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Sex and Estrogen Metabolism in Pulmonary Arterial Hypertension (PAH)

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Abstract

Pulmonary arterial hypertension (PAH) is a progressive, life-limiting disease, characterised by increased pulmonary vascular resistance and right ventricular hypertrophy leading to right heart failure and death. In PAH, a sexual dimorphism is present with a strong sex bias in disease penetrance (4:1 female to male ratio). Despite being more susceptible to the development of PAH, females display improved right ventricular (RV) function and survival rate in comparison to males. As a result, a sex paradox exists and sex hormones have been implicated in the development and progression of PAH, with numerous studies displaying roles for estrogen, its metabolites and receptors in disease progression.

Little is known about the systemic endogenous levels of estrogen and its metabolites, or their relationship to sexual dimorphism in PAH. This is partially due to the fact that current methods to quantify estrogens commonly employ immunoassays, which measure only one metabolite at a time and often over-estimate levels due to cross-reacting antibodies. Therefore, an improved assay to analyse a panel of estrogen metabolites is required to quantify the wide range of endogenous concentrations in females and males. The main aim of this work was to develop, validate and apply a liquid chromatography tandem mass spectrometry (LC-MS/MS) approach to quantify multiple estrogens simultaneously.

Estrogens were extracted from serum or plasma by solid phase extraction on Oasis MCX® cartridges followed by derivatisation using 1-(5-fluoro-2, 4-dinitrophenyl)-4-methylpiperazine (MPPZ). Nine derivatives were quantified using a Shimadzu Nexera X2 interfaced with a QTrap 6500+. Recovery was 90–110% and ion suppression minimal (0 - 30%). The limits of quantification were between 2–6 pg/mL with acceptable precision and accuracy (<15%). Estrogen-MPPZ derivatives also demonstrated minimal degradation upon short-term storage at 15°C (auto-sampler) and longer-term at -20°C (<20%).

Application of this method to clinical samples from various cohorts of PAH and portopulmonary hypertension (PPHTN) patient samples allowed detection of parent estrogens (estrone (E1), estradiol (E2), and its isomer α-estradiol (17αE2)) alongside specific metabolites (16-hydroxyestrone (16OHE1), 16-hydroxyestradiol

(16OHE2), 2-methoxyestrone (2MeOE1), 2-methoxyestradiol (2MeOE2), 4-methoxyestrone (4MeOE1) and 4-methoxyestradiol (4MeOE2)). In summary, sex-dependent differences in estrogen profiles were displayed in comparisons of PAH and non-PAH control samples. In females, E1 and E2 concentrations were reduced in PAH, with an elevation in 16OHE1 and/or 16OHE2, dependent on disease classification. Conversely, in males, E1 and E2 were elevated alongside increased 16OHE1 and, in PPHTN only, 16OHE2. In addition, a proof-of-concept trial of estrogen receptor alpha (ERα) antagonism by fulvestrant administration (intramuscular, 500 mg dose) in five postmenopausal women demonstrated a reduction in 16OHE2 concentrations following 9 weeks.

Comparison of estradiol quantification by LC-MS/MS and immunoassays in two studies provided evidence of over-estimation by immunoassay. In the first, a correlation between the two methods was apparent with lower concentrations consistently quantified by LC-MS/MS than by immunoassay. In the second study, elevated estradiol concentrations following fulvestrant treatment were measured by immunoassay but not by LC-MS/MS. This suggests structural similarities between fulvestrant and estradiol causes cross-reactivity during immunoassay detection, a finding stated in a number of research papers.

Furthermore, *in vitro*, 16OHE2 caused proliferation of rat and human pulmonary artery smooth muscle cells (PASMCs). The proliferative phenotype was confined to female rat PASMCs and female human PAH-PASMCs, with no apparent effect in male rats or in female and male control human PASMCs.

This study is the first to simultaneously quantify estrogen and the bioactive metabolites in PAH patients, providing a sensitive and selective method to quantify endogenous estrogens in human serum or plasma. Its application to a number of PAH cohorts from international collaborators generated evidence of elevated 16-hydroxylation in disease. The novel findings of elevated 16OHE2 in all female patient samples, of reduced concentrations following ERα antagonism in post-menopausal women, and of sex-dependent proliferative effects of this metabolite in PASMCs indicates its potential influence in the pathogenesis of PAH. They also provide evidence that this LC-MS/MS method may be utilised as a clinical tool to monitor estrogen concentrations in patients and for the analysis of estrogen-inhibition therapies in PAH.

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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. The research was carried out in the Institute of Cardiovascular and Medical Sciences, College of Medical, Veterinary and Life Sciences at the University of Glasgow under the supervision of Professor Margaret Maclean. All LC-MS/MS work was carried out in the Mass spectrometry core facility within the Queen's Medical Research Institute at University of Edinburgh under supervision of Professor Ruth Andrew. RNA-sequencing was performed at University of Glasgow Polyomics Facility, Garscube Campus and GlaxoSmithKline, Stevenage with library preparation and sample runs by Julie Galbraith within Polyomics and by myself in GSK under the supervision of Swethajit Biswas and Jackie Meakin.

List of Abbreviations

$^{13}C_3$-16αOHE2	$^{13}C^3$-16-hydroxyestradiol
$^{13}C_6$-4OHE1	$^{13}C^6$-4-Hydroxyestrone
$^{13}C_6$-2OHE2	$^{13}C^6$-2-hydroxyestradiol
$^{13}C_6$-2MeOE1	$^{13}C_6$-2-methoxyestrone
$^{13}C_6$-4MeOE2	$^{13}C_6$-4-methoxyestradiol
2MeOE1	2-methoxyestrone
2MeOE2	2-methoxyestradiol
2MeOE2bisMATE	2-methoxyestradiol-3,17-O, O-bis-sulfamate
2OHE1	2-hydroxyestrone
2OHE2	2-hydroxyestradiol
4OHE1	4-hydroxyestrone
4MeOE1	4-methoxyestrone
4MeOE2	4-methoxyestradiol
11-KT	Keto-androsteinedione
16OHE1	16-hydroxyestrone
16OHE2	16-hydroxyestradiol
6-MWD	6-minute walk distance
A4	Androstenedione
AdvFB	Pulmonary artery adventitial fibroblasts
AF	Adventitial fibroblasts
AhR	Aryl hydrocarbon receptor
AM	Alveolar macrophages
ANGPT1	Angiopoietin 1
ANLN	Anillin Actin Binding Protein
ANXA-5	Annexin 5
APCI	Atmospheric pressure chemical ionisation
APPI	Atmospheric photoionisation
AQP1	Aquaporin 1
BKB2R	Bradykinin B2 receptor
BMI	Body mass index
BMP	Bone morphogenetic proteins
BMPR2	Bone morphogenetic protein receptor type II
BOEC	Blood outgrowth endothelial cells
CAV	Caveolin
cDNA	Complementary DNA
CHD	Coronary heart disease
CO2	Carbon dioxide
COL4	Collagen type IV alpha 1 chain
COMT	Catechol-O-methyltransferase
CS-FBS	Charcoal-Stripped Foetal Bovine Serum
CVD	Cardiovascular disease
CYC1	Cytochrome C1
CYP	Cytochrome p450
CYP19A1	Aromatase
DAPK1	Death Associated Protein Kinase 1
DHEA	Dihydroepiandrostenedione
E1	Estrone
E2	Estradiol
EC	Endothelial cells
EFI2AK4	Eukaryotic translation initiation factor 2α kinase 4

EFNA1	Ephrin A1
EGFR	Epidermal growth factor receptor
ENG	Endoglin
ERs	Estrogen receptors
ERα/ESR1	Estrogen receptor alpha
ERβ	Estrogen receptor beta
ESI	Electrospray ionistaion
ET-1	Endothelin 1
F2R	Coagulation Factor II Thrombin Receptor
FHL2	Four And A Half LIM Domains 2
FOS	Fos Proto-Oncogene
FSH	Follicle stimulating hormone
GATA2	Gata binding protein 2
GDF2	Growth Differentiation Factor 2
GREM1	Gremlin 1
hPASMCs	human pulmonary artery smooth muscle cells
HPCs	Hematopoietic progenitor cells
HRT	Hormone replacement therapy
HTR2B	5-Hydroxytryptamine Receptor 2B
ID	Inhibitor of DNA binding
IgA	Immunoglobulin A
IncRNA	Long non-coding RNA
iPAH	Idiopathic pulmonary hypertension
ITPR1	Inositol 1,4,5-Trisphosphate Receptor Type 1
KCNK3	Potassium channel subfamily K member 3
Kv	Voltage potassium channels
LC	Liquid Chromatography
LOD	Limit of detection
LOQ	Limit of quantitation
LOX	Lysyl Oxidase
LV	Left ventricle
miRNA	micro RNA
mmHG	Millimetres of mercury
mRNA	messenger RNA
MSI	Mass spectrometry imaging
MYCBP	MYC-Binding protein
NADPH	Nicotinamide adenine dinucleotide phosphate
NGS	Next generation sequencing
NO	Nitric oxide
NOX	NADPH Oxidase
Nrf-2	Nuclear erythroid-2 derived factor 2
NT-proBNP	N-terminal B-type natriuretic peptide
NYHA	New York Heart Association
O2	Oxygen
PAH	Pulmonary arterial hypertension
PAHs	polycyclic aromatic hydrocarbons
PAP	Pulmonary artery pressure
PASMCs	Pulmonary artery smooth muscle cells
PCR	Polymerase chain reaction
PGI2	Prostaglandin I2
PH	Pulmonary hypertension
pKa	Ionisation constant
PLK4	polo-like kinase 4

PODN	Podocan
PVCLD	Pulmonary vascular complication in liver disease
PVR	Pulmonary vascular resistance
qRT-PCR	Quantitative real time PCR
RHC	Right heart catheterisation
RIN	Total RNA extraction quality
rPASMCs	Rat pulmonary artery smooth muscle cells
rRNA	ribosomal RNA
RV	Right ventricle
RVSP	Right ventricular systolic pressure
SEM	Standard error of the mean
sGC	Soluble guanylate cyclase
snoRNA	Small nucleolar RNA
SNP	Single nucleotide polymorphism
SOD3	Superoxide dismutase-3
SOX17	SRY-related HMG-box-17
Su/Hx	Sugen-Hypoxic
T	Testosterone
TAPSE	Tricuspid annular plane systolic excursion
TBX4	T-Box-4
TGFBR1	Transforming Growth Factor Beta Receptor 1
TGFBR2A	Transforming Growth Factor Beta Receptor 2A
TMS	Trans-2, 3', 4, 5'-tetramethoxystilbene
TPH-1	Tryptophan hydroxylase 1
TXA2	Thromboxane A2
VEGF	Vascular endothelial growth factor
WHO	World health organisation
WSPH	World Symposium on Pulmonary Hypertension

Chapter 1 Introduction

1.1 The Pulmonary Circulation

Dynamic alterations in pulmonary vascular resistance and blood flow are evident throughout foetal life. In the foetus, alveoli and pulmonary arteries are not required as blood is oxygenated via the placenta. This means the lungs are the final organ to mature *in utero*. In early development the airways act as a template for pulmonary blood vessel development. In later stages of lung development the capillary bed remains essential for alveolar formation, as blood is to be no longer oxygenated through the placenta (Hislop, 2002). A transition of the pulmonary artery occurs at birth when oxygen tension increases causing a reduction in pulmonary vascular resistance (PVR) accompanied by a closure of the ductus arteriosus. This forces blood flow into the pulmonary arteries from the right ventricle (RV).

In the circulation of blood of adults, pulmonary blood flow carries blood to the pulmonary microcirculation. The pulmonary artery pressure (PAP) within the vasculature is a product of cardiac output (CO) and pulmonary vascular resistance (PVR). The pulmonary circulation is a low oxygen and high resistance system. Human lungs are the only organ to receive the entire cardiac output at all times, around 5 litres of blood per minute increasing to around 25 litres upon exercise (Mandegar *et al.*, 2004). Normally, the pulmonary architecture ensures maintenance of the high compliance, low resistance network facilitating gas exchange via an extensive surface area. Major differences exist between the pulmonary and systemic circulation. In the systemic circulation thick walls create high pressure arteries capable of distributing oxygenated blood around the body to peripheral organs. Unlike the pulmonary arteries high pressure is maintained at around 120/80 mmHg (Magder *et al*, 2018). The pressure gradient throughout is 70 – 90 mmHg being around 10-fold higher than those of the pulmonary circulation. Within the systemic circulation, hypoxia results in vasodilation, enabling tissues and organs to receive the energy requirements for normal function via increased perfusion. Here, high vascular tone exists, with vascular resistance being controlled by small muscular arterioles (80%) whereas resistance in the pulmonary circulation is evenly distributed (MacLean *et al.*, 2000).

In the circulatory system, deoxygenated venous blood drains into the right atrium diverging from the upper and lower systemic circulatory systems, via the superior and inferior vena cava respectively. Blood flows into the right ventricle by a diastolic relaxation of the heart, the tricuspid valves then open allowing blood to flow into the right ventricle (RV). Upon systolic contraction, tricuspid valves close and blood is forced through semi-lunar valves into the pulmonary artery for direct oxygenation upon bifurcation into the left and right pulmonary arteries. In pulmonary arteries the systolic pressure is ≤20 mmHg compared to 120 mmHg in systemic arteries. For gaseous exchange, the smooth muscle walls of the artery and minimal and thin which facilitates a low pressure, high resistance system. This allows easier adaption to alterations in CO, maintaining normal exchange rates. Each artery enters the respective lung as intra-lobular pulmonary arteries. The distal intra-lobular pulmonary arteries run in parallel to the bronchus and alveoli creating a complex network of capillaries through which diffusion of carbon dioxide (CO_2) and oxygen (O_2) can diffuse. The pattern of branching runs alongside those of the pulmonary veins but remains distinct and separated from them. The lungs themselves are divided into the left and right lobes which then are subdivided (Figure 1-1).

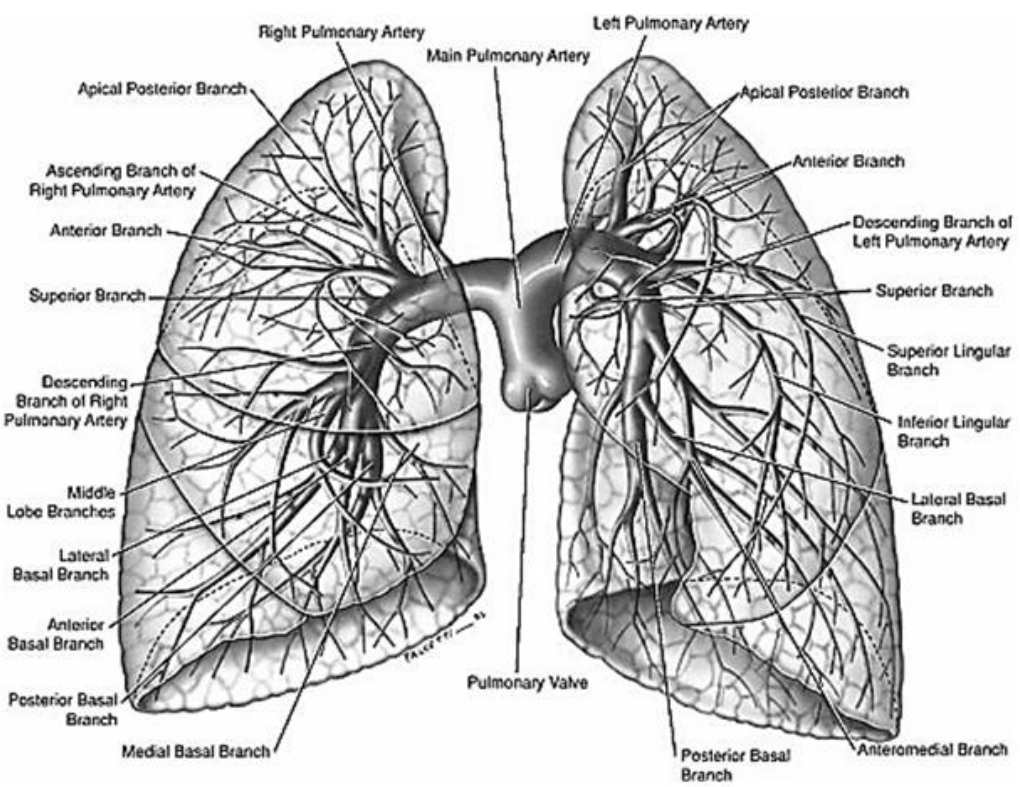

Figure 1-1: Lung Anatomy Distribution of pulmonary arteries in the lobes of both lungs, accessed on thoracickey.com/pulmonary-vasculature, 26/07/2019

The left side of the lung is divided into two lobes, the superior and inferior separated by the oblique fissure. Each lobe receives a branch from the left pulmonary artery. The right lung is divided into three lobes (superior, middle and inferior) separated by interlobular fissures. Each is supplied with a branch from bifurcation of the right pulmonary artery (Garcia-Medina *et al.*, 1990). The distal intra-lobular network continues until terminal alveoli are reached following fifteen orders of branching. Each lung section remains anatomically distinct and hence functionally can act as single entities with distinctive patterns of pulmonary branching and unique bio-molecular activity.

1.1.1 Structure of the Pulmonary Circulation

The structural architecture of the healthy pulmonary vasculature ensures a vast surface area for gaseous exchange within a high compliance, low resistance network. Initial development of the pulmonary vasculature is dependent on the presence of growth factors such as vascular transforming growth factor (VEGF) and transforming growth factor-β (TGF-β). Eventual modelling through migration and proliferative processes results in 3 distinct layers; tunica adventitia, tunica media and the tunica intima. These contain all main functional cellular components within the vasculature: endothelial cells (EC), smooth muscle cells (SMC) and adventitial fibroblasts (AFs), essential for the most vital exchange processes (Chelladurai, Seeger and Pullamsetti, 2012). The pulmonary circulation is characterized by thin walled, large diameter vessels without predominant muscular layer resulting in low resistance and low pressures.

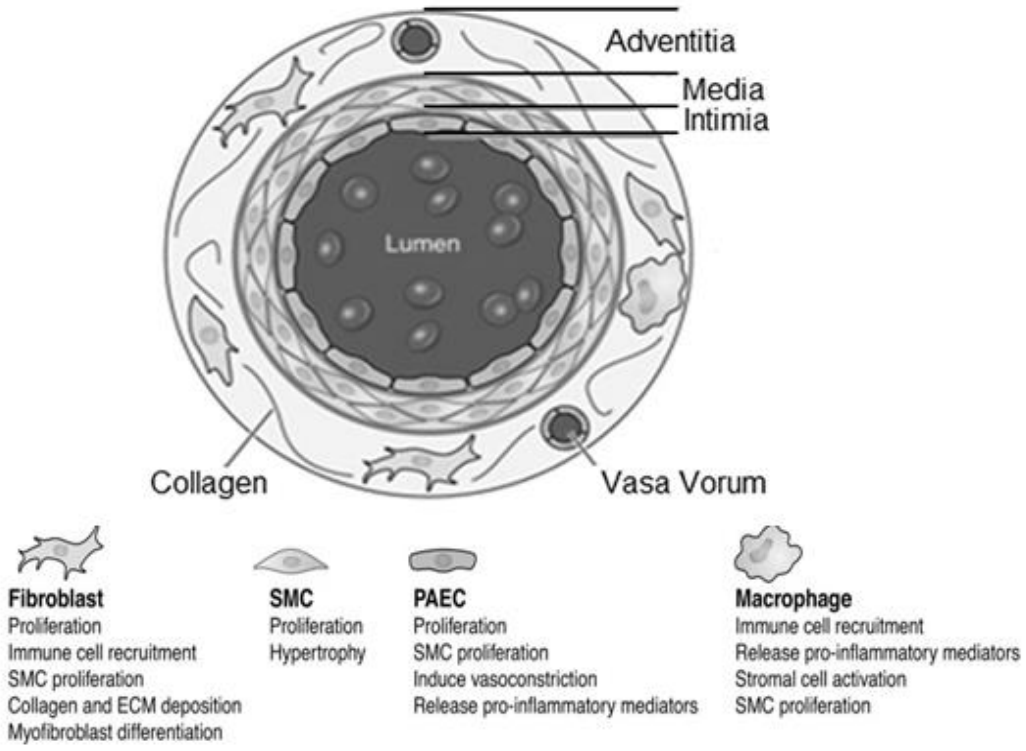

Figure 1-2: Pulmonary arterial wall structure Depicting distinct layering and structural components composing its structural morphology (Adapted from S.C. Pugliese et al, 2015)

The outermost adventitia acts as a collagen matrix upholding the structural integrity and stability of the vessel wall. Within this layer pulmonary artery adventitial fibroblasts (AdvFB) are predominant, and activation of this cell type by environmental stimuli has been identified as an important modulator of vascular modelling (Davi*e et al*, 2006). The medial layer consists of smooth muscle cells (SMC) arranged around the outer intima of the vessel, separated by an internal elastic lamina which regulates vascular tone and blood pressure (Figure 1-2). Under normal physiological conditions pulmonary artery smooth muscle cells (PASMCs) are quiescent, however these are the only cell type capable of switching consecutively between contractile, synthetic and intermediate phenotypes upon stimulation (Stenmark *et al.*, 2011). Finally, the innermost intima layer consists of a thin layer of structurally homogenous ECs lining the lumen and existing as a mono-layer attached to an underlying connective tissue matrix, known as the basement membrane. The ECs are the most vulnerable to external stress and to interactions with external mediators due to their constant physical contact with blood flow. This property may also be crucial in the monitoring and regulation of the luminal environment via the release of agents causing Ca^{2+} sensitization in the smooth muscle (Aaronson *et al.*, 2006). The wall structure changes throughout the orders of pulmonary branching based on the distribution of elastic lamina and degree of

muscularity. Of the 15 orders, 15 – 13 have abundant elastic portions within the tunica media aiding compliance (Elliott and Reid, 1965), 13 – 4 present more SMC layers within the tunica media with these becoming gradually diminished toward order 4. Hence there is partial muscularisation further away from the initial site of bifurcation in the distal vessels approaching the alveoli. By orders 3 -1 the arteries become non-muscularised reaching the capillary network (Jones *et al.*, 2011). Undifferentiated perivascular cells known as pericytes are present within these non-muscularised pulmonary arteries which potentially may contribute toward remodelling by excreting collagen fibres and transforming SMCs (Ricard *et al.*, 2014, Figure 1-3).

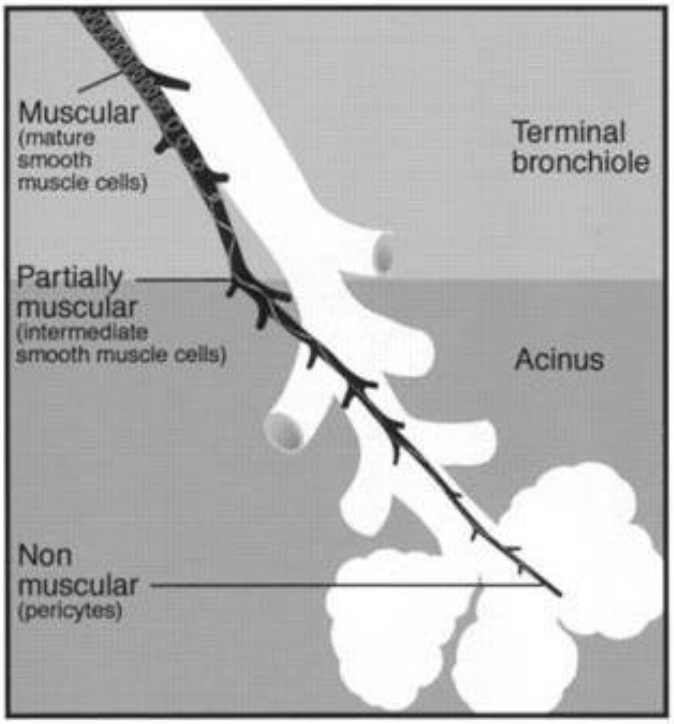

Figure 1-3: Muscularity of the pulmonary arteries within a healthy lung In distal pulmonary arteries the smooth muscle layer is lost in the media and replaced by thin walls composed of endothelial cells and pericytes (adapted from (MacLean *et al*, 2000))

1.1.2 Function of the Pulmonary Circulation

The primary function of the pulmonary circulation is to facilitate gas exchange via the unloading of CO_2 from the blood and upload of O_2 into the blood. Upon respiration, CO_2 is rapidly unloaded from deoxygenated blood whilst O_2 binds to haemoglobin in red blood cells and circulates the upper and lower extremities to sustain essential metabolic processes.

In addition to gaseous exchange, the lung also fulfils important non-respiratory functions. The pulmonary circulation also acts as a primary defence system removing fine particulate matter, toxic gases, micro-organisms and preventing the passage of foreign bodies and pathogens from the respiratory system to the cardiovascular system (Iwasaki *et al,* 2017). As inhaled air is not innocuous this mechanism remains crucial in modulating innate immunity through antimicrobial response mechanisms within the respiratory tract. The upper and lower airways offer protection via anatomical barriers such as the cough reflex and mucociliary apparatus with enzymes and secretory immunoglobulin A (IgA) (Nicod, 2005). In the cardiovascular system the pulmonary circulation also acts as a barrier for lethal thromboembolism and embolic occlusion which may lead to infarction (Barsoum *et al.*, 2014). In addition, the pulmonary circulation acts as a blood reservoir for the left ventricle (LV) (Comroe, 1966).

1.1.3 Regulation of Pulmonary Vascular Tone and Resistance

Pulmonary vascular tone is controlled significantly by the activity of potassium channels within PASMCs. In human PASMCs, voltage-dependent potassium channels (Kv) influence the resting membrane potential mediating vasoconstrictive responses to hypoxia (Gurney *et al.*, 2002). Vasoconstriction in this manner is mediated by calcium mobilisation via voltage sensor activation, resulting in depolarisation of the cell membrane, allowing Kv channels to open and potassium to flow out of the cell, restoring the membrane potential. This in turn restores a low vascular tone.

Additionally, important in this role are ECs which function to sense and mediate signalling to the underlying SMCs. Cross talk between these two cell types occurs upon well-regulated production and release of vasoactive mediators. Vasoconstrictors secreted by ECs include endothelin-1 (ET-1) and thromboxane A2 (TXA2) causing vascular contraction by activation of adrenergic fibres releasing noradrenaline. Conversely, EC-derived vasodilators nitric oxide (NO) and prostaglandin I_2 (PGI_2) cause vascular relaxation via activation of cholinergic fibres releasing acetylcholine. These act in a paracrine manner in the endothelium and in an autocrine manner on the smooth muscle cell layer orchestrating the fine physiological balance in obtaining normal function and vascular homeostasis.

The pulmonary vascular resistance (PVR) is defined as the total peripheral resistance to flow which must be overcome to maintain continuous blood flow through the pulmonary arteries. PVR is inversely proportional to the fourth power of the lumen radius meaning small changes to the lumen size and vascular tone can significantly alter PVR and impact the pulmonary artery pressure (PAP). PVR and total artery compliance (C) quantify the resistive and elastic properties of pulmonary arteries, which allow a steady and pulsatile state for adequate pulmonary artery load. The common calculation of this measure accounts for a trans-pulmonary arterial pressure gradient over flow and total arterial compliance as stroke volume over pulmonary arterial pulse pressure (SV/PAP) (Chemla *et al*, 2015).

1.2 Pulmonary Hypertension

Pulmonary hypertension (PH) is defined as a type of high blood pressure affecting the pulmonary arteries of the lungs, causing damage to the right side of the heart. The disease is complex and devastating in most cases, causing obliterative remodelling and obstruction or total loss of distal circulation. Increased PAP and vascular resistance challenge the RV initiating hypertrophy, increasing the RV cardiac muscle thickness and leading to right ventricular heart failure. As PH embodies a pathophysiological state of diverse aetiologies, grouping of PH into well-defined subcategories aids medical practitioners in preventing mis-diagnosis and in providing suitable treatment options.

1.2.1 WHO and NYHA Clinical Classifications

The World Health Organization (WHO) categorises patients into five distinct functional classifications. These classifications are derived from experts in the field brought together since 1973 by the World Symposium on Pulmonary Hypertension (WSPH). In 2018, the 6th World Symposium on pulmonary hypertension updated how shared histology and vascular pathologies should be defined in updated clinical guidelines (Simonneau *et al.*, 2019). Each classification defines hallmarks of disease based on the aetiology, patient prognosis and therapeutic management (Table 1-1). The WHO classification entails five sub-categorised disorders, which are, pulmonary arterial hypertension (PAH) (Group 1); PH owing to left heart disease (Group 2); PH owing to lung diseases and/or hypoxia (Group 3); chronic

thromboembolic PH (Group 4); and PH with unclear multifactorial mechanisms (Group 5).

The functional classification then assigned to each patient aims to combine symptoms with quality of life data in patients and their daily limitations. This is derived from the New York Heart Association (NYHA) scale of heart failure. Table 1-2 shows that patients may in fact present no daily limitations (Class I) but can also be incapable of breathing comfortably at rest (Class IV). Notably patients from class III represent the widest population of PAH patients from a diverse range in ability and symptoms meaning this should be considered within data extrapolation.

Table 1-1: World Health Organisation (WHO) Clinical Classification of Pulmonary Hypertension Updated: Nice 2018

Group 1: Pulmonary arterial hypertension (PAH)

1.1 Idiopathic
1.2 Heritable
1.2.1 BMPR2
1.2.2 ALK-1, endoglin (with or without hereditary haemorrhagic telangiectasia)
1.3 Drug- and toxin-induced PAH
1.4 PAH associated with:
    1.4.1 Connective tissue disease
    1.4.2 HIV infection
    1.4.3 Portal hypertension
1.4.4 Congenital heart disease
    1.4.5 Schistosomiasis
1.5 PAH long-term responders to calcium channel blockers
1.6 PAH with overt features of venous/capillaries (PVOD/PCH)
1.7 Persistent PH of the newborn syndrome

Group 2: PH due to left heart disease

2.1 PH due to heart failure with preserved LVEF
2.2 PH due to heart failure with reduced LVEF
2.3 Valvular heart disease
2.4 Congenital/acquired cardiovascular conditions leading to post-capillary PH

Group 3: PH due to lung diseases and/or hypoxia

3.1 Obstructive lung disease
3.2 Restrictive lung disease
3.3 Other lung disease with mixed restrictive/obstructive pattern
3.4 Hypoxia without lung disease
3.5 Developmental lung disorders

Group 4: PH due to pulmonary artery obstructions

4.1 Chronic thromboembolic PH
4.2 Other pulmonary artery obstructions

Group 5: PH with unclear and/or multifactorial mechanisms

5.1 Haematological disorders
5.2 Systemic and metabolic disorders
5.3 Others
5.4 Complex congenital heart disease

ALK-1: activing receptor like-kinase-1; BMPR2: Bone morphogenetic receptor type 2; HIV: human immunodeficiency virus; PAH: pulmonary arterial hypertension; PVOD: pulmonary veno-occlusive disease; PCH: pulmonary capillary haemangiomatosis; LVEF: left ventricular ejection fraction.

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

Class I	Patients that do not experience symptoms with ordinary physical activity. Ordinary physical activity does not cause undue dyspnoea or fatigue, chest pain or near syncope.
Class II	Patients with no symptoms at rest but feel uncomfortable and shortness of breath with ordinary activities resulting in limitation of activity. Ordinary physical activity causes undue dyspnoea or fatigue, chest pain or near syncope.
Class III	Patients experience no symptoms at rest with limitation of physical activity. Less than ordinary physical activity causes undue dyspnoea or fatigue, chest pain or near syncope.
Class IV	Patients experience an inability to perform any physical activity without symptoms. These patients manifest signs of right heart failure. Dyspnoea and/or fatigue may be present at rest and increased by almost any physical activity.

1.2.2 Pulmonary Arterial Hypertension (PAH)

Pulmonary arterial hypertension (PAH) is a debilitating disease characterised by an increase in pulmonary artery pressure of over 20 mmHg at rest. Substantial vascular remodelling via excessive smooth muscle, fibroblast and endothelial cell proliferation (Tuder *et al.*, 2013) results in pulmonary artery (PA) occlusion (Perros *et al*, 2005) In 80% of cases patients with severe to end-stage disease develop plexiform regions causing plexogenic arteriopathy (Ogata *et al*, 1993), which manifests in distal pruning of vessels within the lungs. Throughout these pathophysiological alterations, vascular tone increases, causing an elevation of pulmonary pressures and resistance to blood flow. Subsequently, challenging the heart, increasing RV workload, initiating maladaptive hypertrophy, dilating the RV wall and weakening RV function over time leading to right heart failure (Jacobs *et al.*, 2014).

1.2.3 Epidemiology of Pulmonary Hypertension

Occurrence of both idiopathic and familial PAH ranges from 6-10 cases per million in the population whereas the number of those patients developing PAH as a result of other illness is thought to be much higher, although not reported (Barst, 2008). PAH is currently incurable. Its effects on arteries of the pulmonary circulation contribute to the morbidity and mortality of adult and paediatric patients with lung and heart diseases. Recent survival rates suggest the number of patients surviving

the disease following diagnosis stands at 85% after 1 year, 68% after 3 years and 57% after 5 years (Benza *et al.*, 2012). Following successful transplantation, the one year survival rate now exceeds 90% (Galiè, Humbert, *et al.*, 2015). Each class of PAH holds certain levels of severity for individual patients dependent on their symptoms presented in the clinic. Mainly patients are diagnosed based on their physical abilities and on their relative levels of chest pain and fatigue during their day-to-day lives. Table 1-2 describes the four main severity levels related to PAH progression ranging from those with no daily limitation to others who exhibit complete incapacity even at rest.

1.3 Pathobiology of Pulmonary Arterial Hypertension

The pathogenesis of PAH is complex and involves numerous pathophysiological phenotypes including PAEC dysfunction, PASMCs proliferation, apoptosis resistance, metabolic shift (Warburg effect), impaired angiogenesis, phenotypic transition and chronic inflammation (Umar, Rabinovitch and Eghbali, 2012; Austin *et al.*, 2013; Dweik *et al.*, 2014). Evidence from clinical and experimental studies suggests there is a pathogenic transformation in the EC layer, triggered by either an exogenous or endogenous insult (Figure 1-4).

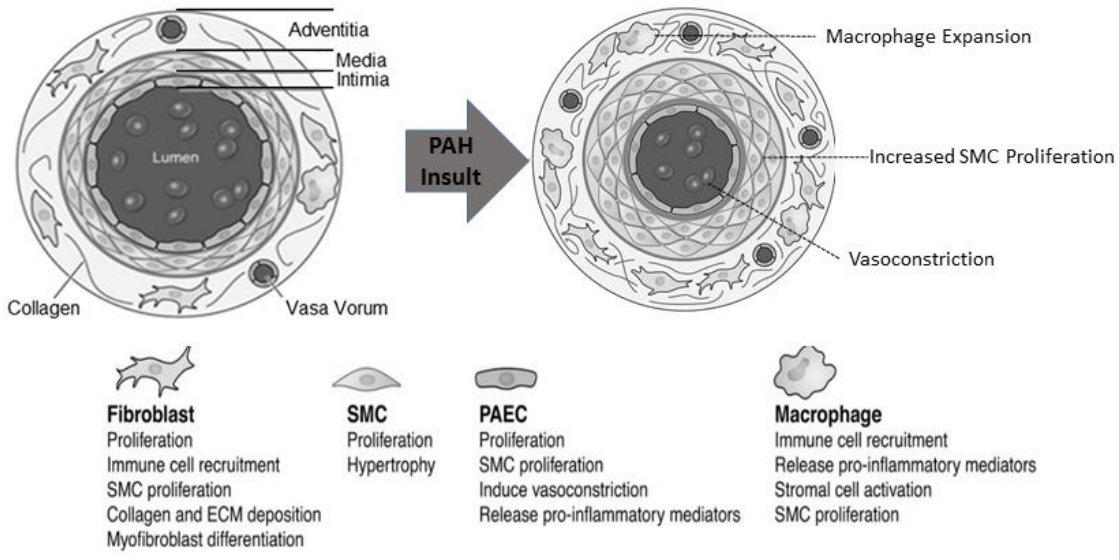

Figure 1-4 Pulmonary arterial remodelling in PAH prolonged exposure to stress factors causes progressive changes in intimal and medial morphology (Adapted from S.C. Pugliese et al, 2015).

Exogenous stimuli include exposure to circulating modulators or exposure to hypoxia causing reduced structural and functional ability (Morrell *et al.*, 2009). Endogenously, heritable genetic dysfunction may cause the switching on/off of

crucial genes within the vasculature. The most prominent within PA relates to the loss of bone morphogenetic protein-II gene mutation (*BMPR2*, 1.3.1.1). Again, this dysfunction disrupts the EC layer, initiating apoptosis which exposes the underlying PASMC layer and leads to its aberrant proliferation (Zhang *et al.*, 2003). In addition, synthesis of modifiers such as endothelin 1 (ET-1) from ECs may occur, further disrupting the PASMC layer (Giaid *et al.*, 1993). ET-1 is a potent vasoconstrictor, predominantly produced and released by endothelial cells which has been reported as being elevated in PAH (Chester and Yacoub, 2014) and contributing to severe phenotypic alterations observed in PAH (Figure 1-4). Simultaneously, in PAH a loss of nitric oxide (NO) production in ECs has been demonstrated (Giaid and Saleh, 1995) contributing to pulmonary vasoconstriction and the excessive growth of PASMCs. Concurrently inflammation of the PA is in part driven by the presence of perivascular macrophages, which expand upon PAH insult (Florentin *et al.*, 2018).

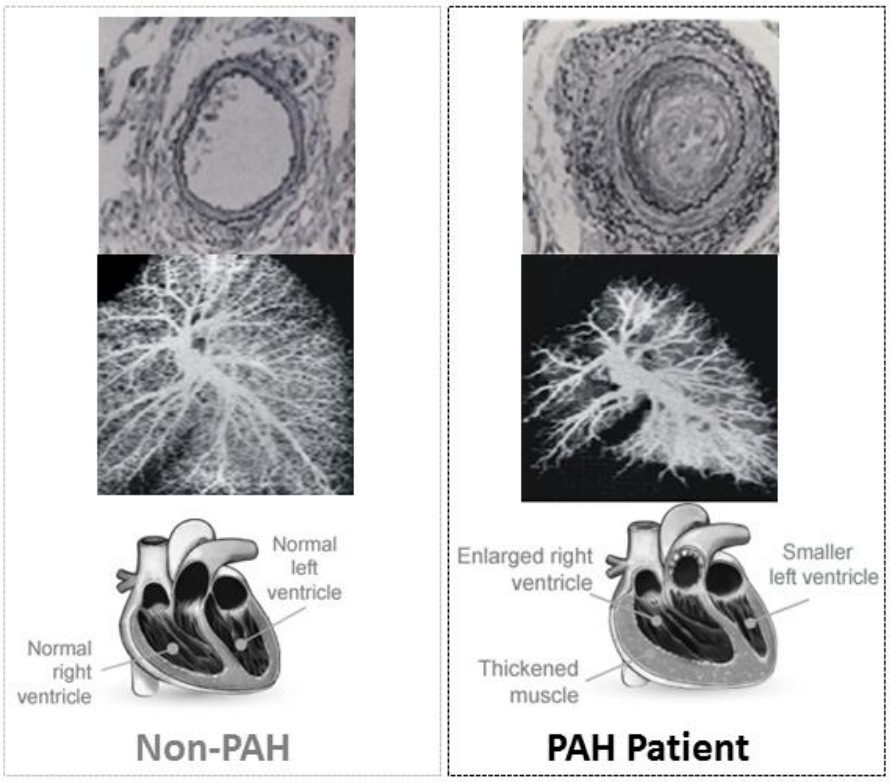

Figure 1-5: Pulmonary vascular histology, arteriograms and heart anatomy Pulmonary vasculature histology section, arteriogram and heart anatomy from a healthy (Non-PAH) adult lung (left) and from a PAH patient lung (right). The extensive loss of peripheral vasculature and consequent strain on the heart are displayed.

All changes result in a direct loss of blood flow within the peripheral vasculature an effect termed “vascular pruning” (Figure 1-5) increasing PAP, pulmonary wedge pressure (PWP) and maladaptive hypertrophy in the heart chambers.

1.3.1 Genetics of PAH

The heritable component within PAH has been recognised since the early 1950s, following observations of repeated occurrence of disease within a single family (Dresdale *et al,* 1951). The precise genetic risk factor contributing to this autosomal inherited trait was then discovered a further 50 years on from this date, showing a mutation in the bone morphogenetic protein receptor type II (*BMPR2*) gene (Deng *et al.*, 2000; Lane *et al.*, 2000). This remarkable finding has paved the way toward understanding the underlying mechanisms of disease manifestation.

1.3.1.1 Bone Morphogenetic Protein Receptor Type II

A mutation in the *BMPR2* gene underpins 80% of heritable PAH (hPAH) and 6-40 % of idiopathic PAH (iPAH) cases, the difference being that hPAH cases present a family history of disease which is not present in iPAH patients. In addition, hPAH and iPAH are histopathologically indistinguishable and clinically present with identical symptoms and characteristics. In each clinical phenotype, females with this mutation are two times more likely to develop hPAH than males (Batton *et al.*, 2018). Interestingly, in hPAH the penetrance of the mutation is only 20% overall, but is sex-dependent with the female to male ratio in hPAH and PAH being 2:5 (Austin *et al*, 2009) A pathogenic variation in *BMPR2* is present in around 14% of male and 42% of female cases. This suggests a sex-dependent secondary risk factor is contributing toward disease manifestation. In addition, the type of *BMPR2* mutation present in individuals also affects disease severity, with this stated to be more influential in males (Liu *et al*, 2012). Presence of an escape (missense) mutation over the active (truncating) mutation results in a dominant negative effect on signalling of bone morphogenetic proteins (*BMP*). Therefore, missense *BMPR2* mutations result in more severe PAH phenotype via a reduction in BMPR2 mediated signalling (Austin *et al*, 2009).

BMPR2 is a serine-theonine receptor kinase member of the TGF-β type II superfamily of type II receptors. This family includes TGF-β, BMPs activins and inhibins. BMPs exhibit a wide range of biological activities including but not limited

to growth regulation, differentiation and apoptotic effects. These are crucial for the development of the pulmonary circulation and signal through pathways involving SMAD, which forms part of the TGF-β type II superfamily (Yang *et al*, 2017) (Figure 1-6). Mediation of BMP signalling effects is dependent on the cell type, with proliferative actions reported in ECs and anti-proliferative effects in PASMCs (Teichert-Kuliszewska *et al.*, 2006). BMPs can signal via both canonical and non-canonical pathways. In canonical signalling they bind to cell surface receptors forming a heterotetrameric complex. These may bind to one of seven type I receptors (*ALK-1*) or to one of four type 2 receptors from the *TGF-β* superfamily, activating phosphorylation of the downstream signalling proteins known as receptor-regulated *SMAD*s (R-Smads). *SMAD1*, *SMAD5* and *SMAD8* are involved in *BMP* signalling. The co-mediator *SMAD* (*SMAD4*) associates with the R-*SMAD*s, with the complex undergoing translocation to the nucleus, where mediation of transcription occurs.

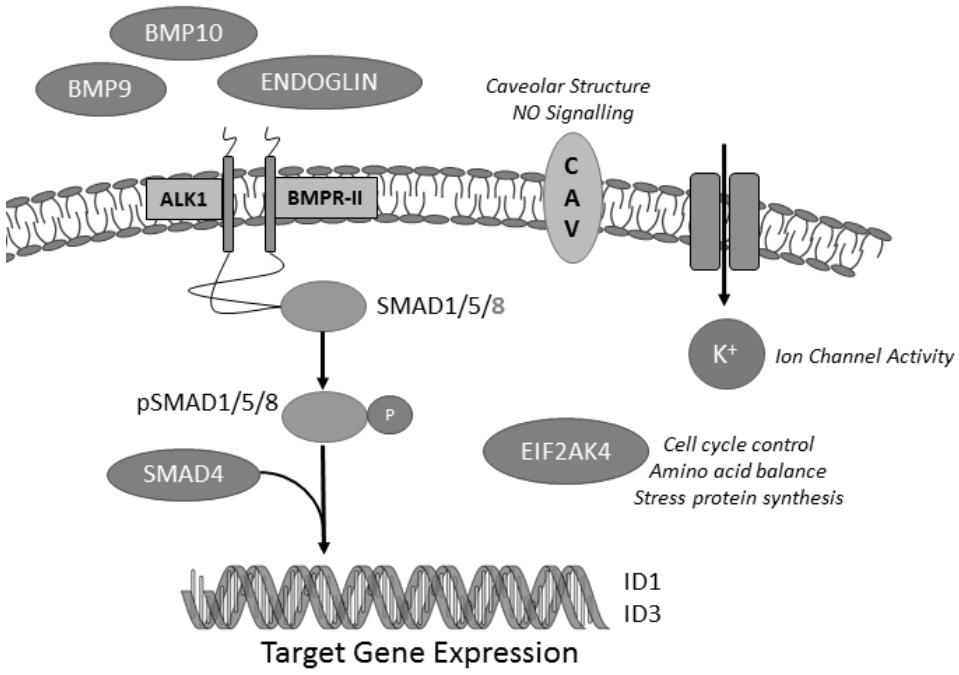

Figure 1-6: BMP signalling BMP9 and BMP10 in the circulation initiate signalling upon binding and phosphorylation of ALK1 by BMPR2. As a result, SMAD 4 forms a complex with SMAD1/5/8 and translocates to the nucleus, regulating genes involved in transcription such as ID1 and ID3. Mutations in BMP signalling components such as SMAD 8, ENDOGLIN, ALK1 BMPR2 are implicated in PAH. In addition, non-directly linked genes such as EIF2AK4 have also been linked to PAH development. CAV caveolin, EFI2AK4 eukaryotic translation initiation factor 2α kinase 4, ENG ENDOGLIN, ID inhibitor of DNA binding, KCNK3 potassium channel subfamily K member 3

Non-canonical *SMAD*-independent pathways exist via activation of the MAP kinase pathway (*MAPK*) or *PI3K/Akt,* P/Rho-GTPases although these signalling routes remain dependent on the extracellular environment (Wang *et al.*, 2014). The pulmonary vasculature appears to be the most prone to the effects of *BMPR2* mutations in the whole body. At the cellular level, within the vasculature, expression of *BMPR2* varies between cell types with a much lower basal expression in PASMCs than ECs (Thenappan *et al.*, 2018). The functionality within each cell type also depends on the proximal or distal location of the vascular cells within the pulmonary artery (Yang *et al.*, 2005). Divergent responses are shown in proximal regions where stimulation of *BMP-4* inhibits proliferation whilst in the distal arteries the opposing effect occurs. In the case of a *BMPR2* mutation, ECs are prone to apoptosis, which exposes the underlying smooth muscle layer to a multitude of growth factors whose production are also increased following mutations (Yang *et al.*, 2005). There is also reduced *SMAD* expression in ECs and PASMCs from hPAH patients presenting with reduced *BMPR2* expression (Yang *et al.*, 2005). Dysfunction of *SMAD* signalling via extracellular regulated kinases (ERK1/2) induces proliferation of PA cells (Yang *et al.*, 2008). Additionally within the vasculature, reduced *BMPR2* signalling and Id1/Id3 have been recognised as important targets; *Id3* regulates the cell cycle in PASMCs and *Id1* also shows crucial roles in PASMC proliferation (Yang *et al.*, 2010). Expression profiles also differ between sex with reduced expression of *BMPR2*, *SMAD1*, *Id1* and *Id3* in female non-PAH PASMCs compared to male non-PAH PASMCs (Mair *et al.*, 2015). The reduced expression profile of *BMPR2* proteins is also replicated in animal models of PAH such as the hypoxia, monocrotaline and Sugen-Hypoxic (Su/Hx) models (Takahas*hi et al*, 2006; Morty *et al*, 2007; Mair *et al*, 2014). In a heterozygous *BMPR2*$^{+/-}$ mouse model of PH the addition of a secondary insult led to a more severe phenotype, in this case serotonin inhibited BMP signalling via SMAD protein exacerbating disease (Long *et al.*, 2006). In mice, 16hydroxyestrone (16OHE1) promotes the development of PAH in the presence of a BMPR2-RX mutation (Chen *et al.*, 2016). This suggests downstream estrogen metabolism may be implicated in the heritable disease, which furthermore may differ based on sex. Despite this pathway being crucially important in PAH, there are still no drugs which target modification of *BMPR2* impairment. Therefore, the ability to reverse *BMPR2* mutations remains a focus for therapeutic development.

1.3.1.2 Newly Identified Genetic Mutations

Advances in sequencing technologies have allowed a greater understanding of the genetics behind PAH leading to the discovery of additional genes related to heritable and idiopathic forms of PAH. Most notably in this century, mutations in *ALK1*, Endoglin, *SMAD9*, *CAV1* and *KCNK3* have been associated with autosomal dominant familial disease and *EIF2AK4* with autosomal recessive (Figure 1-6) (Austin *et al*, 2014). In 2018 at the 6th World Symposium on PAH new sequence variants underlying hPAH were unveiled; *ATP13A3*, *AQP1* and *SOX17* and a critical role for *GDF2*. Here, variants detected in *GDF2* lead to reduced GDF2 production from cells and altered signalling within the BMP pathway (Gräf *et al.*, 2018). *AQP1* was previously implicated in hypoxia-induced PH in pulmonary vascular cells (Lai *et al.*, 2014). Also the implication of *AQP1* was again suggested in hypoxia-induced PAH models following a knockout of this gene which resulted in a reduction of migration and vascular cell apoptosis (Schuoler *et al.*, 2015). *SOX17* was also previously identified as a potential risk gene in PAH associated with congenital heart disease as well as in both idiopathic and heritable cases (Zhu *et al.*, 2018). These findings are significant in contributing toward describing the molecular basis of PAH, indicating potential targets for therapeutic development. The exact interaction of these genes in BMPR2 related and sex-dependent PAH should be investigated.

1.4 Diagnosis of Pulmonary Hypertension

There is no known cure for PH which accounts for a high mortality rate in those patients diagnosed with PAH. In addition, patients often must wait a sustained period for diagnosis, due to the unspecific nature of symptoms, and therefore delayed referral by general practitioners (Connolly and Kovacs, 2012).

The specific pathophysiological insults contributing to PAH remain undetermined with manifesting symptoms such as dyspnoea, impaired exercise tolerance and fatigue which in the majority of cases are attributable to lung diseases and hypoxia. Therefore, diagnosis involves a series of multi-factorial investigations as symptoms mimic those of other conditions, adding a further level of complexity. Initial electrocardiography, chest radiography and pulmonary function tests seek to identify causes of breathlessness. In iPAH, ~85% of cases display evidence of right heart strain on electrocardiography with a reduction in gas transfer and spirometry measures (Kiely *et al*, 2013). If initial symptoms point toward PAH, further tests are

required to determine the cause and disease severity. On average, delay from initial onset of symptoms related to PAH and actual diagnosis of the illness is currently around 2 years due to the unspecific nature of symptoms. The final confirmative diagnosis is given upon referral to a specialist centre where patients undergo an invasive right heart catheterisation (RHC) to confirm increases in the mean PAP >25 mmHg.

1.4.1 Current therapeutic approaches in PAH

The optimal treatment strategy for PAH patients hinges on accurate and specific diagnosis within specialised treatment centres. Often the treatment of group 1 patients, specifically idiopathic and heritable cases, is complicated due to a lack of understanding of the molecular interactions involved. As a monitoring evaluation to treatment response, the six-minute walk test (6-MWD) has been extensively evaluated. This provides a functional and prognostic marker of disease severity which, upon treatment, would be expected to increase following successful assessment in comparison to baseline measurements (Miyamoto *et al.*, 2000).

Current therapies are drug-based approaches aimed at reducing blood pressure and equilibrating the balance between vasoconstriction and vasodilation within the pulmonary arteries, thus improving the quality of life for patients. There remains a high unmet clinical need for advanced understanding and targeting of bio-molecular interactions involved in PAH.

1.4.1.1 Calcium Channel blockers

Patients with a positive vaso-reactivity test upon right heart catheterisation will receive calcium channel blockers. The vasoconstrictive and excessive proliferative response of pulmonary arteries is governed by the availability of intracellular calcium (Ca^{2+}) (Firth, Won and Park, 2013). Ca^{2+} blockers such as diltiazem or nifedipine inhibit the influx via voltage-gated Ca^{2+} channels. This results in a reduction of PAP and vascular tone seen in PAH by preventing hyperpolarisation of SMCs. This treatment is opted for upon a positive vasodilatory test applied during RHC (Tonelli, Alnuaimat and Mubarak, 2010). Roughly 10% of patients respond to the vasodilator test. In anorexigen-associated PAH, the response to Ca^{2+} channel blockers remains favourable although for PAH associated with congenital heart disease, porto-

pulmonary hypertension or HIV the response remains poor (Preston *et al*, 2013). Thus, this treatment option is generally limited to a small minority of PH patients

1.4.1.2 Endothelin receptor antagonists

Endothelin 1 (ET- 1) is a 21-amino acid, vasoactive peptide. Under physiological conditions, ET-1 is produced in small amounts, mainly in endothelial cells, and acts an autocrine/paracrine mediator. The biological effects of ET-1 are mediated by two receptor subtypes, the ETA and ETB receptors. In the vasculature, the ETA receptor is mainly located on vascular smooth muscle cells, whilst ETB receptors are localized to both endothelial and vascular smooth muscle cells. Activation of ETA receptor function promotes vasoconstriction, growth and inflammation whilst ETB receptors located in the pulmonary circulation produce vasodilation, increasing sodium excretion and inhibiting growth and inflammation (McCulloch *et al.*, 1996; Schneider *et al*, 2007). The activation of endothelial ETB receptors stimulates the release of nitric oxide (NO) and prostacyclin (Hirata *et al.*, 1993), prevents apoptosis and plays a minor role in endothelial-dependent vasodilatation (Shichiri *et al.*, 1997). The lungs are the major site for both clearance and production of circulating ET-1: up to 50% of circulating ET-1 is cleared through the ETB receptors (Dupuis, 2001).

In PAH upregulation of the ET-1 system occurs. ET-1 plasma levels are elevated and the increase is correlated with right atrial pressure, pulmonary artery oxygen saturation and pulmonary vascular resistance (Stewart *et al*, 2005). The ET-1 precursor, prepro-ET-1, is also abundantly over-expressed in endothelial cells from patients with PAH and stimulates proliferation of PASMCs and vasoconstriction. The ET-1 receptor antagonists bosentan, macitentan and ambrisentan are currently approved for the treatment of PAH, preventing the aberrant activity of ET-1 observed in patients. Bosentan and macitentan are dual ETA and ETB receptor antagonists, whereas ambrisentan is a selective ETA receptor antagonist (Correale *et al.*, 2018).

1.4.1.3 Prostacyclin Analogues

Prostacyclin is a member of the eicosanoid family of mediators, which include prostaglandins, thromboxanes and leukotrienes. It is a potent vasodilator and inhibitor of platelet aggregation. Prostacyclin is synthesised from arachidonic acid by the actions of cyclo-oxygenase and prostacyclin synthase, primarily in endothelial cells. Dysregulation of prostacyclin metabolic pathways occurs in PAH. A reduction

in prostacyclin levels is observed, characterised by a decrease in urinary metabolites (Christman *et al.*, 1992) and in prostacyclin synthase expression in the lungs of PAH patients (Tuder *et al*., 1999). Several prostacyclin analogues including epoprostenol, iloprost and treprostinil are used clinically in the treatment of PAH (Galiè *et al.*, 2015). In addition to their vasodilator properties, prostacyclin analogues can also have inhibitory effects on PASMC proliferation and migration (Clapp *et al.*, 2002). Hence, prostacyclin agonists and analogues are used to treat severe PAH. The most recent member of this drug class, selexipag, a selective prostacyclin receptor agonist, was approved by the Food and Drug Administration (FDA) for PAH in 2015 (Sharma *et al*, 2016; Kingman *et al.*, 2017).

1.4.1.4 Phosphodiesterase – 5 (PDE-5) inhibitors

The cyclic nucleotides, cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphates (cGMP) are important mediators of pulmonary vasodilation via activation of protein kinase A (PKA) and protein kinase G (PKG), respectively (Montani *et al.*, 2009). NO is a well-established mediator involved in activation of the cGMP pathway. Phosphodiesterases (PDEs) catalyse the hydrolysis of cAMP and cGMP and promote vasodilation by increasing the concentration of both cyclic nucleotides intracellularly. PDE-5 is cGMP selective and is highly expressed in the lung (MacLean *et al.*, 1997). Inhibitors of PDE5 promote vasodilation via cGMP and are effective in PAH management. PDE5 inhibitor drugs approved for the treatment of PAH include sildenafil, tadalafil and vardenafil (Knott *et al.*, 2017; Rashid *et al.*, 2017).

Despite ongoing work to enhance drug discovery efforts in PAH, monotherapy remains unsatisfactory with poor clinical responses and an undesirable mortality rate of ~15% per year in PAH patients (M. Humbert *et al.*, 2010; M. Humbert *et al.*, 2010)

1.4.2 Combination Therapy in PAH

Combination therapy maximises therapeutic potential by targeting multiple pathways in PAH, improving the efficacy and reducing toxicity of drug modulators. In recent meta-analysis studies these therapies have shown to reduce clinical worsening by ~35% in comparison to conventional monotherapies (Lajoie *et al*, 2017). Drug combinations are capable of improving the functional status and quality

of life in patients who remain unresponsive to conventional monotherapy. Therefore, the optimal strategy for combining treatments in this manner is being explored to personalise medicinal strategies for a large number of PAH patients.

1.4.3 Therapeutic Advances in PAH

As discussed, therapeutic modulators in PAH reduce its severity and improve symptoms but fail to halt progression of the adverse pathobiology. Therefore, deterioration of the patient's condition remains an issue often requiring a lung transplant following years of therapy. Coinciding with technological advances in metabolomics, the goal of precision medicine and personalised therapy would be a desirable prospect in the near future to further extend life expectancy of patients with early to end-stage PAH (Savale *et al.*, 2018). For this reason, a number of novel therapeutic options are being explored to pharmacologically target vascular remodelling with anti-proliferative, pro-endothelial function and antioxidant actions. In addition, more robust and effectively-designed phase 2 trials and adequately powered phase 3 trails should be considered allowing earlier access to patients (Graham *et al*, 2018). A small number of these include: soluble guanylate cyclase (sGC) stimulators, nuclear erythroid-2 derived factor 2 (Nrf-2) activation and inhibition of aromatase.

1.4.3.1 Soluble guanylate cyclase stimulator

Soluble guanylate cyclase (sGC) is the primary receptor for NO. On binding to NO, sGC catalyses the synthesis of cGMP, which promotes vasodilation but also inhibits vascular smooth muscle cell proliferation, leukocyte recruitment, platelet aggregation and vascular remodelling. There is dysregulation of NO production, sGC activity and cGMP degradation in PAH (Stasch *et al*, 2013). This enzyme is a direct target to activate the NOcGMP pathway; targeting of sGC stimulation by riociguat has consistently improved response to exercise following treatment in pulmonary hypertension patients (Lian *et al*, 2017). This occurs via vasodilation within the pulmonary arterial bed thus improving hemodynamics and vascular tone.

1.4.3.2 Nrf-2 Activators

Suppressing the activation of pro-inflammatory cells via *Nrf-2* activation has shown promise by targeting damaging oxidative stress pathways thereby decreasing TGF-

β1 levels, inhibiting inflammation and alleviating pulmonary vascular remodelling (Che*n et al*, 2017). One potential pharmacological compound, bardoxolone methyl has shown pre-clinical benefit as a *Nrf-2* activator in PH models (White *et al.*, 2018). This semi-synthetic triterpenoid promotes release of *Nrf-2* from its negative regulator, Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm which supresses nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Wilkin*s et al*, 2018). A small 16-wk study reports significantly improved 6-MWD in analysis of 24 WHO group I PAH patients enrolled in an ongoing phase 2 LARIAT trial (NCT02036970) (Oudi*z et al*, 2015). This benefit of this therapy, if successful, would be its ability to target multiple cell types relevant to PH, including ECs, SMCs and macrophages.

1.4.3.3 Aromatase Inhibition in PAH

Aromatase is an enzyme involved in the aromatization reaction of androgens (testosterone) to estrogens (estradiol) in estrogen-producing cells. A number of studies suggest genetic variants in the promoter region of the aromatase gene to be detrimental in PAH (Tofovic *et al*, 2016). Inhibition via anastrozole or metformin in the hypoxic mouse and sugen5416/hypoxic rat, has reduced PAH and ventricular hypertrophy *in vivo* (Mair *et al*, 2014; Dean *et al*, 2016). In the clinical setting, anastrozole reduced circulating E2 levels resulting in increased 6-MWD in comparison to the placebo group (Ka*wut et al*, 2016). Additionally, in a proof of concept trial of fulvestrant inhibition of aromatase in PAH, estrogen levels were reduced following treatment. Notably, 16hydroxyestradiol (16OHE2) was also shown to be significantly decreased following treatment alongside hematopoietic progenitor cells which, in turn, increased stroke volume and 6-MWD, improving clinical outcomes (Kawut *et al.*, 2019). This will be discussed further in 1.5.6 and in Chapter 7.

1.5 The Influence of Sex in Pulmonary Hypertension

In the 1950s, Dresdale first documented an increased frequency of female PAH patients compared to men (Dresdale *et al*, 1951). The increased prevalence of PAH in females remains evidenced by recent epidemiological studies highlighting that 70-80% of PAH patients are female (Badesc*h et al*, 2010). This is in contrast to other demographics, which have changed over time i.e. age of diagnosis ranges from 50-65 years in contemporary registries compared to 36 years previously reported

(Hoeper *et al*, 2014). As the age of diagnosis has increased, patients are presenting with more co-morbidities, and are therefore more difficult to manage clinically. However, reasons underlying the imbalanced female to male ratio regarding disease prevalence remain obscure. Conversely, male PAH patients exhibit poorer survival than female patients (Jacobs *et al*., 2014), leading to investigation of the phenomenon known as the 'sex paradox' in pulmonary hypertension. Recent studies suggest that males have poorer adaptive remodelling of the right ventricle (RV) in response to increased afterload - this may explain sex differences in survival in PAH (Foderaro *et al*, 2016). Such paradoxical observations suggest a role for complex sex hormone signalling and processing pathways in the development and progression of PAH. This sex paradox also seems to be dependent on the ageing with the onset of menopause being a risk factor in female disease, perhaps due to altered estrogen production at menopausal age. The influence of sex hormones in women presenting with PAH is further emphasised by the increased onset of PAH during pregnancy and/or post-partum period (Tere*k et al*, 2013). While treatment guidelines now suggest that female PAH patients should not take estrogen-based contraceptives (Hemnes *et al.*, 2015) current PAH therapies do not take sex bias into account despite recent studies having shown sex differences in treatment response (Gable*r et al*, 2012; Shapiro *et al*, 2012). For example, Gabler and colleagues reported that ET-1 receptor antagonists had greater efficacy in females than males. A more recent study found increased likelihood of response to the PDE5 inhibitor, Tadalafil, in the treatment of PAH, in men compared with women (Mathai *et al.*, 2016). Sex-specific heterogeneity in treatment response may reflect differences in PAH pathobiology and affords the opportunity to inform individual treatment decisions and provide a basis for exploring potential differences in mechanisms of disease between sexes.

1.5.1 Estrogen Synthesis

Estrogens are pleiotropic hormones synthesised on demand in some tissues from the major circulating adrenal steroids dehydroepiandrosterone (DHEA), andostenediol (A5) through androstenedione (A4) and testosterone (T) via the enzyme aromatase (CYP19A1). Aromatase is the key enzyme in production of estrone and estradiol from androgens. Synthesis of aromatase occurs in the ovaries of premenopausal women. This gonadal site expression is regulated by follicle stimulating hormone (FSH) and cAMP. Extra gonadal production may also be

increased in post-menopausal women and men in sites such as adipose tissue with regulation reported via glucocorticoids and androgens (Zhao *et al.*, 2016; Hetemäki *et al.*, 2017). In addition, aromatase expression is not restricted to these sites, with expression present in a wide variety of tissues including lung, skin fibroblasts, bone, and the brain (Nelson *et al*, 2001). Importantly, aromatase expression has been identified within the vasculature in pulmonary arterial smooth muscle cells (Mair *et al.*, 2014). At extra-gonadal sites such as the brain, bone or breast, local levels of estrogens can be high in some cases, acting as potential sources of paracrine and autocrine effects in tissues and cells *(*Hood *et al*, 2016; Mair *et al*, 2019). Estrone (E1) and estradiol (E2) are the predominant circulating female sex steroids with multiple functions throughout the body (Figure 1-7).

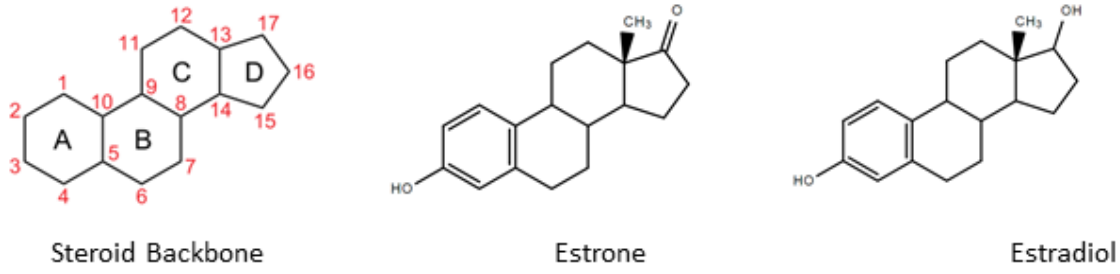

Figure 1-7 Chemical structure of the steroid backbone, estrone and estradiol Figure shows the steroid backbone of all estrogen molecules, estrone and estradiol structures.

Production of the main estrogenic compounds varies widely in men and women. In women, estrogen homeostasis is highly dependent on factors such as age, menstrual cycle and pregnancy. In men, synthesis may increase upon ageing, as increased synthesis of estradiol can occur via aromatase upregulation (Martin *et al*, 2014). Upon ageing in men, aromatase upregulation may occur via age-associated fat mass increase even alongside the decrease in bioavailable testosterone for conversion to estrogenic steroids (Vermeulen *et al.*, 2002). Generally, estradiol is the most abundant estrogenic hormone in pre-menopausal women, whereas estrone becomes the more prevalent in post-menopausal women and in males, being generated from adrenal androstenedione. Isomers of estradiol exist in α and β configurations; 17β- estradiol (E2) refers to the main bioactive version whilst 17α-estradiol is generally thought to be less active (Tofovic *et al*, 2010). The third most common form in humans, estriol (E3 or 16OHE2), can be produced from E2 or from estrone via the 16-hydroxyestrone (16OHE1) intermediate (Thomas and Potter, 2013). Estriol increases throughout pregnancy, being generated in the placenta. Aromatic oxidation of estrone and estradiol generates hydroxy- and methoxylated

metabolites, but the exact circulating level of each remains largely unknown and under-investigated (Simpson *et al*, 2003). Estrogen metabolites vary in their biological activity with some being relatively inactive or exhibiting protective effects whilst others modify pathophysiology of disease bearing potentially mitogenic debilitating effects. The circulating levels of bioactive metabolites are assumed to be lower than those of the main circulating estrogens (Table 1-3).

Table 1-3: Reference ranges of concentrations of estrogens in human plasma

Group	Age (Y)	Estrone (pg/mL)	Estradiol (pg/mL)	Estriol (pg/mL)
Female Children	0-15	0 - 200	ND - 40	ND
Premenopausal	18-55	17 - 200	15 - 350	< 80
Pregnancy	-	>187	188 - 7192	> 2100
Postmenopausal	>55	7 - 40	<10	ND
Male Children	0-18	ND - 38	ND - 38	ND
Males	>18	10 - 60	10 - 40	< 70

Children<18 Y; ND=Not detected; Y=years. Concentration guidelines from Mayo medical laboratories (https://www.mayocliniclabs.com/test-catalog/Clinical+and+Interpretive/84230 and www.mayocliniclabs.com/test-catalog/Clinical+and+Interpretive/81711. accessed 15/07/2019);

1.5.2 Estrogen Metabolism

Upon aromatisation within these sites, E1 and E2 remain in equilibrium via interconversion by 17βHSD1 and 17βHSD2 enzymes, catalysing reduction or oxidation respectively at the C17 position, with the balance largely favouring estrone formation (Figure 1-8). Further metabolism occurs via cytochrome p450 (CYP) enzymes, generating bioactive hydroxyl metabolites upon oxidation of the parent molecules at the C16, C4 and C2 positions, listed in order of reactive preference. The 2- and 4-hydroxy-estrone and estradiol metabolites (2OHE1, 4OHE1, 2OHE2 & 4OHE2) are rapidly converted (t/2= 90 minutes (Ball *et al.*, 1983)) to 2- and 4-methoxy–estrone and estradiol metabolites (2MeOE1, 4MeOE1, 2MeOE2 and 4MeOE2) by the action of catechol-O-methyltransferase (COMT). Hydroxy and methoxy-estrone and estradiol metabolites are also maintained in constant equilibrium by the 17βHSD1 and 17βHSD2 enzymes prior to oxidative metabolism and removal from the body by the liver.

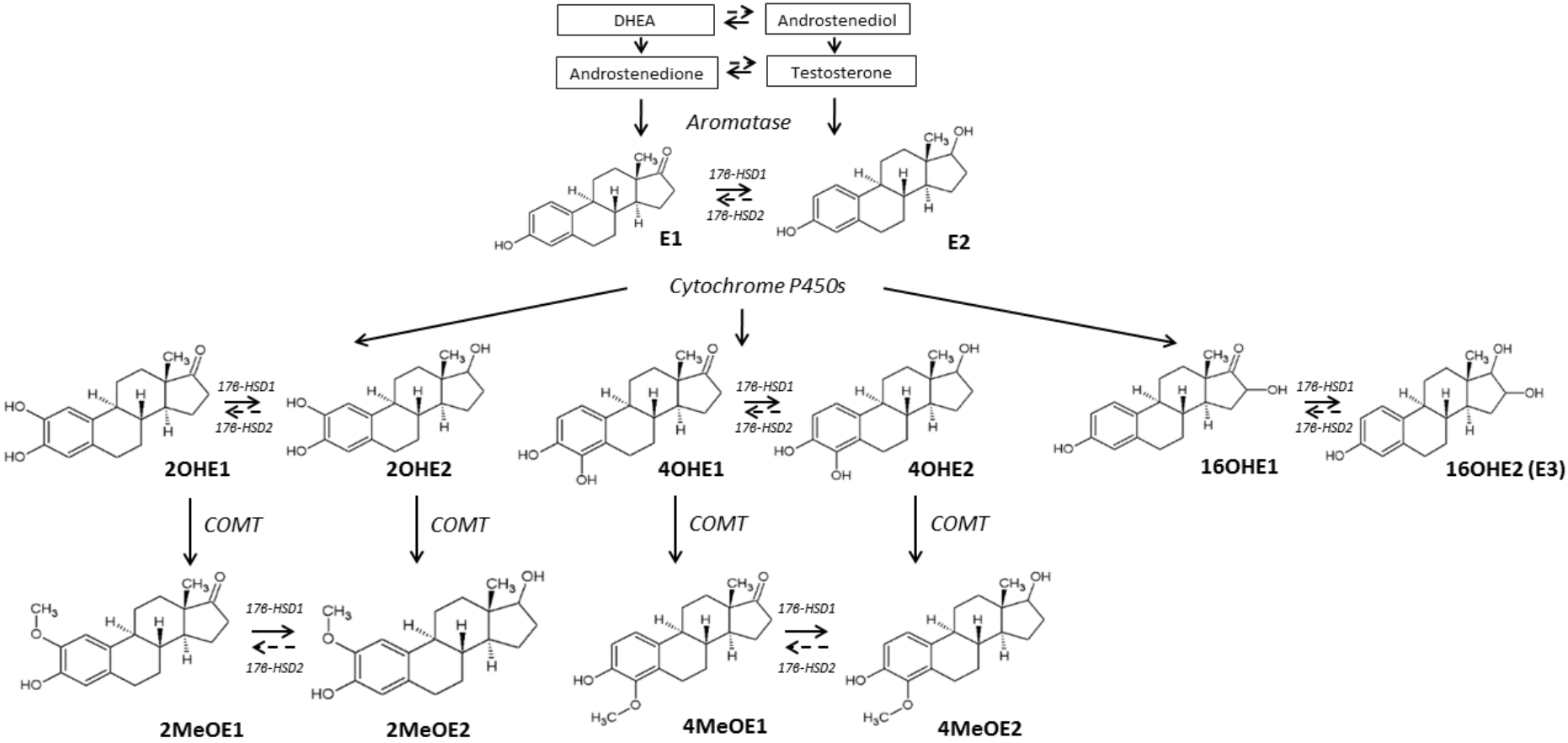

Figure 1-8: Endogenous estrogen metabolism pathway Metabolism of endogenous estrogen from sex hormone substrates dehydroepiandrosterone (DHEA), andostenediol (A5), androstenedione (A4) and testosterone (T). Oxidative metabolism of estrone (E1) and estradiol (E2) at C2, C4 and C16 positions by cytochrome P450 enzymes leads to the generation of hydroxyestrogen metabolites (2OHE, 4OHE & 16OHE). The 2OHE and 4OHE metabolites are rapidly converted to the methoxyestrogens by catechol-O-methyltransferases (COMT). E1 and E2 metabolites are maintained in equilibrium through the actions of 17β-hydroxysteroid 1 & 2 enzymes.

1.5.2.1 Oxidative Metabolism: Cytochrome P450 Enzymes

A number of cytochrome P450 enzymes facilitate the hydroxylation of estrogen to form metabolites which vary in their estrogenic activity and binding affinities to estrogen receptors. All of the CYP1 enzymes (CYP1A1, CYP1A2 and CYP1B1) are regulated by the aryl hydrocarbon receptor (AhR) (Nebert *et al.*, 2004). In their role of metabolising estrogens, CYP1A1, CYP1A2, CYP3A4 and CYP3A5 preferentially metabolise estradiol in the 2' position aromatic carbon within the steroid backbone, producing 2OHE2, whilst CYP3A4 catalyses formation of the 4OHE2 via 4' metabolism (Modugno *et al*, 2003). Contrastingly, CYP1B1 exhibits the capacity to catalyse formation of 2OHE2 and 16OHE2 but preferentially activates the 4OHE2 pathway upon hydroxylation of estradiol. For estrone metabolism, a similar pathway is observed with the 16-hydroxylation pathways being activated predominantly by CYP1A1, CYP2C19 and CYP3A5 (Cribb *et al.*, 2006). Here, CYP1B1 preferentially activates 4OHE1 production (Lee *et al.*, 2003).

1.5.2.2 Phase 2 Metabolism: Catechol-O-methyltransferase

The 2 & 4 – hydroxylated estrogen metabolites known collectively as catechol estrogens are rapidly metabolised by methylation by the activity of Catechol-O-methyltransferase (COMT). The activity of this particular enzyme governs the deactivation efficiency of mitogenic 4-hydroxylated estrogens (Cavalieri *et al*, 2016) and also has the ability to produce methoxyestrogens with therapeutic benefits (Docherty *et al*, 2019). Further conjugation of catechol estrogens also occur by sulfation and glucuronidation.

1.5.3 Estrogenic Signalling

In the endocrine system, estrogens primarily act as modulators of reproductive function, with the last few decades highlighting a wider functional role within alternative organ systems. Estrogens are small lipophilic compounds circulating in blood as free compounds or bound to the sex hormone binding globulin (SHBG). SHBG regulates the circulating concentrations of these steroids and their bioavailability at target tissues. At target cells, estrogen enters the cytosol of cells via diffusion to mediate effector responses. Intracellularly, the response can occur via direct or indirect

genomic signalling (Figure 1-9). Genomic signalling pathways include the activation of estrogen responsive elements (ERE) by estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). Alternatively, estrogens may initiate rapid non-genomic actions, directly altering protein signalling to initiate a cellular response (Umar, Rabinovitch and Eghbali, 2012). Evidence also exists for a third estrogen receptor named G-protein coupled estrogen receptor 1 (*GPER),* thought to be implicated in both rapid non-genomic estrogen responses and in transcriptional regulation (Scaling *et al*, 2014).

1.5.3.1 Estrogen Receptor Signalling

Nuclear estrogen receptors (ERs) are modular proteins encoded by two distinct genes: ERα (*ESR1*) and ERβ (*ESR2*). In the well-characterized genomic pathway (Figure 1-9) signalling cascade activation occurs via these receptors. Structurally ERα and ERβ both display sequence homology comprising of five distinct domains, two activation domains within the N-terminal and ligand-binding domains that regulate the transcriptional activity of ER (Kum*ar et al*, 2011). Due to alternative splicing of the RNA encoding the receptors several isoforms of each also exist with at least three ERα and five ERβ isoforms discovered to date. The unbound ERs are inactive, present as dimers in the peri-membrane, mitochondria and nucleus. They are rendered active through conformational changes caused by estrogen binding, a pivotal step initiating their cellular action (Yaşar *et al.*, 2017). Active ERs undergo homodimerization binding to DNA sequences of 13 bp EREs in target promoters. Upon dimerization translocation of ER to the nucleus occurs with generation of binding surfaces for interactions with co-regulatory proteins, stabilising the binding associations (Muya*n et al*, 2015). Although structurally similar and with similar functionality, ERα and ERβ have distinct, often opposing effects in gene transcription outcomes (Barros *et al*, 2011).

1.5.3.2 Non-Genomic Signalling

Accumulating evidence suggests estrogen also mediates intracellular events via non-genomic signalling. These actions occur rapidly without direct binding of ER to deoxyribonucleic acid (DNA) and are linked to activation of protein-protein interactions via various protein signalling cascades within the cytoplasm (Björnström *et al*, 2005). Such cascades initiate transcription factor complexes that can contact intra-cellular DNA regulating by a large number of estrogen responsive genes not containing EREs

(Kassi *et al*, 2010). Evidence also supports the fact that GPER located on the plasma membrane, binds E2 with high affinity, rapidly altering Ca^{2+} mobilisation (Improta-Brears *et al.*, 1999), stimulating cAMP production (Aronica *et al*, 1994) and activates epidermal growth factor receptor (EGFR) transactivation (Filardo *et al*, 2002) and extracellular signal-regulated kinases (ERK1/2) in a variety of extra-gonadal cells (Kim *et al.*, 2016).

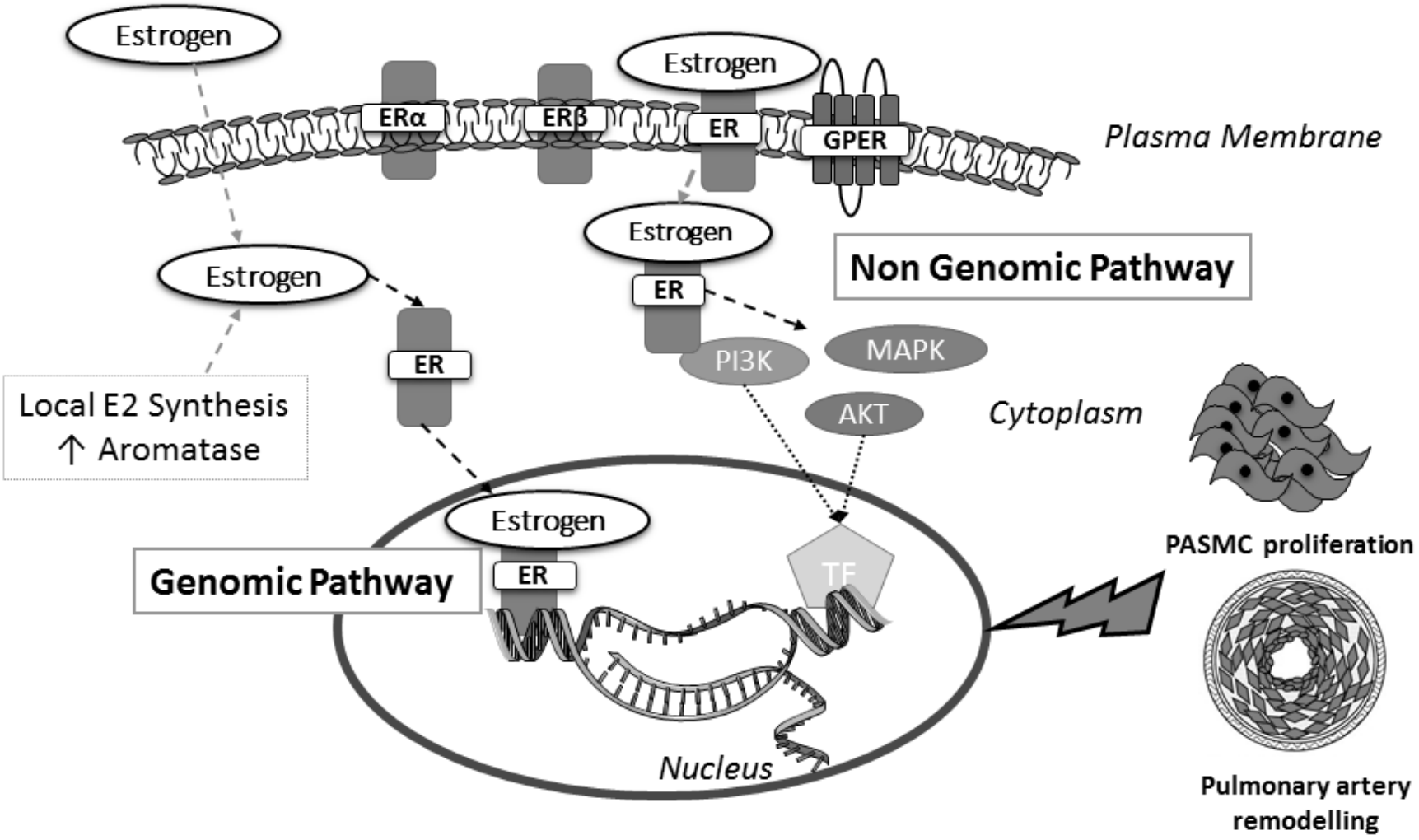

Figure 1-9: Estrogen signalling pathways mediated by estrogen and ERs resulting in characteristic PAH phenotypes Genomic and non-genomic signalling represent the two main pathways of transcriptional dysregulation in PAH. AKT, protein kinase; ER, estrogen receptor; MAPK, mitogen-activated protein kinase; PI3K, phophoinositide-3-kinase

1.5.4 Estrogen in Disease Pathobiology

Epidemiological and experimental studies implicate estrogens in a number of diseases, with the potential roles of bioactive metabolites becoming more prominent. For example, in the cancer and cardiovascular fields (Figure 1-10) E2, 16OHE1, 4OHE1 and 4OHE2 have been implicated in disease progression, whereas 2OHE1, 4OHE1, 4MeOE1, 2MeOE2 and 4MeOE2 have shown protective roles. 16OHE2 presents less extensively in the literature, but was reported some time ago to stimulate tumour growth via estrogen receptors in breast cancer (Lippman *et al*, 1977). The remaining 2MeOE1 is thought to be inactive (Tofovic *et al*, 2010).

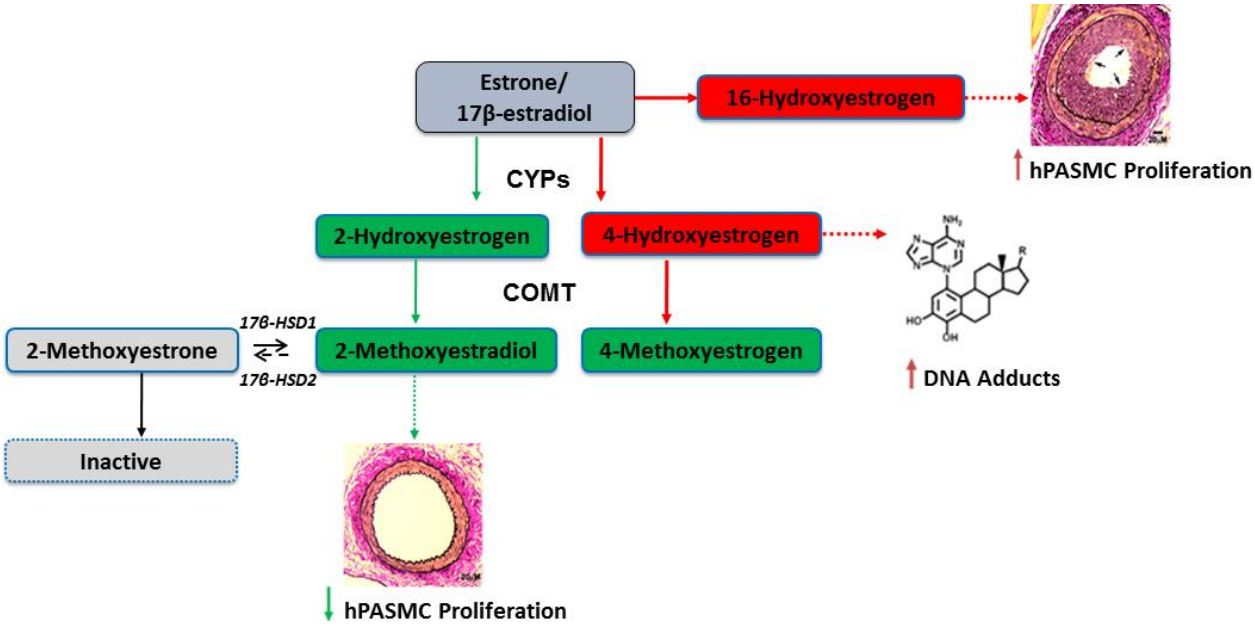

Figure 1-10: Estrogen metabolite pathogenesis in cardiovascular disease and cancer Estrone and estradiol are converted to harmful pro-proliferative metabolites shown in red and protective metabolites shown in green. 2-methoxyestrone reports as biologically inactive is highlighted grey.

