPASMCs, revealing a prominent role of 17βHSD type 2. Interestingly, in male patient hPASMCs, the metabolism of 17βE2 appeared to be slower compared to female patient hPASMCs, as assessed by the amount of the parental hormone, indicating reduced activity of the 17βHSD type 2, albeit the difference in the amount of E1 was not statistically significant. Similar observations were reported previously (Johansen, 2014). Although E1 is significantly less biologically active compared to 17βE2 due to lower affinity for ER, its production might have a significant role in PAH pathogenesis. The conversion to E1 might increase the pool of precursor for oestrogen metabolising enzymes, which generally do not distinguish between their substrates, resulting in increased production of hydroxylated metabolites of E1. Although these metabolites might be converted to OHEs, through the activity of 17βHSD type 1, the enzyme kinetic parameters are less favourable compared to 17βHSD type 2. Moreover, the role of geometrical isomerisation of the 17βE2 to 17αE2 might also be important in the pathogenesis of PAH, as the amount of this metabolite was significantly increased in female patient hPASMCs. Although previously, 17αE2 was considered devoid of oestrogenic effects, recent lines of evidence indicate otherwise. The research in the production and biological effects of the 17αE2 in the lung is therefore merited.

Although the notion of an LC-MS/MS technique to quantify oestrogen metabolism *in vitro* and ascertain the effects of other environmental and endogenous insults involved in the development of clinical PAH on oestrogen metabolism is attractive, unfortunately, the transfer of the method from aqueous to media sample matrix has proven to be more difficult than previously warranted. Indeed, the cell media highly affected the LC-MS/MS system. The presence of matrix components affected the detection of metabolites despite the use of guard columns to remove any unwanted sample matrix constituents. It might therefore be

beneficial to assess how the samples might be further purified or attempt to culture cells in a different media, which might exert less adverse effects on the LC-MS/MS system. Furthermore, our results indicated that the limit of detection of certain metabolites, such as OHEs, needs to be addressed and improved if the LC-MS/MS method is to be used to reliably quantify these metabolites.

Chapter V

5 General Discussion

5.1 General Discussion

More than 60 years ago, the female predominance in the patient population with PAH was recognised. The extent of female susceptibility to develop PAH observed in recent surveys and registries revealed that female sex may be even more important than previously supposed (Humbert et al., 2006;Escribano-Subias et al., 2012;Ling et al., 2012;Badesch et al., 2010). Namely, the incidence of the disease in post-menopausal women is approaching that of men (Hoeper et al., 2013), indicating that the reducing levels of endogenous female sex hormones might be protective. Young female patients exhibit better pulmonary haemodynamics compared to age-matched men (Shapiro et al., 2012). Hence, it appears that albeit endogenous 17βE2 may be facilitating the pathophysiological processes associated with the onset of clinical PAH, 17βE2 might be protective against changes in haemodynamic parameters, such as mean PAP and vascular resistance. Additionally, menopause was reported to lessen the differences in the pulmonary haemodynamics between men and women (Ventetuolo et al., 2014). Despite female sex being considered the main risk factor for the development of clinical PAH in humans, women generally exhibit better outcome and survival compared to men (Shapiro et al., 2012). This could be due to a better response to therapy (Gabler et al., 2012) or better RV compensation (van de Veerdonk et al., 2011;Swift et al., 2015). Hence, the understanding of the role of female sex hormones in the onset and progression of PAH is of utmost importance.

Protective properties of exogenous 17βE2 were reported in the classical cHx and MCT experimental models of PH, where exogenously administered 17βE2 attenuated hemodynamic and remodelling parameters in these animals (Lahm et al., 2012;Yuan et al., 2013). In these models female animals developed less severe experimental phenotype, whereas female sex was associated with the development of severe experimental phenotype in novel experimental models. The latter include the SERT+ and S100 Ca^{2+} binding protein A4 overexpressing mouse models, where ovariectomy protects against the onset of PH (Greenway et al., 2004;White et al., 2011b;White et al., 2011a;Dempsie et al., 2011). Locally, stimulation of hPASMCs with 17βE2 was associated with augmented proliferation of these cells (White et al., 2011a;White et al., 2012). Indeed, PASMCs expressed aromatase and can synthesise 17βE2, and the inhibition of the local synthesis of oestrogens in PAs, documented in (Mair et al., 2014), was shown to reduce progression of established PH phenotype in cHx and Su5416/Hx animal models. The study provided evidence of direct role of endogenous oestrogens in the pathogenesis of PAH. The presence of numerous