Elevated estrogen levels in the serum and plasma of women have been associated with increased risk of breast (Travis *et al*, 2003; Santen *et al,* 2015), endometrial (Brinton *et al.*, 2016; Dallal *et al.*, 2016) and ovarian cancers (Zahid *et al.*, 2014; Trabert *et al.*, 2016), whilst in males estrogen-androgen imbalances are thought significant in the development of aggressive prostate cancers (Black *et al.*, 2014). Emerging evidence implicates estrogen metabolism in the aetiology of diabetes (Gupte *et al*, 2015) and may explain why in breast cancer, obesity has proven to be a major contributing risk factor (Cleary *et al*, 2009). Clinically, three main scenarios link

estrogen bioactivity to a worsened prognosis: in late menopause when site specific estrogen production and metabolism becomes more prominent; in cases of hormone replacement therapy use where metabolic dysfunction occurs via increased exogenous supply; and thirdly in the presence of specific single nucleotide polymorphisms (SNPs) in the aromatase gene resulting in increased circulating plasma estradiol levels (Santen *et al*, 2015). All scenarios lead to exacerbation of breast cancer symptoms. Most literature addresses E2 and E1; the bioactive estrogen metabolites remain less studied but are now gaining more prominence in each field. For example, higher 16-hydroxy estrogen production indicates a risk factor for diseases such as prostate cancer (Black *et al.*, 2014) and mammary tumours, with an emphasis on the 2/16-hydroxyestrogen ratio (Obi *et al.*, 2011).

1.5.5 Estrogen in the Cardiovascular System

It has become apparent that estrogen receptors (ER) impact a multitude of biological functions including inflammatory response and cardiovascular function (Bado *et al.*, 2017). ERα, ERβ and GPER are present within cardiac cells such as cardiomyocytes, endothelial cells and myocytes playing essential roles in energy balance and glucose homeostasis (Luo *et al*, 2016). In comparison to its pathogenic effect in estrogen sensitive cancers, in pre-menopausal women presenting with cardiovascular disease, estradiol has a protective role (Knowlton *et al*, 2012). Clinical manifestations reveal pre-menopausal women are at less risk from cardiovascular disease (CVD) than men, suggesting an underlying protective role of E2 in women. Conversely in post-menopausal woman the protective benefit is lost upon decline of circulating E2, further strengthening the hypothesis that ageing plays a pivotal role in female CVD (Giordan*o* *et al*, 2015). E2 induced attenuation of vasoconstrictive mediators in the aorta and coronary arteries and inhibition of cellular proliferation demonstrate the protective role of E2 in cardiac pharmacology (Dubey *et al*, 2003). However, exogenous estrogen supply, via environmental or metabolically driven stimuli, contribute to non-physiological fluctuations in circulating estrogen levels which can be detrimental to the cardiovascular system. For example, hormone replacement therapy (HRT) use in early post-menopause demonstrated an increased risk of coronary heart disease (CHD) and effects on arterial function (Bhatt *et al.*, 2015). In humans ERα mediates the protective effect, being increased in the hearts of patients with dilated cardiomyopathy

compensating for the loss of cardiac function (Mahmoodzade*h et al*, 2006). The cardio-protective effects of ERα genomic signalling may also be regulated by vascular endothelial growth factor (VEGF), a pro-survival factor for ECs (Xiao *et al.*, 2014). In transgenic ER knockout mouse models, the ratio of ERα/ERβ represents an important balance whose dysregulation may drive the development of metabolic diseases (Lee *et al*, 2012). ERβ knockout in mice proves detrimental, driving hypertension and adaptive hypertrophy (Forster *et al.*, 2004). In agreement with these studies, GPER knockout also demonstrates cardiac dysfunction via oxidative stress pathways (Wang *et al.*, 2018).

1.5.6 Estrogen in PAH

In PAH two main sex paradoxes exist. Firstly, E2 has been shown to be protective in some experimental PAH studies, whilst in human PH registries uniformly display a susceptibility within females. In these cases exogenously administered estradiol was shown to improve PAH pathogenesis (Yuan *et al.*, 2013) and this topic is further explored in section 1.5.7. Conversely, when studying endogenous estrogens, aromatase inhibition displays pathogenic effects (Mair *et al.*, 2014; Dean *et al.*, 2016; Kawut *et al.*, 2016). Despite the clinical prevalence of PAH in women, males present worsened phenotypes exhibiting higher mortality rates (Paulin *et al*, 2012). Therefore, the complexities associated with estrogen signalling in the pulmonary vasculature and systemic circulation should not be understated. To solve this paradox, a number of recent clinical studies have started to unravel the mechanistic underpinning of enzymes involved in estrogen production and metabolism to define their action as disease modifiers.

A comprehensive study has implicated elevated estradiol levels in male patients with PAH, a finding correlating with poorer clinical outcomes (Corey E Ventetuolo *et al*, 2015). Here, lower DHEA, DHEA/T ratios alongside elevated E2 associated with shorter 6-MWD. The next study also discussed differences in RV function due to E2 metabolism with a genetic variant in *CYP1B1* identified in postmenopausal women. In addition testosterone was shown to be associated with worsened RV function in males (Ventetuolo *et al.*, 2016). In idiopathic PAH cohorts, this finding in postmenopausal women was indicative of increased aromatisation of androgens via a genetic variant

in the gene encoding aromatase, raising circulating estrogen levels (Baird *et al.*, 2018). Single nucleotide polymorphisms (SNP) in aromatase and the *ESR1* gene has also been established predisposing individuals to porto-pulmonary hypertension via increased E2 (Roberts *et al.*, 2009).

Metabolism to bioactive metabolites has also been implicated in higher penetrance of disease manifestation. Mainly the action of various CYP enzymes has come into question, and over-expression of these enzymes mediated by AhR plays a role in cell growth related to estrogen metabolism and inflammation (Kwapiszewska *et al.*, 2019). In females, gene expression data implicates *CYP1B1* as detrimental in clinical cases (E. D. Austin *et al,* 2011). Relatively recently this estrogen metabolising enzyme was identified as a modifier of PAH (West *et al*, 2008). Subsequent to this finding, a polymorphism in CYP1B1 was discovered in patients with a BMPR2 mutation also implicating the 16-hydroxylation pathway in mutation carrier females (Ventetuolo 2016). Interestingly these findings correlate with the predominance of PAH within females. More recently at the ATS conference in 2019 the same group presented information on a gene named *TBX4*, identified as a regulator of CYP1B1 activity providing intriguing mechanistic data in PAH pathogenesis, BMPR2 signalling and estrogen metabolism. In metabolism of E1, the 16-hydroxylation pathway also proves detrimental in PAH. Both *in vitro* and *in vivo* research has linked 16OHE1 to cell proliferation and vascular remodelling, significant phenotypical hallmarks of PAH (White *et al*, 2012; Chen *et al*, 2016; Hood *et al*, 2016). 16OHE1 exhibits higher binding affinities and estrogenic effect than parent molecules at classical estrogen receptors (Chen *et al.*, 2016), potentially activating the classical genomic signalling cascade and playing a pathogenic role in the pulmonary circulation. In this setting, increased levels in urine (Franke *et al.*, 2011) also coincide with induction of smooth muscle proliferation within the pulmonary arteries. Here, 16OHE1 was identified as being elevated in obese mice , a further risk factor to PAH (Mair *et al.*, 2019). Alternatively, metabolites like 2MeOE2 have demonstrated protective effects via destabilisation of HIF1-α and decreasing mitogenic proliferative effects within lung fibroblast cells in an apoptotic manner (Docherty *et al*, 2019).

In PAH, the three main estrogen receptors involved in pathogenesis localise in the smooth muscle layer of the pulmonary arteries. In the majority of literature, ERα

remains negatively associated with PAH exhibiting high expression levels in lungs of female iPAH patients (Rajkumar *et al*, 2010) and (Rajkumar *et al.*, 2010; Wright *et al.*, 2015). High expression levels may increase the rate of hPASMC proliferation and remodelling through various non-canonical pathways. There may be increased ERβ in male PAH patient PASMCs in comparison to control cells, whilst ERα is more abundant in female PAH patients PASMCs in comparison to controls (Wright *et al*, 2015). This suggests differential sex- dependent estrogen signalling in PAH. ERβ may play a more beneficial role in protection against PAH modulating angiogenesis through the production of various angiogenic factors such as vascular endothelial growth factor (VEGF) and nitric oxide (NO) upon E2 treatment (A*wad et al*, 2011). It has been suggested that targeting ERβ in future therapeutics strategies may lead to the development of PAH treatments (Umar *et al*, 2012). The third class and more recently discovered transmembrane GPER, binds estrogen causing rapid non-genomic effects (Filardo *et al*, 2002). Relations between GPER and high blood pressure in ageing males and females suggests a beneficial role in the pulmonary vasculature and RV (Austin *et al.*, 2013) although the full functionality of this membrane bound receptor remains undefined and poorly understood.

Therefore, the potential consequences of estrogenic hormone imbalance within the body, at site-specific locations in both females and males, prompts investigation of the circulating and tissue specific estrogens with the aim of developing targeted therapeutic modulators within this sex hormone pathway.

1.5.7 Estrogen in Experimental PAH

In vivo studies generally employ the use of the monocrotaline, hypoxic and sugen-hypoxic PAH models in rodents. All recapitulate some aspects of the disease status in humans. In the monocrotaline model, administration of the toxic alkaloid induces and increases cellular proliferation at variable degrees throughout the proximal and distal PAs. The hypoxic model exposes animals to hypobaric or normobaric hypoxia and doubles the mean PAP, vascular remodelling and inflation but RV failure remains absent. Finally, the sugen-hypoxic (Ab*e et al*, 2010) model employs a vascular endothelial growth factor receptor 2 (VEGFR2) blockade by injection of Sugen 5416 in conjunction with hypoxia. This model remains unique in its formation of plexiform

lesions within the lung resulting in RV failure, which mirrors human disease (Colvin *et al*, 2014).

Treatment with anastrozole, the aromatase inhibitor, has shown remarkable therapeutic potential in the Sugen-hypoxic, *BMPR2* mutant mouse (Chen *et al.*, 2017) and hypoxic rat models, displaying successful reversal of PH phenotypes in female rats which correlates with restored BMPR2 signalling via reduced plasma E2 levels (Tofovic *et al*, 2013; Mai*r et al*, 2014). This was further confirmed in the clinical trial mentioned in section 1.6.6 whereby anastrozole treatment was safe and effective in improving symptoms in PAH patients (Kawut *et al.*, 2016). These findings together warrant conducting a larger phase II trial. This has been funded by NIH (https://clinicaltrials.gov/ct2/show/NCT03229499).

A microarray study of distal pulmonary arteries in mice over-expressing the serotonin transporter suggested dysfunction of estrogen metabolism via CYP1B1 (White *et al*, 2011). This is consistent with the clinical studies suggesting polymorphisms causing overexpression of *CYP1B1* exist in PAH (Austin *et al*, 2009; Rober*ts et al*, 2009; Ventetuolo *et al*, 2016). In addition, ovariectomy, and hence removal of endogenous estrogens, in transgenic mice expressing the serotonin transporter (SERT+), diminished the severity of PAH phenotypes. (White *et al*, 2011). There is increased *CYP1B1* expression and increased urinary 16OHE1 levels in the pulmonary circulation of the hypoxic rat and sugen hypoxic mouse (White *et al*, 2012). This study also demonstrated that administration of 16OHE1 (1.5 mg/kg/day) promotes PAH development, increasing vascular proliferation. These harmful effects were successfully reduced by treatment with Trans-2,3',4,5'-tetramethoxystilbene (TMS), a selective *CYP1B1* inhibitor, presenting *CYP1B1* as a potential therapeutic target in PAH. Furthermore, the increased *CYP1B1* and 16OHE1 induced Nox-dependent ROS generation and decreased *Nrf-2* antioxidant systems contributing to oxidative damage and proliferative processes involved in PAH pathogenesis (Hood *et al.*, 2016).

A number of other studies which have examined the effects of exogenously administered estrogens have implied estrogen has both mitogenic and non-mitogenic effects in disease. Clinically, females repeatedly demonstrate a less severe PH phenotype than males whilst in hypoxic rat models increased pulmonary vascular

remodelling and hypertrophy are greater in male rats over female (Rabinovitch *et al.*, 1981). In male rats, endogenous estrogen therapy decreases haemodynamic and remodelling parameters within the PA suggesting beneficial roles of estradiol in the hypoxia induced model (Lahm *et al*, 2012). The protective signalling of E2 in this manner is suggested to occur via ERβ as inhibition of this ER reverses the protective effects of E2 in the RV (Lahm *et al.*, 2012). Frump et al described increased ERβ expression in pulmonary artery endothelial cells which, via *HIF1-α*, *HIF2-α* and prolyl hydroxylase 2 (*PHD2*) signalling initiated a protective response in PAH (Frump *et al.*, 2018). Also this group showed that Grem1 acts as a BMP agonist in the lung being increased by hypoxia and reduced by E2 (Frum*p et al*, 2017).

These effects also differentiate between the pulmonary circulation and the right ventricle. Much less is known about the role of E2 in the RV, however the potential for E2 mediated reversal of hypertrophy (Nadadur *et al*, 2012; Wang *et al*, 2019) and restoration of RV function (Umar *et al*, 2012) are reported. This may be expected, as females often adapt more efficiently to hypertrophy than males, which may be exerted due to increased circulating E2 levels. A correlation between E2 levels and RV function has in fact been established (Ventetuolo *et al.*, 2011). This study also emphasises the importance of *CYP1B1* induced estrogen metabolism whereby E2 metabolism and androgen signalling were linked to RV morphology in a sex specific manner.

An evaluation of E2 and E2 metabolites in plasma from patients with PA is required to address the inconsistencies and controversies derived from the pre-clinical studies. The complex function of estrogens may be dependent on receptor distribution/expression and metabolism.

1.6 Quantification of Estrogen in Bio-fluids

It remains of great importance to quantify circulating levels of endogenous estrogen in healthy individuals and in disease cohorts for diagnostics, monitoring and biomarker identification. Classically, immunoassays were the “gold standard” technique for this application however in recent years analytical approaches have come to the fore with advances in sample preparation and analytical technologies.

1.6.1 Immunoassays

Measurement of circulating estrogens in clinical diagnostics, research and monitoring classically employed enzyme linked immunoassays (ELISAs) and radioimmunoassay (RIAs) (Stanczyk *et al*, 2010; Jaque *et al.*, 2013) largely focussing on E2 and E1. These approaches are generally chosen due to their low cost and routine use (Riley *et al*, 2016). Both rely on the action of antigen (estrogen) binding to specific antibodies; for ELISA, the detection of this interaction is accomplished via incubation with a substrate(s) known to emit a measurable product; for RIA, radioactive scintillation counting is applied. As a result, these methods can lack selectivity, being dependent on antibody characteristics, often exhibiting cross reactivity between isomeric estrogens and other species. This problem is particularly marked when measuring lower levels (<20 pg/mL). High selectivity at low concentrations is a critical requirement for accurate analysis of estrone and estradiol particularly in men and older women, and the same rigour is needed to assay low level bioactive estrogen metabolites (Santen *et al.*, 2007; Santa *et al.*, 2013). Several studies for more abundant compounds such as cortisol, glucagon and vitamin D illustrate an imprecision between reported concentrations and a bias for false positives using immunoassays over a number of analytical methods (Ketha *et al.*, 2014; Cross and Hornshaw, 2016); this has led to the Endocrine Society issuing a consensus statement recommending avoidance of immunoassays (Handelsman *et al*, 2013). Therefore, development of robust analytical techniques capable of enhanced and simultaneous quantification of panels of estrogens at low circulating concentrations becomes justified and here hyphenated mass spectrometry techniques have led the way.

1.6.2 Analytical Approaches

The journey of estrogen quantification records measurement in a variety of diverse matrices such as topical creams, water and in various pharmaceutical agents (Nováková *et al.*, 2004; Wang *et al.*, 2006; Yilmaz *et al*, 2017) and began using high performance liquid chromatography (HPLC) analysis. This type of LC is commonly coupled to spectrophotometric detectors such as diode array detectors (DAD), and, in analysis of estrogens in tablets detection limits of 0.5 µg/mL (Yilmaz *et al*, 2017) were achieved. However, it has proved difficult to transfer such methods for use with more

complex matrices, such as serum or plasma due to insufficient sensitivity. The vast majority of analytical technologies available for clinical analysis of estrogens employ gas chromatography mass spectrometry (GC-MS) and in more recent years, with the evolution of narrower bore liquid chromatography columns with smaller particle sizes, liquid chromatography tandem mass spectrometry (LC-MS/MS). Both approaches benefit from the use of stable isotope internal standards (IS) and have increased levels of specificity unrivalled by ELISAs and RIAs. Initially attempts to transfer to MS approaches were hampered due to sensitivity issues which can now be resolved by newer instrumentation, advantageous in *both* their increased selectivity and sensitivity (Faupel-Badger *et al.*, 2010; Stanczyk *et al*, 2010; Handelsman *et al.*, 2014), with a number of successful approaches published some including the bioactive metabolites. The critical and defining factors underpinning improvements in speed, sensitivity and reliability are tabulated and discussed below (Table 1-4, Table 1-5).

Table 1-4: Estrogen (unconjugated) quantification by GC-MS/MS

Analyte	Matrix	Extraction Type	V (mL)	Derivatisation Agent	Inj V (µL)	Column	MS	Mode (+/-)	LOQ (pg/mL)	Ref
E1, E2	Serum	LLE	1	TMSI	1	TR-50MS, 5% phenyl-arylene, 30 m × 0.25 mm (0.25 µm)	Ion Trap EI-MS/MS	+	13-21	(Prokai-Tatrai, *et al.*, 2010)
E1, E2	Serum	LLE & SPE	0.25	PFBHA PFBO	NS	DB-17HT, 50% phenylmethyl polysiloxane 15 m × 0.25 mm (0.15 m)	Triple Quad CI-MS/MS	-	0.5	(Nilsson *et al.*, 2015)
E2	Serum	SPE	1	PFBO PFBHA MSTFA	NS	DB-17 fused silica, Dimensions NS	Triple Quad CI-MS/MS	-	1.9	(Santen *et al.*, 2007)
E2	Plasma	SPE	1	PFBC MSTFA	1	(50% phenyl)-methylpolysiloxane phase, 15 m × 0.25 mm (0.25 µm)	Triple Quad CI-MS/MS	-	2.5	(Schweingrube *et al.*, 2007)
E1, E2 16OHE 16EpiOHE2 16KetoOHE2 17EpiOHE2 2,4OHE 2OHE-3ME, 2,4MeOE	Urine	SPE	2	EOC PFP	2	MXT-1, Silcosteel-treated stainless steel, 30 m × 0.25 mm (0.25 µm)	Single Quad EI-MS/MS	+	20-500	(Chung *et al.*, 2011)

Chemical Ionisation (CI); estrone (E1); estradiol (E2); ethoxycarbonlyation (EOC); Gas Chromatography (GC); 2, 4, 16-hydroxyestradiol (2, 4, 16-OHE2); 2, 4, 16-hydroxyestrone (2, 4, 16-OHE1); 2-hydroxyestrone-3-methyl ether (2OHE-3ME); 16β-Hydroxy-17β-estradiol (16epiOHE2); 16-oxo-17β-estradiol (16ketoOHE2); 16α-hydroxy-17α-estradiol (17epiOHE2); 17α-estradiol (17epiestradiol); Injection Volume (Inj V); Liquid Liquid Extraction (LLE); methoxyestrogens (MeOE); N-methyl pyridinium-3-sulfonyl N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA); Not stated (NS); pentadecafluorooctanoyl chloride (PDFO); pentaflurobenzoyl hydroxylamine hydrochloride (PFBHA); perfluorobenzoyl chloride (PFBO); Solid phase extraction (SPE); Tandem mass spectrometry (MS/MS); N-(trimethylsilyl)imidazole(TMSI); Volume (V).

Table 1-5: Estrogen (unconjugated) quantification by LC-MS/MS

Analyte	Matrix	Extraction Type	V (mL)	Agent	Inj V (µL)	LC	Column	Mobile Phase (A/B)	MS	Mode (+/-)	LOQ (pg/mL)	Ref
E2	Serum (Pooled)	LLE	0.15	None	20	HPLC	PoroShell 120 SB-C18, 50 x 2.1 mm (2.7 µm)	MeOH/H_2O (+ 0.1% FA or 2.5 mM NH_4OH)	API 5000 Triple Quad ESI vs APCI vs APPI-MS/MS	+/-	0.14 - 0.68	(Keski-Rahkonen *et al.*, 2013)
E2	Serum	LLE	0.2	DMIS	25	UHPLC	Phenyl-Hexyl, 100 x 2.1 mm (1.7 µm)	H_2O/MeOH+ C_7H_8	API 5000 Triple Quad APPI-MS/MS	+	0.5	(Keski-Rahkonen *et al.*, 2015)
E1, E2, 16OHE2	Serum (Mouse)	Online LLE	0.1	None	1000	HPLC	Supelcosil LC-8-DB, 250 x 4.6 mm (5 µm)	MeOH/H_2O + C_7H_8	API 5000 Triple Quad APPI-MS/MS	-	3-5	(Mcnamara *et al.*, 2010)
E1, E2	Serum	LLE	2	PS	20	HPLC	Phenyl-Hexyl 150 x 2.0 mm (3-µm)	H_2O: (CH_3CN+H_2O) + 0.1% FA	API 4000 Triple Quad-ESI-MS/MS	+	10	(Xu and Spink, 2008)
E1, E2	Serum	LLE	0.2	DS	50	HPLC 2D set-up	C1 cartridge + Gemini Phenyl 100 x 2.0 mm (3 µm)	H_2O/MeOH + 10 nmol/L H_2O/CH_3CN + 10 nmol/L	API 4000 Triple Quad-ESI-MS/MS	-	1	(Kushnir *et al.*, 2008)
E1, E2	Plasma	LLE	0.5	DS	15	HPLC	Synergi, 150 x 2.0 mm (4µMax-RP)	CH_3CN/H_2O + 0.1% FA	API 3000 Triple Quad APCI-MS/MS	+	6.3 - 11.9	(Nelson *et al.*, 2004)

Table 1-5: Estrogen (unconjugated) quantification by LC-MS/MS

Analyte	Matrix	Extraction Type	V (mL)	Agent	Inj V (µL)	LC	Column	Mobile Phase (A/B)	MS	Mode (+/-)	LOQ (pg/mL)	Ref
E1, E2 16OHE 2, 4OHE	Serum	*LLE	0.5	N/A	10	HPLC	ZORBAX C18, 250 x 4.6 mm (5 µm)	CH_3CN/H_2O	API 3000 Tandem Quad axle ESI-MS/MS	-	10-15	*(Gao et al*, 2015)
E1, E2	Serum	LLE + Strata X-SPE	1	PED PPZ MPED MPPZ	3	HPLC	YMC-C8, 150 x 2.0 mm (5 µm) + Pro C18 RS, 150 x 2.0 (5 µm)	$CHCl_3$ /MeOH	API 2000 Triple Quad ESI-MS/MS	+/-	0.55 - 9.2	(Nishio *et al.*, 2007)
E1, E2	Serum	LLE	0.5	DS	30	UHPLC	Poroshell 120 SB-C18, 30 x 2.1 mm (2.7 mm) + Zorbax SB-C18, 50 x 2.1 mm (1.8 µm)	H_2O/MeOH + 0.2% FA	6500 Triple Quad ESI-MS/MS	+	1 - 4	(Ke *et al.*, 2014)
E2	Serum	LLE	0.29	None	5	Micro LC	YMC Triart 50 x 0.5 mm (3 µm)	H_2O/MeOH + 0.05% NH_4OH	6500 Triple Quad ESI-MS/MS	-	3	(Yi *et al*, 2018)
E1, E2	Serum	LLE	0.5	None	100	HPLC	Supelguard LC-8-DB, 20 x 3.0 mm + LC-8-DB, 330 × 2.1 mm (3 µm)	H_2O/MeOH: CH_3CN + NH_4F	5500 Triple Quad ESI-MS/MS	-	0.2 – 0.4	(Fiers *et al.*, 2012)
E1, E2, 16OHE2	Serum	LLE	0.1	DS	20	UHPLC	RP18, 50 x 2.1 mm (1.7 µm)	MeOH/H_2O + 0.2% FA	4500 Triple Quad ESI-MS	+	5	(Yuan *et al.*, 2019)

Table 1-5: Estrogen (unconjugated) quantification by LC-MS/MS

Analyte	Matrix	Extraction Type	V (mL)	Agent	Inj V (µL)	LC	Column	Mobile Phase (A/B)	MS	Mode (+/-)	LOQ (pg/mL)	Ref
E2 16OHE2 MeOE2 2, 4OHE2	Serum	LLE	0.1	NMPS	1	nano AQUITY UHPLC	BEH130 C18 150 x 100 mm (1.7 µm)	H_2O/CH_3CN + 0.1% FA	TSQ Vantage Triple Quad	+	0.5 - 5	(Wang *et al.*, 2015)
E1, E2	Serum/ urine	LLE	0.01	NBCOCL DNBF	10	HPLC	YMC-Pack Pro C18 RS, 150 x 4.6 mm (5 µm)	MeOH /H_2O	ThermoQuest Finnigan LCQ APCI-MS	-	2000 - 3000	(Higashi *et al.*, 2006)
E1, E2, 16OHE, 16Epi & 17Epi - OHE2, 16Keto - OHE2, 2, 4OHE 2, 4MeOE	Serum	LLE	0.1	MIS DS PS P	25	HPLC	Ascentis Express C18, 150 x 3.0 mm (2.7 µm)	H_2O/CH_3CN + 0.1% FA	Orbitrap ESI-MS/MS	+	0.2 - 100	(Li *et al.* 2015)
E1, E2, 16OHE, 16Epi - OHE2, 16Keto - OHE2, 17Epi - OHE2, 2,4OHE 2OHE-3ME 2,4MeOE	Serum (pooled)	*LLE	2	C1-NA-NHS	5	UHPLC	XDB-C18, 50 x 2.1 mm (1.8 µm)	CH_3CN/H_2O + 10 mmol/L $NH_4CH_3CO_2$	TOF APCI-MS/MS	-	360 - 2340	(Yang *et al*, 2008)

Table 1-5: Estrogen (unconjugated) quantification by LC-MS/MS

Analyte	Matrix	Extraction Type	V (mL)	Agent	Inj V (µL)	LC	Column	Mobile Phase (A/B)	MS	Mode (+/-)	LOQ (pg/mL)	Ref
E1, E2 16OHE 2,4OHE2 MeOE2 4OHE1 2MeOE1	Plasma	SPE	0.25	BMP	10	HPLC	Zorbax Extend-C18, 150 x 4.6 mm (5 µm)	H_2O/CH_3CN + 0.1% FA	6420A Triple Quad ESI-MS/MS	+	0.3 – 3.6	(Khedr *et al.* 2016)
E2	Saliva	PPE + Online SPE	0.1	None	200	HPLC	Shim-pack XR-ODS, 75 x 3 mm (2.2 µm)	H_2O/MeOH + 2 mM $NH_4CH_3CO_2$	API 5000 Triple Quad APCI-MS/MS	+	1	(Gao *et al.*, 2015)
E1, E2	Serum	SPE	0.5 - 1	P	100	HPLC	CD-C18 150 x 3 mm (3 µm)	CH_3CN: CH_3OH + HCOOH	API 5000 Triple Quad ESI-MS/MS	+	0.5 - 1	(Yamash *et al.*, 2007)
E2	Serum	SPE	3	DS	25	HPLC	Zorbax Eclipse ZDBC18, 150 x 2.1 mm (5 µm)	H_2O/CH_3CN + 1 mL/L CH_3COOH	API 4000 Triple Quad ESI-MS/MS	+	1	(T*ai et al,* 2004)
E1, E2, 16OHE2	Serum	Online SPE	0.1	2-FMP	300 trap/ elute	HPLC	Kinetex XB-C18, 100 x 2.1 mm (2.6 µm)	H_2O + 2.5% FA /MeOH + 20 mM NH_4HCO_2	8050 Triple Quad ESI-MS/MS	+	3 - 7	(Beinhauer *et al.*, 2015)
E1, E2	Plasma	SPE	0.5 - 2	FMPTS	20	UHPLC	BEH C18 50 x 2.1 mm (1.7 µm)	Isocratic H_2O/MeOH + 0.1% FA	5500 Triple Quad ESI-MS/MS	+	2	(Faqehi *et al.*, 2016)

Table 1-5: Estrogen (unconjugated) quantification by LC-MS/MS

Analyte	Matrix	Extraction Type	V (mL)	Agent	Inj V (µL)	LC	Column	Mobile Phase (A/B)	MS	Mode (+/-)	LOQ (pg/mL)	Ref
E1, E2, 16OHE2	Saliva	SPE	0.25	None	30	UFLC-XR	BEH C18 XP, 100 x 2.1 mm (2.5 µm)	H_2O/CH_3CN + 0.1 mM NH_4F	5500 Triple Quad ESI-MS/MS	-	1	(Li *et al.*, 2018)
E2	Plasma	SPE	0.5	None	NS	UHPLC	HSS T3 C18, 100 x 2.1 mm (1.8 µm)	CH_3CN/H_2O	TQ-S ESI-MS/MS	+	2	(Kumar *et al*, 2011)
E2	Serum	Online - SPE	0.25	None	20	UHPLC	C18 SB, 30 x 2.1 mm (1.8 µm)	H_2O/MeOH	TQ-S ESI-MS/MS	-	3	(Keevil *et al.*, 2014)
E1, E2, 16OHE2	Serum	SLE	0.1	None	90	HPLC	Kinetex C18 100 x 3.0 mm (2.6 µm)	H_2O/MeOH 10% NH_4OH (Post Column)	5500 Triple Quad ESI-MS/MS	-	1 - 30	(Wooding *et al.*, 2015)
E1, E2 16OHE	Serum	Deprotein-ation	0.2	None	600	HPLC	LC-8-DB, 3.3 cm × 3.0 mm (3 µm)	MeOH/H_2O	API 5000 Triple Quad ESI-MS/MS	-	1 - 2	(Guo *et al*, 2008)

*Data reported for unconjugated estrogen quantification, Atmospheric pressure chemical ionisation (APCI); atmospheric pressure photoionisation (APPI); 3-bromomethyl-propyphenazone (BMP); 1,2-dimethylimidazole-5-sulfonyl chloride (DMIS); 2,4-dinitrofluorobenzene 2,4-dinitrofluorobenzene (DNBF); dansyl chloride (DS); electrospray ionisation (ESI); Estrone (E1); Estradiol (E2); 2, 4, 16-hydroxetradiol (2, 4, 16-OHE2); 2, 4, 16-hydroxetrone (2, 4, 16-OHE1); 2-hydroxyestrone-3-methyl ether (2OHE-3ME); 16β-hydroxy-17β-estradiol (16epiOHE2); 16-oxo-17β-estradiol (16ketoOHE2); 16α-hydroxy-17α-estradiol (17epiOHE2); 17α-estradiol (17epiestradiol); 1-methylimidazole-2-sulfonyl (MIS); Injection Volume (Inj V); Liquid Chromatography (LC); Liquid Liquid Extraction (LLE); methoxyestrogens (MeOE); methanol (MeOH); 1-(2,4-dinitro-5-fluorophenyl)-4,4- dimethylpiperazinium iodide (MPPZ); Mass Spectrometry (MS); 4-nitrobenzoyl chloride (NBCOCL); N-methyl-nicotinic acid N-hydroxysuccinimide ester (C1-NA-NHS); Not Stated (NS); pyridine-3-sulfonyl chloride (PS); picolinoyl carboxylate (P); N'-(5-fluoro-2,4-dinitrophenyl)-N,N-dimethyl-1,2- ethanediamine (PED); 1-(2,4-dinitro-5-fluorophenyl)-4-methylpiperazine (PPZ); Solid phase extraction (SPE); trifluoracetic acid (TFA); tandem quadrupole mass spectrometry (TQ-S) 1,2- dimethylimidazole-5-sulfonyl chloride; 2-fluoro-1-methyl- pyridinum p-toluene sulfonate (FMP); Ultra Flow – LC (UF-LC); Ultra high performance–LC (UHPLC); Volume (V).

1.6.3 Mass Spectrometry

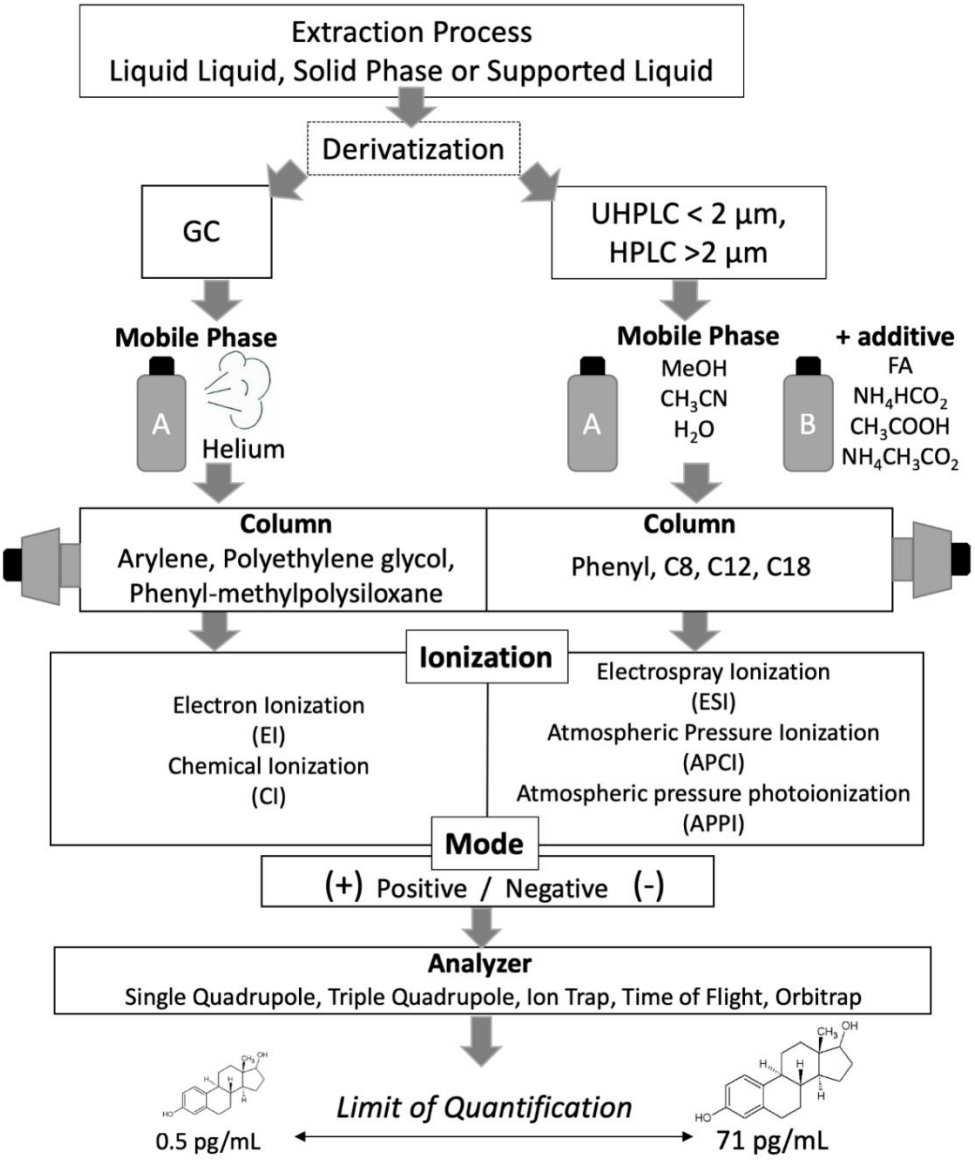

Figure 1-11: Schematic workflow for quantification of estrogen by mass spectrometry

Mass spectrometers interfaced with GC and LC have both been successfully applied to analyse estrogens in plasma and/or serum, with a number of methods being reported as successful (Table 1-5 and Figure 1-11). Single quadrupoles were initially developed to allow one mass filter to be applied for selection of a single *m/z* ratio, known as the precursor ion. Advances in this technology introduced triple quadrupoles, allowing double mass filtering of precursor ions and their breakdown fragments, providing a much higher selectivity with less interference from co-eluting matrix components and increasing the signal to noise ratio. Triple quadrupole MS operating in multiple reaction mode (MRM) rather than conventional selected ion

monitoring (SIM) allows enhanced selectivity over wide dynamic ranges improving accuracy and precision of an assay. Quantitation with high resolution or accurate mass analysers such as time of flight are still in their infancy, held back by poorer quantitative performance due to narrower dynamic ranges. Success with these analysers is variable, with Time-of-Flight instruments to date performing poorly (Table 1-5) but some valuable methods are coming forward with Orbitrap® technology.

1.6.3.1 GC-MS (/MS)

GC-MS has been the method of choice for analysis of sex steroids in aquatic environmental samples. In GC, the mobile phase is an inert gas (usually helium) and the stationary phase is a viscous liquid that coats the walls of the capillary column. Analytes must vaporise and then dissolve into the gas phase upon injection onto the GC column, and this is achieved efficiently for steroids following chemical derivatisation. The derivatised steroids are resolved on the capillary column based on their relative affinities for the stationary phase that coats the inner walls and the temperature gradient applied to the GC oven. Stationary phases with phenyl groups have been most commonly used, as the creation of Π- Π interactions with the estrogenic A-ring enables resolution of more challenging mixtures. Peaks following GC separations are generally narrower compared with LC, and accordingly, resolution of isomers is easier. Chemical derivatisation is applied with a range of reagents reported in (Table 1-4). For MS analysis, electron impact (EI) and chemical ionisation (CI) have been used. Of these, CI in tandem MS instruments are favoured in the literature (Table 1-4). This is due to improved sensitivity in combination with electron capturing halogenated derivatisation reagents. Almost exclusively in GC-MS/MS analyses of estrogens at low concentrations the MS is set to negative ion mode except upon use of ion trap or single quadrupole systems where studies have highlighted elevated estrogen levels within clinical samples, but this method, using an ion trap instrument, achieved a limit of quantification (LOQ) of 13-21 pg/mL (Prokai-Tatrai *et al*, 2010) which would be insufficient for certain patient cohort subgroups (Table 1-3). Urinary analysis of estrogen and its bioactive metabolites by GC typically involves extended sample preparation (two-phase extractive approach) (Chung *et al.*, 2011). For analysis of E1 and E2 in serum, sample extractions in conjunction with a derivatisation show reliable detection Also in this setting, the use

of a two-phase extractive approach yielded the lowest detectable concentrations. A study exploiting this approach via GC-CI-MS/MS has shown potential in the analysis of E1 and E2 to 0.5 pg/mL in 250 μL of serum from rodents (Nilsson *et al.*, 2015), with 2 pg/mL being reported albeit from a high volume of 2 mL pre-pubertal serum (Courant *et al.*, 2007); volumes below 500 μL are desirable for routine clinical research. The evolution of triple quadrupole systems has aided this improvement, but complex sample preparation remains necessary in GC-MS/MS applications. Approaches using solid phase extractions coupled to derivatisation seem the most efficient for single extractive approaches with application to 1.9 pg/mL from 1 mL of post-menopausal serum (Santen *et al.*, 2007) and 2.5 pg/mL in an application note from 1 mL serum. Notably, methods have not been reported for the catechol and hydroxyl metabolites of estrogens in plasma by GC approaches, although in principle this should be achievable as detection has been demonstrated in urinary samples (Chung *et al.*, 2011). Although GC-MS/MS inherently allows enhanced chromatographic resolution, its routine application suffers from time consuming runs (30 min – 1 h), extensive sample preparation procedures, use of high temperatures that may be detrimental to thermos-labile compounds and complex fragmentation of precursor ions applied within the MS source. LC-MS applies atmospheric pressure chemical ionisation (APCI), atmospheric photoionisation (APPI) or electrospray ionistaion (ESI) to generate the ions and these soft ionisation techniques generate the charged molecular ion. Thus, there has been much interest in bringing LC-MS methods to the fore as the approach can be faster, with chromatographic column technology advances improving resolution without the need to convert estrone and estradiol to thermally labile derivatives.

1.6.3.2 LC-MS (/MS)

LC-MS/MS is fast becoming the favoured approach for steroid analysis in clinical laboratories worldwide consequent to the development of more sophisticated and efficient MS systems. Chromatographically, for these applications LC columns usually consist of a reversed phase used in conjunction with a polar mobile phase. C-18 columns remain the column of choice within the literature (Welch *et al*, 2005; Nishio *et al.*, 2007; Yang *et al.*, 2008; Keski-Rahkonen *et al.*, 2013; Ke *et al.*, 2014; Beinhauer *et al*, 2015; Li *et al*, 2015; Wan Li Gao *et al.*, 2015; Wang *et al*, 2015; Li, Li *et al*, 2018) due to their enhanced retention capabilities and the robust

manufacturing consistency. Chemical alterations to bonded stationary phases within LC columns, again using interactions with the aromatic ring, and smaller particle sizes of the packing materials allows clearer distinction of isomeric structures, aiding in retention and selectivity of isomeric estrogen metabolites. It is important to recognise that when studying a family of related molecules that several estrogens may fragment to the same ion, but this can be overcome with chromatographic separation. These possibilities must be excluded or planned for upon method validation. Efficient separation that avoids isobaric interferences is also required between isotopologues [M+2]. Distinction by time of shared product ions generated upon MS fragmentation, between ^{13}C labelled estrogens and endogenous isobars for example, will avoid overlap of signals. Within chromatographic separation, pairings of a wide variety of mobile phase and stationary phases are cited each with their own individual benefit. Most commonly reversed phase chromatography is performed with biphasic mixtures of methanol or acetonitrile and water (R. E. Nelson *et al.*, 2004; Keski-Rahkonen *et al.*, 2013) whilst one has been reported using both acetonitrile and methanol (Nishio *et al.*, 2007). Additions of formic acid, ammonium formate or acetic acid are common to promote the formation of positively charged ions. These polar solvent combinations generally achieve the highest resolving power between isomeric structures under gradient elution. Given the low abundance of estrogens, it is unsurprising to see triple quadrupole instruments, with their improved signal to noise and range dominating over single quadrupole, in the majority of published LC-MS/MS assays for estrogen analysis (Table 1-5). Limits of quantitation by LC-MS/MS in the literature are within the range 0.5 - 10 pg/mL for E1 and E2 and 0.5 - 100 pg/mL for metabolites in positive ionisation mode, usually from 0.1 - 2 mL of serum or plasma (Table 1-5).

Current MS technologies coupled to LC employ three main soft ionisation modes: electrospray ionisation (ESI), atmospheric pressure chemical ionisation (APCI) and with atmospheric pressure photoionisation (APPI) the latter being the most recently developed. All can generate positive and negative ionisation dependent on the charge due to (de)protonation by mobile phase modifiers or coupled by derivatisation. In general, ESI formation of charged droplets plays a major role, creating competition for the ionisation of estrogens versus charged species in endogenous mixtures, tending to result in more complex ionisation mechanisms

being subject to ion suppression (Jessome *et al*, 2006). Nonetheless positive mode ESI analyses remains the most prominent application, at least for derivatised samples (Table 1-5).

Examples include low limits of detection (0.5 – 2.4 pg/mL) for metabolite panels in a study of breast cancer patients (Khedr *et al*, 2016). Negative mode is more commonly used in non-derivatised samples (Mcnamara *et al.*, 2010; Santa *et al.*, 2013), capitalising on the presence of a phenol within the estrogen structure with methods generally reporting limits of ~1 pg/mL for E1 and E2. As an alternative, APCI is less liable to ion suppression due to a lesser probability of charge interference from the corona discharge needle to the analyte. APPI is similar but uses photons from a discharge lamp to aid ionisation of molecules, which respond poorly to ESI. Although reported advantages of APCI/APPI over ESI exist, the majority of routine analytical assays applied in clinical laboratories use ESI, which may be a deterrent to regular exchange between MS interfaces and hindering their extended application. Nevertheless, reported applications of APCI methodologies for estradiol have low LOQs to 0.5 pg/mL but when the metabolites are included within assays sensitivity generally falls due to lower dwell times for each scan. Limits for APPI are reported as 3 – 5 pg/mL for E1, E2 and E3 in mouse serum (Mcnamara *et al.*, 2010). A comprehensive comparison of all ionisation and polarity combinations for estradiol analysis by Rahkonen et al clearly shows APPI in negative mode to have the lowest LOQ at 0.14 – 0.68 pg/mL, but as of yet this is not widely reproduced or available. Low concentrations in pooled serum were detected in negative APPI mode using ammonium hydroxide additives (Keski-Rahk*onen et al*, 2013). However upon application to a clinical cohort samples (200 μL) a derivatisation approach in conjunction with APPI was applied by the same group, quantifying limits of 0.5 pg/mL for human female serum (Keski-Rahkonen *et al.*, 2015). Notably, addition of 2D chromatography boosts sensitivity for E1 and E2, for example reducing LOQs from 10 to 1 pg/mL (Kushnir *et al.*, 2008; Xu *et al*, 2008). Application of this to newer MS technologies applying APPI ionisation might allow a further boost in quantification capabilities.

Alternative LC approaches such as ultraflow LC (UFLC-MS) might be applied, allowing reduction of flow rates (1 - 100 µL/min) and inner column dimensions (ID;

0.1 and 1.0 mm). Advantages include the ability to use smaller volumes of solvents which is of economic and environmental benefit, widening dynamic ranges and, importantly, potential for an improvement to sensitivity and reduction in contamination of the MS instrument (Needham, 2017). A further application of microflow introduces an Ion Key source from Waters, allowing direct infusion of microflow UHPLC into the MS, offering an improvement to sensitivity and chromatographic performance and robustness. This has been exemplified in a technical report for estrone, estradiol and estriol; reporting non-derivatised LOQs of 1 pg/mL using negative-ESI with ammonium hydroxide as a mobile phase modifier for deprotonation illustrating clear analytical advantages over non-derivatised methods by conventional ESI-LC-MS/MS methods (Needham, 2017). In another study by Wang et al using nano-UHPLC technology for analysis of serum from older men, E2 LOQs reached 0.5 pg/mL for estradiol and E2 metabolites and 5 pg/mL for E2 catechol metabolites (Wang *et al.*, 2015); this demonstrated the benefit of low flow in conjunction with triple quadrupole MS in lowering quantification limits within metabolite panels. However, this approach is not yet in routine use. Overall, a wider application of LC-MS/MS than GC-MS/MS for quantification of estrogen and its bioactive metabolites in plasma, serum and more recently in saliva has been demonstrated.

1.6.3.3 Internal Standards

An important feature of mass spectrometry (MS) analytical methods for estrogen quantification is the availability of stable isotope labelled internal standards (IS), giving a retention time match to both derivatised and non-derivatised estrogens. Addition at constant concentrations within the assay accounts for extraction loss at all stages. ^{13}C-labels allow additional selectivity over deuterium-labelled standards, since they are highly unlikely to be removed during processing. Deuterium can be removed through either deuterium-hydrogen exchange under acidic conditions or, depending on the positions of the labels, during derivatisation reactions. By GC and LC, the retention time of ^{13}C-labelled standards are well aligned whereas deuterated IS may differ slightly, probably due to isotope effects on hydrogen vs deuterium bonding interactions with the stationary phase. The slight differences in retention time that arise with deuterium labels are exaggerated when the number of heavy labels is increased and may lead to less accurate quantitation by not compensating

well for matrix effects (Wang *et al*, 2006). However, deuterium labelled standards are applied in a number of studies, generally being less expensive in comparison to the ^{13}C-labelled versions (Kushnir *et al.*, 2008; Mcnamara *et al.*, 2010; Ke *et al.*, 2014; Li *et al*, 2015; Khedr *et al*, 2016). Retention of the stable isotope labels in the product ion generated for detection by mass spectrometry is desirable to enhance specificity, but labels can be lost in fragmentation, leaving ions identical in *m*/*z* to the analyte. $C_{3\text{-}6}$-labelled standards are now available for all estrogens shown in Figure 1-8. Multi-labelled standards, and preferably in excess of two labels, e.g. $^{13}C_3$ and $^{13}C_6$ are desirable to avoid interference with natural isotopologues (Wang *et al.*, 2015).

1.6.4 Sample Preparation

Prior to analytical quantification, estrogens must be efficiently extracted from the matrix of choice. In the case of LC-MS/MS analysis, ion suppression arises due to sample components, such as phospholipid and salt interference. Phospholipids remain the number one cause of diminished signal responses for analytical applications by LC-MS/MS. GC does not suffer from this phenomenon due to the high-energy nature of its ionisation source, although remaining matrix components can cause undesirable deposits in the injector, the start of the column or in the source and will lead reduced sensitivity and poor peak shapes. For either approach, sufficient removal of interfering compounds and lowering of background noise by sample pre-treatment is of paramount importance when approaching estrogen assay development.

1.6.4.1 Serum and Plasma Analysis

Plasma and serum were widely used for the studies discussed (Table 1-4, Table 1-5). Within larger scale metabolomics studies the variance in blood profiles between the two have been highlighted, and is most prominent in proteomics (Dunn *et al*, 2011). This is in part due to the differences in preparation of the two prior to analysis. Plasma is a liquid which makes up 55% of human blood volume, containing fibrinogen and albumin, two factors which aid in normal clotting and retain fluid in the bloodstream, respectively. Serum is a fluid which does not contain the same clotting factors and blood cells as plasma. Removal of these is achieved by

centrifugation which, may add additional variation to the sample preparation process. Specialised tubes are designed for preparation of both bio-fluids which might also affect the study samples. For estrogen analysis the difference should be less substantial but should be considered as both serum and plasma samples will be analysed within this thesis (serum in Chapter 3 & 4, plasma in Chapter 5, 6 & 7)

1.6.4.2 Liquid-liquid extraction (LLE)

LLE provides an inexpensive approach to extract estrogens from the sample matrix exploiting their relative solubility in organic solvents. LLE has been used extensively and as a result is most common for the analysis of E1 and E2 in serum (Fiers *et al.*, 2012; Riley *et al*, 2016). Ultimate recovery and suppression of LLE approaches are dependent on the choice of extraction solvent for individual assay needs. Solvents for extraction of estrogen into the upper organic phase include methyl *tert*-butyl ether (MTBE), ethyl acetate, diethyl ether, dichloromethane or mixtures of these organic solvents. Ethyl acetate is most commonly reported with high recoveries (Gallagh*er et al*, 1958; Dighe *et al*, 2004) and alongside, MTBE it has demonstrated high efficiency for sex steroids yielding a clean extract that avoids precipitation upon derivatisation and fully recovering estrone and estradiol (Keski-Rahkonen *et al.*, 2011; Wang *et al*, 2016). From Table 1-5, LLE has been extensively applied to E1 and E2 assays reporting low detection limits (0.001 – 5 pg/mL). However, for bioactive metabolites results are variable, with higher limits than alternative sample preparation techniques (5 – 360 pg/mL). Additional drawbacks of LLE for routine testing also relate to its time-commitment and the potentially hazardous exposure to high volumes of organic solvents. Also, sample loss in test tubes and plastic plates has been noted using LLE (Keski-Rahkonen *et al.*, 2013). Consequently, the chance of inter-day imprecision is greater than for automated methods (Juhascik *et al*, 2014), as it is normally performed manually.

1.6.4.3 Solid Phase Extraction (SPE)

Off-line solid phase extraction (SPE) is an attractive alternative to LLE, often employed for analysis of estrogen in water (i.e. for processing larger sample volumes) but also with effective application in the clinical setting in saliva, serum and plasma (Nelson *et al*, 2004; Tai *et al*, 2004; Li *et al,* 2018). SPE extraction

cartridges come embedded with a range of solid packing materials, which chemically separate the components of interest from the biological samples. Varieties of bed are commercially available containing reversed, normal, ion exchange or adsorption packing materials.

For recovery of estrogens from aqueous sample matrices, reversed and ion exchange phases are recommended which seem effective for clean up within plasma samples (Table 1-5). In principle, an SPE column containing an alternative packing material to the chromatography column holds advantages in improving sample clean-up. On-line SPE methods are available although less frequently applied due to lack of specialised equipment. These link extraction processes directly to LC-MS/MS instruments minimising manual sample manipulation steps and, once the elution programme is optimised can improve recovery and sensitivity (Čelić *et al.*, 2017). Columns such as Strata X, C18 and Oasis HLB® include hydrophobic compartments optimal for the lipophilic nature of steroid hormones. HLB® operate over a wide range of pH values suitable for all compound classes. These columns have been applied widely to aquatic samples however high recovery of estrogens from biologically complex matrices such as plasma can be associated with undesirable ion suppression for both E1 and E2 (Faqehi *et al.*, 2016). A study by Faqehi et al 2016 suggested the use of Oasis MCX®, a cartridge housing a mixed mode cation exchange reverse phase bed, providing opportunities for additional sample clean-up prior to elution of the estrogen. Use of C_8 polypropylene columns applying conditioning including 0.1% trifluoroacetic acid and sample cleaning with water and the same additive has also shown successful recovery and diminished ion suppression for a panel of 10 estrogens (Khedr and Alahdal, 2016). Moving forward with SPE, newer products eliminate the need for conditioning and equilibration steps, and availability of 96-well plates allows potential automation for robotic liquid handling systems. The main disadvantage of SPE for routine clinical analyses associates with the cost, as cartridges remain expensive. Moreover, coupling SPE and derivatisation can introduce undesirable method transfer steps and also losses depending on the type of collection container required to avoid adhesion (glass *vs* plastic). Glass inserts for 96-well plates are expensive and only available for lower elution volumes.

1.6.4.4 Supported Liquid Extraction (SLE)

Supported liquid extraction (SLE) opens doors to new approaches for extraction but, as yet, methods for estrogen analysis have been scarcely published in the scientific literature, unlike with other steroids (Stirrat *et al.*, 2018). This strategy shows promise in recent company application notes (Blair *et al.*, 2016) with successful application to androgen profiling for diseases such as congenital adrenal hyperplasia (Fiet *et al.*, 2017). SLE applies the same solvent affinity principles as LLE whereby analytes are separated based on their partitioning into one solvent over another immiscible solvent. The support material consists of diatomaceous earth, a natural silica element (~90% silica), being an ideal material to absorb aqueous samples. This extraction employs similar solvents to those in section 2.6.1. One application for analysis of E1, E2 and E3 from 100 µL of plasma in the SLE 96-well format shows potential with low limits of 1, 3 pg/mL for E1, E2 and a higher limit of 30 pg/mL for E3. Testing this extraction method with the full panel of metabolites on more sensitive MS platforms is still awaited. Again, SLE can be fully automated in 96-well formats but, again, there are challenges in interfacing with containers suitable for derivatisation, meaning non-derivatised method development would be favoured, which might prove challenging for certain metabolite groups.

1.6.5 Derivatisation

Derivatisation can be necessary prior to analysis of estrogens by MS, but with different goals for GC and LC. In the case of GC it is necessary to enhance volatility, and the introduction of halogen atoms allows CI approaches to enhance sensitivity (Díaz-Cruz *et al.*, 2003). For LC, estrogens can be analysed in their natural form creating negative ions through proton loss from the phenol group. Derivatisation is often employed, aiding formation of charged ions or generating permanently charged species, increasing the mass of the molecular ion and increasing sensitivity, with further benefits to specificity. In GC-MS/MS, the process adds poorly volatile reagents which cannot be easily removed. In both systems, reagents of derivatisation reactions can build up in the chromatographic column or within components of the mass spectrometer, decreasing assay robustness. In LC, care must be taken to divert initial flow to waste to keep the sources and mass

spectrometer as clean as possible, while in GC frequent inlet liner cleaning or replacement is required.

1.6.5.1 GC approaches

In GC-MS/MS derivatisation, at the 3' A ring position is favoured as reactions at the saturated aliphatic D ring provide no improvement of sensitivity over non-derivatised samples, illustrated by pentafluoropropionyl (PFP) or trimethylsilyl (TMS) derivatives for water analysis by GC-MS/MS (Lee *et al*, 1998; Mol *et al*, 2000). The generation of PFB derivatives is the most commonly reported approach for estrone and estradiol analysis in serum, but cumbersome sample preparation steps have hampered its routine use (Courant *et al.*, 2007; Santen *et al.*, 2007; Nilsson *et al.*, 2015).

1.6.5.2 LC approaches without derivatisation

Development of analytical workflows or sufficient sensitivity without derivatisation is challenging for clinical applications of estrogen, although they are desirable due to sample preparation being shorter and with fewer steps to introduce variation. Further automation of derivatisation by commercial robots is challenging to couple with robotic SPE/SLE workflows. However, a number of methods for estrone and estradiol using LC-MS/MS are beginning to surface (Table 1-5) as instrument technology improves. Methods achieving LOQs to compete with derivatisation approaches have been reported using ammonium fluoride or ammonium hydroxide as a mobile phase modifier. This addition promotes the formation of negative ions in a neutral environment (Fiers *et al.*, 2012; Li, Li and Kellermann, 2018). Recent analyses of estradiol report low LOQs, for example 2 pg/mL (7.3 pmol) using a UHPLC System coupled to a Xevo TQ-S (S. K*umar et al*, 2011). Non-derivatised methods are yet to be extended to include bioactive estrogen metabolites. If optimised successfully, validation of such assays would permit simplified sample preparation with the possibility of higher precision and throughput.

1.6.5.3 LC Approaches with Derivatisation

Derivatisation remains necessary for the majority of estrogen LC-MS assays to overcome poor ionisation, ion suppression or insufficient signal intensity at low

abundance. In the reactions reported, introduction of easily ionisable groups or pre-charged moieties improves sensitivity permitting the use of lower volumes of sample. As in GC-MS, the hydroxyl group of the phenolic A ring in the 3' position should be targeted for improved sensitivity, for the entire analyte panel.

Successful derivatisation methods commonly reported for analysis of E1 and E2 include use of dansyl chloride (S. S.-C. Tai *et al*, 2004; Chang *et al*, 2010; Vitku *et al*, 2015; Blair *et al*, 2016), *N*-methyl-nicotinic acid *N*-hydroxysuccinimide ester (W. C. Y*ang et al*, 2008), 2-fluoro-1-methylpyridinium-*p*-toluenesulfonate (Faqehi *et al.*, 2016), methyl-1-(5-fluoro-2, 4-dinitrophenyl)-4,4-dimethylpiperazine (Nishio *et al.*, 2007) isomers of 1,2-dimethylimidazole-sulfonyl chloride (Escrig-Doménech *et al.*, 2013; Li *et al*, 2015), picolinoyls (Yamashita *et al.*, 2007), pyridine carboxylates (Yamashita *et al.*, 2007), pyridine-3sulfonyl (Xu *et al*, 2008) and *p*-nitrobenzyl (Penning *et al.*, 2010) derivatives. Of these, dansyl chloride has been the most common approach. However, the specificity of the fragment ions of dansyl chloride derivatives is hindered for isobaric estrogen metabolite species since the product ions generated are identical, hailing from the derivative (Chang *et al.*, 2010; Li *et al*, 2015; Vitku *et al.*, 2015). This is similar for alternative derivatives such as BMP (Khedrv *et al*, 2016), whereby these methyl-propyphenazone derivatives generate identical product ions for seven estrogens whilst differing by *m/z* 15 for the catechol metabolites.

This has been partially overcome by use of MPPZ and C1-NA-NHS, where there are a range of product ions, but still they remain identical for certain groups of metabolites (Yang *et al.*, 2008), since isomers undergo similar fragmentation patterns. These potentials for interference underpin a requirement for thorough evaluation of chromatographic methods, both to measures broad panels of estrogens, but also to eliminate possible co-elutants that may be mis-identified and lead to reporting of levels that are too high. It should not be forgotten that estradiol and estrone only differ by 2 mass units so $^{13}C_2$ isotopologues will cross-signal if product ions are identical. Double, triple or isomeric derivatisation must be assessed during method development as it is not uncommon for multiple aliphatic and phenolic hydroxyl groups to be derivatised within the reaction especially within 16-hydroxy- and catechol estrogens. (Nishio *et al.*, 2007). Finally, if derivatisation is

deemed necessary the stability of derivatives may also be less than the original estrogen and this must be studied to ensure practical laboratory workflows e.g. FMP derivatives degrade following 48 hours at -20 °C but remain stable at -80°C (Faqehi *et al*, 2016). Dansyl chloride derivatives have been reported to be stable over a 7-day period in patient plasma (Nelson *et al.*, 2004). In the majority of current literature, such information is lacking for many derivatisation approaches. Derivatisation techniques are still less preferred in the clinical setting, due to the addition of another complexity within sample preparation inevitably contributing error and increased turn-around time.

1.7 Hypothesis and aims

Pulmonary arterial hypertension (PAH) is a severe, debilitating and life-limiting disease, which has no cure. The increased female predominance suggests a sexual dimorphism potentially caused by estrogen and its metabolism. The principal aim of this PhD research was to develop a liquid chromatography tandem mass spectrometry (LC-MS/MS) technique capable of simultaneous quantification of estrogen and its bioactive metabolites in human plasma and serum, to overcome issues faced in the field with immunoassay quantification. I hypothesis that upon application of the LC-MS/MS approach estrogen profiles may be altered between non-PAH controls and PAH patient samples, in a sex-dependant manner, with elevations associating with disease phenotype and haemodynamic parameters. In addition, novel metabolites detected may functionally alter PASMC behaviour contributing to PAH pathophysiology. In PASMCs, an untargeted, unbiased RNA-sequencing analysis may identify transcripts of interest in female PAH for further investigation in disease pathogenesis. These aims were investigated using the following approaches:

1. Development and Validation of an LC-MS/MS Approach to Quantify Estrogen and its Metabolites in Human Plasma (Chapter 2)

2. Preliminary Quantification of Estrogens in PAH and Investigation of the In Vitro actions of Bioactive Estrogen Metabolites (Chapter 3)

3. Estrogen Metabolism in Patients with PAH (Chapter 4)

4. Estrogen Metabolism and the Menstrual Cycle in PAH (Chapter 5)

5. Estrogen Metabolism in Portopulmonary Hypertension (PPHTN) (Chapter 6)

6. Proof of Concept Clinical Trials: Estrogen in PAH (Chapter 7)

7. Untargeted Transcriptome-Wide Analysis of Human Pulmonary Artery Smooth Muscle Cells (Chapter 8)

Chapter 2 Development of an approach to quantify estrogens and their metabolites by LC-MS/MS in human plasma

2.1 Introduction

A number of successful approaches have been reported for quantification of estradiol and estrone in biofluids such as serum, plasma, saliva and urine with a lesser number for the bioactive metabolites (Table 1-4, Table 1-5). It remains of high importance to develop analytical techniques capable of measuring estrogen levels within clinical cohorts. PAH epidemiologically covers a wide population with ranging characteristics, from premenopausal and postmenopausal women to men of all ages showing signs of estrogen-sensitive disease. This presents analytical challenges for estrogen quantification by analytical technologies which must be successfully overcome within method development (Paulin *et al*, 2012; Foderaro *et al*, 2016). As estrogens are isomeric and isobaric in structure, possessing the same formula and mass but alternative chemical structures, this means efficient detection and separation of compounds by LC-MS/MS must be achieved for accurate identification. In addition, estrogens are neutral molecules which do not ionise well within the mass spectrometer, therefore mobile phase additives and ionisation modes in negative or positive ion mode formations have been explored in the literature (Table 1-5). Another way to overcome this challenge is to add a permanent charge by means of chemical derivatisation reactions, appending a charged moiety to all estrogens hence improving their ionisation by formation of charged droplets in electrospray ionisation, aiding fragmentation within the source. Finally, low circulating levels in men and postmenopausal women present a challenge in the detection of low level endogenous estrogens. To assess concentrations below 20 pg/mL, an efficient extraction method from plasma should be found to reduce ion suppression from phospholipids and salts, which would reduce quantifiable levels within plasma. For these reasons, a sensitive and selective method must be developed to allow confident detection and identification at low levels. Here, liquid chromatography tandem mass spectrometry was the analytical method of choice due to the wider range of literature presented by application of this technique (Table 1-5).

2.2 Aims

The aim of this chapter was to develop and validate an LC-MS/MS approach for the quantification of estrogen in human plasma.

2.3 Materials and methods

2.3.1 Standards and solvents

Estrone (E1), 17β-estradiol (17βE2), 17α-estradiol (17αE2), 2-hydroxyestrone (2OHE1), 4-hydroxyestrone (4OHE1), 16-hydroxyestrone (16OHE1), 2-hydroxyestradiol (2OHE2), 4-hydroxyestradiol (4OHE2), 16-hydroxyestradiol (16OHE2), 2-methoxyestrone (2MeOE1), 4-methoxyestrone (4MeOE1), 2-methoxyestradiol (2MeOE2) and 4-methoxyestradiol (4MeOE2) were from Steraloids, Inc (Newport, USA). Internal standards (2,3,4-$^{13}C_3$-estrone ($^{13}C_3$-E1), 2, 3, 4-$^{13}C_3$-estradiol ($^{13}C_3$-E2)), formic acid (FA) triethylamine (TEA), Glycidyltrimethylammonium Chloride (GTMAC) and methyl iodide (CH_3I; ≥99%) were purchased from Sigma-Aldrich, Inc. (St. Louis, USA). 13,14,15,16,17,18-$^{13}C_6$-4-Hydroxyestrone ($^{13}C_6$-4OHE1), 13,14,15,16,17,18-$^{13}C_6$-2-hydroxyestradiol ($^{13}C_6$-2OHE2), 2,3,4-$^{13}C_3$-16-hydroxyestradiol ($^{13}C_3$-16αOHE2), 13,14,15,16,17,18-$^{13}C_6$-2-methoxyestrone ($^{13}C_6$-2MeOE1) and 13,14,15,16,17,18-$^{13}C_6$-4-methoxyestradiol ($^{13}C_6$-4MeOE2) were purchased from CK Isotopes Limited (Leicestershire, UK). Certified estrone (1 mg/mL in methanol; 1mL) and 17β-estradiol (1 mg/mL in CH_3CN; 1mL) were from Cerilliant (Sigma Aldrich, Dorset, United Kingdom). 1-(5-Fluoro-2, 4-dinitrophenyl)-4-methylpiperazine (PPZ) was purchase from TCI chemicals (Chuo-ku, Tokyo, Japan). HPLC grade solvents (methanol, acetone, acetonitrile and water) and LCMS grade (acetonitrile and water) solvents were obtained from Fisher Scientific UK Limited (Leicestershire, UK).

2.3.2 Instrumentation

Tuning and fragmentation analysis of estrogen derivatives at high concentrations was performed on a TSQ Quantum Discovery MS coupled to a Surveyor autosampler, with a Surveyor MS pump (Thermofinnigan, Waltham, USA) operated using Xcalibur software version 2.0. Structural formulae of fragment ions formed from estrogen derivatives were determined by high-resolution MS using a Synapt G2Si instrument (Waters Corp., Manchester, UK). High-sensitivity quantification was performed on a QTrap 6500+ (Sciex, Warrington, UK) interfaced to a Shimadzu Nexera X2 (Shimadzu UK Limited, Buckinghamshire, UK) liquid chromatograph

operated using Analyst software v1.5.1. Data were analysed using MultiQuant v. 3.0.

2.3.3 Plasma samples

Male and female human plasma for method development and validation were purchased from TCS Biosciences (Buckingham, UK), stored at -80°C. This plasma was prepared from the blood of healthy human donors, collected into anticoagulant (citrate phosphate dextrose adenine) and screened for possible infections. For initial method application, plasma from a small cohort of four female PAH patients was collected with ethical approval and informed consent in the Queen Elizabeth University hospital, Glasgow by Dr Colin Church.

2.3.4 Standard Solutions

Endogenous estrogens and internal standards were dissolved in methanol and stored at -80°C. Serial dilutions of the stocks and internal standard (100 pg) were prepared on the day of use to give a suitable range of working solutions (10 pg/mL to 0.0001 µg/mL).

2.3.5 Sample Derivatisation

2.3.5.1 Derivative Selection

Glycidyltrimethylammonium Chloride (GTMAC) derivatisation (Gouw et al. 2002)

Derivatised standards were individually prepared by serial dilution (10 µg, 1 mg/mL) of each estrogen into a 1.5 mL vial and reducing to dryness with oxygen-free nitrogen (OFN). GTMAC (0.5 µL), TEA (5 µL) and ethanol/water (50 µl ; 1:1) was added. The solution was capped and incubated (60°C, 1 hour). The solution was dried down under OFN with the dried residue re-dissolved in acetonitrile (1 mL).

MPPZ derivatisation (Nishio *et al.*, 2007)

Derivatised standards were individually prepared by serial dilution (10 µg, 1 mg/mL) of each compound together with PPZ stock (10 µL, 1 mg/mL), ACN/H_2O/MeOH (20 µL; 40:35:25), sodium bicarbonate (10 µL, 1M) and of acetone (70 µL) in to a 1.75 mL glass vial. The solution was capped and incubated (60°C, 1 hour; Reaction one). CH_3OH: H_2O (500 µL, 1:1) was added to quench the reaction and reduced to dryness under OFN. The dried residue was further reacted with methyl iodide (100 µL) and incubated (60°C, 30 minutes; Reaction two). Then the CH_3I was reduced to dryness with OFN and re-dissolved in H_2O/methanol (1 mL of 1:1). The solutions of estrogen derivatives were directly infused directly into the MS for tuning.

2.3.5.2 Non-Derivatised Solutions

Estrogen standards (1µg/mL) were transferred to individual 1.75 mL vials. Steroids were dried before being redissolved in mobile phase for assessment of their response in comparison to derivatised steroids by LC-MS/MS analysis (n=3).

2.3.5.3 MS Tuning and fragmentation

All estrogens were derivatised prior to tuning via direct infusion on the Quantum TSQ discovery triple quadrupole mass spectrometer with structures confirmed by high resolution MS using a Synapt G2Si instrument (Waters Corp, Manchester, UK) fitted with an ESI source in positive mode. For the latter, samples (0.1 µg/mL) dissolved in H_2O: CH_3CN (70:30, 70 µL) were infused 2 µL/min (Harvard Apparatus UK) at a spray voltage 3.0 kV, sampling cone voltage 40 V and source temperature 100 °C. Data were collected in full scan mode and MS2 spectra (*m/z* 50 - 1200) in resolution mode. Tandem mass spectra were generated in the trapping region of the ion mobility cell using collision energy 40 V, with argon as the collision gas (40.0 psi). Instrument calibration was performed using 0.05M sodium formate. Lock mass correction was applied to precursor masses.

2.3.5.4 MS Quantification Parameters

The 6500+ Mass Spectrometer used for method development and quantitation was operated in positive electrospray (ESI) mode as follows: curtain gas (40.0 psi),

collision gas (medium), ion spray (5500 V), temperature (700 °C), nebulizer gas (60.0 psi) and heater gas (40.0 psi). For the detection of product ions by MS, molecular ions were isolated by their Q_1 parent mass and subjected to collision induced dissociation (CID), in scanning mode using a range from *m/z* 450 to 640. Conditions for multiple reaction monitoring (MRM) were optimized by auto-tuning during infusion of the estrogen metabolites and IS (1 µg/mL). The collision energy for each compound was optimised to achieve maximal sensitivity to detect quantifier and qualifier ions.

2.3.5.5 Optimisation of MPPZ derivatisation conditions

Reaction conditions were optimised in standards and plasma extracts. The efficiencies of Reactions 1 and 2 were measured at incremental temperatures, time points and CH_3I volumes, 40 - 60°C, 40 – 60 minutes and 40 – 160 µL respectively. Additionally, the reaction base catalysts were tested alongside the base pH, reaction solvent and concentration of PPZ derivative. The steps involving ACN/H_2O/MeOH (20 µL, 40:35:25) and of CH_3OH:H_2O (500 µL; 1:1) were removed as this addition was not essential to the reaction process. Finally, redissolving of estrogen derivatives in various compositions of H_2O/CH_3OH & CH_3CN was applied.

2.3.5.6 Optimisation of MPPZ reaction for catechol estrogens

Derivatisation to achieve signals of the required sensitivity to detect 2OHE1, 4OHE1, 2OHE2 and 4OHE2 (i.e. the catechol metabolites) proved to be the most challenging. Optimisation of the MPPZ derivatisation method was pursued to find suitable conditions by testing alternative base catalyst for reaction one. A number of conditions were tested: pH and concentration of sodium bicarbonate and different base catalysts (pyridine, *N*-diethylanaline, *N*-ethylisopropylamine, 4-dimethylpyridine, triethylamine and a mixture of 1:1 of certain bases). For reaction two, incremental temperatures, time points and Ch_3I volumes, 40 - 60°C, 40 – 60 minutes and 40 - 160 µL respectively were tested.

2.3.5.7 Derivatisation - Final conditions

Derivatised standards were prepared in 1.75 mL vials by addition of of acetone (70 µL), PPZ stock (10 µL, 1mg/mL) and sodium bicarbonate (10 µL, 1M). This method

was used for nine estrogens/metabolites, with derivatisation of 2 and 4-hydroxyestrogens showing increased efficiency using *N, N*-diethylaniline as a base catalyst. Solutions were capped, vortexed and incubated (60°C, 1 hour). Samples were reduced to dryness under OFN. The dried residue was methylated by addition of methyl iodide (100 µL) and incubated (40°C, 2 hours). Then it was reduced to dryness with OFN and re-dissolved in of 70:30 water/methanol (70 µL) for transfer to an LC vial with injection of 30 µL for analysis.

2.3.6 Chromatographic conditions

2.3.6.1 Chromatographic Conditions for Nine Estrogens

Optimal chromatographic conditions giving separation of estrogen metabolites were found using an ACE excel 2, C18-PFP (2.1 x150 mm; 2 µm) column at an oven temperature of 25°C. The mobile phase consisted of water and acetonitrile at a 90:10 starting composition plus FA (0.1%). A gradient elution was applied (1 - 12 minutes 90:10, 12 - 15 minutes 50:50 and 15 – 18 minutes 90:10) at a flow rate of 0.5 mL/minute. The autosampler was maintained at a constant temperature (14°C) with samples usually being injected at a volume of 30µL onto the QTrap 6500+ LC-MS/MS (Sciex) interfaced to a Shimadzu Nexera X2 liquid chromatography system.

2.3.6.2 Chromatographic Conditions for Catechol Estrogens

Two Ace Excel 2 C18-PFP column (150 x 2.1 mm 2µm + 20 x 2.1 mm 2µm; HiChrom, Reading, England) were coupled at an oven temperature of 20°C. A gradient solvent system of water: acetonitrile (90:10), containing FA (0.1%, 0.5 mL/min) was diverted to waste for the initial 9 minutes followed by elution for a further 4 minutes at 90:10, then with a gradient over 3 minutes until final conditions of water: acetonitrile (90:10) containing FA (0.1%, 0.5 mL/min) were achieved. Injection volume was 30 µL.

2.3.7 Extraction Efficiency

2.3.7.1 Optimisation of Extraction Method

The aim was to extract 13 estrogens and split the extract to derivatise both the panel of 9 and 4 catechol estrogens using the manufacturer’s guide protocol as a starting

point. However, significant ion suppression was identified upon application to plasma meaning the protocol required optimisation. The two groups of metabolites required separate testing as a result. Therefore, from here group one refers to estrone, estradiol, 16-hydroxyestrone, 16-hydroxyestradiol, 2-methoxyestrone, 4-methoxyestrone, 2-methoxyestradiol and 4-methoxyestradiol, whereas group two refers to 2-hydroxyestrone, 4-hydroxyestrone, 2-hydroxyestradiol and 4-hydroxyestradiol known collectively as the catechol metabolites.

Therefore, the extraction protocol was assessed, evaluating the benefits of addition of a second wash step and alteration of elution conditions for groups one and two.

2.3.7.2 Extraction Method for Nine Estrogens

Aliquots of female plasma were subject to centrifugation (8000 g, 4°C, 20 minutes) with the sediment discarded. Sample volumes (0.5 mL) were adjusted to 1 mL with water and enriched with internal standards (100 pg). Standard solutions were added into water (1 mL). SPE using Oasis® MCX (3 cc/60 mg, Waters, Wilmslow, UK) extraction cartridges was performed under gravity. Prior to loading the sample, the cartridges were conditioned and equilibrated with methanol (2 mL), followed by water (2 mL). The diluted sample was loaded and allowed to pass through the cartridges and the eluate discarded. The cartridges were washed first with aqueous FA (2% v/v, 2 mL) and then with methanol (30% v/v, 2 mL) and eluates discarded. Steroids were eluted in methanol (100%; 2 mL). Extracts were reduced to dryness under OFN (40°C) and the residues were derivatised as above.

2.3.8 Extraction Efficiency

2.3.8.1 Recovery Assessment

Recoveries of steroids from water and plasma were assessed by comparison of mean peak areas of derivatives (1 ng; n=6) following extraction of steroids in pre-spiked samples, divided by those in post-spiked samples.

2.3.8.2 Matrix Effect

Ion suppression or enhancement of signal of derivatives in plasma extracts was assessed by comparing signal intensity of derivatised estrogen standards post spiked into extracted plasma with that of standard solutions at the same concentration (1 ng; n=6).

2.3.9 Assay Validation

For assay validation, FDA bioanalytical method validation guidelines for industry were followed. In addition, tutorial reviews on validation of LC-MS/MS methods were used to obtain an overview of current validation guidelines (Kruve *et al.*, 2015, 2015).

2.3.9.1 Specificity

Chromatograms were inspected at the retentions times of analytes and IS, in both mixed and individually-derivatised samples for possible interfering peaks from endogenous components of plasma.

2.3.9.2 Linearity

Blank samples and aliquots containing estrogens (1, 2, 6, 10, 20, 40, 100, 200, 500, 1000, 2000 pg/mL) and internal standards (200 pg/mL) were prepared as stated previously and analysed to create calibration curves. A lack-of-fit test was applied to replicate (n=6) calibration curves to determine if the regression line fit well across the calibration range (Kruv*e et al*, 2015).

2.3.9.3 Limits of detection and quantification

Replicate aliquots (0.001, 0.006, 0.01 ng and 1, 6, 10 p/ mL; n=6) of estrogens and internal standards (0.2 ng; 200 pg/mL) were analyzed as above. The limit of quantification (LOQ) was calculated, as that amount affording precision and accuracy of ~20%, with the limit of detection (LOD) assigned as the lowest calibration point with a signal to noise ratio >10.

2.3.9.4 Accuracy, Precision and Bias

The intra- and inter-day assay precision and accuracy were assessed using amounts of standard (2 & 200 or 2000 pg/mL) and spiked plasma (100 pg/mL of metabolites only) prepared on the same day and different days, respectively (n=6). The precision was calculated as the Relative Standard Deviation (RSD) (standard deviation/mean x 100), and % accuracy was the Relative Mean Error (RME; ((mean measured value - theoretical value)/theoretical value) x 100); precision and accuracy were accepted with RSD/RME <20% at LOQ and <15% above. The bias of the calibration was tested against certified reference material for E1 and 17βE2; Bias, Average Interday concentration – reference material concentration.

2.3.9.5 Derivative Stability

Stability of derivatives of standards and enriched plasma (200 pg/mL) was assessed following storage in the autosampler (15°C) and on short-term storage in the freezer (-20 and -80°C) by reinjection after 1, 4, 8 and 31 days, where appropriate.

2.3.9.6 Assay Robustness

Assay robustness was reported following the analysis of several larger batches of clinical plasma samples. Here, we examined retention times of analytes for any changes to peak shape and intensity whilst observing the inter-day performance of the Shimadzu Nexera system.

2.3.10 Method Application

The presence of estrogens and their metabolites was assessed, and their amounts quantified in human plasma from healthy female pooled controls (n=3) and individual female PAH patients (n=4) using the validated approach.

2.4 Results and Discussion

2.4.1 Sample Derivatisation

A pre-screen of derivatives in the laboratory prior to my PhD project identified Glycidyltrimethylammonium Chloride (GTMAC) and methylpiperazine (MPPZ) as suitable derivatives for the estrogen panel.

2.4.1.1 Derivative Selection

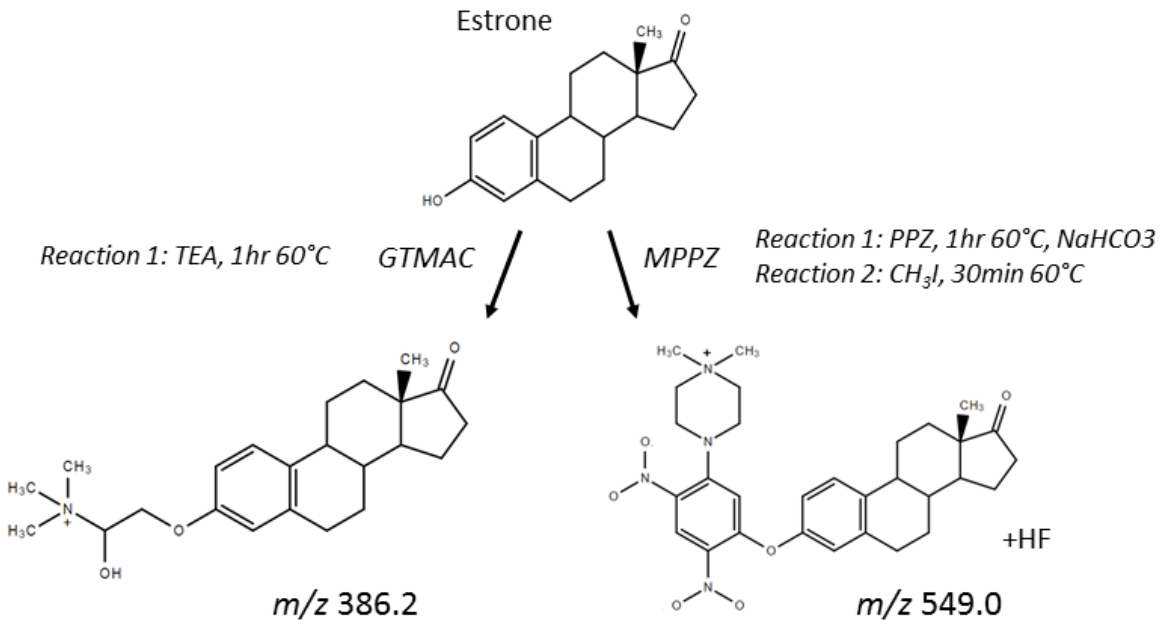

Figure 2-1: Derivatisation reagent selection Expected derivatisation of estrone with Glycidyltrimethylammonium Chloride (GTMAC) catalysed by Triethylamine (TEA), and with methylpiperazine (MPPZ) catalysed by sodium bicarbonate ($NaHCO_3$) in reaction one followed by quaternization by methyl iodide (Ch_3I) in reaction two creating the charged moieties detected by MS analysis.

Upon tuning both derivatives gave similar intensities of product ions for all 13 estrogens of interest at high concentrations.

Table 2-1: Ion transitions from derivative tuning Tuning of estrogen, GTMA and MPPZ derivatives on the Quantum system yielded a number of parent and fragment ions for transfer to the sciex 6500+ mass spectrometer

EST-GTMA	Q1 Mass	Q3 Mass	EST-MPPZ	Q1 Mass	Q3 Mass
E1	386.2	145.0	**E1**	549.071	502.3
E1	386.2	309.2	**E1**	549.071	72.0
E2	388.2	145.0	**E2**	551.075	504.3
E2	388.2	311.2	**E2**	551.075	58.1
2OHE1	402.2	190.1	**E2**	551.075	72.0
2OHE1	402.2	172.1	**2OHE1**	565.024	251.4
2OHE1	402.2	116.4	**2OHE1**	565.024	58.0
4OHE1	402.3	116.2	**2OHE1**	565.024	72.0
4OHE1	402.3	158.4	**4OHE1**	565.024	251.4
4OHE1	402.3	246.3	**4OHE1**	565.024	58.0
16OHE1	402.2	145.0	**16OHE1**	565.024	251.4
16OHE1	402.2	325.2	**16OHE1**	565.024	58.0
16OHE1	402.2	163.0	**16OHE1**	565.024	72.0
2OHE2	404.2	118.1	**2OHE2**	567.08	251.0
2OHE2	404.2	158.1	**2OHE2**	567.08	281.2
2OHE2	404.2	100.0	**2OHE2**	567.08	114.2
4OHE2	404.3	116.1	**2OHE2**	567.08	58.0
4OHE2	404.3	158.2	**4OHE2**	567.08	251.0
4OHE2	404.3	101.1	**4OHE2**	567.08	281.2
16OHE2	404.2	145.0	**4OHE2**	567.08	58.0
16OHE2	404.2	163.0	**16OHE2**	567.08	251.0
16OHE2	404.2	58.2	**16OHE2**	567.08	58.0
MeOE1	416.2	175.0	**MeOE1**	579.02	280.1
MeOE1	416.2	193.0	**MeOE1**	579.02	58.0
MeOE1	416.2	58.1	**MeOE1**	579.02	72.0
MeOE1	416.3	148.9	**MeOE1**	579.02	250.0
MeOE2	418.3	193.1	**MeOE2**	581.03	280.0
MeOE2	418.3	175.0	**MeOE2**	581.03	250.0
MeOE2	418.3	58.1	**MeOE2**	581.03	72.0
MeOE2	418.3	149.2	**MeOE2**	581.03	58.0

Estrone (E1); Estradiol (17 α/ β E2); 2, 4 or 16-hydroxyestrone (2, 4 or 16 OHE1); 2, 4 or 16-hydroxyestradiol (2, 4 or 16 OHE2); 2 or 4-methoxyestrone (2 or 4-MeOE1); 2 or 4-methoxyestadiol (2 or 4-MeOE2); 2,3,4-$^{13}C_3$-estrone ($^{13}C_3$E1); 2,3,4- $^{13}C_3$-estradiol ($^{13}C_3$E2);

The most dominant ions (Table 2-1) were chosen for comparison of derivative signals. At high concentrations (2 ng/mL), lower peak area intensities were observed for estradiol-GTMAC upon comparison to the MPPZ derivative, and furthermore methoxyestrogens co-eluted on the C18_PFP column. These analytes could not be

separated even marginally by column alteration or changes to the oven temperature (Figure 2-2, MeOE1 & MeOE2)

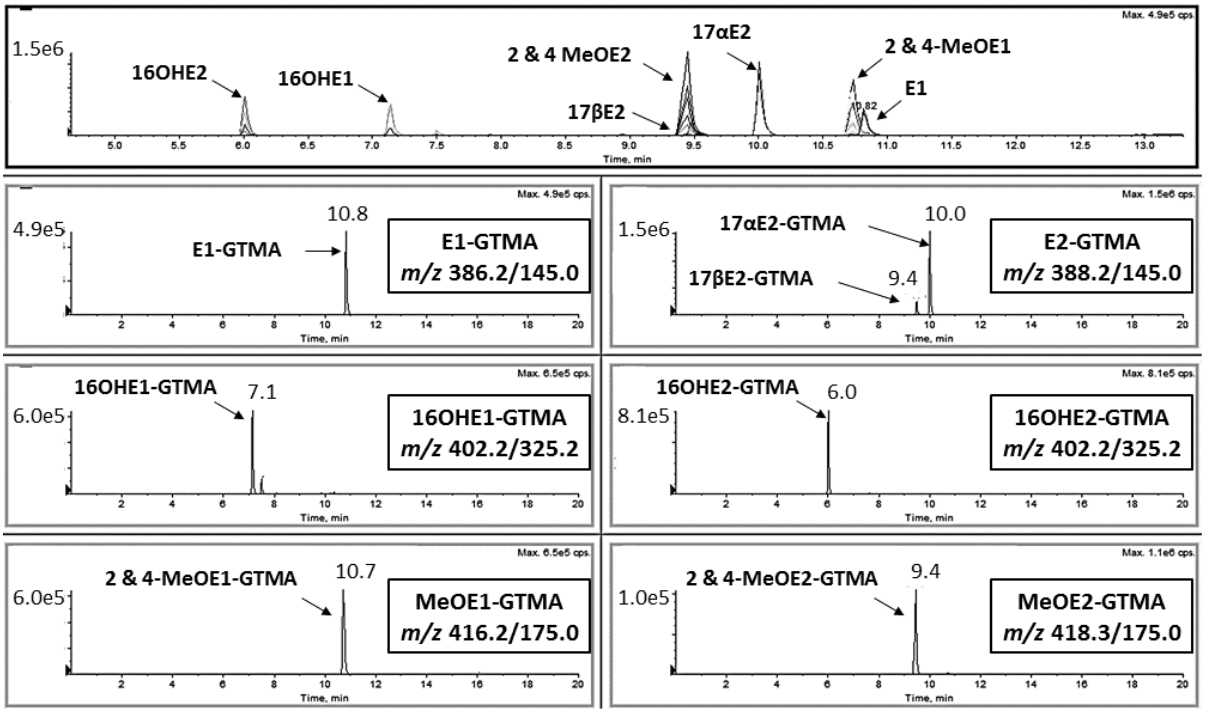

Figure 2-2: Mass chromatograms of Glycidyltrimethylammonium (GTMA) derivatives of estrogens and their metabolites following analysis of an extracted solution of standards Total Ion Chromatograms and the corresponding extracted ion chromatograms showing resolution of derivatives of estrone (E1), estradiol (17α & 17βE2), 16-hydroxyestrogens (16OHE1 & 16OHE2) and methoxyestrogens (2MeOE1, 4MeOE1, 2MeOE2 & 4MeOE2) by retention time and mass transition.

As well as co-elution and reaction efficiency issues for estradiol, poorer signal abundances for the panel of nine metabolites were observed. In addition, peaks were not detected for the more challenging 2-Hydroxyestrone catechol estrogen metabolites (Figure 2-3).

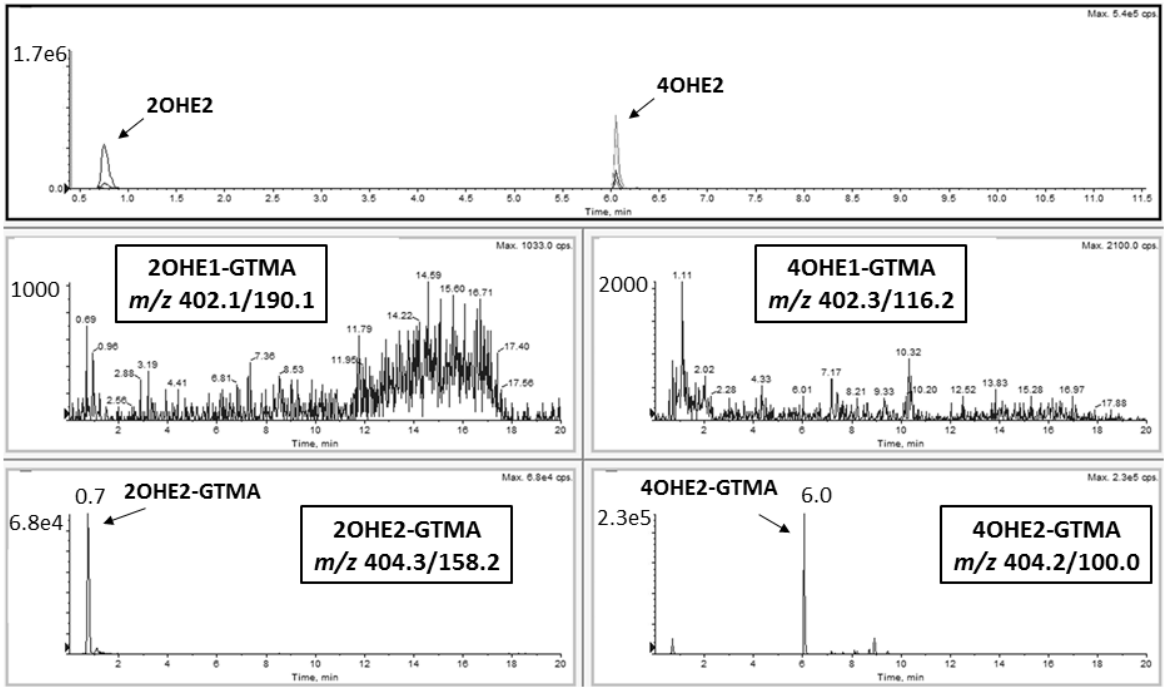

Figure 2-3: Total and extracted ion chromatograms of Glycidyltrimethylammonium (GTMA) derivatives of catechol estrogens following analysis of an extracted solution of standards The estrone metabolites 2-hydroxyestrone (2OHE1), 4-hydroxyestrone (4OHE1) and the estradiol metabolites 2-hydroxyestradiol (2OHE2), 4-hydroxyestradiol (4OHE2) were derivatised at 1000 pg/mL. Figure illustrating challenges in detecting adequate intensities of catechol metabolites even at high concentrations, identified by mass transitions and retention time (minutes).

Therefore, as MPPZ displayed the capability of derivatising this panel of 13 estrogens with a higher efficiency over GTMAC, MPPZ was chosen for the next step of method development.

2.4.1.2 Comparison of estrogens analysed without derivatisation and following MPPZ Derivatisation

Additionally, for comparison, derivatisation with MPPZ successfully showed the ability to improve detection over non-derivatised estrogens by improving ionisation in positive mode electrospray ionisation mode resulting in increased sensitivity, x11 for E1, x885 for αE2, x2628 for βE2, x2 for 16αOHE1, x19 for 16βOHE2, x3 for 2MeOE1, x5 for 4MeOE1, x3 for 2MeOE2, x6 for 4MeOE2, x5 for 13C3E1 and x200 for 13C3E2 (Table 2-2).

Table 2-2: Ion transitions and peak areas for Non-Derivatised and MPPZ-derivatised estrogen metabolites in +ESI-MS/MS

Analyte	Non-Derivatised Transition	Non-Derivatised Retention Time (Rt)	Non-derivatised Peak Area	MPPZ-analyte Peak Area
E1	271-133.0	13.87	1,362,422	15,093,845
17αE2	273 -77.1	12.77	26,231	24,889,945
17βE2	273 -77.1	12.31	9416	25,915,650
2OHE1	287.1-103.0	*ND*	*ND*	107,737
4OHE1	287.1-103.0	*ND*	*ND*	54,841
16OHE1	287.1-103.0	11.79	6,444,815	12,696,601
2OHE2	289.1 -123.0	*ND*	*ND*	125,984
4OHE2	289.1 -123.0	*ND*	*ND*	20,711
16OHE2	289.1-123.0	10.64	494,407	9,363,390
2MeOE1	301.2-189.0	14.44	1,776,717	6,054,850
4MeOE1	301.2-189.0	13.85	1,677,147	8,028,092
2MeOE2	303.2-137.0	13.05	1,115,710	3,404,368
4MeOE2	303.2-137.0	12.27	1,575,590	9,175,856

(E1); estradiol (17 α/ β E2); 2, 4 or 16-hydroxyestrone (2, 4 or 16 OHE1); 2, 4 or 16-hydroxyestradiol (2, 4 or 16 OHE2); 2 or 4-methoxyestrone (2 or 4-MeOE1); 2 or 4-methoxyestadiol (2 or 4-MeOE2); Not detected (ND); retention time (Rt)

Since this test was performed in early 2016, a number of non-derivatised methods using ammonium formate in negative MS mode have been published and discussed in conferences, showing improved signal response of non-derivatised estradiol. Therefore, this may be an alternative method to boost the detection of non-derivatised estrogens, which should be tested for the metabolite panel.

2.4.1.3 MS tuning and fragmentation of Estrogen – MPPZ Derivatives

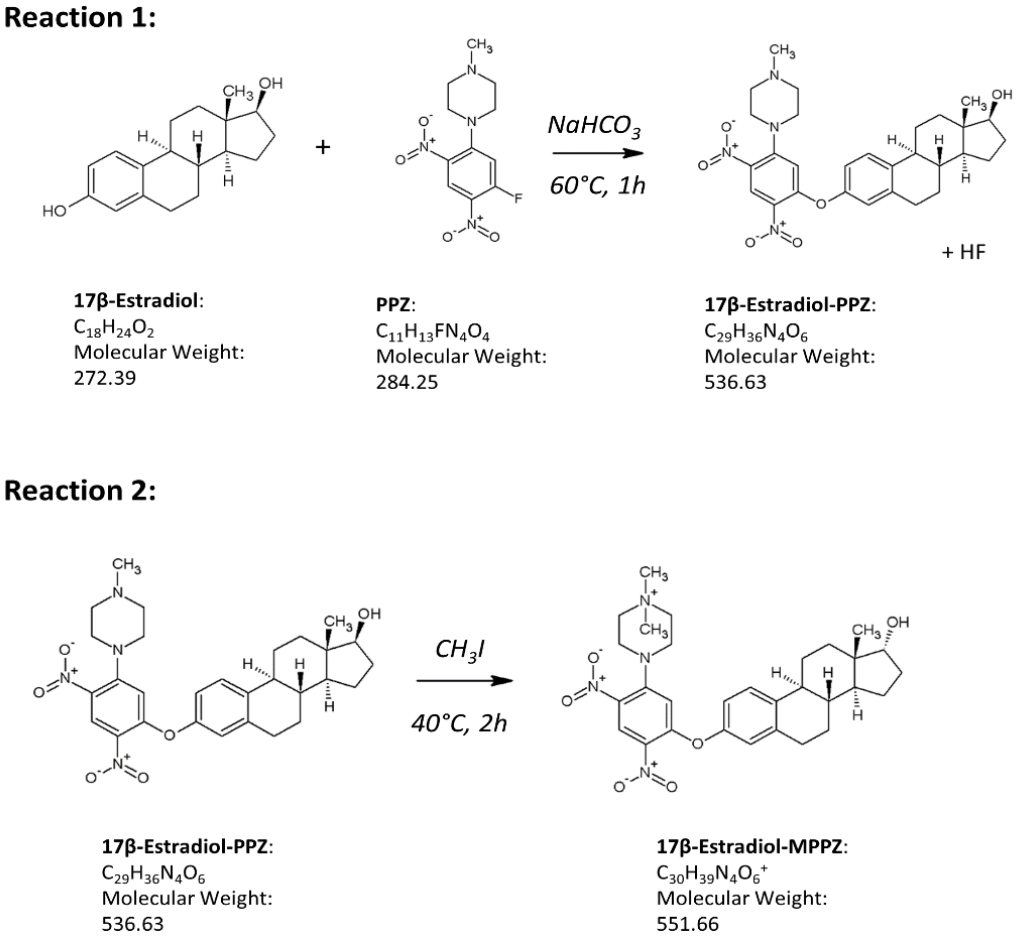

Figure 2-4: Formation of methylpiperazino derivatives of phenolic estrogens Example of 17β-estradiol with 1-5-fluoro-2,4-dinitrophenyl)-4-methylpiperazine (PPZ) followed by reaction with methyl iodide (CH_3I) to form the charged moiety

All estrogen metabolites possess phenolic functional groups that are potential targets for derivatisation by aromatic neutrophilic substitution, with additional ketone and/or hydroxyl groups present for estrone and estradiol metabolites, respectively. The phenolic group in the 3' position of the A ring is most suitable for derivatisation of the entire metabolite panel, allowing clear mass distinction between steroids with ketones (estrone metabolites) and hydroxyls (estradiol metabolites) in the 17' position of the D Ring. Figure 2-4 illustrates the two-step MPPZ reaction, with 17β-estradiol allowing formation of a permanently positively charged derivative. PPZ is a derivatisation agent particularly well suited to steroid analysis by LC-ESI-MS/MS and has been applied to alternative matrices such as serum from pregnant women for commonly analysed E1 and E2, but not for the bioactive metabolites (Nishio *et*

al., 2007). It reacts rapidly and specifically with phenols to give 3-O-[2,4-dinitro]-5-(4-methylpiperazino) phenylestrogens. A permanent positive charge is then appended by subsequent quaternarisation of the piperazine amino group using methyl iodide (Higashi and Shimada, 2004). Intense precursor and product ion signals in ESI-MS were observed, with the anticipated molecular ions of a singly-derivatised species shown in Table 2-1 which formed for all standards tested. Fragmentation patterns of all estrogen-MPPZ derivatives (Figure 2-5) were interrogated by accurate mass spectrometry and putative structures assigned, (Table 2-3) with measured masses within 10 ∆ppm of their theoretical values except in a small number of cases where signal intensity of fragments was low. Analyte-specific product ions were assigned for E1 and E2, although, certain metabolites generated similar fragments through the loss of the derivative function. Quantifier and qualifier ions were assigned, but the common fragments produced by some of the metabolites led to an absolute need for chromatographic separation between isotopologues and isobars.

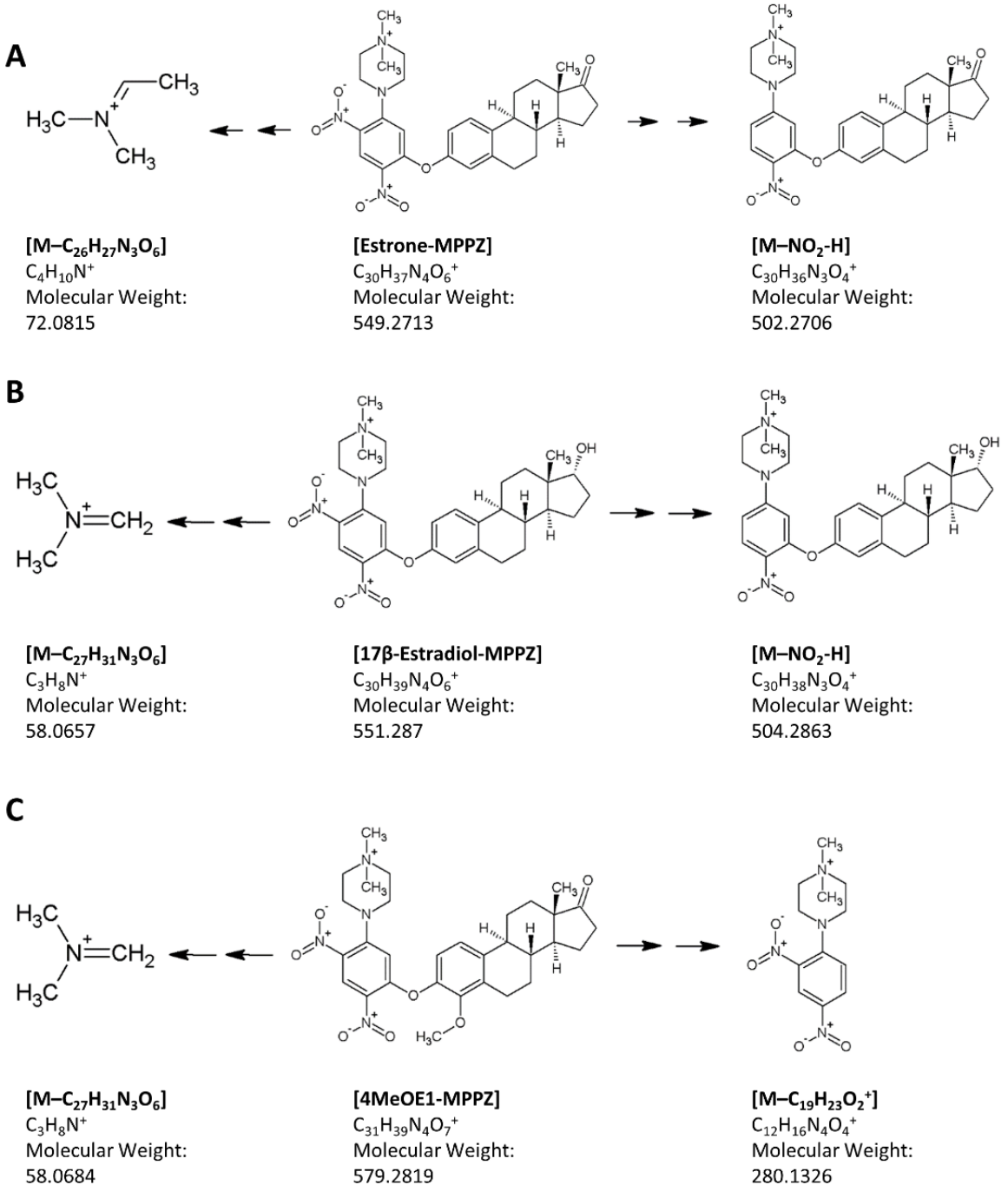

Figure 2-5: Proposed fragmentation patterns following accurate mass analysis Estrogen methylpiperazino derivative fragments assessed for (A) estrone (B) 17β-estradiol and (C) 4-methoxyestrone infused at 1 µg/mL

Table 2-3: Liquid Chromatograpy- Tandem Mass Spectrometry parameters for estrogen-MPPZ derivatives, Optimized tuning conditions for analysis of methylpiperazino derivatives of estrogen metabolites

Analyte-MPPZ	Accurate Mass Precursor Ion	Molecular Formula Precursor Ion	Theoretical Precursor Ion Mass	Observed Product Ion Mass	Proposed Product Ion Molecular Formula	Product Ion Δ ppm
E1	549.2713	$C_{30}H_{37}N_4O_6$	[a]502.27	[a]502.2706	$C_{30}H_{36}N_3O_4$	0.04
			[b]72.08	[b]72.0815	$C_4H_{10}N$	2.77
17αE2	551.2870	$C_{30}H_{39}N_4O_6$	[a]504.29	[a]504.2863	$C_{30}H_{38}N_3O_4$	0.19
			[b]58.07	[b]58.0657	C_3H_8N	1.72
17βE2	551.2870	$C_{30}H_{39}N_4O_6$	[a]504.29	[a]504.2863	$C_{30}H_{38}N_3O_4$	0.19
			[b]58.066	[b]58.0657	C_3H_8N	1.72
2OHE1	565.2662	$C_{30}H_{37}N_4O_7$	[a]251.13	[a]251.1276	$C_{12}H_{17}N_3O_3$	2.78
			[b]58.07	[b]58.0651	C_3H_8N	8.61
4OHE1	565.2662	$C_{30}H_{37}N_4O_7$	[a]251.13	[a]251.1274	$C_{12}H_{17}N_3O_3$	0.39
			[b]58.07	[b]58.0673	C_3H_8N	29.27*
16OHE1	565.2662	$C_{30}H_{37}N_4O_7$	[a]58.07	[a]58.0657	C_3H_8N	1.72
			[b]250.12	[b]250.1184	$C_{12}H_{16}N_3O_3$	2.79
2OHE2	567.2819	$C_{30}H_{39}N_4O_7$	[a]251.13	[a]251.1274	$C_{12}H_{17}N_3O_3$	1.99
			[b]281.12	[b]281.1252	$C_{12}H_{17}N_4O_4$	1.06
4OHE2	567.2819	$C_{30}H_{39}N_4O_7$	[a]251.13	[a]251.1265	$C_{12}H_{17}N_3O_3$	1.59
			[b]281.12	[b]281.1251	$C_{12}H_{17}N_4O_4$	0.71
16OHE2	567.2819	$C_{30}H_{39}N_4O_7$	[a]58.07	[a]58.0655	C_3H_8N	1.72
			[b]250.12	[b]250.1187	$C_{12}H_{16}N_3O_3$	1.59
2MeOE1	579.2819	$C_{31}H_{39}N_4O_7$	[a]58.07	[a]58.0661	C_3H_8N	8.61
			[b]280.12	[b]280.1170	$C_{12}H_{16}N_4O_4$	0.35
4MeOE1	579.2819	$C_{31}H_{39}N_4O_7$	[a]280.12	[a]280.1209	$C_{12}H_{16}N_4O_4$	13.56*
			[b]58.07	[b]58.0652	C_3H_8N	6.88
2MeOE2	581.2975	$C_{31}H_{41}N_4O_7$	[a]250.12	[a]250.1176	$C_{12}H_{16}N_3O_3$	5.99
			[b]58.07	[b]58.0639	C_3H_8N	29.28*
4MeOE2	581.2975	$C_{31}H_{41}N_4O_7$	[a]250.12	[a]250.1190	$C_{12}H_{16}N_3O_3$	0.39
			[b]58.07	[b]58.0652	C_3H_8N	6.88
$^{13}C_3$E1	552.2814	$^{13}C_3C_{27}H_{37}N_4O_6$	[a]505.28	[a]505.2806	$^{13}C_3C_{27}H_{36}N_3O_4$	0.00
			[b]388.20	[b]388.2017	$^{13}C_3C_{22}H_{25}N_2O_2$	0.00
$^{13}C_3$E2	554.297	$^{13}C_3C_{27}H_{39}N_4O_6$	[a]507.30	[a]507.2970	$^{13}C_3C_{27}H_{38}N_3O_4$	1.37
			[b]390.22	[b]390.2183	$^{13}C_3C_{22}H_{27}N_2O_2$	2.56
$^{13}C_6$4OHE1	571.2864	$^{13}C_6C_{24}H_{37}N_4O_7$	[a]251.13	[a]251.1268	$C_{12}H_{17}N_3O_3$	0.39
			[b]58.07	[b]58.0661	C_3H_8N	1.72
$^{13}C_6$2OHE2	573.3020	$^{13}C_6C_{24}H_{39}N_4O_7$	[a]251.13	[a]251.1269	$C_{12}H_{17}N_3O_3$	0.00
			[b]281.12	[b]281.1251	$C_{12}H_{17}N_4O_4$	0.71
$^{13}C_3$16αOHE2	570.2919	$^{13}C_3C_{27}H_{39}N_4O_7$	[a]58.066	[a]58.0655	C_3H_8N	1.72
			[b]72.08	[b]72.0812	$C_4H_{10}N$	1.39
$^{13}C_6$2MeOE1	585.3020	$^{13}C_6C_{25}H_{39}N_4O_7$	[a]58.07	[a]58.0661	C_3H_8N	8.61
			[b]280.12	[b]280.1163	$C_{12}H_{16}N_4O_4$	2.85
$^{13}C_6$4MeOE2	587.3177	$^{13}C_6C_{25}H_{41}N_4O_7$	[a]58.07	[a]58.0655	C_3H_8N	1.72
			[b]280.12	[b]280.1163	$C_{12}H_{16}N_4O_4$	2.85

*Fragments with low signal intensity following infusion were associated with higher ppm values. Voltage (V); estrone (E1); estradiol (17 α/ β E2); 2, 4 or 16-hydroxyestrone (2, 4 or 16 OHE1); 2, 4 or 16-hydroxyestradiol (2, 4 or 16 OHE2); 2 or 4-methoxyestrone (2 or 4-MeOE1); 2 or 4-methoxyestadiol (2 or 4-MeOE2); 2,3,4-$^{13}C_3$-estrone ($^{13}C_3$E1); 2,3,4- $^{13}C_3$-estradiol ($^{13}C_3$E2); 4-hydroxyestrone ($^{13}C_6$-4OHE1); 13,14,15,16,17,18-$^{13}C_6$-2-hydroxyestradiol ($^{13}C_6$-2OHE2); 2,3,4-$^{13}C_3$-

estriol ($^{13}C_3$-16αOHE2); 13,14,15,16,17,18-$^{13}C_6$-2-methoxyestrone ($^{13}C_6$-2MeOE1) and 13,14,15,16,17,18-$^{13}C_6$-4-methoxyestradiol ($^{13}C_6$-4MeOE1)

The MPPZ derivatives of E1 and 17βE2 fragmented to give two specific product ions m/z 549 →502 and 551→504, Figure 2-5, by loss of NO2 (m/z 47). These masses demonstrated an increment of M+3 for their stably labelled counterparts at m/z 505.3 and m/z 507.3, respectively, supporting the presence of the steroidal A ring. Smaller fragments of m/z 72 and m/z 58 were also formed, lacking stable isotope functions and common across several of the metabolite derivatives. These result through loss of [$M^-C_4H_{10}N^+$] and [$M^-C_3H_8N^+$] respectively from the methylated piperazine structure (Figure 2-5). For methoxyestrogens, product ions of m/z 280 and 250 were observed, again without mass increments in the labelled species and were thus less specific for analytes. The difference of unit mass between the fragment ions of m/z 250/251 and m/z 280/281 between methoxy and hydroxy metabolites respectively suggests a block to proton migration from the 2' or 4' position of estrogen due to the methylated group.

In addition, a number of dominant product ions were detected using high resolution mass spectrometry which were not seen in original fragmentation analysis by the Quantum Instrument (Table 2-4). These breakdown patterns might be useful for future directions, as discussed in Chapter 9 section 9.2.1.

Table 2-4: Dominant product Ions determined by high resolution mass spectrometry

Estrogen-MPPZ	Parent ion *m/z*	Current Product Ion *m/z*	High Resolution Product ions *m/z*
16OHE1	565.266	58.0	518.27
16OHE2	567.282	58.0	520.28
2MeOE1	579.282	58.0	532.28
2MeOE2	581.298	250.0	534.00
4MeOE1	579.282	280.0	532.00
4MeOE2	581.298	250.0	534.00
Catechol Estrogens			
2OHE1	565.266	251.1	281.00 + 524.00
4OHE1	565.266	251.1	281.00 + 518.00
2OHE2	567.282	251.1	520.00
4OHE2	567.282	251.1	520.00
Internal Standards			
$^{13}C_6$4OHE1	571.286	251.1	281.00 + 518.00
$^{13}C_6$2OHE2	573.302	251.1	526.00
$^{13}C_3$16OHE2	570.292	58.1	523.29
$^{13}C_6$2MeOE1	585.302	58.1	538.30
$^{13}C_6$4MeOE2	587.318	58.1	540.00 + 250.00

2, 4 or 16-hydroxyestrone (2, 4 or 16 OHE1); 2, 4 or 16-hydroxyestradiol (2, 4 or 16 OHE2); 2 or 4-methoxyestrone (2 or 4-MeOE1); 2 or 4-methoxyestadiol (2 or 4-MeOE2); 2,3,4-$^{13}C_3$-estrone ($^{13}C_3$E1); 2,3,4- $^{13}C_3$-estradiol ($^{13}C_3$E2); 4-hydroxyestrone ($^{13}C_6$-4OHE1); 13,14,15,16,17,18-$^{13}C_6$-2-hydroxyestradiol ($^{13}C_6$-2OHE2); 2,3,4-$^{13}C_3$-estriol ($^{13}C_3$-16αOHE2); 13,14,15,16,17,18-$^{13}C_6$-2-methoxyestrone ($^{13}C_6$-2MeOE1) and 13,14,15,16,17,18-$^{13}C_6$-4-methoxyestradiol ($^{13}C_6$-4MeOE1)

2.4.1.4 Optimization of MPPZ Reaction

The efficiency of reaction one was not improved by alterations in reaction time, temperature, reagent volume, concentration and pH. However, in reaction two, the methylation reaction which quaternises PPZ-estrogen derivatives to form the charged MPPZ moiety was amenable to improvement. Reaction efficiency and reproducibility was improved by increasing reaction time (30 to 120 min) and decreasing the reaction temperature (60 to 40°C) (Figure 2-6).

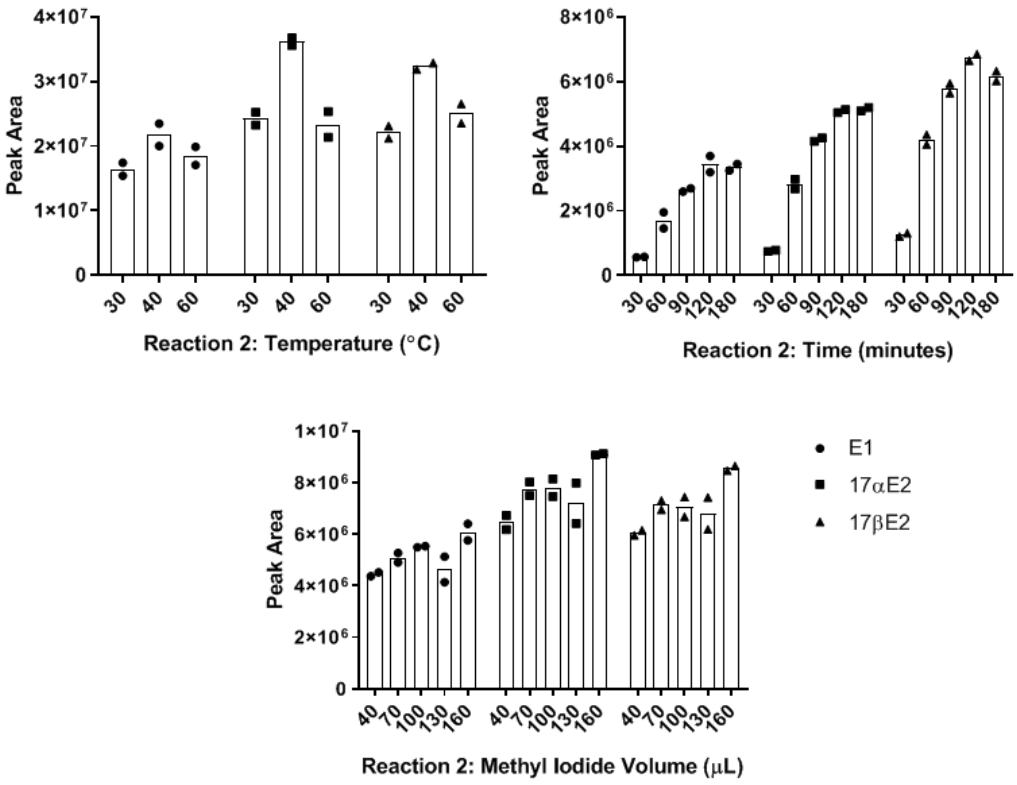

Figure 2-6: Optimisation of the reaction with methyl iodide Effect of altering reaction temperature, time and volume of methyl iodide on the reaction efficiency. Data only shown for the main circulating estrogens as the pattern was similar for metabolites including the catechol estrogens. Data displayed as Mean bars with data points. N=2 duplicates.

Following the initial observation that temperature and time impacted the overall reaction efficiency, the impact of changing the reaction conditions to 40ºC temperature and 120 minutes reaction time on the signal and reproducibility were tested more robustly in n=6 samples.

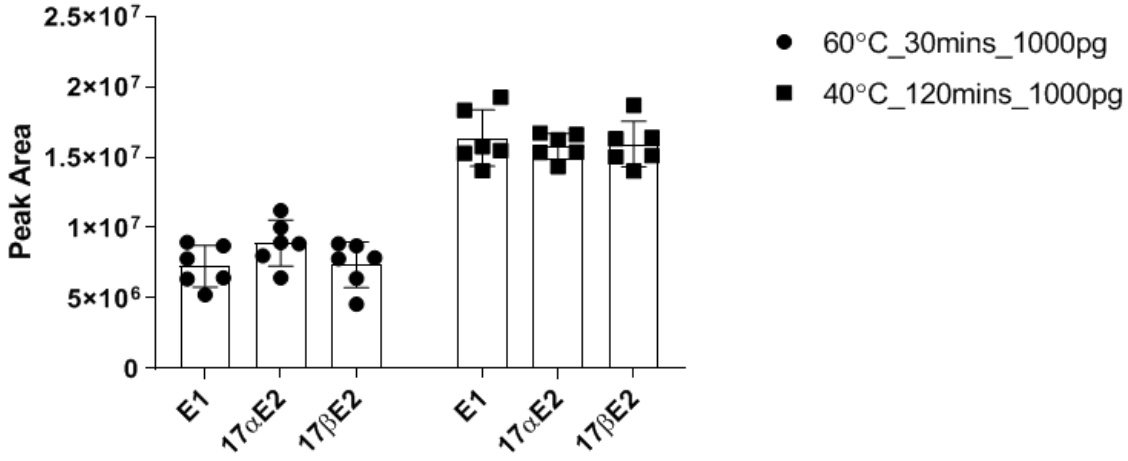

Figure 2-7: Improvement in efficiency of reaction with methyl iodide Optimisation of reaction two improved the signal intensity of the estrogen-MPPZ derivative. Data only shown for the main circulating estrogens as the pattern was similar for metabolites including the catechol estrogen reactions. Data displayed as Mean ± SEM. N=6 replicates.

This test confirmed signal shape, intensity and stability of derivatives were improved by modification of reaction two conditions. Following this, the response of estrogen-MPPZ derivative signals was additionally improved by optimisation of the composition of the reconstitution solution for transfer to LC vials. Samples in mobile phase of higher organic proportions (70:30 H_2O:CH_3CN) improved signal intensities, most likely through increased solubilisation of the derivative. Of note, the use of plastic 96-well microtitre plates for the analysis of MPPZ-estrogen derivatives was attempted to improve throughput but not pursued or recommended, as when, used inconsistent signal responses for spiked samples were observed and derivative stability was poorer.

2.4.1.5 Optimisation for Catechol Estrogens

Upon lowering catechol estrogen concentrations below 500 pg/mL signals intensities became low and difficult to detect. For catechol metabolites double peaks were present upon chemical derivatisation, presumably due to isomeric mono derivatisation; this occurs as derivatisation may progress at both hydroxyl groups in the aromatic A ring 2 and 4' position (Figure 2-8).

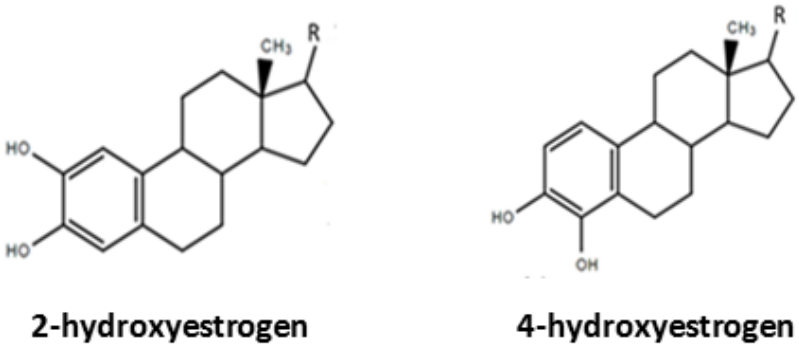

Figure 2-8: Catechol Estrogen Structure has two potential sites of derivatisation Catechol estrogens produced via hydroxylation in the 2' or 4' aromatic A ring position of estrone (R represents a ketone) and estradiol (R represents a hydroxyl group) respectively.

Structurally, positioning of hydroxyl groups creates steric hindrance of the 3' hydroxyl group. At lower estrogen concentrations this effect could be more pronounced causing a lack of reactivity. Therefore, optimisation of reaction conditions to favour single derivatisation on the A-ring was attempted. Initially, a number of reaction bases were tested to determine if this affected the reaction efficiency (Figure 2-9).

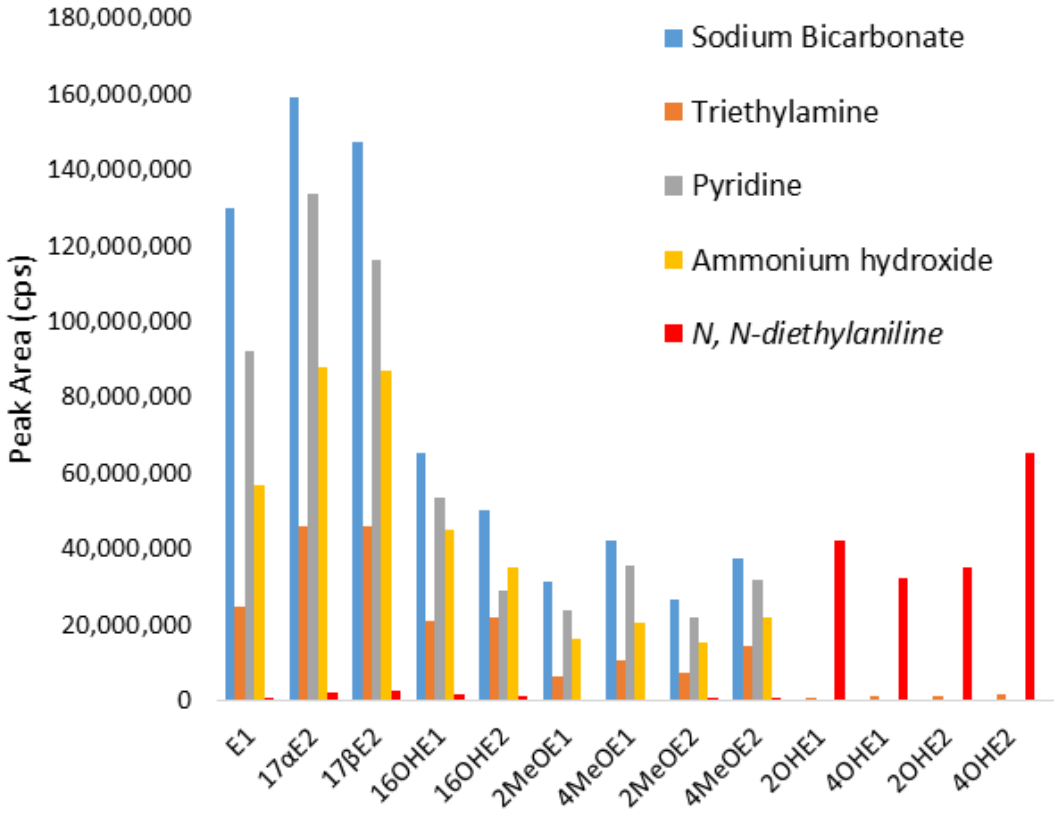

Figure 2-9: Screen of base catalysts to derivatise catechol estrogens Selection of a base catalyst to improve reaction efficiency. Screening of sodium bicarbonate (blue), trimethylamine (orange), pyridine (grey), ammonium hydroxide (yellow) and *N, N*-diethylaniline (red) shows the catechol metabolites are derivatised more efficiently in the presence of *N*-diethylanaline whereas the remaining panel efficiently react in the presence of sodium bicarbonate

Of these base catalysts, *N, N*-diethylaniline (*N,N*-DEA) proved to be the most efficient in derivatising the catechol estrogen metabolites. This boost in reaction efficiency was only observed for the catechol metabolites, not the alternative panel of nine estrogens (Figure 2-9). A clear trend in the reactivity of the panel of nine estrogens and the catechol metabolites can be observed under reaction with PPZ. Chromatographically the boost in detection was clearly apparent upon comparison of the original reaction conditions with $NaHCO_3$ to those using *N,N*-DEA. Derivatisation using this method allowed a limit of detection of 20 pg/mL in unextracted standard solutions. Conditions for E1, E2, 16OHEs and MeOEs were not changed following this test. A combination of reaction base catalysts within the derivatisation also did not facilitate the reaction between group one and group two metabolites. Therefore we were forced to adopt two separate derivatisation approaches for the two groups of estrogens.

2.4.2 Chromatographic conditions

2.4.2.1 Chromatographic Separation of Nine Estrogens

Chromatographic conditions were assessed on five LC columns representing a variety of stationary phases (Acquity Atlantis T3, ACE Ultra Core Super C18, ACE Excel Super C18, ACE Excel C18-AR and ACE Excel C18-PFP, all 150 mm in length). Separation could not be achieved on the Acquity Atlantis T3, which contained C18 groups on particle sizes of 3 µm, whereas the smaller particles of 2.5 µm in the ACE Ultra Core Super C18, allowed partial but incomplete peak separation. The ACE Excel Super C18, with its unique encapsulated bonding technology and ACE Excel C18-AR, with its integrated phenyl capacity chosen for added selectivity for aromatic compounds, still did not resolve the methoxyestrogen metabolites. The ACE Excel C18, with integral pentafluorophenyl functionality added further selectivity via its π-π interactions and was the only column able to successfully resolve the methoxyestrogen metabolites alongside the wider panel, with typical retention times shown in Figure 2-10.

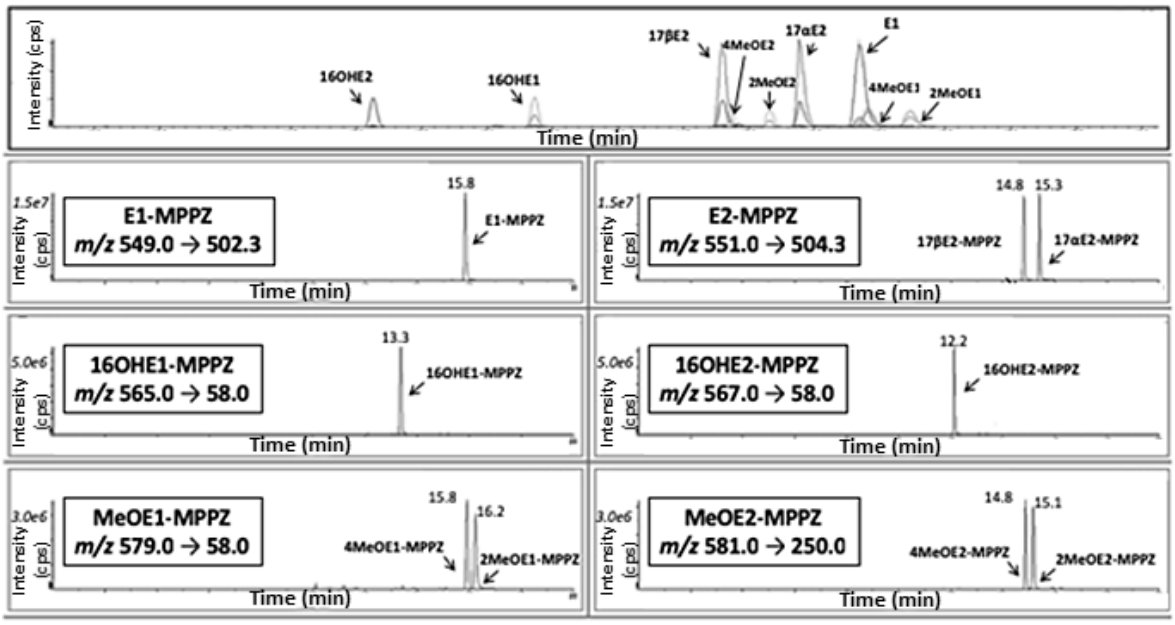

Figure 2-10: Mass chromatograms of methylpiperazino (MPPZ) derivatives of estrogens and their metabolites following analysis of an extracted solution of standards Total Ion Chromatograms and the corresponding extracted ion chromatograms showing resolution of derivatives of estrone (E1), estradiol (17α & 17βE2), 16-hydroxyestrogens (16OHE1 & 16OHE2) and methoxyestrogens (2MeOE1, 4MeOE1, 2MeOE2 & 4MeOE2) by retention time and mass transition, 1000 pg/mL. Counts per second; CPS.

2.4.2.2 Chromatographic Separation of Catechol Estrogens

Catechol estrogen metabolites were analysed both individually and in a mixed solution to confirm retention time and separation efficiency. Following optimisation of the gradient elution, oven temperature and column set-up separation was achieved for 2- and 4-OHE2 with partial separation was achieved for 2- and 4 -OHE1 (Figure 2-11).

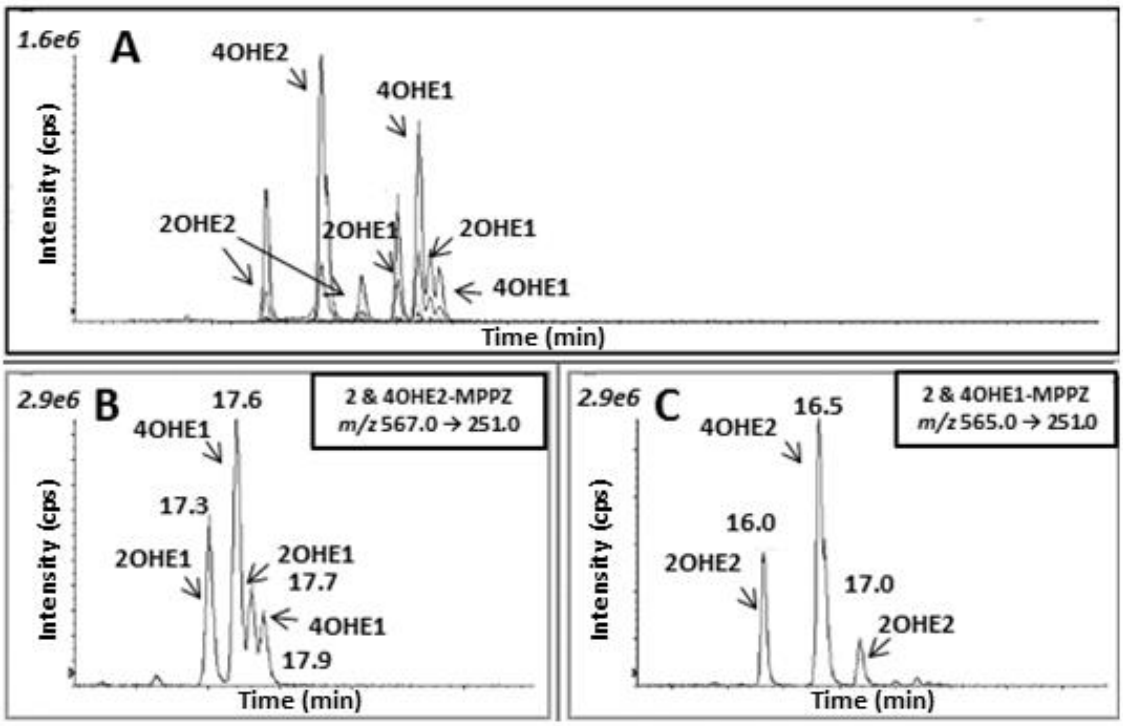

Figure 2-11: Total and extracted ion chromatograms of derivatives of catechol estrogens methylpiperazino (MPPZ) derivatives of catechol estrogens. (B) The estrone metabolites 2-hydroxyestrone (2OHE1), 4-hydroxyestrone (4OHE1) and (C) the estradiol metabolites 2-hydroxyestradiol (2OHE2), 4-hydroxyestradiol (4OHE2) at 1000 pg/mL. Figure illustrating challenges in separating catechol metabolites by mass transitions and retention time (minutes). Counts per second; CPS.

2.4.3 Extraction efficiency

It became apparent via initial tests that the same extraction approach could not be used for the panel of nine estrogens and the catechol estrogen metabolites. Therefore, the extraction of nine metabolites and the catechol estrogens were assessed separately, applying the respective derivatisation protocols discussed in section 1.5.1.5.

2.4.3.1 Recovery of Nine Estrogens from plasma and assessment of Matrix Effects

Liquid-liquid extraction of plasma has been applied to recover estrogens (e.g. using dichloromethane or ethyl acetate) however these have generally given inconsistent results with poor recovery (Wehner *et al*, 1979; Wooding *et al*, 2015). Supported Liquid extraction (SLE) is fairly new in its application to biological samples with few reported methods in the literature for estradiol. One paper emerged showing low limits of 1 pg/mL for estrone and estradiol whilst 16OHE2 limits were higher at 30 pg/mL (Wooding *et al.*, 2015). This suggests for low level estrogen metabolites, the extraction method lacks the clean-up options offered by SPE for removal of phospholipids and salts, which may interfere with the derivatisation reaction and LC-MS/MS quantification. After consideration, Oasis HLB and Oasis MCX cartridges were selected for comparison. HLB columns are suitable for a wide range of acidic, basic and neutral analytes whereas MCX cartridges offer a more selective column, with ion exchange capability, which remains stable in organic solvents. Recovery of estrogen and metabolites using Oasis® MCX separation columns gave a high recovery (93 - 108%) of 9 target metabolites, justifying its choice for further method development.

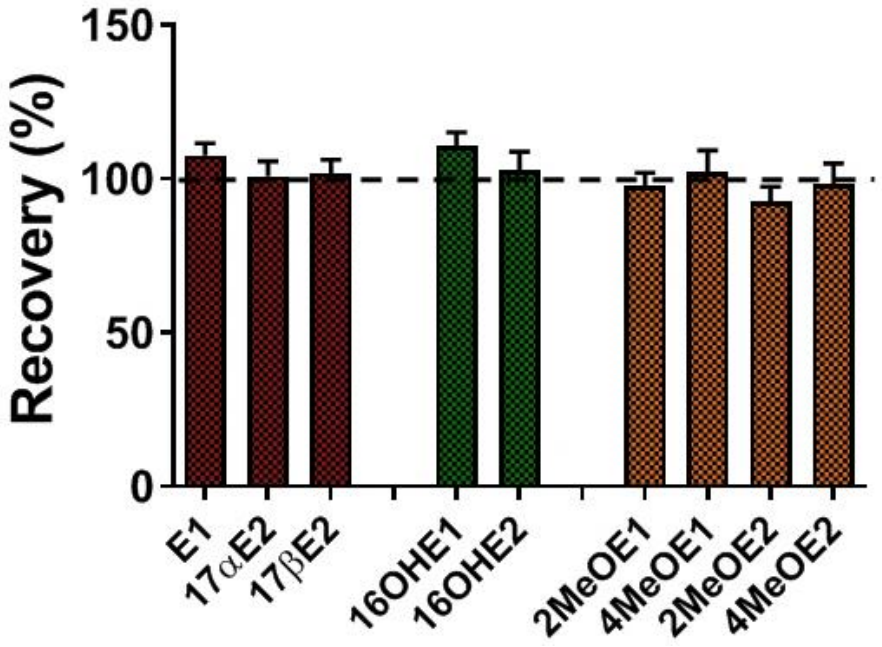

Figure 2-12: Recovery of nine estrogens from plasma Using the MCX generic extraction protocol a high recovery of nine estrogens was achieved. Detection was conducted following derivatisation with MPPZ. Data displayed as Mean ± SEM. N=6 replicates

Upon further testing, significant ion suppression was identified in the extract suggesting a substantial matrix effect. Subsequently, ion suppression was minimised by testing a further cartridge wash step (0 to 100% methanol, Table 2-5) and evaluation of the elution composition (0 to 100% methanol, Table 2-6).

Table 2-5: Optimisation of wash step with methanol for nine estrogens Percentage estrogen signal recovered following 0 – 100% methanol wash in extraction.

	Estrogen Signal Abundance, n=2 (%)								
% Methanol Wash	**E1**	**17αE2**	**17βE2**	**16OHE1**	**16OHE2**	**2MeOE1**	**4MeOE1**	**2MeOE2**	**4MeOE2**
No Wash	*37*	*37*	*29*	*38*	*31*	*44*	*44*	*41*	*42*
0	21	21	26	13	17	23	18	21	20
5	151	170	176	123	140	144	145	151	173
10	151	166	196	120	144	54	152	138	186
15	136	142	223	128	67	35	74	89	95
20	144	161	179	109	116	53	122	124	147
25	43	37	54	33	31	20	37	38	42
30	**100**	**86**	**114**	**69**	**67**	**106**	**94**	**98**	**103**
35	81	86	114	67	93	50	56	66	85
40	85	75	75	40	42	41	54	60	60
45	104	117	143	79	95	98	105	106	141
50	15	17	25	12	16	8	14	14	18
55	163	186	207	126	150	172	175	160	200
60	136	166	171	90	71	85	155	114	171
65	86	89	111	15	9	49	83	59	96
70	27	25	39	3	2	13	19	11	23
75	23	5	34	4	3	13	17	10	17
80	6	4	6	2	1	4	3	2	2
85	3	1	2	1	1	2	1	1	1
90	3	2	3	2	1	1	2	1	2
95	3	1	3	2	1	1	1	0	1
100	3	0	1	1	0	1	0	0	0

Estrone (E1); estradiol (17 α/ β E2); 16-hydroxyestrone (16 OHE1); 16-hydroxyestradiol (16 OHE2); 2 or 4-methoxyestrone (2 or 4-MeOE1); 2 or 4-methoxyestadiol (2 or 4-MeOE2).

Interestingly, cleaning the column cartridge with a second wash altered the estrogen profile significantly with estrogens beginning to be washed off the column at 70 – 100% (Table 2-5). Addition of a 30% methanol wash optimal wash step reduced ion suppression to an improved level for the nine estrogens. Next, the elution profile was tested between 55 – 100% to determine if the 16-hydroxyestrogen signal could

be improved whilst maintaining the signal of the main circulating estrogens and the methoxyestrogens (Table 2-6).

Table 2-6: Optimisation of elution with aqueous methanol for the nine estrogens Percentage estrogen signal recovered following 55 – 100% methanol elution in extraction.

	Estrogen Signal Abundance, n=2 (%)								
% Methanol Elution	**E1**	**17αE2**	**17βE2**	**16OHE1**	**16OHE2**	**2MeOE1**	**4MeOE1**	**2MeOE2**	**4MeOE2**
55	1	1	1	6	22	0	0	0	0
60	1	1	1	6	22	0	0	0	0
65	1	1	1	6	22	25	17	38	29
70	43	69	61	77	65	72	77	76	89
75	167	219	228	129	119	176	179	169	218
80	179	214	229	119	88	167	178	135	212
85	134	161	141	84	83	123	149	107	151
90	84	85	101	43	42	85	87	60	90
95	169	199	196	93	96	155	192	132	182
100	**99**	**110**	**107**	**72**	**70**	**103**	**95**	**94**	**97**

Estrone (E1); estradiol (17 α/ β E2); 2 or 4 or 16 -hydroxyestrone (2 or 4 or 16 OHE1); 2 or 4 or 16 -hydroxyestradiol (2 or 4 or 16 OHE2); 2 or 4-methoxyestrone (2 or 4-MeOE1); 2 or 4-methoxyestadiol (2 or 4-MeOE2).

Upon testing, the 100% methanol wash remained the only condition not causing significant signal enhancement for estrone, estradiol and the methoxyestrogens. Of note, alternative conditions for 16OHE1 and 16OHE2, applying a 30% methanol wash and 95% methanol elution, reduced ion suppression by 21% and 26%, respectively. This, however, negatively influenced the other metabolites, which demonstrated a significant boost in signal for estrone, estradiol and methoxyestrogen metabolites (E1 169%, αE2 199%, βE2 196%, 2MeOE1 155%, 4MeOE1 192%, 2MeOE2 132% and 4MeOE2 182%). Therefore, these conditions were deemed unsuitable for this panel of metabolites. The suppression of the 16-hydroxyestrogens was accepted, as internal standards could correct the quantitative approach. Therefore, those compositions with the lowest ion suppression were chosen for the second wash (30% methanol) and elution (100% methanol). Upon testing in six replicates, ion suppression of derivatives was reduced significantly for all analytes compared to initial conditions (Figure 2-13).

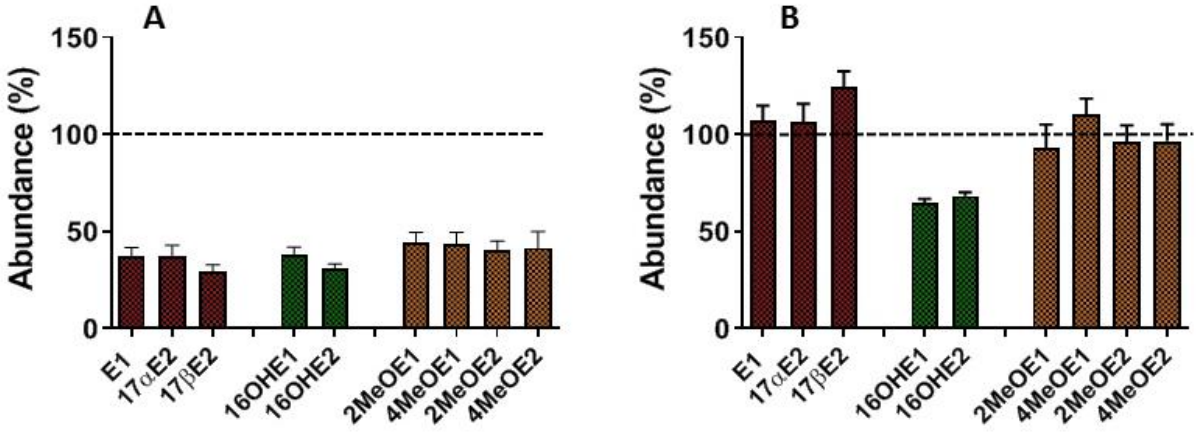

Figure 2-13: Reduction of extraction ion suppression Abundance of estrogen signals using the generic extraction protocol (A) and the optimised protocol including an additional 30% methanol wash and 100% methanol elution (B). Data displayed as Mean ± SEM. N=6 replicates

2.4.3.2 Recovery and Matrix Effect for Catechol Metabolites

Application of the MCX extraction approach for catechol estrogens became challenging. Initial attempts applying the above mentioned protocol, in conjunction with the *N*-*N*-DEA base, produced lower but acceptable recovery rates of 70-75% (Figure 2-14).

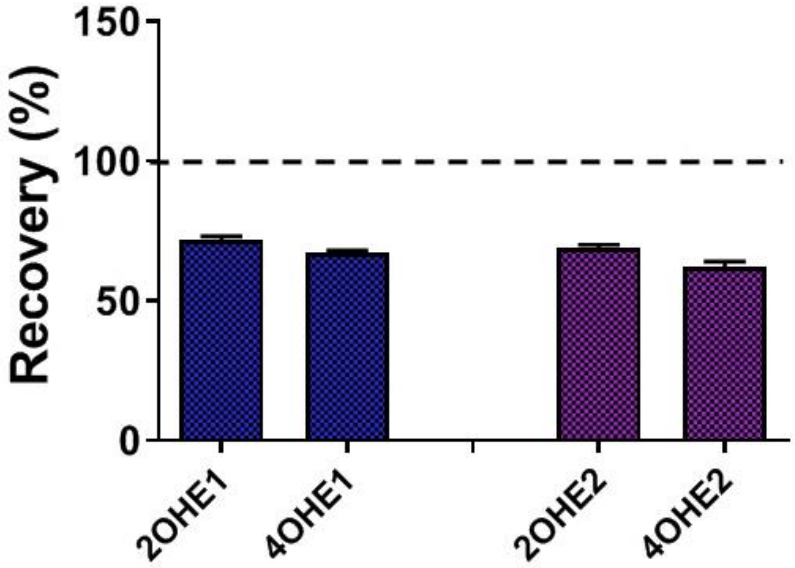

Figure 2-14: Recovery of catechol estrogens from plasma Using MCX generic extraction protocol an acceptable recovery of catechol estrogens of interest was achieved applying MPPZ-with *N,N*-DEA derivatisation. Data displayed as Mean ± SEM. N=3 replicates

Unfortunately, significant ion suppression was detected for all metabolites, which would deter the quantitative analysis. Therefore, to improve this, a similar extraction optimisation process was applied, as with the batch of nine metabolites with an additional clean-up step utilising 0-70% methanol (Table 2-7). Also, on this occasion, the slightly stronger organic solvent acetonitrile was tested at the same percentages.

Table 2-7: Optimisation of the wash step with methanol and acetonitrile for catechol estrogens Percentage estrogen signal abundance following additional wash steps with 0 – 100% methanol and acetonitrile during extraction.

	Catechol Estrogen Signal Abundance, n=2 (%)			
% Methanol Wash	**2OHE1**	**4OHE1**	**2OHE2**	**4OHE2**
No Wash	*7*	*6*	*7*	*6*
0	6	4	5	4
10	5	4	7	5
20	1	1	2	1
30	6	5	7	5
40	12	10	13	10
50	16	14	17	13
60	**20**	**22**	**22**	**21**
70	1	1	1	1
% Acetonitrile Wash	**2OHE1**	**4OHE1**	**2OHE2**	**4OHE2**
0	19	11	20	13
10	20	12	21	15
20	14	7	16	11
30	**20**	**12**	**23**	**14**
40	3	4	10	11
50	1	1	2	1
60	0	0	1	1
70	1	0	2	1

2 or 4,-hydroxyestrone (2 or 4 -OHE1); 2 or 4 -hydroxyestradiol (2 or 4 -OHE2).

The washes that gave the highest signal intensities and lowest ion suppression in plasma were 60% methanol wash (2OHE1 80.5%, 4OHE1 77.9%, 2OHE2 79.9% & 4OHE2 79.7%) and 30% ACN wash (2OHE1 81%, 4OHE1 79%, 2OHE2 81% & 4OHE2 83%) but they were not able to decrease ion suppression to below 80%. As both wash steps displayed similar intensities, the 60% methanol wash and 30% acetonitrile were then used prior to testing a 70-100% methanol/acetonitrile elution

(Table 2-8). This step aimed to identify if alteration of the organic composition could encourage elution of additional catechol estrogens from the column sorbent whilst avoiding interference with phospholipids.

Table 2-8: Methanol and acetonitrile elution optimisation for catechol estrogens Percentage estrogen signal abundance following 70 – 100% methanol or acetonitrile elution in extraction.

	Catechol Estrogen Signal Abundance, n=2 (%)			
60% Methanol Wash				
% Methanol Elution	**2OHE1**	**4OHE1**	**2OHE2**	**4OHE2**
50	0	0	0	0
60	0	0	0	0
70	1	1	1	1
80	0	0	1	0
90	8	2	11	7
100	15	5	22	17
30% Acetonitrile Wash				
% Acetonitrile Elution	**2OHE1**	**4OHE1**	**2OHE2**	**4OHE2**
50	15	22	12	11
60	10	26	16	14
70	16	14	16	18
80	12	13	9	11
90	10	15	14	13
100	10	13	13	11

2, 4, -hydroxyestrone (2, 4 -OHE1); 2, 4 -hydroxyestradiol (2, 4 -OHE2).

Again, these alterations did not decrease ion suppression sufficiently with a significant ion suppression remaining in plasma extracts. Therefore, detection of catechol metabolites in clinical samples would be hindered by lowered signal (Figure 2-15).

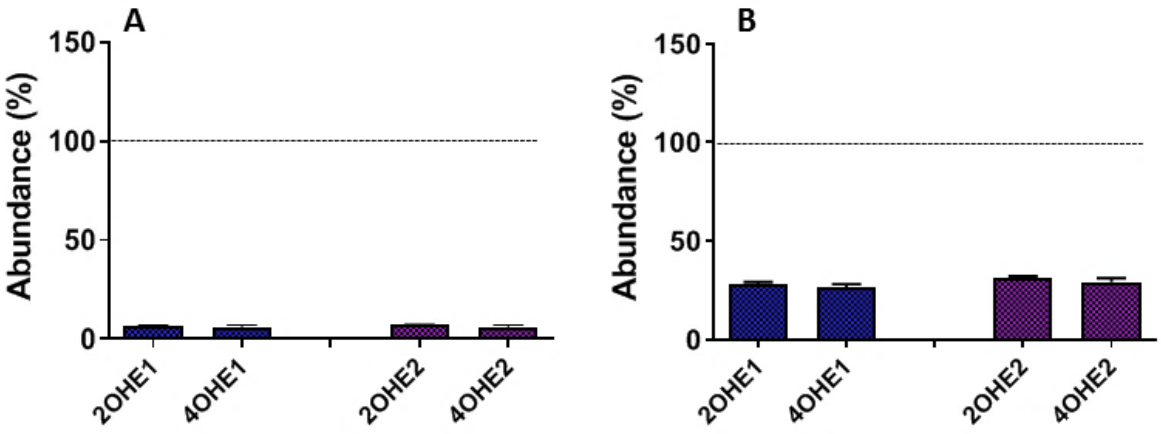

Figure 2-15: Suppression of signals of catechol estrogens in plasma extracts Using the MCX generic extraction protocol a high degree of suppression was present (A) Upon optimisation suppression was reduced only by 10-15% (B), with detection achieved by applying the MPPZ-*N,N*-DEA derivatisation. Data displayed as Mean ± SEM. N=3 replicates

As a result, an alternative extraction protocol for use alongside the modified MPPZ-*N,N*-DEA derivatisation for catechol metabolites is required but this was not pursued.

2.4.4 Future Directions for Catechol Estrogens

For inclusion of catechol estrogen metabolites (2OHE1, 2OHE2, 4OHE1 & 4OHE2) in our LC-MS/MS workflow, an alternative extraction protocol should be tested. A high degree of ion suppression was present for the four metabolites using Oasis MCX SPE, with no improvement following adjustment of wash and elution steps. In the literature, catechol metabolites have been successfully extracted using LLE approaches with one reported SPE method using Chromabond C8 columns (Chapter 1, Table 1-5). Due to the manual aspect of LLE and the potential for easier automation of SPE in the future, an SPE or alternative column-based approach such as SLE would be preferential. Bond Elut C18 cartridges may provide additional retention of non-polar estrogens with application of alternative sample clean-up. Also, from unpublished conference posters a number of potential extraction methods have appeared. Biotage Evolute ABN extraction may be a promising strategy for extraction of the catechol metabolites. This extraction was tested at high concentrations (5000 pg/mL) from cell media applying an ethyl acetate elution (L. Gilligan et al, BMSS 2019). If an alternative SPE approach were adopted, newer

columns which avoid the need for column conditioning would be tested to improve sample preparation time and reduce the use of high solvent volumes within the laboratory. Another alternative may be SLE extraction, using load-wait-elute workflows such as Biotage Isolute SLE providing protein and phospholipid free extracts to improve ion suppression. These columns remove the need for additional clean-up steps due to the water immiscible solvents employed which efficiently exclude interference from the final eluate. In general this approach is less widely applied for estrogen analysis but would provide a worthwhile comparison due to promising results for extraction of E2 (Table 1-5). Conference posters from unpublished abstracts at MASCL 2018 have applied ISOLUTE SLE+ with a 75/25 ethyl acetate and hexane elution for non-derivatised analysis of E1, E2 and E3. This method reported 95-99% recovery with minimal ion suppression from plasma extractions and LOQs of 0.5, 1 and 5 pg/mL for E1, E2 and E3 respectively. In the LC method, ammonium fluoride (NH_4F) was added to the mobile phase to promote negative ion formation representing a key addition for a number of non-derivatised E2 methods discussed during MSACL 2018. These options could be explored for the catechol metabolites alongside the panel of nine estrogens discussed in Chapter 2.

Another factor to consider is the relatively short half-life of the catechol metabolites in plasma, reported to be ~90 minutes (Austin *et al.*, 2013). Attempts to prevent oxidation during the sample preparation by addition of ascorbic acid should also be assessed (Williamson, Van Orden and Rosazza, 1985; Stremetzne, Jaehde and Schunack, 1997), possibly enhancing detection within clinical plasma samples.

Derivatisation of the catechol metabolites also raised a number of issues upon lowering concentrations of aqueous standards below 500 pg/mL. These were overcome by a number of optimisation experiments, specifically the use of *N*-diethylaniline as a base catalyst in the PPZ reaction, allowing sufficiently low level detection in aqueous standards (Section 2.4.1.5). These metabolites form isomeric mono-derivatives with two peaks representing derivatisation of the 3’ and 2’ or 4’ hydroxyl group of the steroid A ring. In aqueous standards, the reaction efficiency remains constant following optimisation of the time, temperature and volume but this should also be verified in plasma samples. This would ensure that the optimised

MPPZ derivatisation would be a suitable for use alongside an alternate extraction upon successful assessment of recovery and ion suppression.

2.4.5 Assay Validation

Method validation was continued for the panel of nine estrogens (E1, 17αE2, 17βE2, 16OHE1, 16OHE2, 2MeOE1, 4MeOE1, 2MeOE2 and 4MeOE2).

2.4.5.1 Specificity

Baseline chromatographic separation of estrogen derivatives was achieved using standards. When applied to plasma samples, interferences of closely eluting compounds at the retention time of each estrogen were not observed, such as 17α-E2, which displays a different retention time to 17βE2 (Figure 2-10).

2.4.5.2 Linearity and Assay Limits

Stable-isotope labelled estrogens ($^{13}C_3$ and $^{13}C_6$) were selected as internal standards, dependent on availability (Table 2-9). One labelled estrogen per chemical grouping was chosen due to cost constraints but paired internal standards are available for all analytes and are recommended. Derivatives quantified with exact labelled versions for comparison demonstrated a more accurate response, Figure 2-9. A mean R-value > 0.99 for calibration lines was achieved for all derivatives with a weighting of 1/x throughout. A linear relationship was also confirmed as the lines also passed Lack-of-fit tests (Table 2-9).

The linear range achieved is similar to that of methods quantifying E1 and E2 using other sensitive derivatives developed in the past few years for both plasma (Rothma*n et al*, 2011; Faqehi *et al*, 2016) and urinary analysis (Franke *et al.*, 2011).

Table 2-9: Limits of quantitation and linearity of response Assessment of the extraction efficiency of estrogens from plasma. The lower limits are presented with linearity demonstrated over a wide range of concentrations covering physiological levels (n=6)

Analyte	Internal Standard	LOD pg/mL (pg on column)	LOQ pg/mL (pg on column)	Mean R (n=6)	F Calculated (Critical: 3.33)
E1	$^{13}C_3$E1	0.5 (0.21)	1 (0.43)	0.998	0.08
17αE2	$^{13}C_3$E2	1 (0.43)	1 (0.43)	0.990	0.07
17βE2	$^{13}C_3$E2	1 (0.43)	1 (0.43)	0.993	0.08
16OHE1	$^{13}C_3$16OHE2	1 (0.43)	6 (1.30)	0.998	0.13
16OHE2	$^{13}C_3$16OHE2	1 (0.43)	6 (1.30)	0.996	0.10
2MeOE1	$^{13}C_6$2MeOE1	5 (2.17)	6 (1.30)	0.996	0.08
4MeOE1	$^{13}C_6$2MeOE1	5 (2.17)	6 (1.30)	0.996	0.09
2MeOE2	$^{13}C_6$4MeOE2	5 (2.17)	6 (1.30)	0.995	0.21
4MeOE2	$^{13}C_6$4MeOE2	5 (2.17)	6 (1.30)	0.996	0.09

Estrone (E1); estradiol (17 α/ β E2); 2, 4, 16-hydroxyestrone (2, 4, 16 OHE1); 2, 4, 16-hydroxyestradiol (2, 4, 16 OHE2); 2, 4-methoxyestrone (2, 4-MeOE1); 2, 4-methoxyestadiol (2, 4-MeOE2); IS = Internal Standard; LLOD/q = lowest limit of detection/quantitation; ULOQ = Upper level of quantitation If Fcalc < Fcrit then reject the null hypothesis, If Fcalc < Fcrit then reject the null hypothesis; As Fcalc < Fcrit the null hypothesis is accepted meaning there is no lack of fit in these calibration curves

The LOD and LOQ for estrogen MPPZ derivatives (Table 2-9) measured in standard solutions are comparable to previous methods for the analysis of E1 and E2 (*Nelson et al*, 2004; Huang *et al*, 2011; Gao *et al*, 2015; Li *et al*, 2015; Faqehi *et al*, 2016). The volume of plasma (0.5 mL) extracted by SPE for detection of metabolites demonstrated an improvement over recent methods for oncology studies; one study by Yang et al, 2008 used 2 mL of patient serum and a longer extraction protocol, where samples were incubated overnight prior to liquid extraction of estrogens (Yang et al, 2008). Another study applied dansyl chloride and achieved low LOQs of 0.05 – 0.1 pg on column using 200 µL plasma but required specialized equipment

not commonly available within hospitals and clinical laboratories (Kushnir *et al.*, 2008).

2.4.5.3 Accuracy, Precision and Bias

The values for intra- and inter-assay precision and accuracy (Table 2-10) were acceptable being below 20% at the LOQ (0.43 & 4.34 pg on column; 2 and 10 pg/mL) and <15% above this level (43.4 & 434.0 pg on column; 200 or 2000 pg/mL). In addition, acceptable precision was shown for endogenous E1 and E2 and for the six metabolites enriched into healthy pooled female plasma (100 pg/mL). An upward deviation was observed for 4MeOE1 which may have reflected the presence of low endogenous levels of this metabolite. Finally, to test assay bias, calibration data for E1 and E2 was compared to certified reference material (metabolites not available) (Table 2-11). Assay bias was higher at the upper LOQ (ULOQ) when a weighting of 1/x was applied, this could be corrected by the removal of weighting achieving a better fit at higher concentrations (Table 2-11). In general, levels in plasma were anticipated to be below these points. In addition, acceptable precision was shown for endogenous E1 and E2 and for the six metabolites enriched in healthy female plasma samples (100 pg/mL metabolites), except for 4MeOE1.

Table 2-10: Intra and Inter-day accuracy and precision Summary table of the accuracy and precision data showing acceptable intra-day and inter-day reproducibility. For standards, acceptance criteria were met at the LLOQ and ULOQ. For metabolites the mid concentration is shown rather than ULOQ, as this is more relevant to expected levels. For plasma, validation was performed in aliquots from a healthy individual containing endogenous estrone and estradiol and enriched with metabolites.

		Intraday (n=6)			Interday (n=6)		
Metabolite	**Target (pg/mL)**	**Mean (pg/mL)**	**Precision (RSD %)**	**Accuracy (RME %)**	**Mean (pg/mL)**	**Precision (RSD %)**	**Accuracy (RME %)**
E1	2	2.2	8.7	7.4	2.0	8.0	0.3
	2000	1282	14.0	14.1	1116	13.9	6.1
	Endogenous	37	13.3	-	31.4	13.1	-
17αE2	2	2.1	10.6	5.0	0.96	12.1	1.6
	2000	2210	11.2	10.6	2280	6.7	13.9
	Endogenous	ND	-	-	ND	-	-
17βE2	2	2.1	7.6	4.2	2.0	7.6	3.6
	2000	1122	11.5	6.1	932	12.1	5.3
	Endogenous	12	13	-	27.9	12.4	-
16OHE1	10	9.3	3.9	7.4	10.0	1.4	1.8
	200	228	9.4	14.4	196	9.7	1.7
	Plasma + 100	48	6.9	4.8	50.6	11.8	1.2
16OHE2	10	9.7	5.0	3.2	10.1	1.5	1.8
	200	202	7.4	7.4	194	9.9	3.4
	Plasma + 100	56	4.1	12	52.2	4.2	4.6
2MeOE1	10	4.7	10.0	4.1	10.2	9.4	1.5
	200	176	10.6	12.2	203	5.6	3.4
	Plasma + 100	114	4.1	12.0	208	6.3	2.7
4MeOE1	10	10.1	2.7	11.8	9.7	5.7	1.2
	200	220	13.3	8.6	202	6.9	1.6
	Plasma + 100	108	11.5	7.0	120	14.2	20.7
2MeOE2	10	8.8	4.2	11.7	9.5	7.2	5.2
	200	198	14.3	0.8	204	4.1	2.4
	Plasma + 100	108	11.7	7.7	108	14.4	8.5
4MeOE2	10	9.0	5.6	8.8	10.3	2.5	2.6
	200	198	8.9	1.0	114	5.9	6.6
	Plasma + 100	104	4.8	3.2	106	9.6	6.6

Estrone (E1); estradiol (17 α/ β E2); 16-hydroxyestrone (16OHE1); 16-hydroxyestradiol (16OHE2); 2 or 4-methoxyestrone (2 or 4-MeOE1); 2 or 4-methoxyestradiol (2 or 4-MeOE2); RSD, standard deviation/mean x 100, RME %, Relative Mean Error ((mean measured value - theoretical value)/theoretical value x 100); Endogenous levels in female plasma; Plasma + 100 (metabolites spiked in plasma at 100 pg/mL). ND= not detected

Table 2-11: Comparison of standards to certified reference material for E1 and 17βE2, determination of assay bias

		1/X weighting			No weighting		
E1 pg/mL	C_{ref}	$C_{average}$	Bias	%	$C_{average}$	Bias	%
2	2.4	2.2	-0.2	-0.2	-	-	-
20	22.2	20.4	-1.8	-1.8	-	-	-
100	104.6	102.6	-2.0	-2	-	-	-
200	199.7	186.3	-13.4	-13.4	203.7	3.9	1.9
2000	2038.8	2464	425.2	425.2	2206.59	167.8	8.2
Plasma	30.4	31.4	1.0	1	-	-	-
		1/X weighting			No weighting		
E2 pg/mL	C_{ref}	$C_{average}$	Bias	%	$C_{average}$	Bias	%
2	2.1	2.1	0.0	0.0	-	-	-
20	19.0	18	-1.0	-5.6	-	-	-
100	98.7	114.8	16.1	14.0	-	-	-
200	196.3	202.2	5.9	2.9	201.7	5.4	2.7
2000	2092.4	2244	151.6	6.8	2052.4	-39.9	-1.9
Plasma	29.5	27.9	-1.6	-1.6	-	-	-

Estrone (E1); estradiol (17βE2); $C_{average}$, average result from laboratory; Cr_{ef}, reference material; Bias = $C_{average}$ - C_{ref}; Bias (%) = ($C_{average}$ - C_{ref}/$C_{average}$)*100

2.4.5.4 Stability of Derivatives

MPPZ derivatives in extracts of standards and plasma demonstrated suitable stability upon short-term storage of up to 8 days in the auto-sampler (15°C) and upon longer-term storage of 31 days in the freezer (-20°C), with less than 20% degradation. Storage at lower temperatures (-80°C) caused degradation of methoxyestrogens over 31 days (50% response), but was suitable for E1, E2 and 16-OHE1/2 metabolites (Table 2-12).

Table 2-12: Stability of estrogen-MPPZ derivatives in standard and plasma extracts following storage short term for 1, 4 and 8 days in the auto sampler at 15°C and longer term for 4, 8 and 31 days in the freezer at -20°C and -80°C

STANDARDS	**Autosampler (15°C)**			**-20°C**			**-80°C**		
Days	***1***	***4***	***8***	***4***	***8***	***31***	***4***	***8***	***31***
E1	84	86	88	115	103	114	94	119	112
17αE2	88	82	89	102	100	75	86	114	67
17βE2	92	81	86	107	94	111	77	104	88
16αOHE1	93	95	80	90	79	117	91	85	109
16αOHE2	91	106	97	87	76	75	54	84	77
2MeOE1	81	90	85	111	89	103	58	98	96
4MeOE1	83	91	91	109	100	76	97	118	76
2MeOE2	102	93	104	115	108	114	86	119	101
4MeOE2	108	94	104	121	105	108	82	111	83

PLASMA	**Autosampler (15°C)**			**-20°C**			**-80°C**		
Days	***1***	***4***	***8***	***4***	***8***	***31***	***4***	***8***	***31***
E1	104	140	109	129	111	123	123	122	98
17αE2	72	95	92	97	94	71	92	99	58
17βE2	85	104	94	107	91	104	102	100	76
16αOHE1	72	102	91	99	98	87	102	98	81
16αOHE2	108	138	120	116	112	82	116	119	85
2MeOE1	76	106	93	92	86	85	103	99	51
4MeOE1	111	148	119	133	117	94	123	119	75
2MeOE2	81	105	102	105	93	84	105	109	49
4MeOE2	83	109	105	106	94	87	103	106	55

All values expressed as a percentage of the original peak area response at T=0 hours ((T=0 peak area/ T= days peak area) *100)

Upon application of the method to clinical samples, batches were stored in the -20°C freezer for up to 18 days and showed acceptable inter-day reproducibility (Chapter 5 & 6).

2.4.5.5 Assay Robustness

Notably, when running larger batches of the biological samples discussed throughout this thesis inclusion of a 95% CH_3CN column wash every 8-10 runs of patient plasma was beneficial to maintain peak integrity, in particular for the methoxy metabolites as an accumulation of contaminants on column resulted in a wide peak which co-eluted with the methoxyestrogens, usually after 8 – 12 samples (Figure 2-16). This was used in conjunction with diversion of flow to waste for the initial 9 minutes of the gradient.

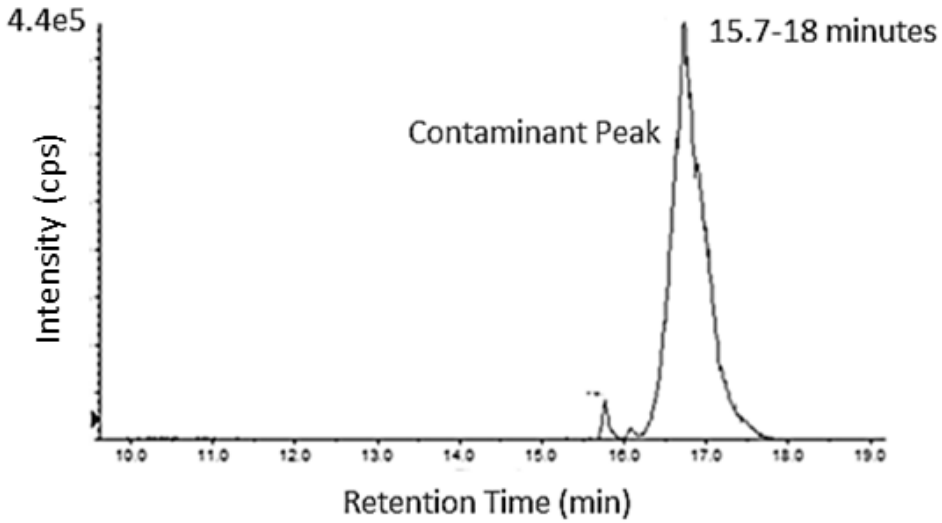

Figure 2-16: LC-MS/MS assay robustness On column contamination build up causes a wide peak over the methoxyestrogen retention time. This can be diminished following application of a 25-minute acetonitrile column wash. It is recommended to run this wash every 8-10 plasma samples. Counts per second; CPS.

Furthermore, to avoid polymerization of the check valves within the Shimadzu Nexera LC system, mobile phase B was altered from 100% ACN to include 10% H_2O (90% ACN - 10% H_2O + 0.1% FA). As a result, the gradient was adjusted to account for this alteration: 1-12 minutes 89:11, 12-15 minutes 56:44 and 15 – 18 minutes 89:11.

2.5 Method Application

The method was applied to a small number of samples from pooled female controls *vs* female PAH patients. Calculated concentrations of estrone and estradiol were within the expected ranges of reported biological concentrations (Table 1-3). Estrogen metabolites were only detected in PAH patient plasma (Figure 2-17).