extrahepatic oestrogen metabolising enzymes indicates the lung is an important site of oestrogen metabolism (Bieche et al., 2007). Increased expression of CYP1B1 was documented in animal models, where more female animals develop experimental PH, and in lung sections derived from IPAH patients (White et al., 2011b;White et al., 2012). The detrimental role of oestrogen metabolism dysregulation in PAH was documented in (Austin et al., 2009), where the authors reported the association of disease penetrance in female *BMPRII* mutation carriers with the levels of urinary oestrogen metabolite, specifically 2OHE1/2 and 16αOHE1. In female C57BL/6 mice, the administration of the latter metabolite resulted in increased haemodynamic and vascular remodelling parameters (White et al., 2012). At least partially, the observed phenotype may be associated with the pro-proliferative effects of 16αOHE1 documented in hPASMCs (White et al., 2012). Interestingly, the products of O-methylation of catechol oestrogens, catalysed by COMT enzyme, opposed proliferation of hPASMCs, as well as lung and cardiac fibroblasts (Dubey et al., 1998;Tofovic et al., 2006;Tofovic et al., 2008b), and even induce apoptosis in ECs (Yue et al., 1997). Due to documented beneficial properties of methoxy oestrogens, increased activity of CYP1A1, which exhibits high affinity for hydroxylation of oestrogens at position C2, and COMT might be protective in cancer (Kisselev et al., 2005) and even PAH. The efficiency of O-methylation depends on the local concentration of catechol oestrogens, with evidence pointing towards inhibition of O-methylation of 4OHE2 by the presence of 2OHE2 (Roy et al., 1990;Zhu and Conney, 1998). Reduced removal of 4OHEs might be directly associated with increased production of ROS, leading to protein and DNA damage. Currently, no direct evidence of the effects 4OHE2 might have on O-methylation of 2OHE2 exists. Nonetheless, in mice, the deletion of *CYP1B1* gene was associated with decreased production of 4-hydroxyoestrogens and increased production of 2-hydroxy and 2-methoxyoestrogens within the lung (Peng et al., 2013a), due to inhibitory effects of 4OHE2 on *COMT* expression. Previously, however, it was reported that 4OHE2 exerts differential effects in female and male hPASMCs (Mair et al., 2015). Namely, the metabolite decreased phospho-Smad1/5/8(9) and Id expression in female hPASMCs while increasing these in males, commensurate with a decreased proliferative effect in male hPASMCs. We showed that stimulation with 2OHE2 and 4OHE2 in male hPASMCs led to a reduction in serum-induced proliferation of these cells. It is possible that the initial resistance of men to develop PAH might be due to the anti-proliferative effects of 2- and 4OHE2, and their respective O-methylated metabolites.

The AhR regulates the expression of xenobiotic-metabolizing enzymes, including CYP450 enzymes. The deletion of AhR is associated with i) the formation of abnormal vascular structures (Lahvis et al., 2000), ii) increased expression of HIF1α and VEGF (Thackaberry et al., 2002), and iii) inability to induce target genes such as *CYP1A1*, *CYP1A2*, and *CYP1B1* upon stimulation with selective AhR agonist (Fernandez-Salguero et al., 1995). The activity of AhR, however, was linked to increased thickness of vessel wall due to abnormal proliferation of SMCs (Sauzeau et al., 2011). Moreover, the expression of AhR was reportedly increased in female hPASMCs derived from PAH patients (Dean et al., 2016). Enhanced expression of the receptor might lead to increased ROS production, as documented in experimental PH animal models (Jernigan et al., 2017), due to enhanced expression of *Phase I* metabolising enzymes (Park et al., 1996). Although female sex is protective in classical experimental models of PH, the oestrogens apparently mediated a detrimental role in the Su5416/Hx model (Tofovic et al., 2012), which therefore recapitulates the human disease in terms of epidemiological data, as female animals develop the disease more frequently compared to males. Female animals exposed to Su5416/Hx insult to establish the experimental phenotype also developed a more severe disease compared to male animals according to (Tofovic et al., 2014).Therefore, we sought to examine the role of the AhR as a plausible key effector in the mechanism driving the onset of PAH phenotype in this animal model, considering the mechanism behind the experimental PH phenotype in this animal model is not completely understood.