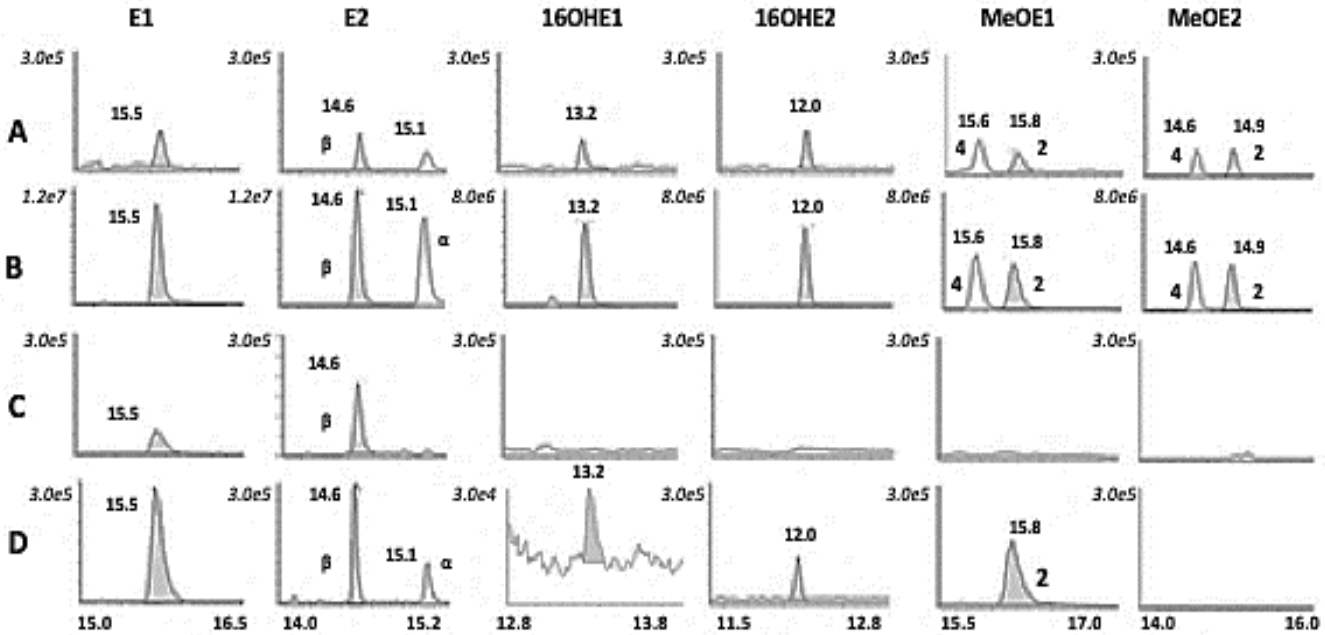

Figure 2-17: Mass Chromatograms of methylpiperazino (MPPZ) derivatives estrone (E1), estradiol (17α & 17βE2), 16-hydroxyestrogens (16OHE1 & 16OHE2) and methoxyestrogens (2MeOE1, 4MeOE1, 2MeOE2 & 4MeOE2) extracted from plasma. Extracted ion chromatograms of derivatised estrogens at (A) lower (1 or 5 pg/sample), and (B) high (1000 pg/sample) concentrations and in plasma (C) from control female subjects and (D) female patients with Pulmonary Arterial Hypertension (PAH). E1 and E2 were detected in control and PAH plasma (0.5 mL) and concentrations fell within the levels expected (20 – 470 pg/mL). Estrogen metabolites were not detected in human control plasma samples, whereas 16OHE1, 16OHE2, 2MeOE1 and 4MeOE1 were detected in a number of PAH patients (7.6 – 200 pg/mL).

2.6 Discussion

In summary, the extraction of estrone, estradiol and six of its bioactive metabolites and quantitation by MPPZ derivatisation allows detection within desired levels in patient samples. This successful development and validation satisfied the aim of this work with the approach comparing favorably with several reported LC-MS/MS reported methods for E1 and E2 with comparable sensitivities, and improved stability of derivatives. Derivatisation of this extended panel of estrogen metabolites for their simultaneous analysis in plasma has not been available routinely in the past for clinical analyses in the PAH field. This approach offers a novel wider view of estrogen metabolism, which appears highly relevant to estrogen-sensitive diseases and will now be applied to larger cohort studies. These findings will next be tested robustly in large patient cohorts. This method development and validation was published in *Analytica Chimica Acta:* Nina Denver, Shazia Khan, Giannis Stasinopoulos, Natalie ZM Homer, Colin Church, Mandy MacLean, Ruth Andrew, Derivatisation enhances analysis of estrogens and their bioactive metabolites in human plasma by liquid chromatography tandem mass spectrometry 2019, Analytica Chimica Acta, DOI: 10.1016/j.aca.2018.12.023.

For catechol estrogens, MPPZ derivatisation with *N*-*N*-DEA was successful allowing detection down to 20 pg/mL in standards. However, extraction of these metabolites using MCX cartridges and applying alternate column washes and elution solvents was not achieved. Therefore, an assessment of extraction modes and cartridges would be justified for this panel of metabolites. Data for the initial analysis was published in a brief article: Nina Denver**,** Shazia Khan, Giannis Stasinopoulos, Natalie ZM Homer, Colin Church, Mandy MacLean, Ruth Andrew, Data for the analysis of catechol estrogens in human plasma by liquid chromatography tandem mass spectrometry, 2019, Data in Brief, DOI: 10.1016/j.dib.2019.103740

Chapter 3 Preliminary Quantification of Estrogens in PAH and Investigation of the *In Vitro* actions of Bioactive Estrogen Metabolites

3.1 Introduction

Pulmonary arterial hypertension (PAH) is a severe, debilitating and life-limiting disease, which has no cure. Diagnosis of PAH is a multi-process evaluation confirmed only by invasive right heart catheterization. The sex paradox in disease epidemiology has implicated estrogen action in the pathogenesis of PAH. Recent studies have implicated sex hormones in postmenopausal PAH women with improved RV function associated with women using hormone replacement therapy (Ventet*uolo et al*, 2011). In addition, elevated estradiol appears in men with PAH (Ventetuolo *et al.*, 2016). Estrogen metabolism may be involved in PAH pathobiology as single nucleotide polymorphisms (SNPs) in *CYP1B1* lead to over-expression and abnormal estrogen metabolism which may underpin the sexual dimorphism in RV failure (Ventetuolo *et al.*, 2016). In PAH, estrogen metabolism has been gaining further prominence in the field, with the exact function and reference range of each estrogen remaining undefined. For this reason, we developed a sensitive and selective LC-MS/MS technique (Chapter 2) for simultaneous analysis of estrogen in human plasma to assess the circulating concentrations of unconjugated estrogens.

3.2 Hypothesis and Aims

I hypothesise that estrogen metabolism may differ between pooled controls and patient samples, with bioactive metabolites presenting a functional role in PASMCs. In this chapter, I aim to conduct a pilot study to test estrogen detection in serum from PAH patients using our developed and validated LC-MS/MS approach. This will allow an evaluation of the quantification parameters: standard curve range and definition of assay limits.

The functional role of bioactive estrogen metabolites quantified by LC-MS/MS will then be assessed *in vitro* in pulmonary artery smooth muscle cells from rats and from an alternative clinical cohort of humans.

3.3 Methods

3.3.1 LC-MS/MS Analysis

3.3.1.1 Study Samples

The study samples were obtained from Royal Papworth Hospital, with ethical permission in collaboration with Professor Nicholas Morrell, Cambridge, United Kingdom. Anonymous information on patient demographics and clinical characteristics were collected from medical records and phone calls. As there was a lack of control serum for this study, human pooled serum was purchased from TCS biosciences (Buckingham, UK), collected from healthy male and female donors in approved collection centres. The pool of serum was from individuals ranging from 24 – 55 years old. All standards, sample preparation and instrumentation used were performed as specified in section 2, 2.3.4.

3.3.1.2 Analytical Criteria

The analytical validity of the batch was determined by the linearity of standard curves and limits of quantitation of the assay as shown in section 2.3.9.2.

3.3.1.3 LC-MS/MS Analysis

LC-MS/MS data was observed using Analyst software v1.5.1 and analysed using MultiQuant, version 3.0 (Sciex, Warrington, UK).

3.3.1.4 Data Analysis

All statistical analyses were performed on IBM SPSS statistics 25. Due to the small sample size and non-normal distribution, data were analysed using non-parametric Mann Whitney test (*p=<0.05) or Kruskal Wallis testing with Bonferroni post-hoc test where appropriate. The limit of quantification values was imputed for all missing values.

3.3.2 *In Vitro* Assessment

All tissue culture experiments were carried out in sterile conditions using a Biological Safety Class II vertical laminar flow cabinet. Cells were housed in a humidified incubator at 37 ºC with a constant supply of 5% CO_2, 95% air. Cells were examined

to confirm healthy morphology and the media was tested using LookOut® Mycoplasma PCR Detection Kit (Sigma Aldrich, UK).

3.3.2.1 Rat Pulmonary Artery Smooth Muscle Cells

rPASMCs were isolated from third-order pulmonary arteries of Wistar Kyoto rats. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, UK) supplemented with antibiotic antimycotic (AA) solution (containing 0.25µg/ml amphotericin B; 100U/mL penicillin; 100µg/ml streptomycin; Sigma-Aldrich, UK) and 10% (v/v) fetal bovine serum (Sera Laboratories International, West Sussex, UK).

3.3.2.2 Human Pulmonary Artery Smooth Muscle Cells

Experimental procedures using human cells conform to the principles outlined in the Declaration of Helsinki. *In vitro* studies were performed using primary culture (passage 4-7) of hPASMCs explanted from the distal pulmonary microvasculature (<1mm external diameter) from non-PAH individuals (control hPASMC) and patients with PAH (PAH-hPASMC). hPASMCs were kindly provided by N.W. Morrell, University of Cambridge, UK with ethical permission. Control hPASMCs were explanted from donor tissue. Non-PAH individuals had no incidence of PAH or pulmonary artery remodelling and the lungs were macroscopically normal. PAH-hPASMCs were obtained at lung or heart-lung transplantations. Only female samples were used in these studies, as specified within the text. Patient details are given in Table 3-1. Samples were stored in liquid nitrogen in 10% dimethyl sulfoxide (DMSO) (v/v) in 10% (v/v) fetal bovine serum (FBS) Dulbecco's modified eagle medium (DMEM). DMSO is a cryoprotectant that aids in reducing cell death during the slow-freezing process to liquid nitrogen (-196ºC). HPASMCs brought up from liquid nitrogen were kept on dry ice before rapidly defrosting the cells by dipping the vial into a 37℃ water bath. Once the contents in the vial had thawed, the exterior of the vial was wiped with 70% ethanol to prevent contamination. Even dispersion of the cells was ensured by pipetting the suspension up and down. Cells were diluted in 10% FBS (v/v; Sera Laboratories International, West Sussex, UK) with 1% antibiotic antimycotic (AA) DMEM at the required cell density. Cells were plated at a density of 10,000 cells per cm^3 in a T75 flask. Cells were then incubated at 37℃, 5% CO_2, 95% air humidified cell culture incubator. Cells were left to adhere for a minimum of 16 hours and thereafter fed fresh media every 24 or 48 hours. DMEM

contains phenol red, a pH indicator which progresses from a red colour to a yellow colour as the pH of the medium decreases, which can be a result of a bacterial infection, cellular death or, more commonly, just a gradual reduction in pH in response to waste products released from the cells. All cell culture medium contained 1% (v/v) AA (10,000 units penicillin, 10 mg streptomycin and 25µg amphotericin B per mL). Penicillin prevents bacterial cell growth by inhibiting cell wall synthesis. Streptomycin is a bacterial protein synthesis inhibitor and amphotericin B is an anti-fungal agent. Cells were utilised for experiments between passages 3-6.

Table 3-1: Cell line characteristics

Sex	Group	Cell line (MP)	Passage	Age	Additional treatment/conditions
Female	PAH	73	5	23	IV prostanoids, warafin, zopliclone, mebevenne, frusemide
		115	5	53	Congenital heart disease
		113	5	45	Unknown
		38	6	39	Idiopathic PAH
		39	4	-	Unknown
		117	3	52	Associated PAH
Female	Control	105	3	57	Emphysema
		77	3	64	Aspirin
		106	4	70	Left lower lobectomy
		83	5	56	Left lower lobectomy
		85	6	64	Angina
Male	Control	103	4	52	Mild bronchiectasis/ adenocarcinoma
		79	4	60	Squamous cell carcinoma
		93	5	75	Lobectomy (lung cancer)
		75	6	-	Unknown

3.3.2.3 Sub-culturing of Human Pulmonary Arterial Smooth Muscle Cells

Cells were grown in T75 flasks until 90-100% confluent in 10% FBS, before sub-culturing for experiments between passage 4 - 7.

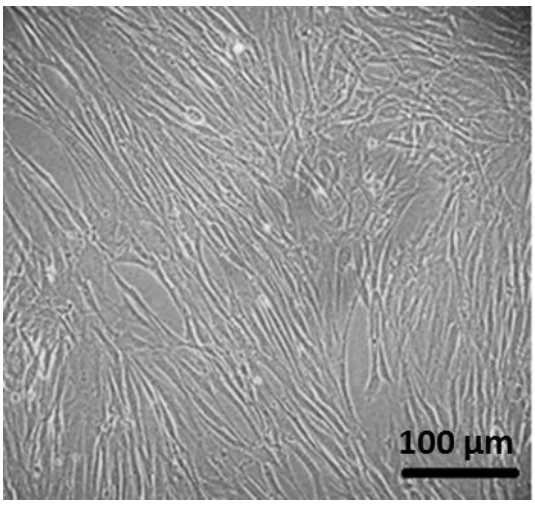

Figure 3-1: Distal human pulmonary arterial smooth muscle cells morphology Photograph of distal 103MP P4 cells grown to confluence in a T75 Flask (x40 magnification).

The culture media was aspirated from flasks and cells washed twice with PBS, 1 mL of trypsin (0.1% (w/v) EDTA in PBS, Life Technologies, Paisley, UK) was added to the flask and coated around the base of the flask upon swirling prior to incubation for 2 minutes. Trypsin cleaves proteins, thereby detaching cells from the flask. Upon detachment, cells develop a round morphology, which can be aided by a gentle tap upon removal from the incubator. Immediately 9 mL of fresh 10% FBS DMEM was added to terminate effects of the trypsin. For Countess automated cell counting cells were split into 6 well plates whilst for the cell counting kit cells were seeded to 96-well plates and grown to the required confluence for experiments. The cell media was replenished the following day and every 24 – 48 hours afterward. 24 Hours prior to stimulation with estrogen metabolites cells were quiesced with 0.2%- FBS in phenol red-free (PRF) DMEM for 24 hours. This serum deprivation or quiescence step prior to experiments allowed cell cycle synchronisation to be achieved. The cell culture media was replenished following 24 hours with the relevant estrogen metabolites added to the wells for cell growth assays.

3.3.2.4 Cell Stimulations

Cells were stimulated with 16OHE2 and 2MeOE1 dissolved in ethanol. Stock solutions were prepared to 1M and stored at -20ºC. Serial dilutions were performed in PRF-DMEM on the day of use for addition to cell culture wells.

3.3.2.5 Cell Proliferation Assays

Countess automated cell counter

Cell proliferation experiments were performed on a Countess II Automated Cell Counter (Life Technologies, UK). The automated cell counter allows assessment of live and dead cells in addition to cell viability and allows capture of cell images.

Rat pulmonary artery smooth muscle cells (rPASMCs) from 6-well plates were washed twice with PBS. To detach cells, 600 µL of trypsin was added to each well with the plate incubated for 5 – 8 minutes. Cells were inspected and transferred to a pre-chilled 1.5 mL Eppendorf tube upon detachment prior to centrifugation at 4900 rcf for 3 minutes. The supernatant was removed with the cell pellet and washed with PBS, ensuring the pellet did not become dislodged. Thereafter, 15 µL PBS was added to each pellet with the Eppendorf tube placed on ice until ready for analysis.

When ready, 15 µL trypan blue (added immediately before viewing as within 3 minutes the solution is toxic to cells) was added with cells re-suspended by mixing. Trypan blue is a cell stain allowing assessment of cell viability as viable cells do not absorb the dye, whilst dead cells are permeable to the dye and appear a dark blue colour. Upon re-suspension cells were transferred to Countess Cell counting slides and inserted to the machine.

Optimization of protocol for hPASMCs

For human PASMCs, the above protocol was optimised to facilitate the increased size and elongated shape compared to small circular rPASMCs. Cell incubation following trypsinisation was increased to 10 - 15 minutes allowing cells to become morphologically similar. Centrifugation was extended to 6 minutes, as the pellet formation was not achieved after 3 minutes. In addition, the Countess parameters for focus adjustment and cell size were altered accordingly.

Cell Counting Kit

As a secondary method, cell proliferation in human cells was also assed using cell counting kit-8, cell proliferation and cytotoxicity assay (Dojindo Molecular Technologies, Inc, USA). Here cells were seeded into 96-well plates and grown as described above, to determine cell number. 10 µL of the CCK-8 solution was added to each well (avoiding bubbles as these interfere with optical density readings). The plate was incubated for 3 hours before the absorbance at 450 nm was measured using a microplate reader.

3.3.2.6 *In Vitro* Statistical Analysis

Data were processed using GraphPad Prism 7 (GraphPad Inc. USA). Data is represented as the mean ± standard error of the mean (SEM). Statistical comparisons were made by comparing the vehicle group to estrogen stimulations using a one-way ANOVA followed by a Dunnett's post hoc test.

3.4 Results

3.4.1 Patient Information

The age, BMI and PAH classification of patients were provided (Table 3-2).

Table 3-2: Patient characteristics

Female	**Control Pool**	**hPAH**	**iPAH**
Subjects	4	8	10
Age (y)	24 – 55	52 ± 17.6	53 ± 7.9
Premenopausal Age (y)	-	39 ± 12.3	47 ± 4.2
Postmenopausal Age (y)	-	65 ± 12.0	60 ± 3.8
BMI $kg \cdot m^{-2}$	-	30 ± 7.3	24 ± 6.7
PAH Classification	-	3	3
Male	**Control Pool**	**hPAH**	**iPAH**
Subjects	4	2	9
Age (y)	(24 - 55)	53 ± 28.3	52 ± 16.4
BMI $kg \cdot m^{-2}$	-	21 ± 2.1	28 ± 5.2
PAH Classification	-	3	3

Pulmonary arterial hypertension, PAH; hPAH, heritable PAH; iPAH, idiopathic PAH; Clinical characteristics shown as Mean ± SD and age range for pooled controls.

3.4.2 Analytical Quality Control

The standard curve for this batch was linear as determined by an r value > 0.9 and limits of detection were displayed at a lower limit of 6 pg/mL for estrogen metabolites in comparison to the more conservative value of 10 pg/mL reported in our published paper. This finding was confirmed upon analysis of the required number of inter-day batches for low levels analysis as explained in Chapter 5.

Table 3-3: Linearity of standard curves

Estrogen-MPPZ	LOQ (pg/mL)	ULOQ (pg/mL)	R Value
E1	2	500	0.996
17αE2	2	500	0.984
17βE2	2	500	0.995
16OHE1	6	200	0.999
16OHE2	6	200	0.999
2MeOE1	6	200	0.999
4MeOE1	6	200	0.999
2MeOE2	6	200	0.998
4MeOE2	6	200	0.998

Estrone, E1; Estradiol, E2; 16-Hydroxyestrone, 16OHE1; 16-Hydroxyestradiol, 16OHE2; 2-Methoxyestrone, 2MeOE1; 4-Methoxyestrone, 4MeOE1; 2-Methoxyestradiol, 2MeOE2; 4-Methoxyestrone, 4MeOE1. Limit of quantification, LOQ; Upper Limit of Quantification, ULOQ.

3.4.3 LC-MS/MS Analysis

E1, E2, 16OHE1, 16OHE2 and 2MeOE1 were detected within serum samples (Figure 3-2). 17αE2, 2MeOE2, 4MeOE1 and 4MeOE2 were not detected in this cohort.

3.4.3.1 Chromatographic Identification

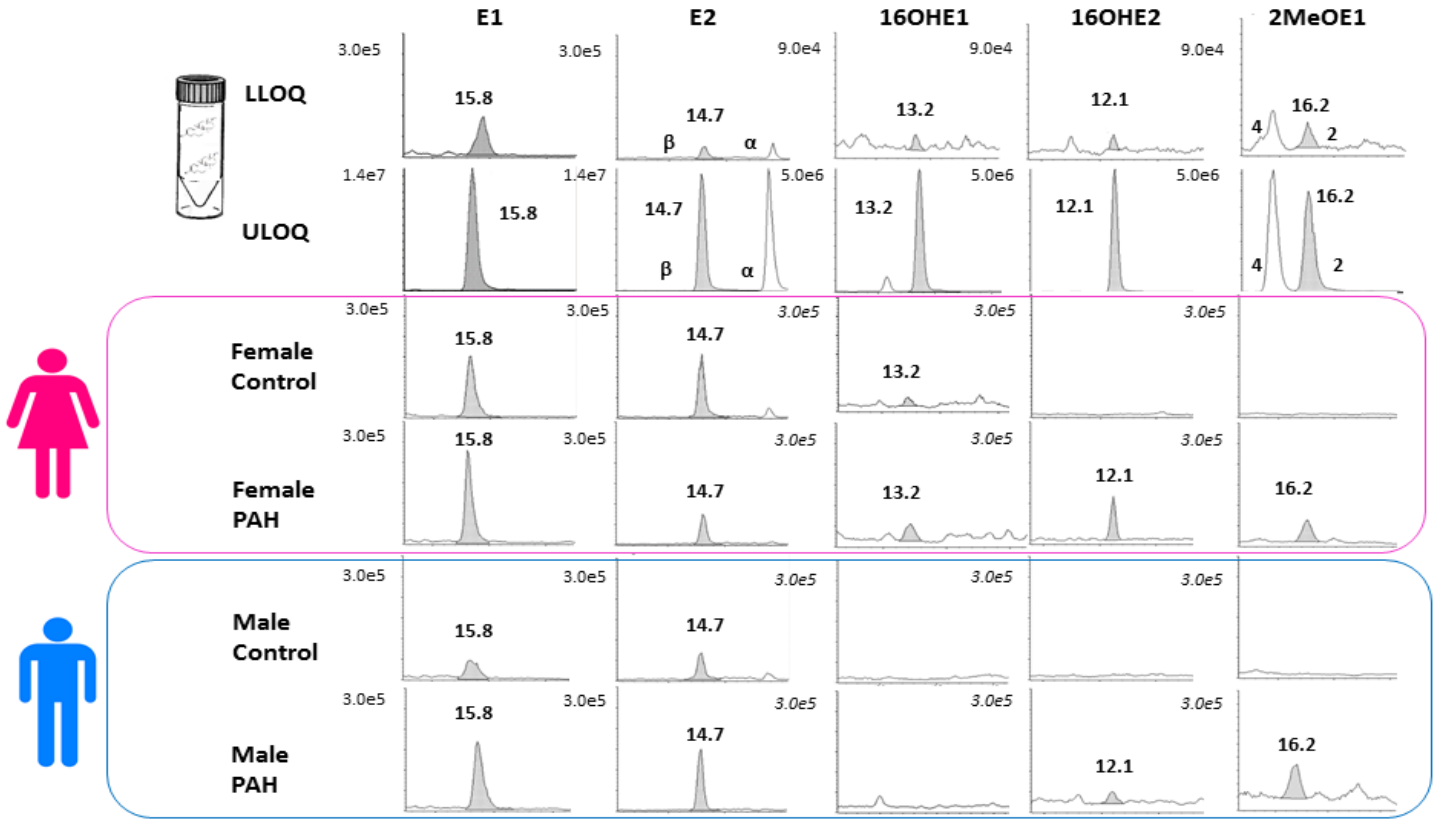

Figure 3-2: Chromatographic identification of estrogen and metabolites detected in serum Estrone (E1), estradiol (E2), 16-hydroxyestrone (16OHE1), 16-hydroxyestradiol (16OHE2) and 2-methoxyestrone (2MeOE1) are shown at 2 pg/mL for E1, E2 and 6 pg/mL for estrogen metabolites and at high concentrations, 1000 pg/mL. Detection in control and PAH samples of female and male serum samples was also achieved.

3.4.3.2 Estrogen Concentrations and PAH

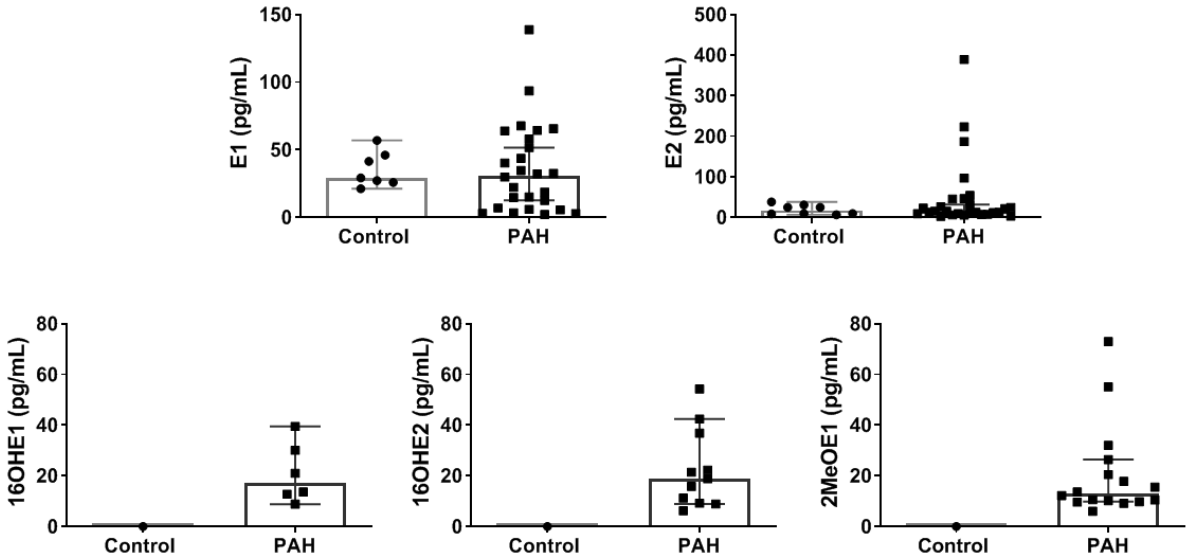

Figure 3-3: Estrogen and disease status Estrogen concentrations in pooled controls vs PAH: Estrone, E1; Estradiol, E2; 16-Hydroxyestrone, 16OHE1; 16-Hydroxyestradiol, 16OHE2; 2-Methoxyestrone, 2MeOE1; Pulmonary Arterial Hypertension, PAH. Data shown as median ± 95% Confidence Interval. N = 8, control; N= 30, PAH.

Estrone and estradiol were detected in controls and patients with PAH while bioactive estrogen metabolites were detected only in PAH patients. In general, a wider range of circulating estrogens are profiled within the PAH cohort (Figure 3-3).

Table 3-4: Concentrations of estrogen in pooled control and PAH patients

(pg/mL)	Controls	PAH	P-Values
E1	47.4 ± 3.3	31.2 ± 6.0	0.28
E2	29.5 ± 3.1	43.7 ± 15.1	0.93
16OHE1	*ND*	9.0 ± 1.4	-
16OHE2	*ND*	12.2 ± 2.2	-
2MeOE1	*ND*	13.9 ± 2.8	-

Concentration of estrogens in Controls vs PAH: Pulmonary arterial hypertension, PAH; Not detected, ND; Estrone, E1; Estradiol, E2; 16-Hydroxyestrone, 16OHE1; 16-Hydroxyestradiol, 16OHE2; 2-Methoxyestrone, 2MeOE1. Estrogen levels shown as Mean ± SEM. Statistics following a Mann Whitney test.

3.4.3.3 Concentrations of Estrogen, Sex and PAH

The data was stratified to examine the possible influence of sex on estrogen profiles in controls and PAH patients (Figure 3-4 and Figure 3-5).

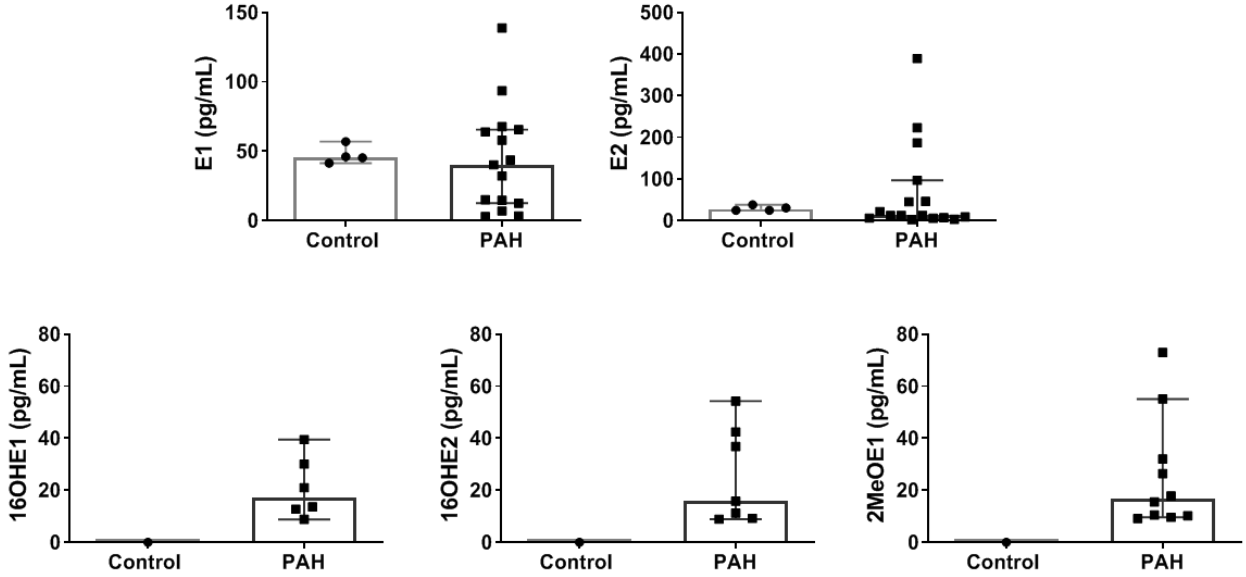

Figure 3-4: Estrogen concentrations in female pooled controls and pulmonary arterial hypertension (PAH) patients Estrogen concentrations in female pooled controls vs PAH: Estrone, E1; Estradiol, E2; 16-Hydroxyestrone, 16OHE1; 16-Hydroxyestradiol, 16OHE2; 2-Methoxyestrone, 2MeOE1, Pulmonary arterial hypertension, PAH. Data shown as median ± 95% Confidence Interval with statistics following a Mann Whitney test. N = 4, control; N= 19, PAH.

In females, E1 and E2 were detected in both controls and PAH patients whilst the bioactive metabolites were only detected in the patient cohort (Figure 3-4). Therefore, in pooled controls bioactive metabolites were not present ≥6 pg/mL, most likely due to the preparation of the pooled serum. In females, there was no difference between controls and female PAH (Table 3-5).

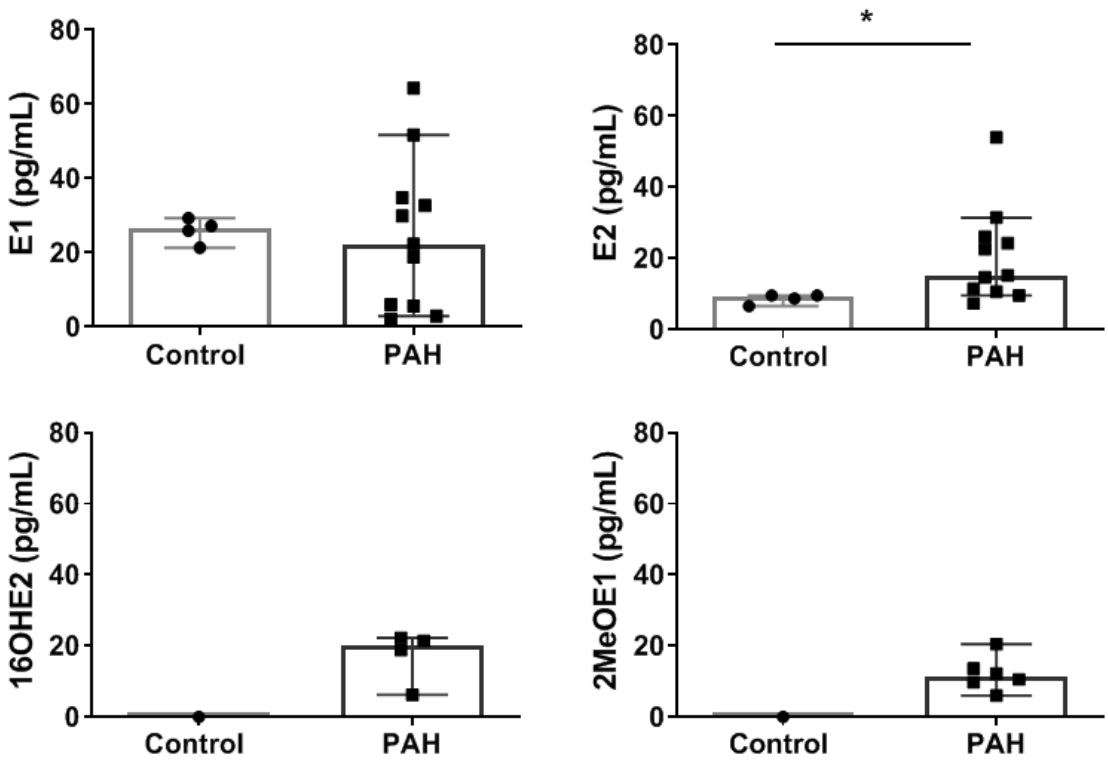

Figure 3-5: Estrogen concentrations in male pooled controls and pulmonary arterial hypertension (PAH) patients Estrogen concentrations in male pooled controls vs PAH: Estrone, E1; Estradiol, E2; 16-Hydroxyestrone, 16OHE1; 16-Hydroxyestradiol, 16OHE2; 2-Methoxyestrone, 2MeOE1; Pulmonary arterial hypertension (PAH). Data shown as median ± 95% Confidence Interval with statistics following a Mann Whitney Test *P<0.05. N = 4, control; N= 11 PAH.

In males, E1 and E2 were also detected in both control and patient groups. Here, 16OHE2 and 2MeOE1 were detected in the PAH patient cohort with levels remaining undetected in the control groups (Figure 3-5). Hence, in controls, bioactive metabolites were not present ≥ 6 pg/mL. E2 concentrations were elevated in the PAH cohort, p=0.01 (Table 3-5).

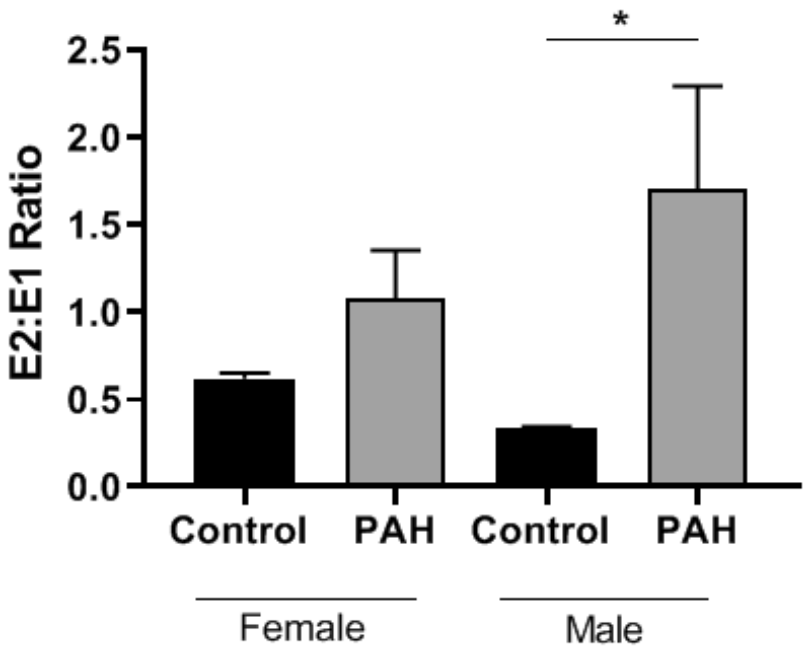

Figure 3-6: E2:E1 ratio as a marker of 17βhsd activity in pooled controls and pulmonary arterial hypertension (PAH) patients from females and males Estradiol, E2: Estrone, E1 ratio in controls and PAH patients from females and males. Data expressed and mean ± SEM. *P<0.05 using a Kruskal Wallis multiple comparison test *P<0.05. Females, N = 4, control; N= 19, PAH; Males, N = 4, control; N= 11 PAH.

The ratio of E2 to E1 was significantly increased in male PAH in comparison to male controls (p=0.03). There was no difference between female controls in comparison to PAH patients (Figure 3-6).

Table 3-5: Estrogen concentrations, sex and PAH

Female (pg/mL)	Controls	PAH	Control vs PAH p-values	Female vs Male PAH p-values
Subjects	4	19		
E1	47.4 ± 3.3 45.7 [43.4 – 51.5]	35.1 ± 8.7 40.2 [13.6 – 65.6]	0.32	0.87
E2	29.5 ± 3.1 27.7 [24.7 – 34.3]	57.1 ± 23.5 12.6 [6.6 – 71.4]	0.29	0.37
16OHE1	*ND*	10.7 ± 2.2 17.3 [12.8 – 30.1]	-	-
16OHE2	*ND*	13.4 ± 3.3 13.5 [9.4 – 39.6]	-	-
2MeOE1	*ND*	16.5 ± 4.3 16.7 [10.2 – 32.0]	-	-
Male (pg/mL)	**Controls**	**PAH**	**P-Values**	
Subjects	4	11	-	
E1	24.9 ± 4.5 24.5 [21.2 – 27.1]	24.5 ± 6.2 22.2 [5.7 – 34.7]	0.95	
E2	8.6 ± 3.3 9.1 [7.6 – 9.6]	20.6 ± 4.0 15.2 [11.0 – 26.1]	**0.01**	
16OHE1	*ND*	*ND*	-	
16OHE2	*ND*	10.1 ± 2.1 20.1 [12.5 – 21.9]	-	
2MeOE1	*ND*	9.3 ± 1.41 12.2 [10.2 – 13.6]	-	

Estrogen Concentrations in female and male control vs PAH including p-values and p-values comparing female vs male concentrations in each group: Estrone, E1; Estradiol, E2; 16-Hydroxyestrone, 16OHE1; 16-Hydroxyestradiol, 16OHE2; 2-Methoxyestrone, 2MeOE1; Pulmonary arterial hypertension, PAH; Not detected, ND. Estrogen levels shown as Mean ± SEM and Median [Q_1-Q_3]. Statistics following a Kruskal-Wallis test with significant increase ($p<0.05$) comparing PAH to controls in bold.

3.4.3.4 Stratification of estrogens: sex and clinical classification

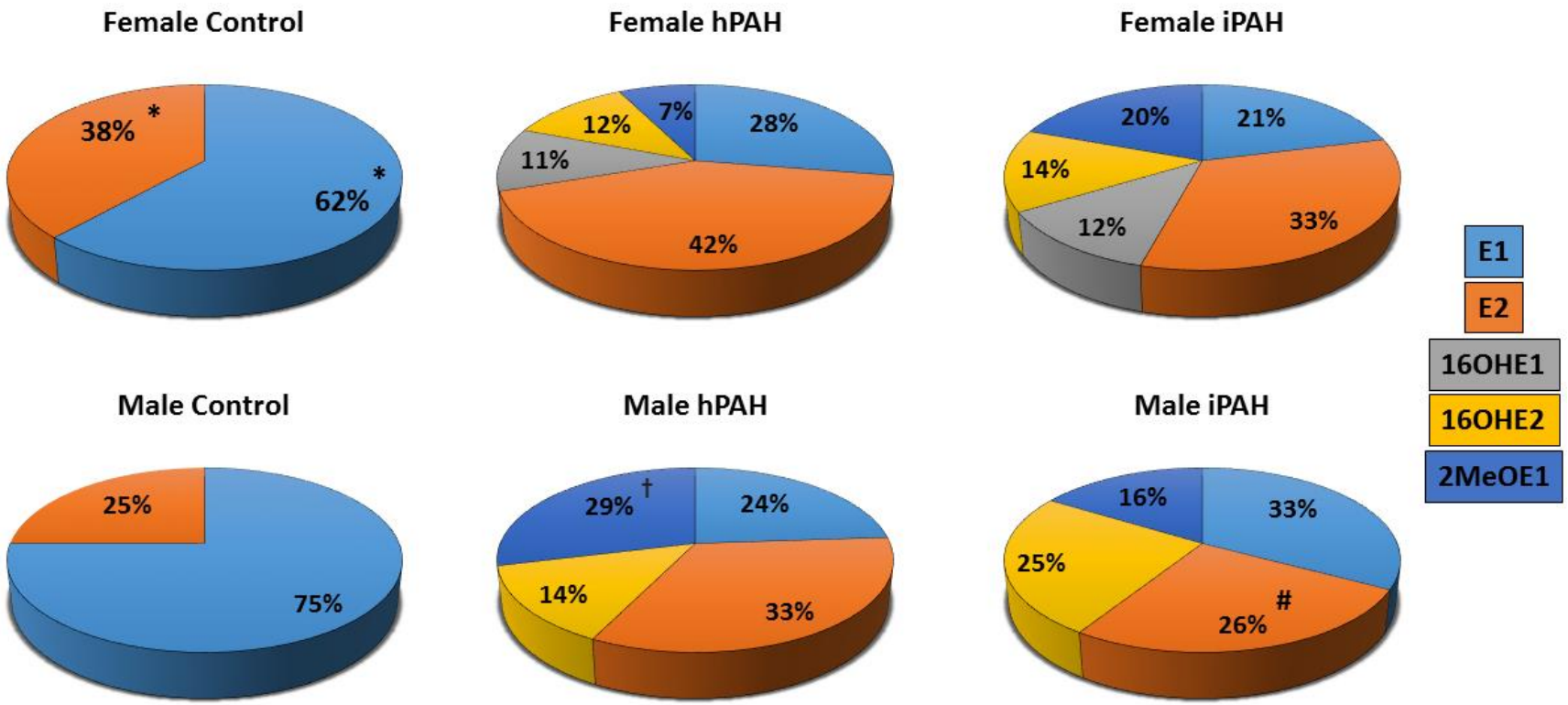

Figure 3-7: Percentage of estrogens and bioactive metabolites detected in controls, heritable and idiopathic PAH Profile of percentage estrogens in disease classification of heritable pulmonary arterial hypertension (hPAH); idiopathic pulmonary arterial hypertension (iPAH) between males and females: Estrone (E1) and estradiol (E2) were detected in both control and patients. Whilst bioactive metabolites were only detected in the patient samples, with 16-hydroxyestradiol (16OHE2) and 2-methoxyestrone (2MeOE1) in both sexes and 16-hydroxyestrone only detected in females. hPAH. * indicates increased in female vs male ($p<0.05$) and # increase in iPAH vs control ($p<0.05$) †trend toward increase hPAH vs control ($p=0.08$) following Kruskal Wallis multiple comparisons with Bonferroni post-hoc tests. . N=4, Control; N=8 hPAH; N=11 iPAH in females and N=4, Control; N=2 hPAH; N=10 iPAH in males.

Altered estrogen profiles were observed dependent on clinical classification and sex (Figure 3-7, Table 3-6). 16OHE1 was only detected in female patients but was not significantly increased in hPAH or iPAH (Table 3-6, Figure 3-7). It should be noted that 16OHE1 was only detected in one of the hPAH patients. In males, E2 was significantly increased in the patient group with a significant increase in iPAH patients compared to controls (p=0.04). Interestingly, E2 concentrations were lower in hPAH than in iPAH. Also, in controls, E1 and E2 were higher in female vs males, as expected (Table 3-7) 2MeOE1 was increased between iPAH patients and controls in males. It should be noted that 16OHE2 was only detected in one of the two hPAH patients therefore this comparison was limited by low statistical power.

Table 3-6: Estrogen, sex and PAH classification

Female	**Controls**	**hPAH**	**iPAH**	**P-Values**
E1	47.4 ± 3.3 45.7 [43.4 – 51.5]	46.0 ± 11.1 58.0 [38.0 – 67.7]	26.9 ± 12.7 14.9 [7.9 – 52.9]	0.15
E2	29.5 ± 3.1 27.7 [24.7 – 34.3]	70.3 ± 31.5 21.9 [12.5 – 186.7]	47.4 ± 34.6 9.0 [4.2 – 45.7]	0.21
16OHE1	*ND*	21.0	21.0 ± 5.9 13.6 [10.8 – 30.1]	0.14
16OHE2	*ND*	22.1 ± 10.8 12.7 [9.2 – 35.1]	24.9 ± 8.6 24.0 [10.2 - 39.6]	0.28
2MeOE1	*ND*	14.2 ± 4.1 10.4 [9.9 – 18.5]	33.8 ± 10.3 24.9 [15.6 – 55.1]	0.16
Male	**Controls**	**hPAH**	**iPAH**	
E1	24.9 ± 4.5 24.5 [21.2 – 27.1]	10.7 ± 8.0 10.7 [2.8 – 18.7]	27.6 ± 7.11 29.8 [5.9 – 43.1]	0.30
E2	8.6 ± 3.3 9.1 [7.6 – 9.6]	15.0 ± 7.6 14.9 [7.3 – 22.6]	21.9 ± 4.8* 15.2 [11.4 – 28.8]	**0.02**
16OHE1	*ND*	*ND*	*ND*	*ND*
16OHE2	*ND*	6.22	20.8 ± 1.1 21.4 [20.1 – 22.3]	-
2MeOE1	*ND*	12.94 ± 0.7† 12.9 [12.2 – 13.6]	13.6 ± 3.4 10.5 [9.8 – 15.5]	**0.05**

Estrogen concentrations in female and male controls vs hPAH vs iPAH: Pulmonary arterial hypertension, PAH; hPAH, heritable PAH; iPAH, idiopathic PAH; Not detected, ND; Estrone, E1; Estradiol, E2; 16-Hydroxyestrone, 16OHE1; 16-Hydroxyestradiol, 16OHE2; 2-Methoxyestrone, 2MeOE. Estrogen levels shown as Mean ± SEM. Overall statistics following a Mann Whitney test or Kruskal-Wallis test with Bonferroni post-hoc tests where appropriate. *p<0.05 increase in iPAH vs control, †·p=0.08 trend toward increase in hPAH vs Controls.

Table 3-7: Estrogen concentrations in male *vs* female groups

Male vs Female P-Value	E1	E2
Control	**0.03**	**0.03**
hPAH	0.27	0.89
iPAH	0.33	0.11

Estrogen Concentrations in female's vs males: Pulmonary arterial hypertension, PAH; hPAH, heritable PAH; iPAH, idiopathic PAH; Not detected, ND; Estrone, E1; Estradiol, E2; 16-Hydroxyestrone, 16OHE1; 16-Hydroxyestradiol, 16OHE2; 2-Methoxyestrone, 2MeOE. Estrogen levels shown as Mean ± SEM. Statistics following a Kruskal-Wallis test with significant differences highlighted in bold.

In summary, a number of novel metabolites were detected in higher concentrations in PAH patients. Little has been published about 16OHE2 and 2MeOE1 with respect to their influence in PAH. Therefore, the functional role of these metabolites was examined *in-vitro* in PASMCs.

3.4.4 *In vitro* assessment of bioactive metabolites

3.4.4.1 Countess Cell Counting: 24-hour stimulation with 16OHE2 and 2MeOE1 in rat PASMCs

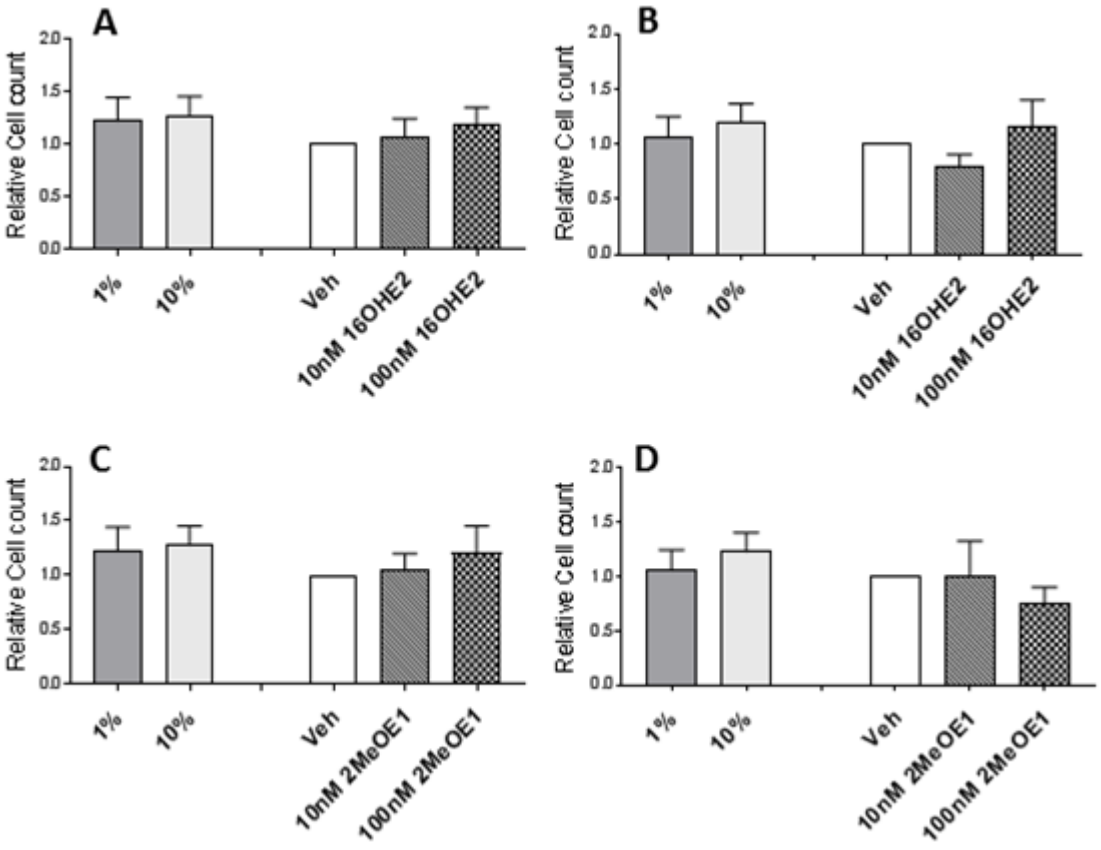

Figure 3-8: No influence of 16OHE2 and 2MeOE1 on cellular proliferation of rat PASMCs over 24 hours Effect of 16OHE2 and 2MeOE1 (10 nM & 100 nM/L) in the presence of 1% FBS over 24 hours in female rPASMCs (A, C) and male rPASMCs (B, D). Vehicle (Veh), 1% (1% FBS), 10% (10% FBS). Data are relative to the vehicle and shown as the mean ± SEM the n=6 per experiment.

Initially incubation of the metabolites for 24 hours was tested to determine the rate of proliferative changes in rPAMSCs (Figure 3-8). After 24 hours, differences in proliferation were not observed in the experimental comparisons between vehicle and 16OHE2 or 2MeOE1 stimulations. Therefore, cells were stimulated with metabolites for 48 hours in all future experiments to allow a further 24 hours for cell cycle progression.

3.4.4.2 Countess Cell Counting: 48-hour stimulation with 16OHE2 and 2MeOE1 in rat PASMCs

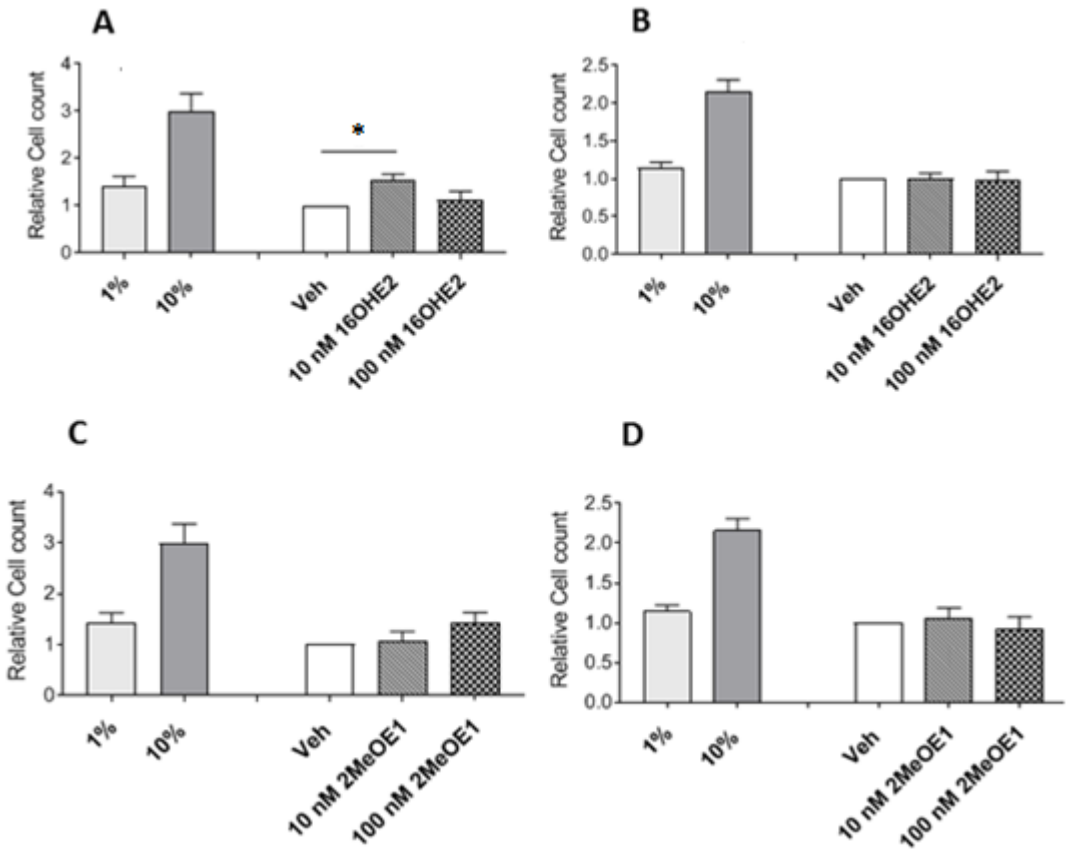

Figure 3-9: Proliferative effect of 16OHE2 and no influence of 2MeOE1 on cellular proliferation of rat PASMCs over 48 hours. Effect of 16OHE2 in female (A) and male (B) rat PASMCs. 2MeOE1 effect in females (C) or male (D) rat PASMCs. Experiments performed in 1% FBS over 48 hours. Vehicle (Veh), 1% (1% FBS), 10% (10% FBS). Data are relative to the vehicle and shown as the mean ± SEM. Statistical analysis compared estrogen stimulations to the vehicle using a Dunnett's multiple comparison; *p<0.05. N=6 per experiment.

Following 48 hours of stimulation with estrogen metabolites, 10nM 16OHE2 induced proliferation in female rat cells (Figure 3-9 A). This effect was not duplicated in males showing a possible sex difference in the action of this estrogen metabolite. As 2MeOE2 has been reported to induce anti-proliferative properties, the possibility of 2MeOE1 producing the same effect was assessed (Figure 3-10).

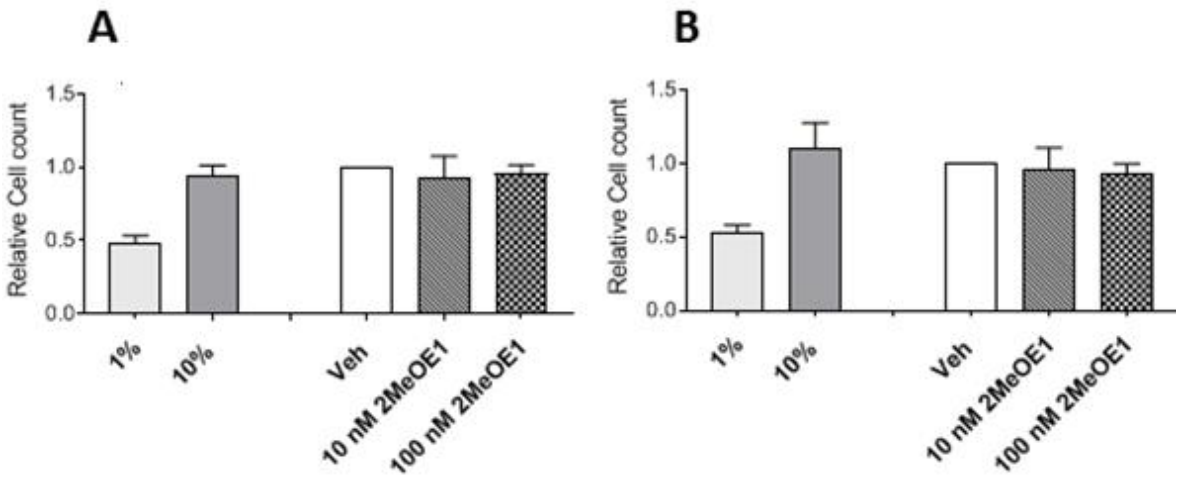

Figure 3-10: No anti-proliferative effect of 2MeOE1 in rat PASMCs over 48 hours Anti-proliferative effect in 10% media over 48 hours in female (A) and male (B) rat PASMCs. Data are relative to the vehicle and shown as the mean ± SEM. Vehicle (Veh), 1% (1% FBS), 10% (10% FBS). Statistical analysis determined by comparing the estrogen stimulations to the relative vehicle group by a Dunnett's multiple comparison. N=6 per experiment.

As 2MeOE1 displayed no proliferative effect, the possibility of an anti-proliferative effect was assessed (Figure 3-10). In both female and male cells no effect of 2MeOE1 stimulation was detected.

3.4.4.3 Optimisation of Countess Automated Cell Counter for Human Cell Proliferation

For accurate analysis of hPASMCs using Countess automated cell counting, the protocol had to be optimised from that used for rat cells. Morphologically, human cells are larger and more elongated than those of rats upon trypsinisation. Due to this difference a large number of cells appeared dead upon counting (Figure 3-11).

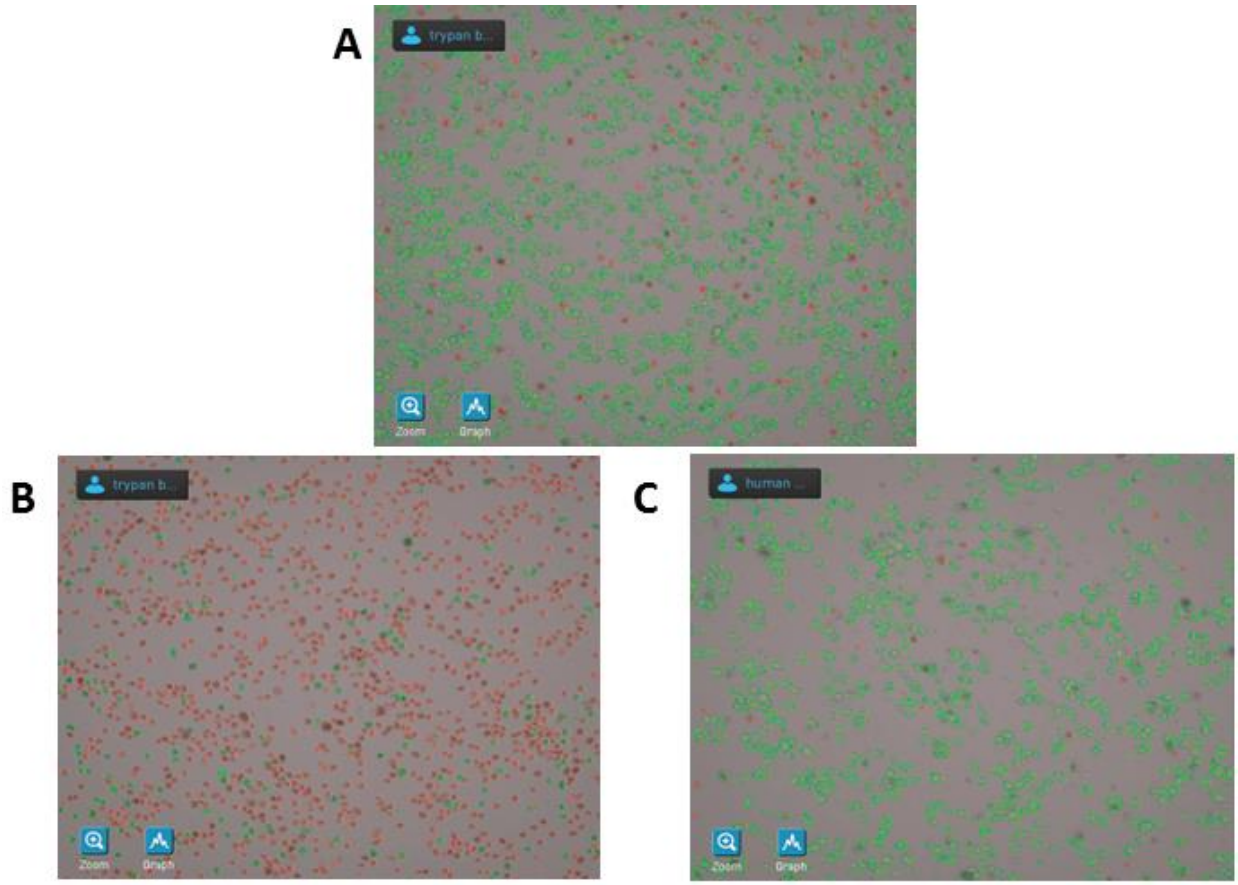

Figure 3-11: Automated cell counts of human and rat cells Cells viewed within the countess slides with rat pulmonary artery smooth muscle cells (A) and human pulmonary artery smooth muscle cells with the initial protocol (B) and following optimisation (C). Living cells are shown in green with dead cells in red.

To obtain a high recorded number of living human cells, a number of alterations were applied to the rat cell protocol. Alterations to the trypsinisation protocol appeared to facilitate a more circular morphology upon detachment, with adjustment of the centrifugation time creating a larger, easier to handle cell pellet. Modifications to the countess autofocus parameters allowed more live cells to be detected upon counting of human cells. This was also assessed by trypan blue exclusion as a measure of cell viability, as prior to optimisation (Figure 1-10 C) viability was poor (<50%). Therefore, improvement to an adequate level was achieved (Figure 3-12).

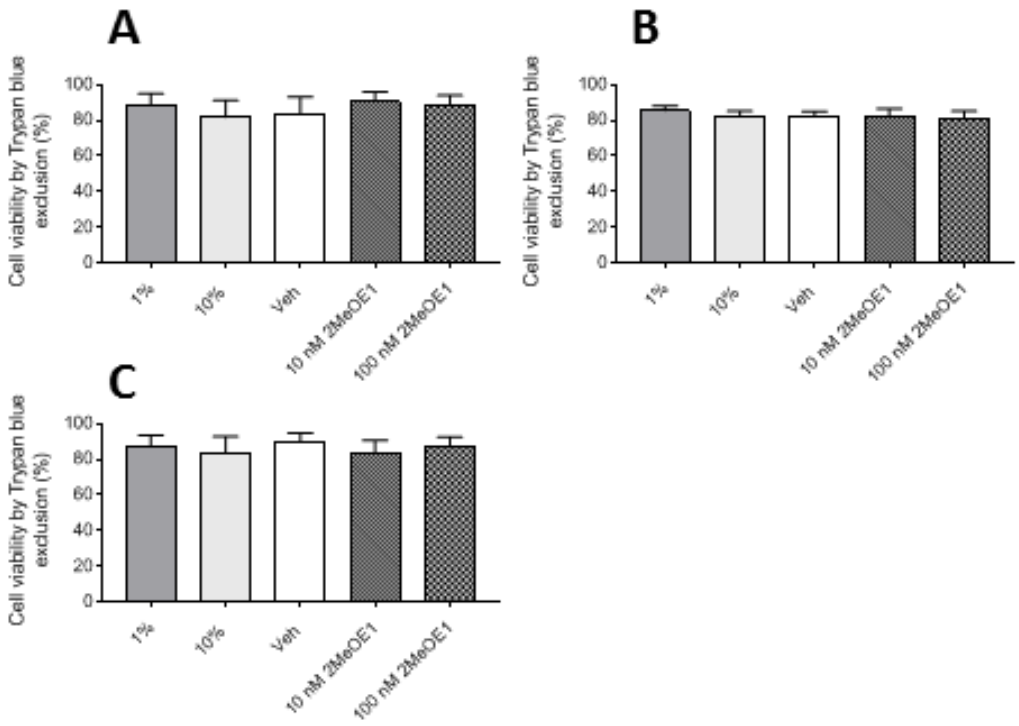

Figure 3-12: Example of cell viability of human cells Example of cell viability for 2MeOE1 stimulations in human female control (A), male control (B) and female PAH (C) cells displaying a high number of viable cells using the trypan blue exclusion method. Intact cell membranes of live cells exclude the uptake of this dye allowing differentiation between live and dead cells. Data shown as mean ± SEM with a viable control and treatment group at 80 – 100%. Vehicle (Veh), 1% (1% FBS), 10% (10% FBS).

3.4.4.4 Countess cell counting: Effect of 48-hour stimulation with 16OHE2 and 2MeOE1 in hPASMCs

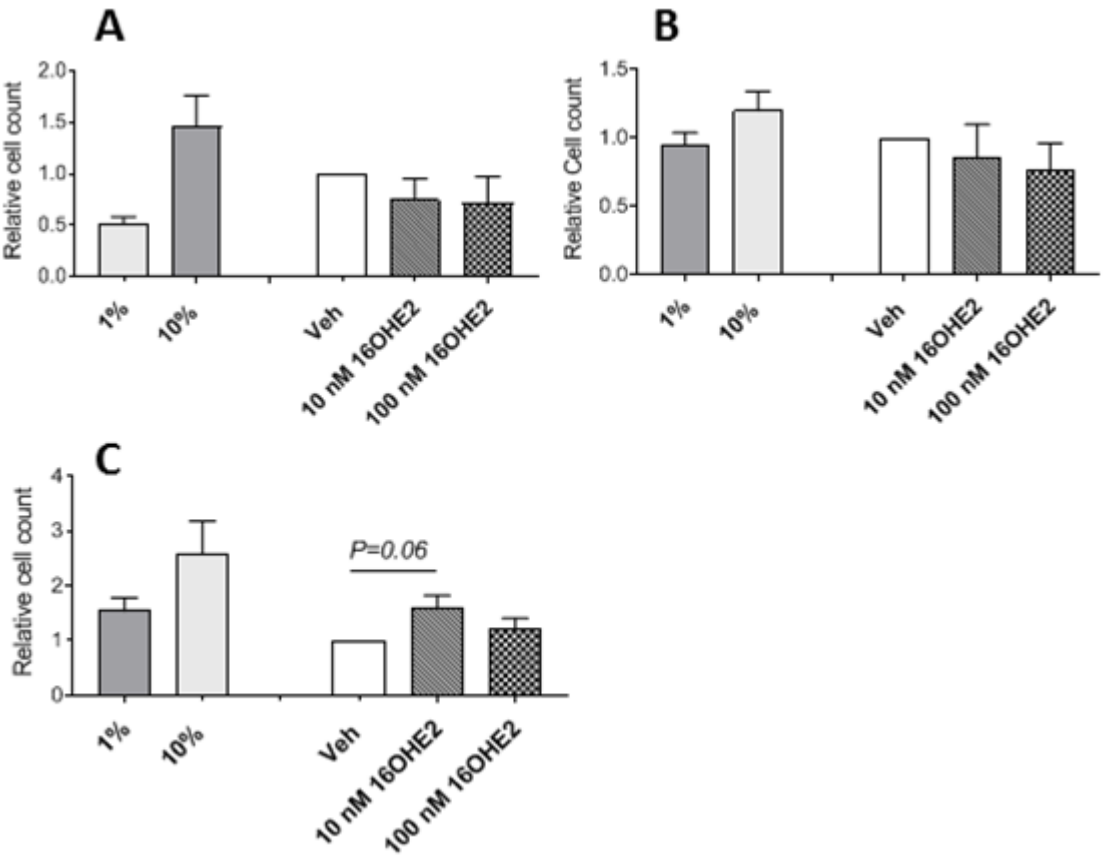

Figure 3-13: Effect of 16OHE2 on human PASMC cellular proliferation, Effect in female control (A), male control (B) and female PAH (C) hPASMCs in 1% FBS over 48-hours. Vehicle (Veh), 1% (1% FBS), 10% (10% FBS). Data are relative to the vehicle and shown as the mean ± SEM with groups Statistical analysis compared the vehicle to treatment groups using a Dunnett's multiple comparison. N=4-6 per experiment

In PASMCs from female and male controls, no proliferative effect was observed. In female PAH cells a trend toward increased proliferation was observed following 10 nM 16OHE2 stimulation (Figure 3-13). Male PAH cells were not studied due to a lack of available human cells lines for this group.

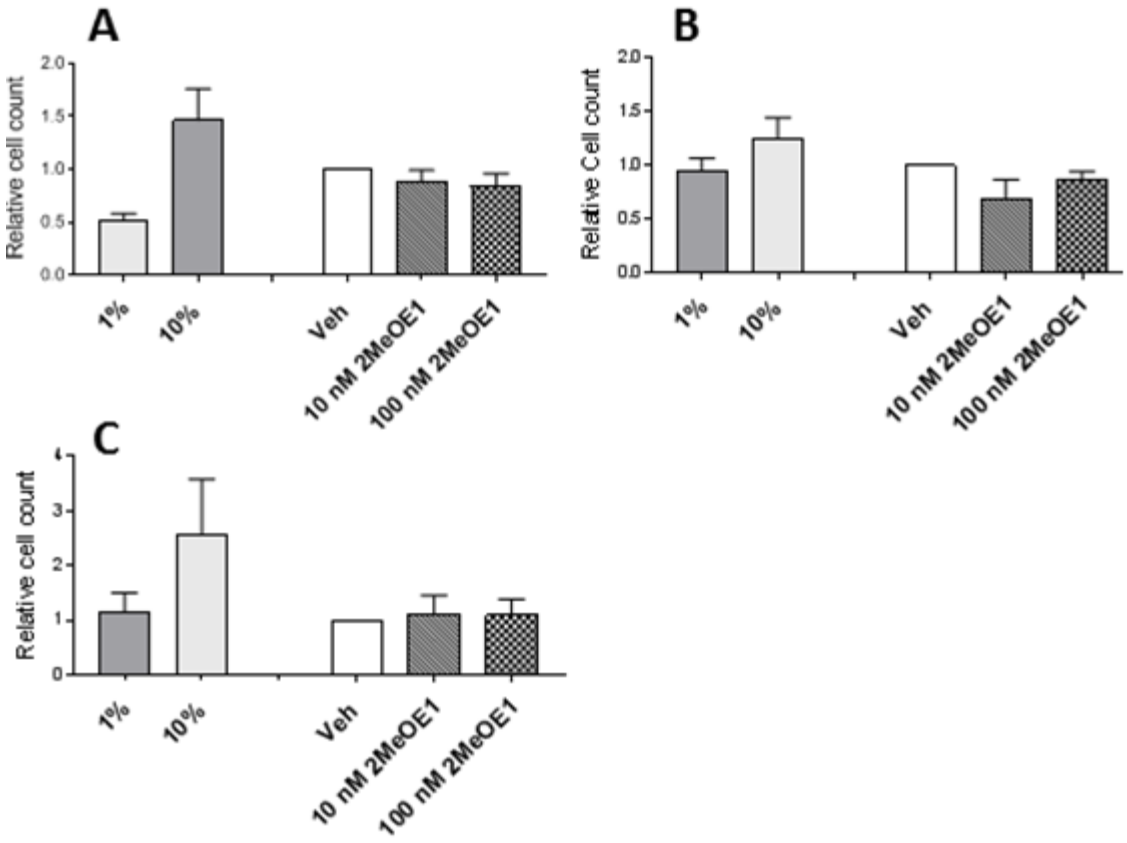

Figure 3-14: No Proliferative effect of 2MeOE1 in human PASMCs over 48 hours Effect in female control (A), male control (B) and female PAH (C) hPASMCs in 1% FBS over 48-hours. Vehicle (Veh), 1% (1% FBS), 10% (10% FBS). Data are relative to the vehicle and shown as the mean ± SEM. Statistical analysis determined by comparing the estrogen stimulations to the relative vehicle group by a Dunnett's multiple comparison N=4-6 per experiment

2MeOE1 did not induce proliferation in female or male control and female PAH PASMCs (Figure 3-14).

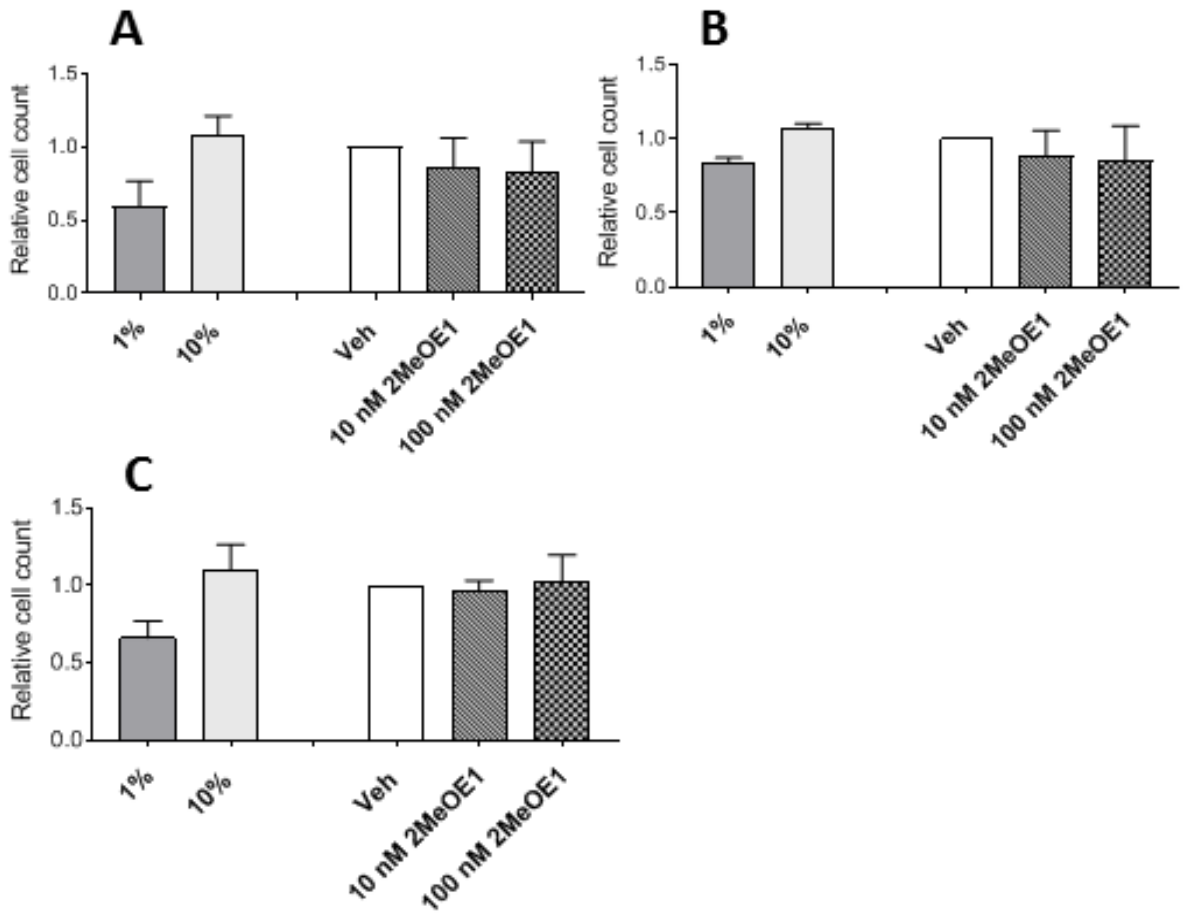

Figure 3-15: No anti-proliferative Effect of 2MeOE1 in human PASMC after 48 hours Effect in female control (A), male control (B) and female PAH (C) hPASMCs over 48-hours in 10% FBS. Vehicle (Veh), 1% (1% FBS), 10% (10% FBS). Data are relative to the vehicle and shown as the mean ± SEM. N=4-6 per experiment

2MeOE1 had no anti-proliferative effect in female or male control PASMCs or female PAH PASMCs (Figure 3-15).

3.4.4.5 Cell Counting Kit: 48-hour stimulation with 16OHE2 and 2MeOE1 in hPASMCs

For human cells, cell counts were additionally tested using a cell counting kit. This method uses a WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) producing a water-soluble dye. Upon addition to cells WST is reduced to an orange formazan directly proportional to the number of living cells. Dye formation is then measured at a wavelength of 450 nm to count the number of cells.

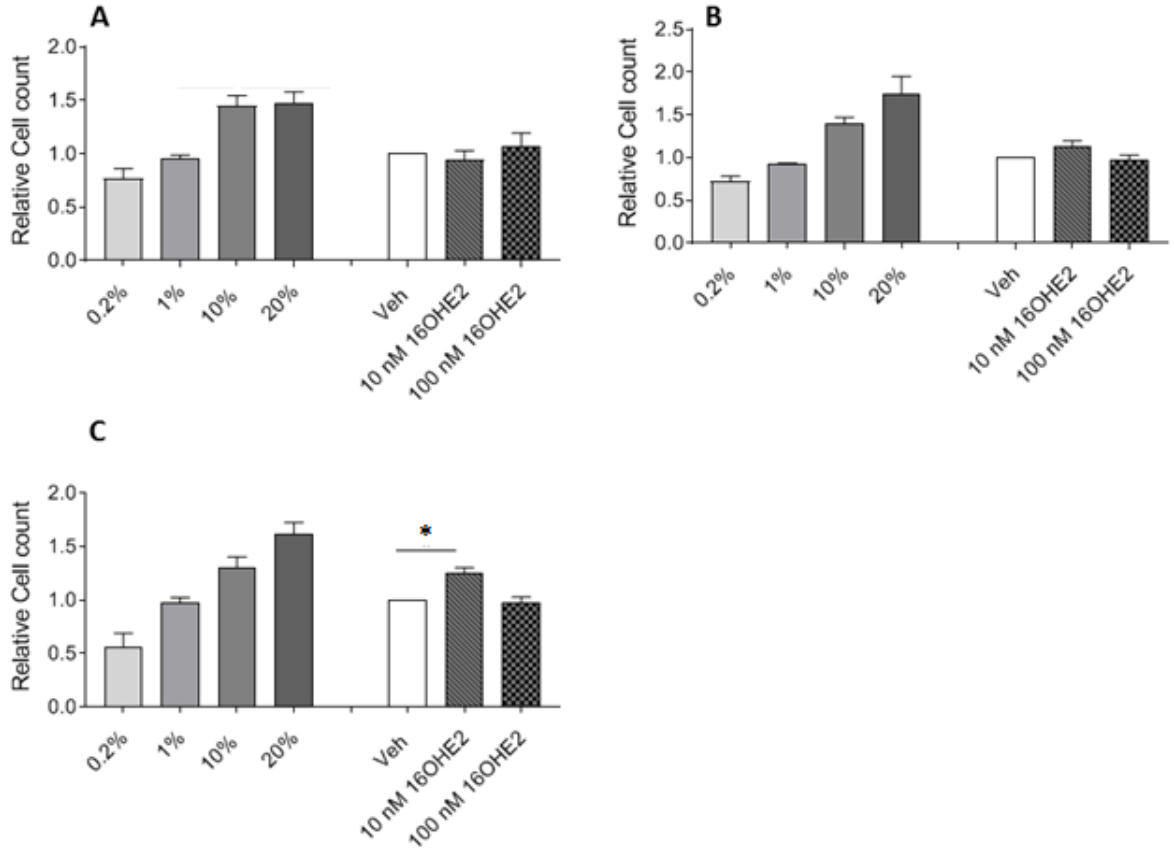

Figure 3-16: Effect of 16OHE2 on human PASMC cell counts Effect in female (A), male (B) controls and female PAH (C) hPASMCs. Experiments performed in 1% FBS over 48 hours with data presented relative to the vehicle and as the mean ± SEM. Vehicle (Veh), 0.2% (0.2% FBS), 1% (1% FBS), 10% (10% FBS), 20% (20% FBS). Statistical analysis determined by comparing the estrogen stimulations to the relative vehicle group by a Dunnett's multiple comparison; *p<0.05. N=3 per experiment.

16OHE2 did not induce proliferation in female and male control PASMCs (Figure 3-16). Intriguingly, in female PAH cells 10nM 16OHE2 induced a proliferative response. This finding is the same as in section 1.5.4.4, where countess automated cell counting suggested proliferation at the same 16OHE2 concentration in female PAH cells.

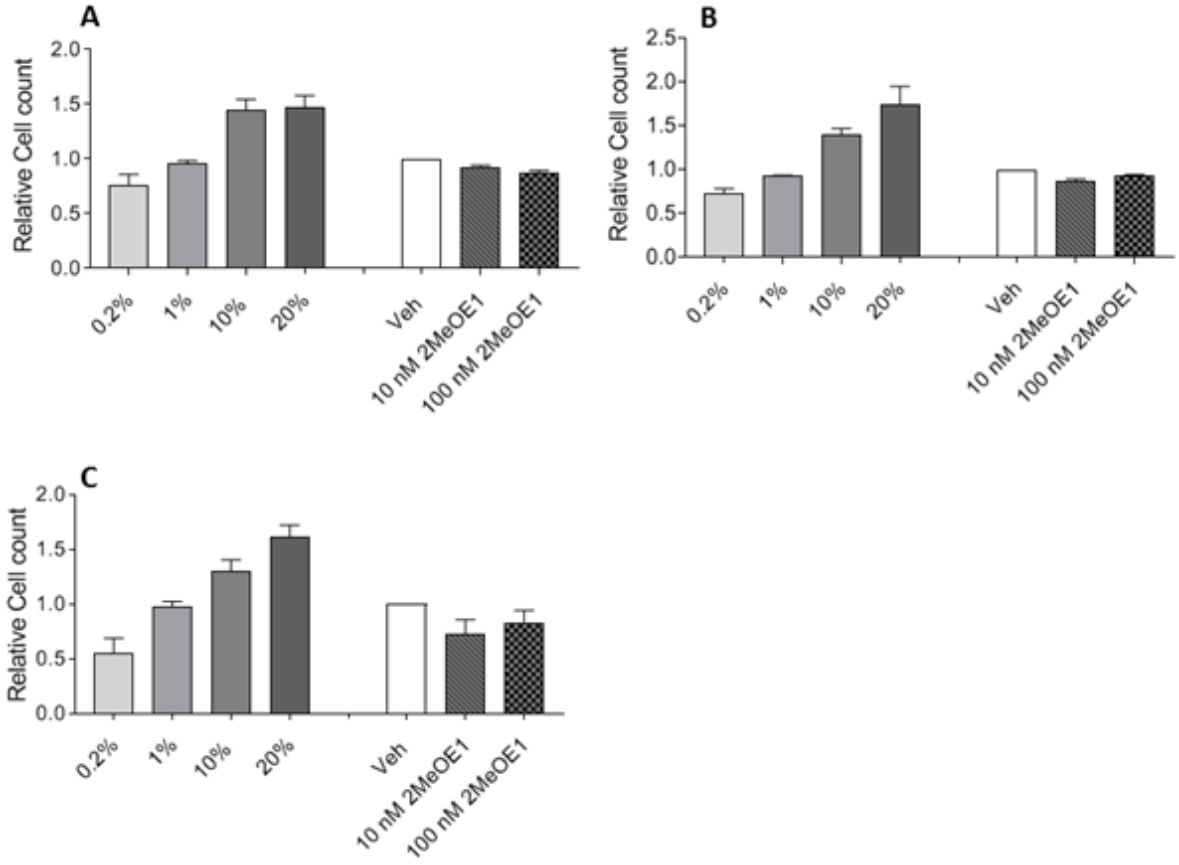

Figure 3-17: Effect of 2MeOE1 on cellular proliferation of human PASMC Effect over 48 hours in female (A), male (B) control and female PAH (C) hPASMCs. Experiments performed in 1% FBS over 48 hours with data presented relative to the vehicle and as the mean ± SEM. Vehicle (Veh), 0.2% (0.2% FBS), 1% (1% FBS), 10% (10% FBS), 20% (20% FBS). Statistical analysis determined by comparing the estrogen stimulations to the relative vehicle group by a Dunnett's multiple comparison. N=3 per experiment.

No proliferative effect was displayed upon stimulation with 2MeOE1 in female or male control PAMCs or female PAH PASMCs (Figure 3-17). This result is also the same as those from countess automated counting in section 1.5.4.4, suggesting 2MeOE1 bears no proliferative function and may be inactive in PASMCs.

3.5 Discussion

PAH is more common in females than males, which has implicated estrogen in PAH pathobiology. However, the role of estrogen, its bioactive metabolites and the molecular events governing their effects remain obscure. Understanding sex differences in the pathobiology of PAH may suggest new treatment strategies for sub-groups of clinical phenotypes. Estrogen or its metabolites may be biomarkers of disease and therefore could lead to development of personalised or stratified medicines targeting this pathway. If successful, these metabolic markers would aim to target patient interventions at an earlier stage of disease progression. (Savale *et al*, 2018). It is therefore of high importance to quantify estrogen in human bio-fluids for biomarker discovery and disease monitoring among clinical cohorts. We challenged the concept that estrogen metabolism might be altered in PAH patients. Here, we have established that our novel and validated LC-MS/MS approach may be used as a platform for quantification of estrogen and bioactive metabolite profiles in human serum. We therefore provide preliminary evidence of abnormal estrogen metabolism in PAH patients. In addition, we also demonstrate that 16OHE2 causes proliferation of female rat and female PAH PASMCs with no effect in male cells, suggesting a potentially important sex-dependent role.