In Chapter 3 we first examined the expression level of *CYP1A1*, in several models of PH utilised within our research group, as unpublished data from microarray analysis revealed that this gene is significantly upregulated in Su5416/Hx model. We demonstrated that the expression of *CYP1A1* gene could be decreased in *in vitro* model using hPASMCs, *Smad1* heterozygous mice and rats exposed to cHx. Our observations corroborate previous work showing that HIF signalling pathway reduces the expression of *CYP1A1*(Vorrink et al., 2014). Recently it was demonstrated that CYP1A1 influences blood pressure regulation through activation of eNOS, NO bioavailability and NO-dependent vasodilation (Agbor et al., 2014). Hence, the decreased expression of CYP1A1 we observed might be associated with increased blood pressure in pulmonary circulation. Interestingly, an association between increased sheer stress and AhR activation was also reported in ECs (Han et al., 2008).

The AhR and HIF1α signalling pathways share a common translocator protein, HIF1β (ARNT), which is required for receptor activation in both instances. Vorrink and colleagues showed that overexpression of HIF1β (ARNT) rescues the interference of Hx with AhR transcriptional transactivation of its target genes (Vorrink et al., 2014). In Su5416/Hx model, however, the expression of *CYP1A1* and *CYP1B1* enzymes was considerably increased, at gene and protein levels, respectively. Considering the reported affinity of murine AhR receptor isoforms for Su5416 approaches that of TCDD (Mezrich et al., 2012), we inferred that the mechanism of Su5416/Hx-induced experimental PH might at least partially entitle the activation of the AhR signalling pathway. Interestingly, increased expression of CYP1A1 and CYP1B1 was previously also reported in the classical MCT-induced PH animal model (Yuan et al., 2013). In primary human hepatocytes, pyrrolizidine alkaloids were shown to regulate the expression of numerous nuclear receptor or ligand-activated transcription factors, including PPARs and AhR, both of which regulate gene expression of CYP enzymes (Luckert et al., 2015). Although the effects of pyrrolizidine alkaloids and their metabolites in the lung have not yet been assessed in terms of their effects on the AhR signalling pathway, evidence suggests that increased expression and/or activity of AhR leads to increased expression of CYP enzymes. Sustained activity of AhR was linked to onset of endothelial dysfunction, hypertension and CVD (Korashy and El-Kadi, 2006;Kopf et al., 2010).

To further determine whether the AhR signalling pathway is activated in Su5416/Hx model of PH, we examined the expression of *AhRR* and *NQO1*, which are both under the transcriptional regulation of AhR. The expression pattern of both genes was similar to that observed for *CYP1A1* and *CYP1B1*, showing that numerous target genes of AhR are induced in the Su5416/Hx model. It would be interesting to determine whether these genes are also induced in the MCT model, considering current, albeit indirect, evidence showed that pyrrolizidine alkaloids affect the AhR expression. We recently published our findings that Su5416 activates the AhR *in vitro* in female patient hPASMCs, resulting in the translocation of the receptor to the nucleus, and leading to the induction of *CYP1A1* and *CYP1B1*, in the Red Journal with an encouraging positive editorial of the findings presented within the paper. The antagonist of the AhR attenuated the gene induction only in female hPASMCs from control subjects, while in patients the AhR antagonist showed no effect. The increased expression of AhR reported (Dean et al., 2016) might be the underlying cause for findings reported in this work.

The interaction of the HIF and AhR signalling pathways appears to be significant in the establishment of the experimental Su5416/Hx model, as both pathways require the translocator protein, HIF1β (ARNT), for full functionality of the respective receptors. We reported herein that the basal expression of proteins regulating the proteasome-mediated degradation of HIF1α, PHD2 and vHL, is significantly increased in male patient hPASMCs, compared to same-sex control cells. Similar observations were also made in male rats treated with Su5416 and exposed to cHx. Contrary, in female cells, we reported opposite trends, with female patient cells exhibiting lower basal expression of PHD2 and vHL compared to control cells. In line with these observations, exposure to cHx only increased HIF1α in female animals. Moreover, herein we showed that the expression of HIF1α might be higher in male Nx animals compared to matched female animals. Additionally, the expression of HIF1α did not change in Su5416/Hx male animals, while in female animals treated with both experimental insults, HIF1α was significantly increased. We also report the basal expression of HIF1α is significantly increased in female patient hPASMCs, probably due to lower expression of proteins regulating the receptor degradation. It appears that a sex dimorphism exists in response to cHx, due to sex differences in the regulation and signalling of HIF1α pathway. Sex differences in the response to cHx in left and right heart ventricles were reported, where females initially were more responsive to Hx, however in long-term, male animals exhibited higher levels of Hx-inducible genes (Bohuslavova et al., 2010). Whether the observed differences in PASMCs in exposure to cHx could be responsible for differences in the severity of disease phenotype and survival remains to be investigated. Equally, it is possible that the initial adverse response to Hx, which could be greater in females, might be associated with the observed dimorphism in the onset of PAH. We recently shown that in Hx conditions, Su5416 decreased the expression of HIF1α in nuclear and cytoplasmic fractions, in hPASMCs derived from female patient subjects (Dean et al., 2017). Although we showed that Su5416 does not have any effect on PHD2 and vHL, which are involved in regulation of, these experiments were conducted in Nx conditions. It would be interesting to repeat these experiments in Hx. Herein, we also demonstrated that in male rats exposure to cHx leads to diminished expression of *AhR*, further indicating that a complex interaction between both pathways exists.

A significant hallmark of pathophysiological changes in the pulmonary circulation of patients with PAH, is the muscularisation of normally non-muscular small PAs. In experimental models of PH, the establishment of disease phenotype often relays on vascular injury and introduction of an insult causing hyper-proliferation of PASMCs. In the

Su5416/Hx model, Su5416 caused proliferation of PASMCs in Nx (Taraseviciene-Stewart et al., 2001). The increased muscularisation of distal small PAs *in vivo*, depends on the increased proliferation of PASMCs, although this was only described in animals which werem exposed to cHx (Ciuclan et al., 2011). Here it was observed that there is increased proliferation of hPASMCs derived from female PAH patients stimulated with Su5416 and Hx reported herein, which is consistent with previous reports (Ciuclan et al., 2011). Our observations, now published in (Dean et al., 2017), indicate that a possible synergy of Su5416 and Hx insults exists, through the increased expression of ARNT (HIF1β). The return of ARNT (HIF1β) to normal levels by the AhR antagonist, suggested the possibility of a synergy of the AhR and HIF signalling pathways through this coupling protein, which was also shown by (Vorrink et al., 2014). As ECs are also intimately involved in the pathogenesis of PAH, we also assessed the effects of Su5416 on PMECs, observing increased apoptosis. In BOECs from clinical patients, Su5416 induced cellular proliferation. We deduced that the initial vascular injury by apoptosis of ECs, and subsequent proliferation of the apoptosis-resistant population of ECs, contributes to the development of PH (Dean et al., 2017).

The interaction of AhR and ER signalling needs to be investigated in the context of understanding the role of AhR signalling in the pathogenesis of disease with exceeding prevalence in females, such as PAH. The interaction of the AhR/ARNT complex with ERs was previously reported by (Ohtake et al., 2003). The authors showed that activated AhR may directly associate with unliganded ERα and ERβ, and the co-activator p300. Hence, also in the absence of oestrogens, the oestrogenic effects, such as induction of VEGF, *c-fos* and oestrogen-dependent cell proliferation, may be mediated through the association of AhR/ARNT/ER to EREs. Since exposure to cHx and 17βE2 have synergistic effects in ECs, resulting in increased proliferation of this cell type (Kleinman et al., 2007), it might be interesting to investigate whether the AhR/ARNT/ER interaction is involved in the synergistic effects of Su5416 and Hx on proliferation of female patient hPASMCs. To determine the role of AhR in the establishment of experimental PH phenotype, the interaction with ER should also be considered.