Estrone and estradiol are metabolised by a variety of CYP enzymes to form both pro- and anti-proliferative metabolites. Inhibition of the CYP1B1 enzyme has been shown to inhibit cellular proliferation via a reduction in estrogen metabolism (White *et al*, 2012; Hood *et al*, 2016). Therefore, bioavailability of estrogen and the bioactive metabolites in the systemic circulation may influence tissue specific actions in the pulmonary circulation. This LC-MS/MS Study demonstrates an increased E2:E1 ratio in male PAH in comparison to pooled controls. In breast cancer studies, this ratio is often used as a marker of 17βHSD activity, increased activity of 17β-HSD1 shown by an elevated ratio is associated with increased levels of E2 and increasing disease risk among postmenopausal women (Gunnarsson *et al.*, 2008). Additionally in lung cancer and endometriosis the same trend has become apparent (He *et al.*, 2016). Therefore, in PAH the same hydroxysteroid dehydrogenase activity might reveal an interesting dynamic in sex-dependent estrogen synthesis upon comparison to real world controls.

Here we provide preliminary evidence that elevated serum concentrations of 16OHE1, 16OHE2 and 2MeOE1 may be present in PAH patients. 16OHE1 was elevated specifically in female PAH. However the lack of real-world control samples and low n numbers within this group makes interpretation of this observation difficult. Previous studies have shown 16OHE1 may induce vascular proliferation of hPASMCs via Nox-induced reactive oxygen species production in human PASMCs (Hood *et al*, 2016). The NOX family of NADPH (nicotinamide adenine dinucleotide phosphate-oxidase) oxidases produce superoxides such as reactive oxygen species (ROS) which have been implicated in pulmonary hypertension. There are five NOX subunits, with Nox1 and Nox4 expression linked directly to PAH pathogenesis (Hood *et al.*, 2017). ROS are important regulators of vascular tone and function (Sanders and Hoidal, 2007). Induction of ROS generation *in-vitro* has been linked to proliferation in previous studies of systemic arterial smooth muscle cells and PASMCs, with suppression of endogenous ROS production promoting apoptosis (DeMaio *et al.*, 2001; Brennan *et al.*, 2003). Studies in humans demonstrate that patients with idiopathic PAH have increased oxidation to superoxides (Galiè, Humbert, *et al.*, 2015). In monocrotaline animals, enhanced NADPH-oxidases increased superoxide production which in the last stages of PAH was produced by neutrophils (Semenkova, Adzerikho and Yatsevich, 2016). In hypoxia models, ROS also mediate disease development and are implicated in vascular remodelling, RV hypertrophy and right heart failure as a result of PAH (Ma *et al.*, 2016). 16OHE1 is also produced in visceral adipose tissue of obese male mice contributing to oxidative stress and vascular proliferation (Mair *et al.*, 2019).

In the current study 16OHE2 and 2MeOE1 are present at higher abundance in both male and female PAH patients in comparison to pooled controls. In males, a significant elevation of estradiol in the PAH group over controls suggests increased bioavailability for conversion to metabolites such as 16OHE2. This may be of interest as recent studies have implicated higher estradiol levels in male PAH, where a 50-fold increased risk of PAH occurred for every 1 unit increase in E2 (Corey E Ventetuolo *et al*, 2016). These findings were earlier linked to postmenopausal women with elevated E2 in PAH patients in comparison to age-matched controls. The elevated levels were also associated with shorter 6MWD and a worsened functional PAH class (Ventetuolo *et al.*, 2011). It is difficult to make this comparison here as age matched controls were not collected However, our study demonstrated

successful detection in patient samples. Therefore, further analysis of extended cohorts in Chapter 4 may provide an insight into these associations.

Since 16OHE2 and 2MeOE1 were abundant in PAH serum and relatively undefined within the literature, an *in vitro* assessment of their proliferative effect in PASMC explanted from rats and humans was investigated. Intriguingly, our *in-vitro* analysis of pulmonary artery smooth muscle cells demonstrated a proliferative effect of 16OHE2 in only female rat cells and in female PAH human cells. No effect was seen in male rat, male human control or female human control cell lines. For the 2MeOE1 studies, this metabolite displays no proliferative or anti-proliferative function in all cell lines tested. As these findings are duplicated between two independent cell counting methods this strengthens the possibility that 16OHE2 may exert a pathogenic influence in PAH pathophysiology and should be further investigated both *in-vivo* and *in-vitro*. In additional proof of concept studies from our lab we have demonstrated a sex-dependent functional effect of 16OHE2 in blood outgrowth endothelial cells (BOECs) (Denver *et al*, 2018). We suggested that 16OHE2 may induce vascular proliferation via dysregulated aryl hydrocarbon receptor (AhR) signalling. The molecular function governing 16OHE2-mediated proliferation differs in male and female BOECs, with increased proliferation observed in female PAH-BOECs and no effect in male cells. In addition, 16OHE2 also induced migration of male PAH-BOECs by 81%, identifying this metabolite as a potential modulator of disease in multiple cell types of importance in PAH. Biological inactivity of 2MeOE1 has been reported in the literature (Tofovic, 2010) which is consistent with our observation that 2MeOE1 did not induce proliferation in female or male cells. Therefore, its elevation in PAH individuals remains intriguing. Functionally, increased 2MeOE1 could be produced via interconversion with 2MeOE2 via 17βHSD enzymes. This conversion would reduce the anti-proliferative effects of 2MEOE2 which have recently been reported as a result of HIF1α disruption (Docherty *et al*, 2019). Intriguingly in cancer studies 17βHSD2 activity promotes conversion of the anti-cancerous agent 2MeOE2 by oxidation to 2MeOE1, also an inactive metabolite in this setting (Newman *et al.*, 2006). Consequently, 2MeOE1 accumulation presents a potential route of disease acceleration via decreases in protective 2MeOE2 concentrations. Secondly, in alternative research fields conversion of inactive 2-methoxyestrone to active steroid hormones has also been reported (Axelson and Sjövall, 1983). This demethylation to mitogenic 4-

hydroxyestrogens should not be excluded as a possibly pathway to PAH upon excess synthesis of 2MeOE1.

3.5.1 Study limitations

The LC-MS/MS studies are limited firstly by a lack of control serum. Here, purchased pooled samples have undergone extensive preparation steps with filtering prior to shipment. The samples were also thawed an additional time to aliquots for analysis. Therefore, the levels in our control cohort do not represent a healthy individual profile. In addition, small sample sizes lower the power of this study for statistical analysis. This factor made comparisons between BMI and additional patient characteristics difficult as this information was not available for all samples. Female menstrual cycle was also not taken into account within the sample collection which may affect the distribution of estradiol concentrations among female patients. Therefore, extension to a wider cohort of serum samples with individual controls would allow an extensive comparison of male and female estrogen levels. Also, further studies might demonstrate if the bioactive metabolites detected are present in wider clinical cohorts. For cell studies, it would be of interest to group patients into the associated PAH classification for tests (i.e. associated PAH vs heritable PAH and idiopathic PAH) and to study male PAH if cell lines become available to facilitate such comparisons.

Chapter 4 Estrogen Metabolism in Patients with Pulmonary Arterial Hypertension (PAH)

4.1 Introduction

Increased endogenous estrogens represent a risk factor in PAH. There is a female predominance in both heritable (hPAH) and idiopathic (iPAH) cases. Heritable PAH (hPAH) is associated with haplo-insufficiency of the bone morphogenetic protein receptor II (BMPR2) gene leading to pulmonary remodelling, but many cases are idiopathic (iPAH) with the underlying cause unknown. Both disease classifications, iPAH and hPAH share pathophysiological features characterised by increased pulmonary vascular resistance and pulmonary artery remodelling as a result of increased vascular tone and cellular proliferation (Machado *et al.*, 2015). A mutation in the *BMPR2* gene underpins the majority of hPAH cases (>80%) but remains implicated in a lesser number of iPAH (6-40%) cases. Females with this mutation are far more likely to develop hPAH than males, with 43% of females compared to 14% of males with a *BMPR2* mutation developing PAH in their lifetime. As a result, the gender dimorphism is well established in both idiopathic and heritable aetiology suggesting a common upstream pathway. The sex paradox may be linked to estrogen metabolism, with mitogenic 16-hydroxylated metabolites promoting disease whilst metabolism via the 2 or 4- hydroxylation pathways presents a less harmful route (West *et al*, 2008; Austin *et al*, 2009; White *et al*, 2012). Indeed, estrogen metabolism to 16-hydroxyestrogens has been linked to exacerbation of BMPR2 associated PAH via modulation of micro RNA signalling (Chen *et al.*, 2016). In this study, 16-hydroxyestrone formation promoted hPAH via miR-29 upregulation within the lung suggesting estrogens may account for the gender disparity among females. The pathogenic effects of 16-hydroxyestrone production were successfully reversed upon treatment with aromatase inhibitors and ER antagonists (X. Chen *et al.*, 2017). In another study within pulmonary artery smooth muscle cells (PASMCs) from females, estrogen-driven suppression of BMPR2 signalling contributed toward a proliferative phenotype in non-PAH individuals (Mair *et al.*, 2015). This may predispose females to development of disease. Large-scale genomic studies may also provide valuable data to unravel the sex based disparity in PAH implicating a number of novel genes in disease such as *TBX4* (Morrell *et al.*, 2019). This gene may be of interest, recently being linked to expression of CYP1B1, modulating PAH progression (Kielt et al., 2019). In lung fibroblasts *TBX4* is found in higher abundance than other cell types regulating a number of pathways known to be involved in PAH pathogenesis further implicating this in *CYP1B1* mediated PAH (Yan *et al.*, 2019). Dysregulated estrogen metabolism between hPAH and iPAH

patients may present further evidence toward defining the sex paradox in PAH. Therefore, simultaneous analysis of estrogen and the bioactive metabolites in bio-fluids from both hPAH and iPAH patients might reveal differences in systemic circulating levels leading to a wider action in PAH progression.

Evaluation of systemic circulating levels of estrogens in patients from different PAH classifications might clarify the role of estrogen in PAH. In the previous chapter, application of the novel LC-MS/MS assay identified a number of metabolites to be elevated in patients in comparison to controls. Therefore, we wished to conduct a pilot study comparing control, heritable and idiopathic PAH in real-world subjects to further define concentration ranges.

4.2 Aim

We hypothesised the estrogen metabolite profile is altered in PAH, in particular among idiopathic PAH subjects contributing to disease phenotype. In this chapter we aim to further investigate circulating estrogens in heritable and idiopathic PAH patients against control samples from healthy non-PAH individuals. Estrogen concentrations will be quantified in serum using our LC-MS/MS assay to determine if dysfunctional estrogen metabolism is present in PAH.

4.3 Methods

4.3.1 Study samples

The study serum samples were obtained from Vanderbilt Medical Centre, Nashville, USA in collaboration with Dr Eric Austin. We performed a ‘proof-of-concept’ study on serum from 12 males with idiopathic PAH (iPAH), 15 males with heritable PAH (hPAH) and 18 male controls (patients without clinical pathology). Serum for 11 women with iPAH, 14 women with hPAH and 17 female controls (42%, 58% & 82% premenopausal, respectively) were also analysed (Table 4-1). Samples were collected with ethical approval and anonymous information on patient demographics and clinical characteristics were derived from medical records and from phone calls.

4.3.2 LC-MS/MS Analysis

Serum samples were subject to LC-MS/MS analysis, as described previously in Chapter 2 (Section 2.3.4).

4.3.3 Analytical Criteria

Percentage differences of the two batches were calculated in calibration standards at the LOQ and for endogenous E1 and E2 in pooled serum samples. A number of alterations were applied in this method to aid in the detection of low level serum metabolites (as in Chapter 6):

- 10% water was added to the acetonitrile mobile phase to improve robustness preventing damage to pump seals.

- Sample transfer steps were removed within the preparation to reduce variability

- One additional point (4 pg/mL) was added to the standard curve to improve quantitation of metabolites at present in low levels.

4.3.4 Data analysis

Plasma concentrations were non-normally distributed with rank order mean values compared using a Mann Whitney test for two groups and a Kruskal-Wallis test with Bonferroni post-hoc analysis for comparison of more than two groups. Correlations between estrogen levels, BMI and age were assessed using Pearson correlations. Data were analysed using SPSS 25 statistical software.

4.4 Results

4.4.1 Patient information

Table 4-1: Clinical characteristics of the Non-PAH controls and heritable or idiopathic PAH patients

Clinical Characteristics	Female Controls	Female hPAH	Female iPAH	P-Values	Male Controls	Male hPAH	Male iPAH	P-Values
Subjects	17	13	11		17	14	12	
Age (y)	38 ± 13.3	43.0 ± 14.3	54 ± 13.8	0.07	35 ± 9.7	40.0 ± 16.7	53 ± 11.7	**0.005**
BMI ($kg \cdot m^{-2}$)	27 ± 7.6	32.6 ± 6.0	30 ± 5.9	0.46	28 ± 8.6	28.8 ± 4.0	31 ± 4.2	0.10
Right Atrial Pressure (mmHg)	-	10.6 ± 8.1	6.7 ± 5.2	0.32	-	11.0 ± 6.6	10.3 ± 5.4	0.83
mPAP (mmHg)	-	59.3 ± 15.8	52.3 ± 15.2	0.41	-	59.5 ± 12.6	49.8 ± 17.6	*0.06*
Cardiac Output (L/min)	-	3.7 ± 1.3	5.6 ± 2.2	**0.03**	-	4.1 ± 0.8	5.0 ± 1.2	**0.05**
PCWP/PAWP (mmHg)	-	9.3 ± 4.5	10.7 ± 5.6	0.68	-	13.2 ± 4.8	14.8 ± 9.7	0.62
PVR (mmHg/l·min)	-	16.2 ± 8.9	9.0 ± 5.9	0.09	-	11.8 ± 4.2	8.6 ± 5.0	0.12
BNP Value (pg/mL)	-	332.5 ± 372.0	132.0 ± 126.3	0.28	-	255.2 ± 439.6	283.1 ± 450.2	0.88

Pulmonary arterial hypertension, PAH; idiopathic PAH, iPAH; Mean pulmonary artery pressure, mPAP; Pulmonary Artery Capillary Pressure/Pulmonary artery wedge pressure, PCWP/PAWP; Pulmonary Vascular Resistance, PVR; B-type natriuretic peptide, BNP; Clinical characteristics shown as mean ± SD. P-values following a Mann Whitney test in control vs iPAH or female vs male clinical characteristics.

Patient information was compared due to differences observed between estrogen concentrations and age in LC-MS/MS analysis (section 4.4.3.5). In females, there was no difference in age between control, hPAH and iPAH groups. However, the iPAH males were older than the male controls. Between each sex, there was no difference in the right heart catheterisation data. Increased cardiac output was displayed on comparison of hPAH to iPAH in females and males.

4.4.2 Analytical validation

Samples were analysed in two LC-MS/MS batches. Therefore, the percentage accuracy and precision difference were assessed at the LOQ for analytical criteria to be met prior to data analysis (Table 4-2). In preparing these samples, a transfer step was removed following elution of estrogen from the SPE column in an attempt to lower the variability at lower concentrations and to improve detection of low-level metabolites. The upper limit of quantification was 200 pg/mL for all estrogens. Revalidation at 2 pg/mL for estrogen metabolites was performed in Chapter 6 (Section 6.3.3, 6.4.2) when sufficient batches had been collated for inter-assay consideration.

Table 4-2: Inter-day precision and accuracy of analytical batches

Estrogen-MPPZ	IS	LOQ (pg/mL)	% Accuracy Difference	% Precision Difference	Mean R (n=2)
E1	$^{13}C_3$E1	2	1.6	14.0	0.998
17αE2	$^{13}C_3$E2	2	6.7	4.5	0.997
17βE2	$^{13}C_3$E2	2	3.9	1.4	0.998
16OHE1	$^{13}C_3$-16OHE2	6	10.0	6.7	0.996
16OHE2	$^{13}C_3$-16OHE2	2	6.6	8.0	0.998
2MeOE1	$^{13}C_6$-2MeOE1	2	17.0	8.0	0.987
4MeOE1	$^{13}C_6$-2MeOE1	6	11.8	16	0.996
2MeOE2	$^{13}C_6$-4MeOE2	6	1.6	2.4	0.998
4MeOE2	$^{13}C_6$-4MeOE2	6	4.6	1.8	0.999

E1; Estrone E2; Estradiol; 16-Hydroxyestrone, 16OHE1; 16-Hydroxyestradiol, 16OHE2; 2-Methoxyestrone, 2MeOE); IS = Internal Standard; LOQ, lower limit of quantitation.

A quality control sample was also run within each batch to confirm reproducibility of the signal response between days of sample preparation and LC-MS/MS analysis. These samples were composed of pooled female and male serum (Table 4-3).

Table 4-3: Inter-day quality control: Blank serum

	Estrogen-MPPZ	Batch 1 Concentration (pg/mL)	Batch 2 Concentration (pg/mL)	% Batch Difference
Female	E1	30.17	32.55	5.4
	17βE2	22.25	27.43	14.7
Male	E1	19.39	21.22	6.4
	17βE2	8.71	10.62	14.0

E1; Estrone E2; Estradiol; RSD, Relative standard deviation.

Metabolites were not detected routinely detected in these pooled samples as expected.

4.4.3 LC-MS/MS Analysis

E1, E2, 16OHE1, 16OHE2 and 2MeOE1 were detected within serum samples. 2MeOE2 was detected in one female control and 3 female hPAH patients but this was not progressed further in the analysis due to low sample numbers. 17αE2, 4MeOE1 and 4MeOE2 were not detected in this cohort.

4.4.3.1 Estrogen Concentrations and Disease Status

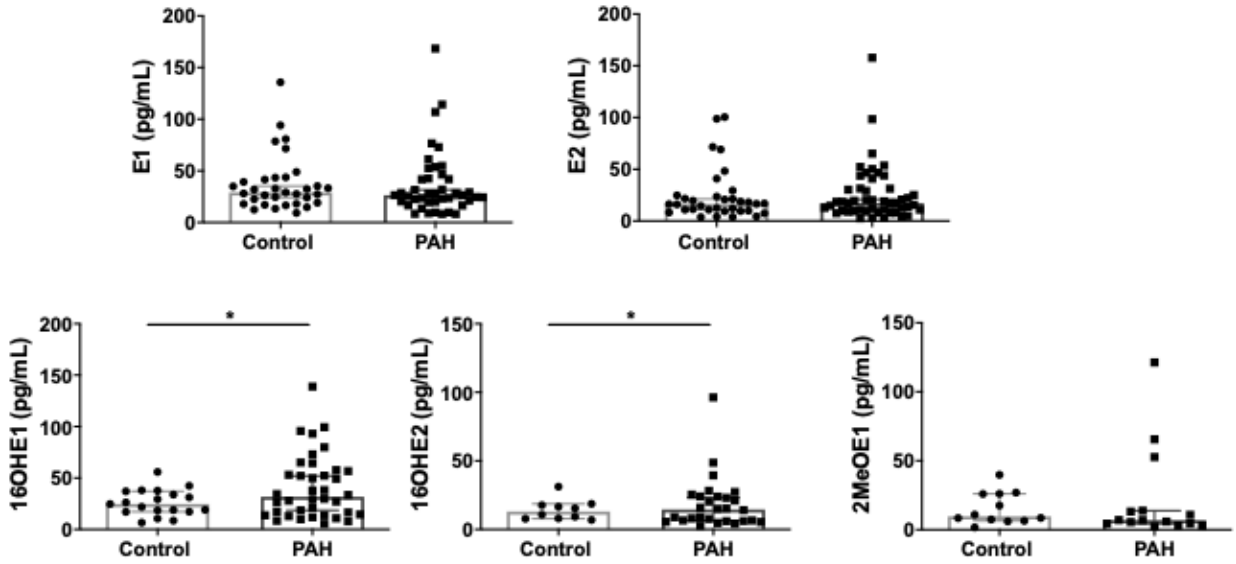

Figure 4-1: Estrogen concentrations and disease status Estrone, estradiol and bioactive estrogen metabolites were detected in controls and PAH patients. Estrone, E1; Estradiol, E2; 16-Hydroxyestrone, 16OHE1; 16-Hydroxyestradiol, 16OHE2; 2-Methoxyestrone, 2MeOE1. Data shown as median ± 95% confidence Interval with statistics following a Mann Whitney test, *p<0.05. n=34, Controls; n=40, PAH.

Upon comparison of controls to PAH, concentrations of 16OHE1 (p=0.03) and 16OHE2 (p=0.02) were increased in the PAH cohort (Figure 4-1).

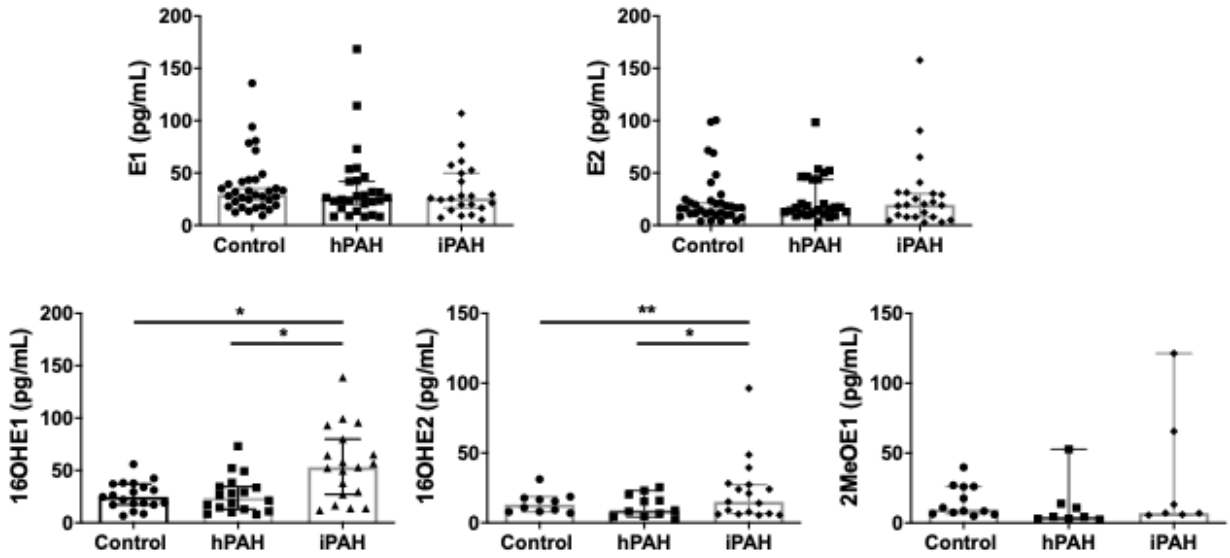

Figure 4-2: Estrogen concentrations and clinical phenotype Estrone, estradiol and bioactive estrogen metabolites were detected in controls and PAH patients. Estrone, E1; Estradiol, E2; 16-Hydroxyestrone, 16OHE1; 16-Hydroxyestradiol, 16OHE2; 2-Methoxyestrone, 2MeOE1. Data shown as median ± 95% confidence Interval with statistics following a Kruskal Wallis with Bonferroni post hoc test, *p<0.05, **p<0.01. n=34 Control, n=27 hPAH, n=23 iPAH.

Following stratification of clinical phenotypes between heritable (hPAH) and idiopathic (iPAH) disease (Figure 4-2), the 16-hydroxymetabolites were found to be elevated among iPAH subjects on comparison to controls (p=0.002, 16OHE1; p=0.001, 16OHE2). iPAH had higher concentrations compared with hPAH patients (p=0.02, 16OHE1; p=0.01, 16OHE2).

4.4.3.2 Estrogen Concentrations, Sex and Disease Status

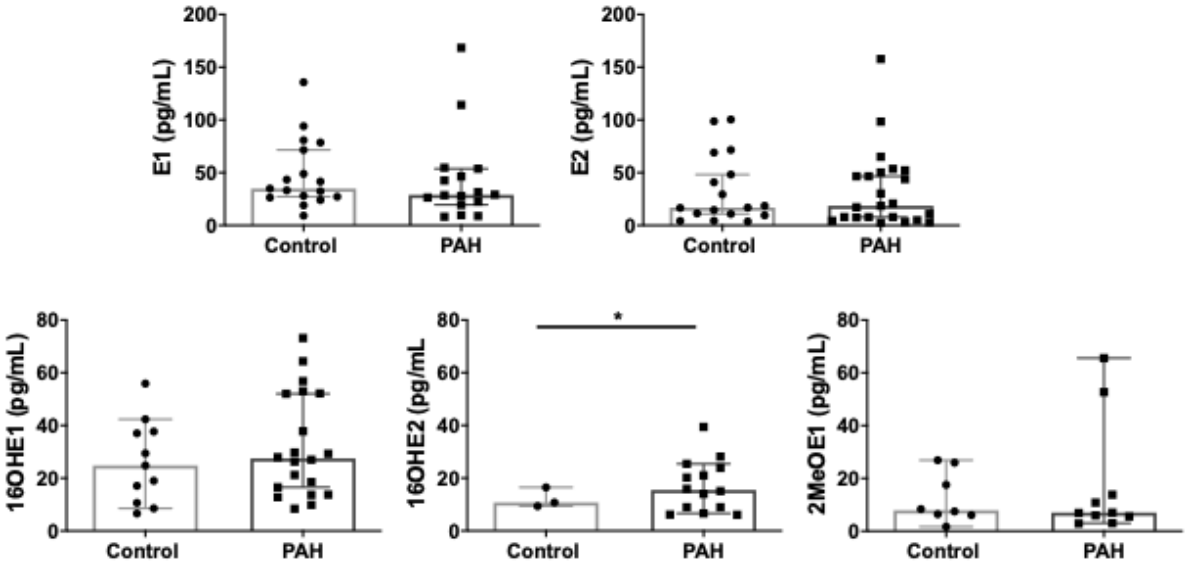

Figure 4-3: Estrogen concentrations in female controls *vs* PAH Estrone, estradiol and bioactive estrogen metabolites were detected in controls and PAH patients. Estrone, E1; Estradiol, E2; 16-Hydroxyestrone, 16OHE1; 16-Hydroxyestradiol, 16OHE2; 2-Methoxyestrone, 2MeOE1. Data shown as median ± 95% CI with statistics following a Mann Whitney test, *p<0.05. n=17, Control; n=24, PAH.

In female PAH, there was an increase in 16OHE2 concentrations compared with controls (Figure 4-3).

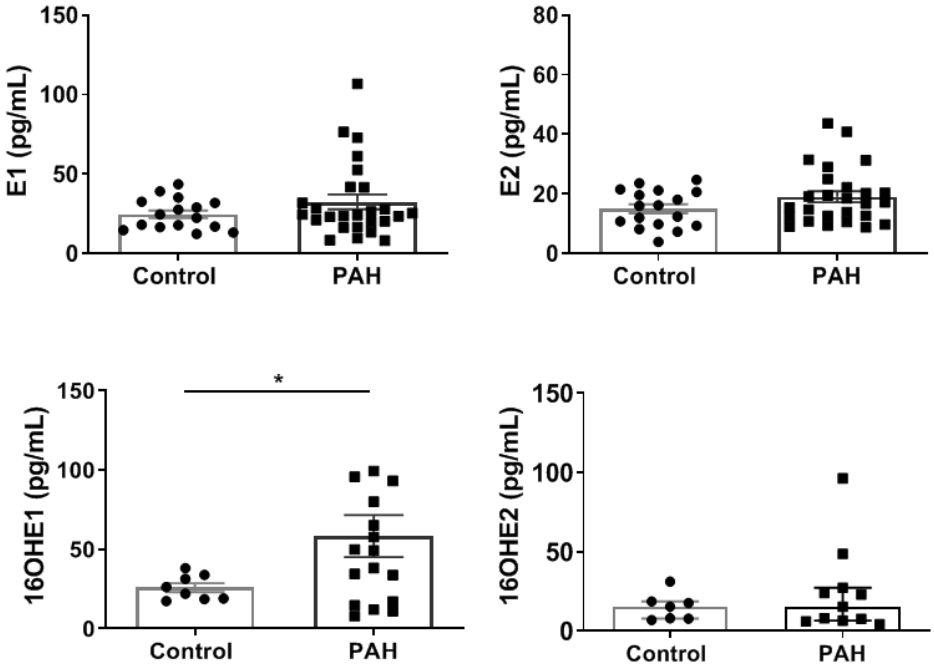

Figure 4-4: Estrogen concentrations in male controls *vs* PAH Estrone, estradiol and bioactive estrogen metabolites were detected in controls and PAH patients. Estrone, E1; Estradiol, E2; 16-Hydroxyestrone, 16OHE1; 16-Hydroxyestradiol, 16OHE2; 2-Methoxyestrone, 2MeOE1. Data shown as median ± 95% confidence Interval with statistics following a Mann Whitney test, *p<0.05. n=17, Control; n=26, PAH.

In male PAH, there was an increase in 16OHE1 concentrations compared with controls. In males, 2MeOE1 was only detected in one PAH patient therefore the data were not presented here for comparisons (Figure 4-4).

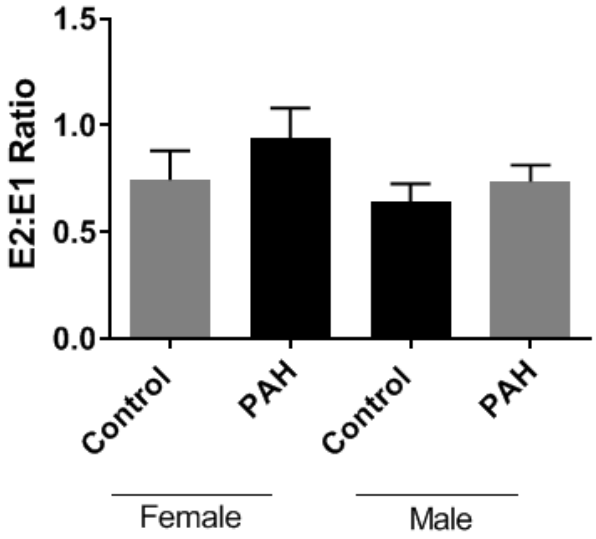

Figure 4-5: E2:E1 ratio as a marker of 17βHSD activity in control and pulmonary arterial hypertension (PAH), females and males Estradiol, E2: Estrone, E1 ratio in control and PAH patients of females and males. Data expressed as mean ± SEM with statistics following a Kruskal Wallis multiple comparison test. Females, n=17, Control; n=24, PAH; Males, n=17, Control; n=26, PAH.

There were no changes in the E2:E1 ratio in female and male PAH patients in comparison to controls (Figure 4-5).

4.4.3.3 Estrogens, Sex and Clinical Classification

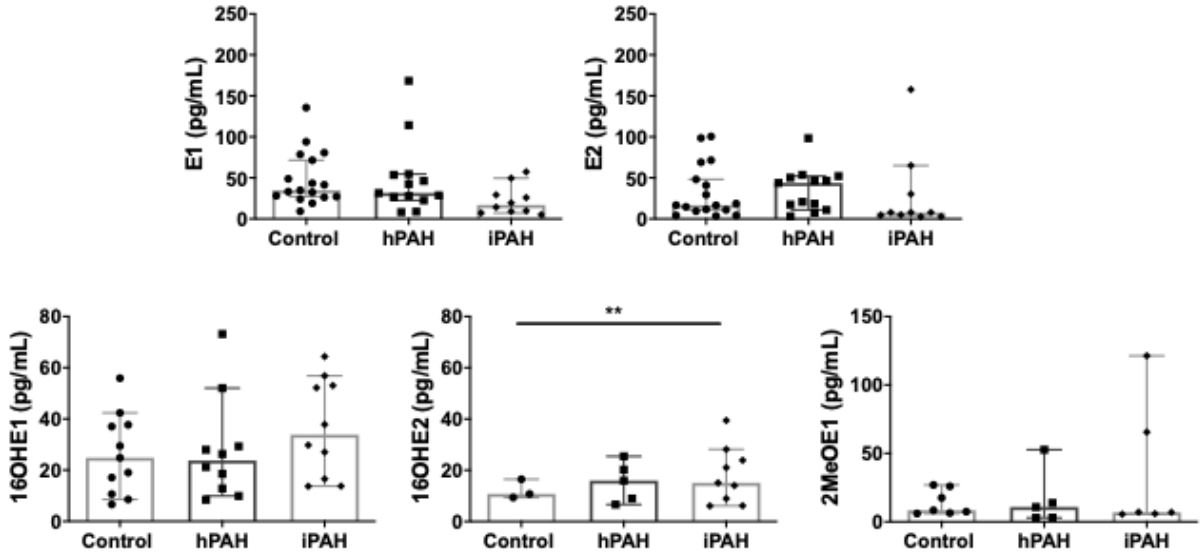

Figure 4-6: Estrogen concentrations in female PAH classifications Estrone, estradiol and bioactive estrogen metabolites were detected in controls and PAH patients. Estrone, E1; Estradiol, E2; 16-Hydroxyestrone, 16OHE1; 16-Hydroxyestradiol, 16OHE2. Data shown as median ± 95% Confidence Interval with statistics following a Kruskal-Wallis test, *p<0.05. n=13 Control, n=13 hPAH, n=11 iPAH.

Upon stratification between heritable and idiopathic disease in females 16OHE2 was shown to be elevated in the iPAH group in comparison to controls (Figure 4-6).

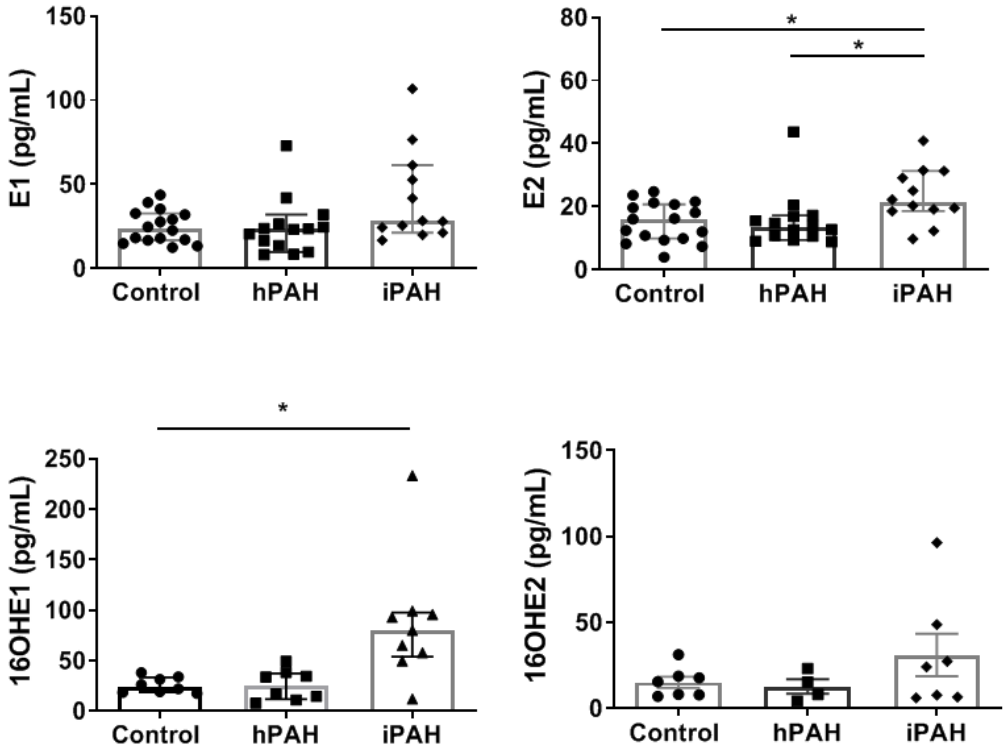

Figure 4-7: Estrogen concentrations in male PAH classifications Estrone, estradiol and bioactive estrogen metabolites were detected in controls and PAH patients. Estrone, E1; Estradiol, E2; 16-Hydroxyestrone, 16OHE1; 16-Hydroxyestradiol, 16OHE2; 2-Methoxyestradiol, 2MeOE2. Data shown as median ± 95% Confidence Interval with statistics following a Kruskal-Wallis test, *p<0.05. n=17, Control; n=14, hPAH; n=12 iPAH.

Upon stratification between heritable and idiopathic disease in males (Figure 4-7), E2 concentrations were found to be higher in the iPAH group in comparison to controls and hPAH patients. For the bioactive metabolites, 16OHE1 was elevated in iPAH in comparison to controls. Data here also suggest that in hPAH, profiles of all estrogens and metabolites resemble those of controls, whereas, for iPAH, E2 and 16OHE1 may be biomarkers of male idiopathic disease.

Table 4-4: Estrogen concentrations, sex and clinical classification

Female	Controls	hPAH	iPAH	Overall P-Values
Subjects	17	13	11	
E1	48.8 ± 8.0 (100)	48.8 ± 12.5 (100)	22.7 ± 5.7* (90)	**0.03**
E2	33.5 ± 8.0 (100)	36.1 ± 7.3 (100)	34.7 ± 15.1 (100)	0.39
16OHE1	26.3 ± 4.7 (65)	28.0 ± 6.4 (77)	36.5 ± 6.0 (91)	0.11
16OHE2	12.2 ± 2.2 (18)	15.4 ± 3.5 (39)	18.1 ± 3.7*** (82)	**0.004**
2MeOE1	14.1 ± 3.5 (41)	16.6 ± 9.3 (39)	35.40 ± 19.7 (55)	0.70
Male	**Controls**	**hPAH**	**iPAH**	**Overall P-Values**
Subjects	17	14	12	
E1	24.8 ± 2.4 (94)	24.6 ± 4.5 (100)	41.9 ± 8.0† (100)	*0.08*
E2	15.1 ± 1.5 (100)	15.5 ± 2.4# (100)	23.3 ± 2.5* (100)	**0.02**
16OHE1	25.8 ± 2.8 (47)	23.47 ± 5.2† (64)	76.8 ± 12.0* (75)	**0.02**
16OHE2	15.1 ± 3.3 (41)	9.7 ± 3.2 (43)	27.7 ± 11.2 (67)	0.23
2MeOE1	18.0 ± 6.5 (29)	3.7 ± 0.6 (21)	13.2 (8)	0.35

Comparison of estrogen concentrations in controls vs hPAH vs iPAH: Pulmonary arterial hypertension, PAH; hPAH, heritable PAH; iPAH, idiopathic PAH; Not detected, ND; Estrone, E1; Estradiol, E2; 16-Hydroxyestrone, 16OHE1; 16-Hydroxyestradiol, 16OHE2; 2-Methoxyestrone, 2MeOE1. Data shown as Mean ± SEM (% Detection by LC-MS/MS). P-values show overall Kruskal wallis values, post hoc test were performed between groups where *indicates $p<0.05$ and **$p<0.01$ in iPAH vs Control, #indicates $p<0.05$ in iPAH vs hPAH and †indicates trends between iPAH and hPAH groups $p\leq0.06$.

In summary, E1 concentrations were decreased in PAH compared to control whilst 16OHE2 was increased in iPAH patients compared with controls and hPAH. In males, E2 was elevated in iPAH compared with controls and hPAH, while 16OHE1 concentrations were elevated in iPAH patients compared with controls. No changes between control, hPAH and iPAH were detected for E2, 16OHE1 and 2MeOE1 in females or 16OHE2 and 2MeOE1 in males.

4.4.3.4 Abnormal Estrogen Metabolism in idiopathic PAH

It became apparent from the analysis in section 4.4.3.3 that hPAH profiles were similar to those of controls. Therefore, to directly assess the circulating estrogens in iPAH patients, we excluded the hPAH group from the analysis (Figure 4-8, Figure 4-9, Table 4-5).

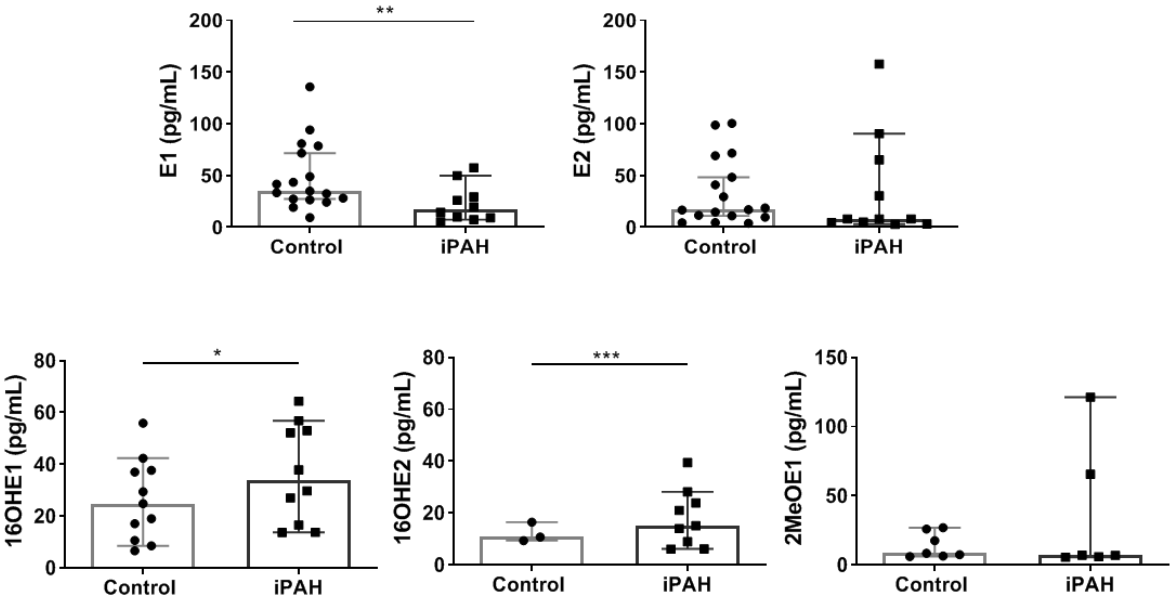

Figure 4-8: Estrogen and female idiopathic PAH Estrone, estradiol and bioactive estrogen metabolites were detected in controls and PAH patients. Estrone, E1; Estradiol, E2; 16-Hydroxyestrone, 16OHE1; 16-Hydroxyestradiol, 16OHE2; 2-Methoxyestradiol, 2MeOE2. Data shown as median ± 95% Confidence Interval with statistics following a Mann Whitney test, *p<0.05, **p<0.01, ***p<0.001. n=17, Control; n=14, hPAH; n=12, iPAH.

In female idiopathic PAH E1 was reduced and 16-hydroxylation to 16OHE1 and 16OHE2 was elevated (Table 4-5). On comparison of iPAH to hPAH patients, E1 remained reduced with only 16OHE2 elevated between the groups.

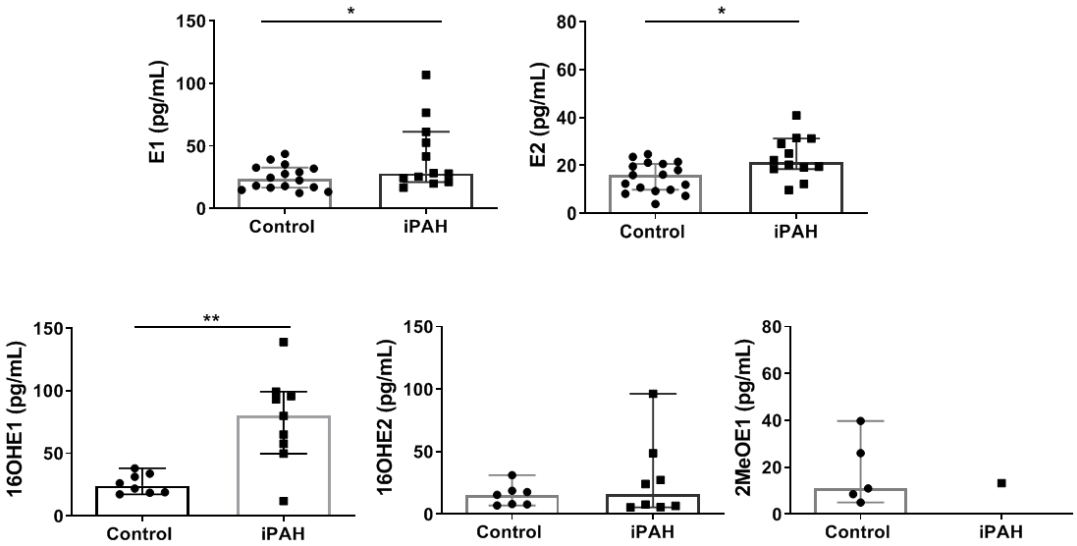

Figure 4-9: Estrogen and male idiopathic PAH Estrone, estradiol and bioactive estrogen metabolites were detected in controls and PAH patients. Estrone, E1; Estradiol, E2; 16-Hydroxyestrone, 16OHE1; 16-Hydroxyestradiol, 16OHE2; 2-Methoxyestradiol, 2MeOE2. Data shown as median ± 95% Confidence Interval with statistics following a Kruskal-Wallis test, *p<0.05, **p<0.01. n=17, Control; n=12 iPAH.

In males, E1, E2 and 16OHE1 were elevated in controls in comparison to iPAH (Table 4-5). On comparison of hPAH to iPAH, the same elevations occurred (Table 4-6).

Table 4-5: Clinical characteristics and estrogen concentrations for controls *vs* iPAH patients

Estrogen Levels (pg/mL)	Female Controls	Female iPAH	P-Values	Male Controls	Male iPAH	P-Values
Subjects	17	11		17	12	
E1	48.8 ± 32.8 34.9 [26.8 – 75.1]	22.7 ± 18.2 16.9 [8.5 – 34.3]	**0.01**	24.8 ± 9.7 23.6 [16.8 – 32.5]	41.9 ± 27.7 28.1 [22.0 – 59.2]	**0.05**
E2	33.5 ± 32.6 16.8 [10.2 – 58.6]	34.7 ± 50.2 7.9 [4.4 – 65.1]	0.29	15.1 ± 6.3 16.0 [9.6 – 31.0]	23.3 ± 8.8 21.3 [18.7 – 30.7]	**0.02**
16OHE1	26.3 ± 15.7 24.8 [10.6 – 37.7]	36.5 ± 19.1 33.8 [15.9 – 54.0]	**0.04**	25.8 ± 7.9 24.1 [18.7 – 33.2]	76.8 ± 36.1 79.9 [53.7-97.5]	**0.01**
16OHE2	12.2 ± 3.8 10.7 [9.34 – 10.7]	18.1 ± 11.2 15.1 [7.6 – 26.1]	**0.001**	15.1 ± 8.6 15.4 [7.8 – 18.7]	27.7 ± 31.6 15.9 [5.8 – 43.4]	0.24
2MeOE1	14.1 ± 9.3 8.4 [6.4 – 25.9]	35.40 ± 48.3 7.0 [5.9 – 79.5]	0.63	18.0 ± 14.6 10.9 [6.7 – 32.9]	13.2 ± 0.0 -	0.19

Direct comparison of estrogen concentrations in controls and idiopathic PAH: Pulmonary arterial hypertension, PAH; iPAH, idiopathic PAH; Estrone, E1; Estradiol, E2; 16-Hydroxyestrone, 16OHE1; 16-Hydroxyestradiol, 16OHE2; 2-Methoxyestrone, 2MeOE1. Clinical characteristics shown as Mean (Range) and data as Mean ± SEM and median [Q_1-Q_3]. Kruskal Wallis p-values on comparison of iPAH to controls where *p<0.05, **p<0.01, ***p<0.001.

Table 4-6: Clinical characteristics and estrogen concentrations for hPAH vs iPAH patients

Estrogen Levels (pg/mL)	Female hPAH	Female iPAH	P-Values	Male hPAH	Male iPAH	P-Values
Subjects	17	13		17	14	
E1	48.8 ± 12.5 31.6 [24.5 – 54.2]	22.7 ± 18.2 16.9 [8.5 – 34.3]	**0.05**	24.6 ± 4.5 23.4 [12.6 – 27.8]	41.9 ± 27.7 28.1 [22.0 – 59.2]	**0.05**
E2	36.1 ± 7.3 43.9 [14.2 – 51.2]	34.7 ± 50.2 7.9 [4.4 – 65.1]	0.24	15.5 ± 2.4 13.3 [10.3 – 17.0]	23.3 ± 8.8 21.3 [18.7 – 30.7]	**0.01**
16OHE1	28.0 ± 6.4 23.8 [12.1 – 35.0]	36.5 ± 19.1 33.8 [15.9 – 54.0]	0.12	23.47 ± 5.2 17.1 [9.4 – 36.3]	76.8 ± 36.1 79.9 [53.7-97.5]	**0.02**
16OHE2	15.4 ± 3.5 15.9 [7.7 – 22.9]	18.1 ± 11.2 15.1 [7.6 – 26.1]	**0.05**	9.7 ± 3.2 6.4 [3.8 – 17.2]	27.7 ± 31.6 15.9 [5.8 – 43.4]	*0.08*
2MeOE1	16.6 ± 9.3 10.9 [3.0 – 33.2]	35.40 ± 48.3 7.0 [5.9 – 79.5]	0.36	3.7 ± 0.6 4.3 [2.4 – 4.3]	13.2 ± 0.0 -	0.44

Direct comparison of estrogen concentrations in controls and idiopathic PAH: Pulmonary arterial hypertension, PAH; iPAH, idiopathic PAH; Estrone, E1; Estradiol, E2; 16-Hydroxyestrone, 16OHE1; 16-Hydroxyestradiol, 16OHE2; 2-Methoxyestrone, 2MeOE1. Clinical characteristics shown as Mean (Range) and data as Mean ± SEM and median [Q_1-Q_3]. Kruskal Wallis p-values on comparison of iPAH to controls where *p<0.05, **p<0.01, ***p<0.001.

Upon comparison of controls and idiopathic PAH patients, sex-specific alterations in parent estrogen and metabolite metabolism emerged. In females, E1 concentrations were decreased in iPAH patients. Elevations in 16-hydroxyestrogen formation in iPAH were observed for both 16OHE1 and 16OHE2 in comparison to controls. E1 and E2 were elevated in males with iPAH compared with controls. Elevated 16OHE1 concentrations were also observed in male iPAH patients compared with controls.

4.4.3.5 Estrogen and Ageing

In females, estrogens were detected in both the premenopausal (<54 y) and postmenopausal (≥54 y) groups (Figure 4-10). Age information was not available for 1 control, 1 hPAH and 4 iPAH subjects.

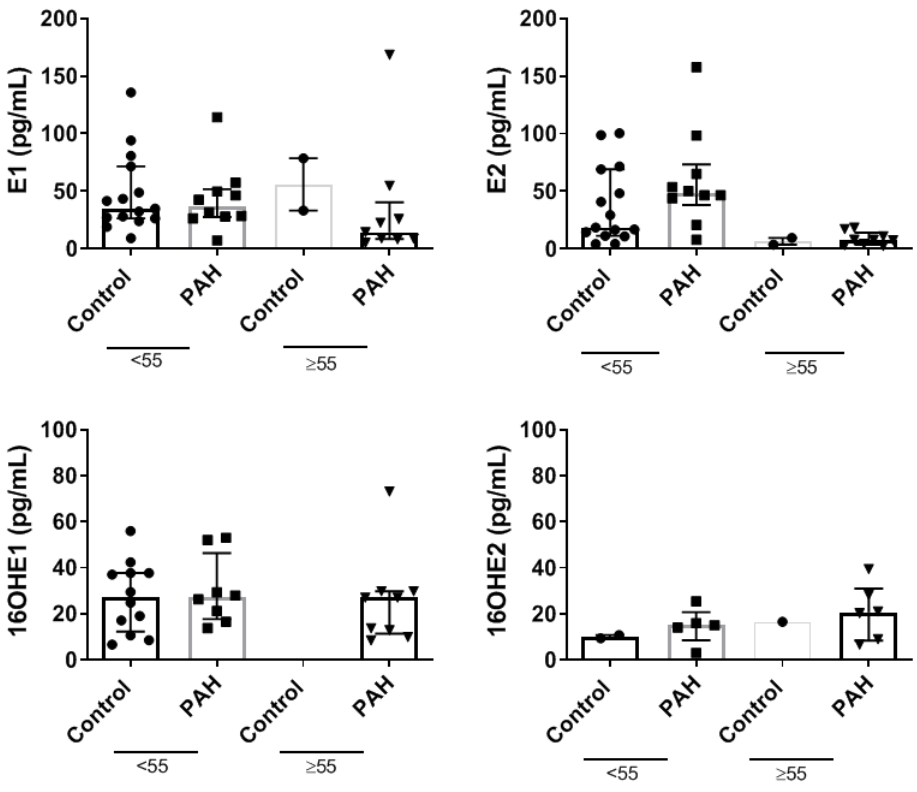

Figure 4-10: Estrogen concentrations and ageing in females Estrone, estradiol and bioactive estrogen metabolites were detected in premenopausal (<55) and postmenopausal (≥55) controls and PAH patients. Estrone, E1; Estradiol, E2; 16-Hydroxyestrone, 16OHE1; 16-Hydroxyestradiol, 16OHE2. Data shown as mean ± SEM. N=14 (<55), 3 (≥55), Control; N=7 (<55), 5 (≥55), hPAH and N=3 (<55), 4 (≥55), iPAH.

In female PAH patients, E2 concentrations decreased following menopausal age as expected. Also, 16OHE1 was not detected in the control group whereas

concentrations between 10 – 80 pg/mL were detected in PAH patients. Applying bivariate analysis there was a correlation between age and 16OHE2 levels in female iPAH patients (R^2 = 0.7, p= 0.04). The evidence presented from this study and in Chapter 3 from UK iPAH samples indicate 16OHE2 as a potentially important metabolite in female PAH. Upon ageing this metabolite may further increase in concentration bearing a mitogenic function in postmenopausal PAH and indeed predisposing females to disease pathogenesis. Therefore, in further studies of plasma in Chapters 5, 6 and 7.it will be of interest to discover if this metabolite is further implicated in female PAH. Within the male cohort, ageing also affects the estrogen concentration profiles (Figure 4-11). Age information was not available for 2 controls and 1 hPAH subject.

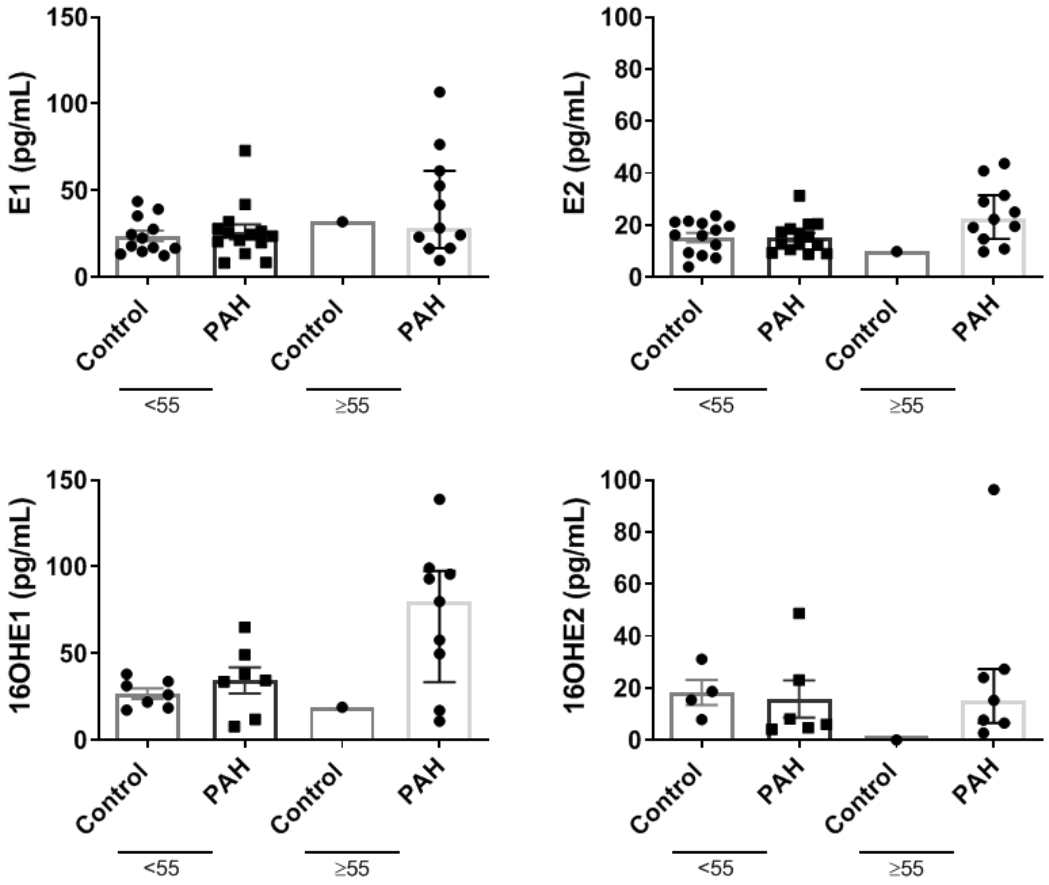

Figure 4-11: Estrogen concentrations and ageing in males Estrone, estradiol and bioactive estrogen metabolites were detected in younger (<55) and older (≥55) controls and PAH patients. Estrone, E1; Estradiol, E2; 16-Hydroxyestrone, 16OHE1; 16-Hydroxyestradiol, 16OHE2. Data shown as mean ± SEM. N=13 (<55), 1 (≥55), Control; N=10 (<55), 3 (≥55), hPAH and N=4 (<55), 8 (≥55), iPAH.

Again, in males, no changes were observed. In older men >55 years, a range of 16-hydroxylated estrogen metabolites was wider in the PAH group on comparison to controls. In younger men <55 years, the mean estrogen concentrations are around

the same level in PAH and controls. The statistical analysis here is underpowered, as only one control subject above the age of 55 years was collected. Therefore, the PAH group was not stratified based on the clinical phenotype for this analysis.

BMI data was available for this cohort, although there was no correlation between increasing BMI and estrogen levels in the control or patient cohort (data not shown). The majority of patients were of a BMI >25, with only one patient below this cut-off, therefore further studies should be conducted to include both lean and obese individuals for comparison.

4.5 Discussion

In this proof of concept study, we display evidence of varying circulating estrogen profiles dependent on sex and subgroup of PAH. Heritable PAH profiles were similar to those of non-PAH controls. Concentrations of estrogen and the bioactive metabolites were altered in idiopathic PAH patients in comparison to both non-PAH controls and hPAH. In comparison to non-PAH controls, E1 was decreased whilst 16OHE1 and 16OHE2 were increased in females, whereas in males E1, E2 and 16OHE1 were increased. In comparison to hPAH, the same pattern occurred in males, whereas in females E1 was reduced with an increase in 16OHE2 concentrations. These results suggest that conversion of parent estrogens toward the 16-hydroxy pathway in females via various CYP enzymes may be of importance. In males, an overall increase in both parent and 16-hydroxyestrogens in males may cause pathogenic effects in idiopathic disease.

Male sex is a risk factor for mortality among PAH patients in two individual registries (Benza *et al.*, 2010; Humbert *et al*, 2010), with around 80% of the population being diagnosed with idiopathic clinical phenotype. However, female sex remains strongly associated with disease susceptibility with a ratio of 4:1 reported in recent studies (Galiè *et al*, 2015). *BMPR2* mutations occur in a large number of heritable PAH and idiopathic PAH, family history of disease is identified in hPAH with no evidence of this history in iPAH. Despite the identification of *BMPR2* mutations in the majority of patients, two clinical studies have been unable to distinguish between carriers from heritable PAH or idiopathic PAH disease classifications (Elliott *et al.*, 2006; Sztrymf *et al.*, 2008). Another study suggests differences may occur in alteration of the *BMPR2/SMAD* signalling axis, resulting in increased proliferation and deficient

apoptosis of PASMCs (Dewachter *et al.*, 2009). Although responses varied dependent on the exact *BMPR2* mutation present, proliferation *in vitro* could be inhibited by BMP4 in idiopathic, but not heritable, patients. Additionally in 2016, Xinping Chen, Eric Austin and colleagues identified that 16OHE1 promotes PAH in heritable *BMPR2* mutation cases via upregulation of miR-29 (Chen *et al.*, 2016). This promotion of hPAH via 16OHE1 associate's disease penetrance with preferential metabolism to the 16-hyroxylation pathway. As a result, analysis of circulating estrogens to further elucidate circulating profiles between heritable and idiopathic PAH patients was desired (White *et al*, 2016). Our LC-MS/MS findings add to this story by displaying patterns in parent estrogens and 16-hydroxylation vary in idiopathic disease but not in heritable.

Estradiol (E2) has been reported as both beneficial and pathogenic in PAH. In male iPAH serum, E2 is increased, a finding consistent with our preliminary study reported in Chapter 3. This is also supported by a previously published study which showed elevated E2 in men with iPAH (Corey E Ventetuolo, Baird, *et al.*, 2016) and a more recent study showing elevated E2 in male patients, which was associated with increased mortality rates in males (Wu *et al.*, 2018). Increased E2 synthesis could be driven by an excess local production of E2 via an abundance of aromatase within the pulmonary circulation (White, Johansen, Nilsen, Ciuclan, Wallace, Paton, Campbell, Morecroft, Loughlin, John D. McClure, *et al.*, 2012a). In postmenopausal females, higher E2 has been associated with better RV systolic function (Ventetuolo *et al.*, 2011) and are reported as elevated iPAH female patients compared with controls (7 *vs* 5 pg/mL) respectively (Baird et al., 2018). Elevated E2 concentrations were also associated with shorter 6MWD and a worsened functional PAH class. The same group also linked a wider action of sex hormone metabolism to PAH via single nucleotide polymorphisms (SNPs) in *CYP1B1,* associating this with RV function, the relationships between RV measures and SNPs identified in PAH may underpin RV failure (Ventetuolo *et al.*, 2016). In our LC-MS/MS study, menstrual variation was not accounted for within the sample collection prior to this study, with reports suggesting estradiol fluctuations are relevant throughout the menstrual cycle in disease (Travis *et al*, 2003). 75% of the females studied here were pre-menopausal with a mean E2 concentration in controls of 34 pg/mL, which might explain why further elevations were not detected, as E2 concentrations fall to <10 pg/mL in postmenopausal women (Chapter 1, Table 1-3). Currently there is no data relating endogenous circulating E2 levels to premenopausal female PAH. A combined study

of female and males adjusted for sex between liver disease controls *vs* portopulmonary hypertension samples shows a specific SNP in the aromatase gene corresponded with increasing estradiol in portopulmonary hypertension (Roberts *et al.*, 2009). Cardioprotective effects of E2 might contribute to the improved survival among females in comparison to males. Improved right ventricular systolic function is improved in healthy and PAH females with higher levels of E2 (Roberts *et al.*, 2009; Ventetuolo *et al.*, 2011). In hypoxia-induced PAH, the protective effects of E2 are mediated by ERα, as shown by inhibition upon receptor inhibition with the 1-methyl-4-phenyl pyridinium (MPP) antagonist (Lahm *et al.*, 2012).

Elevated E1 concentrations in male iPAH patients were identified. Estrone is a more sensitive marker of diabetes in men than estradiol and might also be identified as a useful marker in other diseases (Jasuja *et al.*, 2013). However, in females, E1 concentrations were decreased in iPAH which may suggest increased activity of CYP enzymes and further conversion to bioactive metabolites (Lee *et al.*, 2003). This may be due to already elevated concentrations of E1 among females where no further elevation would be expected or, in fact, could be a sex specific difference in parent estrogen signalling among idiopathic disease, which should be further explored. E1 has to date been regarded as less estrogenic in activity than E2, serving as a reservoir for E2 production. In male control cells, the MacLean group have shown E1 to have no effect on proliferation (White *et al*, 2011), although it may be that in PAH cells E1 can induce accelerated proliferation and, hence, disease worsening. However, in breast cancer elevated serum estrone in postmenopausal women has been linked to ER-positive breast cancer (Miyoshi *et al.*, 2003). This particular study was carried out among Japanese women. In premenopausal women, an association between the 2OHE/16OHE ratio has been established but remains poorly defined in relation to disease severity in ER-positive tumours (Arslan *et al.*, 2009; Sampson *et al.*, 2016).

Polymorphisms in *CYP1B1* have been observed in PAH, associated with functional differences in 16-hydroxylation, cancer risk and E2-mediated carcinogenicity (Cribb *et al.*, 2006; Zahid *et al.*, 2014). A *CYP1B1* SNP identified in a healthy cohort was also implicated in hPAH penetrance and oncogenesis affected by sex- and race-specific differences in RV function. In addition, levels of DHEA were reduced in PAH leading to the hypothesis that steroidogenic hormones such as mineralocorticoids and glucocorticoids may be implicated in disease but have yet to be defined (Badlam

et al, 2018). In that study, urinary 2OHE:16OHE metabolite ratios were associated with right ventricular ejection fraction (RVEF) dependent on race but did not mediate the genetic variation to disease severity in females. Whereas, in males, SNPs modulated serum testosterone levels and RV measures in men (Badlam and Austin, 2018), a finding which further emphasises the sex-dependent estrogen metabolism in PAH.

16OHE1 concentrations were higher in male iPAH patients compared with female iPAH patients. 16OHE1 concentrations were not detected within male samples in the cohort described in Chapter 3 of this thesis. However, a number of improvements were applied to the method in the time between these two studies to improve robustness and, indeed, successful method validation was achieved prior to this study. We can see that concentrations detected in the Papworth hospital cohort study were relatively low and have been improved within this study. In males, elevated 16OHE1 levels were observed in iPAH patients compared with hPAH and controls. No changes in 16OHE1 were observed in female PAH until comparison was limited to only controls and iPAH patients. This suggests 16OHE1 could be a biomarker of idiopathic disease in females. 16OHE1 can be derived directly from estrone (via CYP3A4), this may also contribute to the elevated 16OHE1 in male and female iPAH patients, who have high levels of estrone. Conversion of E2 to 16OHE1 by CYP1B1 may contribute to the pathogenesis of experimental pulmonary hypertension and can cause proliferation of hPASMCs via increased oxidative stress (White *et al.*, 2012; Hood *et al.*, 2016). Hence the presence of increased 16OHE1 at levels shown to mediate hPASMC proliferation (Hood *et al.*, 2016) in male iPAH patients could have significant effects on the development of PAH. In addition, elevations in E2 and 16OHE1 have been demonstrated in obese male mice (Mair *et al.*, 2019). In that study, increased production of 16OHE1 was shown to contribute toward oxidative stress which is perhaps of importance in the male iPAH setting. It appears on comparison of 16-hydroxylation to 2-hydroxylation within the literature that the former represents the mitogenic pathway. Increased 16-hydroxyestrogens are linked to risk factors of disease via increased proliferation through estrogen receptor signalling. In addition they may also produce a genotoxic effect via formation of unstable DNA-Adducts (Austin *et al.*, 2013). 16-hydroxyestrogens also promote heritable PAH via microRNA-29 upregulation (Chen, Talati, Joshua P Fessel, *et al.*, 2016), meaning even if we do not see elevated levels in heritable disease the presence of this metabolite may alter gene

transcription in BMPR2 mutation cases. In heritable PAH, the 2/16 hydroxyestrogen ratio may also be abnormally affected in mutation carriers. Experimentally, mice exposed to hypoxia display increased 16OHE1 in urine (E. D. Austin *et al*, 2009). In our LC-MS/MS studies, we are unable to measure the catechol estrogen metabolites presenting a drawback for comparison to this literature. However, it remains interesting that 16OHE1 concentrations would be elevated in comparison to the non-mitogenic 2-hydroxylation pathway, subsequently affecting *BMPR2* status as we see mean concentrations of 28 pg/mL in female hPAH and 23.4 pg/mL in male hPAH, which may indeed be higher than 2-hydroxyestrogen levels. In additional *in vivo* studies 16OHE1 induced PH phenotypes in female mice, only within genetically susceptible mice (BMPR2 mutants), providing further evidence of 16OHE1 contributing to PAH pathogenesis (White *et al.*, 2012; Fessel *et al*, 2013).

Through direct conversion from parent estrogen by CYP enzymes (1A1, 1A2, 2C8, CYP3A's), 16OHE2, also known as estriol (E3), is normally produced in abundance during pregnancy. In pregnancy, substantial maternal mortality rates of 56% occur in women with PAH (Jaïs *et al.*, 2012), but it is unknown if disease worsening could be related to elevated bioavailability of estrogens alongside other cardiac changes, such as increased heart rate, size and RV strain (Olsson *et al*, 2016). Interconversion by dehydrogenation of 16OHE2 to 16OHE1 may also occur. 16OHE2 levels were increased in female iPAH patients compared with controls and hPAH whilst no changes in 16OHE2 were observed in male PAH patients. This result suggests sex and PAH sub-group-dependent alterations in E2 metabolism in PAH patients. This is likely to be due to increased CYP enzyme and/or altered CYP1B1-mediated 16-hydroxylation activity (4, 10). 16OHE2 is a potent ER agonist, capable of cellular proliferation and has been associated with cancer risk and E2-mediated carcinogenicity (Castagnetta *et al.*, 2004). However, its effects in PASMCs are unknown within the literature, with Chapter 3 of this thesis showing sex-dependent proliferation in female rat and human cells. This suggests increased levels of 16OHE2 in female PAH patents may induce vascular remodelling and contribute toward development of PAH. The correlation of 16OHE2 concentrations with age in this study may also be a contributing factor toward predisposition to PAH in post-menopausal females. Unpublished proof of concept studies from our lab also suggest that in BOECs cells, 16OHE2 induces proliferation and migration in a sex-dependent manner. BOECs are a subpopulation of endothelial cells found in peripheral blood and generated from circulating endothelial progenitors. Collection

of these cells by Nicholas Morrel et al has allowed study of molecular mechanisms underlying endothelial dysfunction, where availability of primary endothelial cells from patients with vascular disorders has hindered analysis (Ormiston *et al.*, 2015). Proliferation induced by 16OHE2 occurred in female BOECs with migration in male BOECs, further demonstrating the sex-dependent role of 16-hydroxyestrogens. These results suggest that 16OHE2 may contribute to the development of PAH specifically in females. In general, heritable PAH profiles tended to be comparable to the control groups. Changes in E2 metabolite profiles may be a potential biomarker of idiopathic disease where CYP activity might also be implicated.

2MeOE1 was present in higher abundance within serum collected from iPAH patients. One paper defines the bis-sulfamoylated derivative of 2-methoxyestradiol, 2-methoxyestradiol-3,17-O, O-bis-sulfamate (2- MeOE2bisMATE), as a potent anti-proliferative agent and anti-angiogenic activity *in vitro* and inhibition of tumour growth *in vivo* (Newman *et al.*, 2006). In addition, this derivative is not prone to deactivation by 17βHSD 1 & 2 activity in the same manner as 2MeOE2. To the best of our knowledge this type of experiment has not been performed in PAH to determine if the therapeutic benefits might apply in the pulmonary vasculature. However, demonstration of the therapeutic potential of 2MeOE2 has begun to emerge. In human PASMCs, higher basal levels of HIF1α may increase female susceptibility of PAH, and that inhibition of this gene and microtubular disruption of cells by 2MeOE2 holds anti-proliferative and therapeutic benefits (Docherty *et al,* 2019). An alternative therapeutic route in PAH associated with estrogen metabolism may be to harness the anti-proliferative effect of 2MeOE2. 2MeOE2 has been shown to be anti-proliferative, showing a number of potential therapeutic roles in hormone sensitive cancers. 2MeOE2 may interfere with normal microtubule function by binding to tubulin and inhibiting its polymerisation (Kamath *et al.*, 2006). This blocks mitosis and inhibits endothelial adhesion and migration (Mabjeesh *et al.*, 2003). 2MeOE2 effectively inhibits HIF-1 protein levels disrupting HIF transcriptional activity which reverses tumour metastasis, angiogenesis and improves patient prognosis (Masoud *et al*, 2015). This finding might explain why this metabolite is not widely detected among patients in this cohort.

Whether any of the estrogens mentioned are candidate biomarkers in female or male idiopathic disease has yet to be ascertained. Estrogen metabolism may be integral to a number of hormone sensitive diseases leading to improved target

discovery and therapeutic interventions in PAH management. Idiopathic and heritable PAH may share common vascular histopathology, but this study suggests sex and estrogen metabolism imbalances may discriminate between the two sub-groups. In this study, we have provided evidence for abnormal estrogen metabolism within idiopathic PAH. In particular, increase 16-hydroxylation was demonstrated in patients highlighting a pathway already implicated in disease. Alongside the *in vitro* evidence gathered in Chapter 3, further investigation of the signalling mechanism and inhibition of 16–hydroxylation might advance PAH therapies, which do not currently target the gender disparity.

4.5.1 Study Limitations

This study is limited by small sample numbers but warrants larger cohort studies to correlate metabolite levels with PAH severity, clinical phenotype, age, BMI and additional clinical characteristics such as right ventricular pressures and 6-minute walk distances.

Chapter 5 Estrogen metabolism and the menstrual cycle in PAH

5.1 Introduction

Female predominance in PAH has been known for several decades. Data deriving from recent interest in sex hormones in the development and progression of PAH has substantially increased our understanding of this complex epidemiology. Although disease is predominant among females, males suffer a higher mortality from PAH. Particularly in heritable and idiopathic PAH, a clear female predominance persists, with the risk of disease development increasing with age (Pugh *et al*, 2010). The higher prevalence and lower mortality among females may be explained by a protective effect of estradiol in the RV. A number of factors associated with being female may predispose individuals to disease. For example, lung dimensions and control of breathing differ between the sexes. Natural anatomical differences are present between males and females with females having higher ratios of large to small airway and higher lung maturation than males at birth (Lomauro *et al*, 2018). Males however do have larger lungs than women meaning a larger number of alveoli and greater alveolar surface area (Thurlbeck *et al*, 1982). In females estradiol has positive effects on the production of foetal surfactant and on alveogenesis during neonatal and prepubertal periods. In females, decreasing concentrations of E2 with ageing has been linked with respiratory disorders with the balance between estrogen and progesterone being important in worsening hypoxemia and hypocapnia (Naeije *et al*, 2014).. This detrimental effect was not observed in premenopausal women (Behan *et al*, 2008). Hypocapnia, which is defined as a state of reduced carbon dioxide in blood, usually results from hyperventilation but more importantly has been linked to poor survival in PAH (Hoeper *et al.*, 2007). In addition, higher concentrations of circulating estrogen throughout the menstrual cycle could add to the beneficial effect on the RV and detrimental effect in the pulmonary vasculature, in agreement with PAH literature (Austin *et al.*, 2013). Estrogenic compounds exclusive to females increases circulating levels, medications such as birth control or hormone replacement therapy may influence the overall systemic circulating concentrations. These exogenous estrogens may contribute to the disease insult in the vasculature and/or protective function in the heart (de Jesus Perez *et al*, 2011), discussed further in Chapter 1, section 1.5. In addition, higher bioavailable estradiol has been associated with improved RV systolic function in women using hormone therapy (Ventetuolo *et al.*, 2011).

In recent studies, elevated plasma estradiol has been demonstrated in men and postmenopausal women with PAH and is thought to increase the risk and severity of this devastating disease in each population (Ventetuolo et al., 2016; Baird et al., 2018). Also, in portopulmonary hypertension, elevated estradiol has been reported in subjects with a specific SNP in the aromatase gene (Roberts *et al.*, 2009), which will be discussed further in chapter 6 of this thesis. These findings implicate elevated estrogen synthesis in PAH and increased estradiol production in the circulation, leading to increased bioactive metabolite production. Depending on the metabolic pathway affected, (namely 2, 4 or 16 hydroxylation by a variety of CYP enzymes) this might influence the detrimental and beneficial balance in the clinical setting. There has been a substantial growing amount of evidence to suggest that metabolites of estrone and estradiol may have independent and important influences on onset and progression of PAH (Tofovic *et al*, 2010; White *et al.*, 2012; Chen *et al.*, 2016; Hood *et al.*, 2016; Docherty *et al.*, 2018; Docherty *et al*, 2019; Mair *et al.*, 2019). These studies implicate specific metabolites within the 16-hydroxyestrogen pathway as being detrimental (Chen *et al.*, 2016; Hood *et al.*, 2016; Mair *et al.*, 2019), whilst metabolites such as 2-methoxyestradiol have potentially beneficial roles in the PAH setting (Tofovic *et al.*, 2010; Docherty *et al*, 2019). The study by Tofovic et al also demonstrates the potential importance of 17βHSD enzymes in converting metabolites with biological inactivity to form metabolites with therapeutic or detrimental roles at target tissues. For example, conversion of E1 to the more potent E2 via the action of 17βHSD1 stimulates development of hormone sensitive breast cancer (Ayan *et al.*, 2012). Also, 2MeOE2 a metabolite with potential beneficial roles in the pulmonary vasculature (Docherty *et al*, 2019), can be converted to the reportedly inactive 2MeOE1 by 17βHSD2, resulting in a loss of the anti-proliferative function of 2MeOE2 (Tofovic *et al.*, 2006). In addition, elevated amounts of *CYP1B1* gene or mutations of its gene are also implicated, with inhibition of this enzyme showing benefit within preclinical models of PAH (Austin *et al*, 2009; White *et al,* 2012). This finding could be important within females as the ability to prevent formation of mitogenic metabolites whilst preserving menstruation via circulating estradiol in premenopausal females may lead to preserved RV function and extended health benefits (Austin *et al.*, 2013).

In premenopausal women with PAH the relationship between estrogen and disease remains poorly understood. During the menstrual cycle, estrogen concentrations fluctuate between the first and last day of cycle, which lasts for 25 to 36 days (Figure

5-1). This may influence the development of PAH, with its severity depending on estrogen levels throughout the cycle. In the initial 12-day follicular phase levels of estrogen and progesterone are low. Towards day 12, follicles are produced in the ovary producing estrogen. The ovulatory phase then begins with a surge in hormone levels, normally lasting 4-5 days. Finally, in the luteal phase from approximately day 16, estrogen and progesterone levels remain high until dropping significantly upon menstruation. The normal range of E2 during this time is 15 – 350 pg/mL with concentrations varying throughout the day and cycle. The relation of the estrogen metabolite fluctuation across the cycle in healthy females and PAH patients remain undefined.

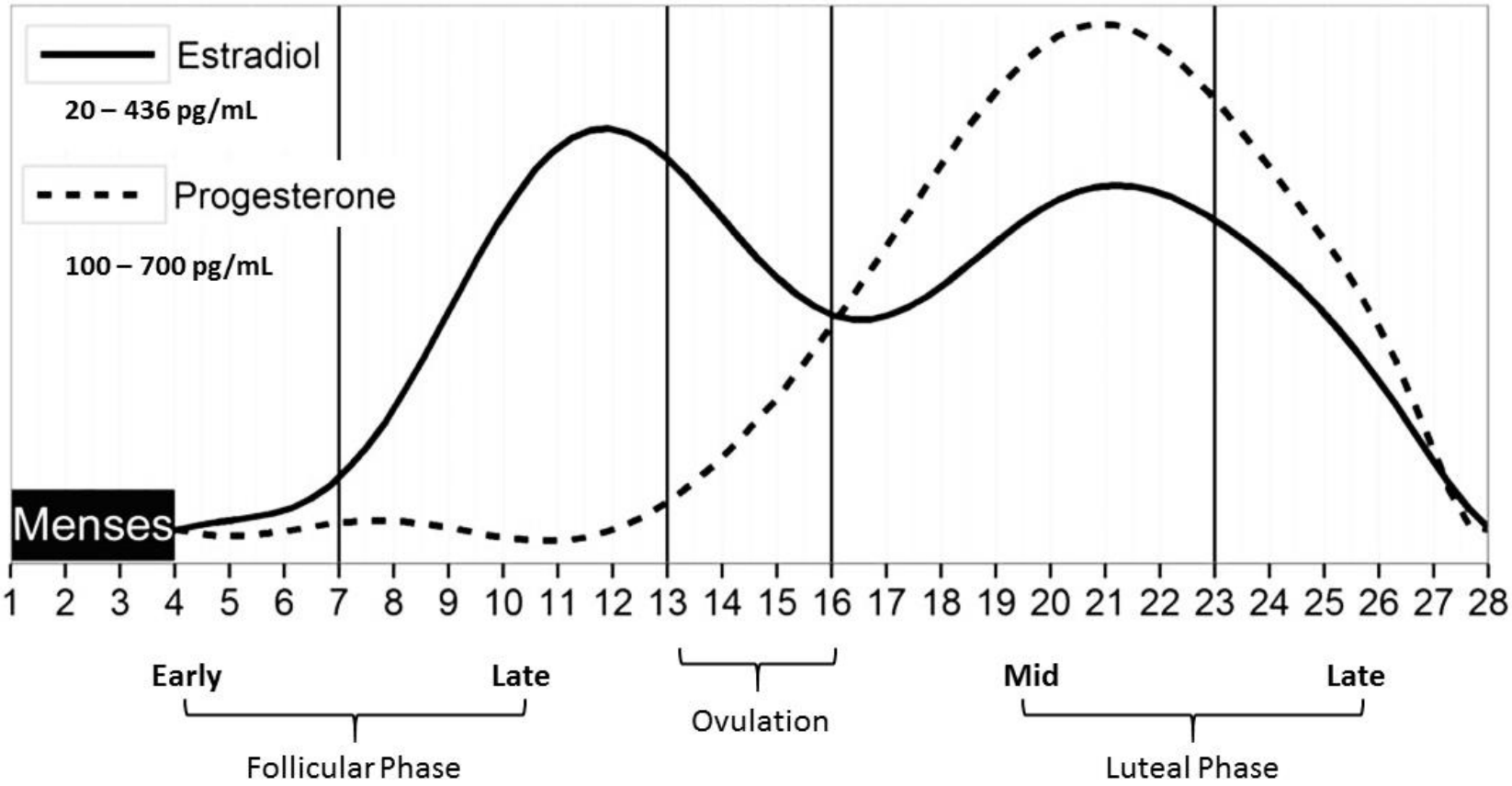

Figure 5-1: Menstrual cycle in healthy premenopausal females Throughout the menstrual cycle concentrations of estradiol and progesterone vary significantly over the 24 days from menstruation. Estradiol concentrations elevate in the late follicular phase, lowering upon ovulation when progesterone increases. Estradiol remains at high concentrations in the mid and late luteal phase until menstruation. (adapted from Tenan, Hackney, & Griffin, 2016).

5.2 Hypothesis and Aims

We hypothesised that, in premenopausal women with PAH, estrogen and bioactive metabolite concentrations will be increased which may relate to PAH severity, and this may differ across the menstrual cycle. The main aim of this chapter was to determine if there are variations in concentrations of estrogen metabolites during the menstrual cycle in control and PAH females using LC-MS/MS.

5.3 Methods

5.3.1 Study samples and data collection

Plasma samples were collected with ethical approval and obtained from Rhode Island Hospital, USA in collaboration with Dr Corey Ventetuolo. We performed a proof-of-concept study on premenopausal women with plasma from 20 control and 13 PAH subjects. Samples were collected once per week on the same day for 4 weeks.

5.3.2 LC-MS/MS Analysis

All standards, sample preparation and instrumentation used were performed prior to amending the method by addition of certified standards, as described in Chapter 2, section 2.3.4.

5.3.3 Analytical Criteria

The biological samples were run over 6 analytical batches, and therefore the accuracy and precision of the method and the lower limit of detection for estrogen metabolites were rechecked. Inter-day precision and accuracy at the LOQ were calculated alongside linearity values as samples were studied across six LC-MS/MS runs.

5.3.4 Data Analysis

LC-MS/MS data was observed using Analyst software v1.5.1 and analysed using MultiQuant, version 3.0 (Sciex, Warrington, UK). Statistical analyses were performed on IBM SPSS statistics 25. Data were log-transformed to apply normal distribution for multivariate analysis. Comparisons between estrogen levels in

controls vs PAH were compared by unpaired t-tests and across the menstrual cycle weeks were compared by Bonferroni post-hoc tests (*p=<0.05). Estrogen ratios in controls and PAH were compared using a Student's t test.

5.4 Results

5.4.1 Analytical Criteria

This batch of clinical samples was the first run following successful method validation (Chapter 2), where the limit of quantification for metabolites was set at 10 pg/mL. However, upon extended analysis of these batches suitable accuracy and precision was demonstrated at a LOD, 6 pg/mL, achieved by using calibration curves over a narrower range (Table 5-1,

Table 5-2); the narrower range was assigned with knowledge from the increased number of data points available to define a reference range. This provided justification to reduce the LOQ for the estrogen metabolites. The assay was also linear over this range with an acceptable r value >0.98 and no lack-of-fit identified across the curve range (Table 5-1).

Table 5-1: Inter-day linearity and lack of fit tests

Analyte-MPPZ	Lower Limit (pg/mL)	Mean R	F-calculated
E1	2 (0.43)	0.993	0.016
17αE2	2 (0.43)	0.999	0.013
17βE2	2 (0.43)	0.999	0.023
16OHE1	6 (2.17)	0.998	0.019
16OHE2	6 (2.17)	0.999	0.023
2MeOE1	6 (2.17)	0.998	0.014
4MeOE1	6 (2.17)	0.998	0.015
2MeOE2	6 (2.17)	0.998	0.017
4MeOE2	6 (2.17)	0.988	0.021
	Critical F value (95% CI)		2.18

If Fcalculated > Fcritical then reject the null hypothesis, As Fcalculated < Fcritical the null hypothesis is accepted meaning there is no lack of fit in these calibration curves; Dof = degrees of freedom; MSS = Mean Sum of squares; LOF = Lack of fit; Fcalculated = MSS (LOF)/MSS (error); Estrone (E1); estradiol (17 α/ β E2); 16-hydroxyestrone (16OHE1); 16-hydroxyestradiol (16OHE2); 2 or 4-methoxyestrone (2 or 4-MeOE1); 2 or 4-methoxyestradiol (2 or 4-MeOE2).

Table 5-2: Accuracy and precision of inter-day curves Calibration curves were compared at the lower and upper limits of quantification

		Inter-day (n=4-5)		
Analyte	**Target (pg/mL)**	**Mean (pg/mL)**	**Precision (RSD %)**	**Accuracy (RME %)**
E1	2	1.9	10.9	4
	1000	974.5	12.0	2.6
	Endogenous	49.2	13.9	-
17αE2	2	1.9	8.7	1.3
	1000	1024.3	3.1	2.4
	Endogenous	-	-	-
17βE2	2	2.2	7.6	8.2
	500	493.7	7.6	1.3
	Endogenous	50.8	13.5	
16OHE1	6	4.9	6.8	11
	500	496.6	7.9	0.7
	Plasma + 100	101.3	8.2	1.3
16OHE2	6	5.7	8.7	5.5
	100	99.3	8.4	0.7
	Plasma + 100	103.8	6.5	3.9
2MeOE1	6	6.2	8.5	3.2
	100	96.0	8.6	4.0
	Plasma + 100	98.6	3.9	1.4
4MeOE1	6	5.8	13.5	1.9
	100	98.6	7.5	3.5
	Plasma + 100	113.6	17.6	13.0
2MeOE2	6	5.5	9.3	7.3
	100	98.6	8.8	1.4
	Plasma + 100	89.7	8.7	10.3
4MeOE2	6	5.7	4.7	5.5
	100	93.3	8.8	6.7
	Plasma + 100	109.6	8.7	9.7

Estrone (E1); estradiol (17 α/ β E2); 16-hydroxyestrone (16OHE1); 16-hydroxyestradiol (16OHE2); 2 or 4-methoxyestrone (2 or 4-MeOE1); 2 or 4-methoxyestradiol (2 or 4-MeOE2); RSD, standard deviation/mean x 100, RME %, Relative Mean Error ((mean measured value - theoretical value)/theoretical value x 100); Endogenous levels in female plasma; Plasma + 100 (metabolites spiked in plasma at 100 pg/mL).

This was the first, larger batch of plasma samples run using this assay and a contaminant build-up on column peak was discovered, which masked the methoxyestrogens (Chapter 2, section 2.4.5.5) after about 8-10 plasma injections. Thus, further steps to optimise the wash step were taken in response and included in the analyses presented in Chapters 4, 6 and 7.

5.4.2 Patient Information

Sample information for this study is currently limited as the statistical analysis remains ongoing with collaborators. Information on patient age, BMI and cardiac parameters would have been useful for association with estrogen concentrations. For the purpose of this chapter, we have been unblinded to case and control status and the menstrual week following our LC-MS/MS analysis.

Table 5-3: Characteristics of pre-menopausal women

	Control	**PAH**		**Total**
Subjects	20	13		33
Week Code	**Coding Information**		**Menstrual phase**	
F	2 days since menstruation		Early Follicular	31
S	1 week from F		Ovulation	32
T	2 weeks from F		Mid Luteal	31
L	3 weeks from F		Late Luteal	31
	Total samples			125

5.4.3 LC-MS/MS Analysis

In premenopausal plasma all 13 estrogens included within the assay were detected and quantified among controls and cases, but not in every individual.

5.4.3.1 Estrogen and Disease Status in Premenopausal Women

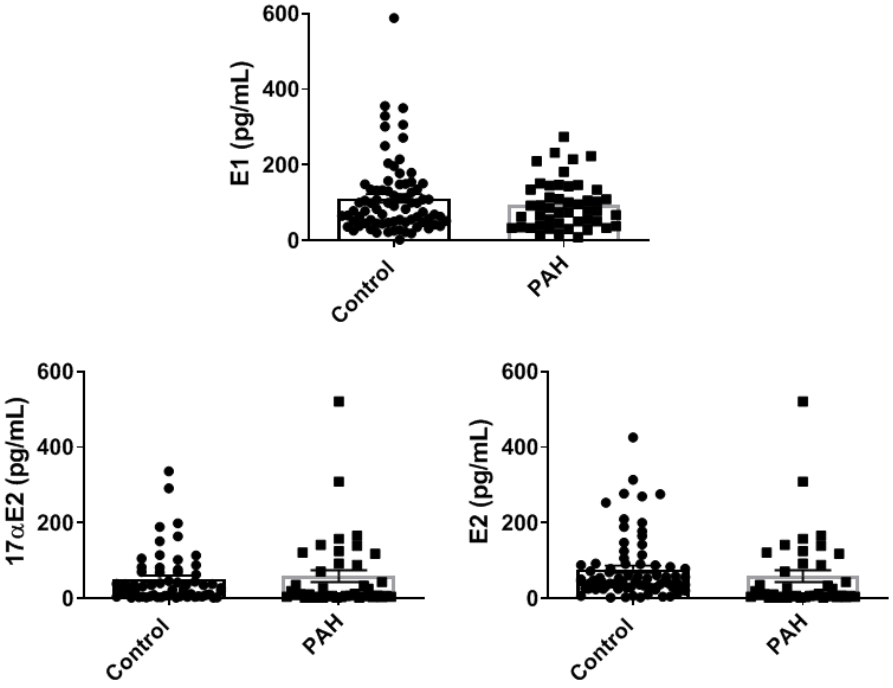

Figure 5-2: Parent estrogens in premenopausal controls and patients with pulmonary arterial hypertension (PAH) Estrone, E1; alpha-estradiol, 17αE2 and Estradiol, E2 in control and PAH patients. Data expressed as mean ± SEM. n=78, Controls; n=47 PAH.

A wide range of circulating concentrations were present for each parent estrogen, prior to accounting for menstrual phase. Differences were not seen in the concentrations of parent estrogens detected in controls and patients (Figure 5-2).

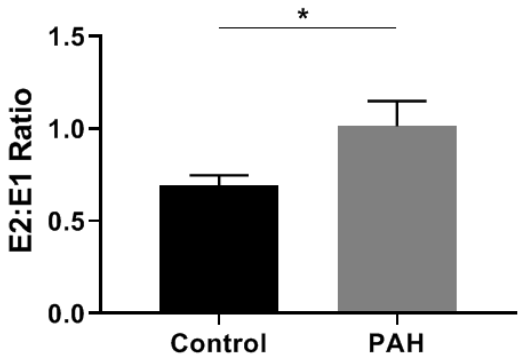

Figure 5-3: E2:E1 Ratio as a marker of 17βHSD activity in premenopausal control subjects and patients with pulmonary arterial hypertension (PAH) Data expressed as mean ± SEM. P<0.05 using a Student's t-test to compare groups. n=78, Controls; n=47 PAH.

The ratio of E2 to E1 concentration was significantly increased among the PAH group (Figure 5-3).

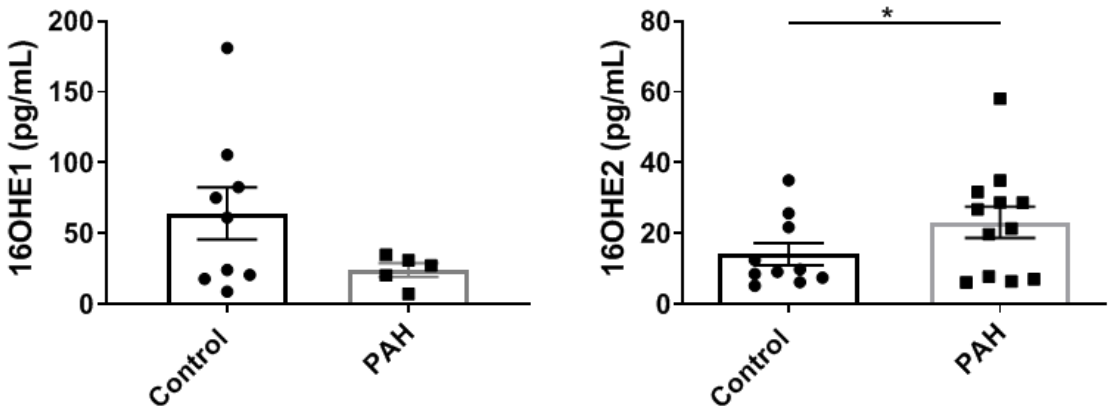

Figure 5-4: 16-Hydroxyestrogen metabolites in premenopausal control subjects and patients with pulmonary arterial hypertension (PAH) 16-Hydroxyestrone, 16OHE1 and 16-Hydroxyestradiol, 16OHE2 in control and PAH patients. Data expressed as mean ± SEM. n=78, Controls; n=47 PAH.

The 16-hydroxylation of parent estrogens in this study appear in a relatively small number of individuals: 16OHE1 (n=12, 5) and 16OHE2 (n=10, 12) in controls and PAH respectively. Intriguingly, 16OHE2 was elevated among PAH patients in comparison to controls, (Figure 5-4, Table 5-4).

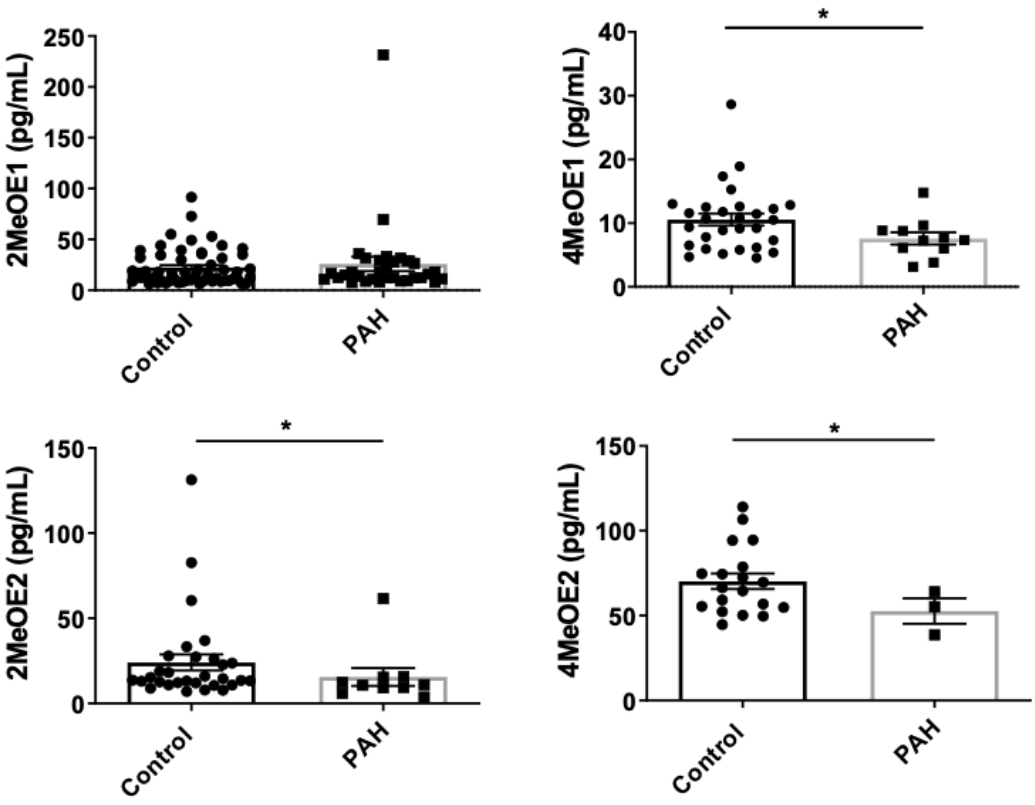

Figure 5-5: Methoxyestrogen metabolites in premenopausal control subjects and patients with pulmonary arterial hypertension 2-methoxyestrone, 2MeOE1; 4-methoxyestone, 4MeOE1; 2-methoxyestradiol, 2MeOE2 and 4-methoxyestradiol, 4MeOE2 in control and PAH patients. Data expressed as mean ± SEM. n=78, Controls; n=47 PAH.

Conversion to methoxyestrogens via 2- or 4-hydroxylated precursors occurs in a higher number of controls and patients by comparison to 16-hydroxylation, (Figure 5-4). There was a decrease in circulating concentrations of 4MeOE1, 2MeOE2 and 4MeOE2 in PAH patients (Figure 5-5). No differences were seen in 2MeOE1 concentrations in comparison to controls and PAH.

The percentage detection of the assay was also assessed (Table 5-5) for improvement of metabolite detection in future studies.

Table 5-4: Estrogen concentrations in controls and pah

	Control	PAH	p-value
Subjects	79	47	
E1	117.4 ± 11.6 83.0 [48.4 - 138.4]	94.5 ± 9.2 79.4 [39.4 - 134.9]	0.26
17αE2	52.1 ± 8.8 35.2 [6.7 - 79.0]	59.1 ± 14.4 11.4 [4.6 - 91.3]	0.60
17βE2	77.3 ± 9.4 44.8 [29.3 - 89.3]	76.7 ± 9.1 55.1 [28.1 – 130.6]	0.36
16OHE1	103.71 ± 30.5 61.3 [19.3 - 94.6]	24.2 ± 4.9 27.4 [13.7 - 33.0]	0.18
16OHE2	14.15 ± 3.2 9.51 [7.2 - 22.8]	23.14 ± 1.5 24.1 [7.3 – 31.0]	**0.02**
2MeOE1	20.6 ± 2.3 14.5 [9.4 - 33.3]	25.3 ± 6.8 15.4 [10.9 – 28.3]	0.96
4MeOE1	11.4 ± 0.9 10.6 [6.3 - 12.6]	8.5 ± 0.4 7.7 [6.7 – 9.3]	**0.02**
2MeOE2	24.1 ± 2.9 13.7 [10.9 - 25.6]	16.8 ± 2.5 11.0 [9.3 – 15.6]	**0.02**
4MeOE2	70.2 ± 19.9 66.4 [54.8 - 78.7]	52.7 ± 1.9 55.3 [38.6 – 55.3]	**0.01**

Estrone, E1; alpha-Estradiol, 17αE2; Estradiol, E2; 16-Hydroxyestrone, 16OHE1; 16-Hydroxyestradiol, 16OHE2; 2-methoxyestrone, 2MeOE1; 4-methoxyestone, 4MeOE1; 2-methoxyestradiol, 2MeOE2 and 4-methoxyestradiol, 4MeOE2. Data expressed as mean ± SEM and median [Q_1-Q_3]. Statistics were performed on the log values using multivariate analysis with a Bonferroni post hoc test.

Table 5-5: Percentage of estrogen detected by LC-MS/MS in control and PAH subjects.

% Detected Estrogen	Control	PAH
Subjects	79	47
% E1	100	100
% 17αE2	78	85
% 17βE2	98	100
% 16OHE1	14	11
% 16OHE2	13	26
% 2MeOE1	73	68
% 4MeOE1	36	19
% 2MeOE2	41	19
% 4MeOE2	24	7

Estrone, E1; alpha-Estradiol, 17αE2; Estradiol, E2; 16-Hydroxyestrone, 16OHE1; 16-Hydroxyestradiol, 16OHE2; 2-methoxyestrone, 2MeOE1; 4-methoxyestone, 4MeOE1; 2-methoxyestradiol, 2MeOE2 and 4-methoxyestradiol, 4MeOE2.

5.4.3.2 Menstrual Cycle, Estrogen and Disease Status

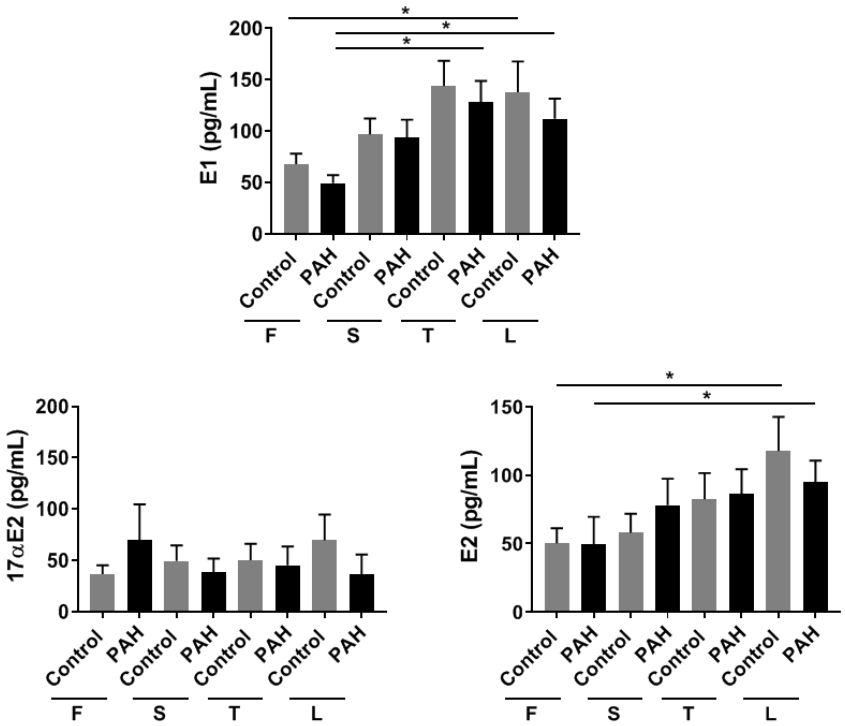

Figure 5-6: Parent estrogens during the menstrual cycle in controls and patients with pulmonary arterial hypertension (PAH) Estrone, E1; alpha-estradiol, 17αE2 and Estradiol, E2 in control and (PAH) patients within two days of menstrual cycle week 1, W1; week 2, W2; week 3, W3 and week 4, W4. Data expressed as mean ± SEM. Statistical analysis was performed using a multivariate analysis with Bonferroni post hoc tests. n=20, Control; n=13, PAH; n=31, F; n=33, S n=31, T; n=31, L.

E1 was elevated over the menstrual cycle from week 1–3 and week 1-4; this correlates with timing of the luteal phase in the final fourteen days of the menstrual cycle. In PAH patients, E1 is also elevated from week 1 to 3. Estradiol was elevated between week 1 and 4 in controls and PAH cases (Figure 5-6). These elevations correlate with natural rises in estrogen concentrations throughout the menstrual cycle.

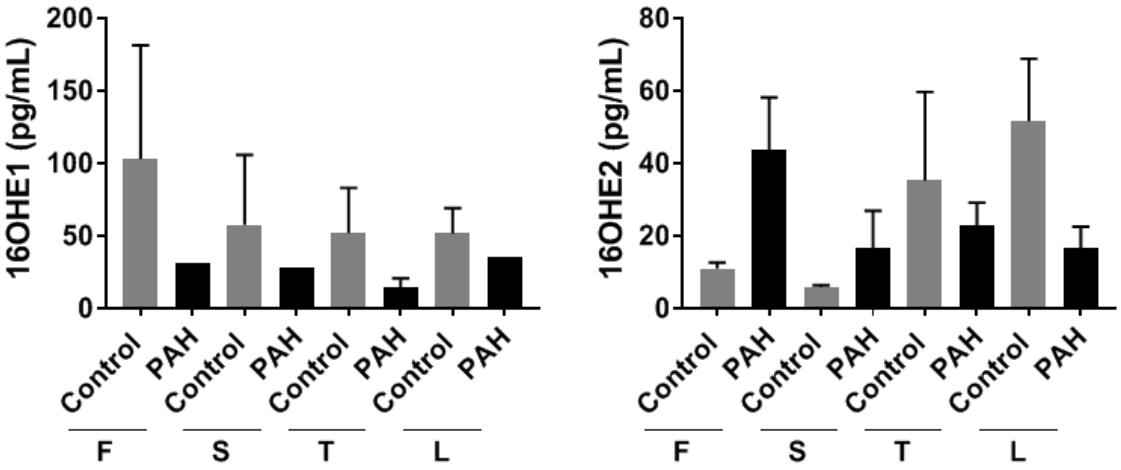

Figure 5-7: 16-Hydroxyestrogen metabolites during the menstrual cycle in premenopausal control subjects and patients with pulmonary arterial hypertension (PAH) 16-Hydroxyestrone, 16OHE1 and 16-Hydroxyestradiol, 16OHE2 in control and PAH patients. Data expressed and mean ± SEM. n=20, Control; n=13, PAH; n=31, F; n=33, S n=31, T; n=31, L.

With respect to 16-hydroxyestrogen metabolites, 16OHE1 and 16OHE2 did not change not change across the cycle (Figure 5-7).

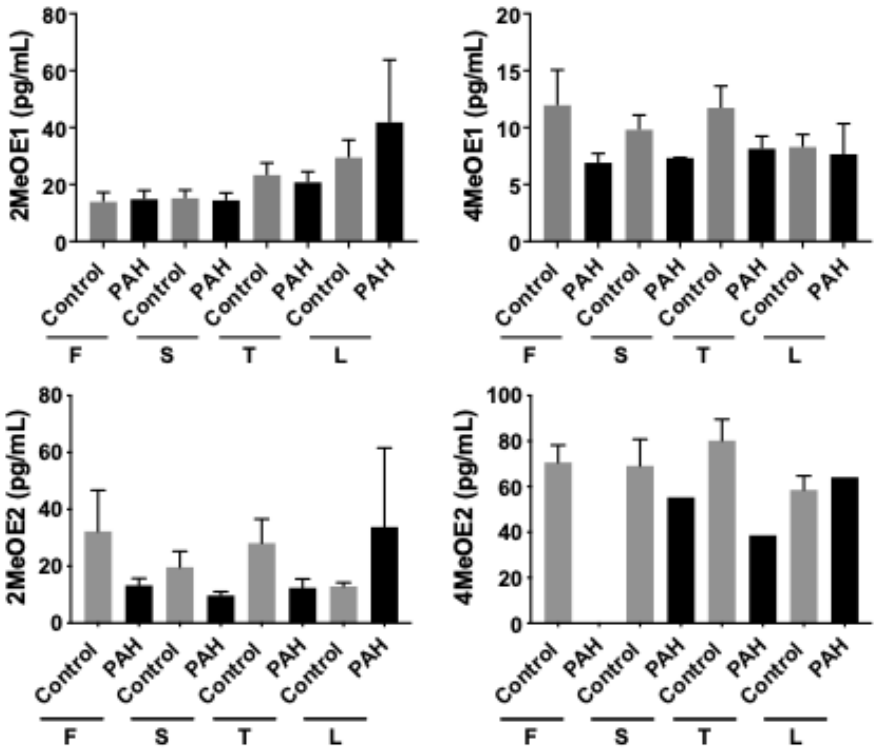

Figure 5-8: Methoxyestrogen metabolites during the menstrual cycle in premenopausal controls and pulmonary arterial hypertension (PAH) patients 2-methoxyestrone, 2MeOE1; 4-methoxyestone, 4MeOE1; 2-methoxyestradiol, 2MeOE2 and 4-methoxyestradiol, 4MeOE2 in control and pulmonary arterial hypertension (PAH) patients within two days of menstrual cycle week 1, W1; week 2, W2; week 3, W3 and week 4, W4. Data expressed and mean ± SEM. n=20, Control; n=13, PAH; n=31, n=31, F; n=33, S n=31, T; n=31, L.

There were no changes in methoxyestrogen concentrations across the cycle. (Figure 5-8).

5.5 Discussion

In summary, comparison of the entire dataset shows elevated 16OHE2 with reduced 4MeOE1, 2MeOE2 and 4MeOE2 in premenopausal PAH patients in comparison to controls. In addition, the E2:E1 ratio demonstrates increased 17βHSD1 activity in PAH patients over controls. Upon analysis by menstrual week, E1 and E2 concentrations increased during the menstrual cycle in female controls and patients. No changes in estrogen metabolites were detected across the cycle.

We show that in premenopausal females, estrone and estradiol concentrations appear unchanged in controls and PAH patients, prior to sub-analysis at each menstrual week. We did however demonstrate increased E2:E1 ratios in PAH which may also be of interest in disease pathogenesis. In oncology, activation of 17βHSD1 impacts on cellular migration and stimulation of breast cancer tumour growth (Aka *et al.*, 2012). In this setting, 17βHSD1 converts E1 to the more potent E2, a finding which has been loosely linked to PAH pathogenesis (Tofovic *et al*, 2010). Increased activity of 17βHSD1 may also impact estrogen receptor signalling and subsequent transcriptional activity, with inhibition of the increased activity emerging as a novel therapeutic target in hormone-sensitive cancers (Ayan *et al.*, 2012). Upon stratification of parent estrogens by the menstrual week, E1 and E2 significantly increased in concentration from week 1 to 4 among the control group. These elevations follow the expected pattern in healthy females (Figure 5-1). In PAH patients, E1 increased from weeks 1 to 3 & 4, and E2 is increased from week 1 to 4. These elevations correlate with transition from the follicular to the luteal phase of the menstrual cycle. It may be of interest that in PAH E1 concentrations are higher in week three than week one, suggesting an earlier peak of high circulating E1 for the final 14 days. In comparison with controls, there were no changes in estrogen concentrations in PAH at any point of the cycle.

With respect to bioactive metabolites, there was no change in 16OHE1, but we did see an elevation of 16OHE2 among PAH patients in comparison of the entire cohort, controls vs PAH. Elevated 16OHE2 among females appears to be in agreement with Chapters 3, 4 and 6 of this thesis, which also demonstrated elevated 16OHE2 among female PAH sub-groups. In the literature, 16OHE1 has been linked to pathogenesis in PAH (Chen *et al.*, 2016; Hood *et al.*, 2016). Preliminary evidence from proof of concept studies show 10nM 16OHE2 may cause PASMC proliferation

in female rat cells and in female PAH cells (Chapter 3, section 3.3.2). Further proof-of-concept studies in blood outgrowth endothelial cells (Denver *et al.*, 2018), illustrate a sex-dependent effect of 16OHE2. In this setting, 1 nM 16OHE2 initiates proliferative responses in female BOECs but has a migratory effect in male BOECs. These effects may be mediated by dysregulated aryl hydrocarbon receptor (*AhR*), CYP enzymes and *Nrf-2* as further discussed in Chapter 4.

2MeOE2 and 4MeOE2 are significantly reduced among PAH patients with no change in 4MeOE1 or 2MeOE1 concentrations in comparison to the entire cohort, (controls *vs* PAH). A reduction in methoxyestrogen production might suggest a build-up of 2- or 4-hydroxyestrogen (catechol) metabolites in premenopausal females, but we are currently unable to detect these metabolites by this assay. In this pathway, conversion to 4-hydroxyestrogens presents a less favoured pathway (~5%) in comparison to 2-hydroxylation, and overall these pathways are thought to account for 50% of overall E2 metabolism (Tofovic *et al*, 2010). CYP enzymes which convert parent estrogens to the 2-, 4- & 16-hydroxyestrogens may provide a route of therapeutic modulation that proves to be important in premenopausal women. Clinical trials into the feasibility of *CYP1B1* immunotherapy in patients with breast, ovarian, prostate, colon and rectal cancers using a plasmid DNA of CYP1B1, ZYC300, encapsulated in biodegradable poly-DL-lactide-coglycolide microparticles has been evaluated showing promising safety and tolerability among patients (Luby *et al*, 2008). As CYP1B1 is not expressed in the adult liver and does not having a major role in hepatic metabolism, it may present a promising target. In cancer research, CYP1B1 protein expression is increased in almost all carcinogenic tumours with almost no expression in breast tissues of healthy adults. This is true except in females where in ureter, fallopian tube, breast, and uterus, expression is present, which may predispose women to pathogenesis (Maecker *et al.*, 2003). Application of ZYC300 in advanced cancer patients showed this therapeutic strategy was well tolerated and safe with patients developing anti-CYP1B1 immunity and, in turn, responding better to conventional cancer therapies (Gribben *et al.*, 2005). A number of experimental studies have demonstrated pharmacological inhibition of CYP1B1 to have beneficial effects on cardiac RV hypertrophy and pulmonary vascular remodelling (White *et al.*, 2012; Dempsie *et al.*, 2013; Johansen *et al.*, 2016; Mair *et al.*, 2019). The possible application of ZYC300 or alternative means of targeting *CYP1B1* as an adjuvant therapy in PAH given over-expression of *CYP1B1* in disease should not be overlooked.

Due to reduced methoxyestrogen production in the overall premenopausal cohort, a dysfunction or inherited alteration in COMT could also be present, thus leading to inadequate detoxification of catechol metabolites (Shimada *et al.*, 1996). Such dysfunctional activity may result in an increase abundance of proliferative metabolites and a reduction in formation of the protective effect of 2MeOE2 leading to reduced anti-proliferative action, as demonstrated in the Shimada et al study of estrogen sensitive cancers. COMT overexpression also attenuates E2-induced proliferation in treatment of uterine leiomyomas, inhibiting ERα signalling and reducing HIF1α levels and aromatase expression in human leiomyoma cells (HLCs) (Salama *et al.*, 2006, 2009). These cancer studies may have implications in PAH as the same pathways have been implicated in sex-dependent pathophysiology of PAH (Docherty *et al*, 2019). The hypothesis that there may be reduced production of methoxyestrogens in PAH, is also in line with this LC-MS/MS study. In addition, 2MeOE2 may exert feedback inhibition of CYP1B1 activity in turn reducing production of pathogenic 4- and 16- hydroxyestrogens in healthy individuals (Dawling *et al*, 2003).

5.5.1 Study Limitations

This study is relatively small with a lack of patient information such as age and BMI alongside a lack of clinical characteristics which would allow further interrogation of the data. As this was one of the first plasma batches run by this method, the optimisation of column washes between clinical samples was applied in batch 2 and 3 of this analysis. Some amendments were made after these batches to improve robustness e.g. sample transfer steps in the extraction process were removed, mobile phase adjustments and certified standards purchased after this study. These changes improved metabolite detection in later studies as shown by comparison of the % detection tables in this chapter and those in Chapters 4 & 6 (Table 5-5, Table 4-4, Table 6-4) and a reduction in metabolite limits of quantification seen in the Chapter 4 and 6 studies.

Chapter 6 Estrogen metabolism in Portopulmonary Hypertension (PPHTN)

6.1 Introduction

As defined by the World Health Organisation (WHO), portopulmonary hypertension (PPHTN) falls under sub-group four of group 1 PAH, which outlines PAH associated with other systemic diseases. Chronic liver disease is a cause of pulmonary vascular disease with PPHTN being diagnosed when PAH is present in the setting of portal hypertension. The prevalence of PPHTN is not directly related to the presence of underlying liver disease, with the main risk factor attributed to end stage liver disease and portal hypertension (Rodríguez-Roisin *et al.*, 2004). PPHTN occurs in approximately 2-16% of patients with portal hypertension, and survival remains poor despite targeted therapies (Hadengue *et al.*, 1991; Kawut *et al.*, 2008; Sithamparanathan *et al.*, 2017). It remains unknown whether patients with PPHTN share disease characteristics with those with other forms of PAH (Kawut *et al.*, 2005). From the Kawut et al studies (Kawut *et al*, 2005, 2008), PPHTN patients were at higher risk of death than patients with iPAH despite having lower vascular resistance and improved cardiac output. In addition, hepatitis C infection decreased the risk of PPHTN in patients with advanced liver disease whilst female sex and autoimmune hepatitis were associated with increased risk of PPHTN. Female sex remains prevalent in PPHTN with far fewer males developing the disease phenotype. The link between sex and both idiopathic and heritable PAH is well known, with a well-established dimorphism in disease onset and severity (Loyd *et al.*, 1995; Foderaro *et al*, 2016). However PPHTN is the first form of PAH associated with an alternate condition also shown to have a high female predominance (Kawut *et al.*, 2008). In addition, young women with autoimmune hepatitis may also have increased risk of PPHTN compared with those with advanced liver disease from other causes (Castaño *et al*, 2008). Therefore, comparison of estrogen and the bioactive metabolites in both liver disease and PPHTN cohorts may present an insight into female predominance given the recent implication of estrogen in PAH (Docherty *et al.*, 2018).

In recent years, the number of alcohol-related liver disease cases has dramatically increased, and evidence suggests alcohol is now the most commonly abused drug in the world, with research linking chronic abuse to serious lung infections (Yeligar *et al.*, 2016). Despite this, little is known on the interaction of alcohol abuse with lung health, or any sex differences in risk. Alcohol-induced myocardial oxidative stress may occur via ERα-induced catalase activity generating higher ethanol-derived

acetaldyhde in the female heart (El-Mas *et al*, 2019). This emphasises the vulnerability of this growing group of chronic alcohol abuser to cardiac dysfunction, presenting an area of research which may expand in the coming years. It has also become apparent that racial differences are also present in the manifestation of respiratory diseases, with African American patients presenting with high mortality (Vaz Fragoso *et al.*, 2014). This study and others investigating the relationship between race and disease are generally conducted in the USA. Literature shows higher prevalence for chronic bronchitis and emphysema are higher in White-Americans than in African-Americans or Hispanics. In a recent study, racial and ethnic differences were shown to exist in PAH subtypes and severity also associating with treatment response. Here, African Americans were 2.5x more likely to be diagnosed with congenital heart defect-associated PAH compared to Caucasian and Hispanics whilst Hispanics remained twice as likely to have PAH associated with congenital heart disease compared to Caucasians (Al-Naamani *et al.*, 2017). The exact driving force behind pathogenic modifications in PAH remains unknown with current studies ongoing to elucidate factors contributing to racial differences in PAH, which may extend to PPHTN cohorts.

Evidence has also implicated bone morphogenetic protein 9 (BMP9) in PPHTN. BMP9, a ligand for the TGFβ superfamily with a selective affinity for *BMPR2/ALK 1* complex, is significantly reduced in PPHTN patients but not in other forms of PAH (Toshner *et al*, 2019). Interestingly, BMP9 is produced predominantly within the liver with potential autocrine/paracrine function within the hepatic reticuloendothelial system (Miller *et al.*, 2000). In an autosomal inherited disorder which affects the blood vessels, hereditary haemorrhagic telangiectasia (HHT), 80% of cases presented with mutations in *ALK1* (Yan *et al.*, 2006). In patients undergoing liver transplantation due to intrahepatic manifestations in HHT genetic sequencing revealed that a mutation in *ALK-1* was present in all patients (Argyriou *et al.*, 2005). In PAH patients these mutations in *BMPR2* and *ALK1* have been commonly reported (Austin *et al*, 2014). As this thesis discussed previously the potential for estrogen and its metabolism to affect *BMPR2* mutations via increased miR-29 could be hypothesised in PAH. Therefore, determination of estrogen concentrations in PPHTN may demonstrate potential biomarkers of importance acting on similar pathways. Initial studies predict genetic variation in estrogen signalling and cell growth regulators are associated with increased risk of PPHTN (Roberts *et al.*, 2009). In the 2009 study, a high-throughput candidate gene approach was

employed to identify a number of SNPs in genes associated with estrogen signalling, cell growth/apoptosis and oxidative stress in PAH. This identified a genetic variation in ERα and aromatase, which was a risk factor for PPHTN but independent of sex. An association between the high-risk aromatase allele (rs7175922) and increased estradiol concentrations was identified. This finding implicates increased estradiol in the pathogenesis of PPHTN. However, the circulating bioactive metabolite concentrations remain undefined.

6.2 Hypothesis and Aims

We hypothesise that circulating parent estrogen and estrogen metabolites are elevated in portopulmonary hypertension in comparison to normal liver disease control subjects. In this chapter we aim to further investigate circulating estrogen and metabolite profiles in liver disease controls and portopulmonary hypertension patients using LC-MS/MS.

6.3 Methods

6.3.1 Study samples

Plasma samples were obtained from 234 liver disease patients acting as controls and 32 patients with portopulmonary hypertension with ethical approval in collaboration with Steven Kawut MD at the University of Pennsylvania, USA. Samples were collected from liver disease controls (n=154, n=80) and portopulmonary hypertension cases (n=20, n=12) males and females, respectively, for LC-MS/MS analysis. Information on patient demographics and clinical characteristics were collected in person, from medical records and from phone calls.

6.3.2 LC-MS/MS Analysis

Plasma samples were subjected to LC-MS/MS analysis, as described previously in Chapter 2 (Section 2.3.5.7, 2.3.7.2). A number of changes were applied to this study to improve robustness and detection:

- New metabolite standards were purchased from Steraloids for estrogen metabolites and fresh stock solutions prepared to 1 mg/mL as described in Chapter 2, section 2.3.4.

- 10% water was added to the acetonitrile mobile phase to improve robustness preventing damage to pump seals.

- Sample transfer steps were removed within the preparation to reduce variability

- One additional point (4 pg/mL) was added to the standard curve to improve quantitation of metabolites at present in low levels.

6.3.3 Analytical Criteria

Inter-day precision and accuracy at the LOQ were calculated alongside linearity values, as samples were studied across six LC-MS/MS runs, as described in chapter 2 (Section 2.3.9.4). Blank samples and aliquots containing estrogens (0, 0+IS, 2, 4, 6, 10, 20, 40, 100, 200, 500 pg/mL) and IS (200 pg/mL) were analysed.

The upper limit of quantification was determined to align with the highest concentration of each estrogen present in study samples.

6.3.4 Data analysis

Plasma concentrations were non-normally distributed, with order ranked mean values compared using rank order Kruskal Wallis tests. The LOQ was substituted for non-detected values within the statistical analysis. The frequencies of detected estrogens within sample groups with Fisher tests to compare frequencies between LD-Controls and PPHTN. Correlations between measured estrogens and age were assessed using Pearson correlations; here data were not imputed for missing values. Data were analysed using SPSS 25 statistical software.

6.4 Results

6.4.1 Patient information

The cases enrolled in the study of Pulmonary Vascular Complications of Liver Disease (PVCLD) with PPHTN were chosen based on the evaluation criteria stated, whereby these patients must have: 1) mean pulmonary artery pressure > 25 mm Hg, pulmonary wedge pressure <15 mm Hg and pulmonary vascular resistance >240 dynes.$s^{-1}cm^{-5}$ as measured by right heart catheterisation and 2) no additional aetiology for pulmonary hypertension. Cases included as Liver disease controls had a right ventricular systolic pressure < 40 mm Hg and an absence of RV hypertrophy or dysfunction. Patients with HIV infection or the presence of more than moderate aortic or mitral vulvar disease or left ventricular dysfunction were excluded from the study. For LC-MS/MS analysis, 234 controls and 32 cases were provided for analysis. In liver disease controls 36% of females were premenopausal whilst none of the female PPHTN cases were premenopausal, with all cases between the age of 56 and 69 years (Table 6-1). The ages of the males acting as liver disease controls and PPHTN cases within the study covered a wider range of 31 – 74 and 35 – 73 years of age, respectively.

Table 6-1: Clinical characteristics of patients with portopulmonary hypertension and those acting as controls with liver disease

Female	LD-Controls	PPHTN	P-Values
Subjects	80	12	
Age (y)	55 ± 10.8	61 ± 4.9	0.16
Premenopausal Age (y)	44 ± 9.8	- -	-
Postmenopausal Age (y)	62 ± 4.3	61 ± 4.9	0.49
Race			
Caucasian	57	8	
African American	10	1	
Hispanic	11	3	
Asian	0	0	
Other Race	2	0	
Male	**LD-Controls**	**PPHTN**	**P-Values**
Subjects	154	20	
Age (y)	58 ± 8.1	54 ± 9.4	0.12
Race			
Caucasian	108	16	
African American	14	2	
Hispanic	28	1	
Asian	2	0	
Other Race	2	1	

Pulmonary arterial hypertension, PAH; Portopulmonary Hypertension PPAH, heritable PAH; Clinical characteristics shown as Mean ± SD. P-values following Mann Whitney U tests.

6.4.2 Analytical validation

Since several assay parameters were adjusted for this study the inter-day accuracy and precision were assessed over the six analytical batches to reassess the limit of quantification for sample analysis. The upper limit of the calibration was set following data analysis, depending on the highest concentration quantified for each estrogen (Table 6-2).

Table 6-2: Inter-day assessment of assay limits Revalidation of assay at the LLOQ and ULOQ based on range of concentrations within liver disease and portopulmonary hypertension patients

		Inter-day (n=5-6)		
Analyte	**Target (pg/mL)**	**Mean (pg/mL)**	**Precision (RSD %)**	**Accuracy (RME %)**
E1	2	2.0	10.1	0.4
	500	500.2	3.2	0.1
17αE2	2	1.9	10.8	4.1
	100	92.5	6.1	4.4
17βE2	2	2.0	9.1	1.2
	200	201.7	2.7	0.9
16OHE1	4	4.1	7.1	1.4
	100	99.4	9.8	0.6
16OHE2	2	1.9	8.7	6.8
	100	91.7	5.4	8.3
2MeOE1	2	1.8	8.8	9.5
	100	97.1	4.6	2.9
4MeOE1	4	3.9	9.5	2.0
	100	101.8	11.7	1.8
2MeOE2	4	4.2	7.1	3.8
	100	94.4	6.3	5.6
4MeOE2	4	4.2	9.4	5.3
	100	96.4	6.4	3.6

Estrone, E1; Estradiol, E2; 16-Hydroxyestrone, 16OHE1; 16-Hydroxyestradiol, 16OHE2; 2-Methoxyestrone, 2MeOE1; 4-Methoxyestrone, 4MeOE1; 2-Methoxyestradiol, 2MeOE2; 4-Methoxyestradiol, 4MeOE2; lower limit of quantitation, LLOQ; upper limit of quantification, ULOQ; Relative standard deviation, RSD; Relative mean error, RME.

6.4.3 LC-MS/MS Analysis

LC-MS/MS analysis detected E1, 17αE2, E2, 16OHE1, 16OHE2, 2MeOE1, 2MeOE2 and 4MeOE2 within liver disease controls and portopulmonary hypertension patients. 4MeOE1 was not detected in any samples within this cohort.

17αE2 is thought to be inert in disease but was monitored as an isomer of the active β-isomer, E2.

6.4.4 Estrogens and Portopulmonary Hypertension

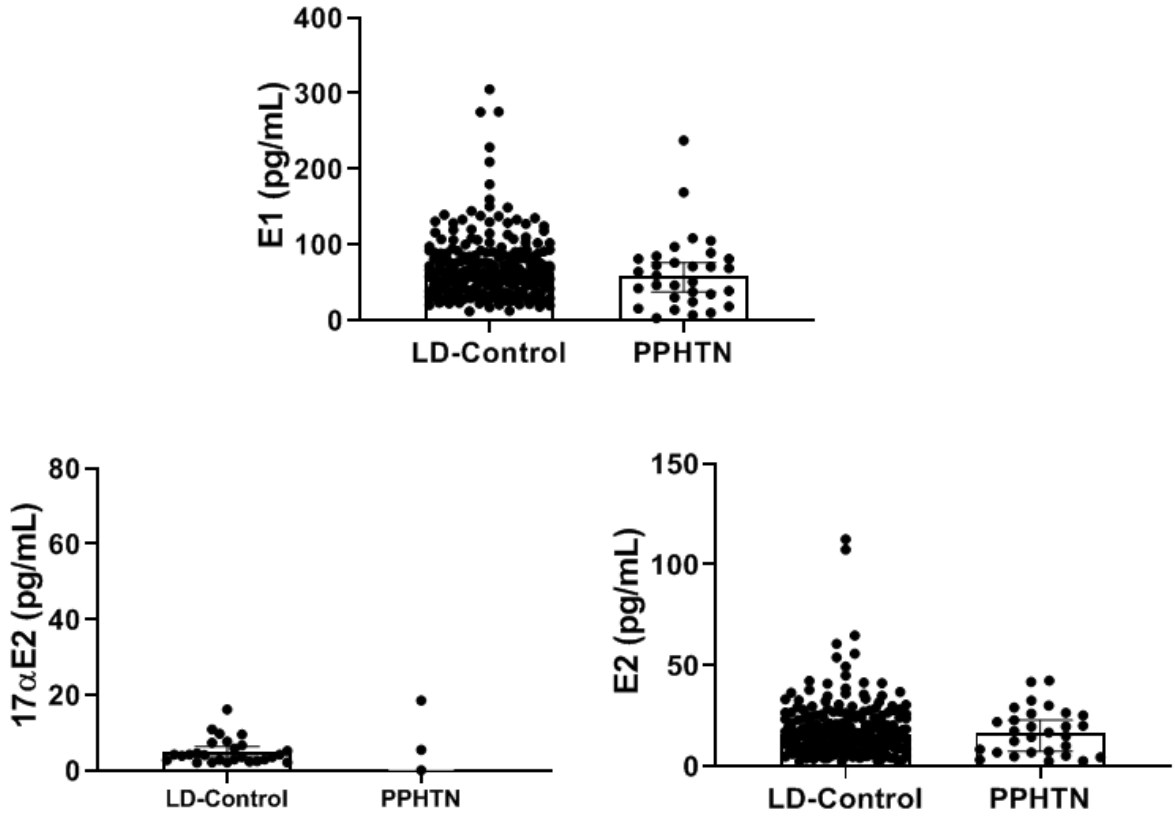

Figure 6-1: Parent estrogens in patients with portopulmonary hypertension (PPHTN) and those acting as liver disease controls Estrone, E1 and Estradiol, E2 in liver disease control (LD-Control) and PPHTN. Data expressed as median ± 95% Confidence interval. Groups compared using a Mann Whitney Test. n=234, LD-Control; n=32 PPHTN, Females and n=80, LD-Controls; n=13, PPHTN.

There were no differences in E1, 17αE2 and E2 concentrations among LD-controls and PPHTN patients (Figure 6-1).

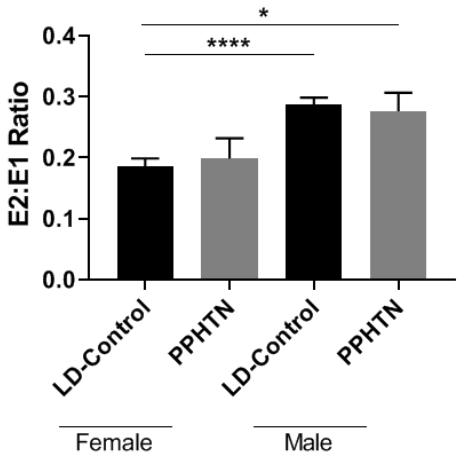

Figure 6-2: E2:E1 ratio as a marker of 17βHSD activity in females and males Estradiol, E2: Estrone, E1 ratio in liver disease controls (LD-Controls) and portopulmonary hypertension (PPHTN) patients. Data expressed and mean ± SEM. *P<0.05, ****P<0.0001 using a Kruskal Wallis multiple comparison test. n=80, 152, LD-Controls; n= 13, 20 PPHTN, female, male respectively.

The ratio of E2 to E1 was significantly increased in males in comparison to females (Figure 6-2). In this setting, the ratio was increased in male liver disease control (p=<0.0001) and PPHTN (p=0.04) in comparison to female liver disease controls.

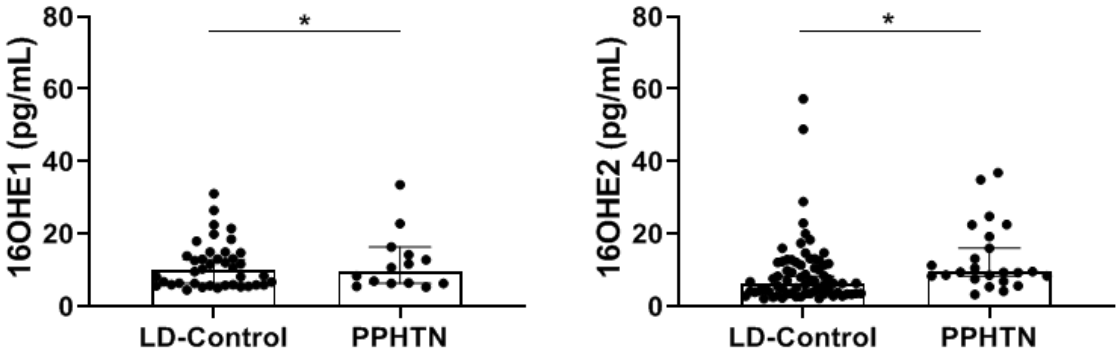

Figure 6-3: 16-hydroxyestrogen metabolites in patients with portopulmonary hypertension (PPHTN) and those acting as liver disease controls 16-Hydroxyestrone, 16OHE1 and 16-Hydroxyestradiol, 16OHE2 in liver disease control (LD-Control) and PPHTN. Data expressed as median ± 95% Confidence interval. Groups compared using a Mann Whitney Test *p<0.05. n=234, LD-Control; n=32 PPHTN.

16OHE1 and 16OHE2 are elevated in PPHTN in comparison to LD-controls (Figure 6-3). The conversion of parent estrogens via the 16-hydroxylation pathway may therefore be elevated within PPHTN patients.

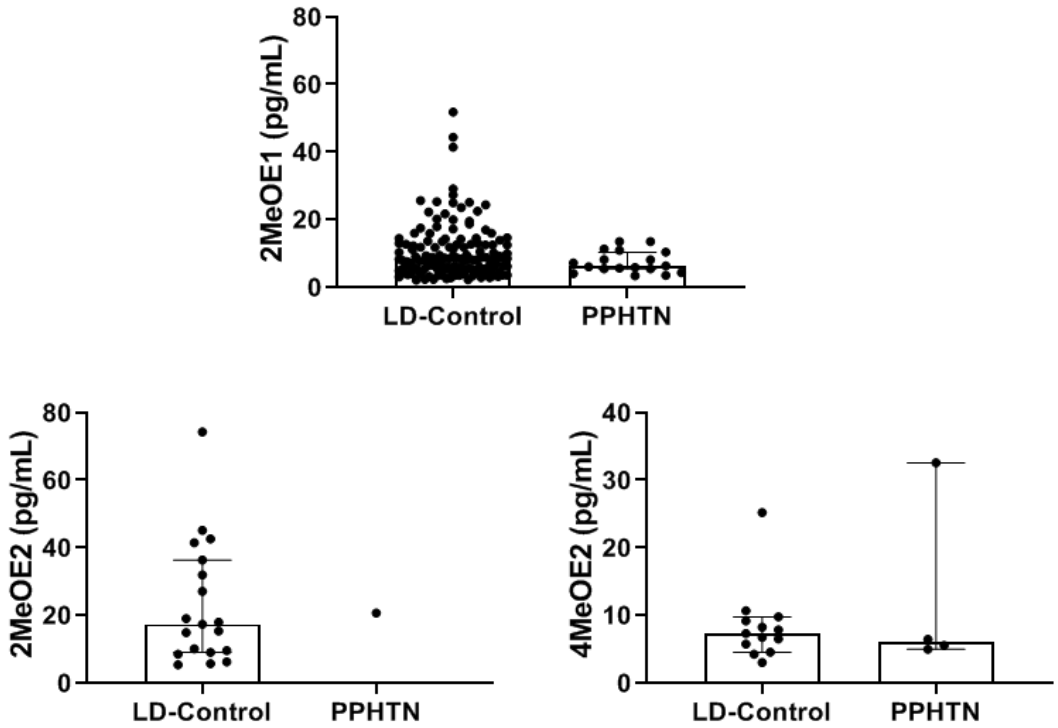

Figure 6-4: Methoxyestrogen metabolites in patients with portopulmonary hypertension (PPHTN) and those acting as liver disease controls 2-methoxyestrone, 2MeOE1; 4-methoxyestone, 4MeOE1; 2-methoxyestradiol, 2MeOE2 and 4-methoxyestradiol, 4MeOE2 in liver disease control (LD-Control) and PPHTN. Data expressed as median ± 95% Confidence interval. Groups compared using a Mann Whitney Test *p<0.05. n=234, LD-Control; n=32 PPHTN.

The conversion of parent estrogens to methoxyestrogens via the 2 and 4-hydroxylation pathway remains unchanged between PPHTN patients. Notably, 2MeOE2 was only detected in one PPHTN patient (Figure 6-4).

A summary of the data from the total cohort (Table 6-3) and frequencies of measured metabolites (Table 6-4) illustrates the differences between the LD-Controls and PPHTN subjects.

Table 6-3: Estrogens and liver disease phenotype

	LD-Controls	PPHTN	P-Values
Subjects	234	32	
Estrogen Levels (pg/mL)			
E1	68.8 ± 2.8 62.3 [41.3 – 86.6]	62.8 ± 8.7 58.9 [30.0 – 80.8]	0.16
17αE2	5.2 ± 0.6 4.1 [2.7 – 7.1]	11.9 ± 6.5 11.9 [5.4 – 11.9]	0.38
E2	18.1 ± 1.0 15.8 [8.9 – 23.9]	16.9 ± 2.1 16.5 [6.7 – 25.5]	0.51
16OHE1	11.7 ± 1.4 8.7 [5.9 – 14.3]	11.8 ± 2.1 9.5 [6.2 – 14.7]	**0.01**
16OHE2	9.5 ± 1.1 6.5 [3.4 – 12.1]	13.1 ± 1.9 9.3 [7.6 – 18.4]	**0.00**
2MeOE1	10.5 ± 0.7 8.3 [5.1 – 12.9]	7.3 ± 0.7 6.1 [5.1 – 10.4]	0.29
2MeOE2	23.0 ± 4.2 17.3 [9.0 – 36.3]	20.6 -	0.29
4MeOE2	8.1 ± 1.4 7.0 [4.8 – 9.3]	12.4 ± 6.7 6.0 [5.1 – 26.0]	0.21

Liver Disease Control, LD-control; Portopulmonary hypertension, PPHTN; Not detected, ND; Estrone, E1; Estradiol, E2; 16-Hydroxyestrone, 16OHE1; 16-Hydroxyestradiol, 16OHE2; 2-Methoxyestrone, 2MeOE1; 2-Methoxyestradiol, 2MeOE2; 4-Methoxyestradiol, 4MeOE2. Data shown as Mean ± SEM and Median [Q1 – Q3]. P-values following a rank order Kruskal Wallis tests.

Table 6-4: Percentage of Estrogen Detection by LC-MS/MS in Portopulmonary Hypertension (PPHTN) and those acting as Liver Disease Controls (LD-Controls)

	LD-Controls	PPHTN
Subjects	234	32
% Measured		
E1	100	97
17αE2	12	6
17βE2	96	91
16OHE1	23	44
16OHE2	32	75
2MeOE1	61	56
2MeOE2	8	3
4MeOE2	6	12

Liver Disease Control, LD-control; Portopulmonary hypertension, PPHTN; Not detected, ND; Estrone, E1; Estradiol, E2; 16-Hydroxyestrone, 16OHE1; 16-Hydroxyestradiol, 16OHE2; 2-Methoxyestrone, 2MeOE1; 2-Methoxyestradiol, 2MeOE2; 4-Methoxyestradiol, 4MeOE2. Data shown as % estrogens detected by LC-MS/MS in sample subjects.

Stratification of estrogen concentrations might discriminate sex-dependent alterations in estrogen metabolism.

6.4.5 Estrogen, Sex and Portopulmonary Hypertension

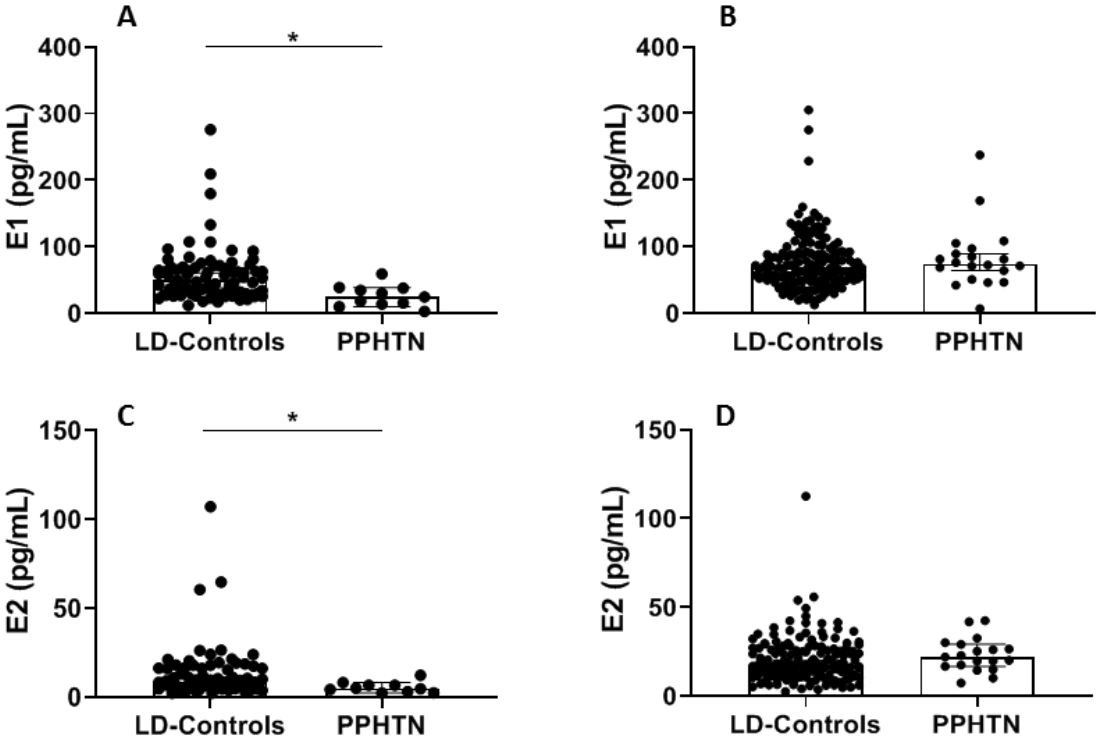

Figure 6-5: Parent estrogens in female and male patients with portopulmonary hypertension (PPHTN) and those acting as liver disease controls Estrone, E1 and Estradiol, E2 in females (A, C) and males (B, D) with liver disease control (LD-Control) and PPHTN. Data expressed as median ± 95% Confidence interval. Groups compared using a Mann Whitney Test *p<0.05. Females: n=80, LD-Controls; n=13, PPHTN. Males: n=154 LD-Controls, n=20, PPHTN.

In females, E1 and E2 concentrations were significantly reduced among the PPHTN patients in comparison to LD-Controls. In males, there were no differences in the concentrations of E1 and E2 in the LD-Controls in comparison to PPHTN patients (Figure 6-5).

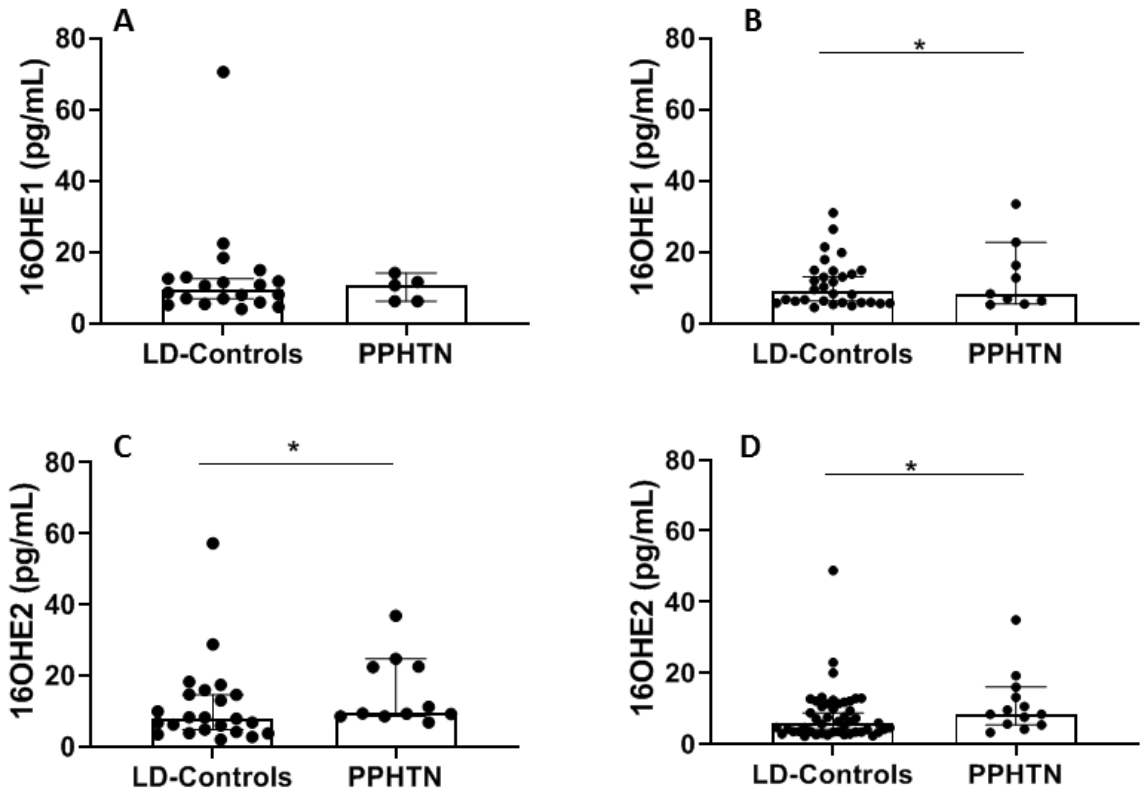

Figure 6-6: 16-Hydroxyestrogens in female and male liver disease controls and portopulmonary hypertension patients 16-Hydroxyestrone, 16OHE1 and 16-Hydroxyestradiol, 16OHE2 in females (A, C) and males (B, D) with liver disease control (LD-Control) and PPHTN. Data expressed as median ± 95% Confidence interval. Groups compared using a Mann Whitney Test *p<0.05. Females: n=80, LD-Controls; n=13, PPHTN. Males: n=154 LD-Controls, n=20, PPHTN.

In females, 16-hydroxylation of parent estrogens to 16OHE2 appeared increased in PPHTN patients. In contrast, there were no differences in concentrations of 16OHE1 between female LD-controls and PPHTN patients. In males, 16-hydroxylation of parent estrogens to both 16OHE1 and 16OHE2 was present in PPHTN patients, with elevated concentrations in comparison to LD-controls (Figure 6-6).

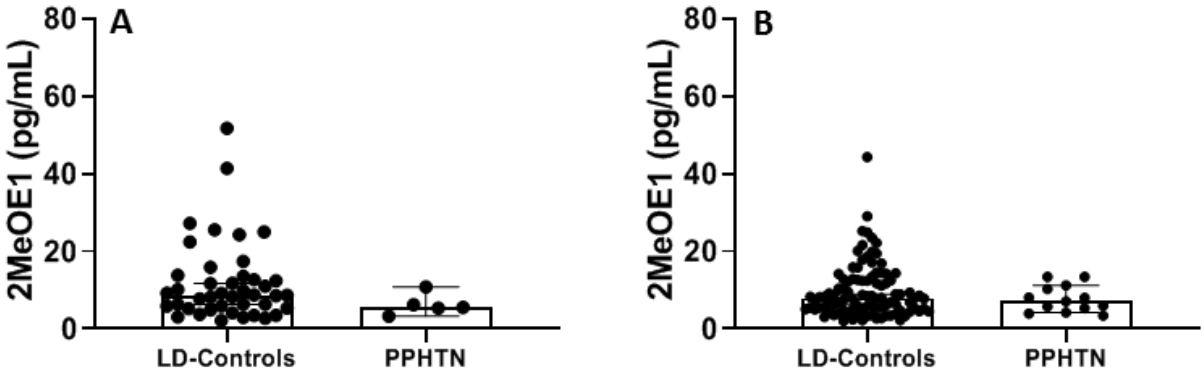

Figure 6-7: 2-Methoxyestrone in female and male patients with portopulmonary hypertension (PPHTN) and those acting as liver disease controls 2-methoxyestrone, 2MeOE1 in females (A) and males (B) with liver disease control (LD-Control) and PPHTN. Data expressed as median ± 95% Confidence interval. Groups compared using a Mann Whitney Test *p<0.05. Females: n=80, LD-Controls; n=13, PPHTN. Males: n=154 LD-Controls, n=20, PPHTN.

In females (Figure 6-7, A), 2MeOE1 was reliably detected but no differences in concentration were detected between LD-Controls and PPHTN patients. In males, methoxyestrogens were not altered between LD-Controls and PPHTN patients (Figure 6-7, B).

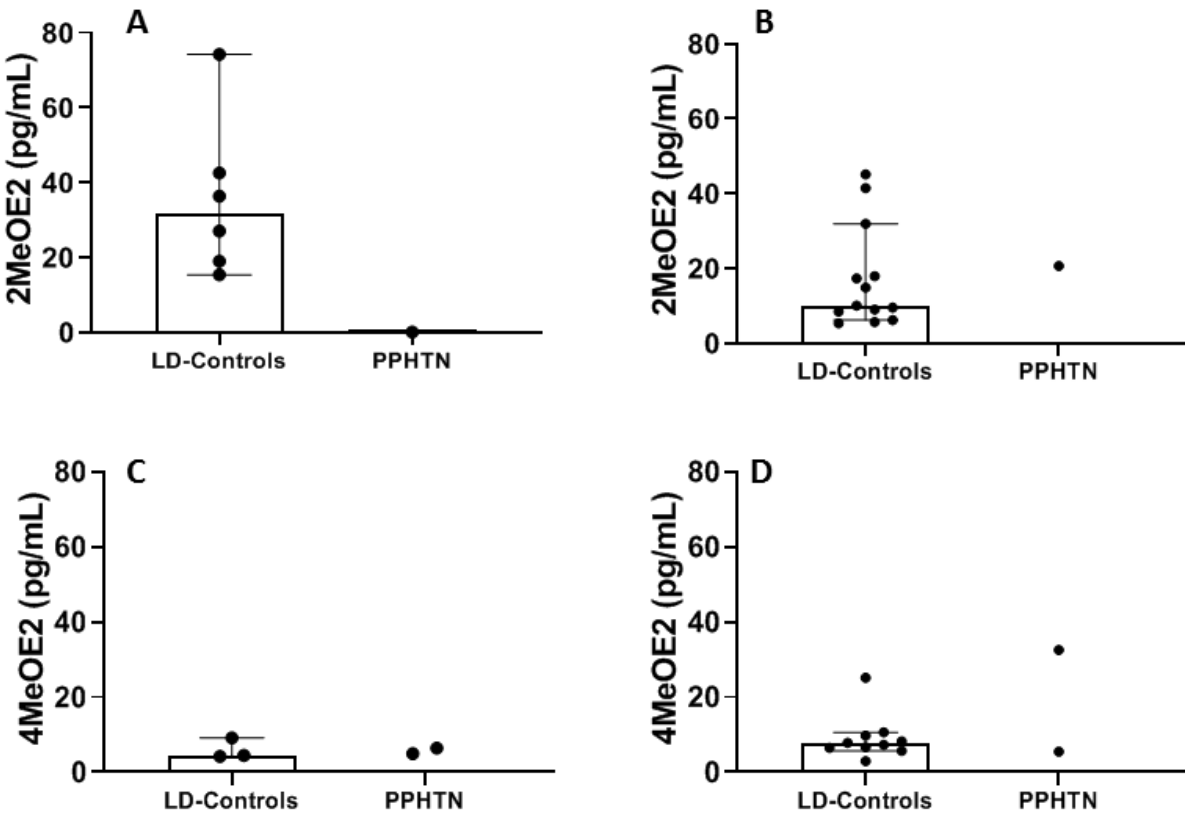

Figure 6-8: 2 & 4 Methoxyestradiol in patients with portopulmonary hypertension (PPHTN) and those acting as liver disease controls 2-Methoxyestrone, 2MeOE1 and 4-methoxyestradiol in females (A, C) and males (B, D) with liver disease control (LD-Control) and PPHTN. Data expressed as median ± 95% Confidence interval. Groups compared using a Mann Whitney Test *p<0.05. Females: n=80, LD-Controls; n=13, PPHTN. Males: n=154 LD-Controls, n=20, PPHTN.

In females (Figure 6-8, A, C) 2MeOE2 remained undetected within the PPHTN patient group, while 4MeOE2 was detected in both groups infrequently. In males (Figure 6-4 Figure 6-8, B, D), 2MeOE2 was detected only in one PPHTN patient and 4MeOE2 in two PPHTN patients.

The frequency of detection in female and male liver disease controls and portopulmonary hypertension also displays differences between each group (Table 6-5).

Table 6-5: Percentage Detection of estrogens in female and male patients with portopulmonary hypertension (PPHTN) and those acting as liver disease controls

	Female		Male	
	LD-Controls	**PPHTN**	**LD-Controls**	**PPHTN**
Subjects	80	12	154	20
% Measured				
E1	100	92	100	100
17αE2	10	17	13	0
17βE2	89	83	99	95
16OHE1	26	42	21	45
16OHE2	30	92	33	65
2MeOE1	54	42	65	65
2MeOE2	8	0	8	5
4MeOE2	5	17	7	10

Liver Disease Control, LD-control; Portopulmonary hypertension, PPHTN; Not detected, ND; Estrone, E1; Estradiol, E2; 16-Hydroxyestrone, 16OHE1; 16-Hydroxyestradiol, 16OHE2; 2-Methoxyestrone, 2MeOE1; 2-Methoxyestradiol, 2MeOE2; 4-Methoxyestradiol, 4MeOE2. Data shown as % estrogens detected by LC-MS/MS in sample subjects.

In summary, data shows sex-dependent estrogen profiles in LD-Controls and PPHTN patients (Table 6-6).

Table 6-6: Estrogens, sex and liver disease phenotype

	Female LD-Controls	Female PPHTN	P-Values	Male LD-Controls	Male PPHTN	P-Values
Subjects	80	12		154	20	
Estrogen Levels (pg/mL)						
E1	57.4 ± 4.6 51.0 [29.0 – 69.3]	25.6 ± 4.9 24.2 [13.4 – 37.3]	**0.00**	76.2 ± 3.4 68.6 [49.1 – 90.8]	83.2 ± 10.9 73.9 [53.9 – 94.9]	0.47
17αE2	4.8 ± 0.9 4.0 [2.5 – 7.2]	11.9 ± 6.5 12.0 [5.4 – 12.0]	0.43	5.3 ± 0.8 4.1 [2.7 – 7.1]	ND -	*0.08*
E2	13.4 ± 1.9 9.0 [5.5 – 16.4]	5.6 ± 0.9 4.9 [2.9 – 7.1]	**0.02**	20.3 ± 1.1 17.6 [11.5 – 26.7]	23.0 ± 2.1 21.7 [16.5 – 28.9]	0.24
16OHE1	13.3 ± 3.1 10.6 [6.4 – 13.9]	9.7 ± 1.6 10.7 [6.2 – 12.9]	0.30	10.8 ± 1.2 8.2 [5.9 – 14.5]	13.1 ± 3.2 8.3 [5.9 – 20.0]	**0.01**
16OHE2	11.9 ± 2.4 8.1 [4.3 – 15.6]	15.4 ± 2.9 9.4 [8.6 – 22.5]	**0.00**	8.2 ± 1.1 5.7 [3.3 – 11.4]	11.2 ± 2.4 8.3 [5.4 – 14.5]	**0.00**
2MeOE1	11.9 ± 1.6 8.8 [5.4 – 13.6]	6.2 ± 1.2 5.5 [4.3 – 8.5]	0.21	9.8 ± 0.7 7.9 [5.0 – 12.8]	7.7 ± 1.0 7.1 [4.8 – 10.7]	0.75
2MeOE2	35.7 ± 8.8 31.7 [18.0 – 50.5]	ND -	0.33	17.1 ± 3.8 10.0 [7.3 – 24.9]	20.6 -	0.57
4MeOE2	5.7 ± 1.2 4.7 [4.3 – 8.1]	5.7 ± 0.7 5.7 [4.9 – 5.7]	0.12	9.1 ± 1.9 7.5 [6.3 – 10.0]	19.0 ± 13.5 19.0 [5.5 – 19.0]	0.63

Liver Disease Control, LD-control; Portopulmonary hypertension, PPHTN; Not detected, ND; Estrone, E1; Estradiol, E2; 16-Hydroxyestrone, 16OHE1; 16-Hydroxyestradiol, 16OHE2; 2-Methoxyestrone, 2MeOE1; 2-Methoxyestradiol, 2MeOE2; 4-Methoxyestradiol, 4MeOE2. Data shown as Mean ± SEM and Median [Q_1-Q_3]. P-values show overall value from mean rank Kruskal-Wallis tests.

In females, E1 and E2 reductions alongside 16OHE2 elevations are linked to PPHTN status. Whereas in males, 16-hydroxyestrogen production is elevated, with higher concentrations of 16OHE1 and 16OHE2 in PPHTN patients (Table 6-6).

In comparison of estrogen concentrations between females and males, E1 and E2 were each increased in males ($p<0.01$) within the liver disease cohort. Similarly, in the PPHTN cohort, comparison based on sex also demonstrated elevated E1 and E2 in males ($p<0.01$). Opposite to these changes, the estrogen metabolite 16OHE2 was elevated in females compared to males ($p=0.01$) in the PPHTN cohort. This suggests an imbalance of circulating parent estrogen in patients presenting with liver disease and PPHTN whereby the estrogen metabolite 16OHE2 is elevated in males and females.

As all PPHTN subjects were of postmenopausal age, there was no comparison of pre- to post-menopausal age between the groups. Applying bivariate analysis there was also no correlation between age and estrogen concentrations (Data not shown).

6.4.6 Advantages of LC-MS/MS over Immunoassay for Clinical Studies

In the PVCLD2 study, plasma was analysed from males and females with 234 controls with liver disease (N=154 males; N=80 females, 59% females postmenopausal) and 32 PPHTN patients (N=20, males; N=12, 100% females postmenopausal). Samples were shipped for analysis by the Roche Diagnostics estradiol immunoassay and as well as for analysis by our validated LC-MS/MS approach (Table 6-7).

Table 6-7: Estradiol quantification by immunoassay and lc-ms/ms in liver disease controls and PPHTN in the PVCLD2 study

E2 Technique	**Liver Disease Controls (pg/mL)**	**PPHTN (pg/mL)**
IA	47.2 (32.8 - 60.3)	40.0 (18.6 - 60.2)
LC-MS/MS	15.52 (8.92 - 23.80)	16.54 (6.73 - 25.10)

Immunoassay, IA; Liquid Chromatography Tandem Mass Spectrometry, LC-MS/MS. Data shown as median (IQR)

E2 was detected by immunoassay in 203 samples and by LC-MS/MS detection in 254 of the 266 samples. E2 concentrations were reported as higher when measured by immunoassay than when assayed by LC-MS/MS. Pearson's test demonstrated a positive correlation between the two methods ($R=0.71$, $p<0.001$, Figure 6-9).

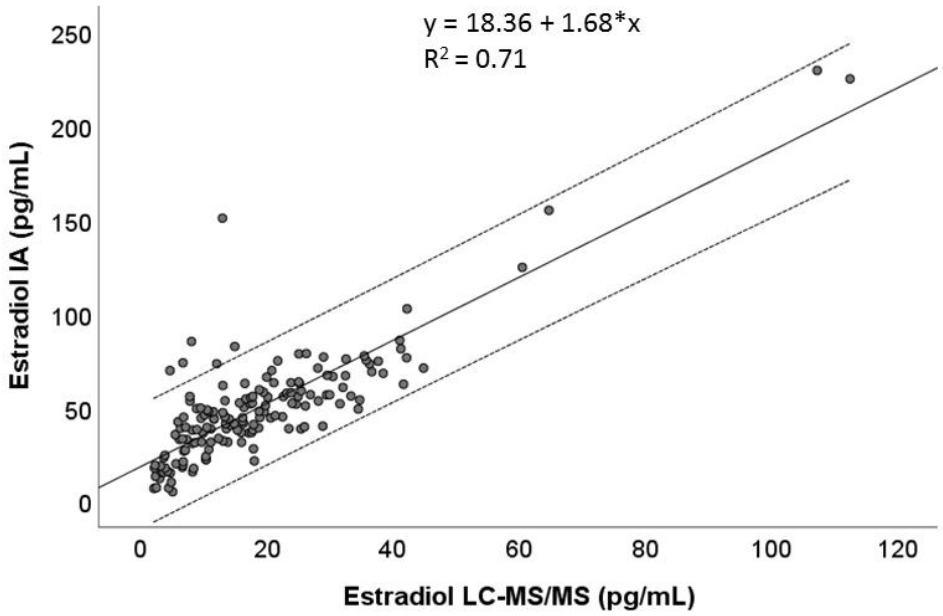

Figure 6-9: Correlation plot of estradiol quantified by immunoassay (IA) and liquid chromatography tandem mass spectrometry (LC-MS/MS). Data presented as values detected by both assays, dotted lines represent the 95% CI for individual data points. Data for immunoassay provided by Nadine al-Naamani and Steven Kawut from the PVCLD2 study team.

The criteria for comparison of the two quantitative measurements in a Bland-Altman plot was rejected, as results from the two methods were significantly different ($p<0.001$, paired sample t-test). Findings from the comparative E2 measurements in PVCLD2 samples are in agreement with a number of studies in the literature showing a positive bias in immunoassay techniques, generally caused by cross reactivity of estrogenic isomers or estrogen analogues which increase the quantified concentrations of estrogen (Handelsman and Wartofsky, 2013). This inaccuracy and around three fold overestimation within my study results in false positives. This issue has been recognised as a substantial problem in clinical and diagnostic studies, with a move toward LC-MS/MS methods advised (Ohlsson *et al.*, 2013; Rosner *et al.*, 2013; Handelsman *et al.*, 2014).

6.5 Discussion

In this study, we found a number of interesting findings implicating increased 16 hydroxylation of parent estrogens in PPHTN. In the overall cohort, increases in both products of the 16-hydroxylation pathway occurred: 16OHE1 and 16OHE2 concentrations were elevated within PPHTN patients. Upon stratification for sex, comparing the PPHTN patients to LD-Controls, E1 and E2 concentrations were

decreased in females with PPHTN, with an increase in 16OHE2 production whereas, in males with PPHTN only elevation in 16OHE1 and 16OHE2 concentrations were observed. Also, within males the E2:E1 ratio was increased, indicative of increased 17βHSD1 production. The increase was present in male LD-Controls and PPHTN in comparison to female LD-Controls. Finally, analysis of the Caucasian population within the study demonstrated that females of this ethnicity show decreased E1, E2 in PPHTN. However, in Caucasian males only increased 16OHE2 was displayed within PPHTN patients, with no change in 16OHE1.

In 2009 the first study of pulmonary vascular complication in liver disease (PVCLD) were performed by Kawut et al (Roberts *et al.*, 2009). This cohort was composed of 536 patients evaluated for liver transplant or pulmonary hypertension in seven centres around the US between 2003 and 2006. Female sex and autoimmune hepatitis were both associated with an increased risk of PPHTN. In 31 PPHTN cases compared with 131 liver disease controls a genetic study associated a variety of genes associated with estrogen signalling and cellular growth/apoptosis involved in PAH were present in PPHTN cases. Genetic variations in genes coding for estrogen receptor 1, aromatase, phosphodiesterase 5, angioprotein 1 and calcium binding protein 4 were associated with PPHTN. Most significantly, two SNPs in aromatase (rs1902584 and rs7175922) were detected which may influence the downstream conversion of androgens to parent estrogens. Upon adjustment of estradiol and the rs7175922 genotype for sex, increasing estradiol concentrations were still displayed in 4 PPHTN patients, identifying this genetic variation as high-risk and implicating estadiol production via aromatase SNPs in PPHTN.

Included within this chapter, the PVCLD2 study marks a continuation of this project with samples collected from an alternative cohort 10 years later than the initial PVCLD study. We aimed to simultaneously determine the concentrations of estrogen and bioactive metabolites within this additional cohort using our validated LC-MS/MS approach described in Chapter 2 with the revalidated limit of quantification (Section 6.3.3 & 6.4.2), following alterations to certain assay parameters.

In this cohort, sex-dependent estrogen differences emerged with a reduction in E1 and E2 in females. Intriguingly, this was accompanied by an increase in 16OHE2 concentrations suggesting an increased conversion of estradiol to its 16-

hydroxylated metabolites. 2MeOE2, a metabolite described as protective in PAH (Docherty *et al*, 2019), was not detected in PPHTN females. In males, there were no changes in E1 and E2 concentrations, but elevations of both 16OHE1 and 16OHE2 were detected in PPHTN cases. Estrogen concentrations were already elevated in males, with a mean concentration of 76.2 pg/mL in LD-Controls and 83.2 pg/mL in PPHTN. These elevations may be caused by genetic variance in metabolism enzymes or by impaired metabolic clearance. Therefore, a further rise may be unexpected in this scenario as healthy circulating male concentrations are expected to be in the range of 10 – 60 pg/mL, (Chapter 1, Table 1-3). The genetic analysis of these PVCLD2 individuals remains unexplored with further analysis currently ongoing with the Kawut group in the US. Therefore, it will be interesting to find out if any alterations in E1 and E2 exist in conjunction with the presence of the aromatase SNPs described in their 2009 publication (Roberts *et al.*, 2009).

Additional evidence for sex-dependent estrogen elevations was shown on comparison of each individual cohort: with males showing no changes in parent estrogens (only a 3-6 pg/mL mean elevation in PPHTN) and increased 16-hydroxyestrogen production. In females, decreased parent estrogens associated with increased 16OHE2 in PPHTN patients. It should be noted that 79% of women within the entire cohort were postmenopausal (56% LD-controls and 100% PPHTN cases), meaning ageing may have an influence in these findings. Nevertheless, recent studies of PAH suggest elevated E2 in males which may also be linked with lower DHEA concentrations via increased conversion of androgens to estrogens in men (Ventetuolo *et al.*, 2016). This emphasises the importance of hormonal imbalances in male PAH being in line with literature hypothesising dysregulated sex steroid metabolism and increased E2 in the pathogenesis of male PAH (Tofovic and Jackson, 2016). Therefore, our finding of elevated 16OHE2 in males might suggest preferential metabolism toward the 16-hydroxylation pathway as being important in male disease. The E2:E1 ratio also indicated a higher activity of 17βHSD1 in males with liver disease compared with females. A high E2/E1 ratio is correlated with proliferation of breast cancer cells (Chen *et al*, 2012), increasing the risk of male breast cancer (Brinton *et al.*, 2015). In liver disease, genetic variants in 17βHSD13 play a role in non-alcoholic fatty liver disease via its enzymatic activity (Ma *et al.*, 2019). In this case, SNP in 17βHSD13 associated with the extent of liver ballooning and inflammation. In liver cirrhosis, abnormalities in 17βHSD activity also occur via the decreased hepatocytes, and in a small number of hepatocellular carcinoma

cases, have been linked with increased 17βHSD2 activity. However the biological significance of this has yet to be determined (Narasaka *et al.*, 2000). The biological interplay between genetic variants, 17βHSD enzymes and estrogen metabolism imbalance may play an important role in male patients and should be further investigated in future studies of PAH and PPHTN pathogenesis.

As previous chapters have suggested, the 16-hydroxyestrogen pathway appears predominantly in patients with PAH, in this case within the PPHTN patients. 16OHE2 increases in females with PPHTN and higher concentrations are seen in female patients in comparison to males. This demonstrates increased abundance of 16OHE2 in diseased females with a potential role in pathogenesis. 16OHE2 is not widely defined in PAH literature and therefore to an even lesser extent in PPHTN. In our proof-of-concept studies, 16OHE2 initiates proliferation in female PASMCs and BOECs while lacking any effect in male cells. This proliferative response may occur via oxidative stress pathways, dysregulating *AhR* signalling and consequently altering CYP enzymes involved in the metabolism of parent estrogens (N Denver et al., 2018; N Denver et al, 2019). This finding may also correlate with increasing activity of the CYP1B1 enzyme in PAH. This particular CYP was elevated in PAH in a microarray study of lung from female mice over-expressing the serotonin transporter with further confirmation of elevations in PASMCs from iPAH patients at the mRNA and protein level. (White et al., 2012, 2011). The White et al 2012 study also demonstrated an increase in 16OHE1 associated with increased pulmonary hypertension in hypoxic mice following administration for 28 consecutive days (White et al., 2012). 16OHE1-treated mice were compared to vehicle treated controls, demonstrating increased urinary 16OHE1, right ventricular hypertrophy, right ventricular systolic pressures and vascular remodelling. Inhibition of CYP1B1 in hypoxic and Sugen hypoxic mice reversed these indicators of disease. These studies were performed in females, although it remains undetermined if increased CYP1B1 could also lead to elevated 16OHE2 production in PAH, a finding which has been displayed throughout this thesis among idiopathic PAH, premenopausal PAH and PPHTN. Therefore, these studies suggest that 16OHE2 production may be upregulated in females with PAH.

Elevated E2 and lower DHEA plasma concentrations associated with PAH in men (Ventetuolo *et al.*, 2016). A second recent study demonstrated increased E2 and higher E2/testosterone ratios, as well as lower testosterone and progesterone

associated with increased risk of PAH (Wu *et al.*, 2018). Fifty-five of ninety-five patients enrolled in this study died during follow-up, with elevated E2 in these male patients being associated with increased mortality. Further extension of these studies may show increased estrogen metabolism in male disease with a potential dysfunction in *CYP1B1,* which may account for alterations displayed in 16-hydroxylation of PPHTN patients.