The research presented in this thesis was enabled by the NC3Rs, which is dedicated to promoting the development of new research technologies and refinement of existing models and techniques to minimise the number of animals in research and improve the welfare of animals, whose use might not be completely avoidable. Currently, *in vitro* models offer the

advantage measuring the responses to stimulation and/or inhibition of individual molecular processes in real time, to determine the contribution of specific molecular processes to disease development (Benam et al., 2015). In Chapter 4 we sought to develop a LC-MS/MS technique to be used to quantify local oestrogen metabolism by hPASMCs. Although the circulatory levels of the parental oestrogens have been well-established, the levels of specific oestrogen metabolites were not addressed in the context of PAH pathogenesis. Moreover, as the importance of local oestrogen synthesis and metabolism were recently demonstrated and recognised (Mair et al., 2014;White et al., 2012), and oestrogen metabolites reportedly have protective and pathogenic effects in the pulmonary circulation, the quantification of local levels of oestrogen metabolites was warranted. We aimed to present the first quantification of oestrogen metabolism in hPASMCs. However, as the concentrations of some metabolites were below the methods' limit of detection, the complete quantification of oestrogen metabolism *in vitro* was not reliable. Significant differences in the metabolism of parental oestrogen hormones were detected. Our findings indicated that in PAH the activity and/or expression of the 17βHSD type 2 enzyme might be increased, as the conversion of 17βE2 to E1 was increased. The consequences of high levels of E1 could be three-fold. Firstly, as numerous oestrogen-metabolising enzymes exhibit similar affinity for E1 and 17βE2, it is plausible that increased formation of E1 could be driving increased production of oestrone metabolites, such as 16αOHE1. Importantly, compared to 16αOHE2, 16αOHE1 metabolite has greater pro-proliferative character (White et al., 2012). Secondly, increased conversion of the 17βE2 to less biologically active E1, could also diminish the protective effects of 17βE2 reported in the heart (Frump et al., 2015;Liu et al., 2014). Thirdly, the role of 17βHSD type 2 is not solely restricted to the conversion of 17βE2 to E1, as the enzyme can convert all metabolites of oestradiol to the corresponding oestrone metabolites, which are considered to be largely devoid of protective properties in cardiovascular cells. (Tofovic et al., 2008b). The importance of the 17βHSD pathway was shown to impose significant effects in the experimental MCT model in rats, where preventive treatment with 2MeOE1 attenuated the development of experimental phenotype, as well as cardiac and vascular remodelling (Tofovic et al., 2008b). Increased conversion of protective oestradiol metabolites to less active oestrone metabolites might therefore be an important aspect of oestrogen metabolism dysregulation in PAH. Although the expression of 17βHSD type 2 might be increased in hPASMCs derived from male subjects with clinical PAH phenotype, the quantitation of oestrogen metabolites using the novel LC-MS/MS approach, the amount of unmetabolized 17βE2 was greater in male patient hPASMCs metabolised compared to female patient hPASMCs. It would be beneficial to assess the activity of 17βHSD type 2 in hPASMCs, as

it is possible that the presence of polymorphic variants exhibiting increased metabolic rate might result in observed increased formation of E1 in female patient cells. Similar suggestions were made regarding the expression of *CYP1A1* and *COMT*, where the presence of such variants might lead to accumulation of hydroxylated oestrogen metabolites, and increased production of oestrogen quinones and semi-quinones (Kisselev et al., 2005).

We also observed that geometrical isomerisation of 17βE2 to 17αE2 was increased in female patient cells. The geometric isomer of oestradiol was long considered to convey little hormonal activity. Although, the literature on the effects of 17αE2 in humans is very limited, there are reports of its pro-proliferative effects in human neoplasms (Papendorp et al., 1985, Hajek et al., 1997). In breast cancer cell lines, 17αE2 was shown to bind to ERs with high affinity, resulting in i) increased expression of progesterone receptor, ii) reversal of inhibition of cellular proliferation, and iii) increased DNA polymerase activity (Edwards and McGuire, 1980). Furthermore, 17αE2 may also affect aromatase activity (Hoffmann et al., 2002). Although the authors did not provide a possible mechanism, others have suggested that 17αE2 may affect the levels of sex hormones by inhibiting the 5α-reductase, which catalyses the conversion of testosterone to the more potent dihydrotestosterone (DHT), in skin fibroblasts (Munster et al., 2003). The physiological function remains undetermined as 17αE2 appeared to mediate vasorelaxation (Salas et al., 1994). It would be therefore interesting to assess the effects of stimulation with 17αE2 on i) aromatase activity in hPASMCs and ii) vasoconstriction in isolated intra-pulmonary arteries.

Although we, in part, succeeded to quantify oestrogen metabolites produced in hPASMCs upon stimulation with 17βE2, we observed that the method is extremely sensitive to the presence of impurities present in the samples. Indeed, the analysis of numerous spent media samples resulted in significant decline in the sensitivity of the LC-MS/MS method, probably due to deterioration of column condition brought upon sample impurity. Moreover, the detection limit of OHEs was considerably higher than the detection limit for parental oestrogens or MeOEs. Hence, more work is warranted to establish ranges within which the method would produce reliable quantification of these metabolites. Further, it was considered that the composition of the cell media and serum used in these experiments might not be most suitable for LC-MS/MS approach, as the technique heavily relies on the use of sensitive LC and chemical derivatisation of oestrogen metabolites. We therefore suggest that if local oestrogen metabolism *in vitro* should be further investigated, efforts must be made

to try and replace the currently used cell growth media with more inert matrix or to make the method more robust.

HPLC/flux analyses were used to investigate how the use of a common medicine prescribed for the treatment of PAH, treprostinil, affects oestrogen metabolism in hPASMCs. Prostacyclin analogues, which are currently used to manage moderate to severe PAH affect i) vasorelaxation and vasodilation of PAs (Narumiya et al., 1999, Wise, 2003), and ii) PASMC proliferation (Clapp et al., 2002; Yang et al., 2010). Recently it was shown that therapeutic effects of treprostinil might present sex bias, with females showing better anti-proliferative response to the therapeutic compared to males (Murphy, 2017). Endogenous PGI_2 and its stable metabolite to activate certain PPAR isoforms (Forman et al., 1995;Nemenoff et al., 2008), directly (Forman et al., 1997) or indirectly, though the IP receptor (Falcetti et al., 2007). PPARs affect oestrogen metabolism by i) competitive inhibition of ERs (Keller et al., 1995), ii) increased degradation of ERα (Qin et al., 2003), iii) increased expression of 17βHSD type 1 expression (Corton et al., 1997;Davis et al., 1994), and iv) modulation of the expression of oestrogen metabolising enzymes (Corton et al., 1997). Recently published evidence suggests that in human lung fibroblasts, treprostinil inhibits proliferation of these cells through activation of PPARβ (Ali et al., 2006). Our results indicate that treprostinil could affect oestrogen metabolism in male patient hPASMCs, through increased activity of 17βHSD type 2, indicating the used prostacyclin analogue might affect the expression of this enzyme.

The unbiased metabolomic screen of female control and patient hPASMCs stimulated with 17βE2 revealed significant changes in the levels of L-kynurenine, a catabolite of tryptophan which is the precursor of 5-HT, in female patient cells treated with 17βE2, where treatment resulted in catabolite diminished concentration. The identification of L-kynurenine in the context of PAH is important, as tryptophan serves as the precursor for the formation of 5-HT, acting at the 5-HT(1B) receptor and 5-HT transporter (SERT or 5-HTT) to mediate constriction and proliferation of PASMCs (Maclean and Dempsie, 2010). Interestingly, the implication of L-kynurenine in PAH might be two-fold. Firstly, L-kynurenine metabolism produces molecules with vasoactive properties (Wang et al., 2010). Secondly, the parental molecule is a known AhR ligand (Nguyen et al., 2014). Importantly, we showed that 5-HT may affect the pathogenesis of PAH in two ways: directly through activation of the 5-HT1B receptor (MacLean, 2007;MacLean et al., 2004;Morecroft et al., 2005), and indirectly

through the degradation of tryptophan precursor, leading to formation of AhR ligands (Denison and Nagy, 2003;DiNatale et al., 2010).