Initial studies from this PVCLD2 cohort by Al-Naamani et al lead to further implication of changes in sex hormone metabolism in PPHTN (Al-Naamani *et al.*, 2019); analysis of 33 PPHTN cases vs 166 LD-Control subjects displayed alterations in urinary estrogen concentrations. In PPHTN, an unexpected finding saw a lower urinary ratio of 2OHE1/16OHE1, hence suggesting a higher production of 16OHE1 and lower production of 2OHE1 in PPHTN cases than in liver disease controls. This finding is in part supported within our current plasma study with an overall increase of 16OHE1 in the entire cohort. Upon stratification for sex, it was clear that 16OHE1 was elevated in male PPHTN patients. The 2OHE1/16OHE1 ratio is a commonly applied approach in oncology to determine the risk of hormone-sensitive disease; 2-hydroxylation of E1 and E2 presents a less mitogenic pathway than 16-hydroxylation. One study reported lower concentrations of 2OHE, 2/16OHE ratios and higher 16OHE1 in serum from breast cancer patients (Ho *et al.*, 1998). The lowering of this ratio has more recently been confirmed as a biomarker of estrogen-sensitive breast cancer (Kabat *et al.*, 2006; Arslan *et al.*, 2014). In PAH, this ratio has also been implicated; lower urinary ratios among PAH-*BMPR2* mutation carriers suggest elevated 16OHE1 production in PAH (Austin *et al*, 2009). The circulating concentrations of these metabolites has not previously been determined. Our finding that 16OHE1 is increased in PPHTN is of interest as it presents a potential biomarker for PAH. Lowering of urinary/plasma 2/16-hydroxyestrogen ratios may also be useful as a predictor of PAH. 16OHE1 has previously been shown to cause proliferation of hPASMCs, induce pulmonary hypertension in the mouse and is associated with induction of experimental pulmonary hypertension in the obese mouse (Hood et al., 2016; Mair et al., 2019; White et al., 2012). In a study by Mair et al (Mair *et al.*, 2019), E2 was decreased in plasma from normoxic obese male mice in comparison to lean controls. However, in visceral adipose tissue (VAT), CYP1B1 and 16OHE1 were increased and may have contributed to oxidative stress, effects that were attenuated by both anastrozole and TMS. This somewhat supports the situation reported here, i.e. an

increase in 16OHE1 in male PPHTN patients, although BMI parameters are still blinded in these patients. This suggests further studies into the action of CYP1B1 in PAH including BMI data are warranted.

Finally, in relation to ethnicity, the Caucasian population presented the greatest changes in circulating estrogens, with decreased E1, E2 and increased 16OHE2 in females and increased 16OHE2 in males with PPHTN compared to LD-Controls. The comparison of additional ethnic groups were hindered by low numbers of African-American and Hispanics within the PPHTN group, likely due to the rare nature of PPHTN in general and subsequent rare nature in the ethnic minority sub-groups. However, this is one of the largest and first studies of PPHTN with very strict case and control inclusions which elucidates a number of estrogens as altered in disease status and hence opens questions for other populations of PPHTN patients.

6.5.1 Study Limitations

This study may have been limited by blinding of additional clinical characteristics for the purpose of this thesis. However, this will be analysed out with this chapter by the PVCLD2 research team in the USA in due course.

Chapter 7 Proof-of-concept Clinical Trials: Estrogen in Pulmonary Arterial Hypertension

7.1 Introduction

In the treatment of sex hormone-sensitive disease two possible therapeutic strategies are commonly adopted: estrogen receptor inhibition and aromatase inhibition. These endocrine therapies are treatments of choice for a number of hormone-sensitive cancers. Drugs such as anastrozole (an aromatase inhibitor) and fulvestrant (an ERα inhibitor) are being favoured over compounds such as tamoxifen (an ERα inhibitor), as tamoxifen has been related to increased risk of endometrial cancer and ischemic cerebrovascular events via estrogen-like activity (Lancet *et al*, 1998). For decades tamoxifen, known as Nolvadex®, was considered the first line treatment for hormone-responsive breast cancers. The nonsteroidal anti-estrogen reduces breast cancer recurrences in postmenopausal women and, since its first trials, has been further extended to treat premenopausal women and men. It emerged that longer use of tamoxifen, for 10 years, reduced the risk of breast cancer recurrence by a further 25% over those women who ceased treatment after 5 years, showing that extended treatment periods are therapeutically beneficial (Davies *et al.*, 2013). However, tamoxifen can display side effects including hot flashes, fatigue, and an increased risk of blood clots and endometrial cancer, meaning the benefit to risk ratio must be seriously considered prior to prescription (Robert *et al*, 1997). The risk is amplified in premenopausal women as early onset menopause can occur. Third generation selective estrogen therapies are now competing with traditional endocrine therapies due to improved efficacy and reduced side effects. Fulvestrant, commonly known as Faslodex®, is a potent selective estrogen receptor down-regulator with a novel mechanism of action, effectively shown to block proliferation of breast cancer cells in estrogen and progesterone-positive cancers (Lee *et al*, 2017). This treatment option is effective in the postmenopausal patient cohort, showing a higher efficacy than alternative treatment routes. The chemical structure is also unique (Figure 7-1), being a 7α-alkylsulphinyl analogue of estradiol. This allows highly-competitive binding to the ER, with an 89% binding affinity over estradiol (Wakeling *et al*, 1988), which disrupts ER dimerization and nuclear localisation. The use of conventional antibody-based immunoassay techniques for quantification of estradiol levels in patients receiving fulvestrant has resulted in false positives for estradiol concentrations (Elguero *et al*, 2014). This is due to the structural similarities between the two molecules. Therefore, LC-MS/MS has been suggested as the gold standard approach for clinical assessment of estradiol concentrations in postmenopausal subjects, further emphasising the need

for accurate and precise analytical methods for use in routine clinical applications (Owen *et al.*, 2019).

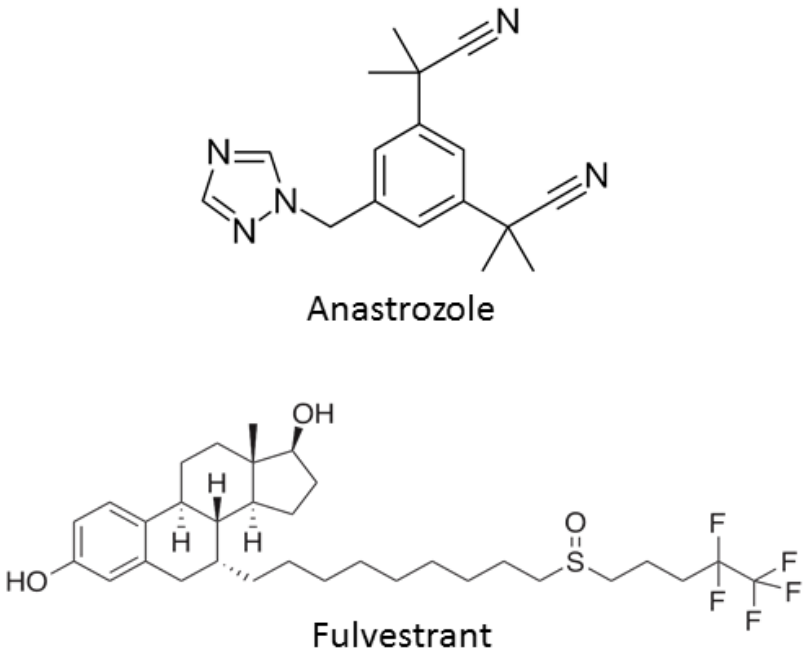

Figure 7-1: Chemical structure of anastrozole and fulvestrant Structure of α, α, α', α'-Tetramethyl-5-(1H-1, 2, 4-triazol-1-ylmethyl)-m-benzenediacetonitrile, anastrozole and the 7α-alkylsulphinyl analogue of estradiol, fulvestrant.

Anastrozole, known as Armidex®, is also a drug commonly used to treat ER-positive breast cancer in postmenopausal women (Figure 7-1). Anastrozole inhibits aromatase which converts androgens to estrogens. Clinical trials have shown that anastrozole significantly reduces tumour progression in comparison to tamoxifen in terms of drug-free survival, non-musculoskeletal adverse effect and prevention of contralateral breast cancer in postmenopausal women (Mokbel, 2003). In the hypoxic mouse and Sugen 5416/hypoxic rat models of PAH, aromatase inhibition via anastrozole only shows benefit in females with reduced remodelling of pulmonary arteries, lower RV pressures and less RV hypertrophy; males had no benefit from treatment (Mair *et al.*, 2014). This sex-dependent effect might in part be due to altered aromatase expression, as males have lower aromatase levels in the lungs of mice and in addition human PASMCs derived from postmenopausal women display increased aromatase expression over age-matched male cells (Mair *et al.*, 2015) Further to this finding, sex-dependent *BMPR2* expression has also

been described, with 25% lower expression in female human-derived lymphocyte cells compared to males and a 15% lower expression in whole lungs of ovariectomised female FVB/NJ mice compared to males. This occurred with exposure to the same concentrations of E2 in each, leading to the conclusion that the effect on *BMPR2* expression is mediated via canonical estrogen receptors (Austin *et al.*, 2012). Data from Chen and Austin et al also demonstrate fulvestrant and anastrozole treatment to be highly effective in PAH over tamoxifen (X. Chen *et al.*, 2017). Animal studies using BMPR2 mutant mice showed that fulvestrant and anastrozole caused reversal of PAH and inhibition of markers of metabolic defects such as homeostatic-insulin resistance, here 16OHE1 was used to demonstrate the mitogenic effects via increased superoxide production, as this metabolite is more estrogenic than E2, and in patients, E2 has been shown to be preferentially metabolised to 16OHE1 (Chen *et al.*, 2017). Sex-specific efficacy and potential PAH sub-group efficacy may relate to estrogen, estrogen metabolism and the consequent effect on BMPR2 signalling (Austin *et al.*, 2012; Prins *et al.*, 2019).

A further study in female BMPR2 transgenic mice demonstrated that combining fulvestrant and anastrozole reduced pulmonary artery remodelling and lowered RV pressures, due to increased inhibition of estrogen signalling (Hajri *et al.*, 2002). Comparisons of fulvestrant to anastrozole in a phase three trial for breast cancer show fulvestrant provides superior efficacy and remains the preferred treatment option for ER positive breast cancers. Therefore, fulvestrant is recommended in postmenopausal women who have not received previous endocrine therapies (Robertson *et al.*, 2016). Histologically-confirmed estrogen receptor-positive or progesterone receptor-positive patients, or both, were given fulvestrant (n=230, intramuscular injection 500 mg) or anastrozole (n=232, orally 1 mg), a placebo group was also assessed. Interestingly, fulvestrant had a superior efficacy and was the preferred treatment option for patients, increasing survival rates by around 3 months. Clinically, the use of fulvestrant in the treatment of PAH has yet to be examined. However the blockage of ER-mediated transcriptional activity and receptor degradation seems relevant to improved clinical characteristics of disease. On the other hand, pilot data from a clinical trial of 18 male and female PAH patients, randomised in a 2:1 fashion receiving 1 mg/day oral anastrozole or placebo for 3 months, demonstrated significantly decreased estradiol levels in patients whilst increasing 6-MWD. This did not show any increase in quality of life or RV function for patients (Kawut *et al.*, 2016). Additionally, an ongoing multi-centre study of

anastrozole treatment in PAH (PHANTOM, NCT03229499) aims to further examine the efficacy of treatment over 1 year with secondary end points of PAH.

7.2 Hypothesis and aims

For each of the above-mentioned studies, analysis of circulating levels of multiple estrogens has not been conducted. We predict levels of mitogenic bioactive metabolites might also be affected by PAH therapies. The aim of this chapter was to quantify estrogens in patients treated with fulvestrant and anastrozole by LC-MS/MS.

7.3 Methods

7.3.1 Study Samples and Data Collection

Samples from both studies were obtained in collaboration with Steven Kawut at Perleman School of Medicine, University of Pennsylvania, Philadelphia, USA. All the clinical aspects of the study were carried out by Kawut et al while the LC-MS/MS and data analysis was performed in Edinburgh by Nina Denver.

Fulvestrant Trial: The trial protocol was approved by the institutional review board (NCT02911844). Five postmenopausal women (>50 years) were recruited with PAH. Patients included had not menstruated in the preceding 12 months or undergone bilateral oophorectomy; those excluded were using hormone therapy or warfarin and had a history of breast cancer or liver disease. Collection was approved at sites covered by the University of Pennsylvania's IRB with sample collection at baseline (0 days, before first dose) and following 9 weeks (63 days) of administration of a 500 mg dose intramuscularly, on days 0, 14, 28 and 56 of the study.

Anastrozole Trial: The trial was approved by the institutional review board (NCT 1545336). The samples shipped for LC-MS/MS analysis were remaining aliquots from this study entitled "Anastrozole in Pulmonary Arterial Hypertension: A Double Blind, Placebo Controlled Trial" (Kawut *et al.*, 2016). Patients included in the study were men aged 18 or older and postmenopausal women diagnosed with PAH. Patients were excluded if treated with estrogen or anti-hormone therapy, or had a history of breast cancer. Two thirds of the subjects were given a 1 mg tablet of anastrozole while the remaining one third received placebo tablets, decided by

random assignment. Tablets were to be taken orally, once daily in the morning. Patients were assessed at baseline (0 days) and following 6 and 12 weeks. Samples for LC-MS/MS analysis were from baseline and week 12.

A number of clinical end-points were also assessed for both studies over the 9-week fulvestrant and 12-week anastrozole study. Characteristics such as 6MWD, plasma NT-proBNP, HPCs, blood biomarkers and echocardiographic measurements (right ventricular (RV) systolic pressure, stroke volume and tricuspid annular plane systolic excursion (TAPSE)) were recorded.

7.3.2 LC-MS/MS Analysis

Fulvestrant Trial: Analysis was performed with the adapted method to improve robustness, as in Chapter 2 (2.3.9.6) also mobile phase B consisted of 90% acetonitrile/10% water + 0.1% formic acid. The gradient was adjusted to ensure retention of analytes was in the same timeframe as other studies. Calibration curves were prepared (2, 6, 10, 20, 100, 200, 500, 1000 + 200 pg/mL internal standard). Certified standards were used for E1 and E2. Also, within the sample preparation the transfer following elution was removed, an optimisation adopted to improve reproducibility at lower concentrations and avoid sample loss. 500 µL of plasma was available for each subject (n=5).

Aromatase Trial: Analysis was performed using the original published LC-MS/MS method and was prior to optimisation of the transfer steps and mobile phase. Calibration curves were prepared (2, 10, 20, 40, 100, 200, 500, 1000, 2000 + 200pg/mL internal standard). 100 µL to 500 µL of plasma was available for each of the subjects (n=5 placebo, n=10 anastrozole), which had been previously thawed. The appropriate dilution factor was taken into account when calculating final pg/mL estrogen concentrations.

7.3.3 Data Analysis

Plasma concentrations were non-normally distributed with values compared using Wilcoxon Sign Rank Tests. No data input was used for missing values. Data were analysed using SPSS 25 statistical software.

7.4 Results

7.4.1 Patient Information

Fulvestrant Trial

Table 7-1: Baseline patient characteristics of subjects in fulvestrant trial

Patient	1	2	3	4	5	Median
PAH Class	CTD	iPAH	HIV	iPAH	CTD	
Age	52	58	52	55	66	55
BMI (kg/m²)	32	27	42	31	33	31.7

Body Mass Index, BMI; Congenital heart defect, CTD; idiopathic pulmonary arterial hypertension, iPAH; Human immunodeficiency Virus, HIV

The median age range of study subjects was 55 (range 52 – 66). Three women were non-Hispanic white and two were African-American with a body mass index of 31.7 kg/m^2 (range 27 – 42), (Table 7-1).

Anastrozole Trial

Table 7-2: Baseline patient characteristics of subjects randomised to placebo and anastrozole

Patient	Placebo	Anastrozole
Subjects	5	10
Females	2	7
Males	3	3
Age y	59 ± 10	61 ± 13
BMI (kg/m^2)	33 ± 9	29 ± 6
PAH diagnosis		
iPAH	2	5
Systemic Sclerosis	2	2
HIV	1	1
Congenital Systemic to pulmonary shunt	0	2

Body Mass Index, BMI; idiopathic pulmonary arterial hypertension, iPAH; Human immunodeficiency Virus, HIV

In the placebo group (Table 7-2), all five subjects were white non-Hispanic with two females and three males. In the treatment group seven were white non-hispanic, one was Hispanic and three were African-American with seven females and three males.

7.4.2 LC-MS/MS Analysis

7.4.2.1 Quality Control

Fulvestrant Trial

Calibration curves were linear from 2 – 500 pg/mL for E1, E2 and from 2 – 100 pg/mL for 16OHE1, 16OHE2 and 6 – 200 pg/mL for methoxyestrogen metabolites. In pooled plasma, E1 was detected at 28.8 pg/mL in females and 10.5 pg/mL in males. E2 was detected at 32.96 pg/mL in females and 9.4 pg/mL in males. Estrogen metabolites were not detected within these pooled samples, as expected.

Anastrozole Trial

Calibration curves were linear from 2 – 500 pg/mL for E1, E2, 2-2000 pg/mL for 17βE2, 2 – 500 pg/mL for 16OHE1, 16OHE2 and 10 – 500 pg/mL for the methoxyestrogens. In pooled plasma, E1 was detected at 26.3 pg/mL in females and 14.2 pg/mL in males. E2 was detected at 34.6 pg/mL in females and 10.3 pg/mL in males. Estrogen metabolites were not detected within these pooled samples, as expected.

The concentrations of E1 and E2 in pooled plasma samples were similar between both trial batches. Also, in both batches, no estrogen metabolites were detected, as expected.

7.4.3 Estrogen in Patients treated with Fulvestrant

In the five postmenopausal patients, estrogens were detected at baseline and follow-up after 9 weeks (Table 7-3). In this cohort, estradiol levels were lower than estrone, as expected. Elevated 16-hydroxyestrogen concentrations were present, which might be indicative of their disease status. The changes following fulvestrant treatment were analysed (Table 7-4, Figure 7-2, Figure 7-3) to assess efficacy of this treatment.

Table 7-3: Baseline and follow up estrogen levels by LC-MS/MS

	Baseline (pg/mL)					Follow-Up (9 Weeks, pg/mL)				
Patient	**E1**	**17αE2**	**E2**	**16OHE1**	**16OHE2**	**E1**	**17αE2**	**E2**	**16OHE1**	**16OHE2**
1	29.86	10.38	5.64	60.20	22.82	19.84	8.70	3.94	36.98	9.66
2	21.44	29.60	2.02	58.70	17.18	15.18	10.40	0.76	28.9	6.86
3	34.58	3.50	6.84	43.58	3.82	47.82	9.80	7.80	34.26	1.72
4	31.02	3.24	3.24	ND	ND	22.96	ND	0.38	9.38	ND
5	22.24	17.3	2.94	2.64	16.34	64.76	31.9	2.68	1.18	14.94

Not detected, ND; Estrone, E1; Estradiol, E2; 16-Hydroxyestrone, 16OHE1; 16-Hydroxyestradiol, 16OHE2

Table 7-4: Analysis of estrogen levels following treatment with Fulvestrant

	Baseline Mean (pg/mL)	9 Weeks Mean (pg/mL)	Mean Change (pg/mL)	P value
E1	27.83 ± 2.6	34.11 ± 9.5	+6.28	0.69
17αE2	12.80 ± 4.9	15.20 ± 12.8	+2.4	0.14
17βE2	4.14 ± 0.9	3.11 ± 1.3	-1.02	0.50
16OHE1	41.28 ± 12.0	22.14 ± 7.1	-19.14	0.22
16OHE2	15.04 ± 3.6	8.30 ± 2.5	-6.75	*0.06*
Total	101.1	82.9	-30.8	

Estrone, E1; Estradiol, E2; 16-Hydroxyestrone, 16OHE1; 16-Hydroxyestradiol, 16OHE2. P-values from signed rank tests comparing 9-week estrogen concentrations to baseline. Data shown as the total mean concentration ± SEM

Overall, there was an 18% reduction in total circulating estrogens following fulvestrant treatment (Table 7-4). Notably, there was a trend toward decreasing 16OHE2 among these patients, with a 6.75 pg/mL reduction among the five patients. If we consider the response for each patient in spaghetti plots (Figure 7-2), the general profile decreased although one patient showed increased E1 and E2 concentrations, with two patients showing increased 17αE2.

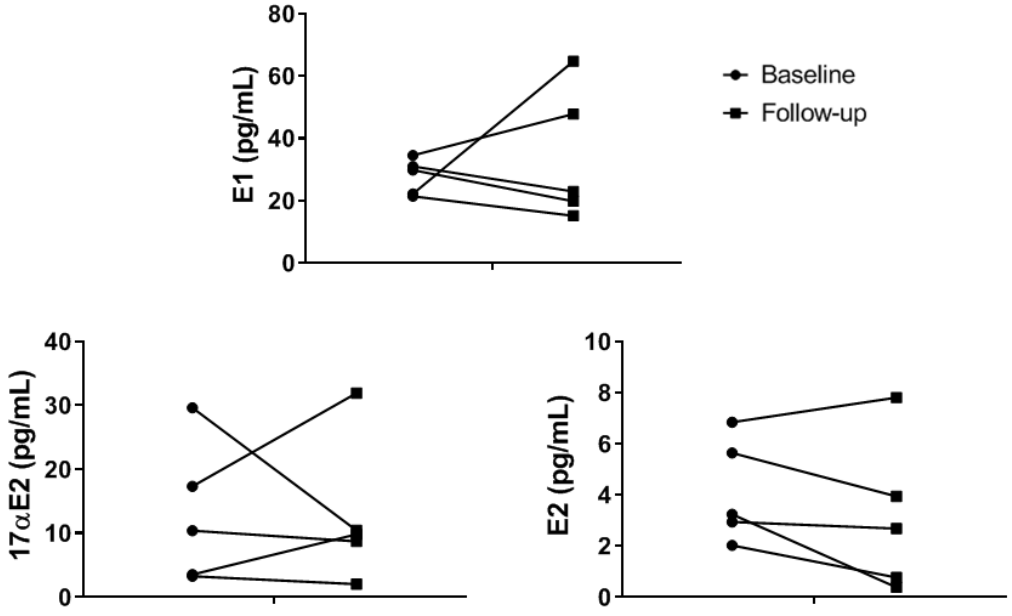

Figure 7-2: Parent estrogens before and after fulvestrant treatment Estrone, E1; alpha-estradiol, 17αE2 and Estradiol, E2 at baseline (circles) and follow-up of 9 weeks (squares) following fulvestrant treatment.

For bioactive estrogen metabolites, 16-hydroxyestrone was detected in five patients and 16-hydroxyestradiol was detected in four of the five patients (Figure 7-3). Favourable profiles appeared with a reduction in the majority of patients.

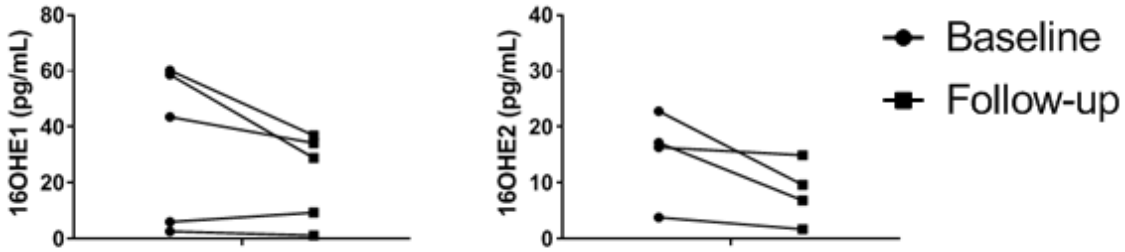

Figure 7-3: Estrogen metabolite concentrations before and after fulvestrant 16-Hydroxyestrone, 16OHE1 and 16-Hydroxyestradiol, 16OHE2 at baseline (circles) and follow-up of 9 weeks (squares) following fulvestrant treatment.

There was a reduction in 16OHE1 and 16OHE2 in four of the five patients. 16OHE2 was not detected in one patient at baseline or follow-up. Interestingly, the reduction in 16OHE2 was significantly correlated when the group in Philadelphia applied a Spearman's test with additional clinical characteristics collected throughout the study. There was a significant correlation between 16OHE2 and hematopoietic progenitor cells (HPCs) after treatment with fulvestrant (r=0.9, p=0.04) in four patients (Data not shown – Analysed by PVCLD2 study team). A number of additional clinical characteristics recorded during the studies are reported (Table 7-6). This study has now been published (Kawut et al., 2019).

7.4.3.1 Advantages of LC-MS/MS over Immunoassay in Patients Treated with Fulvestrant

In the Fulvestrant trial plasma samples were analysed by LC-MS/MS as well as being shipped for analysis by the Roche Diagnostics estradiol immunoassay, allowing comparison between the two methods for estradiol analysis (Table 7-5).

Table 7-5: Estradiol quantification by immunoassay and LC-MS/MS in postmenopausal women at baseline and following fulvestrant treatment

	Technique	Baseline (pg/mL)	Week 9 (pg/mL)
Patient 1	IA	25.64	33.31
	LC-MS/MS	5.64	3.94
Patient 2	IA	20.66	29.55
	LC-MS/MS	2.02	0.76
Patient 3	IA	27 .52	57.32
	LC-MS/MS	6.84	7.8
Patient 4	IA	17.93	35.34
	LC-MS/MS	3.24	0.38
Patient 5	IA	21.44	42.99
	LC-MS/MS	2.94	2.68

Immunoassay, IA; Liquid Chromatography Tandem Mass Spectrometry, LC-MS/MS; picogram per millilitre, pg/mL.

There are noticeable differences in the concentrations determined by immunoassay and LC-MS/MS at baseline and post-fulvestrant treatment, with higher E2 concentrations determined by IA (Table 7-5). In postmenopausal women, the use of immunoassays has been questioned due to a lack of sensitivity and overestimation of estradiol levels due to cross-reactivity (Handelsman and Wartofsky, 2013). In breast cancer studies, fulvestrant has also been shown to interfere with immunoassay measurements in postmenopausal women (Elguero, Patel and Liu, 2014; Owen *et al.*, 2019). Due to structural similarities between fulvestrant and estradiol the cross-reactivity of immunoassays is enhanced causing false elevations. This interference by the structural analogues may occur within the Fulvestrant trial immunoassay, as we see elevated concentrations following 9 weeks of treatment with IA but not LC-MS/MS. In the application of LC-MS/MS, greater selectivity within chromatographic development can rule out these interferences. For example, in our method we analyse both the alpha and beta isomers of estradiol and fulvestrant would have a different SRM if present in the extract. Therefore, from similarities between observations in Table 7-5 with the literature data we hypothesise that reports suggesting higher immunoassay quantification of E2 on comparison to LC-MS/MS due to cross-reactivity are true.

7.4.3.2 Baseline and Follow-up Clinical Characteristics of Patients receiving Fulvestrant

Table 7-6: Baseline and follow-up clinical endpoints for patients receiving fulvestrant

	N	Baseline	Follow-up	Median Change	P
Six-minute walk distance, m					
	5	347 [336 – 488]	365 [357-519]	31 [10–32]	0.50
Echocardiography					
Tricuspid annular plane systolic excursion, mm	4	19.5 [14.5-28.5]	25 [18-27.5]	2 [-3-5.5]	0.47
Right ventricular systolic pressure, mm Hg	3	86 [35-100]	87 [40-89]	1 [-11-5]	1.0
Right ventricular index of myocardial performance	4	0.29 [0.26-0.66]	0.52 [0.37-0.82]	0.095 [-0.04-0.2]	0.47
Stroke volume, mL	4	56 [36, 73.5]	62.2 [48, 79.9]	6.4 [2.2, 16.2]	*0.07*
Biomarkers					
NT-proBNP, pg/mL	5	33.8 [28.1-1542]	70.3 [26.9 – 2150]	42.1 [6.7 – 114]	0.14
HOMA index	5	3.9 [1.4-6.1]	3.0 [1.7-4.6]	0.3 [-1.0 – 0.6]	0.89
		187 [157-227]			
Total cholesterol, mg/dL	5	185[167-203]	185 [167-203]	-2[-16-2]	0.42
High-density lipoprotein, mg/dL	5	45 [40-51]	47 [40-60]	9[2-9]	0.13
Triglycerides, mg/dL	5	139 [107-196]	133 [123-137]	-16[-30-3]	0.42
Low-density lipoprotein, mg/dL	5	112 [87-122]	108 [100-110]	-12[-17—4]	0.22
IL-6, pg/mL	5	2.8 [2.4 – 2.9]	1.8 [1.2 – 2.9]	-0.7 [-1.8 – 0.06]	0.35
(18F-FES) uptake	5	0.73 [0.62-1]	0.69 [0.64-1.03]	0.04 [-0.09-0.7]	0.69

Adapted from (Kawut, Pinder, Naamani, Palevsky, Fritz, K Akaya Smith, *et al.*, 2019). Data shown as the median and interquartile ranges with p-values comparing baseline to follow-up by Wicoxon signed ranks tests.

7.4.4 Estrogen in Patients Treated with Anastrozole and Placebo

In postmenopausal women and men, estrogens were detected in both the anastrozole and placebo groups. Patient number 6 displayed lower concentrations than expected within the baseline visit particularly for E1 and E2 (Table 7-7).

Table 7-7: Estrogen concentrations in anastrozole and placebo groups

Patient	Baseline (pg/mL)					Follow-Up (12 Weeks, pg/mL)				
Anastrozole	**E1**	**17αE2**	**E2**	**16OHE1**	**16OHE2**	**E1**	**17αE2**	**E2**	**16OHE1**	**16OHE2**
1	10.8	385.9	ND	ND	ND	3.1	42.4	6.0	49.5	ND
2	129.0	8.78	36.8	ND	14.8	ND	7.83	3.9	ND	6.7
3	40.6	1255.2	290.3	62.7	4.1	ND	16.1	ND	3.1	3.1
4	21.0	54.6	3.9	23.4	4.4	5.6	17.8	ND	ND	ND
5	172.4	2.02	37.5	ND	ND	48.3	18.8	42.9	ND	ND
6	ND	85.7	ND	ND	ND	ND	4.6	ND	137.1	ND
7	196.5	48.3	95.1	93.4	15.2	14.9	27.0	33.1	ND	16.2
8	26.4	4.9	ND	ND	ND	ND	64.0	6.8	43.0	ND
9	26.3	99.9	6.62	77.1	ND	4.2	230.3	ND	ND	25.0
10	35.9	5.5	16.06	35.4	ND	ND	28.7	3.0	14.12	ND
Placebo	**E1**	**17αE2**	**E2**	**16OHE1**	**16OHE2**	**E1**	**17αE2**	**E2**	**16OHE1**	**16OHE2**
11	52.5	31.95	43.15	183.45	31.08	47.16	52.38	25.8	59.92	31.8
12	146.6	724.4	170.7	ND	55.4	119.24	84.52	88.88	ND	30
13	100.94	8.52	68.52	39.1	ND	67.68	13.7	34.4	27.1	5.78
14	118.82	58.58	36.3	18.08	9.94	170.2	461.3	43.2	ND	53.2
15	31.12	40.92	9.88	13.6	29.16	42.72	531.7	19.34	19.42	19.44

Not detected, ND; Estrone, E1; alpha-Estradiol, 17αE2; Estradiol, E2; 16-Hydroxyestrone, 16OHE1; 16-Hydroxyestradiol, 16OHE2; pg/mL, picogam per millilitre.

Table 7-8: Mean change in anastrozole and placebo Groups Differences in baseline and 12-week estrogen concentrations (pg/mL).

Anastrozole	**Baseline**	**12 Week**	**Total**	**p-value**
E1	73.2 ± 24.1	15.2 ± 8.5	-64.75	*0.08*
17αE2	195.1 ± 123.2	45.8 ± 21.2	-149.32	0.39
E2	69.5 ± 69.5	15.9 ± 7.1	-43.41	0.39
16OHE1	58.4 ± 13.0	60.9 ± 26.5	+2.5	0.89
16OHE2	8.1 ± 2.9	10.6 ± 4.4	+2.5	1.00
Total	405.9	150.5	-261.1	
Placebo	**Baseline**	**12 Week**	**Total**	**p-value**
E1	90.0 ± 21.2	89.4 ± 24.4	-17.4	1.00
17αE2	172.8 ± 138.1	228.7 ± 110.5	+17.8	0.82
E2	65.7 ± 27.9	42.3 ± 12.3	-22.5	1.00
16OHE1	63.5 ± 33.7	35.5 ± 12.4	-28.2	0.79
16OHE2	31.4 ± 9.3	28.0 ± 7.8	+2.0	0.35
Total	423.4	423.9	-48.4	

Estrone, E1; Estradiol, E2; 16-Hydroxyestrone, 16OHE1; 16-Hydroxyestradiol, 16OHE2. Data shown as the total mean concentration ± SEM in anastrozole (n=10) and placebo (n=5) with statistics shown following a Wilcoxon mean ranks test.

Table 7-9: Comparison of anastrozole to placebo treatment at baseline and week 12

Anastrozole vs Placebo	**p-value Baseline**	**p-value Week 12**
E1	0.69	*0.08*
17αE2	1.00	0.15
E2	1.00	*0.08*
16OHE1	0.56	0.40
16OHE2	0.71	*0.06*

Estrone, E1; Estradiol, E2; 16-Hydroxyestrone, 16OHE1; 16-Hydroxyestradiol, 16OHE2. Data shows p-values comparing total estrogen levels in anastrozole vs placebo groups at baseline and following 12 weeks of treatment. Statistics performed using a Wilcoxon mean ranks test.

Following anastrozole treatment there was a 63% reduction in total pg/mL estrogen concentrations in the anastrozole group and a 0% change in placebo between baseline and 12-week follow-up (Table 7-8). However, the reduction of each was not significant for each individual estrogen with only E1 showing a trend to be reduced following treatment. In comparing estrogen concentrations in the placebo and anastrozole group at baseline there were no changes in estrogens (Table 7-9). The concentrations of estrogens in week 12: E1, E2 and 16OHE2 concentrations tended to be reduced in the anastrozole patients in comparison to placebo.

However, the proof-of-concept study was not powered sufficiently for these to reach significance being designed to show potential modifications upon treatment.

The response of individual patients was analysed as spaghetti plots (Figure 7-4, Figure 7-5)

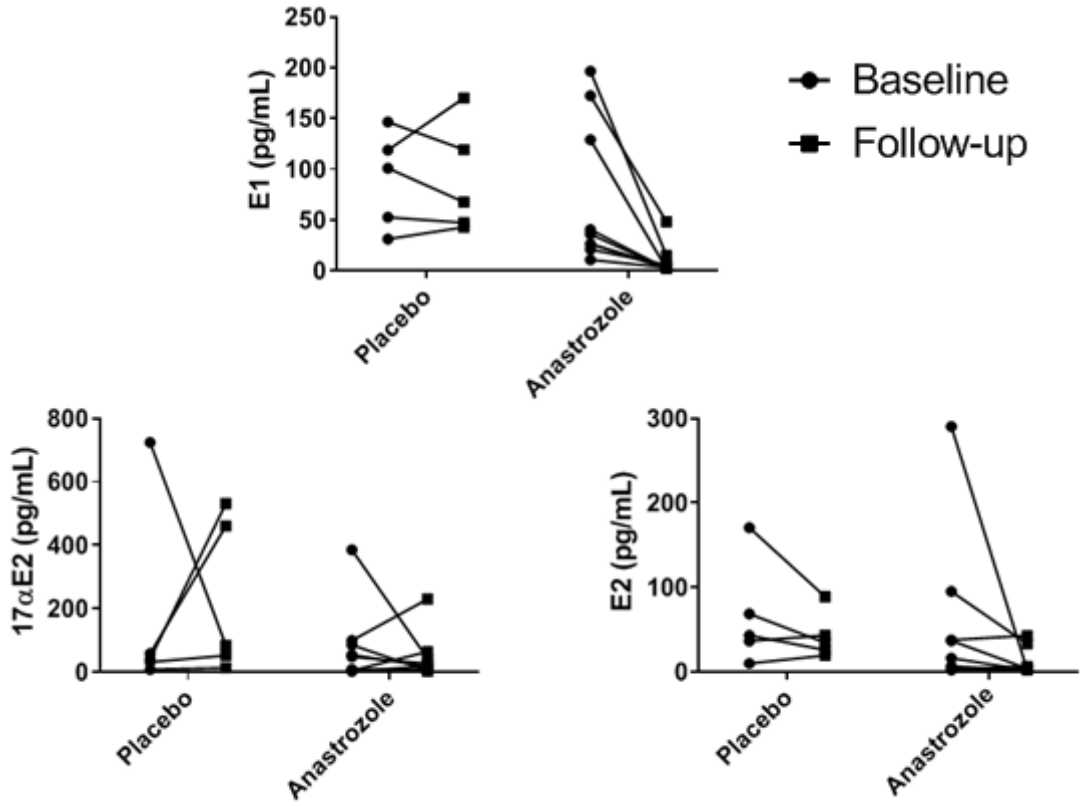

Figure 7-4: Parent estrogen concentrations before and after placebo or anastrozole treatment Estrone, E1; Estradiol, E2; alpha-estradiol, 17αE2 in patients treated with placebo (left) and anastrozole (right) at baseline (circles) and follow-up of 12 weeks (squares).

The general profile displayed a general reduction in E1 and E2 concentrations among the anastrozole-treated group. For two patients an increase in 17αE2 was displayed, note the patient with 1255 pg/mL in baseline to 16.1 pg/mL in the follow-up was omitted from the graph but showed a large decrease in concentration to 16 pg/mL.

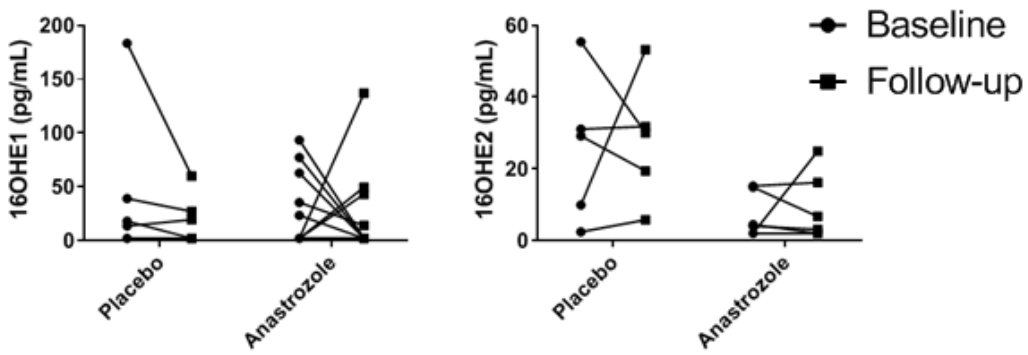

Figure 7-5: 16-Hydroxyestrogens before and after placebo or anastrozole treatment 16-Hydroxyestrone, 16OHE1; 16-Hydroxyestradiol, 16OHE2 Estrone in patients treated with placebo (left) and anastrozole (right) at baseline (circles) and follow-up of 12 weeks (squares).

For the 16-hydroxy- metabolites (Figure 7-5), 16OHE1 was reduced in seven anastrozole-treated patients and increased in three. 16OHE2 was increased in one patient, decreased in 4 patients and remained below the assay limit (2 pg/mL) in five patients. The total concentrations of 16OHE1 and 16OHE2 appeared to be 2.5 pg/mL higher upon treatment, a change which was not significant.

There were no changes in clinical biomarker levels of NT-proBNP, Insulin, HOMA, IL-6 and VEGF in the placebo or anastrozole groups. The 6MWD did improve for these patients following 12 weeks anastrozole treatment while in the placebo group this remained stable or decreased slightly (Kawut *et al*, 2019).

7.5 Discussion

From the LC-MS/MS analysis both fulvestrant and anastrozole display overall efficacy in reduction of circulating estrogen concentrations. Fulvestrant demonstrated promising therapeutic potential in this small proof-of-concept study. One patient did display clinical worsening, but a number of additional clinical conditions were involved in this deterioration. Fulvestrant appeared to have an influence on bioavailable estradiol and 16-hydroxyestrogens, in particular showing a trend toward decreasing 16OHE2. The associations between higher 6MWD, increasing stroke volumes and decreasing HPCs and 16OHE2 show potential benefit associated with this treatment. In alternative studies, E2-regulated bioactivity has previously been shown to stimulate HPCs causing proliferation via ERα, and abnormal CYP signalling and metabolism to 16-hydroxyestrogens might be involved in this (Masuda *et al.*, 2007). HPCs promote vascular remodelling via endothelium activation and injury (Asosingh *et al*, 2012,) which is consistent with our observation that 16OHE2 may induce migration and proliferation of endothelial cells from PAH patients (Denver *et al.*, 2018). As fulvestrant decreased both HPCs and 16OHE2, this supports the hypothesis that there is a role for ER-induced signalling in PAH which should be further explored. These results were published as a research letter in Annals of the American Thoracic Society (Kawut et al., 2019).

From the study of anastrozole treatment in PAH, anastrozole reduces circulating estrogens, potentially having less impact on the bioavailability of 16-hydroxyestrogen metabolites. E1 concentrations showed a trend toward reduction with lower concentrations in all patients. Of note, the analysis of estrogen metabolites was hindered by analytical challenges, such as the variability between sample volumes provided, samples at volumes <200 µL caused metabolite concentrations to fall nearer or below the assay detection limit. In addition, the previous thawing of samples may have resulted to additional alterations in estrogen profiles. Finally, this study was performed prior to additional method changes which improved robustness and the limits of detection. Overall the study was in agreement with the published work showing a 40.4 pg/mL reduction in E2 concentrations over 12 weeks in anastrozole-treated patients (Kawut *et al.*, 2016). However, this was not significant due to increasing E2 concentrations in three patients. 16OHE1 was also increased within three patients, suggesting that anastrozole may not fully block estrogen production in these individuals. This finding could be more pronounced in

men within the study due to high plasma concentrations of bioavailable testosterone (Ronde *et al.*, 2011). Interestingly there was a pattern beginning to emerge between estrogen levels in placebo and anastrozole groups when comparing baseline and 12 weeks. This suggested that individuals receiving anastrozole tended toward lower concentrations of E1, E2 and 16OHE2 than those receiving the placebo dose. These observations require confirmation in larger, fully powered, clinical trials linking the findings to clinical characteristics for the patients was not possible due to study blinding. Within the publication by Kawut et al (Kawut *et al.*, 2016) reports increases in the 6-minute walk distances following anastrozole at a level which approached clinical significance, which the group suggested was due to low power to detect small changes. Also, the trial was conducted over a 12-week period which may might suggest further changes may occur over longer periods. We showed in Chapter 4 that abnormal parent estrogen and bioactive metabolite concentrations were prominent within idiopathic patients. Here, half of the patients treated with anastrozole were idiopathic meaning it would be of interest to define these within the analysis and perhaps conduct a study to analyse the effects of ER antagonists and aromatase inhibition within patients sub-divided into heritable and idiopathic patient groups. It was suggested within the anastrozole trial that aromatase therapy may target local estrogen synthesis and therefore have a less defined impact on circulating E2 (Kawut *et al.*, 2016). We know aromatase is over-expressed in pulmonary artery smooth muscle cells from females (Mair *et al.*, 2014) which may suggest there is an important local function of estrogens in target tissues. Sample collection for the multi-centre (PHANTOM) trial by Kawut et al remains ongoing and should provide a more comprehensive analysis of the efficacy of anastrozole in PAH (NCT03229499).

The reduction of bioactive metabolites specifically in 16-hydroxylation within these studies are the first to be reported in PAH following fulvestrant treatment. It may well be that reduction of metabolites enhancing PAH progression provides a more effective route of therapeutic management. As E2 may present a cardio-protective role in women to an unknown degree it might well be appropriate to inhibit formation of only the bioactive metabolites, hence reducing their mitogenic roles whilst preserving the beneficial effects of E2 on right ventricular function. Among premenopausal women this type of treatment would be preferential to avoid early onset of menopause, which in itself remains a risk factor for disease (Scorza *et al.*, 2002). For example, inhibition of CYP1B1 might reduce the formation of detrimental

16OHE and 4OHE. This enzyme has been implicated in both clinical and experimental PAH by a number of studies (Austin *et al*, 2009; White *et al*, 2011; White *et al*, 2012; Dempsie *et al*, 2013; Ventetuolo *et al*, 2016; Johansen *et al*, 2016; Mair *et al*, 2019). CYP1B1 is over-expressed in PAH patient pulmonary arteries and in the pulmonary arteries from experimental models of disease (White *et al.*, 2011). Polymorphisms in the CYP1B1 gene may modify estrogen metabolism, *BMPR2* mutations, miRNA-29 and disease penetrance among heritable cases, as discussed previously in Chapters 3 and 4. CYP1B1 has also been shown to promote PAH by induction of polycyclic aromatic hydrocarbons (PAHs) activated via the aryl hydrocarbon receptor (AhR) (Bansal *et al.*, 2014). *In vivo,* this research in knockout and wild type mice shows CYP1B1 plays a major role in mitochondrial dysfunction via overproduction of reactive oxygen species, leading to chemical damage. The CYP1B1-specific inhibitor 2, 3′, 4, 5′-tetramethoxystilbene (TMS) reverses damage caused by over-expression. In additional studies, TMS also attenuates PAH in hypoxia-induced and Sugen hypoxic rodent models of disease (White *et al*, 2012). The therapeutic effect here was hypothesised to occur via a reduction in mitogenic 16-hydroxylation of parent estrogens (White *et al*, 2012). TMS is a selective inhibitor of CYP1B1 activity which should be further tested in PAH. For clinical use structural modulations may be necessary to reduce the long hydrocarbon chain breakdown, enhance efficacy and detection within target tissues.

My LCMS/MS studies in Chapters 3, 4, 5 and 6 have identified a number of metabolites that are elevated in PAH. Elevations in the 16-hydroxylation pathway appear to be important in disease status. In particular, increased 16OHE2 among females appear consistent across all of these studies. The specific functional role of 16OHE2 in PAH remains to be defined locally within the pulmonary vasculature. Preliminary evidence suggests that 16OHE2 may be proliferative in female BOECs (Denver *et al.*, 2018) and PASMCs (Chapter 3), perhaps mediated by ROS generation. Hydroxylation through the action of CYP1B1, CYP1A1, CYP1A2, CYP2C8 and CYP3A isoforms govern the conversion of E2 to 16OHE2, with polymorphism accounting for dysfunctional metabolism in disease (Austin *et al*, 2009). In this context it would be interesting to discover if reduction in 16OHE2 levels would reverse PAH phenotypes or improve haemodynamic parameters *in-vivo.* Interestingly in cancer, 16OHE2 has been reported as a potent ER agonist, capable of cellular proliferation to the same extent as E2 in human MCF-7 cell lines (Gupta *et al*, 1998). The effects of 16OHE1 in PAH has been more widely defined (Chen *et*

al., 2016; Hood *et al.*, 2016; Docherty *et al.*, 2018). Chen et al show 16OHE1 to promote the development of heritable PAH in cases where *BMPR2* mutations are present. Within the lungs of transgenic mice miR-29 expression was higher altering molecular and functional energy metabolism in the presence of 16OHE1. The pathogenic effect was rescued by miR-29 antagonism. In lung tissue derived from human hPAH patient's increased miR-29 expression was also present. This micro RNA may be therapeutically targeted using a miR-antagonist. Hood et al also demonstrated 16OHE1 as a mitogenic and harmful metabolite stimulating Nox-induced reactive oxygen species generation. Hence, activating proliferation within human PASMCs and in PAH promoting vascular remodelling. Interestingly within this study the pathogenic effect of 16OHE1 in human PASMC function was blocked by CYP1B1 inhibition particularly among females. Sex hormone abnormalities in the 16-hydroxylation pathway therefore appear highly relevant in PAH. 16OHE1 may provide a promising target pathway for modulation in PAH patients. 16OHE1 binds covalently to the estrogen receptor alpha via Schiff base formation, a sub class of imines with a general formula of RR'C=NR" (Swaneck *et al*, 1988). This means 16OHE1 could also bind DNA forming adducts although this has yet to be further studied in hormone-sensitive disease settings. 16-Hydroxyestrogen concentrations are also modulated by interconversion by 17βHSD, which could present another important modulatory pathway in PAH upon further definition of 16OHE1 and 16OHE2 action.

Overall, from the data presented within this chapter, fulvestrant appears to be a promising new therapy. In this small n=5 study there was a reduction in estrogen concentrations except in one patient. In the four patients who benefited from treatment, the improvement correlated 16-hydroxyestrogen metabolism, HPCs and 6-minute walk distances which supports the role of estrogen receptor signalling in PAH. Anastrozole treatment was also well tolerated and reduced estrogens within the majority of patients. This treatment showed reduction of bioactive estrogens in the majority of patients which did not correlate to clinical characteristics. Anastrozole did however increase 6-minute walk distances displaying a potential to improve patient quality of life. For both anastrozole and fulvestrant, larger and longer clinical trials would be beneficial to assess clinically important endpoints and to determine the true efficacy of both compounds. It might also be of interest to test combination therapy in larger clinical trials to assess the therapeutic potential of blocking estrogen production and signalling in PAH. This could be important in

postmenopausal women and men whereas CYP1B1 inhibition might prove more effective in pre-menopausal women.

7.5.1 Study Limitations

The fulvestrant study was limited by the small size and open-label and uncontrolled design making it difficult to directly attribute the findings to the study intervention. The short duration of the study may also have impacted on the results. The promising results gained from this study suggest that further clinical trials in PAH are warranted to fully evaluate efficacy in postmenopausal women. The anastrozole study was also limited by small size and in addition for LC-MS/MS analysis extracted samples were not from the same volumes (100 – 500 µL) this added to the inter-sample variability as the assay requires 500 µL for adequate detection, and this was detrimental to quantification. In addition, these samples had been thawed prior to the study which may cause alterations to the circulating estrogen profiles. Therefore, further studies to evaluate the effect of anastrozole treatment are warranted.

Chapter 8 Untargeted Transcriptome analysis of genes involved in PAH

8.1 Introduction

The transcriptome includes the total set of transcripts within a cell, including messenger RNA (mRNA), small RNA and non-coding RNA. Actively-expressed genes within cells reflect the developmental stage or physiological conditions with transcriptome analysis allowing interrogation of the gene expression and regulation of the genome (Wang *et al*, 2009).

8.1.1 Transcriptomic analysis

Transcriptome analysis can be employed using a number of techniques which have progressed over the past seventeen years to improve throughput and efficiency. Microarrays were introduced in 2002. These hybridisation-based methods were first tested on mammalian genomes (Murphy *et al*, 2002). Microarrays are commonly used for genome-wide expression studies, working by hybridisation of fluorescently-labelled cDNA to high-density oligo-arrays. The technique itself is inexpensive but suffers from a number of limitations such as limited detection ranges and difficulty in comparing results from different experiments. Tag sequencing methods such as massively parallel signature sequencing (MPSS) and serial analysis of gene expression (SAGE) were then developed to overcome these problems (Reinartz *et al.*, 2002; Coyne *et al.*, 2004). Instead of hybridisation, MPSS and SAGE techniques first sequence part of the mRNA with the products then mapped to known genes from databases. This determines the presence of genes and their abundance within the cell. The main drawbacks are the expense of conducting large scale studies and also the time-consuming nature of the protocol and analysis combined. More recently, next generation sequencing (NGS) was invented, most notably the Illumina Solexa Genome Analyser in 2007. A move toward RNA-sequencing has allowed innovative insight of the transcriptome compared to conventional techniques (Kulski, 2016). RNA-sequencing allows not only a more comprehensive and sensitive way to measure gene expression than micro-array assays but also permits analysis of individual splicing variants (Bryant *et al*, 2012) and allelic-specific expression (Raghupathy *et al.*, 2018). Additionally, it has the ability to discover novel genes and transcripts, as it does not require previous knowledge of a gene. Therefore, RNA-sequencing has been favoured over microarrays in recent years for transcriptome characterisation due to growing confidence in literature and databases available from the technique.

8.1.2 Sample Preparation Methods

In preparation for sequencing, ribosomal RNA (rRNA) must be efficiently removed from samples. rRNA represents the most abundant component of total RNA isolated from human cells which should be removed prior to transcript/gene detection (Zhao *et al.*, 2018). For library preparation, a number of approaches may be utilised, with Illumina driving the market in NGS kits. Two standard approaches known as poly-A selection and RNA depletion may be employed for separation of mRNA from rRNA and removal of rRNA, each coming with their own advantages and disadvantages. Poly-A selection efficiently separates mRNA from rRNA via selection of polyadenylated tails. These polyadenylated tails are added at the end of transcription and protect the molecule from enzymatic degradation in the cytoplasm, aiding in transcription termination and export of mRNA from the nucleus and translation. This method relies on the use of oligo (dT) primers attached to beads which isolate the protein-coding polyadenylated RNA transcripts (Zhao *et al.*, 2014). As the most commonly applied method, this is cost effective, requiring a lower sequencing depth and offers greater exonic coverage. However, this method does not allow detection of non-poly (A) transcripts such as micro RNA (miRNA), small nucleolar RNA (snoRNA) and some long non-coding RNA (IncRNA) therefore providing less information about immature transcripts. The second rRNA depletion method, known as ribosomal depletion (e.g. RiboZero in Illumina kits), may also be adopted to remove of rRNA by hybridisation to complimentary biotinylated oligo probes. These probes are then removed by extraction with streptavidin-coated magnetic beads before purification of remaining RNA for sequencing (Kumar *et al.*, 2017). In comparison to poly(A) selection methods, RiboZero enables detection of small and non-polyadenylated RNA, detection of long and short transcripts and application to prokaryotes. However this approach affords greater intronic reads, requiring a greater sequencing depth and is considerably more expensive than poly(A) selection techniques (Petrova *et al.*, 2017). Both library preparation methods are commonly automated for increased accuracy and reproducibility in sample preparation, ensuring minimal introduction of errors and higher throughput of samples.

8.1.2.1 Transcriptome studies in PAH

In PAH, a number of microarray and RNA-sequencing studies have allowed an unbiased look at differential whole genome gene expression patterns from lung tissue (generally representing end-stage disease) and isolated or circulating cells.

In studies of ECs from the lungs of idiopathic PAH patients from two individual cohorts, significant fold changes in 10 genes were found, with no mutation of *BMPR2* detected within patients. Reductions of both Ephrin A1 (*EFNA1*) and collagen type IV alpha 1 chain (*COL4*) expressed were detected across the studies (Rhodes *et al.*, 2015). *COL4* is a major component of vascular basement membranes, with its loss associated with loss of the normal healthy cell phenotype. *EFNA1* is an EC guidance molecule: the loss of expression is associated with a higher RVSP, RVH and greater degree of distal artery loss in transgenic mice compared to those with the *EFNA1* gene. Upon siRNA knockdown of *BMPR2 In vitro* in ECs, the ability of ECs to produce *EFN1* and *COL4* was reduced. This suggests a potential route of endothelial dysfunction which may be targeted by modulation of the BMPR2 receptor function. In comparison of frozen lung tissues and isolated fibroblasts from systemic sclerosis (SSc), pulmonary fibrosis, PAH and normal controls, three genes of interest Podocan (*PODN*), Lysyl Oxidase (*LOX*) and Four and A Half LIM Domains 2 (*FHL2*) were prominent in the analysis. Aberrant mRNA expression of the major histocompatibility complex and transporter 1 was also observed in SSc-PAH and iPAH patients. These patient groups displayed alterations in HLA expression (Hsu *et al.*, 2011). Aberrant expression of genes associated with antigen presentation resulting from infiltration of immune cells suggest inflammation and innate immunity may play a role in PAH development.

A large multi-centre clinical study of peripheral blood extracts from 1038 PAH cases (908 with idiopathic PAH) and 6385 non-PAH controls identified a number of novel gene variants in PAH patients following sequencing (Gräf *et al.*, 2018). Over-expression of ATPase 13A1 (*ATP13A1)*, aquaporin 1 (*AQP1*) and SRY-related HMG-box-17 (*SOX17*) were identified. In PAH, a deletion affecting the *BMPR2* locus occurred in 23 cases with deleterious variants in a number of other genes previously reported in PAH identified, such as *SMAD9* (4 cases), Potassium Two Pore Domain Channel Subfamily K Member 3 (*KCNK3*) (4 cases) Activin A Receptor Like Type 1 (*ACVRLI*) (9 cases) and T-Box-4 (*TBX4*) (14 cases).

These studies highlight the wide-ranging genetic alterations present in PAH pathobiology. *ATP13A1* was expressed in EC, PASMCs and BOECs, with loss of the gene causing inhibition of proliferation and increasing cellular apoptosis in endothelial cells. Literature supports a hypothesis stating EC signalling triggers initiation of PAH phenotypes (Teichert-Kuliszewska *et al.*, 2006). However studies in isolated cells remain limited, with the majority of studies conducted in lung homogenate, circulating cells and via laser capture microdissection directly from tissues (Hoffmann *et al.*, 2016). These methods are useful for direct analysis of cells excluding the effects of cell culture, passaging and freeze/thaw cycles. Elimination of these steps reduces variability between biological samples. However, PA cells are the main site of pulmonary vascular pathophysiological alteration in PAH manifestation, meaning their transcriptomic profile is of importance in disease pathobiology.

Microarray studies in PAH-PASMCs have identified changes in gene expression heavily related to cellular growth/proliferation and regulation of the cell cycle pathways (Yu *et al.*, 2015). A study comprised of cells collected in a controlled manner split between non-PAH, hPAH and iPAH classifications (n=3 each) found 227 genes with commonly dysregulated expression patterns in both hPAH and iPAH in comparison to controls. Most significantly, as identified by Ingenuity pathway analysis, polo-like kinase 1 (PLK1) increased in hPAH with a further increase in iPAH. This gene is expressed in cancers involved in tumour growth and proliferation, presenting a potential therapeutic target which may be applicable to PAH treatments. Other prominent genes altered in this study were bradykinin B2 receptor (*BKB2R*), polo-like kinase 4 (*PLK4*), Anillin Actin Binding Protein (*ANLN*), Angiopoietin 1 (*ANGPT1*), Death Associated Protein Kinase 1 (*DAPK1*), superoxide dismutase-3 (*SOD3*). Also, in an Affymetrix microarray study, PASMCs isolated from normal controls and IPAH were treated with BMP-2 (200nM for 24hr), a number of genes related to cellular growth and apoptosis showed divergent expression patterns (Fantozzi *et al.*, 2005). Following BMP-2 treatment, 523 genes were upregulated and 540 downregulated in iPAH patients on comparison to controls. These changes suggest BMP-mediated gene regulation is significantly altered in PASMCs from iPAH patients. This study by Fantozzi et al (Fantozzi *et al.*, 2005) shows genes such as caveolin 2 (*CAV2),* MYC-Binding protein *(MYCBP),* Fos Proto-Oncogene *(FOS),* Annexin 5 (*ANXA5),* cytochrome C1 *(CYC1),* gata binding protein 2 *(GATA2),* Transforming Growth Factor Beta Receptor 1 *(TGFBR1),*

Transforming Growth Factor Beta Receptor 2A *(TGFBR2A),* 5-Hydroxytryptamine Receptor 2B *(HTR2B),* Coagulation Factor II Thrombin Receptor *(F2R) and* Inositol 1,4,5-Trisphosphate Receptor Type 1 *(ITPR1)* were prominently dysregulated in iPAH, being either positively or negatively associated with pulmonary arterial pressure of patients.

These studies demonstrate the genetic diversity in PAH with the exact pathobiology behind PA remodelling remaining undefined. Transcriptomic studies in PASMCs from PAH patients may reveal important changes in the medial layer of the pulmonary vascular wall, which heavily contributes to advancing proliferation and PA occlusion. No studies to date have compared male and female controls or diseased transcriptomes in hPASMCs.

8.1.3 Hypothesis and Aims

PASMCs located in the longitudinal area of pulmonary arteries are phenotypically modified in PAH. These cells exhibit uncontrolled proliferation and metastasis upon original insult, the identity of which in the large majority of PAH cases remains unknown. Therefore, we wished to conduct a transcriptome-wide analysis to identify differential gene expression and therefore pathways involved in female PAH, and subsequently compare baseline differences in gene expression between male and female PASMCs.

8.2 Materials and methods

8.2.1 Pulmonary artery smooth muscle cell culture

All tissue culture procedures were carried out in sterile conditions using a Biological Safety Class II vertical laminar flow cabinet. Cells were maintained in a humidified incubator at 37^{o}C with a constant supply of 5% CO_2, 95% air. All cultures were examined under a phase contrast inverted microscope (Leitz Diavert, Germany) to assess morphological characteristics.

8.2.2 hPASMCs

Human pulmonary artery smooth muscle cells (hPASMCs) were derived and supplied by Professor Nicholas W. Morrell, University of Cambridge as discussed in

Chapter 6. Patients for this study were from idiopathic, heritable and associated PAH clinical classifications. For control subjects, PAH and underlying lung conditions were absent. All cell lines were tested for mycoplasma contamination prior to sub-culturing for experiments.

Table 8-1: Cell line information

		Code	Passage	Age (y)	PAH Classification/Cause of Death
Female	PAH	FPAH1	6	39	Idiopathic PAH
		FPAH2	5	45	Associated PAH
		FPAH3	6	30	Heritable PAH
		FPAH4	3	52	Associated PAH
Female	Control	FC1	6	56	Lower lobectomy/lung cancer
		FC2	5	57	COPD/Emphysema
		FC3	6	64	Emphysema
		FC4	6	64	Angina
Male	Control	MC1	5	52	Adenocarcinoma
		MC2	6	60	Squamous cell carcinoma
		MC3	6	78	Unknown

Characteristics of patients from whom Human pulmonary artery smooth muscle cell were derived. PAH, pulmonary arterial hypertension, Chronic obstructive pulmonary disease (COPD).

8.2.3 Stimulation of hPASMCs

All hPASMCs were utilised for these experiments between passages 4-7 with the cell passage prior to culture shown in Table 8-1. Cells were maintained in 75cm^3 culture flasks containing 10% (v/v) FBS (Sera Laboratories International, West Sussex, United Kingdom), 1% (v/v) antibiotic antimycotic solution (10,000 units penicillin, 10mg streptomycin and 25µg amphotericin B, Sigma Aldrich, Dorset, United Kingdom) in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, Paisley, United Kingdom). DMEM was routinely replenished every 24 hours. hPASMCs were split on reaching ~90% confluency. This involved aspirating the DMEM off the PASMCs, washing with sterile PBS followed by addition of 1mL trypsin/EDTA solution (0.1% (w/v) EDTA in PBS, Life Technologies, Paisley United Kingdom). Media was warmed to 37°C. The culture flask was then placed in the incubator for 1-2 minutes to aid the trypsinisation process. Trypsin is a proteolytic enzyme which dissociates the adherent PASMC from the T75 culture dish. Once detached the PASMCs were re-suspended in 9mL of 10% FBS DMEM, thereby neutralising the trypsin and halting the trypsinisation process. 1mL of cell suspension was transferred to a 10cm dish containing 9mL of DMEM. Following

growth to 80% confluence cells were quiesced in 0.2% FBS for 24 hours. Cells were then stimulated in 1% FBS for a further 24 hours to allow comparison of cell groups.

8.2.3.1 RNA Harvest

hPASMCs were collected from the 10cm plates at the experimental end by placing the plate immediately on ice. The media was aspirated, and cells washed with ice cold PBS, 5 mL. Following this, 700 µL of Qiazol lysis reagent was added to each plate. Qiazol is a phenol/guanidine-based reagent providing an efficient cell lysis method for harvesting total RNA. Scraping of cells was performed with the culture dish still on ice using a plastic scraper; the resultant cell lysates were then collected in a pre-chilled 1.5mL Eppendorf. Directly afterward cells were homogenised by vortex. Samples were then stored at -80°C until RNA extraction.

8.2.3.2 Protein Harvest

For protein harvest, hPASMC were also collected from 10cm plates at the experimental end-point by placing the plate on ice immediately. The media was aspirated, and cells washed with ice cold PBS, 5 mL. Following this, 100 µL of ice-cold radio immune precipitation assay (RIPA, Thermofisher Scientific, Loughborough, UK) buffer supplemented with protease inhibitors (0.1mmol/L PMSF, 1µg/ml soybean trypsin inhibitor and 1µg/ml benzamidine) was added to the culture dish. RIPA buffer lyses whole cells and allows proteins to be released and solubilised. Protease inhibitors help to prevent proteolysis, dephosphorylation and denaturation of the protein sample. Cell culture plates remained on ice, then cells were scraped using a sterile plastic scraper with cell lysates collected in a pre-chilled 1.5 mL Eppendorf. Samples were then left on ice for 30 minutes to allow disassociation of protein complexes and stored at -80 ºC until required for validation experiments.

8.2.4 RNA Preparation

To increase yield, RNase Zap (Invitrogen, UK) was applied to all benches, pipettes and consumables used in the extraction of RNA. In addition, sterile filter tips were used to prevent contamination or degradation of RNA samples.

8.2.4.1 RNA Extraction

RNA was extracted using a Qiagen RNeasy RNA extraction kit following the manufacturer's guidelines. Briefly, RNA was precipitated using ethanol and applied to a RNeasy spin column. High salt buffers bind total RNA to the silica embedded within the column. Samples were washed with RWT buffer prior to on-column DNase digestion for 30 minutes, allowing removal of DNA contamination. Samples were twice washed with RPE buffer with the spin column transferred to a 1.5 mL RNase free tube. Upon elution of RNA in 42 µL RNase free water, samples were aliquoted with 20 µL sent for RNA-Sequencing analysis (performed by the Glasgow polyomics facility), 2 µL used for quantitation of RNA (Nanodrop analysis) and the remainder (20 µL) was converted to cDNA for qPCR experiments.

8.2.4.2 Quality control

The concentration and quality of extracted RNA from each sample was determined spectrophotomtrically (NanoDrop, ND-1000 spectrophotometer (Thermo Scientific, UK). The absorbance of RNA was determined at 260 nm and absorbance of protein at 280 nm and data calculated using themodified Beer-Lambert Law. The ratio of 260/280 was calculated and a ratio over ≥2 was indicative of good RNA integrity. Following sample shipment to the Polyomics facility quality was further tested using a Bioanalyser, eukaryote Total RNA Analysis (v2.6, Agilent Technologies).

8.2.4.3 Illumina sequencing of mRNA

1 µg RNA was prepared for sequencing in the Glasgow Polyomics facility. The cDNA library was created using the Illumina TruSeq Stranded mRNA sample preparation kit in accordance with the manufacturer's protocol. cDNA was then processed on the NextSeq 500 System, 75 bp single end run with 35 million counts per sample.

8.2.5 Data Analysis

8.2.5.1 Sequence data

Raw reads from sequencing were trimmed of contaminating sequencing adapters and poor-quality bases using Cutadapt (v1.13). Bases were assessed on their Phred score, used to determine the quality of nucleobases generated; values below 28

were trimmed. The quality of reads was assessed by Fastqc (v0.11.4), before and after trimming.

8.2.5.2 Alignment

Reads were pseudo-aligned to the transcriptome using Kallisto (v0.43.0) which generates counts of data for each transcript. Resulting count matrices were created with the Bioconductor package TXImport (v1.4.0).

8.2.5.3 Differential expression

The differential expression of gene transcripts for the analysis of each group was assessed using the Bioconductor package DESeq2 (v1.16.1).

8.2.5.4 Pathway mapping

To understand experimental results in biological context, pathway mapping was performed using Ingenuity Pathway Analysis (IPA) Software (v2.3). Analysis of the data included the false discovery rate (FDR) and/or Benjamin Hochberg (B-H) correction. The p-adjusted value was set as 0.1 within the core analysis to give an adequate number of genes for pathway analysis. However, a focus was given to genes with a significance of $p<0.05$.

8.2.5.5 Quantitative Real Time-Polymerase Chain Reaction

Quantitative real time PCR (qRT-PCR) was used to investigate mRNA expression using the ViiA7^{TM} Real time PCR system with samples prepared using TaqMan® Universal Mastermix II (Life Technologies, UK). Genes highlighted during the IPA analysis were chosen for the initial validation experiments. Specific dual-labelled TaqMan primer probe sets were purchased from Thermofisher. Conditions for thermal cycling were 50 ºC for 2 mins, 95 ºC for 10 minutes, 95 ºC for 0.15 minutes and 60 ºC for 1 minute. Relative changes in gene expression were quantified using the comparative Ct ($2^{-\Delta\Delta Ct}$) method (Bustin *et al.*, 2009). Using this method, the signal from treated samples was compared to that from a control sample following normalization of both to a housekeeper gene. The optimal housekeeping gene was selected based on the optimal expression stability using NormFinder software (Andersen, Jensen and Ørntoft, 2004). Ct Values are calculated from a log-linear curve whereby PCR signal is plotted against the cycle number. The average Ct from

duplicate samples was then calculated. The quantity detected in a certain number of cycles is proportional to the initial amount of transcript in each sample. Therefore, to determine the quantity of transcripts for each gene, the Ct values from each sample are normalised by subtracting the mean Ct of the chosen reference gene, giving ΔCt. The concentration of specific gene transcripts in samples relative to controls were normalised again via subtraction to obtain ΔΔCt values. Finally, the relative expression was given by raising 2 to the power of the negative value of ΔΔCt ($2^{-\Delta\Delta Ct}$) for all samples.

Table 8-2: List of TaqMan primers

Gene name	Assay ID
HLA-A	Hs01058806_g1
HLA-B	Hs00818803_g1
HLA-C	Hs00740298_g1
TAP2	Hs00241060_m1
TAPBP	Hs00917451_g1
DDR1	Hs01058430_m1
PPP1R11	Hs00606032_g1
GATA2B	Hs00231119_m1
GAPDH	Hs02786624_g1
B2M	Hs00187842_m1
18S	Hs03003631_g1
ACTB	Hs01060665_g1

Human Leukocyte antigen A, B, C, *HLA-A, B & C*; Transporter 2, *TAP2*; Tapasin, *TAPBP*; Discoidin Domain Receptor Tyrosine Kinase 1, *DDR1*; Protein Phosphatase 1 Regulatory Inhibitor Subunit 11, *PPP1R11*; GATA Binding Protein 2, *GATA2B*; Glyceraldehyde-3-Phosphate Dehydrogenase, *GAPDH*; Beta-2-Microglobulin, *B2M*; 18S ribosomal RNA, *18S*; Beta-actin, *ACTB*.

8.3 Results

8.3.1 RNA extraction and Quality Control

To perform RNA-sequencing experiments a total of 1 µg of RNA was recommended. Therefore, the concentration was estimated by Nanodrop and quality checked by Bioanalyser prior to library preparation and sequencing (Table 8-3).

Table 8-3: Nanodrop and bioanalyser quantification of rna extracts from hPASMCs

Sample	Passage	260/ 280	260/ 230	Nanodrop ng/µL	Bioanalyser ng/µL
Male Control					
MC1	6	2.1	1.74	210.8	241.6
MC2	7	1.94	1.53	101.9	100.7
MC3	7	2.04	1.86	86.4	93.2
Female Control					
FC1	7	2.07	2.17	107.6	105.9
FC2	6	2.08	2.15	171.9	181.61
FC3	7	2.06	1.99	82.7	83.9
FC4	7	2.05	2.14	329.3	140.6
Female PAH					
FPAH1	7	2.04	1.18	110.7	126.0
FPAH2	6	2.04	2.14	140.2	161.4
FPAH3	7	2.06	2.1	127.3	134.9
FPAH4	4	2.08	2.04	166.1	180.6

Male control, MC; Female control, FC; Female pulmonary arterial hypertension, FPAH.

In order to perform RNA-sequencing experiments the extracted RNA must be of a high quality. The total RNA extraction quality (RIN) obtained was higher than the recommended quality of 8.0 (Figure 8-1, D). The lack of a peak indicative of contamination between 18S and 28S in the spectra further indicated RNA from cells free of bacterial infections, indicative of high-quality clean RNA samples (Figure 8-1, A, B & C). Therefore, all RNA was of sufficient quality to proceed with next generation sequencing with results not being skewed by low quality RNA.

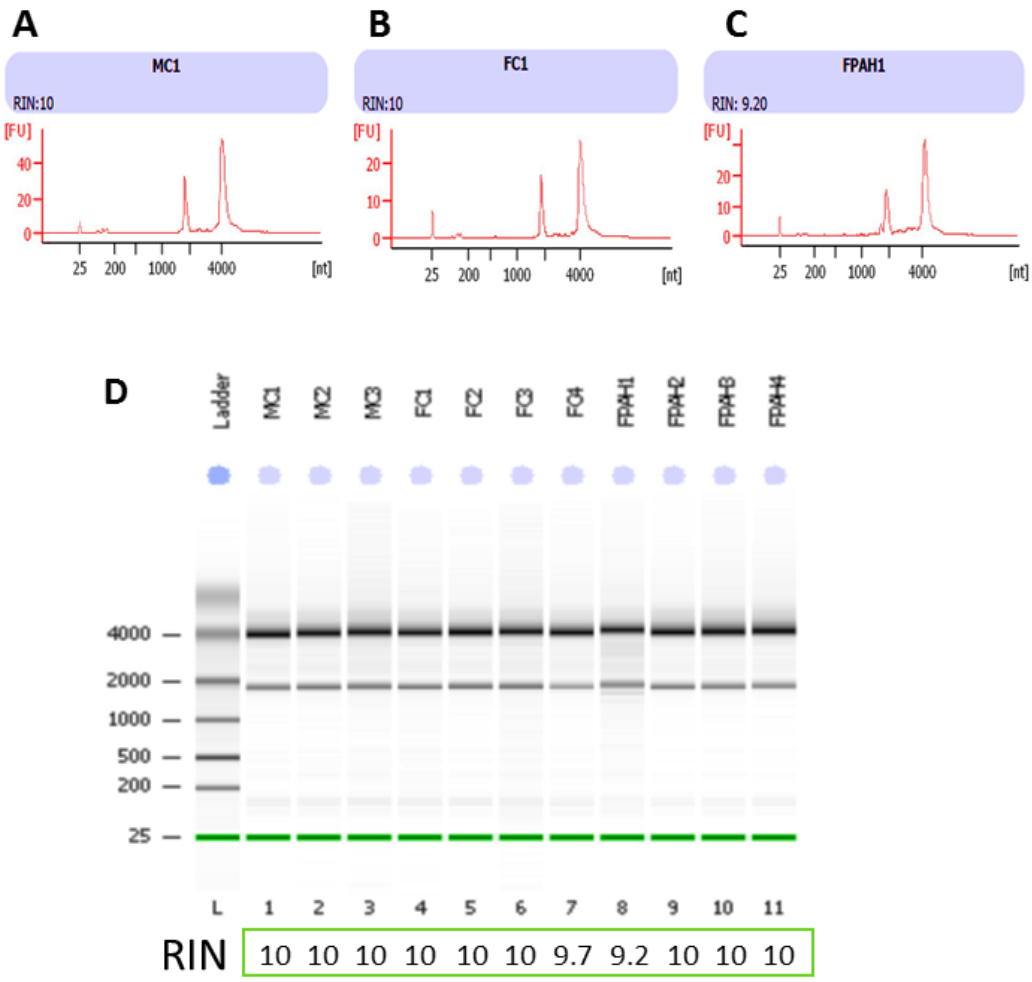

Figure 8-1: Quality control of RNA extracted from hPASMCs, the trace of RNA from cells of A) male control, B) female control and C) female PAH patient, all showing a strong 18S and 28S peak following extraction. D) The virtual electrophoretic gel representing the high quality of RNA extracted from hPASMCs. RNA integrity numbers (RIN) equal to or over eight are considered indicative of samples of high integrity.

8.3.1.1 Sequencing Report

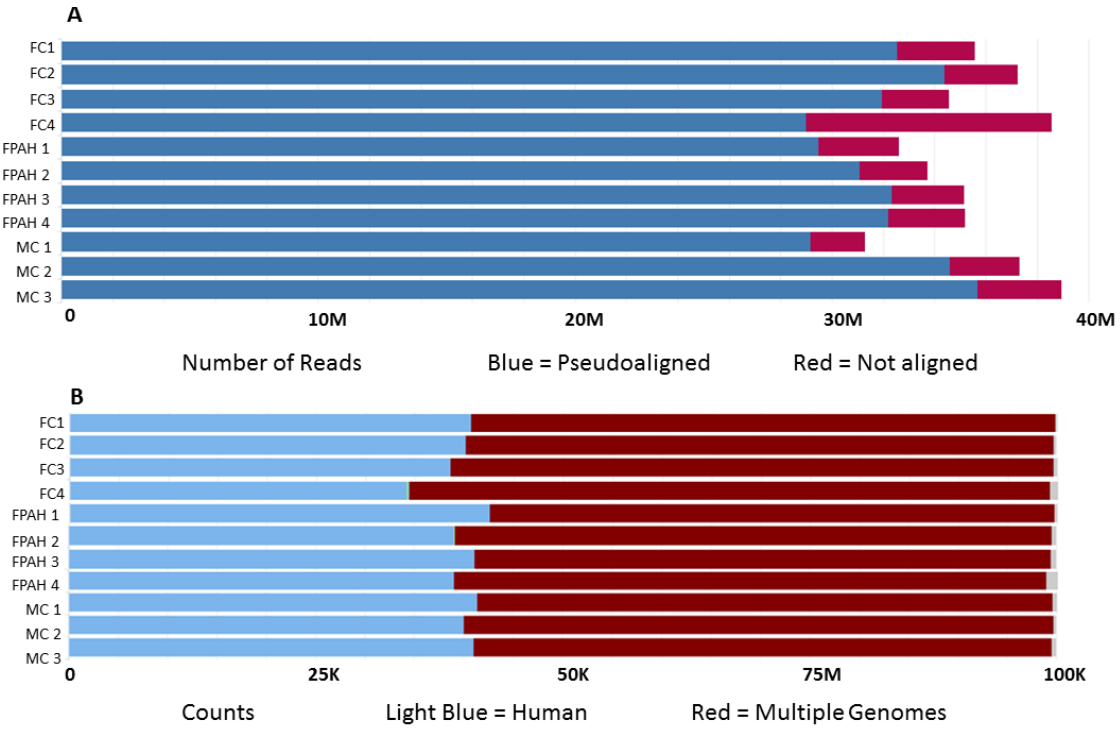

Figure 8-2: Alignment of trimmed reads Quantification of transcript abundance from RNA-Seq data (A) and library screening of sequences in FastQ format against a sequence database to determine the composition of libraries (B).

Multi-Quality Control reporting (mulitiqc.info) generated graphical representations of sequence-read alignments following trimming (Figure 8-2). The number of reads for the aligned genes are in the region of 28 – 35M. As expected, reads were assigned to human transcripts with a proportion aligning to multiple hit genomes (small unspecific fragments of RNA). No reads were aligned to mycoplasma transcripts, which confirmed results of our initial cell culture tests. The multi hit genome represents short unspecific sequences which align to a number of sequences in the genome. Principal component analysis was performed to identify correlations between the experimental groups (Figure 8-3).

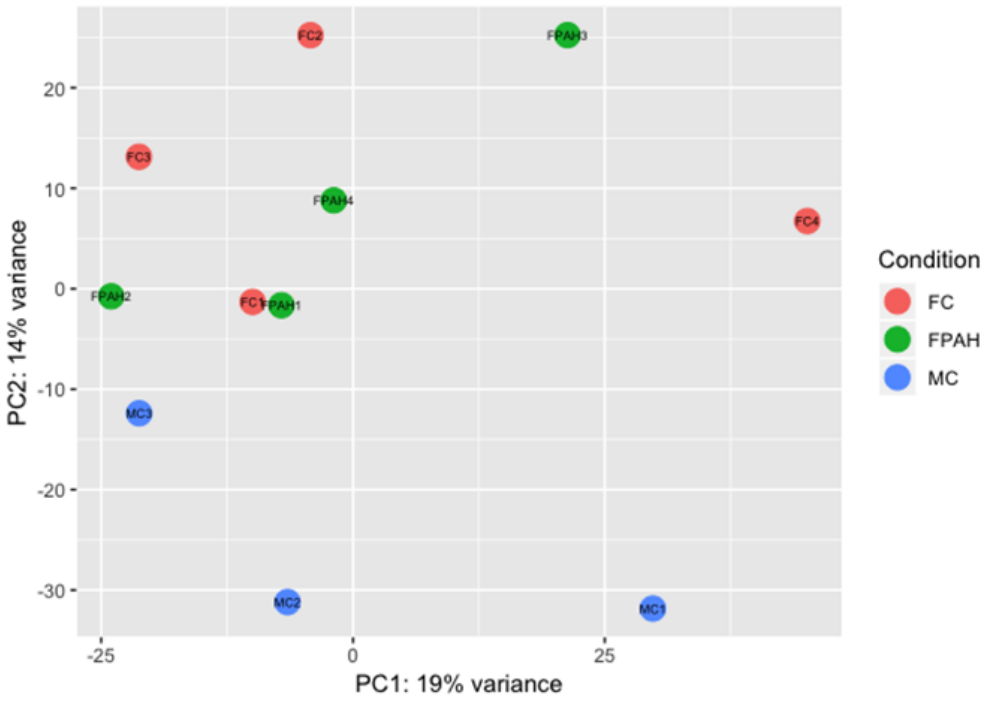

Figure 8-3: Principal Component Analysis (PCA) of experimental groups Comparison of RNA sequenced from female control (FC, Red), female pulmonary arterial hypertension patients (FPAH, Green) and male control (MC, blue) pulmonary arterial smooth muscle cell lines (PASMCs).

Data was evaluated using PCA plots (Figure 8-3) to determine associations between samples. The sequenced samples loosely grouped depending on the experimental condition, with a difference between female and male PASMCs. Notably, sample FC4 appeared as an outlier in all PCA tests. Also, the hPAH patient (FPAH3) was more variable than the associated and idiopathic patients, with one female control grouping closer to PAH patients (FC1; lower lobectomy for lung cancer).

8.3.2 Differentially Expressed Genes and Functional Analysis

In this study, only genes that have been characterised in the IPA database were analysed. Genes that have not been yet characterized were omitted from the analysis.

8.3.3 Patterns of Gene Expression

Female PAH *vs* Female Control

At the p-adjusted value of 0.05 few genes were differentially expressed between control and patients, with only 18 falling within this significance level (11 up and 7 down). This may be attributed to the low level of FBS (1%) within cell culture to simulate a stressed, serum starved environment. Therefore, to ensure an adequate number of genes for pathway analysis the p-adjusted significance level was adjusted to 0.1 giving 33 significantly altered genes (9 up and 24 down) in female controls vs female PAH (Table 8-4).

Table 8-4: Gene expression in female PAH *vs* female control PASMCs Log_2 (LogFold) change >1 ranked in order of fold change magnitude in hPASMCs

Upregulated in PAH			Downregulated in PAH		
LogFold Change	**P-Adjusted**	**Gene**	**LogFold Change**	**P-Adjusted**	**Gene**
9.62	0	*NFKBIL1*	-24.9	0.03	*TUBB*
9.04	0.08	*CNOT3*	-24.25	0.04	*PPP1R11*
8.99	0.02	*SCO2*	-23.11	0.07	*TRIM26*
8.64	0	*CYFIP1*	-22.85	0.08	*AL356432.3*
8.58	0.02	*B3GNTL1*	-22.77	0.08	*RPL18AP3*
8.43	0	*VPS52*	-22.46	0.08	*C6orf47*
7.26	0	*TAPBP*	-22.02	0.1	*TAP2*
6.17	0.08	*DNAH11*	-21.97	0.1	*MED16*
5.19	0.08	*RPL23AP87*	-19.17	0.08	*HLA-E*
			-18.16	0.1	*ZBTB22*
			-12.63	0	*DDR1*
			-11.98	0	*RPP21*
			-10.33	0.06	*GATA2B*
			-9.57	0	*HLA-A*
			-9.16	0.08	*MRM1*
			-8.18	0	*HLA-C*
			-8.05	0	*CSNK2B*
			-7.24	0	*LRRN1*
			-7.05	0.01	*CSNK2B*
			-6.42	0.05	*TAPBP*
			-6.34	0	*PRRC2A*
			-5.86	0	*HLA-B*
			-5.05	0.05	*MICA*
			-4.03	0.1	*TSEN34*

Male control *vs* Female control

In comparison of female and male control PASMCs (Table 8-5) a higher number i.e. 67 genes were differentially expressed at the 0.05 significance level (29 up and 38 down). However, to ensure consistent analytical parameters to the female comparison a significance level cut-off of 0.1 was applied giving 113 differentially expressed genes (41 up and 72 down).

Table 8-5: Gene expression in male control vs female control PASMCs Log_2 (LogFold) change >1 ranked in order of fold change magnitude in hPASMCs

Upregulated in Males			Downregulated in Males		
LogFold Change	**P-Adjusted**	**Gene**	**LogFold Change**	**P-Adjusted**	**Gene**
12.30	0.00	*RPS4Y1*	-24.70	0.00	*HSPA1A*
11.90	0.02	*KDM5D*	-23.70	0.00	*IER3*
9.80	0.00	*EIF1AY*	-22.70	0.00	*PPP1R18*
8.89	0.00	*DDX3Y*	-22.40	0.00	*RNF5*
8.34	0.07	*HLA-A*	-22.20	0.00	*C6orf47*
8.15	0.00	*TXLNGY*	-22.20	0.00	*RPP21*
7.74	0.01	*HLA-K*	-21.60	0.00	*MICB*
7.06	0.00	*PRKY*	-21.40	0.00	*B3GALT4*
6.80	0.10	*PSORS1C1*	-10.30	0.00	*PSMB9*
6.60	0.10	*PGA5*	-9.73	0.00	*HLA-A*
6.28	0.07	*HSPA1L*	-8.47	0.00	*PSMB9*
6.26	0.07	*CSF3R*	-8.31	0.01	*CSNK2B*
6.22	0.00	*ZFY*	-8.28	0.01	*HLA-C*
6.20	0.00	*UTY*	-7.68	0.10	*FAM45BP*
6.15	0.00	*EEF1GP1*	-7.52	0.00	*HLA-B*
6.09	0.00	*PSORS1C1*	-7.43	0.02	*ADD2*
6.01	0.00	*SLC24A3*	-5.98	0.07	*HLA-H*
5.44	0.00	*PRRC2A*	-5.95	0.02	*BRD2*
4.99	0.04	*ITIH5*	-5.62	0.00	*LRRN1*
4.36	0.00	*WDR46*	-5.06	0.00	*CSNK2B*
4.25	0.00	*CSNK2B*	-4.86	0.01	*HLA-B*
4.24	0.00	*AC008038.1*	-4.84	0.03	*TAPBP*
4.23	0.00	*SDHAP3*	-4.76	0.00	*CSNK2B*
4.02	0.04	*ZBTB9*	-4.72	0.00	*TENM3*
3.91	0.02	*PENK*	-4.39	0.10	*NEFM*
3.76	0.04	*FMO3*	-4.38	0.00	*ANK3*
3.66	0.01	*LNX1*	-4.23	0.00	*PRRC2A*
3.59	0.00	*RPS9*	-4.08	0.00	*CLEC2D*
3.48	0.10	*RARRES2*	-3.46	0.00	*TAPBP*
3.22	0.05	*CHRNE*	-3.08	0.08	*ANKRD12*
3.19	0.02	*DHX16*	-3.00	0.03	*CCND2*
3.04	0.00	*LRRC15*	-2.62	0.08	*EPHX2*

2.56	0.10	*KCNS2*	-2.57	0.05	*FAM3C2*
2.47	0.04	*CRLF1*	-2.52	0.01	*SYNGR3*
2.42	0.04	*ADAMTS8*	-2.47	0.04	*STK33*
1.85	0.00	*S100A3*	-2.42	0.10	*RGS5*
1.77	0.10	*STMN3*	-2.41	0.01	*ZFX*
1.57	0.07	*BIRC5*	-2.36	0.07	*FAM13C*
1.57	0.10	*PTGS1*	-2.25	0.10	*ZP3*
1.45	0.08	*EBP*	-2.18	0.08	*RB1CC1*
1.18	0.05	*RPA2*	-2.13	0.10	*PRKD3*
			-2.11	0.04	*SUSD5*
			-2.09	0.01	*FLRT2*
			-2.01	0.01	*ATRX*
			-2.01	0.07	*LRRK2*
			-1.95	0.02	*PLEKHA1*
			-1.91	0.04	*SPATA6*
			-1.85	0.08	*ESPNL*
			-1.83	0.00	*YPEL2*
			-1.83	0.10	*PEG10*
			-1.83	0.10	*BBX*
			-1.80	0.08	*PNRC2*
			-1.75	0.03	*ACAP2*
			-1.75	0.05	*HDAC9*
			-1.71	0.08	*RANBP2*
			-1.65	0.10	*LACC1*
			-1.57	0.00	*ZMYND8*
			-1.56	0.09	*ZNF462*
			-1.55	0.04	*PRUNE2*
			-1.55	0.10	*IGF2BP3*
			-1.54	0.07	*EMB*
			-1.54	0.07	*CDK19*
			-1.54	0.08	*DNAJC13*
			-1.51	0.05	*SASH1*
			-1.49	0.04	*WASF3*
			-1.47	0.09	*NBPF19*
			-1.42	0.04	*VPS13B*
			-1.31	0.09	*ROCK1*
			-1.26	0.03	*UTRN*
			-1.17	0.10	*DDX3X*
			-1.14	0.05	*SAMD4A*

8.3.4 Gene Ontology of Statistically Significant Genes

The pairwise (FPAH *vs* FC or MC *vs* FC) differentially expressed genes were categorised following analysis using Ingenuity Pathway Analysis (IPA).

In summary, IPA analysis identified a number of genes associated with antigen presentation pathways as being downregulated in female PAH vs female controls and male control vs female control PASMCs. A number of genes were also related to cell growth, proliferation and cardiotoxicity pathways. Potential upstream regulation of immune response subunit degradation was predicted to occur via estradiol signalling and estrogen receptor alpha (*ESR1*).

8.3.4.1 Canonical Pathways Affected by Differential Gene Expression

Female PAH vs Female Control

In female PAH *vs* female control cells, a number of canonical pathways were highlighted as dysregulated in PAH PASMCs with a high degree of representation of the antigen presentation and autoimmunity pathways (Table 8-6).

Table 8-6: Top canonical pathways in control *vs* female PAH

Canonical Pathway	p-Value	Overlap (%)
Antigen Presentation Pathway	8.9e-12	15.8
Dendric Cell and Natural Killer Cell Crosstalk	1.15e-07	5.6
Autoimmune thyroid disease	4.85e-07	8.3
Graft vs Host disease signalling	4.85e-07	8.3
Allograft rejection signalling	4.87e-06	4.7

The top hits for disease and functional pathways modified in PAH PASMCs also displayed a number of gene transcripts associated with inflammation in disease, cellular dysfunction and antigen presentation and a number of cardiotoxic roles (Table 8-7).

Table 8-7: Top functions in Female Control vs PAH

Disease and Biofunctions	Number of Genes
Connective tissue disorder	16
Immunological disease	19
Inflammatory Disease	16
Inflammatory Response	16
Organismal injury and Abnormalities	25
Molecular and Cellular Functions	**Number of Genes**
Cell Death and survival	5
Cellular Compromise	5
Antigen Presentation	4
Cellular Growth and Proliferation	7
Cellular Function and Maintenance	8
Cardiotoxicity	**Gene**
Congenital Heart Anomaly	↑ *DNAH11*
Pulmonary Arterial Hypertension	↓ *DDR1*
Cardiac Dilation	↑ *CNOT*
Cardiac Arrythmia	↓ *HLA-B*
Cardiac Fibrosis	↑ *CNOT*

In addition to the disease and biofunctions in Table 8-7, a number of genes associated with liver damage and hyper-proliferation were highlighted.

Male Control vs Female Control

In male control *vs* female control cells, the differentially-expressed canonical pathways also displayed a high association with antigen presentation and autoimmunity pathways (Table 8-8).

Table 8-8: Top canonical pathways in female *vs* male controls

Canonical Pathway	p-Value	Overlap (%)
Antigen Presentation Pathway	3.75e-07	13.2
Protein Ubiquitination Pathway	8.32e-05	2.6
Cytotoxic T Lymphocyte-mediated Apoptosis of Target Cells	2.67e-04	9.4
Dendric Cell and Natural Killer Cell Crosstalk	4.19e-04	4.5
Autoimmune Thyroid Disease	8.89e-04	6.2

Again, the top disease and functions in male controls highlighted immunological disease and inflammatory genes. A number of genes were also related to cell signalling, development and growth (Table 8-9).

Table 8-9: Top functions in female *vs* male control

Disease and Biofunctions	Number of Genes
Connective Tissue Disorder	25
Immunological Disease	42
Inflammatory Disease	31
Inflammatory Response	30
Organismal Injury and Abnormalities	83
Molecular and Cellular Functions	**Number of Genes**
Cell-To-Cell Signalling and Interaction	14
Cellular Assembly and Interaction	17
Cellular Development	29
Cellular Function and Maintenance	17
Cellular Growth and Proliferation	30
Clinical Chemistry and Haematology	**Number of Genes**
Increased Levels of Haematocrit	1
Increased Levels of Red blood Cells	1
Cardiotoxicity	**Number of Genes**
Heart Failure	1
Cardiac Enlargement	1
Cardiac Infarction	1
Cardiac Dysfunction	1
Cardiac Pulmonary Embolism	1

In summary, the pathways affected most in both the female PAH and male controls in comparison to female controls were those heavily involved in immune system modulation (Graft-vs-Host, autoimmune thyroid disease signalling, dendritic cell maturation, and antigen presentation pathway).

8.3.4.2 Pairwise Comparisons

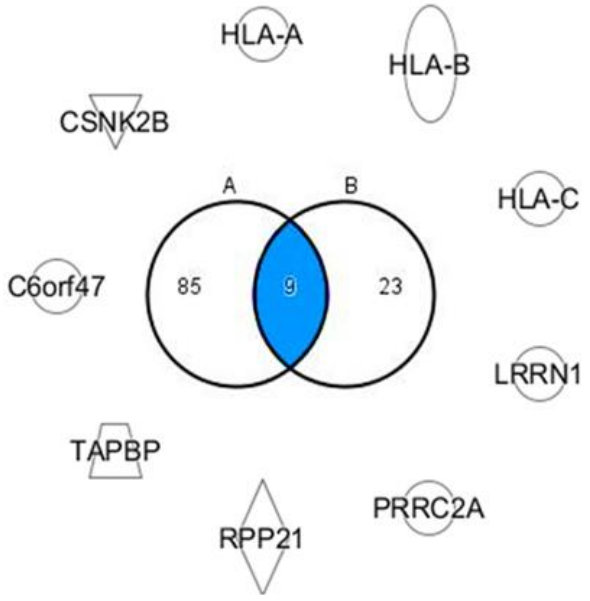

Figure 8-4: Venn diagrams of multiple comparisons The genes are divided into comparison A (Male control *vs* female control PASMCs) and comparison B (female PAH *vs* female control PASMCs). The Venn diagram shows the number of genes unique to each comparison (A = 85, B= 23 Genes) and genes that are constitutively changed across both comparisons (9 genes).

A Venn diagram (Figure 8-4) displays changes in human leukocyte antigens (HLA-A, B, C); Tapasin (*TAPBP*); Ribonuclease P/MRP Subunit P21 (*RPP21*); Proline Rich Coiled-Coil 2A (*PRRC2A*); Leucine Rich Repeat Neuronal 1 (*LRRN1*); Casein Kinase 2 Beta (*CSNK2B*) and Chromosome 6 Open Reading Frame 47 (*C6orf47*) were common between the experimental multiple comparisons of Female PAH vs female control, and Male control vs female control. A number of these common genes were also involved in immune system modulation (*HLA-A, HLA-B, HLA-C* and *TAPBP*). There were 85 genes distinct to male control *vs* female control and 23 to female PAH *vs* female control.

8.3.5 Immune Response Compromised in Female PAH

From IPA analysis, a number of molecules comprising the major histocompatibility complex (MHC1) sub-unit are down-regulated in female PAH *vs* female control. The MHC class I and class II proteins play a role in the immune system, presenting peptides on the cell surface for recognition by T cells (Wieczorek *et al.*, 2017). The most polymorphic MHC Class I proteins, also known as human leukocyte antigens (*HLAs*), are expressed from three distinct gene regions *HLA-A, HLA-B* and *HLA-C*. Together these play an important role in immune surveillance interacting with CD8+T and natural killer (NK) cells. This locus of genes is located on chromosome 6, regulating immune control and homeostasis. There is reduced expression of two subunit components in female PAH in comparison to female controls (p<0.1, Figure 8-5).

Female Control vs Female PAH

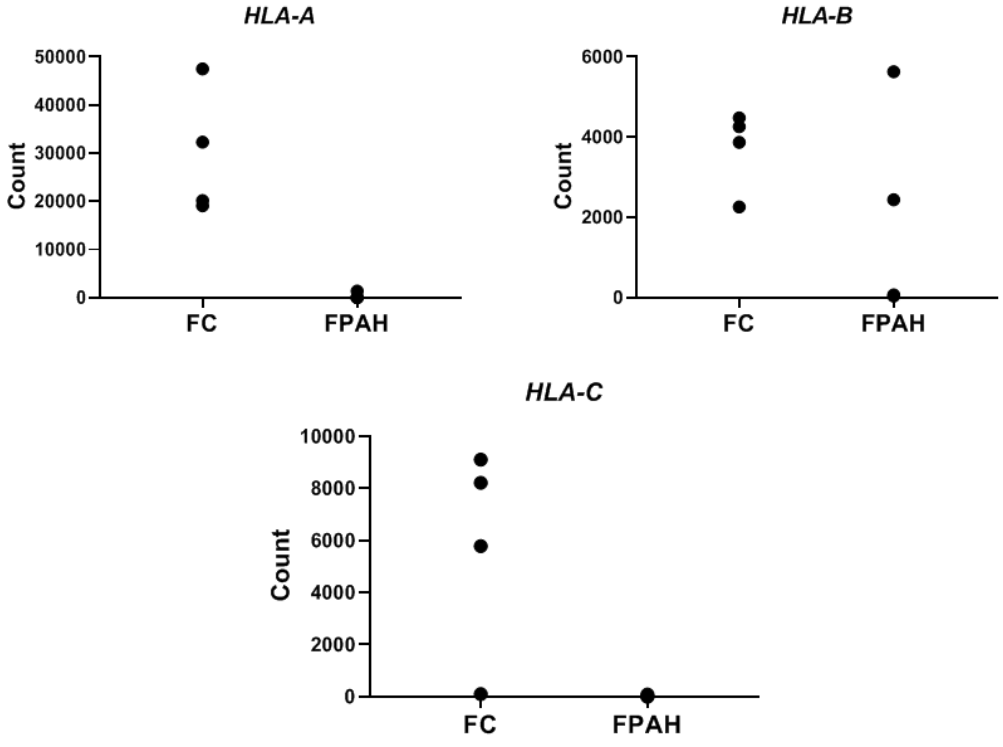

Figure 8-5: *MHC1* Subunit reduced in hPASMCs derived from female PAH compared to female non-PAH Reduction in *HLA-A* and *HLA-C* transcripts in all female PAH cells. *HLA-B* transcripts were reduced in only associated PAH patients (not iPAH and hPAH patients) in comparison to female non-PAH cells. FPAH. Female Non-PAH, FC; Female pulmonary arterial hypertension, FPAH; pulmonary artery smooth muscle cell, PASMC; human leukocyte antigen, HLA. n=4, Non-PAH; n=4, padj<0.1.

Transcripts of the MHC Class 1 subunit composed of *HLA-A* and *HLA-C* genes are down-regulated in cells from female PAH PASMCs. HLA-B displays more variability

with no reduction in the idiopathic and heritable PASMCs, only reducing in PASMCs derived from two “associated” PAH patients. This represents a limitation of the study with low n numbers between the experimental groups and added variability based on additional diseases in controls and a variety of disease classifications in PAH patients.

8.3.6 Overlay with Oncology: HLA genes dysregulated in female PAH PASMCs show relevance in cancer pathobiology.

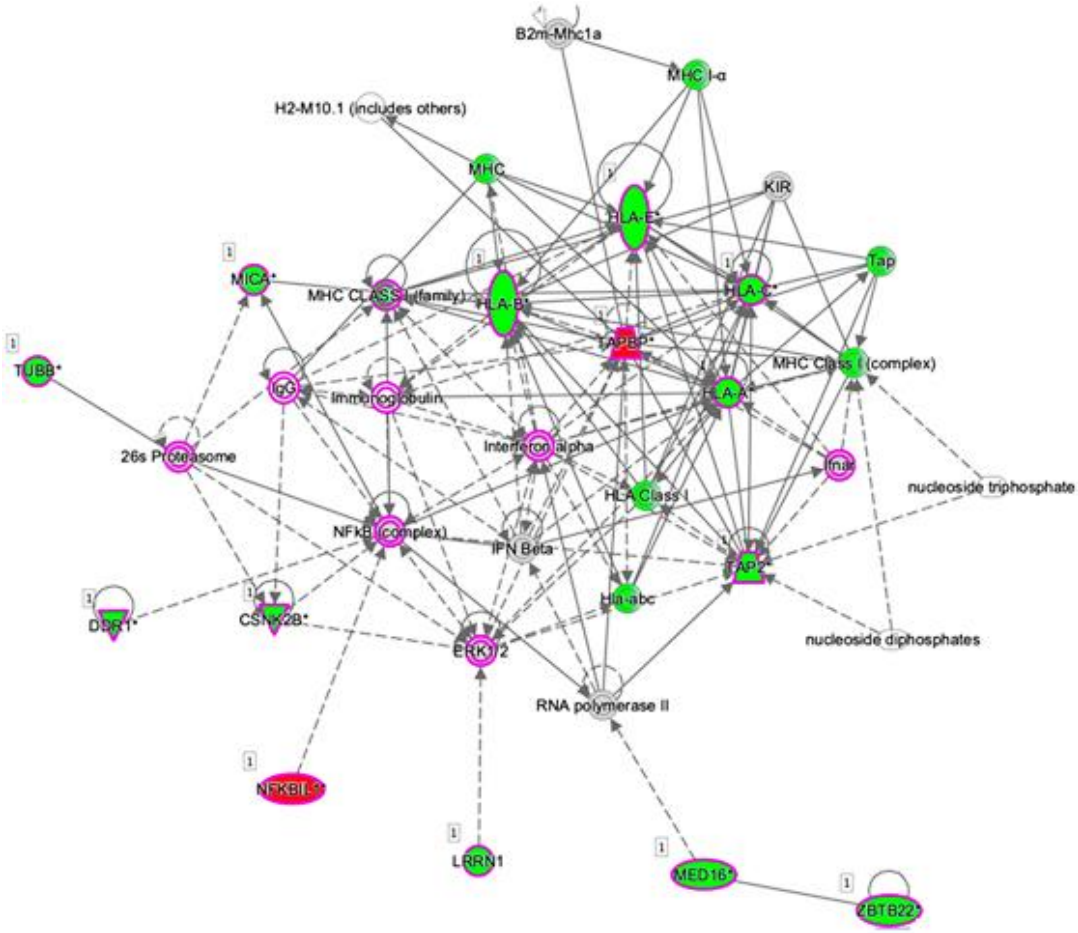

Figure 8-6: Overlay with oncology genes in female PAH PASMC signalling Gene ontology in female PAH overlaps with genes identified in pathways of cancer metastasis and progression with downregulation of MHC class 1 subunits and predicted upregulation of TAPBP and NFKBIL1. Green indicates downregulated genes and red upregulated genes.

A number of genes related to the MHC 1 subunit are implicated in oncology (Figure 8-6). Overlaying the oncology findings to this RNA-sequencing dataset displayed 21 genes associated with female PAH PASMCs.

8.3.7 Potential Upstream Regulators Driving Differential Expression in hPASMCs

8.3.7.1 17β-estradiol and ESR1 and Aberrant Genes in Female PAH

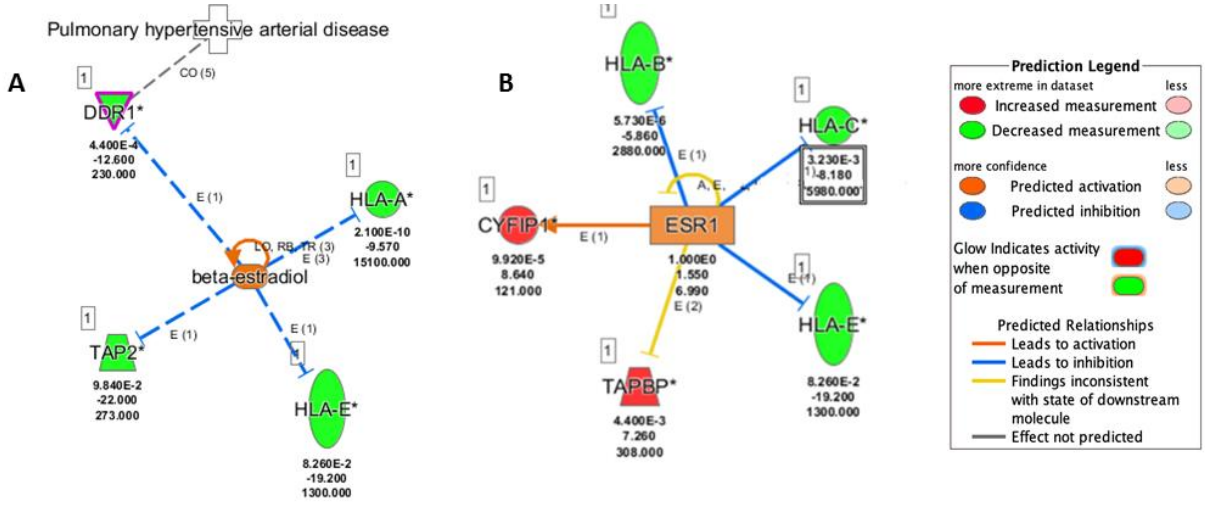

Figure 8-7: Estradiol and ESR1 predicted as upstream regulators of immune response genes downregulated in PAH The network displays relationships between the upstream regulators and their target molecules in female PAH PASMCs with estradiol (A) and estrogen receptor α (B). Green represents a predicted downregulation and red a predicted upregulation of mRNA.

In female PAH PASMCs (Figure 8-7), predicted upstream activation of 17βE2 may result in inhibition of *HLA-A*, *HLA-E*, *TAP2* and *DDR1.* Interestingly, predicted inhibition of *DDR1* by estradiol in female PAH was suggested by the database. In addition, *ESR1* (ERα) was also predicted to be activated in female PAH-PASMCs. Activation then leads to the reduction in *HLA-B*, *HLA-C* and *HLA-E* and overexpression of *CYF1P1* in PASMCs. However, the activation of *TAPBP* in this study was inconsistent with current literature findings. This was further assessed by qPCR experiments (Section 8.3.10.2).

8.3.8 Potential Role of Human Leukocyte Antigen Downregulation in the Pulmonary Vasculature

HLA components of the MHC class 1 complex have been prominent in the pathway analysis of this study. Auto-immunity of PASMCs may be lost in female PAH and male controls. This loss of functionality may be exacerbated by upstream estradiol signalling via ESR1 (Figure 8-7) causing functional modification via aberrant gene expression (Figure 8-8).

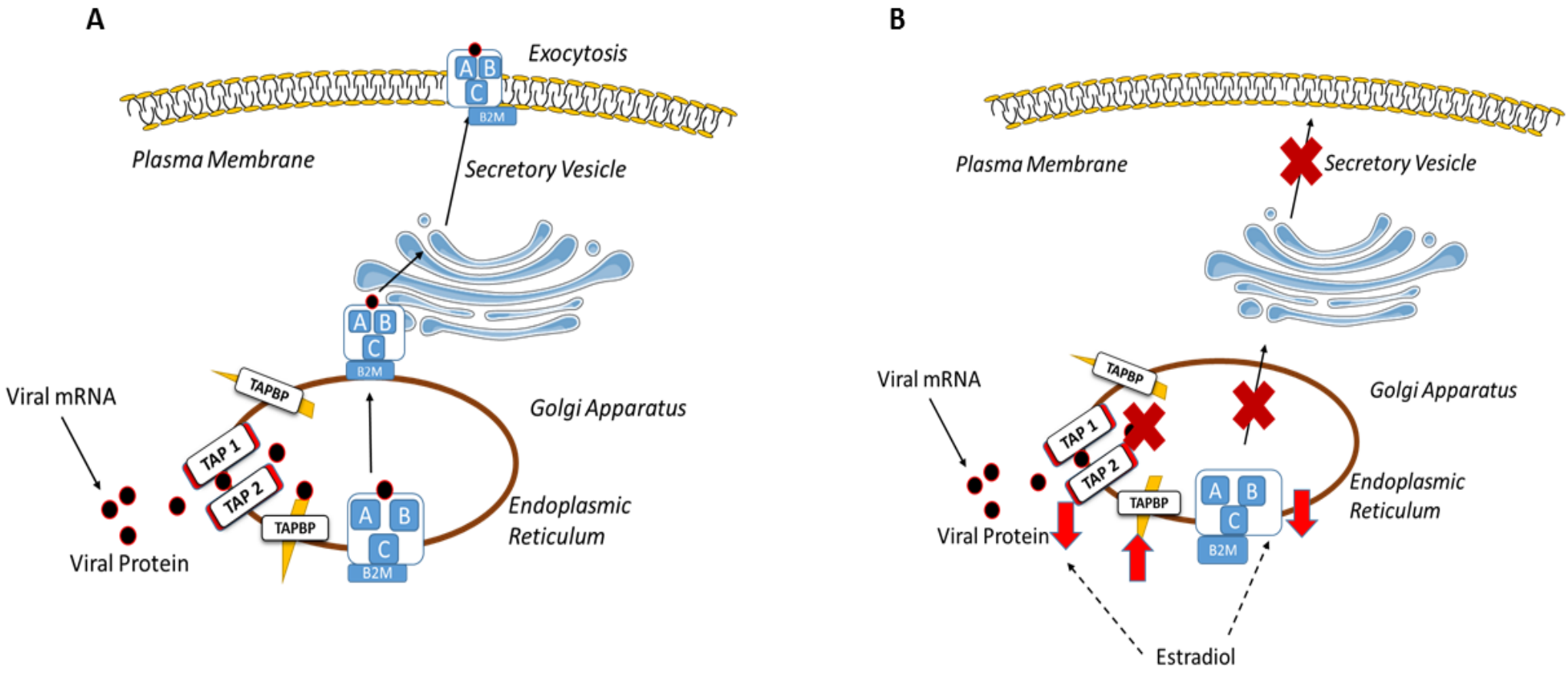

Figure 8-8: Potential consequences of HLA reduction in PASMCs: loss of innate immunity Human leukocyte antigen (HLA) class I molecules subunit downregulation in Female PAH and Male Non-PAH cells. In female control individuals (A) the MHC class 1 subunit (HLA-A, HLA-B and HLA-C) binds viral proteins and translocates from the endoplasmic reticulum to the plasma membrane for removal from the cell by exocytosis. In male controls and female PAH PASMCs (B) MHC class 1 is downregulated resulting in a loss of this function in the pulmonary artery which may contribute to disease pathogenesis.

The assembly of the HLAs (HLA-A, B & C) with B2M occurs on the endoplasmic reticulum (Figure 8-8). Following assembly, the complex is loaded with peptides formed via degradation of internal proteins. The antigenic peptides are transported to the ER lumen via tapasin transporter proteins named TAP1 and TAP2 for secretion by exocytosis (TAP 2 is also downregulated in female PAH, Table 8-4). In patients deficient of TAP, HLA class 1 complexes are peptide-free and retained between the endoplasmic reticulum and the Golgi compartment, causing a loss of innate immunity (Figure 8-8, B).The RNA-sequencing study within this chapter identified estradiol and ESR1 as potential modulators for reduction of HLAs and TAP in PASMCs (Figure 8-7).

8.3.9 Sex Differences in Control PASMCs

The MHC class 1 subunit genes (*HLA-A*, *HLA-B* and *HLA-C*) are downregulated in male non-PAH PASMCs upon comparison to female controls (Figure 8-9). Again, the variation represents a limitation of the study with low n numbers between the experimental groups and added variability based on additional diseases in controls and a variety of disease classifications in PAH patients.

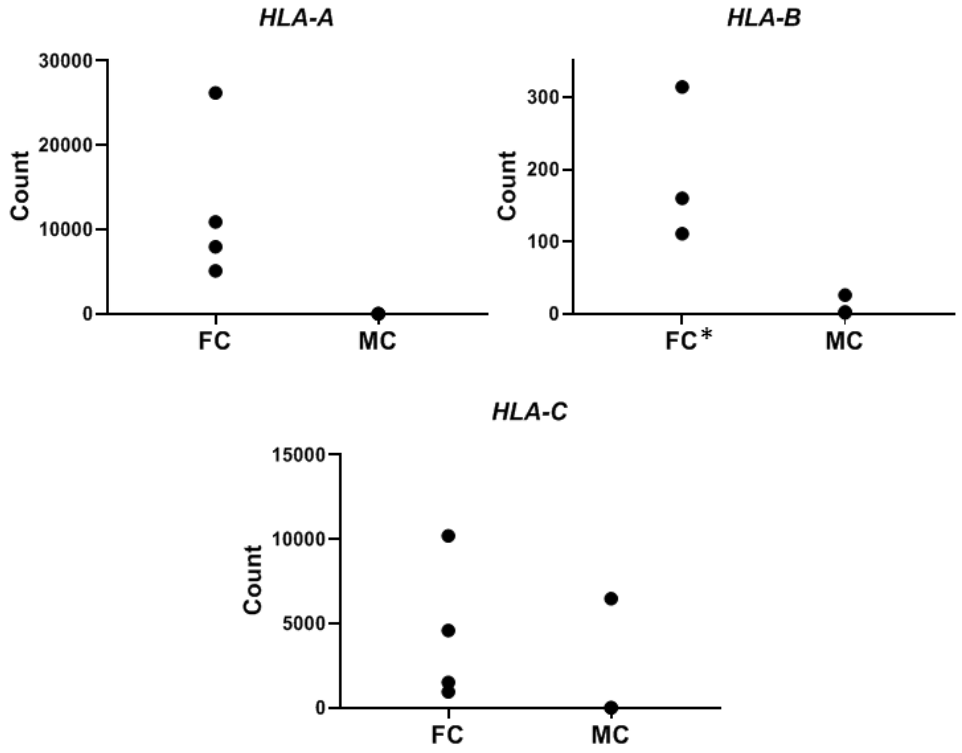

Figure 8-9: *MHC1* subunit reduced in PASMCs derived from Male non-PAH compared to female non-PAH Differences between males and females; HLA-A and HLA-B in all male non-PAH cells (*indicates one value excluded from graph due to 11000-fold decrease in male compared to female control). A reduction in HLA-C was displayed in 2/3 male non-PAH PASMCs. FPAH. Female Non-PAH, FC; Male non-PAH, MC; pulmonary artery smooth muscle cell, PASMC; human leukocyte antigen, HLA.n=4, Female Non-PAH; n=3, Male Non-PAH, padj<0.1.

In male controls, alternative top hit gene transcripts were over-expressed, such as Ribosomal Protein S4 Y-Linked 1 (*RPS4Y1*), Lysine Demethylase 5D (*KDM5D*), Eukaryotic Translation Initiation Factor 1A Y-Linked (*EIF1AY*) and male specific Y-linked gene transcripts for DEAD-Box Helicase 3 Y-Linked (DDX3Y).

8.3.10 Validation of Differentially Expressed Genes

To confirm the fold change of differential gene expression, RNA was tested by qPCR. Ideally these findings would be confirmed in an independent cohort of PASMCs at a later date. Firstly, a stable housekeeping gene was sought for accurate normalisation (Figure 8-10).

8.3.10.1 Housekeeping Gene

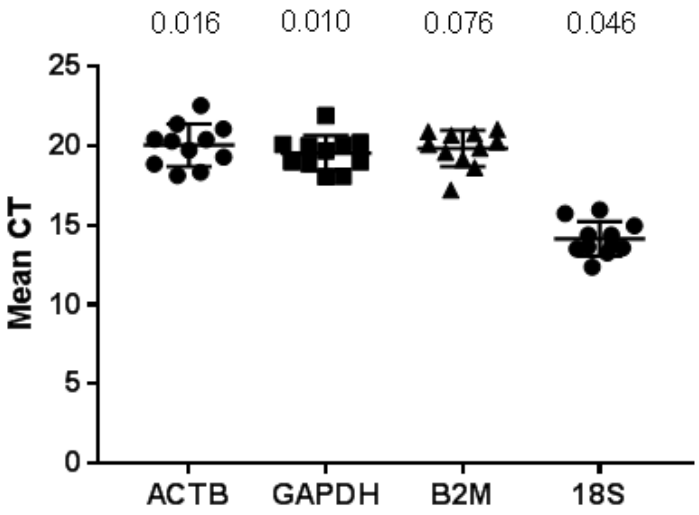

Figure 8-10: Stable reference gene for qPCR analysis NormFinder analysis identifies Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the most stable for accurate qPCR quantification with the lowest stability value (0.01) over beta-actin (ACTB, 0.16), beta-2-microglobulin (B2M, 0.076) and 18S ribosomal RNA (18S, 0.046).

GAPDH was chosen as a reference gene, displaying stability in amount within RNA extracted from hPASMCs. (Figure 8-10).

8.3.10.2 qPCR of Seven Genes Highlighted within IPA Analysis

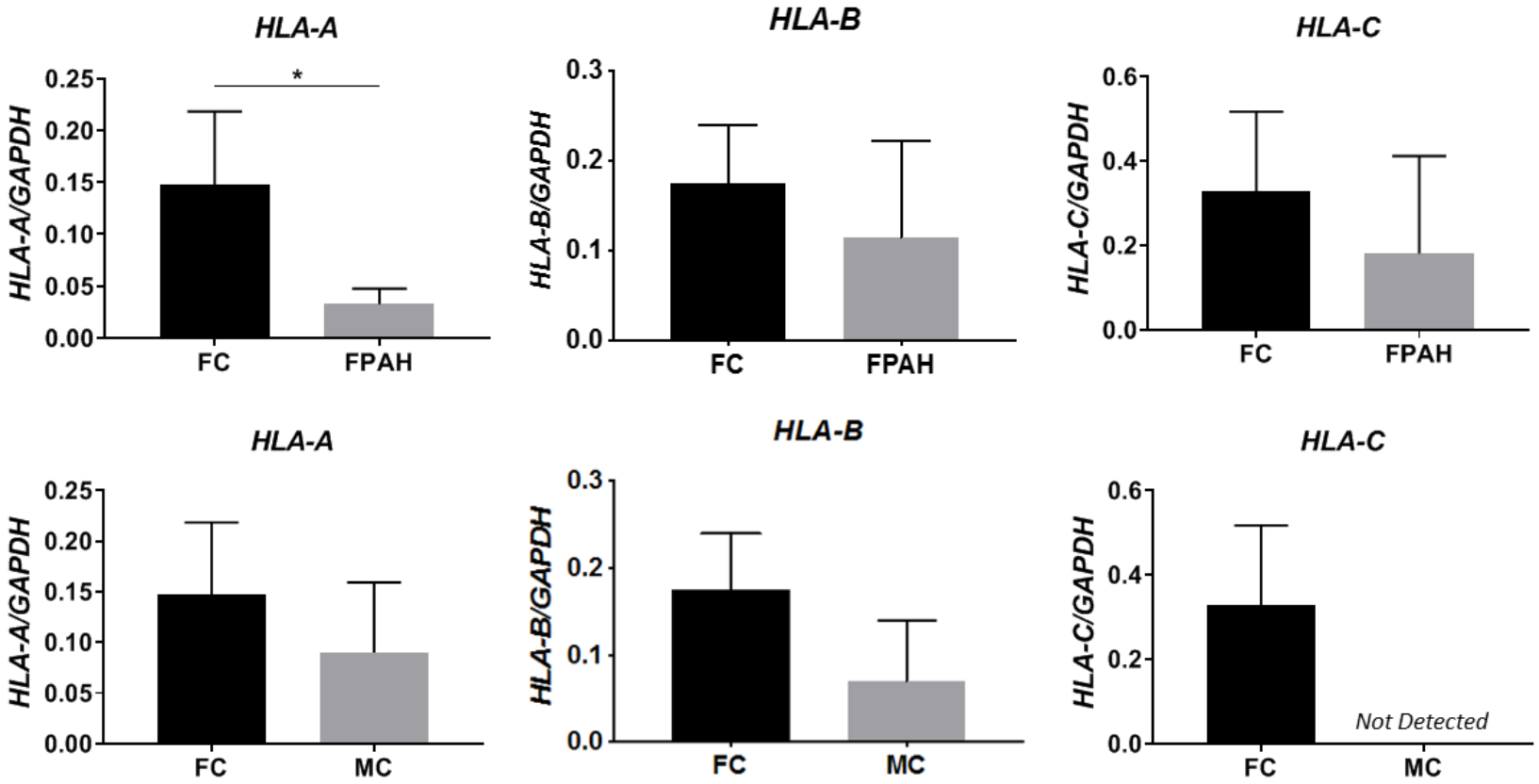

Figure 8-11: qPCR validation of MHC Class I subunits Validation of human Leukocyte Antigens; HLA-A, HLA-B and HLA-C in Female Control, FC; Female pulmonary arterial hypertension, FPAH; Male Control, MC PASMCs. Data expressed as mean ± SEM with comparison by a Student's t test; Female Control; n=4, Female PAH, n=4, Male Control, n=3.

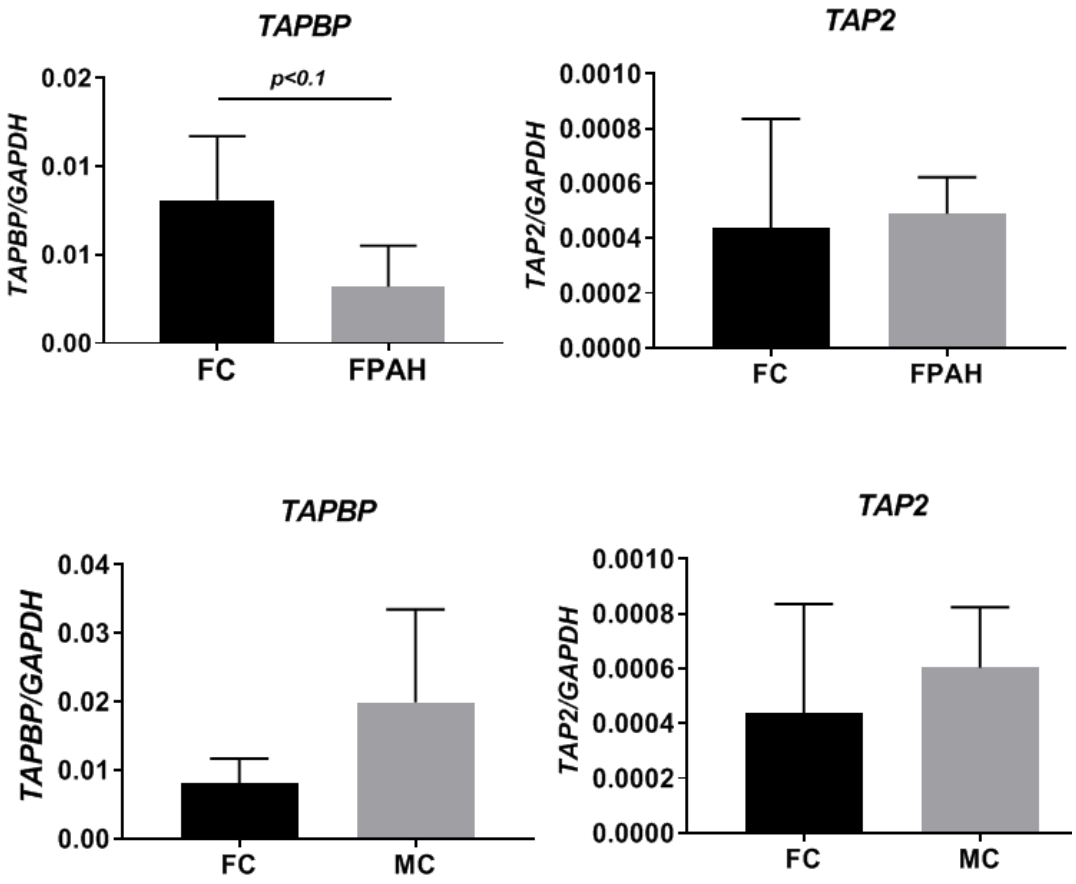

Figure 8-12: qPCR validation of *TAPBP* and *TAP2* in hPASMCs Validation of TAPBP, TAP2 and ESR1 in Female Control, FC; Female pulmonary arterial hypertension, FPAH; Male Control, MC PASMCs. Data expressed as mean ± SEM with comparison by Student's t test; Female Control; n=4, Female PAH, n=4, Male Control, n=3.

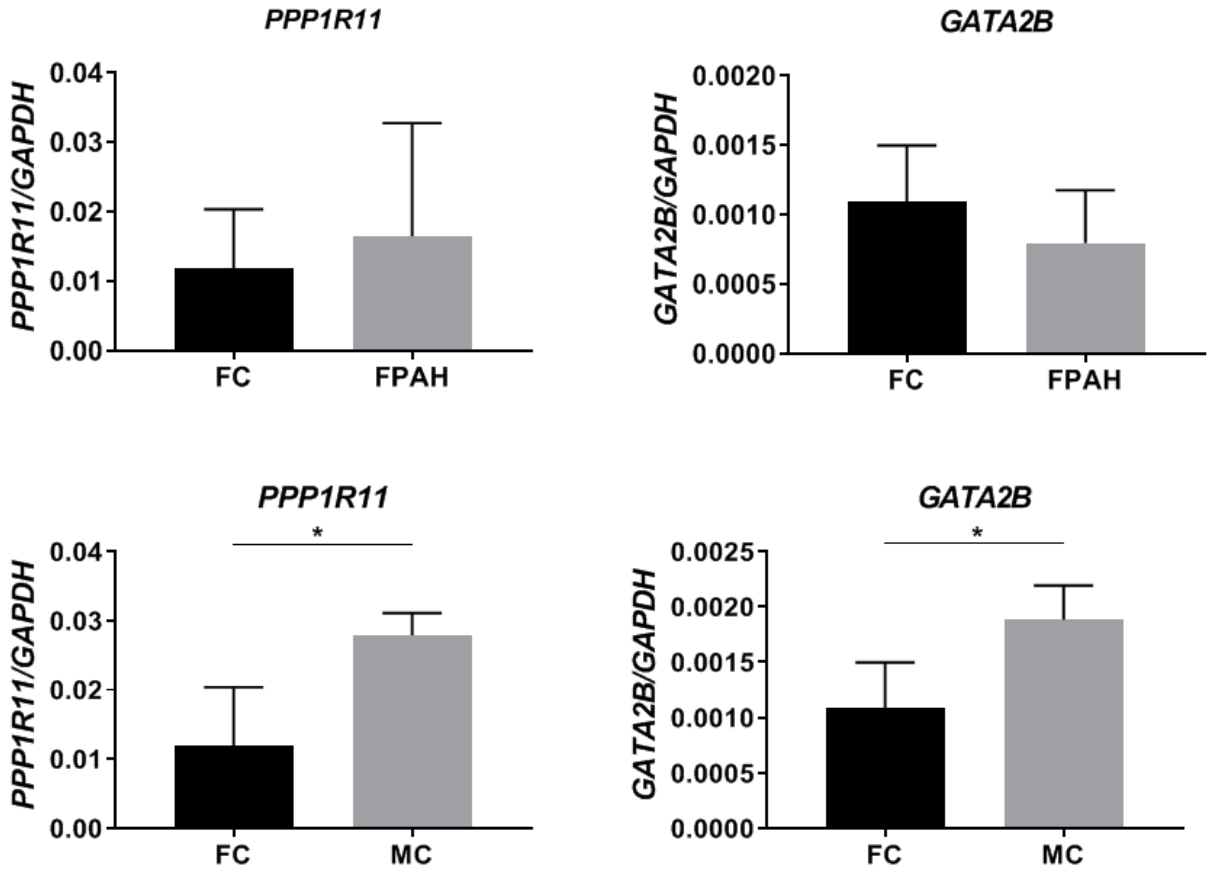

Figure 8-13: qPCR validation of *PPP1R11* and *GATA2B* in hPASMCs Validation of *PPP1R11* and *GATA2B* in Female Control, FC; Female pulmonary arterial hypertension, FPAH; Male Control, MC PASMCs. Data expressed as mean ± SEM with statistics following a student's t test; Female Control; n=4, Female PAH, n=4, Male Control, n=3.

In female PAH PASMCs, decreased expression of *HLA-A* transcript was shown in comparison to female controls by qPCR which replicates the RNA-sequencing findings. In males, the reduced expression of *HLA-C* in comparison to female controls was also replicated between the RNA-Sequencing and qPCR (Figure 8-11). For additional HLA subunits, changes were not detected by qPCR. The transcript reduction of *TAPBP* was inconsistent with sequencing findings with the initial IPA analysis showing an overexpression of this gene in PAH-PASMCs (Figure 8-7). *TAP2* also did not show any changes between experimental comparisons using qPCR. Therefore, sample numbers should be increased to provide further validation.

The expression of *GATA2B* transcripts in female PAH was unchanged by qPCR, however sample numbers values were low. *PPP1R11* expression was also reduced in female PAH cells (Table 8-4) by RNA-sequencing but remained unchanged following qPCR (Figure 8-13). Addition of sample numbers to this experiment is needed to determine if discrepancies in PASMC comparisons is due to reduced power.

8.3.11 Discussion

This study was conducted to assess the global gene expression changes in human PASMCs between female and male non-PAH and also in female PAH patients. Sequencing identified downregulation of MHC class 1 subunits (*HLA-A, HLA-B* and *HLA-C*) alongside transporters *TAPBP* and *TAP2* in female PAH vs female controls. The same was found in male control vs female control PASMCs. Following IPA analysis, estradiol and *ESR1* were implicated as upstream regulators of a number of the HLAs and *TAP2* genes in female PAH. This suggests female PAH patients may be at higher risk of cellular dysfunction and that non-PAH control males may be predisposed to immune escape mechanism within the pulmonary vasculature leading to proliferative phenotypes observed in disease. The qPCR experiments showed a degree of replication with sequencing findings. However numbers for these experiments should be increased to reduce the variability and to confirm gene expression in a larger number of cell lines from additional patients. The analysis of gene changes in a second cohort of cell lines would also be preferential.

The MHC region of the genome has been associated with a higher number of diseases than any other (Trowsdale et al, 2013). The MHC Class 1 subunit provides protection via receptor families recognising the HLA-1 ligands *HLA-A, B & C,* such as NK cells, and killer-cell immunoglobulin-like receptors (KIRs), expressed on NK cells and leukocyte immunoglobulin-like receptors (*LILRs*), expressed on antigen presenting cells, monocytes, macrophages, B-Cells, T Cells and NK cells (Borges and Cosman, 2000). Therefore, a loss of these MHC Class 1 subunit genes expressed in female PAH vs female controls and in male control vs female control PASMCs may represent a susceptibility to PASMC dysfunction. In human cancers, a loss or down-regulation of MHC Class I (*HLA-A*, *HLA-B* and *HLA-C*) has been demonstrated in a number of malignancies representing an important immune escape mechanism (Aptsiauri *et al.*, 2013). Throughout each stage of cancer progression, tumour cells become more abundant exploiting a variety of immune escape mechanisms causing abnormal expression patterns and increased proliferative and migratory phenotypes (Delgobo and Frantz, 2018). The MHC class 1 complex may be degraded due to the loss of the TAP transporter, with the same effect reported to be shown in *B2M* negative cells (Pamer and Cresswell, 1998). In our sequenced PASMCs, *B2M* was present at stable levels (Figure 8-10) suggesting the reduction of *TAP2* shown by sequencing may have been responsible for degradation of the MHC class 1 subunit. Further studies have linked the reduced expression of MHC class 1 subunits and loss of function to lung cancers as a secondary consequence of disease insult which remains unrelated to the growth of cancers or survival of patients (Korkolopoulou *et al.*, 1996). However, in the early stages of breast cancer, disruption of TAP expression decreases peptide delivery to avoid $CD8^{+}$T cell recognition, hence altering the processing and presentation of tumour antigens and progressing malignant transformation of cells via immune escape mechanisms (Johnsen *et al.*, 1998; Matsui *et al.*, 2002; Setiadi *et al.*, 2007).

HLA- findings in cancer may also translate to PAH, as loss of *HLA-B* and *HLA-C* have been linked to severe cardiovascular complications such as HIV-PAH (Jarrett and Barnett, 2017). HIV infection severely affects peptide generation, transport and, most importantly for comparison to this sequencing study, the MHC Class I molecules. Increased susceptibility to lung disease and infection also occurs via the viral downregulation of HLA-A, B and C by HIV variants (Ende *et al.*, 2018). There are few immunogenic studies of PAH. However, given the association with HIV and the relevance of immune disorders and inflammation these studies have become

more widespread in recent years (Mouthon, Guillevin and Humbert, 2005). It has become clear in specific clinical cases that infective viruses can accumulate in the pulmonary vasculature, causing pathophysiological alterations.

For example, pathogenic endothelial cell antibodies can initiate PAH development via dysfunctional T- and B- lymphocytes in PAH lesions (Nicolls *et al.*, 2005). HLA class 1 antigens also play an important role in modulation of natural killer (NK) cell function. Impairments of NK cell function are related to pathobiology of PAH (Ormiston *et al.*, 2012). A loss of binding sites for NK cells to MHC class molecules can lead to cytotoxicity advancing vascular remodelling. The Ormiston et al study demonstrated differences in subsets of NK cells in PAH *vs* healthy donors; $CD56^{dim}$/CD16 were decreased, $CD56^{bright}$/$CD3^{-}$ were unchanged and $CD56^{-}$/$CD16^{+}$ were increased, a finding similar to HIV patients leading to impaired capacity to lyse dysfunctional tumour cell lines and HIV-infected autologous target cells. The same study utilised the monocrotaline rat and chronic hypoxia mouse models of PAH to confirm defects in NK cells which mimicked findings from human disease. In HIV-1, expression of HLA- A, B & C is compromised in infected cells rendering them susceptible to NK cell-mediated killing, with the non-classical ligands HLA-E,G & F also being impaired (Hölzemer *et al*, 2017). This somewhat supports our findings of aberrant MHC subunit expression in the medial layer of the pulmonary vasculature. The decreased level of MHC subunits in female PAH and male control PASMCs may impair NK cell binding leading to advanced proliferative phenotypes.

IPA analysis also implicated estradiol and ESR1 as potential upstream regulators of MHC Class 1 subunit gene expression reductions. In breast cancer studies, HLA expression is inversely correlated with the expression of ESR1 in normal luminal and breast cancer cells (Lee *et al.*, 2016). Estradiol has been implicated in immunosuppressive activities affecting the viability, phenotype, endocytosis and inflammatory cytokine expression in RAW264.7 macrophage cells (Yang *et al.*, 2016). These studies demonstrate a role for both ESR1 and estradiol in antigen presentation and innate immunity which has yet to be extended to PASMCs from control subjects and PAH patients. In female PAH PASMCs, the predicted activation of estradiol signalling also generated a inhibitory connection with Discoidin Domain Receptor Tyrosine Kinase 1 (*DDR1*), a protein which binds a number of collagen related proteins facilitating cell migration, survival proliferation and differentiation (Leitinger *et al*, 2014), which has been linked to PAH. Overexpression of DDR1 has

been shown in multiple cancers, positively regulating tumour progression via increased proliferation rates (Belfiore *et al.*, 2018). Therefore, it may be of interest that within female-PAH PASMCs that estradiol was predicted to cause a reduction of *DDR1*, leading to a protective role among female patients. Imatinib, which is currently in clinical trials of PAH, inhibits tyrosine kinases such as *DDRs* (Ghofrani *et al.*, 2010). The interaction between estradiol and DDR1 activity should be further investigated in females.

8.3.11.1 Future Directions

This study indicates that immune deficiency may indirectly affect the pathogenesis of female PAH and predispose males to disease insult in serum-reduced environments imitating conditions of environmental stress. The downregulation of the MHC1 Class subunit may be linked to cell dysfunction and the inability of cells to maintain a healthy proliferation and apoptosis rates throughout cell cycle stages. In the male controls, each patient displayed a carcinoma prior to explant with one patient's additional disease being unknown. The presence of carcinoma may explain the similarity with female PAH with downregulation of MHC class 1 subunits already linked to several cancers (Korkolopoulou *et al.*, 1996; Johnsen *et al.*, 1998; Aptsiauri *et al.*, 2013; Garrido *et al.*, 2016). Therefore, the presence of the MHC Class 1 subunit reduction in female PAH may be more significant. Another study shows that female predominance of chronic thromboembolic pulmonary hypertension without the presence of deep vein thrombosis (CTEPH) exists in Japan and is associated with HLA subunits (Tanabe *et al.*, 2005). Tanabe et al suggest, this female prevalence in CTEPH is uncommon in the Western world, demonstrating genetic mutations in HLA are associated with ethnicity. Caucasian populations generally display low incidence of HLA modulation; the ethnicity of patient cell lines used in this chapter remains unknown but may account for variability in expression levels for future studies. The qPCR results of our study remain varied although it remains reassuring that a number of the HLA genes showed the same trends upon the targeted analysis (Figure 8-11). In light of the variation, it would be beneficial to extend these validations to a larger number of cell lines and to cell lines in an alternative cohort when cells become available. This would allow a clearer definition of gene expression changes between our experimental conditions.

In addition, a literature review of the top three overexpressed genes in female PAH compared to female controls (Nuclear Factor of Kappa Light Polypeptide Gene Enhancer in B-Cells Inhibitor-Like 1 *(NFKBIL1),* CCR4-NOT Transcription Complex Subunit 3 (*CNOT3) and* SCO cytochrome c oxidase assembly protein 2 *(SCO2)*, (Table 8-4)) revealed potential directions for future targeted analysis. *NFKBIL1* was significantly overexpressed in female PAH-PASMCs; this gene regulates immune response similarly to a number of genes heavily associated with disease status in this chapter. *NFKBIL1* lies within the MHC Class 1 region of chromosome 6 and is associated with a mild phenotype for chronic thromboembolic pulmonary hypertension (CTEPH) (Kominami *et al.*, 2009). The Kominami et al study implies HLA and non-HLA transcript abundance located in the HLA region of chromosome 6 associated with PAH are highly dependent on the ethnic groups studied, which may be important in identifying immune response dysfunction in PAH subgroups. *CNOT3* is one of the major cellular mRNA deadenylates, linked to various cellular processes such as bulk mRNA degradation, miRNA-mediated repression, translational repression during translational initiation and general transcription regulation. Interestingly, *CNOT3* overexpression has been identified as enhancing proliferation in cultured human and mouse intact murine heart cardiomyocytes (Zhou *et al.*, 2017). In cardiomyocytes, degradation of anti-proliferative gene transcripts occurred in a *CNOT3*-dependent manner, displaying an unknown role for mRNA degradation in the progression of cardiac cell proliferation. It would be of interest to determine whether CNOT3 caused the same proliferative function in female PAH PASMCs. Finally*, SCO2* is essential for assembly of the catalytic core of cytochrome-c oxidase (COX) at the protein level with mutations in *SCO2* leading to severe deficiency of COX in the heart, brain and muscle. *SCO2* mutations leading to COX deficiencies have been described in one patient case from a Brazilian female infant presenting cardioencephalomyopathy (Gurgel-Giannetti *et al.*, 2013). The clinical consequence of the mutation in the female infant was severe phenotype and early death. This case report in the literature may demonstrate an indirect link with PAH, as in PAH cyclooxygenase-2 (*COX-2*) deficiencies leading to detrimental pulmonary vascular consequences in patients with hypoxemic lung disease or pre-existing PAH (Fredenburgh *et al*, 2009). In addition COX assembly factors are linked to cell proliferation in lung, breast and nasopharyngeal carcinomas by favouring metabolic reprogramming toward cell proliferation (Rak *et al.*, 2016). Therefore, investigation of a link between *SCO2* and *COX* deficiencies in PAH may lead to

definition of proliferative reprogramming in the pathobiology observed in female patients.

In male control PASMCs, a literature review of the top hit gene transcripts correlated with disease. *KDM5D* over-expression has been linked to cardiovascular disease, in proteomic studies (Mokou *et al,* 2019). In patients with cardiovascular disease over-expression of the protein levels were accompanied by a reduction in levels of the substrate trimethylated lysine 4 of histone H3 (*H3K4me3*). Subsequent *in vitro* functional studies applying a *KDM5* inhibitor on endothelial cells demonstrated a reduction in proliferation, migration and tube forming ability. Also over-expression of the *EIF1AY* transcript in hPASMCs is of interest as this transcript is also over-expressed in hPAH patients (Davies *et al.*, 2012). This implies over-expression of gene transcripts linked to disease in male control hPASMCs may predispose to severe phenotypes and worsened mortality in cardiovascular disease.

8.3.11.2 Study Limitations

This study was limited by small statistical power in each group for sequencing. Each non-PAH control may have induced additional added variability due to the presence of additional medical conditions such as carcinomas, angina and emphysema. Also, it was not possible to collect four patients from the same PAH classification, which would have been desirable in the final analysis. Within the analysis there were several genes with increased expression in associated PAH that remained unchanged in idiopathic and heritable RNA. Therefore, grouping of patient classifications may provide invaluable data. Finally, due to the rare nature of this disease in males, male PAH cells were not available and only three non-PAH cell lines were available.

Chapter 9 General Discussion

In this thesis, we provide evidence of sex-dependent estrogen production among PAH patients using our validated LC-MS/MS approach. In males, elevations in parent estrogens are present in idiopathic disease with further elevations in 16-hydroxyestrogens in idiopathic and portopulmonary disease. In females, parent estrogens are reduced in concentration in PAH patients with a subsequent increase in 16-hydroxyestrogen production suggesting an overexpression or SNP in genes encoding CYP enzymes may be important. Most significantly, 16OHE2 is elevated in samples from premenopausal and postmenopausal PAH patients with reduced concentrations in postmenopausal women correlating with improved 6-MWD. These findings suggest 16OHE2 may be a biomarker of disease and therapeutic response in female disease. In studies exploring the functional role of 16OHE2 in PASMCs, a sex-dependent proliferative action was demonstrated. *In vitro* 16OHE2 caused proliferation only in female cells explanted from rats and humans with no effect in male cells. This further emphasises a prominent function for this steroid and enzymatic pathway in the deterioration of cardio-pulmonary health in female PAH. Further definition of genetic alterations in PASMCs via a transcriptome wide analysis of human cells from female PAH patient's *vs* female controls demonstrated differences in gene transcripts. This study implicates a possible immune escape mechanism in the abnormal function of PASMCs from PAH patients. In a sex comparison of male control to female control PASMCs, we also demonstrated a loss of immune recognition subunits and an overexpression of gene transcripts previously linked to increased cardiovascular disease and hPAH in male control PASMCs. These gene transcriptional differences may lead to the severe phenotypes of PA remodelling and higher mortality rates among males than females. Taken together these findings indicate targets for future validation experiments and begin to explore the underlying genetic mechanisms of PAH and differences between the sexes.

9.1 Analytical Challenges in Measurement of Estrogens

A number of challenges are encountered when measuring low abundance, isomeric endogenous steroids in biological matrices. The isomeric and isobaric nature of estrogens of interest results in concerns over co-elution of similar structures by LC with similar breakdown product ions by MS analysis. In addition, all estrogens are

uncharged structures at neutral pH, meaning ionisation in the MS source can be inefficient without addition of mobile phase modifiers or derivatisation of estrogens to form permanently charged or chargeable species, improving ionisation efficiency within the ESI source. Finally, low circulating concentrations in males and postmenopausal women and a wide range of concentrations due to high estrogen concentrations in premenopausal women present another challenge for accurate analysis across a wide linear range. Therefore, definition of the expected ranges in the types of sample cohorts encountered is desirable moving forward.

A thorough validation of sample preparation using Oasis MCX SPE coupled with MPPZ derivatisation was performed (Chapter 2). This method allowed distinction of isomeric and isobaric estrogens by chromatographic separation and mass fragmentation with improved ionisation efficiency following derivatisation. Our studies of plasma and serum from PAH patients and controls (described in Chapters 3 – 7), have demonstrated the capability of our LC-MS/MS approach to quantify nine endogenous estrogens and bioactive metabolites. Upon initial validation of the technique, in agreement with analytical guidelines and tutorials, a number of further adjustments were required to allow analysis of estrogen metabolites within clinical samples (Section 9.1.2). This demonstrates the importance of routine analytical testing and validation within samples from healthy individuals and the patients of interest.

In samples from non-PAH controls, liver disease and multiple classifications of PAH patients the full detection of analytes could be fully assessed with calibration ranges for each group suggested for future studies (Chapters 4 – 7). Age and sex represent the most important variables in setting steroid reference ranges, therefore the ranges of estrogens detected for each cohort could be split based on menopausal age and sex (Eisenhofer *et al.*, 2017). Detection of nine estrogens was achieved in plasma, with five estrogens detected in serum. This difference might be due to the extraction approach which was optimised in pooled plasma aliquots. The full panel of nine estrogens was detected only in premenopausal females. This might also be expected, as parent estrogen concentrations are higher in women aged ~ 18 – 55 years, meaning we would expect to see higher production of metabolites than in males and post-menopausal females. In general, 17αE2 was undetected in serum, with 2MeOE2, 4MeOE2 and 4MeOE1 appearing less frequently in serum than in plasma. In serum, preparation of blood following collection by centrifugation

removes clotting factors such as fibrinogen and thus the preparation of serum between laboratories may differ slightly. Plasma on the other hand, is composed of both serum and the clotting factors, with collection in tubes containing anticoagulants allowing storage of samples for many years. In this thesis, both serum and plasma were assessed by LC-MS/MS. However, the exact collection sample protocols remain unknown. There was variation dependent on the collection, sample handling, storage, number of years stored and shipment of the samples. Hence, we have not directly compared estrogen concentrations between studies.

9.1.1 Estrone and Estradiol Quantification

During serum and plasma studies, estrone and estradiol concentrations were well defined at the lower limit of 2 pg/mL and routinely measured in the majority of samples. The derivatisation of E1 and E2 using the optimised MPPZ protocol (Chapter 2) was also successfully applied for analysis of E1 and E2 in adipose tissue from breast cancer patients (Laforest *et al.*, 2019) and cell medium (Laio Y, MRes in progress). This demonstrates the versatility of the analytical approach upon extraction optimisation from the matrix of choice. As the initial PPZ reaction is specific to the 3' hydroxyl A ring of estrogen, glucocorticoids (cortisol and cortisone) with a ketone in this position could be included without interference from the reaction process allowing detection of concomitant non-derivatised steroids, extending the number of steroids for simultaneous analysis. In addition, more recently in unpublished work, testosterone (T), dehydroepiandrostenedione (DHEA), androsteinedione (AN), keto-androsteinedione (11-KT) and alpha-androstenedione (5α-KT) were added to this method, allowing analysis of aromatase substrates simultaneously alongside the glucocorticoids and parent estrogens. This could be applied for further investigations in PAH, allowing analysis of the upstream pathway prior to estrogen production: in particular DHEA, androstenedione and testosterone analysis would be of high interest in the PAH field. This broadens the scope of our derivatisation method for application to biomarker discovery and clinical monitoring for alternative estrogen sensitive diseases.

9.1.2 Estrogen Metabolite Quantification

Validation of the method was achieved using pooled plasma and subsequent detection of estrogen metabolites in more variable clinical samples. This proved to

be more challenging with intermittent detection in initial studies (Chapter 3 & 5). This prompted review of assay parameters (Chapter 4 & 6). These resulting improvements were visible following the introduction of a wash step following 8-10 plasma injections, addition of 10% water to the acetonitrile mobile phase, removal of sample transfer steps within the extraction process and narrowing of the linear calibration range for postmenopausal and male samples. Following reassessment, the lower limits were revalidated from 10 pg/mL for all metabolites to 2 pg/mL for 16OHE2, 2MeOE1 and 4 pg/mL for 16OHE1, 2MeOE1, 4MeOE1, 2MeOE2 and 4MeOE2 in calibration standards. It may be possible to further improve detection of estrogen metabolites in calibration standards and plasma via a number of adjustments to the method.

9.2 LC-MS/MS Method Development

9.2.1 Validated Estrogen Metabolites

To improve the detection of estrogen metabolites in plasma and serum, a number of approaches may be tested. Firstly, addition of an internal standard for 16OHE1 would be of high importance. This metabolite has appeared elevated in multiple PAH cohorts analysed within this thesis (Chapter 3, 4 and 6); internal standard addition would allow a more accurate quantification of 16OHE1 in future studies. We can see from metabolite LOQs reported earlier in this chapter (Section 9.1.2) that those with labelled internal standards incorporated into the method (16OHE2 & 2MeOE1) display lower detection limits and improved detection of endogenous species at low levels. Secondly, larger volumes, perhaps 1 mL, of plasma could be extracted using SPE to demonstrate if increasing volume would increase detection of metabolites at lower concentrations, reducing the number of missing values within our statistical analyses. This would require some additional testing as noise would increase due to increased background from plasma samples and larger SPE columns may be required, increasing cost and time. This effect should be assessed in each transition of the MRM method. Extraction of higher volumes would also be dependent on the volume of plasma or serum available from collaborators, which can be a limiting factor. Finally, upon characterisation of the fragment ions by high-resolution mass spectrometry for the validated estrogens and the catechol metabolites, a number of dominant transitions appeared in time of flight MS, which were not fragmented upon tuning on the Quantum instrument before transfer of tuning parameters to QTRAP

5500 then to the 6500+ upon installation (Chapter 2, Table 2-4). High-resolution analysis demonstrated product ions of higher masses to the dominant 58.0, 250.0 & 280.0 *m/z* currently used for metabolite detection. These larger ions may be more specific including a larger proportion of the estrogen-MPPZ structure. In addition, larger product ion formation on fragmentation of the parent ions may improve detection at lower concentrations via improvement of the signal over noise. This may be advantageous for analysis within postmenopausal women and men and modification of the tune parameters could be explored.

9.2.2 Catechol Estrogen Metabolites

For inclusion of catechol estrogen metabolites (2OHE1, 2OHE2, 4OHE1 & 4OHE2) in our LC-MS/MS workflow, an alternative extraction protocol should be tested as discussed in Chapter 2, section 2.4.4. The 4-hydroxyestrogen pathway is detrimental in a number of cancers causing DNA damage upon oxidation (Yasuda *et al*, 2017). In contrast, the 2-hydroxylation pathway presents a protective route which, upon comparison to 16-hydroxyestrogen ratios, can provide a marker of cancer risk (H*o et al*, 1998). More recently the ratio has also been implicated in the presence of familial PAH (Austin *et al*, 2009). In premenopausal samples analysed in Chapter 5, we show reduced production of methoxyestrogens among PAH patients compared to controls. This raises the question of abundant hydroxyestrogen production via reduced COMT activity. The exact concentrations of the catechol metabolites in PAH remains undefined. Therefore, assays for their analysis in the disease setting are of high importance.

9.3 Estrogen Metabolism in PAH

As PAH is more common in women than men, the main hormone in premenopausal women, E2, was initially thought to encourage development of the pathophysiological phenotype. However a number of pre-clinical studies have suggested a protective role of E2 in the PA, with less severe PAH in female rats over males (Lahm *et al.*, 2012; Umar *et al*, 2012; Frump *et al.*, 2017, 2018). The protective role of E2 in animals was demonstrated in hypoxia but not during normoxia, suggesting the anti-proliferative effect is highly dependent on the study context, which may also be the case in proliferative studies. Contrary to this protective role in preclinical models, a number of publications indicate a pathogenic

function of E2 in experimental PAH. This has become known as the ‘sex paradox’ in the PAH field (Foderaro *et al*, 2016). E2 induces the proliferation of human PASMCs, contributing to PA remodelling (White *et al.*, 2012). In the PA, over-expression of aromatase is documented creating a locally synthesised source of E2 over and above that delivered by the systemic circulation (Mair *et al.*, 2014). In an animal study also reported in the Mair et al 2014 publication, inhibition of E2 production via anastrozole treatment in two models of PAH (hypoxic mouse and Sugen 5416/hypoxic rat) reversed the PAH phenotypes. Interestingly, the effect was only relevant in females which raises the question of whether blocking E2 or its metabolite formation underpins the beneficial effects. The findings from anastrozole treatment in animals was translationally relevant when a clinical trial of 18 patients showed decreased E2 and improved clinical endpoints such as improved 6-MWD following anastrozole treatment (Kawut *et al.*, 2016). This has yet to be confirmed in larger clinical trials. Despite these studies, and the exact role of E2 remains undefined, with worldwide research remaining ongoing in clinical studies.

Most relevant to work within this thesis are recent studies that show E2 to be elevated in male iPAH (Ventetuolo *et al.*, 2016), a finding replicated in another study showing elevated E2 in male patients that were associated with increased mortality rates (Wu *et al.*, 2018). This supports findings from Chapter 3 & 4 of this thesis, showing elevated E1 in males with iPAH. Elevated E1 and E2 in this study may be linked to a pathogenic role in male PAH which potentially contributes to poorer RV adaption. In contrast, females showed no changes in circulating E2 concentrations between controls and patients (Chapter 3, 4, 5 & 6), although these decrease in circulating E2 in female PPHTN. In a combined study from 2009 of female and male patients with portopulmonary hypertension *vs* liver disease acting as controls, patients estradiol associated with a specific SNP in aromatase when data was adjusted for sex in portopulmonary hypertension patients (Roberts *et al.*, 2009). Presence of these SNPs may be essential in promoting dysfunctional estrogen signalling among PAH patients. The cardioprotective effects of E2 may contribute to the improved survival among females in comparison to males. Interestingly, in females, higher E2 levels improve right ventricular systolic function in both healthy individuals and PAH patients (Roberts *et al.*, 2009; Ventetuolo *et al.*, 2011). In all clinical studies presented in this thesis, we demonstrate unchanged E2 concentrations among PAH patients on comparison to controls, with the E2 concentrations quantified in Chapter 5 falling in line with ranges encountered across

the stages of a healthy menstrual cycle. It may be that local production or accumulation of E2 in tissues or adipose pose a greater threat to PAH pathogenesis (Tofovic *et al.*, 2012).

In Chapter 4, we demonstrate that reduction of E1 concentrations in female serum coincides with elevated 16-hydroxyestrogens (16OHE1 and 16OHE2) in iPAH patients suggesting over-expression of CYP enzymes. SNPs in *CYP1B1* can be detrimental for health. For example SNP in *CYP1B1* has been associated with poorer RV function, the relationship between RV measures and the incidence of SNPs associated with PAH which may underpin RV failure (Corey E. Ventetuolo *et al.*, 2016). The presence of *CYP1B1* SNPs can lead to dysfunctional metabolite formation, with over-production of pro-proliferative metabolites, which may be key in solving the sex hormone paradox in pre-menopausal females with metabolites being more pathogenic effect than E2 itself. This finding is supported within this thesis, which repeatedly shows increased 16-hydroxylation in PAH.

In our studies, elevations in 16OHE1 levels were detected in iPAH males and females in comparison to controls (Chapter 4) with elevations also detected in male PPHTN patients in comparison to the liver disease controls (Chapter 6). Production of 16OHE1 can be direct from estrone (via CYP3A4) or via overexpressed CYP1B1. Metabolites formed by this route can cause proliferation of hPASMCs via increased oxidative stress (White *et al*, 2012; Hood *et al.*, 2016). The presence of increased 16OHE1 at the equivalent of 0.3 nM shows a dose-dependent relationship may occur in hPASMCs, shown to proliferate in the presence of 1 nM 16OHE1 (Hood *et al.*, 2016). This finding in male iPAH patients should be further tested and could have significant effects on the development of PAH. In addition, 16OHE1 may upregulate miRNA-29 expression in the presence of a *BMPR2* mutation altering energy metabolism and molecular function and contributing to PAH (Chen *et al.*, 2016). Production of 16OHE1 in this case may also be linked with an earlier finding by this group which demonstrated additional genetic mutations in the *CYP1B1* allele and highlighted the importance of these enzymes in the progression of PAH (Austin *et al*, 2009). Finally, a recent publication found visceral adipose tissue *CYP1B1* expression and 16OHE1 concentrations were increased in obese male mice and may have contributed to increased oxidative stress, effects that were attenuated by both anastrozole and TMS (Mair *et al.*, 2019). This finding suggests BMI may play an important role in the production of proliferative metabolites and hence PAH

pathobiology. In the clinical studies presented here, a correlation between BMI and 16OHE2 was demonstrated in control males, but this should be confirmed in larger cohort studies.

9.3.1 16OHE2 in PAH

The conversion of E1 and E2 to produce 16OHE2 occurs via cytochrome P450 enzymes. 2-Hydroxylation presents the prominent pathway in conversion of E1 and E2. However, in disease the balance is thought to be shifted toward less prominent and potentially harmful pathways such as 4 & 16- hydroxylation (Tsuchiya *et al*, 2005). 16-Hydroxyestrogens are formed via a number of CYP enzymes such as CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2C8, CYP3A4, CYP3A5, and CYP3A7. In a number of experimental studies, CYP1B1 is overexpressed in PAH and linked to increased 16OHE1 production in a number of studies (Johansen et al., 2016; Mair et al., 2019; White et al., 2012). Upon production of 16OHE1 or 16OHE2 via hydroxylation, interconversion can occur by dehydrogenation reactions facilitated by 17βHSD enzymes. The production rate of 16OHE2 in PAH patients and its functional role in PA cells remains undefined. Overall, this thesis has introduced the novel finding that sexual dimorphism of estrogen production exists and that 16OHE2 could contribute to disease in female iPAH via proliferation of PASMCs.

In Chapters 3 - 6 of this thesis, production of 16OHE2 was elevated in PAH patients compared to study controls. This finding is important and also correlates with the *in vitro* work in human and rat PASMCs, which demonstrated a proliferative effect of 16OHE2 in cells explanted from females but not males (Chapter 3). Intriguingly, elevations of 16OHE2 occur almost exclusively in females, with the exception of PPHTN patients. In iPAH patients (Chapter 4), 16OHE2 was elevated in comparison to controls and hPAH meaning this metabolite may be a biomarker of idiopathic disease with the ability to distinguish hPAH from iPAH. The metabolite also correlated with ageing in female controls and iPAH patients. This suggesting there may be an increase in the metabolite formation during transition from pre- to post-menopause in females. This hypothesis should now be confirmed in larger cohort studies of iPAH patients. In premenopausal women (Chapter 5) 16OHE2 concentrations are elevated in PAH vs non-PAH controls; in this case the majority of PAH cases were also idiopathic. In PPHTN cases (Chapter 6), 16OHE2 is elevated in the overall cohort, which upon stratification was consistent between both

males and females. Furthermore, reduction in circulating concentrations of 16OHE2 following treatment with fulvestrant in postmenopausal PAH females indicated that this metabolite may act as a potential therapeutic marker of response (Chapter 7). The reduced circulating concentrations of 16OHE2 correlated with a reduction in HPCs and improvement in the 6-MWD observed in the study (Kawut *et al.*, 2019). The growing evidence and similarity between multiple cohorts from these studies implicates 16OHE2 elevations in disease which may be caused by genetic variation and SNPs in genes encoding CYP enzymes yet be revealed (Ventetuolo *et al.*, 2016).

Also, an interesting translational link between 16OHE2 and pregnancy should be investigated in PAH. Pregnancy represents a major risk factor in PAH patients. In current guidelines, women with PAH are recommended to avoid pregnancy due to mortality risk (Olsson and Channick, 2016). Maternal mortality of 56% represents a high percentage of women with PAH who fail to survive pregnancy (Jaïs *et al.*, 2012). During pregnancy, circulating levels of 16OHE2 and the unconjugated parent estrogens soar above normal healthy circulating concentrations due to placental production (Falah *et al.*, 2015). Therefore, we hypothesise a link between elevated estrogen, in particular 16OHE2, and additional cardiac changes such as increased heart rate, size and RV strain promote the adverse effects of pregnancy in PAH patients (Olsson *et al*, 2016). As we have shown the ability of 16OHE2 to increase proliferation in PASMCs (Chapter 3), we suspect the high production of 16OHE2 throughout pregnancy can also contribute to the increased growth of plexiform lesions within the lung as a result of increased EC proliferation (Assaggaf *et al*, 2017).

In our *in vitro* assessment of 16OHE2 (Chapter 3), human PASMCs from female and male controls displayed no proliferative effect in response to treatment, whilst in PASMCs from male rats, proliferation was again not evident. This finding demonstrates a sex bias toward a proliferative role in PASMCs in only human females with PAH and female rats. Experimental stimulations were performed in 1% FBS to stimulate a serum-reduced state of cell stress, possibly indicating 16OHE2 exerts a proliferative phenotype upon initial disease onset or upon cellular stress. The canonical or non-canonical signalling cascade activated remains unknown. I hypothesies that the signalling mechanism may be similar to proof-of-concept studies in BOECs from our lab (Denver *et al.*, 2018). In BOECs, proliferation

occurred following 1 nM 16OHE1 stimulation in female BOECs. A signalling mechanism via dysregulated *AhR*, CYP enzymes and *Nrf-2* deactivation was proposed following analysis of gene amplification in PAH *vs* control BOECs. A sex-dependent effect of 16OHE2 function was also demonstrated in BOECs with proliferation in females and migration in males. Migration studies were attempted in human PASMCs but not reported in this thesis as they require further optimisation of the scratch assay and imaging for conclusions to be drawn. It may also be possible to measure migratory effects via transwell migration assays. These experiments would be beneficial and may explain the functional role behind our observation of elevated 16OHE2 in male PPHTN patients.

9.3.2 Transcriptome Wide Analysis

In PASMCs, we aimed to take an unbiased view of the differences between female control and female PAH cells using RNA-sequencing. Although there are limitations (Chapter 8, section 8.3.11.2) this study found some interesting differences. We show evidence of dysfunctional immune response components in PASMCs from female PAH over 24 hours in a reduced serum environment (1% FBS). The HLA components of the MHC Class 1 subunit were downregulated in female PAH PASMCs, with a potential role of upstream estradiol and ESR1 activation leading to reduced HLAs being identified. This remains interesting as activation of ESR1 by a variety of estrogen metabolites may also occur. We have demonstrated in alternative studies that increased estrogen metabolism occurs in iPAH in comparison to controls which exerts a proliferative role in PASMCs.

Deactivation of immune escape mechanisms in female PAH may contribute to dysfunctional cell activity. In female PAH patients, the inability of cells to prevent infection may contribute to the increased proliferation seen in PASMCs at 48hr in the presence of relevant estrogen concentrations (Chapter 3, Section 3.3.2). It will be interesting to discover if metabolites elevated in PAH status as determined by LC-MS/MS activate ESR1 signalling cascades. This association, if established, may further link estrogen action, hPASMC proliferation and PAH progression. This also applies to alternative pathways of PAH, all of which should be studied in greater detail to confirm if a link may be established.

This explorative study to find new genes of interest in female PAH was also the first study to compare male and female control PASMCs, with the aim of identifying baseline sex differences and potential disease susceptibility. The comparison of non-PAH PASMCs of both sexes found a number of interesting differences in immune regulation and cell cycle progression which may predispose males to disease. It should be noted that within our study the majority of male controls exhibited carcinomas, which may underlie the risk of more serious conditions. Also, overexpression of the *EIF1AY* transcript in males on comparison to female PASMCs represents an interesting finding as this transcript is over-expressed in hPAH patients (Davies *et al.*, 2012). In the presence of 10 ng/mL TGF-β1, hPAH cells which displayed a *BMPR2* mutation were resistant to the anti-proliferative effect of TGF-β1 observed in controls, resulting in over-representation of transcripts such as *EIF1AY.* Also, the over-representation of the *KDM5* gene whose over-expression has been linked to cardiovascular disease should be studied in greater detail (Mokou *et al.*, 2019). The similarities in profiles related to PAH and cardiovascular disease in male control PASMCs represent a factor predisposing males to higher mortality than females.

9.4 Future Directions: Estrogens in PAH

In general, it would now be useful to conduct controlled larger cohort clinical studies for comprehensive analysis of estrogen metabolites by LC-MS/MS in relation to ageing and BMI in control individuals and PAH patients. This would hopefully reinforce the relationship between estrogen profile and the sexual dimorphism in PAH and confirm the proposed biomarkers or therapeutic modifiers of disease. In addition, studies, which correlate estrogen metabolism and abundance of genetic transcripts from individual plasma samples, may be beneficial to establish a link between genetic mutations and the predicted 16OHE2 elevations. For example, plasma samples could be analysed by LCMS/MS for elevations in 16OHE2 with RNA extraction from the same samples using a Qiagen miRNeasy serum/plasma kit for purification of total RNA for sequencing or qPCR studies. Estrogen metabolites identified as elevated in PAH throughout this thesis (Chapters 3-7) should be tested for activity in experimental models of PAH. These studies could confirm the sex-dependent proliferative action of 16OHE2 in PAH*. In vivo* experiments comparing normoxic and hypoxic animals with vehicle or 16OHE2-administered groups could be performed. This could demonstrate differences in the

pathogenic influence of the metabolite and further our understanding of the effect in PA remodelling and haemodynamic parameters. To expand data from animal studies, analysis of tissues relevant to PAH (heart and lungs) by transcriptomics and imaging approaches could identify site-specific expression, information that is lacking in the current LC-MS/MS work. These studies could identify the possibility of local estrogen production in PAH which may also vary dependent on patient sex. Lastly, following the sequencing analysis of PASMCs, targeted assessments should now follow this work to confirm changes and pathways in relation to PAH. In general, for RNA-sequencing with increased replicates for future validations would be recommended to confirm all gene transcript alterations and activated pathways discussed.

9.5 Translational Impact

The translational relevance of this experimental research in the PAH field is substantial. I have identified a number of potential biomarkers of PAH, which could be routinely analysed in the clinic by collection of serum or plasma. Sex-dependent elevations in 16-hydroxyestrogen production above the normal non-PAH ranges indicate signs of idiopathic or portopulmonary disease. These elevations might also distinguish hPAH and iPAH upon validation in larger cohort studies, a distinction which has been impossible histologically. The final diagnosis of the PAH subgroup would remain dependent on a number of additional clinical diagnostic tests. However, in the clinical setting our LC-MS/MS approach could also be utilised to assess human bio-fluids from alternative estrogen sensitive diseases. For example, in cancers, elevated serum and plasma estrogen concentrations also correlate with disease status and prognosis (Cauley *et al.*, 2003; Santen *et al*, 2015).

A number of chapters provide mounting evidence of increased 16-hydroxyestrogen production in PAH. In our study of postmenopausal women treated with fulvestrant, we demonstrate the estrogen metabolite 16OHE2 may be a biomarker of treatment response in PAH (Chapter 7). The improvement of 6-MWD and reduced 16OHE2 concentrations among women should be further explored. This also emphasises the need for further investigation of polymorphisms in genes encoding CYP enzymes and overexpression in tissues relevant to PAH. Future studies investigating the safety, tolerability and effectiveness of CYP1B1 inhibition in cardiovascular diseases, including PAH, are encouraged. Our finding of increased 16-hydroxylation

among pre- and post-menopausal women with no changes in E2 demonstrate that inhibition of pro-proliferative metabolite formation can be more effective as a treatment in PAH than conventional aromatase inhibition. Among pre-menopausal women the inhibition of CYP enzymes would be preferential allowing preservation of circulating E1 and E2. The preservation of parent estrogens would allow continuation of the menstrual cycle, preventing early onset of menopause, which may in fact pose a greater risk in PAH cases. In addition, the potentially cardio-protective benefits of E2 should not be overlooked. The protective effect of E2 on RV function should be further studied in women in comparison to men. In abnormal estrogen metabolism, multiple pathogenic pathways are activated by CYP1B1 activity (Austin et al, 2009; Austin et al, 2009; Tofovic, 2010; White *et al*, 2012; Zahid *et al*, 2014; Ventetuolo *et al*, 2016; Yan *et al*, 2019). Future studies will also address the molecular mechanisms associated with CYP1B1 pathogenesis and specifically the effects of 16OHE1 and 16OHE2 on cellular signalling pathways. In addition, our LC-MS/MS method should be tested and applied to rodent plasma, which would provide the possibility to track the alterations of estrogen metabolism upon treatment of PAH rat models with CYP inhibitors, experiments which would be translationally relevant in pre-clinical drug development. In cancer, a phase I clinical trial assessing the feasibility, safety and tolerability of CYP1B1 immunity in patients with breast, ovarian, prostate, colon and renal cancer using ZYC300 has been evaluated with promising results (Gribben *et al.*, 2005; Luby *et al*, 2008). This plasmid DNA which codes for an inactive form of CYP1B1, stimulates immune response generating cytotoxic T lymphocyte against CYP1B1. As we have also shown immune escape mechanisms to be deficient in female PAH PASMCs, a means of activating immune response mechanism in PAH may be useful. More relevant to the PAH field, another method of inhibiting CYP1B1 and pro-proliferative estrogen metabolism is by TMS administration. This strategy has been investigated *in vitro* and *in vivo* displaying ability to reduce PA remodelling by cell apoptosis while improving haemodynamic parameters in PAH animals. (White *et al.*, 2012; Johansen *et al.*, 2016). TMS has a 50-fold selectivity of CYP1B1 inhibition over CYP1A1 and a 500-fold selectivity over CYP1A2 (Chun *et al.*, 2009), although this method of CYP1B1 inhibition has yet to be evaluated in clinical cohorts of PAH patients. We anticipate that future therapies targeting the estrogen pathway in PAH will show promising therapeutic effects on the basis of evidence shown throughout this thesis and by research conducted by our collaborators and others in the PAH field. As a result, we strongly encourage the

development of therapies that target estrogen and its metabolism in this devastating cardiovascular disease.

In a small number of individuals, we have also shown that age and BMI correlate with increased 16OHE2 production. In female controls and iPAH, ageing and elevated 16OHE2 concentrations positively correlate. In contrast, males display an increasing BMI, which positively correlates with elevated 16OHE2 concentrations. Clinically, extension patient characteristic and estrogen profiles based on age, BMI and sex would be of great value to allow grouping of estrogen concentrations to multiple PAH parameters. This grouping would identify possible risk factors of disease to guide the exploration of personalised therapy approaches in PAH treatment. Alongside our LC-MS/MS approach, advances in metabolomics applications for the analysis of small molecules in further profiling epidemiology markers would be useful. We could develop a non-invasive blood test for PAH diagnostics and monitoring which would avoid the use of invasive right heart catheterisation and at the same time provide valuable information for personalised therapies. As I have demonstrated, estrogen concentrations vary between idiopathic and heritable patients. Further emphasis of differences in these currently indistinguishable patient subgroups should be identified. The understanding of small molecules such as lipids, amino acids, eicosanoids, ROS and hormones involved in PAH development to *BMPR2* mutations remain crucial. The ultimate goal of these studies would be to distinguish biomarkers and epidemiology markers for precision personalised medicine in PAH clinics, with the aim of improving survival and life-quality for individuals living with this severe life-limiting disease.

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