In Chapter 4 we summarised the findings of the effects of oestrogen metabolites in *in vitro* model of PAH. Protective effects of 2MeOE2 were reported *in vitro*, where 2MeOE2 was shown to attenuate the proliferation of several vascular cell types (Fotsis et al., 1994; Barchiesi et al., 2006; Tsukamoto et al., 1998; Tofovic et al., 2009b; Nishigaki et al., 1995), and *in vivo*, where 2MeOE2 prevented and attenuated the development of experimental PH phenotype in monocrotaline, bleomycin-induced and Su5416/Hx model (Tofovic et al., 2009b; Tofovic et al., 2008b; Tofovic and Rafikova, 2009). Previously, treatment with 17βE2 was reported to increase the expression of p27/Kip1 animals and cultured PASMCs exposed to Hx (Xu et al., 2010), where p27/Kip is a tumour suppressor which negatively regulate cells' progression through cell cycle. Authors of (Barchiesi et al., 2006) also reported that the inhibition of neointima formation by 2MeOE2 depends on upregulation of p27/Kip1 expression in VSMCs. Our data showed that treatment with 2MeOE2 resulted in arrested proliferation in control hPASMCs of both sexes, whilst only female patient hPASMCs responded to treatment. We further showed that in female control cells, stimulation with 2MeOE2 may result in increased expression of p27/Kip1. As the protective effects of 2MeOE2 might be dependent on the effects the metabolite exerts on the regulation of cells' progression through cell cycle, it might be worthy of examining the basal level of p27/Kip1 expression in PASMCs derived from control and patient subjects of both sexes. This might help to determine whether poorer survival prognosis in men could be associated with poorer response to protective metabolites. Additionally, in females, the protective character of 2MeOE2 might depend on the induction of PGIS in ECs and hPASMCs. Our findings indicated that in male cells, the mechanism through which 2MeOE2 might attenuate proliferation of male control hPASMCs might depend on the effects on BMPRII signalling pathway. Hence, it appears that 2MeOE2 might affect multiple pathways. Additionally, we have shown that the anti-proliferative effects observed for 2OHE2 are mediated through the formation of 2MeOE2, as inhibition of COMT enzyme reversed the inhibitory effects on proliferation. Partially, 2OHE2 might also activate classical ER. The inhibition of proliferation by 4OHE2 was found to be mediated through classical oestrogen signalling pathways, as inhibition of COMT had no significant effect. The enzyme was shown to favour 2-O-methylation over 4-O-methylation, due to unfavourable position of the 4-O-hydroxyl substrate in the catalytic site (Männistö and Kaakkola, 1999). Although 4OHE2 was shown to mediate its biological effects through pathways independent of classical ER (Das et al.,

1997), our data indicates certain effects might be mediated through activation of ERs, which is in accordance with reports by (Martucci and Fishman, 1976). The oestrogenic activity of 4OHE2 through activation of ER might be prolonged due to the slow dissociation rate of the metabolite (Zhu and Conney, 1998; Barnea et al., 1983). The activity of COMT in the liver was shown to be sex-dependent, with males exhibiting higher COMT activity compared to females (Boudikova et al., 1990). Therefore, due to significant activity of COMT in the lung, the expression of this enzyme should be assessed in terms of possible differences between the sexes. Moreover, an assay to assess the activity of this enzyme in hPASMCs is also warranted.

5.2 Limitations of the Study

There are several aspects of limitation for the research work presented herein. Firstly, the pathological onset and progression of PAH is a highly complex process, involving several cellular types, as described in Chapter 1.2.5. Although the pathological mechanisms that could contribute to the onset of PAH in animal models, as well in humans, were investigated in SMCs and ECs, the research presented was limited by the unavailability of an established co-culturing or conditioning technique. Unfortunately, the use of a single cell type cell culture is not appropriate as the natural interactions between cell populations, which are present within the lung and might be important for the disease process, were not taken into consideration.

Secondly, when investigating the effects of oestrogens in males and females, one should be aware that it is not known how the isolation of a cell type from a tissue affects the sexual aspect of the cells. Whether the cultures of isolated SMCs or ECs derived from a female/male subject retain the characteristics that they have within the intact organ within a functioning body is not known. Hence, *in vitro* research presents a valid supportive technique, however, only *in vivo* experiments might offer conclusive results when investigating the role of sex in PAH.

Thirdly, the chemiluminescent technique employed to investigate the levels of protein expression levels presents three major disadvantages. Firstly, narrow linear signal response range, which differ between exposures of film and hence make it difficult to compare band intensities from different exposures, secondly, reciprocity failure, which may cause the faint and strong bands to be underrepresented on film, and thirdly parallax effects, which can

reduce image clarity and sharpness. Therefore, the use of a fluorescence detection-based system would be more appropriate to avoid the named disadvantages associated with protein quantitation. Additionally, the quantitation of certain proteins was hindered by the low expression levels in individual cell types or by the availability of a suitable monoclonal antibody to be employed in tissue samples.

Fourthly, the study might have been partially limited by the selection of the housekeeping genes used for normalisation of RT-qPCR results. Especially, in the instances where the difference in the gene of interest was marginal, the variability of the housekeeper gene might affect the final result. Although, the variability of the housekeeper gene expression was assessed for each of the experiments, and was found to be acceptable, additional value would be added if another housekeeper gene would be employed, with documented stable expression.

Lastly, the work presented herein was limited by the lack of time and access to working LC-MS/MS equipment, in order to optimise the culture conditions, which would not hinder the following steps in derivatisation of oestrogen metabolites and allow us to lower the limit of detection.

5.3 Concluding Remarks

In summary, we have provided evidence for the importance of the AhR signalling pathway in experimental PH, showing how activation of this signalling pathway might result in initial vascular injury, increased VSMCs proliferation and dysregulation of oestrogen metabolism. We have further shown that exposure to cHx might elicit differential response depending on sex. Hence exposing the important question of AhR pathway interaction with HIF and ER signalling pathways.

The detection of oestrogen metabolism in hPASMCs using a novel LC-MS/MS approach has revealed that significant differences in the metabolism of 17βE2 exist between control and patient hPASMCs of both sexes. The most significant finding shows altered conversion of 17βE2 to its enantiomer 17αE2 and E1, indicating the possible role of 17βHSD type 2 and dehydrogenase in PAH. Further investigation of oestrogen metabolism dysregulation is therefore warranted.

5.4 Future Perspective

Future studies examining the interaction of AhR, hypoxia-inducible factor (HIF) and oestrogen signalling pathways are strongly encouraged, as evidence of synergistic effects of these pathways have been reported (Kleinman et al., 2007;Dean et al., 2017). The knowledge of how these pathways interact might be of critical importance in the understanding of the onset and progression of experimental and clinical PAH.

Inhibition of AhR signalling pathway attenuated experimental PH in Sugen 5416 and chronic hypoxia model (Dean et al., 2017), which is currently considered as the model which most accurately recapitulates the essential hallmarks of PAH pathogenesis. The role of AhR signalling in the classical monocrotaline model should be investigated, possibly to determine whether the inhibition of AhR activity might be protective against disease phenotype as shown in (Dean et al., 2017).

Studies of the role of 17β-hydroxysteroid dehydrogenase type 2 PAH are highly encouraged. Understanding the role of 17β-oestradiol to oestrone conversion may be of critical importance due to its role in the conversion of protective oestrogen metabolites to metabolites exhibiting no protective properties. A study of 17β-hydroxysteroid dehydrogenase type 2 inhibition might offer additional insight in the dysregulation of oestrogen metabolism in pulmonary arterial hypertension.

Although promising, the novel liquid chromatography tandem mass spectrometry to quantify oestrogen metabolism in *in vitro* model of PAH using PASMCs, requires further optimisation in terms of eliminating the any impurities and compounds that might interfere with sample preparation and analysis. Moreover, method optimisation might be required for this novel approach to be used as a high-through put screening method to identify novel therapeutic targets.

Due to significant role of endothelial cells in the pathogenesis of pulmonary arterial hypertension, we suggest that the expression of CYP1A1 should be addressed in blood-outgrowth ECs of control and patient subjects. Furthermore, the effects of silencing CYP1A1 on nitric oxide synthase and nitric oxide bioavailability in the BOECs from patients with PAH should also be investigated, as CYP1A1 was shown to affect NO-dependent blood pressure regulation through metabolism of polyunsaturated fatty acids in a CYP1A1 knock-out mouse model (Agbor et al., 2014).

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